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Potential and pitfalls of non-viral mRNA delivery for ocular therapies

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Nothing is impossible.

The word itself says, I'M POSSIBLE

-Audrey Hepburn

Ghent, March 15th 2019

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LIST OF ABBREVIATIONS

2-AP	2-aminopurine
AAV	Adeno-associated virus
ABV	Adult bovine vitreous
Ad	Adenovirus
ADAR	RNA-specific adenosine deaminase
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
ANT	Ascl1+NMDA+Tamoxifen
ARCA	Anti-reverse cap analog
ASC	Apoptosis-associated speck-like protein
Ascl1	Achaete-scute homologue 1
ASD	Anterior segment disorders
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BRB	Blood-retinal barrier
BrdU	Bromodeoxyuridine
Cabp	Calcium-binding protein
CAG	CMV enhancer/chicken β-actin
CBA	Chicken β-actin
CD44	Cluster of differentiation 44
CIS	Cone inner segment
cKO	conditional Knockout
CLQ	Chloroquine
CMV	Cytomegalovirus
CNTF	Ciliary neurotrophic factor
COS	Cone outer segment
Crb	Crumbs homologue
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Crx	Cone-rod homeobox
DAPI	4',6-diamidino-2-fenylindool
Dh	Hydrodynamic diameter

Dkk	Dickkopf WNT signaling pathway inhibitor
DLL	Delta like canonical Notch ligand
DLS	Dynamic light scattering
DMEM	Dulbecco’s Modified Eagle’s Medium
DR	Diabetic retinopathy
dsRNA	double-stranded RNA
DUBA	Deubiquitinating enzyme A
EDTA	Ethylenediaminetetraacetic acid
EF	Endotoxin-free
EGF	Epidermal growth factor
eGFP	enhanced Green fluorescent protein
EGFR	Epidermal growth factor receptor
eIF	eukaryotic translation Initiator factor
ELISA	Enzym-linked immunosorbant assay
ERG	Electroretinogram
ERG	electroretinography
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FCS	Fluorescence correlation spectroscopy
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
fLuc	firefly Luciferase
fSPT	fluorescence Single particle tracking
GAA	Glacial acetid acid
GAD	Glutamate decarboxylase
GAG	Glycosaminoglycans
GCL	Ganglion cell layer
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
GMP	Good manufacturing process
GNAT	Guanine nucleotide binding protein alpha transducing
GS	Glutamine synthetase
HA	Hyaluronic acid

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF	Human foreskin fibroblasts
HRE	Hypoxia-responsive element
HSA	Human serum albumin
HuC/D	ELAV like RNA binding protein 3/4
i.p.	Intraperitoneal
IFN	Interferon
IFNAR	Interferon-α/β receptor
IGF-1	Insulin-like growth factor 1
IKK	IκB kinase
IL	Interleukin
ILM	Inner limiting membrane
INL	Inner nuclear layer
IPL	Inner plexiform layer
iPSC	induced Pluripotent stem cell
IRF	Interferon regulatory factors
IS	Inner segments
ISGF	IFN-stimulated gene factor
ISG	IFN-stimulated gene
IVT	*In vitro* transcribed
IVTR	Intravitreal
JAK	Janus kinase
LCA	Leber's Congenital Amaurosis
LGP	Laboratory of genetics and physiology
LIF	Leukemia inhibitory factor
LNP	Lipid nanoparticles
LPD	Liposome-protamine-DNA
LV	Lentivirus
m1ΨU	N^1-methylpseudouridine
m1ψU	methylpseudouridine
m5C	5-methylcytidine
MAVS	Mitochondrial antiviral-signalling protein
MDA	Melanoma differentiation-associated protein
MFI	Mean fluorescence intensity

MG	Müller glia
MIO-M1	Müller cell line Moorfields/Institute of Ophtalmology-Müller 1
miRNA	microRNA
MNU	N-methyl-N-nitrosourea
mRNA	messengerRNA
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MW	Molecular weight
MyD88	Myeloid differentiation primary response gene 88
NA	Nucleic acid
NEMO	NF-κB essential modulator
NeuN	Neuronal Nuclei (Hexaribonucleotide Binding Protein-3)
NF	Nuclease-free
NFL	Nerve fiber layer
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NLR	NOD-like receptors
NLRP	NOD-like receptor pyrin domain containing
NMDA	N-methyl-D-aspartate
NMU	N-methyl-N-nitrosourea
NP	Neuroprotection
Nrl	Neural retina leucine zipper
NSE	Neuron-specific enolase
NT	Neurotrophin
NTC	Non-treated control
NV	Neovascularisation
OAS	2’-5’-oligoadenylate synthetase
OIR	Oxygen-induced retinopathy
ONL	Outer nuclear layer
OPL	Outer plexiform layer
ORF	Open reading frame
OS	Outer segments
OTC	Optimal cutting temperature compound
Otx2	Orthodenticle homeobox2
PABP	Polyadenylate binding proteins

PAX6	Paired box 6
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
pDNA	plasmid DNA
PEDF	Pigment epithelium-derived factor
PEG	Polyethylene glycol
PKC	Protein kinase C
PKR	dsRNA dependent protein kinase
PR	Photoreceptor
Prox1	Prospero homeobox 1
PRR	Pattern recognition receptor
PRS	Photoreceptor segments
RA	Retinoic acid
RD	Retinal degeneration
RGC	Retinal ganglion cell
RIG-I	Cytoplasmic retinoic acid-inducible gene I
RLBP	Retinaldehyde-binding protein
RLR	RIG-I-like receptors
Rnase L	Ribonuclease L
ROS	Reactive oxygen species
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RT	Room temperature
s2U	2-thiouridine
SD	Standard deviation
sgRNA	synthetic guide RNA
Shh	Sonic hedgehog
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLN	Solid lipid nanoparticles
SR	Subretinal
ssRNA	single-stranded RNA
STAT	Signal transducer activator of transcription
SV	Sonicated vitreous

TBE	TRIS/Borate/EDTA
TBK1	TANK-binding kinase 1
TGF-β	Transforming growth factor-β
TIR	Toll/Interleukin (IL)-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRIF	TIR domain-containing adaptor inducing IFN-β
TrkB	Tyrosine receptor kinase B
TSA	Trichostatin-A
TYK2	Tyrosine kinase 2
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VIM	Vimentin
VR	Vitreoretinal
VV	Vaccinia Virus
Wnt	Wingless/Integrated
WT	Wilde type
α-AA	α-aminoadipate
ΨU	pseudouridine

Aim and outline of this thesis

Retinal cell degeneration is a leading cause of vision loss and irreversible blindness in many ocular diseases, including retinitis pigmentosa, diabetic retinopathy and glaucoma. Loss of sight influences the quality of life of the patient and entails an economic burden, both for individuals and for the larger healthcare system. Despite ongoing efforts to find new treatments, there are currently few effective options in the clinic.

Given the benefits that the retina offers as a target tissue, it has been at the forefront of clinical gene therapy leading to the recent approval of the first gene therapy product for an ocular disease. At the moment, the delivery of therapeutic genes to the retina is limited to the use of replication-deficient viral vectors. However, the risk of insertional mutagenesis and induction of unwanted immune responses still remain critical for their safe application. On the other hand, non-viral vectors have been intensively investigated for plasmid DNA (pDNA) delivery as a safer alternative. Despite their many advantageous properties, non-viral gene therapeutics have not yet reached clinical trials for retinal diseases. One of the biggest obstacles for effective non-viral gene therapy in the retina is the delivery of the transgene across the nuclear membrane. In this regard, the delivery of messengerRNA (mRNA) to the retina has a major advantage over plasmid DNA (pDNA) as it is completely functional in the cytoplasm. Indeed, recent investments in improving mRNA synthesis and stability have enabled a wide range of applications surpassing the potential that was once foreseen for DNA-based medicine.

Therefore the principal objective of this thesis is to unravel, for the first time, the potential of non-viral mRNA delivery for ocular applications. As it is not our aim to design new delivery vectors, we will make use of well-established commercial available mRNA transfection reagents. With these delivery tools, we will evaluate the capacity of mRNA to transfect different retinal cell types and investigate the most important barriers that might forestall mRNA-based protein expression in the retina.

Chapter 1 provides a general introduction to the field of mRNA-based gene delivery. Although discovered almost 60 years ago, mRNA was long deemed inferior to pDNA-based transfection techniques, owing to its labile nature and short-term protein expression. Its intrinsic capacity to elicit innate immune responses spurred investigations into the use of mRNA for vaccination strategies. However, its use for non-immunological approaches seriously lagged behind. This chapter describes the immune-related hurdles responsible for the limited advance of mRNA-based therapeutics for non-immunotherapy-related

applications and proposes some promising methods to broaden the use of mRNA beyond vaccination strategies.

Chapter 2 comprises a detailed description of the anatomy of the eye and the cellular organization of the retina. One cell type in specific, namely the Müller cell, caught our attention as a promising target for mRNA-based gene delivery to the retina, the reasons for which will be highlighted in this chapter. Besides covering the most intriguing features, which favor their position as therapeutic target, we provide an overview on how to selectively transduce this retinal cell type. In a final section we will summarize the role of Müller cells in mediating retinal neuroprotective and regenerative strategies, two applications which might benefit from mRNA-based transfection.

Convinced of the idea that mRNA holds great promise for retinal neuroprotection or reprogramming methods, we will first investigate the efficiency of *in vitro* mRNA-based transfection in Müller cells and compared this strategy with the well-established pDNA transfections in **Chapter 3**. As intravitreal injections are a very promising administration route to target the inner retina, we will make use of an experimental *in vitro* setup to evaluate the influence of the vitreous on the transfection efficiency and an *ex vivo* model to screen the intravitreal mobility of the complexes. When limited mobility is observed, we will assess whether electrostatical coating with hyaluronic acid can result in the desired physicochemical characteristics to efficiently overcome the vitreal barrier.

Based on previous observations that lipid particles result in an overall better mRNA transfection compared to polymer-based carriers, we aim to maximize mRNA-mediated transfection by means of a well-established lipid-based transfect reagent in **Chapter 4**. Next, we will evaluate nucleotide modification, one of the strategies mentioned in **Chapter 1** to increase the stability of the mRNA and decrease innate immune responses to the synthetic mRNA backbone, in order to obtain a promising tool for *in vivo* application. Finally, we will determine the localization and extent of mRNA expression after administration to bovine retinal explants and *in vivo* injections in mice. In the meantime we will determine the impact of the delivery route by comparing intravitreal and subretinal injections.

In **Chapter 5** we will examine the role of the intracellular innate immune response to in vitro transcribed (IVT) mRNA in another relevant target cell type, namely the retinal pigment epithelium (RPE) cells. To this end we will evaluate the use of small molecules to abrogate the mRNA-induced innate immune response, another important strategy described in **Chapter 1**. Five different small molecules (chloroquine, Pepinh-TRIF, Pepinh-MYD, B18R and 2-AP) will be tested in their antagonistic activities against different elements of the innate immune pathways and in their capacity to increase mRNA transfection efficiency.

Finally, in **Chapter 6** we position the work presented in this thesis within a broader international context and discuss its relevance to the field of gene therapy. First, we describe the current events in ocular gene therapy and consider some challenges that may jeopardize the success of gene therapy in the near future. Next, new and emerging applications are introduced that may revolutionized the field of ocular gene therapy as we know it. In the last part, we aim to envision how the work performed in this thesis could contribute to these new strategies and which areas warrant further investigation.

Chapter 1

A general introduction to non-viral mRNA delivery: addressing the inherent challenges of mRNA

This chapter is published as:

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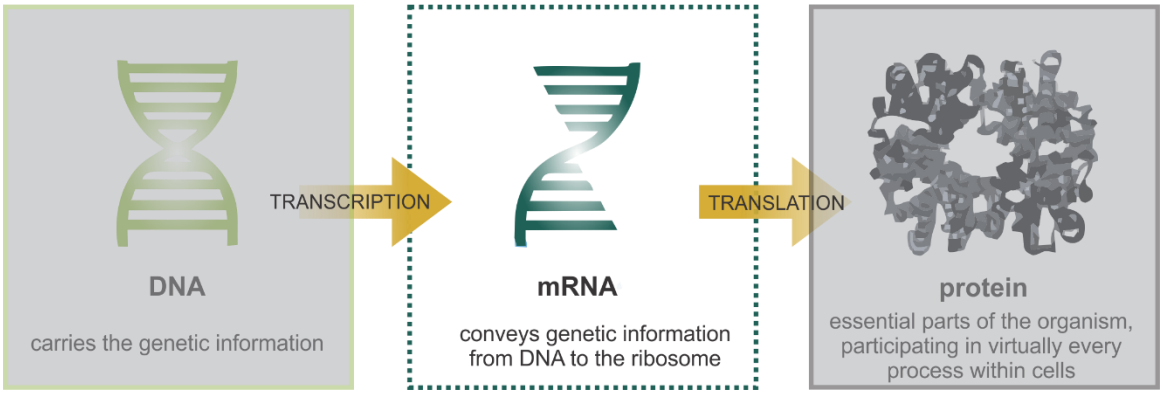
ABSTRACT

In de field of non-viral gene therapy, *in vitro* transcribed (IVT) messenger RNA (mRNA) has emerged as a promising tool for the delivery of genetic information. Worldwide effort is committed to the commercial development of mRNA drugs, with ongoing clinical trials focusing on vaccines and cancer immunotherapy. Although the strong immune-stimulatory effect of mRNA is an added benefit for immunotherapeutic vaccination strategies, it is a major disadvantage for non-immunotherapy related applications. The intrinsic immunogenicity of mRNA was shown to directly interfere with the aimed therapeutic outcome of these applications, as it can seriously compromise the expression of the desired protein. Therefore clinical translation of mRNA-therapeutics was seriously hampered in these areas. This chapter presents an overview of the field of mRNA-therapeutics and the immune-related obstacles that limit mRNA advance for non-immunotherapy related applications. In addition, we suggest some promising methods to reduce this 'unwanted' innate immune response and reflect on recent developments in the use of non-viral mRNA delivery for non-immunogenic purposes.



INTRODUCTION

Recent advances in the field of molecular biology have revolutionized mRNA as a therapeutic. The concept of nucleic acid based therapy emerged in 1990, when Wolff *et al*. reported successful expression of proteins into target organs by direct injection of either plasmid DNA (pDNA) or messenger RNA (mRNA)[1]. Although this pioneering study showed a similar potential of mRNA and pDNA to induce protein expression, it took another 10 years for *in vitro* transcribed (IVT) mRNA to compete with the success of DNA transfection. Initially the use of mRNA as a gene therapeutic was confronted with much skepticism due to its perceived instability and transient nature. However, recent research demonstrating the many advantages of mRNA over pDNA, brought about a new wave of interest into the use of IVT mRNA. A first convenience is that mRNA exerts its function in the cytoplasmic compartment. As a consequence, mRNA activity does not depend on nuclear envelope breakdown, which is a major disadvantage of pDNA transfection. In this regard, mRNA is an ideal candidate for protein expression in non-dividing cells, such as dendritic cells, which are otherwise hard to transfect[2]. Secondly, mRNA, unlike pDNA and viral vectors, lacks genomic integration and thus avoids potential insertional mutagenesis[3]. This provides mRNA with a substantial safety advantage for clinical practice. Thirdly, mRNA production is relatively easy and relatively low-priced, since there is no need to select and incorporate a specific promoter into the transfection construct[4]. Furthermore, since IVT mRNA is synthesized in a cell-free system, the production process, manufacturing material as well as the product quality can be easily standardized and controlled in good manufacturing process (GMP) conditions. GMP manufacturing of mRNA guaranties high batch-to-batch reproducibility and makes it easy to translate mRNA use from bench to bedside[5].

One of the applications in which induction of transient gene expression by mRNA transfection is of great interest is vaccination, in which transcripts encoding a certain antigen are administered directly *in vivo* or *ex vivo* via dendritic cell transfection in order to elicit antigen-specific immune responses[6-9]. Besides the desired immune responses against the antigenic protein encoded by the mRNA, the mRNA itself is often the target of the immune system, making mRNA both the messenger and its own adjuvant. For immunotherapy, this intrinsic immune-stimulatory activity of mRNA is not a limiting factor, as it can increase the potency of the vaccine (as extensively reviewed elsewhere [3, 5, 10-14]). When extending the use of mRNA for applications outside this area, however, innate immune responses against mRNA can seriously compromise its delivery efficiency. To address these issues, this chapter aims to discuss the immune-related hurdles that need to be tackled to allow clinical application of IVT mRNA for non-immunotherapy related applications. We present a summary of the current knowledge of the signal pathways induced by mRNA transfection

and suggest some promising methods to enhance mRNA expression by reducing this 'unwanted' innate immune response. Furthermore, we overview recent developments in the use of non-viral mRNA delivery for non-immunogenic purposes, such as protein-replacement therapies and regenerative medicine applications.

IN VITRO TRANSCRIBED (IVT) MRNA

Interestingly, the production of functional mRNA by *in vitro* transcription was already reported in 1984 by Krieg *et al*[15]. They synthesized mRNA using a phage RNA polymerase and a cloned cDNA template. Following this publication, a high number of technical refinements have been reported and kits for synthesis have been commercialized.

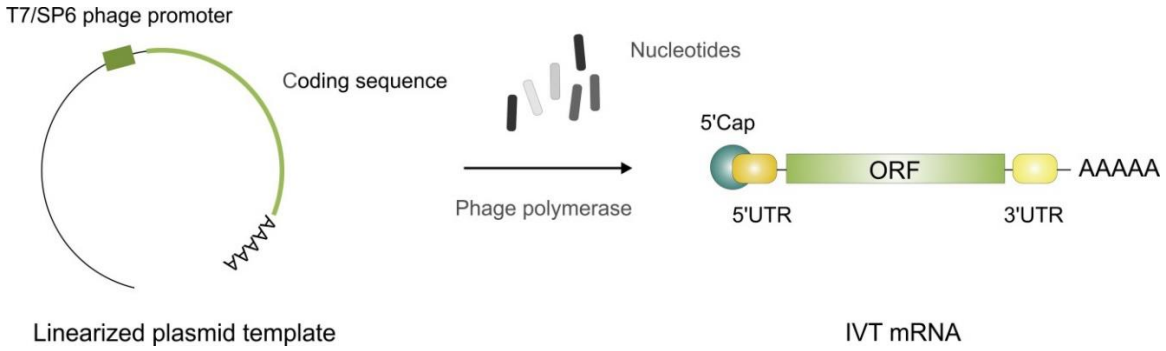


Figure 1 | ***In vitro* transcription of mRNA.** Capping of the mRNA can be done during the *in vitro* transcription reaction by addition of synthetic cap analogues or post-transcriptionally by means of recombinant capping enzymes. The poly(A) tail can be encoded in the template DNA or can be enzymatically added after *in vitro* transcription. IVT, *in vitro* transcribed; ORF, open reading frame; UTR, untranslated region

IVT mRNA is a single-stranded polynucleotide, structurally resembling naturally occurring eukaryotic mRNA. The sequence encoding the desired protein is called the open reading frame (ORF) and is located between two untranslated regions (UTRs). A 5' cap structure and a 3' poly(A) tail flank the mRNA at its extremities (**Figure 1**). Eukaryotic mRNA contains a 7-methylguanosine cap coupled to the mRNA via a 5'-5' triphosphate bridge (m^7GpppN). For efficient translation, binding of the 5'-cap to the eukaryote translation initiation factor 4E (eIF4E) is essential. Binding with the decapping enzymes (DCP1, DCP2, DCPS) on the other hand results in a loss of mRNA activity[16, 17]. IVT mRNA can be capped either post-transcriptionally using recombinant capping enzymes[18] or during the *in vitro* transcription reaction by adding a synthetic cap analogue. The poly(A) tail, a long sequence of polyadenylate residues binds to the polyadenylate binding proteins (PABPs) leading to mRNA circularization, thereby increasing the affinity of eIF4E for the cap structure[19, 20]. This synergistic interaction between the two termini of mRNA plays an important role in the stability of mRNA by limiting both decapping as well as 3' to 5' mRNA degradation.

Although IVT mRNA strongly resembles endogenous mRNA, it is still considered as foreign by the innate immune system. Over the past few years it has become known that the introduction of IVT mRNA into mammalian cells induces activation of several mechanisms of which the natural purpose is to identify and attack viral RNAs.

THE IMMUNE-STIMULATING ACTIVITY OF MRNA

Intracellular mRNA sensing pathways

Knowledge of the mechanisms recognizing and responding to viral intruders has furthered our understanding of the cytosolic sensors involved in innate immunity. These sensors have been shown to be activated mainly by viral nucleic acids, rather than viral proteins[21]. DNA, double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) found in viral genomes, as well as dsRNA-intermediates of viral replication are recognized by so-called pattern recognition receptors (PRRs)[22]. Stimulation of these PRRs activates a downstream cascade of signalling reactions, eventually inducing gene expression of pro-inflammatory cytokines and type I interferons (IFNs). By identifying the structural elements responsible for this activation, insight was gained into the immune-stimulatory activity of IVT mRNA.

Figure 2 summarizes the main pathways involved in mRNA recognition. Two families of PRRs are thought to be responsible for the detection of IVT mRNA: the Toll-like receptors (TLRs) and the cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). TLRs, predominantly but not exclusively expressed in immune cells, are transmembrane receptors with leucine-rich repeats in the extracellular or intra-endosomal region and a signal-transduction or Toll/Interleukin (IL)-1 receptor (TIR) domain in the cytosolic region. Thirteen TLRs have so far been identified in humans and mice together[21, 23, 24]. Their location in the cell seems to correlate to the pathways by which their molecular ligands are processed[25]. Accordingly, the TLRs involved in the recognition of foreign mRNA – TLR3, TLR7 and TLR8 – are located in the endosomal compartment. As such, especially uridine-rich ssRNA was identified as a strong immune inducer, mainly via stimulation of TLR7[26, 27], whereas dsRNA rather activates TLR3[28, 29]. Generally, mRNA is considered ssRNA, causing the foreign IVT mRNA to be mostly recognized by the structurally homologous TLR7 and TLR8 receptors[26, 30]. However, mRNA is also able to form secondary structures, such as hairpins, containing double stranded sequences. These short segments interact with the dsRNA binding protein of the TLR3 signalling cascade, making mRNA a suitable ligand for TLR3 [28, 29].

Following activation, PRRs transmit downstream signalling via specific adaptor molecules. For TLR7 and 8, the required adaptor is the Myeloid differentiation primary response gene 88 (MyD88). TLR3 transmits signals via TIR domain-containing adaptor inducing IFN-β (TRIF)[31]. The adaptor proteins MyD88 and TRIF initiate a signalling cascade that consists of a complex network of signalling molecules. These signalling networks cooperate, integrate and finally converge into the activation of several transcription factors, including nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs) 3 and 7[32].

In addition to TLRs, IVT mRNA can be detected by RLRs, which are cytosolic RNA helicases. These sensors, mainly important in non-immune cells, include RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I has been long thought to specifically detect ssRNA bearing a '5-triphosphate (5'ppp) group[33, 34]. Recent studies however, challenged this hypothesis and demonstrated that activation of RIG-I requires base pairing of the nucleoside carrying the 5'ppp. Evidence was provided that RIG-I is triggered by double-stranded, but not single-stranded, RNA containing 5'ppp[35, 36]. In addition, Goubau *et al.* showed that also 5'-diphosphate (5'pp) dsRNA serves as an RIG-I ligand, thereby concluding that a minimal feature for RIG-I activation is a base-paired RNA with a free 5'pp[37]. Since endogenous RNA is processed and capped before entering the cytoplasm, its 5'ppp group is shielded from detection by RIG-I. IVT mRNA however, if co-transcriptionally capped, yields a significant fraction of uncapped single- and double-stranded molecules, which can trigger RIG-I signalling. The second RLR, MDA-5, is activated by cytoplasmic long dsRNA[38, 39]. Recognition of RNA by RIG-I or MDA5 triggers an ATP-dependent change in the receptor conformation, which allows interaction with the mitochondrial antiviral-signalling adaptor protein MAVS (also known as IPS-1). The obtained complex actuates several proteins to initiate downstream signalling, which similar to the activation of TLRs converges in the activation of several transcription factors. A third member of the RLRs is LGP2 (not depicted in **Figure 2**). LGP2 is much less explored and conflicting data have been published on its role in innate immune signalling. Although LGP2 was initially assumed to negatively regulate RLR-mediated signalling[40, 41], more recent studies revealed a positive role for LGP2 in the regulation of type I IFN responses[42]. Nevertheless, experimental data of further studies are still controversial, with both overexpression and knockdown of LGP2 resulting in type I IFN production[43]. Whether LGP2 mediated signalling can be induced by IVT mRNA remains to be established.

It is clear from the above that both TLR and RLR sensors respond to mRNA stimulation by activation of transcription factors, such as NF-κB, IRF3 and IRF7. Both pathways converge in the activation of the IκB kinase (IKK) complex and the IKK-related kinases TANK-binding kinase 1 (TBK1) and IKKε. The IKK complex, which includes the kinases IKKα

and IKKβ as well as the regulatory subunit IKKγ/NEMO, is responsible for the activation of NF-κB, whereas TBK1 and IKKε phosphorylate and activate IRF3 and IRF7[31, 44, 45]. In unstimulated cells, NF-κB, IRF3 and IRF7 are located in the cytoplasm. Activation by the aforementioned kinases, however, causes them to translocate to the nucleus. Intranuclear, they bind to the type I IFN gene promoter, inducing expression of type I IFNs, in particular IFN-α and IFN-β. NF-κB additionally activates the expression of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-12 (IL-12)[5, 32].

As depicted in **Figure 3**, type I IFNs are secreted in the extracellular environment and bind to the transmembrane IFN receptor complex of the stimulated cell and adjacent cells. This receptor complex in turn induces a downstream transmission of signals through the so-called Janus kinase (JAK)-Signal transducer activator of transcription (STAT) pathway. The STAT proteins, STAT1 and STAT2, are phosphorylated by the Janus kinases JAK-1 and TYK-2, and bind a third factor, IRF-9 to form a transcription activator complex, the IFN-stimulated gene factor 3 (ISGF-3). Upon activation ISGF-3 translocates to the nucleus, where it initiates the transcription of more than 300 IFN-stimulated genes (ISGs). Of these ISGs, many encode for proteins that are components of the signalling pathways themselves, such as PRRs and transcription factors, thus providing an autocrine loop that amplifies IFN production[46]. However, several other ISGs encode for proteins which confer strong anti-viral activity, including dsRNA dependent protein kinase (PKR), 2'-5'-oligoadenylate synthetases (OASs) and RNA-specific adenosine deaminase (ADAR)[47]. It is interesting to point out that both type I IFNs as well as the pro-inflammatory cytokines, not only act in an autocrine fashion, but concurrently activate receptors in adjacent cells via paracrine secretion. As a result, upregulation of PRRs is induced in neighbouring cells, sensitizing them to subsequent exposure to nucleic acids[48, 49].

Recently, Andries *et al.* demonstrated that another PRR family, the NOD-like receptors (NLRs) are also involved in the cytoplasmic recognition of IVT mRNA[50]. They demonstrated an upregulation of caspase-1 after non-viral carrier-mediated delivery of mRNA in respiratory cells. Caspase-1 is a zymogen, typically regulated by NLRs. The NLR most broadly associated with RNA sensing is NLRP3, which has been shown to respond to dsRNA[51]. NLRP3, also known as cryopyrin or Nalp3, forms a multiprotein complex with the adaptor protein ASC and caspase-1. This complex, called "the inflammasome", is responsible for the proteolytic maturation of the IL-1β and IL-18 cytokines. A recent study by Sabbah *et al*. has demonstrated that another member of the NLRs, NOD2, can also serve to detect ssRNA[52].

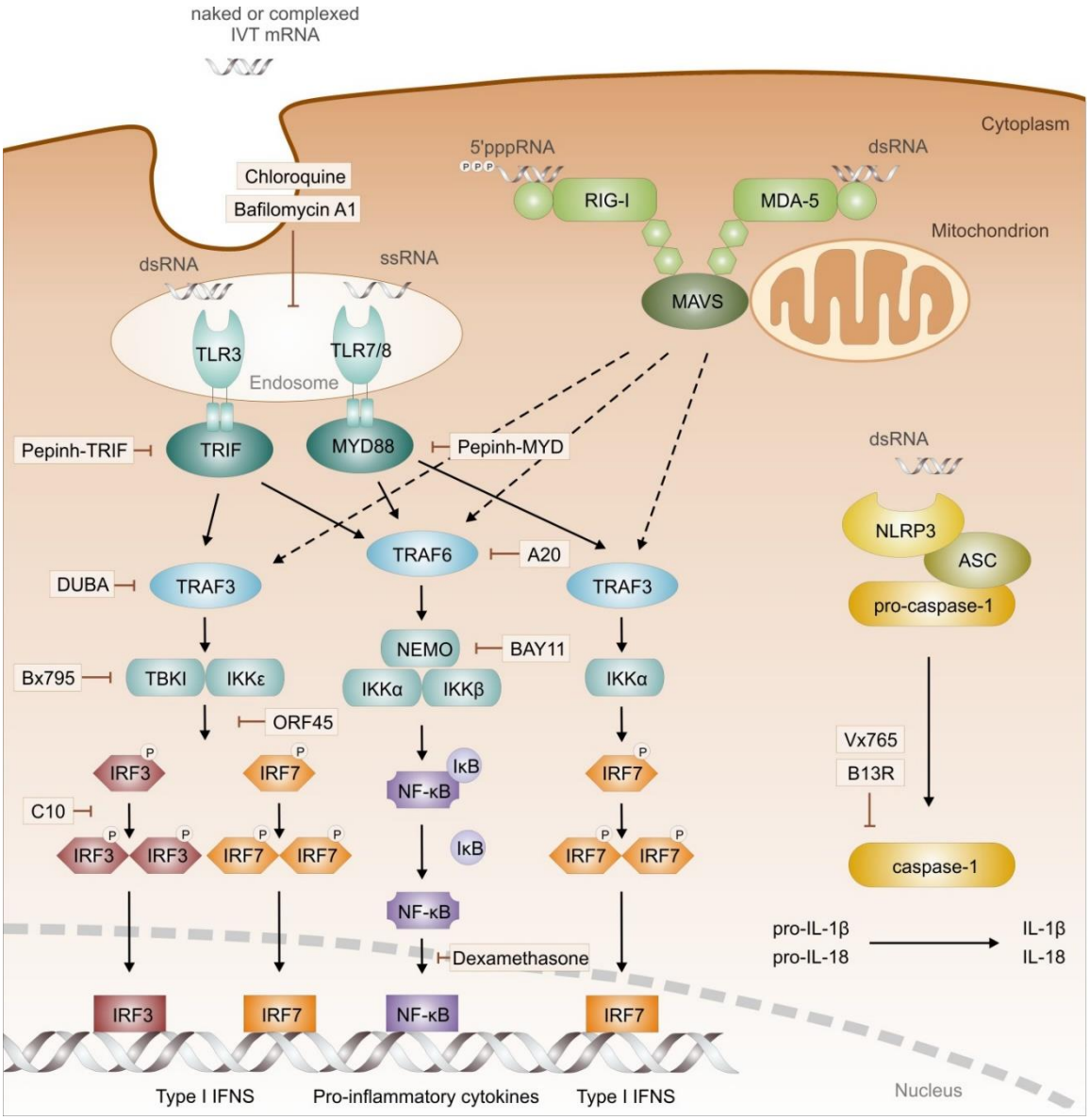


Figure 2 | Innate immune responses to intracellular delivery of IVT mRNA. Synthetic mRNA is recognized by several PRRs, including the endosomal TLR3 and TLR7/8 receptors and the cytoplasmic RIG-I, MDA-5 and NLRP3 sensors. Each PRR interacts with a specific adaptor molecule, which recruits the illustrated signalling molecules and activates downstream transcription factors IRF3, IRF7 and NF-κB. IRF3 and IRF7 regulate the expression of type I IFNs (IFNα and IFNβ), whereas NF-κB additionally controls the production of pro-inflammatory cytokines. Production of type I IFNs and can be inhibited at multiple levels: (i) minimize mRNA recognition through administration of PRR inhibitors, (ii) delivering deubiquitinating enzymes, (iii) inhibiting the adaptor molecules by means of peptide inhibitors, (iv) suppressing transcription factor activation with kinase inhibitors, (v) counteracting transcription factor activity itself and (vi) applying caspase-1 inhibitors to prevent NLRP3-mediated cytokine production. ASC, apoptosis-associated speck-like protein; dsRNA, double-stranded RNA; DUBA, deubiquitinating enzyme A; IFN, interferon; IKK, IκB kinase; IL, interleukin; IRF, interferon regulatory factor; IVT, *in vitro* transcribed; MAVS, mitochondrial antiviral-signalling protein; MDA-5, melanoma differentiation-associated protein 5; MyD88 Myeloid differentiation primary response gene 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor- κB; NLRP3, NOD-like receptor pyrin domain containing 3; RIG-I, cytoplasmic retinoic acid-inducible gene I; ssRNA, single-stranded RNA; TBK1, TANK-binding kinase 1; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adaptor inducing IFN-β

All these intracellular cascades have been shown to interact with each other in a complex network. It is this crosstalk together with the strength, timing and context of stimulation that determines the type and duration of immune responses. Besides this inter-pathway crosstalk, PRR-mediated signalling is regulated by polyubiquitination or deubiquitination of the involved proteins and can therefore be influenced by deubiquitinating enzymes[32].

Unwanted immune responses induced by mRNA recognition

As previously discussed, IVT mRNA-induced immune activation is considered beneficial for vaccination strategies as it can attribute to the desired cellular and humoral immune response. Especially the strong cytokine milieu that results from antigen-encoding IVT mRNA transfection is of particular interest, as this can boost dendritic cell maturation as well as T cell activation[53, 54].

By contrast, for non-immunotherapy related applications this immune-stimulatory activity of IVT mRNA might be a major concern, as was shown in several mRNA-based reprogramming studies[48, 49, 55]. Signalling through the different PRR pathways forces the cells into an overall anti-viral state, affecting the efficiency of mRNA translation and causing RNA degradation. In this anti-RNA response, a key role is played by the ISG encoded proteins (**Figure 3**). To date, three anti-RNA pathways that shoot the messenger have been identified. These comprise the PKR, the OAS and the ADAR system.

PKR (also known as Eif2ak2) is a kinase that phosphorylates the α-subunit of the eukaryotic translation initiator factor 2 (eIF2α). Activation of PKR impairs eIF-2 activity, which results in an inhibition of general mRNA translation and thus stalls protein synthesis[56]. Besides this regulatory translational controlling function, PKR is also involved in various signalling pathways. Active PKR has been shown to provoke release of NF-κB from its inhibitory subunit, IκB, by stimulation of the IKK kinase complex, thereby activating the NF-κB transcription factor and promoting the expression of multiple genes[57]. Finally PKR also induces cellular apoptosis, which serves as a natural process of preventing further viral infection[56, 58].

A second anti-RNA pathway involves the activation of OAS by dsRNA to produce of 2'-5'-oligoadenylates (2-5A) from ATP. These rare 2-5A oligomers have the capacity to induce the catalytical activity of the latent enzyme RNaseL, which causes cleavage of ssRNA, thus promoting RNA degradation[59]. In addition, the cleavage products can again bind and activate cytoplasmic PRRs, thus maintaining and amplifying the type I IFN loop[47, 60].

Another ISG family that influences translation is the adenosine deaminases acting on RNA or ADARs. These genes encode for the ADAR enzyme, which catalyzes RNA editing through site-specific deamination of adenosine (A) to yield inosine (I). By inducing the formation of a weak I:U mismatch, ADARs are capable of destabilizing the RNA molecule. Moreover, conversion of A to I may alter the coding capacity of mRNA and thus the amino acid sequence of the encoded proteins[61, 62]. Surprisingly, however, recent studies found that the absence of ADAR1, one of the three identified ADAR proteins, significantly increases IFN-mediated signalling, suggesting a role for ADAR1 as a suppressor of IFN responses[63, 64]. Presumably, this negative feedback serves to prevent overreaction during viral infection. The mechanism by which ADAR1 impairs type I IFN response has not been thoroughly elucidated. One possibility is that ADAR1 edits the RNA in such a way that it no longer serves as an activator of innate immune signalling and loses its IFN inducing capacity[61, 62]. Another feasible explanation is that the RNA binding activity of ADAR1 is involved in the suppression of IFN signalling. Recently, Yang *et al.* demonstrated that ADAR1 binds dsRNA, thereby limiting cytosolic dsRNA sensing by RLRs[65]. In addition, ADAR1 suppresses activation of both PKR and IRF3, by a mechanism still to be resolved[66].

OAS as well as PKR and ADAR require IFN signalling for induction of their synthesis, but also call for dsRNA to initiate their activation. In this way all three enzymes not only act as RNA-induced effectors but also serve as PRRs for the detection of dsRNA in the cytosol[47]. It is important to note that these are probably not the only ISGs that negatively influence IVT mRNA translation. Likely, additional IFN-induced proteins with similar roles exist, but await further investigation into their specific relevance.

The processes induced by these effectors not only hamper mRNA transfection, but also disfavor cell viability and can eventually result in apoptosis[67]. Besides type I IFNs, up-regulation of caspase-1 by NLR-mediated signalling too is detrimental to cells and causes programmed cell death[50]. This is probably one of the reasons why non-immunotherapeutic mRNA therapies are still in its infancy (as will be more thoroughly discussed below).

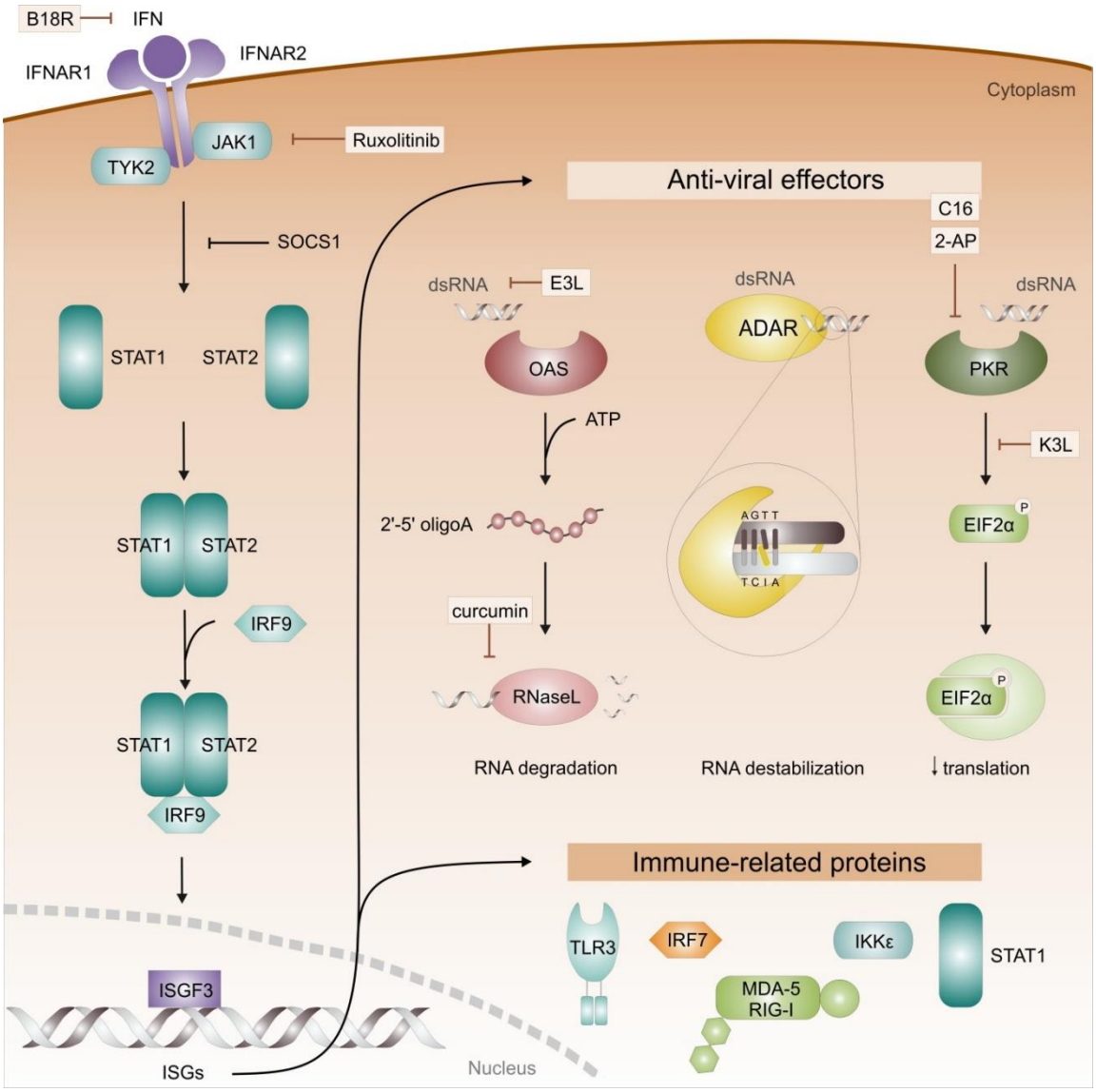


Figure 3 | IFN-mediated signalling. Following their production (cf. **Figure 2**) type I IFNs bind to autocrine or paracrine IFN receptor complexes, composed of IFNAR1 and IFNAR2. Recognition of IFNs stimulates the Jak kinases, Jak1 and Tyk2 to phosphorylate Stat1 and Stat2, which form a transcription activator complex, ISGF3, together with IRF9. ISGF3 activates hundreds of ISGs, including genes encoding for anti-viral effectors OAS, PKR and ADAR. Overall, these create an anti-viral environment, enhancing RNA degradation, causing RNA destabilization and stalling RNA translation. Among the ISGs several genes encode for immune-related proteins, thereby initiating the transcription of a second wave of type I IFNs and amplifying the antiviral response. IFN-induced signalling can be avoided blocking different steps of the signalling cascade: (i) apply IFN-capturing proteins to prevent IFN-receptor binding, (ii) inhibit interferon-induced signalling by means of JAK/STAT inhibitors and (iii) administer molecules which minimize the antiviral action of IFN-induced proteins. 2-AP, 2-aminopurine; ADAR, RNA-specific adenosine deaminase; dsRNA, double-stranded RNA; EIF2α, eukaryotic translation initiator factor 2; IFN, interferon; IFNAR, interferon-α/β receptor; IKK, IκB kinase; IRF, interferon regulatory factor; ISGF3, the IFN-stimulated gene factor 3; IVT, *in vitro* transcribed; JAK1, Janus kinase 1; MDA-5, melanoma differentiation-associated protein 5; OAS, 2'-5'-oligoadenylate synthetase; PKR, dsRNA dependent protein kinase; RIG-I, cytoplasmic retinoic acid-inducible gene I; ssRNA, single-stranded RNA; STAT, Signal transducer activator of transcription; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adaptor inducing IFN-β; TYK2, tyrosine kinase 2

BYPASSING THE INTRACELLULAR INNATE IMMUNE SYSTEM

Owing to the strong immune responses induced by mRNA transfection, the use of IVT mRNA has been mainly limited to therapeutic vaccination approaches. Over the past few years several strategies have been explored to decrease the immune-activating capacity of IVT mRNA in order to promote non-immunogenic applications, such as protein-addition therapies and mRNA-based reprogramming methods. This chapter discusses three possible strategies to evade mRNA-induced immunity: i) optimization of delivery methods to shield the IVT mRNA and control its entry pathway into the cells, ii) modifications on the level of the mRNA template or the IVT mRNA molecule itself or iii) blocking key proteins involved in the intracellular recognition of IVT mRNA and its subsequent signalling cascades.

mRNA delivery methods

Most cell types show only limited cytoplasmic presence of IVT mRNA after spontaneous uptake of the naked transcript[68]. An exception to this are immature dendritic cells, which efficiently take up and accumulate mRNA by macropinocytosis[69]. By contrast, effective delivery of mRNA in other cell types requires alternative delivery methods. In addition to a facilitated uptake, most of these delivery methods have focused on the protection of mRNA against RNase degradation, thus increasing its extra- and intracellular stability. However, also the delivery route (endosomal vs. direct cytoplasmic entry) will determine which PRR the mRNA will encounter on its intracellular journey. Unfortunately, favoring particular delivery routes as a means to protect mRNA against PRR recognition has not been one of the main focus points so far.

Several strategies have been investigated to package the negatively charged mRNA into cationic carriers. These carriers condense the mRNA into positively charged complexes that interact with the negatively charged cell membrane, facilitating mRNA uptake[70, 71]. Both viral and non-viral vectors have been investigated, with a better efficiency for the former but a higher safety and adjustability for the latter. A more detailed overview of the advantages and limitations of both vector strategies is provided in **Chapter 6**. Although knowledge of the cellular pathways involved in vector-mediated mRNA transfection expands by the day, their interaction with cellular components and the subsequent effects on cell function have been strongly overlooked so far. Evidence is emerging which indicates that most carriers exhibit an intrinsic immune-stimulating activity, inducing cell signalling cascades even without mRNA complexation[72].

One very clear example is the oldest and most widely used non-viral mRNA-carrier, protamine. Although this naturally occurring protein is demonstrated to protect the mRNA from degradation, mRNA:protamine complexes were shown to strongly induce innate immune response[73]. Scheel *et al*. indicate that protamine condensed mRNA stimulates the immune system through a MyD88-dependent pathway, suggesting that TLR7 and TLR8 are probably the receptors involved[74]. This immune activating capacity of protamine can be exploited for vaccination strategies, but seemed to inhibit the primary goal of mRNA delivery, i.e. expression of the encoded protein[73]. Other well-investigated mRNA carriers are cationic lipids and polymers. Spontaneous electrostatic interactions condense the mRNA into lipo- or polyplexes respectively[71]. Rejman *et al*. demonstrated that both lipid-based carriers, such as DOTAP/DOPE, and polymers, exemplified by poly-ethylene-imine (PEI) are capable of transfecting mRNA into cells with a higher and longer lasting protein expression found for liposomes than for polymers (the reason being currently unknown)[75]. As for the protamine-RNA complexes, also DOTAP and PEI RNA formulations were shown to be detected by TLR-7 and TLR-8[26, 30]. Furthermore, *Lonez et al.* conclude that multiple cationic lipids, such as stearylamine-liposomes and Lipofectamine, in itself activate intracellular immune pathways, independent of mRNA complexation, resulting in the induction of several pro-apoptotic and pro-inflammatory signalling molecules[76]. The activated immune profile will additionally depend on the particle size, as it has been shown that the immune system distinguishes nanometric and micrometric structures in order to adapt the response to viral or bacterial/fungal organisms[77]. Taken together, these examples show that when mRNA is formulated in particulate delivery systems, the immune-stimulatory effects of the resulting complex will be dictated by both the mRNA molecule, as well as by the nature of the carrier used.

Apart from packaging IVT mRNA into nanoparticles, enhanced uptake has also been achieved by physically disturbing the cell membrane. Methods like micro-injection, electroporation, sonoporation or photoporation shuttle the mRNA directly in the cytosol and thus avoid detection by endosomal RNA receptors[78]. Whether or not mRNA delivery by one of these approaches is a suitable strategy to circumvent endosomal sensing of IVT mRNA remains to be elucidated. Nevertheless, studies in the field of cancer immunotherapy have demonstrated that neither sono- nor electroporation results in strong activation of immune cells. In fact, both techniques require additional stimulation with adjuvants to induce therapeutically beneficial immune responses[79, 80]. This might indicate that cytosolic PRRs are less immunogenic than endosomal TLRs. If so, cytosolic delivery might be the preferred route of administration for non-immunotherapy-related applications.

Modifying the mRNA

In the past few years, considerable efforts have been undertaken to increase the stability of the mRNA transcript by applying modifications to the plasmid template or to the mRNA molecule itself. As these modifications have been extensively reviewed elsewhere, we will only list these modifications that reduce IVT mRNA immunogenicity[5, 11].

First of all, Koski *et al.*, provided evidence that enzymatic 3'-polyadenylation with a minimum of 150 adenosines lowers the immune stimulatory activity of synthetic mRNA[81]. Therefore, apart from increased stability, elongation of the poly(A) tail seems to be a good strategy to temper the immunogenic profile of IVT mRNA. To provide mRNA with a fixed poly(A) tail length, the adenosine residues are mostly encoded in the DNA template, as posttranscriptional polyadenylation yields mixtures of mRNAs with different poly(A) tail lengths.

A second strategy makes use of the observation that uncapped IVT mRNA bears a triphosphate group at the 5' end, which can be detected by the cytosolic RNA sensors RIG-I and PKR[37, 82]. Therefore, shielding the 5'ppp with a synthetic cap analogue can evade immune activation. This can be achieved by addition of an anti-reverse cap analog (ARCA) during the *in vitro* transcription reaction or by means of posttranscriptional capping using recombinant capping enzymes. However, even with these methods it is impossible to accomplish a capping efficiency of 100%[83]. To further reduce the immunogenicity of the remaining uncapped mRNA, a phosphatase treatment can remove any resting triphosphates at the 5' end of the mRNA transcript[84]. Besides capping, also 2'-O-methylation at the penultimate nucleotide of the 5' end has been shown to prevent RIG-I binding and activation[84, 85].

De-immunization of the mRNA construct can be further achieved by the incorporation of naturally occurring modified nucleosides. Kariko and colleagues demonstrated that activation of TLR3, TLR7 and TLR8 can be reduced or completely eliminated with RNA containing 5-methylcytidine (m5C), N6-methyladenosine (m6A), 5-methyluridine (m5U), pseudouridine (ΨU) or 2-thioruridine (s2U)[86]. Two of the modified nucleosides, S2U and ΨU, seem to reduce detection by RIG-I and PKR as well[33, 87]. Additionally, in 2008, ΨU was shown to increase mRNA translation capacity, by improving the overall stability of mRNA and avoiding PKR recognition[88, 89]. In the same vein, *Kormann et al.* indicated that replacement of 25% uridine and cytidine with s2U and m5C substantially reduced binding to PRRs and decreased innate immune activation, leading to an increased protein expression *in vitro* and *in vivo*[90]. A wide variety of these modifications is evaluated in **Chapter 4**, in their ability to provide immunosilent mRNA and increase the mRNA translation potential.

Finally, purification of the IVT mRNA can further mitigate the immune stimulatory properties as was demonstrated by Kariko *et al*. by the removal of dsRNA contaminants through high-performance liquid chromatography purification [91].

Interfering with the signalling downstream pathways

Although a wide range of mRNA delivery techniques and modification strategies have been available for a while, activation of the innate immune cascades still remains a primary concern for mRNA transfections in non-immunogenic applications. In particular, repeated transfections seem to be problematic, as have been demonstrated when using mRNA for cellular reprogramming. The mRNA-triggered immune response seems to hypersensitize transfected cells, as well as neighboring cells to subsequent mRNA exposure, causing cell damage and eventually cell death[48, 49]. In spite of this problem, repeated transfections are often required due to the transient nature of mRNA expression. The strong and detrimental immune responses against foreign mRNA originally serves to detect and prevent the spreading of RNA viruses in such a way that if needed, host cells are sacrificed to prevent further infection. RNA viruses, however, have developed a remarkable diversity of countermeasures to evade immune detection and down-regulate induced responses. Mimicking this viral immune-evasion could therefore be an interesting strategy to bypass the mRNA-triggered immune responses and increase the transfection efficiency of non-viral mRNA based gene delivery systems.

It is known that viruses inhibit innate immunity by avoiding or inhibiting specific immune-related proteins. Genetic analyses have revealed antagonistic activities against virtually all elements of the immune pathways. In this chapter, we aim to address some of these potential target points in order to bypass mRNA triggered innate immunity. Given the redundancy in possible interfering molecules, other examples than the ones listed here might also form a possible evading strategy. Since the innate immune response to mRNA is bimodal, evasion of the response can be divided in two aspects as well: prevention of the initial type I IFNs production (**Figure 2**) and inhibition of the auto- and paracrine effects of type I IFNs (**Figure 3**).

Prevention of type I IFN production

The most obvious approach to escape the negative effects of IFN induction is to intervene in their production. This can be achieved by (i) avoiding mRNA detection and/or (ii) intervene in the mRNA-induced signal transmission. A straightforward strategy to prevent mRNA-mediated IFN production would be to avoid detection in the first place. PRR mediated

recognition of mRNA can be inhibited using small molecules, such as bafilomycin A1 and chloroquine, which can simply be added to the cell culture medium. Bafilomycin A1 acts as an endosomal TLR inhibitor by selectively blocking the vacuolar H^+-ATPase. As a result, bafilomycin increases the acidic endosomal pH, which is thought to be essential for the activation of TLR mediated signal transduction[92-94]. As for bafilomycin A1, the inhibitory activity of chloroquine has been generally ascribed to the inhibition of endosomal acidification. However, Kuznik *et al.* recently demonstrated the effect of chloroquine on the endosomal pH to be negligible at concentrations required for TLR suppression. Instead, they proposed a direct interaction of chloroquine with nucleic acids, which causes a conformational change and makes the nucleic acid ligand unavailable for TLR recognition[94].

A second strategy to restrict IFN production is to intervene negatively in the mRNA-induced signal transmission. Over the past years, it has become evident that the activation of innate immune signalling involves ubiquitination of several immune-related proteins. A case in point is the ubiquitin-dependent activation of the RIG-I receptor required for the recruitment of MAVS and the subsequent signalling molecules. Ubiquitination also activates TRAF3 and TRAF6, which in turn activate, respectively, TBK1/IKKε and the IKK complex for subsequent phosphorylation of transcription factors. In addition, the IκB inhibitory protein depends on ubiquitination for its degradation and hence release of NF-κB. Considered together, administration of deubiquitinating enzymes could negatively regulate innate immunity[95]. Examples in this regard, include the deubiquitinating enzyme A (DUBA) and A20, which inhibit IRF3 and NF-κB activation respectively by deubiquitinating TRAF3 and TRAF6.

Alternatively, mRNA-induced signal transmission can be diminished by interfering with the PRR-adaptor molecule interaction. Pepinh-TRIF and Pepinh-MYD are two peptide inhibitors which contain specific domains of the adaptor molecules TRIF and MyD88. Administration of Pepinh-TRIF and Pepinh-MYD therefore competitive reduces interaction between these adaptor molecules and their respective TLRs[96, 97]. Another technique to interrupt mRNA-induced signalling is through the administration of kinase inhibitors. Since the IKK complex and the IKK-related kinases, TBK1 and IKKε are responsible for the activation of NF-κB and IRF3/7 respectively, related inhibitors can minimize the ensuing IFN and cytokine production. BX795, a potent inhibitor of TBK1 and IKKε, has been shown to suppress the phosphorylation of IRF3, and thus activation of the IFN production[98]. In this regard, Awe *et al*. recently compared BX795 with an inhibitor of the IKK complex, BAY11, in their ability to increase mRNA-mediated protein expression by suppressing the innate immune response[99].

Besides inhibiting the adaptor molecules and the kinases evolved in innate immune signalling, the transcription factors itself can also be targeted. A variety of small molecule NF-κB antagonists are available, repressing cytokine and IFN expression. An example of this is dexamethasone, which is often used as a positive control for NF-κB inhibition[100]. Dexamethasone has been shown to counteract NF-κB activity in many cell types through upregulation of its cytoplasmic inhibitor IκB, thereby reducing the amount of NF-κB translocating to the nucleus[101-103]. Recently, Bhattacharryya *et al.* indicated this inhibition to be dependent on the type of TLR activated and the specific adaptor protein involved[104]. Another small molecule, phenylmethimazole (also known as C10) has been reported to block transcriptional activity of IRF3. Courreges *et al*. describe the molecular basis for this inhibition, which seems to be a prevention of dsRNA-induced IRF3 translocation and homodimerization[105]. The observation that C10 blocks IRF3 transactivation is consistent with prior studies which demonstrate C10-mediated inhibition of the TLR3-regulated IRF3/IFN-β/STAT signal pathway[106, 107]. In the same way, establishment of a cellular anti-RNA state can be prevented through inhibition of IRF7. By impairing the phosphorylation and nuclear translocation of IRF7, the ORF45 protein of Kaposi's sarcoma-associated herpesvirus blocks activation of type I IFN induction. Mechanistically, ORF45 acts as a decoy substrate for TBK1/IKKε and thus competitively inhibits IRF7 phosphorylation[108, 109].

Inhibition of the IFN-induced effects

A second approach to quelling interferon-mediated immune activation is to inhibit the effects induced by IFN production. Again several options can be explored to accomplish this inhibition. The first is to block IFN transduction by inhibiting engagement with its receptor. IFN binding proteins or neutralizing antibodies compete with the cellular IFN receptor by capturing the secreted IFNs. As a consequence, they avert not only the autocrine IFN amplification loop, but also the induction of IFN-triggered signalling in neighbouring cells. The only IFN binding protein whose use has been extensively published is the Vaccinia Virus (VV) encoded B18R protein. B18R is a decoy receptor, specific for type I IFNs of various species that has been shown to increase cell viability during mRNA-based reprogramming protocols[55, 110].

A second strategy to prevent IFN-induced effects is to inhibit IFN induced signal transduction. Proteins that interfere with the JAK/STAT signalling pathway will inhibit production of IFN effectors, such as PKR and OAS, but they will also suppress the upregulation of PRRs and transcription factors, thus reducing the second-wave IFN production. A commonly used JAK inhibitor is the small molecule ruxolitinib[111]. Ruxolitinib potently inhibits the phosphorylation of JAK1 and can therefore interrupt IFN-JAK-STAT

signalling in mRNA-stimulated cells. In 2011, ruxolitinib was approved by the U.S. Food and Drug Administration for the treatment of myeolofibrosis, which underscores its potential use for clinical applications[112, 113].

A third strategy to inhibit IFN response is to counter the action of the IFN-induced effectors. Accordingly, Gupta *et al*. recently discovered a specific, potent inhibitor of the human RNase L. Curcumin, a naturally occurring antioxidant, was shown to non-competitively inhibit RNase L, presumably by inducing a switch in the conformation of the enzyme, leading to a complete loss of its activity[114]. Likewise, PKR function can also be interrupted. For instance, Carroll *et al*. demonstrated inhibition of eIF2α activation by the Vaccinia virus protein, K3L. They revealed that K3L shows structural similarities to the eIF2α molecule and competes with eIF2α for its phosphorylation by PKR, thereby preventing inhibition of the protein synthesis[115, 116]. Another potent PKR inhibitor, which has been widely used for signalling analysis, is 2-aminopurine (2-AP). Attachment of 2-AP to the ATP-binding site of PKR prevents autophosphorylation of the protein kinase, thus inhibiting subsequent phosphorylation of eIF2α[117, 118]. More recently, Jammi *et al.* identified an even stronger PKR inhibitor, known as C16. As for 2-aminopurine, this small molecule inhibits RNA-induced PKR autophosphorylation and rescues the PKR induced translation blockade[119, 120]. Besides a direct inhibition of the IFN-induced effectors, some compounds prevent effector activation by sequestering dsRNA, as is described by Xiang *et al.* for the VV E3L protein. Since both PKR and 2-5A synthase require activation by dsRNA, sequestration of dsRNA by the E3L protein will hamper induction of both effectors [115]. Similarly, a cell-permeable peptide (PRI) containing a motif of the dsRNA binding domain of PKR has been reported to prevent PKR activation by sequestering of dsRNA molecules[121].

As is clear from the above, the intracellular immune responses are generated in cascades. Hence, proteins interfering at one level of a cascade will also influence distant signalling, leading to an even stronger immune-inhibition. In addition, one protein may inhibit different components of the immune signalling cascades. As such, *Xiang et al*. have demonstrated that besides dsRNA sequestration and direct inhibition of PKR, the E3L protein also prevents activation of IRF3, thereby not only blocking the second, but also the first wave of IFN production[115]. In the same way, 2-aminopurine was shown to impair nuclear translocation of phosphorylated IRF3, in addition to its inhibitory effect on PKR[118]. Another molecule which has recently been shown to target more than one element of the intracellular pathways is the anticancer drug, sunitinib. Although commonly known as an inhibitor of the vascular endothelial growth factor receptor (VEGF-R) and the platelet-derived growth factor receptor (PDGF-R), Jha *et al*. reported *in vivo* inhibition of both PKR and RNase L by sunitinib, due to a kinase homology between both effectors[122, 123].

As discussed above, activation of PRRs can also result in the production of pro-inflammatory cytokines. These cytokines amplify the innate immune response to mRNA recognition and some of them negatively influence cell viability as they induce apoptosis. Cytokine-mediated signal transduction can be blocked in the same way as for IFN inhibition, i.e. restriction of cytokine production, prevention of receptor binding and inhibition of the cytokine-induced signalling pathways. To illustrate, production of IL-1 and IL-18 can be prevented by inhibition of their proteolytic maturation[124]. Since both cytokines require caspase-1 to activate their premature form, inhibitors of caspase-1 such as the VV B13R protein and the small molecule VX-765 prevent synthesis and secretion of both cytokines (**Figure 2**)[125]. As for IFNs, several viruses also secrete proteins that serve as decoy receptors to sequester extracellular cytokines and impede interaction with cellular cytokine receptors[126]. Furthermore, most cytokines are induced by activation of the NF-κB pathway. Therefore, the aforementioned NF-κB inhibitors will also decrease cytokine production.

It should be noted that instead of using classic small molecule inhibitors, every aspect of the IFN defense could also be targeted for inhibition by means of small interfering RNAs (siRNAs)[86, 127, 128] or short hairpin RNAs (shRNAs)[86, 129, 130]. siRNA and shRNA are short artificial dsRNA molecules used to silence gene expression via RNA interference by homology to the targeted gene. Although silencing by siRNA and shRNA has been initially considered sequence specific, Kariko *et al.* recently demonstrated suppression of nontargeted mRNA expression as well. In this paper evidence is provided that both siRNA and shRNA induce type I IFN signalling through TLR3 and activate sequence-independent inhibition of gene expression[131]. Therefore, their use to enhance non-viral mRNA transfection seems contradictory, as they trigger innate immunity per se. In this respect, the use of microRNAs (miRNAs) could be considered as well. Since miRNAs have a natural role in regulating inflammatory responses, the chances at immune activation might be lower. In fact, Drews *et al.* observed absolutely no induction of a significant immune response when transfecting mouse fibroblasts with a mix of pluripotency-promoting miRNAs[48]. Nonetheless, a better understanding of the precise regulatory roles of miRNAs in innate immune signalling is needed in order to unravel their potential in manipulating the intracellular pathways.

Another more general strategy to neutralize the innate immune responses is the use of monoclonal antibodies targeting either signalling molecules or their receptors. Owing to the inefficient transport of monoclonal antibodies across cellular membranes, this method will mainly target extracellular elements of the innate immune system, such as the IFN receptor expressed on the surface of the cell or the circulating type I IFNs themselves. Inhibition of intracellular components of the immune pathways might be possible by integrating the antibody to the delivery vehicle.

Finally, we wish to stress that the list of potential immune-inhibitors is tremendously increasing and it is beyond the scope of this review to sum up all commercially available or virus-related inhibitors. Therefore, we have attempt to exemplify every possible strategy with at least one inhibitory molecule.

CURRENT STATE OF NON-IMMUNOTHERAPY RELATED MRNA APPLICATIONS

So far, vaccination and cancer immunotherapy are the only fields in which mRNA-based therapeutics are reaching clinical trials. Although mRNA has garnered broad interest for its utility in other medical indications, clinical translation has been hampered by its immunogenicity, limited stability and transient nature. The finding that the immune-stimulatory activity of RNA could be tempered by incorporation of modified nucleosides was crucial to extend the applicability of mRNA into other areas than immunotherapy[86].

Table 1 | Examples of in vivo mRNA-based protein-replacement studies.

Refs	Application	mRNA	Frequency	Innate immune evasion		
				Delivery method	Modification	Signalling inhibitors
134	Reporter assay Hypoxic stress	Luc Hsp70	Single	Lipid-based	Non-modified mRNA	None
135	Melanoma	BAX	5 daily injection	Lipid-based	Non-modified mRNA	None
90	Congenital lung disease	SPB	Twice weekly aerosol	Aerosolisation of naked mRNA	m5C and s2U	None
136	Anaemia	EPO	Once weekly injection	Lipid/ polymer-based (TransIT®)	ΨU	None
137	Asthma	FOXP3	Single and repeated spraying (5 times)	Intratracheal high-pressure spraying of naked mRNA	m5C and s2U	None
138	Myocardial infarction	VEGFA	Single injection	Lipid-based (RNAiMAX®)	m5C and ΨU	B18R
139	Olfactory nerve disfunctions	BDNF	Once daily	Polymer-based	m5C, 2sU and ΨU	None

Currently, the potential of IVT mRNA is being explored for a variety of applications, ranging from inherited or acquired metabolic disorders to regenerative medicine. The first

study in which IVT mRNA is used for the replacement of a deficient protein *in vivo*, was published in 1992. In this work, Jirikowski and colleagues demonstrated that direct injection of vasopressin-encoding mRNA in the hypothalamus of vasopressin-deficient rats led to the production of significant plasma levels of vasopressin and temporary reversed their diabetes insipidus[132]. For about a decade, this remained the only mRNA-based paper demonstrating the feasibility of using IVT mRNA to express therapeutic proteins *in vivo*. Advances in the optimization of IVT mRNA and the many conveniences coupled to its use, reinstituted mRNA as a possible method for protein replacement therapies. Ever since, studies have attempted *in vivo* mRNA administration targeting a variety of tissues (some examples are summarized in **Table 1**). A more recent overview of all mRNA-based protein-replacement studies can be found in the recent review of Zhong *et al*.[133]

Strikingly, although most studies prove their awareness of the immune-stimulatory activity of IVT mRNA by using modified mRNA, only Zangi *et al*. make use of an additional immune-inhibiting compound, B18R, however without stressing the function of this molecule[138]. Whether or not supplementation with immune-inhibiting molecules could enhance the level and duration of mRNA expression and thereby advance protein-replacement therapies, warrants further investigation and will be evaluated in **Chapter 5**. Furthermore, it is important to note that not all the aforementioned molecules can evidently be used in an *in vivo* setting. Obviously, prior to clinical application, the toxicological profile of the selected therapeutic components should be determined diligently. As most signalling pathways are critical elements of cell physiology, supplementation with immune decreasing molecules should be further advanced with due caution.

Apart from protein-replacement applications, IVT mRNA has also been extensively used in the field of regenerative medicine for the reprogramming of cell fates. In 2007, Yamanaka and colleagues discovered that the expression of only 4 transcription factors could reverse the fate of human fibroblasts towards pluripotency[140]. From then on, researchers tried optimizing the transfection protocol in order to render a safe and stable generation of induced pluripotent stemcells (iPSCs). Yakubov *et al*. were the first to propose a mRNA-based approach as a solution to minimize genome integration as well as to increase reprogramming efficiency. In 2010, they demonstrated that lipid-based mRNA encoding four reprogramming factors could be used to induce expression of pluripotency markers in human fibroblasts[141]. Unfortunately, this study was limited by the absence of pluripotency verification tests, leading to the question whether these iPSCs were able to functionally differentiate into each of the three germ layers. In the same year, Warren *et al*. described mRNA-based reprogramming methodology that rendered iPSCs that met all the molecular and functional pluripotency requirements. In order to enhance the sustainability of the mRNA-mediated

protein expression, the authors searched for approaches to reduce the immunogenic profile of IVT mRNA. To this end, modified IVT mRNA was used, which contained ΨU and m5C and was subjected to a phosphatase treatment. In addition, the cell culture medium was supplemented with the soluble IFN inhibitor B18R to further mitigate innate immune responses[55]. This was in line with a previous protocol published by Angel *et al.*, which demonstrated that a combined knockdown of immune-related proteins with a siRNA cocktail rescues human fibroblasts from the innate immune response triggered by frequent non-modified mRNA transfection, and enables sustained, high-level expression of the encoded proteins. They also suggest that the use of small-molecule immunosuppressants either alone or in combination with siRNA might be a suitable strategy to increase the frequency of mRNA transfections, without compromising cell viability[49]. Since the onset of this initial approach, numerous refinements have been published, each claiming to reach a higher reprogramming efficiency (summarized in **Table 2**). Despite these achievements, cellular reprogramming still faces a lot of technical challenges and requires intensive optimization to become routinely applicable. Recently, Drews *et al*. attributed the lack of reproducibility to severe toxicity and cell death, still caused by activation of the innate immune response even by modified mRNA. In their assays supplementation with a variety of immunosuppressing compounds, including B18R, Pepinh-TRIF and Pepinh-MYD, did not down-regulate the immune response-related genes[48]. Similarly, Awe *et al*. reported that the reprogramming methodology of Warren and colleagues did not completely reduce the mRNA-induced innate immune responses in their experiments. They noticed a significant degradation of their OCT4 encoding mRNA, which could not be prevented by B18R supplementation. Nevertheless, they suggest a different kind of small molecule-based inhibition of the innate immune response, namely the administration of BAY11. Being an inhibitor of the IKK complex, BAY11 diminishes the negative IFN-induced responses, such as decay of the encoding mRNA, thereby stabilizing mRNA expression[99].

Of note, for reprogramming strategies is not desirable to completely block the innate immune system, as a recent study of Lee *et al.* demonstrated a positive effect of TLR3 stimulation on the reprogramming efficiency. The authors discovered a striking difference in the gene expression profiles induced by viral delivery of reprogramming factors compared to other reprogramming methods, suggesting that viral vectors actively contribute to the reprogramming process. Functional studies indicated that the TLR3 pathway is required for efficient induction of pluripotency genes. Stimulation of TLR3 seems to affect the expression and/or distribution of epigenetic modifiers promoting an open chromatin configuration and thus nuclear reprogramming. Although these findings recommend stimulation of the innate immune system for efficient mRNA-based iPSC generation, the authors also note that the level of TLR3 should be balanced, as further stimulation can cause cell death[142].

Table 2 | Examples of mRNA-based reprogramming studies.

Refs	Frequency	mRNA	Innate immune evasion		
			Delivery method	Modification	Signalling inhibitors
[141]	5 daily transfections	Oct4, Lin28, Sox2 and Nanog	Lipid-based (Lipofectamine)	Non-modified mRNA	None
[49]	3 daily transfections	Oct4, Sox2, Klf4 and Utf1	Lipid-based (RNAiMAX)	Non-modified mRNA	SiRNA against IFNB1, Eif2ak2 (PKR) and STAT2
[55]	17 daily transfections	Oct4, Sox2, Klf4, cMyc and Lin28	Lipid-based (RNAiMAX)	m5C and ΨU	B18R
[143]	Single transfection	Oct4, Sox2, Klf4, cMyc, LT	Electroporation	Non-modified mRNA	None
[144]	3 consecutive transfections	Oct4, Lin28Sox2 and Nanog	Lipid-based (RNAiMAX)	Non-modified mRNA	None
[48]	Single transfection	OCT4, Sox2, Klf4 and cMyc	Lipid-based (RNAiMAX)	Non-modified and m5C and ΨU modified mRNA	B18R Chloroquine TSA Pepinh-TRIF Pepinh-MYD
[145]	9 daily transfections	M_3O, Sox2, Klf4, cMyc, Lin28 and Nanog	Lipid-based (RNAiMAX)	m5C and ΨU	B18R
[142]	Max. 17 daily transfections	Oct4, Sox2, Klf4 and cMyc	Non-specified	Modified mRNA (non-specified)	B18R shRNA against TLR3, TRIF and MyD88
[99]	5 daily transfections	Klf4, cMyc, Oct4, Sox2 and Lin28	Lipid-based (RNAiMAX)	m5C and ΨU	B18R, BX795 BAY11
[110]	14-16 daily transfections	Oct4, Sox2, Klf4, cMyc, Lin28 and NDG	Lipid-based (RNAiMAX or Stemfect RNA)	m5C and ΨU	B18R
[146]	Single transfection	1 single VEE RNA replicon,encoding Oct4, Sox2,Klf4, cMyc or GLIS1	Lipid-based (Lipofectamine)	Non-modified mRNA	B18R
[147]	5 daily transfections	Oct4, Sox2, Klf4 and cMyc	Lipid/polymer-based (TransIT®)	Mouse specific synthesized mRNA	None

Considering all of these data, it is clear that the innate immune response represents a big hurdle for advancing non-immunotherapy applications. Especially when multiple mRNA transfections are required, unmodified IVT mRNA induces severe cytotoxicity, making repeated transfections over time almost impossible. The studies presented thus far provide the basis for further investigations into other immunosuppressing strategies. Use of other chemical compounds, as suggested above, either alone or in combination, may allow frequent mRNA transfections and robust expression of the encoded protein.

CONCLUDING REMARKS

IVT mRNA transfection is a versatile and promising tool for the delivery of genetic information. Unprecedented advances in controlling the stability of IVT mRNA have reestablished mRNA interest for a wide range of potential applications. However, the fact that IVT mRNA, despite its strong resemblance to naturally occurring mRNA, can be recognized by the innate immune system, presumably plays an important role in its applicability. For vaccination approaches, the inflammatory cytokine production resulting from mRNA-induced immune-stimulation might add to the effectiveness of the evoked immune response. For non-immunotherapy approaches, however, the story becomes quite different. In this chapter, we discussed a number of important considerations that should be taken into account when using IVT mRNA for non-immunogenic applications, such as protein-replacement therapy or cellular reprogramming.

Firstly, whether or not the induced innate immune response will affect the therapeutic outcome of the mRNA delivery will likely depend on the required mRNA application frequency, which in its turn is determined by the intended application. So far, mRNA-based reprogramming protocols require about 12 daily transfections, whereas transfection frequencies for long-term treatment of congenital diseases still remain to be elucidated.

Secondly, as soon as mRNA is delivered using a chemical or physical delivery method, the vehicle or technique will also play an undeniable role in the induction of innate immune responses. Besides influencing the mRNA uptake mechanism and as such favoring or avoiding contact with specific mRNA sensors, increasing evidence indicates that most RNA-carriers possess an intrinsic immune-stimulating activity, inducing cell signalling cascades independent of mRNA complexation.

Thirdly, over the years, considerable efforts have been made to unriddle mRNA recognition pathways and limit the immune-stimulatory activity of IVT mRNA. Besides the well-known modifications that can be made to the mRNA molecule itself, a number of potential immune-inhibitors have been identified and are currently under investigation. This

chapter has focused on the different players involved in innate immunity signalling, all of which are potential targets to shut down to enhance the level and duration of mRNA expression. In this regard, it is worth mentioning a couple of side notes. For one thing, the inhibition of only one key molecule of a signalling pathway might be nullified as its function can be superseded by a connected pathway. Therefore, simultaneous inhibition on different levels of the mRNA recognition should be considered, as exemplified by several RNA based viruses. In addition, evidence is emerging that the innate immune response might not be all bad for mRNA-based reprogramming purposes. Such observations prompt further investigation and will likely require fine-tuning the balance between immune suppression and immune stimulation. Furthermore, clearly not all combinations of immune-inhibitory strategies that are feasible in an *in vitro* cell culture setting (*e.g.,* reprogramming of isolated stem cells) can be translated into the *in vivo* situation (*e.g., in situ* protein replacement). As most signalling pathways are involved in many other regulatory aspects of cells as well, care should be taken when one interferes with these critical elements, in order to avoid side effects. What is more, *in vivo* application of inhibitory molecules faces the same challenge as mRNA therapy per se: targeted delivery. So far, research on how to efficiently deliver molecules to the target cell type and avoid systemic exposure is still pending.

Moreover, it is highly likely that not all elements in the mRNA recognition pathways have been identified thus far. Since research into the cell type dependent reaction to intruding mRNA molecules is still in its infancy, there is bound to be limited insights available. Finally, we wish to stress that although these innate immune responses might appear to limit the use of mRNA for non-immunotherapy applications, as evidenced by the fact that clinical IVT mRNA-therapy is still very much in its infancy, these responses do not solely occur in response to mRNA. Long before the discussion of mRNA-induced immune triggering arose, we knew about TLR9 ligation of CpG-rich pDNA, also resulting in the secretion of type I IFN and IL-12[148-150].

Although the use of mRNA has been extensively investigated over the past few years, non-immunotherapy-related *in vivo* applications are merely at the beginning of development. In this regard the use of small molecule immune-inhibitors might bring non-immunogenic mRNA strategies to a higher level (as examined in **Chapter 5**). For protein replacement therapies in specific, substantial improvements will be required in the delivery of mRNA to efficiently target the desired cell type and ensure a duration of protein production that benefits patient compliance. Even though we still have a long way to go before mRNA can be used as an off-the-shelf drug further insight into the major hurdles compromising mRNA-based protein expression, as presented in this chapter, might provide new inspiration for the therapeutic development of mRNA.

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REFERENCES

1. Wolff, J.A. et al. Direct gene transfer into mouse muscle in vivo. *Science* **247**, 1465-8 (1990).
2. Leonhardt, C. et al. Single-cell mRNA transfection studies: delivery, kinetics and statistics by numbers. *Nanomedicine* **10**, 679-88 (2014).
3. Pascolo, S. Vaccination with messenger RNA (mRNA). *Handb Exp Pharmacol*, 221-35 (2008).
4. Tavernier, G. et al. mRNA as gene therapeutic: how to control protein expression. *Journal of controlled release : official journal of the Controlled Release Society* **150**, 238-247 (2011).
5. Sahin, U., Kariko, K. & Tureci, O. mRNA-based therapeutics--developing a new class of drugs. *Nat Rev Drug Discov* **13**, 759-80 (2014).
6. Conry, R.M. et al. Characterization of a messenger RNA polynucleotide vaccine vector. *Cancer Res* **55**, 1397-400 (1995).
7. Martinon, F. et al. Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. *Eur J Immunol* **23**, 1719-22 (1993).
8. Tang, D.C., DeVit, M. & Johnston, S.A. Genetic immunization is a simple method for eliciting an immune response. *Nature* **356**, 152-4 (1992).
9. Hoerr, I., Obst, R., Rammensee, H.G. & Jung, G. In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. *Eur J Immunol* **30**, 1-7 (2000).
10. Kallen, K.J. & Thess, A. A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs. *Ther Adv Vaccines* **2**, 10-31 (2014).
11. Van Lint, S. et al. The ReNAissanCe of mRNA-based cancer therapy. *Expert Rev Vaccines*, 1-17 (2014).
12. Pollard, C., De Koker, S., Saelens, X., Vanham, G. & Grooten, J. Challenges and advances towards the rational design of mRNA vaccines. *Trends Mol Med* **19**, 705-13 (2013).
13. Kreiter, S., Diken, M., Selmi, A., Tureci, O. & Sahin, U. Tumor vaccination using messenger RNA: prospects of a future therapy. *Curr Opin Immunol* **23**, 399-406 (2011).
14. Pascolo, S. The messenger's great message for vaccination. *Expert Rev Vaccines* **14**, 153-6 (2015).
15. Krieg, P.A. & Melton, D.A. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res* **12**, 7057-70 (1984).
16. Li, Y. & Kiledjian, M. Regulation of mRNA decapping. *Wiley Interdiscip Rev RNA* **1**, 253-65 (2010).
17. Coller, J. & Parker, R. Eukaryotic mRNA decapping. *Annu Rev Biochem* **73**, 861-90 (2004).
18. Martin, S.A., Paoletti, E. & Moss, B. Purification of mRNA guanylyltransferase and mRNA (guanine-7-) methyltransferase from vaccinia virions. *J Biol Chem* **250**, 9322-9 (1975).
19. Wells, S.E., Hillner, P.E., Vale, R.D. & Sachs, A.B. Circularization of mRNA by eukaryotic translation initiation factors. *Mol Cell* **2**, 135-40 (1998).
20. Gallie, D.R. A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene* **216**, 1-11 (1998).

21. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783-801 (2006).
22. Kawai, T. & Akira, S. Innate immune recognition of viral infection. *Nat Immunol* **7**, 131-7 (2006).
23. Akira, S., Takeda, K. & Kaisho, T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* **2**, 675-80 (2001).
24. Kumar, H., Kawai, T. & Akira, S. Toll-like receptors and innate immunity. *Biochemical and biophysical research communications* **388**, 621-625 (2009).
25. Yamamoto, A., Kormann, M., Rosenecker, J. & Rudolph, C. Current prospects for mRNA gene delivery. *Eur J Pharm Biopharm* **71**, 484-9 (2009).
26. Heil, F. et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303**, 1526-9 (2004).
27. Diebold, S.S. et al. Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. *Eur J Immunol* **36**, 3256-67 (2006).
28. Alexopoulou, L., Holt, A.C., Medzhitov, R. & Flavell, R.A. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732-8 (2001).
29. Kariko, K., Ni, H., Capodici, J., Lamphier, M. & Weissman, D. mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem* **279**, 12542-50 (2004).
30. Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. & Reis e Sousa, C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**, 1529-31 (2004).
31. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nat Rev Immunol* **4**, 499-511 (2004).
32. Yoneyama, M. & Fujita, T. Recognition of viral nucleic acids in innate immunity. *Reviews in medical virology* **20**, 4-22 (2010).
33. Hornung, V. et al. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* **314**, 994-7 (2006).
34. Pichlmair, A. et al. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**, 997-1001 (2006).
35. Schlee, M. et al. Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* **31**, 25-34 (2009).
36. Schmidt, A. et al. 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proc Natl Acad Sci U S A* **106**, 12067-72 (2009).
37. Goubau, D. et al. Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5'-diphosphates. *Nature* **514**, 372-5 (2014).
38. Pichlmair, A. et al. Activation of MDA5 requires higher-order RNA structures generated during virus infection. *J Virol* **83**, 10761-9 (2009).
39. Zust, R. et al. Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. *Nat Immunol* **12**, 137-43 (2011).
40. Rothenfusser, S. et al. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* **175**, 5260-8 (2005).

41. Komuro, A. & Horvath, C.M. RNA- and virus-independent inhibition of antiviral signaling by RNA helicase LGP2. *J Virol* **80**, 12332-42 (2006).
42. Satoh, T. et al. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc Natl Acad Sci U S A* **107**, 1512-7 (2010).
43. Liniger, M., Summerfield, A., Zimmer, G., McCullough, K.C. & Ruggli, N. Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling involving LGP2. *J Virol* **86**, 705-17 (2012).
44. Ikeda, F. et al. Involvement of the ubiquitin-like domain of TBK1/IKK-i kinases in regulation of IFN-inducible genes. *EMBO J* **26**, 3451-62 (2007).
45. Israel, A. The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harb Perspect Biol* **2**, a000158 (2010).
46. Sen, G. & Sarkar, S. The interferon-stimulated genes: targets of direct signaling by interferons, double-stranded RNA, and viruses. *Interferon: The 50th Anniversary* (2007).
47. Sadler, A.J. & Williams, B.R. Interferon-inducible antiviral effectors. *Nat Rev Immunol* **8**, 559-68 (2008).
48. Drews, K. et al. The cytotoxic and immunogenic hurdles associated with non-viral mRNA-mediated reprogramming of human fibroblasts. *Biomaterials* **33**, 4059-4068 (2012).
49. Angel, M. & Yanik, M. Innate immune suppression enables frequent transfection with RNA encoding reprogramming proteins. *PloS one* **5** (2010).
50. Andries, O. et al. Innate immune response and programmed cell death following carrier-mediated delivery of unmodified mRNA to respiratory cells. *Journal of controlled release : official journal of the Controlled Release Society* **167**, 157-166 (2013).
51. Kanneganti, T.D. et al. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem* **281**, 36560-8 (2006).
52. Sabbah, A. et al. Activation of innate immune antiviral responses by Nod2. *Nat Immunol* **10**, 1073-80 (2009).
53. Heufler, C. et al. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur J Immunol* **26**, 659-68 (1996).
54. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245-52 (1998).
55. Warren, L. et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell stem cell* **7**, 618-630 (2010).
56. Garcia, M.A., Meurs, E.F. & Esteban, M. The dsRNA protein kinase PKR: virus and cell control. *Biochimie* **89**, 799-811 (2007).
57. Kumar, A., Haque, J., Lacoste, J., Hiscott, J. & Williams, B.R. Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. *Proc Natl Acad Sci U S A* **91**, 6288-92 (1994).
58. Balachandran, S. et al. Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* **13**, 129-41 (2000).

59. Silverman, R.H. Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. *J Virol* **81**, 12720-9 (2007).
60. Borden, E.C. et al. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* **6**, 975-90 (2007).
61. George, C.X., John, L. & Samuel, C.E. An RNA editor, adenosine deaminase acting on double-stranded RNA (ADAR1). *J Interferon Cytokine Res* **34**, 437-46 (2014).
62. Samuel, C.E. ADARs: viruses and innate immunity. *Curr Top Microbiol Immunol* **353**, 163-95 (2012).
63. Hartner, J.C., Walkley, C.R., Lu, J. & Orkin, S.H. ADAR1 is essential for the maintenance of hematopoiesis and suppression of interferon signaling. *Nat Immunol* **10**, 109-15 (2009).
64. Qiu, W. et al. ADAR1 is essential for intestinal homeostasis and stem cell maintenance. *Cell Death Dis* **4**, e599 (2013).
65. Yang, S. et al. Adenosine deaminase acting on RNA 1 limits RIG-I RNA detection and suppresses IFN production responding to viral and endogenous RNAs. *J Immunol* **193**, 3436-45 (2014).
66. Pfaller, C.K., Li, Z., George, C.X. & Samuel, C.E. Protein kinase PKR and RNA adenosine deaminase ADAR1: new roles for old players as modulators of the interferon response. *Curr Opin Immunol* **23**, 573-82 (2011).
67. Pichlmair, A. & Sousa, C.R.E. Innate recognition of viruses. *Immunity* **27**, 370-383 (2007).
68. Lorenz, C. et al. Protein expression from exogenous mRNA: uptake by receptor-mediated endocytosis and trafficking via the lysosomal pathway. *RNA Biol* **8**, 627-36 (2011).
69. Diken, M. et al. Selective uptake of naked vaccine RNA by dendritic cells is driven by macropinocytosis and abrogated upon DC maturation. *Gene Ther* **18**, 702-8 (2011).
70. Pathak, A., Patnaik, S. & Gupta, K. Recent trends in non-viral vector-mediated gene delivery. *Biotechnology journal* **4**, 1559-1572 (2009).
71. Tros de Ilarduya, C., Sun, Y. & Düzgüneş, N. Gene delivery by lipoplexes and polyplexes. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences **40**, 159-170 (2010).
72. Dewitte, H., Verbeke, R., Breckpot, K., De Smedt, S.C. & Lentacker, I. Nanoparticle design to induce tumor immunity and challenge the suppressive tumor microenvironment. *Nano Today* **9**, 743-758 (2014).
73. Fotin-Mleczek, M. et al. Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. *J Immunother* **34**, 1-15 (2011).
74. Scheel, B. et al. Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. *Eur J Immunol* **35**, 1557-66 (2005).
75. Rejman, J., Tavernier, G., Bavarsad, N., Demeester, J. & De Smedt, S. mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers. *Journal of controlled release : official journal of the Controlled Release Society* **147**, 385-391 (2010).
76. Lonez, C., Vandenbranden, M. & Ruysschaert, J.M. Cationic lipids activate intracellular signaling pathways. *Adv Drug Deliv Rev* **64**, 1749-58 (2012).

77. Rettig, L. et al. Particle size and activation threshold: a new dimension of danger signaling. *Blood* **115**, 4533-41 (2010).
78. Wang, W., Li, W., Ma, N. & Steinhoff, G. Non-viral gene delivery methods. *Curr Pharm Biotechnol* **14**, 46-60 (2013).
79. Dewitte, H. et al. The potential of antigen and TriMix sonoporation using mRNA-loaded microbubbles for ultrasound-triggered cancer immunotherapy. *J Control Release* **194**, 28-36 (2014).
80. Bonehill, A. et al. Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. *Mol Ther* **16**, 1170-80 (2008).
81. Koski, G.K. et al. Cutting edge: innate immune system discriminates between RNA containing bacterial versus eukaryotic structural features that prime for high-level IL-12 secretion by dendritic cells. *J Immunol* **172**, 3989-93 (2004).
82. Nallagatla, S.R. et al. 5'-triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science* **318**, 1455-8 (2007).
83. Grudzien-Nogalska, E., Jemielity, J., Kowalska, J., Darzynkiewicz, E. & Rhoads, R.E. Phosphorothioate cap analogs stabilize mRNA and increase translational efficiency in mammalian cells. *RNA* **13**, 1745-55 (2007).
84. Pardi, N., Muramatsu, H., Weissman, D. & Kariko, K. In vitro transcription of long RNA containing modified nucleosides. *Methods Mol Biol* **969**, 29-42 (2013).
85. Wang, Y.L. et al. Structural and functional insights into 5 '-ppp RNA pattern recognition by the innate immune receptor RIG-I. *Nature Structural & Molecular Biology* **17**, 781-U19 (2010).
86. Kariko, K., Buckstein, M., Ni, H. & Weissman, D. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* **23**, 165-75 (2005).
87. Nallagatla, S.R. & Bevilacqua, P.C. Nucleoside modifications modulate activation of the protein kinase PKR in an RNA structure-specific manner. *RNA* **14**, 1201-13 (2008).
88. Kariko, K. et al. Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol Ther* **16**, 1833-40 (2008).
89. Anderson, B.R. et al. Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. *Nucleic Acids Res* **38**, 5884-92 (2010).
90. Kormann, M.S. et al. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat Biotechnol* **29**, 154-7 (2011).
91. Kariko, K., Muramatsu, H., Ludwig, J. & Weissman, D. Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res* **39**, e142 (2011).
92. Hacker, H. et al. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J* **17**, 6230-40 (1998).

93. He, X., Jia, H., Jing, Z. & Liu, D. Recognition of pathogen-associated nucleic acids by endosomal nucleic acid-sensing toll-like receptors. *Acta Biochim Biophys Sin (Shanghai)* **45**, 241-58 (2013).
94. Kuznik, A. et al. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *J Immunol* **186**, 4794-804 (2011).
95. Rajsbaum, R. & Garcia-Sastre, A. Viral evasion mechanisms of early antiviral responses involving regulation of ubiquitin pathways. *Trends Microbiol* **21**, 421-9 (2013).
96. Loiarro, M. et al. Peptide-mediated interference of TIR domain dimerization in MyD88 inhibits interleukin-1-dependent activation of NF-{kappa}B. *J Biol Chem* **280**, 15809-14 (2005).
97. Toshchakov, V.U., Basu, S., Fenton, M.J. & Vogel, S.N. Differential involvement of BB loops of toll-IL-1 resistance (TIR) domain-containing adapter proteins in TLR4- versus TLR2-mediated signal transduction. *J Immunol* **175**, 494-500 (2005).
98. Clark, K., Plater, L., Peggie, M. & Cohen, P. Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. *J Biol Chem* **284**, 14136-46 (2009).
99. Awe, J.P., Crespo, A.V., Li, Y., Kiledjian, M. & Byrne, J.A. BAY11 enhances OCT4 synthetic mRNA expression in adult human skin cells. *Stem Cell Res Ther* **4**, 15 (2013).
100. Rowland, T.L. et al. Differential effect of thalidomide and dexamethasone on the transcription factor NF-kappa B. *Int Immunopharmacol* **1**, 49-61 (2001).
101. Auphan, N., DiDonato, J.A., Rosette, C., Helmberg, A. & Karin, M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* **270**, 286-90 (1995).
102. Scheinman, R.I., Cogswell, P.C., Lofquist, A.K. & Baldwin, A.S., Jr. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* **270**, 283-6 (1995).
103. Chauhan, D. et al. Identification of genes regulated by dexamethasone in multiple myeloma cells using oligonucleotide arrays. *Oncogene* **21**, 1346-58 (2002).
104. Bhattacharyya, S. et al. TAK1 targeting by glucocorticoids determines JNK and IkappaB regulation in Toll-like receptor-stimulated macrophages. *Blood* **115**, 1921-31 (2010).
105. Courreges, M.C., Kantake, N., Goetz, D.J., Schwartz, F.L. & McCall, K.D. Phenylmethimazole blocks dsRNA-induced IRF3 nuclear translocation and homodimerization. *Molecules* **17**, 12365-77 (2012).
106. Harii, N. et al. Thyrocytes express a functional toll-like receptor 3: overexpression can be induced by viral infection and reversed by phenylmethimazole and is associated with Hashimoto's autoimmune thyroiditis. *Mol Endocrinol* **19**, 1231-50 (2005).
107. Schwartz, A.L. et al. Phenylmethimazole decreases Toll-like receptor 3 and noncanonical Wnt5a expression in pancreatic cancer and melanoma together with tumor cell growth and migration. *Clin Cancer Res* **15**, 4114-22 (2009).

108. Zhu, F.X., King, S.M., Smith, E.J., Levy, D.E. & Yuan, Y. A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. *Proc Natl Acad Sci U S A* **99**, 5573-8 (2002).
109. Zhu, F.X., Sathish, N. & Yuan, Y. Antagonism of host antiviral responses by Kaposi's sarcoma-associated herpesvirus tegument protein ORF45. *PLoS One* **5**, e10573 (2010).
110. Mandal, P.K. & Rossi, D.J. Reprogramming human fibroblasts to pluripotency using modified mRNA. *Nat Protoc* **8**, 568-82 (2013).
111. Kambara, H. et al. Negative regulation of the interferon response by an interferon-induced long non-coding RNA. *Nucleic Acids Res* **42**, 10668-80 (2014).
112. Quintas-Cardama, A. et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood* **115**, 3109-17 (2010).
113. Quintas-Cardama, A. & Verstovsek, S. Molecular pathways: Jak/STAT pathway: mutations, inhibitors, and resistance. *Clin Cancer Res* **19**, 1933-40 (2013).
114. Gupta, A. & Rath, P.C. Curcumin, a natural antioxidant, acts as a noncompetitive inhibitor of human RNase L in presence of its cofactor 2-5A in vitro. *Biomed Res Int* **2014**, 817024 (2014).
115. Xiang, Y. et al. Blockade of interferon induction and action by the E3L double-stranded RNA binding proteins of vaccinia virus. *J Virol* **76**, 5251-9 (2002).
116. Carroll, K., Elroy-Stein, O., Moss, B. & Jagus, R. Recombinant vaccinia virus K3L gene product prevents activation of double-stranded RNA-dependent, initiation factor 2 alpha-specific protein kinase. *J Biol Chem* **268**, 12837-42 (1993).
117. Hu, Y. & Conway, T.W. 2-Aminopurine inhibits the double-stranded RNA-dependent protein kinase both in vitro and in vivo. *J Interferon Res* **13**, 323-8 (1993).
118. Sugiyama, T. et al. Mechanism of inhibition of lipopolysaccharide-induced interferon-beta production by 2-aminopurine. *Mol Immunol* **52**, 299-304 (2012).
119. Jammi, N.V., Whitby, L.R. & Beal, P.A. Small molecule inhibitors of the RNA-dependent protein kinase. *Biochem Biophys Res Commun* **308**, 50-7 (2003).
120. Ingrand, S. et al. The oxindole/imidazole derivative C16 reduces in vivo brain PKR activation. *FEBS Lett* **581**, 4473-8 (2007).
121. Nekhai, S., Bottaro, D.P., Woldehawariat, G., Spellerberg, A. & Petryshyn, R. A cell-permeable peptide inhibits activation of PKR and enhances cell proliferation. *Peptides* **21**, 1449-56 (2000).
122. Jha, B.K. et al. Inhibition of RNase L and RNA-dependent protein kinase (PKR) by sunitinib impairs antiviral innate immunity. *J Biol Chem* **286**, 26319-26 (2011).
123. Jha, B.K., Dong, B., Nguyen, C.T., Polyakova, I. & Silverman, R.H. Suppression of antiviral innate immunity by sunitinib enhances oncolytic virotherapy. *Mol Ther* **21**, 1749-57 (2013).
124. Smith, G.L. et al. Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. *J Gen Virol* **94**, 2367-92 (2013).
125. Kettle, S. et al. Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1beta-induced fever. *J Gen Virol* **78 (Pt 3)**, 677-85 (1997).

126. Mantovani, A., Locati, M., Vecchi, A., Sozzani, S. & Allavena, P. Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines. *Trends Immunol* **22**, 328-36 (2001).
127. Myskiw, C., Arsenio, J., van Bruggen, R., Deschambault, Y. & Cao, J. Vaccinia virus E3 suppresses expression of diverse cytokines through inhibition of the PKR, NF-kappaB, and IRF3 pathways. *J Virol* **83**, 6757-68 (2009).
128. Yoneyama, M. et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* **5**, 730-7 (2004).
129. Allen, I.C. et al. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* **30**, 556-65 (2009).
130. Schmolke, M. et al. RIG-I detects mRNA of intracellular Salmonella enterica serovar Typhimurium during bacterial infection. *MBio* **5**, e01006-14 (2014).
131. Kariko, K., Bhuyan, P., Capodici, J. & Weissman, D. Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3. *J Immunol* **172**, 6545-9 (2004).
132. Jirikowski, G.F., Sanna, P.P., Maciejewski-Lenoir, D. & Bloom, F.E. Reversal of diabetes insipidus in Brattleboro rats: intrahypothalamic injection of vasopressin mRNA. *Science* **255**, 996-8 (1992).
133. Zhong, Z.F. et al. mRNA therapeutics deliver a hopeful message. *Nano Today* **23**, 16-39 (2018).
134. Anderson, D.M. et al. Stability of mRNA/cationic lipid lipoplexes in human and rat cerebrospinal fluid: methods and evidence for nonviral mRNA gene delivery to the central nervous system. *Hum Gene Ther* **14**, 191-202 (2003).
135. Okumura, K. et al. Bax mRNA therapy using cationic liposomes for human malignant melanoma. *J Gene Med* **10**, 910-7 (2008).
136. Kariko, K., Muramatsu, H., Keller, J.M. & Weissman, D. Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin. *Mol Ther* **20**, 948-53 (2012).
137. Mays, L.E. et al. Modified Foxp3 mRNA protects against asthma through an IL-10-dependent mechanism. *J Clin Invest* **123**, 1216-28 (2013).
138. Zangi, L. et al. Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat Biotechnol* **31**, 898-907 (2013).
139. Baba, M., Itaka, K., Kondo, K., Yamasoba, T. & Kataoka, K. Treatment of neurological disorders by introducing mRNA in vivo using polyplex nanomicelles. *J Control Release* **201**, 41-8 (2015).
140. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-72 (2007).
141. Yakubov, E., Rechavi, G., Rozenblatt, S. & Givol, D. Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. *Biochem Biophys Res Commun* **394**, 189-93 (2010).
142. Lee, J. et al. Activation of innate immunity is required for efficient nuclear reprogramming. *Cell* **151**, 547-58 (2012).

143. Plews, J.R. et al. Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach. *PLoS One* **5**, e14397 (2010).
144. Tavernier, G. et al. Activation of pluripotency-associated genes in mouse embryonic fibroblasts by non-viral transfection with in vitro-derived mRNAs encoding Oct4, Sox2, Klf4 and cMyc. *Biomaterials* **33**, 412-417 (2012).
145. Warren, L., Ni, Y., Wang, J. & Guo, X. Feeder-free derivation of human induced pluripotent stem cells with messenger RNA. *Scientific reports* **2**, 657 (2012).
146. Yoshioka, N. et al. Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell stem cell* **13**, 246-254 (2013).
147. El-Sayed, A.K. et al. Pluripotent state induction in mouse embryonic fibroblast using mRNAs of reprogramming factors. *Int J Mol Sci* **15**, 21840-64 (2014).
148. Krieg, A.M. et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546-9 (1995).
149. Halpern, M.D., Kurlander, R.J. & Pisetsky, D.S. Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. *Cell Immunol* **167**, 72-8 (1996).
150. Ballas, Z.K., Rasmussen, W.L. & Krieg, A.M. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol* **157**, 1840-5 (1996).

Chapter 2

Shedding light onto the organ of sight: Müller cells as a target for retinal therapy

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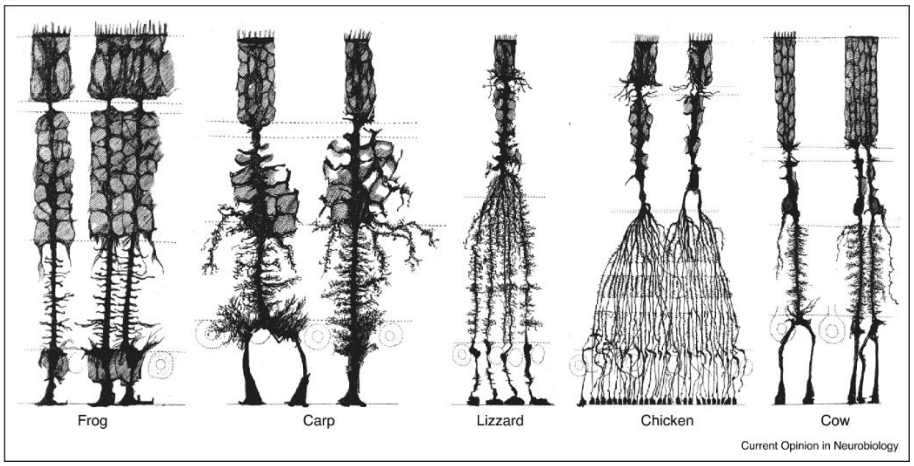
ABSTRACT

One cell type in specific, namely the Müller cell, caught our attention as a promising target for mRNA-based gene delivery to the retina. Müller cells are very specialized glial cells that span the entire retina from the vitreous cavity to the subretinal space. Their functional diversity and unique radial morphology renders them particularly interesting targets for new therapeutic approaches. In this chapter we reflect on various possibilities for selective Müller cell targeting in general and describe how some of their cellular mechanisms can be used in the advantage of retinal neuroprotection. Intriguingly, cross-species investigation of their properties revealed that Müller cells also play an essential role in retinal regeneration. Although many questions regarding this subject still need to be resolved, it is clear that Müller cells have unique characteristics that make them highly suitable targets for the prevention and treatment of numerous retinal diseases.



Graphical abstract | Species specific Müller glia morphologies.[1]

INTRODUCTION TO THE ANATOMY OF THE EYE

The eye is a sophisticated, impressive organ that enables the conversion of light into biological signals which are interpreted by the brain and grant us vision. As it is an easily accessible and immune-privileged tissue, the eye is a very attractive target for gene therapy and a useful model for proof-of-principle as well as clinical studies[2]. The eye can be divided in two anatomical regions: the anterior segment and the posterior segment (**Figure 1**). The anterior segment includes all structures in front of the vitreous and is responsible for the light guidance to the back of the eye. The outer surface of the anterior segment is formed by the cornea, a transparent and avascular tissue, which protects the internal structures against infection and physical damage. The cornea is the primary light-focusing structure of the eye as it refracts and transmits incoming light to the lens. While the shape of the cornea is fixed, the shape of the lens can be adjusted by the ciliary muscles, allowing the eye to focus on objects at various distances. In front of the lens, the iris functions as a diagram controlling the size of the pupil and thus the amount of light reaching the retina[3].

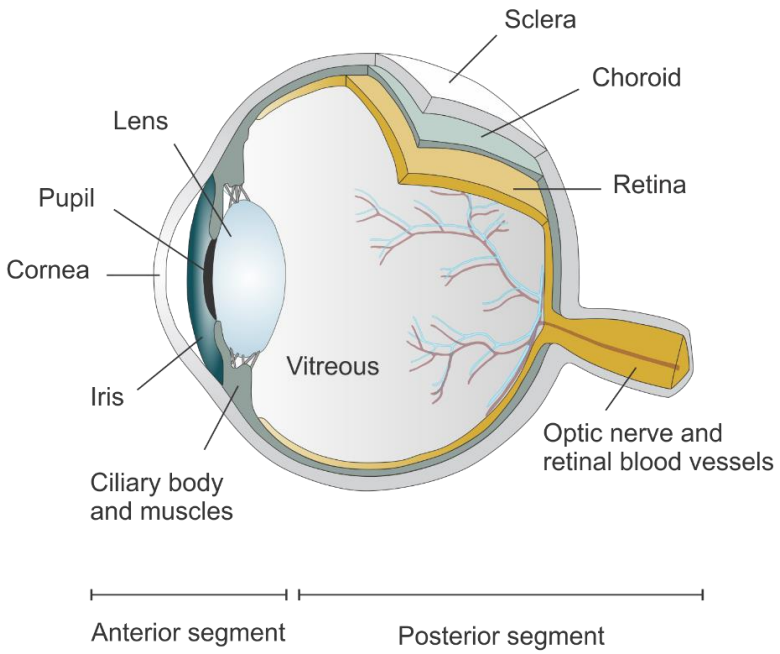


Figure 1 | Schematic representation of the anatomy of the eye.

The posterior segment of the eye is comprised of three layers, the sclera, the choroid and the retina, surrounding the vitreous cavity. The sclera, often referred to as “the white of the eye” is a sturdy outer layer which mainly consists of connective tissue that protects the eye and helps to maintain its shape. Underneath the sclera lies the vascular choroid, that in conjunction with the retinal blood supply, provides oxygen and nutrients to the outer retinal layers. The retina is the inner layer of the posterior segment and is separated from the

choroid by Bruch's membrane. As the retina is one of the main focuses of this dissertation, it is described in more detail below. Finally, the largest part of the eye is taken by the vitreous humor, a transparent gel composed of water, collagen, hyaluronic acid and proteoglycans. A more thorough description of the vitreous is provided in **Chapter 3**.

THE RETINA

The retina is the sensory membrane that lines the inner surface of the back of the eye. Through the fibers of the optic nerve, the retina forms the indispensable link between the eye and the brain, translating light photons into electrophysiological impulses. Over time, it became clear that the retina is a remarkable, complex, layered structure containing a large diversity of cells that form morphologically and functionally distinct circuits working together to encode visual information[4].

The vertebrate retina is divided into two regions, namely the inner and outer retina, each composed of multiple cell layers (**Figure 2**). The outer retina houses the rod and cone photoreceptors, which absorb incoming light and transduce this information to the retinal ganglion cells (RGCs) through three types of interneurons (bipolar, amacrine and horizontal cells) that reside in the inner retina[5]. RGCs join their axons together, forming the optic nerve and function to transfer the visual information gathered by the photoreceptors to the visual cortex[6]. This precise location, where the nerve fibers unite, contains no light-sensing photoreceptors and is therefore referred to as the 'blind spot' of the retina.

Besides photoreceptors and neurons, three main types of glial cells are found in the mammalian retina; the microglia, astrocytes and Müller cells, each with distinct morphological, developmental and antigenic characteristics. Occasionally, a fourth glial cell type, the oligodendrocyte, is identified. However, oligodendrocytes are only observed in the myelinated ganglion cell axons of a few species, including the rabbit [7]. Glial cells do not only provide support to their surrounding neurons, but also serve to maintain retinal homeostasis. As an example, microglia, the blood-derived resident immune cells of the retina, are constantly engaged in the surveillance of their surrounding neural tissue[8]. In this surveilling state, microglia are normally located in the inner retinal layers and display a branched morphology with many sensing processes. In response to retinal disturbance, such as light injury or retinal diseases, microglia become activated and change their morphology[9]. They migrate to the region of injury where they participate in the removal of waste materials and degenerated cells and secrete neuronal survival factors to limit further injury. On the other hand, persistent activation of microglia can also become harmful by the constant recruitment of additional inflammatory cells[10].

Named after their star-shaped cell body, astrocytes are often oriented perpendicularly to the neuronal cell bodies. They most commonly reside in the inner retinal layers, where they accompany the retinal blood vessels. Due to their striking association with the presence and distribution of these retinal blood vessels, it is generally assumed that during development, the astrocytes migrate from the brain through the optic nerve and enter the retina along with the vasculature. In addition, astrocytes are known to aid in the maintenance of the mechanical integrity of the blood retinal barrier (BRB)[8,11]. In the central nerve system it was recently demonstrated that the astrocytes' endfeet form a second barrier surrounding the endothelials cells of the blood brain barrier, the so-called 'glia limitans' or glial limiting membrane. Horng *et al.* demonstrated that this glia limitans induced tight junction formation in response to inflammatory cues and that preventing the formation of this membrane increased the severity of inflammatory diseases[12]. These results have spurred research into the existance of a retinal glia limitans as part of the inner BRB, which could be further explored as target for ocular therapies.

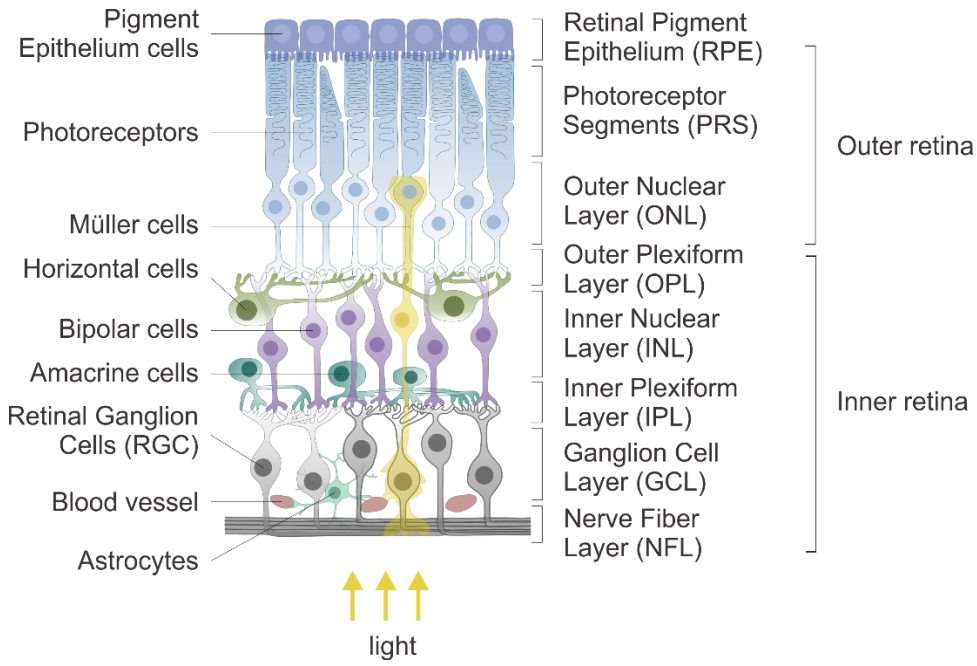


Figure 2 | Cellular organization of the retina.

The predominant glial cell in the retina, representing 90% of all retinal glia, is the Müller cell (**Figure 2**, yellow). Müller cells are radially oriented cells, extending throughout the entire retina. They are organized in a tightly contiguous, orderly pattern that allows them to come into contact with almost all other cell type in the retina[8]. This explains the many functions that Müller cells fulfill, which are summarized later in this chapter.

THE RETINAL PIGMENT EPITHELIUM

The retinal pigment epithelium (RPE) is a contiguous monolayer of pigmented epithelial cells, firmly packed together by tight junctions between the lateral surfaces[13]. Usually not considered a part of the neurosensory retina, the RPE is located between the choroid and the outer segments of the photoreceptors and exerts a multitude of functions. The apical area of the RPE cells consist of microvilli that contact the subretinal matrix enabling interaction with the light sensitive outer segments of the photoreceptors. At their basal side, the RPE is attached to the Bruch's membrane, enabling interaction with the underlying blood vessels of the choroid[14]. During development the RPE is crucial for the functional differentiation of the photoreceptors and interaction between both cell types is essential for visual function[15]. Indeed, mutations in genes expressed in the RPE can result in photoreceptor degeneration and vice versa[16].

At a first glance, the most obvious - and long assumed the only - function of the RPE is the absorption of excess light by means of melanin, which improves image resolution and protects the retina from photo-oxidative stress[17]. However, we now know that the RPE has many other functional characteristics (represented in **Figure 3**) and is vital for the health of the neural retina and choroid vasculature[18]. First, the tight junctions between the lateral surfaces of the RPE cells are part of the BRB[19]. They selectively control the transport of nutrients and metabolites from the blood to the retina and eliminate water and waste products from the retina to the choroidal blood vessels. The barrier function of the RPE also protects the inner retina from systemic influences, thereby granting the eye its immune-privileged status[18]. Second, the RPE is responsible for the phagocytosis of the photoreceptor outer segments, which are continuously shedded and renewed as a result of the photo-oxidative damage[20]. Third, as the photoreceptors do not express a re-isomerase for the retinoids, this process takes place in the RPE. The RPE cells thus contribute to the continuation of the visual cycle by conversion of all-*trans*-retinol to 11-cis-retinal and its redelivery to the photoreceptors[13,21]. Fourth, the RPE is known to produce a large variety of neurotrophic factors and signaling molecules to communicate with or support the maintenance of the photoreceptors[18]. As an example secretion of the vascular endothelial growth factor (VEGF) can induce the formation new blood vessels and restore the retinal oxygen supply in hypoxic conditions. However, overexpression of VEGF can have detrimental effects on the retinal vasculature and may contribute to neurodegeneration. Besides VEGF the RPE also secretes the pigment epithelium-derived factor (PEDF), known for its antiangiogenic properties[22]. A balance between the distinct neurotrophic factor levels is therefore crucial to prevent retinal dysfunction.

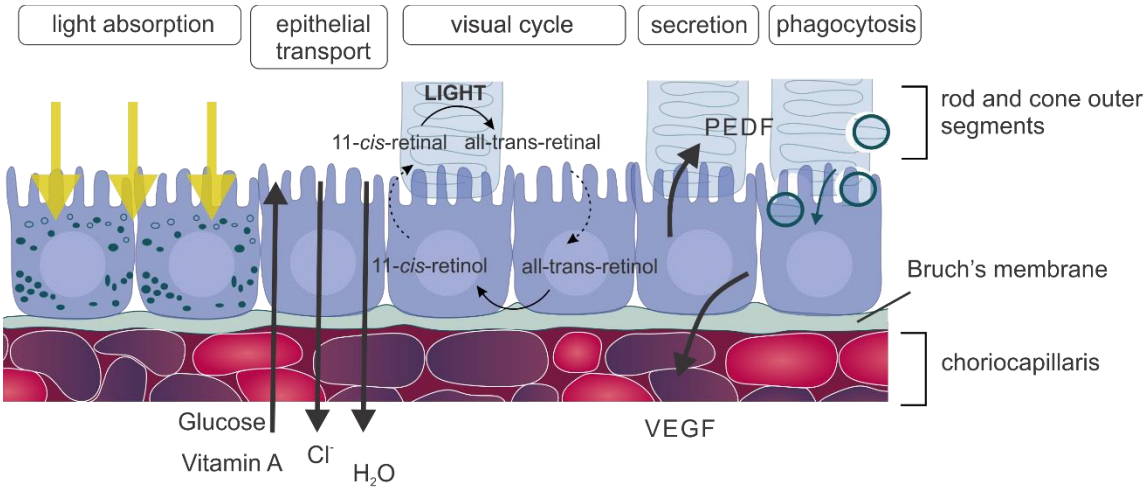


Figure 3 | Summary of RPE functions and structure. Adapted from[26].

Finally, the RPE is responsible for the ion homeostasis of the subretinal space. In addition to the basic transepithelial transport of ions, the cells feature many voltage-dependent ion channels to compensate for the fast changes in the subretinal ion concentrations during light-dependent photoreceptor activity[23-25]. Owing to their prominent involvement in the health of the neuroretina, RPE cells represent an important target in many gene therapy strategies, as will be further evaluated in **Chapter 5**.

THE MÜLLER CELL

In 1851 the German anatomist Heinrich Müller discovered a new cell type in the retina, which he described as thin fibers vertically extending throughout the vertebrate retina[27]. Named after their finder, it has now been demonstrated that Müller cells are of vital importance for the proper functioning of our vision through the support of retinal neurons. Indeed, as mentioned above, Müller cells are one of the most important glial cell type of the retina besides astrocytes and microglia. They stand out because of their unique radial morphology which spans the entire thickness of the retina, extending from the inner limiting membrane (ILM) to the outer nuclear layer, which allows interactions with all retinal neurons. Moreover, Müller glia are in proximity with the vitreous, the blood vessels and the subretinal space and thus represent an anatomical and functional connection between these compartments and the retinal neurons. Each Müller cell is described to be coupled to one cone, about ten rods and a varying amount of inner retinal neurons[28]. This columnar structure represents the smallest functional unit needed for the forward transduction of visual information[29,30]. As core of this column, Müller cells interact with their associated neurons in

a symbiotic way and are responsible for their functional, metabolic and structural support[31]. Therefore, Müller cells are ideally positioned to perform a wide variety of functions in order to maintain retinal homeostasis and initiate a protective response in case of retinal damage[28,29].

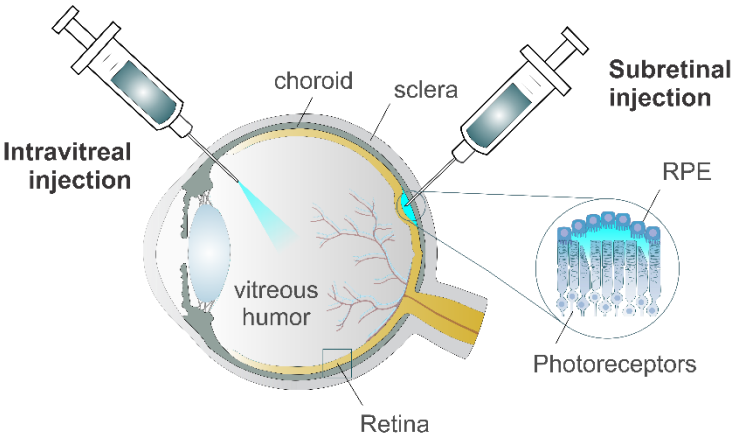


Figure 4 | Overview of the intraocular injection routes. An intravitreal (IVTR) injection is an injection into the vitreous humor of the eye. A subretinal (SR) injection, on the other hand, delivers its cargo in the subretinal space, between the photoreceptors (PRs) and the retinal pigment epithelium (RPE) layer.

Because of their unique anatomic and physiological features, we and others have shown increasing interest in the use of Müller cells as a target for novel therapeutic approaches. Indeed, a lot of their characteristics greatly favor their position for therapeutic purpose and encouraged us to focus on these cells as one of our main targets in this dissertation (cfr. **Chapter 3** and **4**). Firstly, their close contact to the vitreous and the subretinal space make them easy accessible to both intravitreally (IVTR) and subretinally (SR) injected drugs (cfr. **Figure 4**), which benefits various therapeutic applications. Secondly, Müller glia are highly resistant to pathological stimuli, allowing them to survive and remain a relevant target in advanced stages of retinal degenerative diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD)[32,33]. Thirdly, Müller glia are naturally involved in the synthesis and secretion of neuroprotective cytokines and growth factors, positioning them as an ideal target cell for the expression of these substances. Since their many processes ensheath almost every retinal cell type, they are able to secrete neurotrophic factors towards the inner and outer retina, and are therefore perfectly situated for the protection of degenerating neurons in for example glaucoma, diabetic retinopathy or photoreceptor degenerations. Finally, the discovery that Müller cells possess an evolutionarily conserved stem cell potential has opened the door for a range of new therapies that aim to induce self-renewal of the mammalian retina[34-36]. In this case neurons that were lost by trauma or all kinds of retinal diseases could be regenerated by the retina itself.

In the next sections we provide a short summary of the most established Müller cell functions and reflect on their unique response to retinal injury. We discuss how some of their natural characteristics can be used for the benefit of retinal therapy and how to avoid undesirable side effects by selectively targeting Müller cells with gene therapy. Finally, we summarize the current application of Müller cells for retinal neuroprotection and regeneration.

Müller cell functions

Structural, functional and metabolic support to maintain retinal homeostasis

There is a wide range of important functions performed by the Müller cells, which nearly all assist in the functional, metabolic or structural support of retinal neurons (**Figure 5**). In the healthy retina, Müller cells participate in the establishment of the BRB, of which the integrity is essential for the health, functioning and immune privilege of the retina[19]. The BRB consists of an inner and an outer barrier, the former represented by the tight junctions of the inner retinal vasculature and the latter by tight junctions between the RPE. These junctions highly restrict the movement of fluid and molecules between the blood and the retina and prevent entry of pathogens and other potentially harmful agents into the retinal tissue[37]. Müller cells were shown to enhance this barrier function since their selective ablation in transgenic mice resulted in severe BRB breakdown[38,39]. The precise mechanism by which they reinforce the BRB properties is not completely elucidated, but includes the secretion of factors such as PEDF and thrombospondin-1, that increase the tightness of the endothelial barrier[40,41].

Besides their contribution to the BRB function, Müller cells are directly responsible for the light conduction in the retina. Since the photoreceptors are found at the outermost layer of the retina (see **Figure 5** No 7 and 8), light has to pass all retinal layers before reaching its target. This retinal organization seems counter-intuitive since massive loss of light intensity could be expected due to light scattering by the multiple layers of retinal cells. Franze *et al*., however, discovered that the incident light is actually collected by Müller cells, which act as living optical fibers that guide the light through the retinal tissue towards the photoreceptors[42]. Thanks to their funnel-shaped endfeet and increasing refractory index along the different retinal layers, light reflection is reduced and a high intensity signal is transported to the photoreceptors. It has been suggested that Müller cells are also crucially involved in the integrity maintenance of the photoreceptor outer segments, which are essential for proper light detection and visual function and are continuously being renewed[43]. Müller cells are reported to phagocytose cone outer segments[44,45] and contribute to the

assembly of new outer segment discs[46,47]. Finally, similar to the RPE, they also participate in the recycling of the retinal chromophore, by converting cone-specific all-*trans*-retinal to 11-*cis*-retinol. This chromophore is then returned to the cones in order to restart the visual cycle (see **Figure 5A**)[48].

The strong connection with their surrounding neurons allows Müller cells to involve in synaptic activity. During neurotransmission, Müller cells are responsible for the fast clearance of glutamate in the synaptic spaces, thereby protecting retinal neurons against excitotoxicity (see **Figure 5C**)[49,50]. Glutamate in Müller cells is also used for the production of glutathione, a crucial antioxidant that protects the retina against oxidative stress. When oxidative stress occurs, Müller cells rapidly release glutathione, a molecule that prevents neuronal damage by neutralizing reactive oxygen species (ROS)[50,51]. Moreover, they are the primary site of glycogen storage in the retina[31] and in times of need, they address this storage to provide the neurons with glucose[32,52]. As many other glial cell types, Müller cells mainly rely on anaerobic glycolysis, even when sufficient oxygen is present. This metabolic feature allows them to save oxygen for retinal neurons and renders them less susceptible to anoxia[32]. Furthermore, via anaerobic degradation of their own glucose, Müller cells produce large amounts of lactate, which is preferentially taken up by photoreceptors as an alternative energy source[53,54]. Finally, Müller cells are enriched with numerous ion channels in order to regulate the electrolytic balance. During neuronal activity neurons release potassium (K^+) ions in the synaptic spaces, that can be taken up by Müller cells, which in their turn redistribute the excess K^+ into the fluid-filled spaces outside the neuroretina (i.e. the blood, the vitreous humor and the subretinal space), thereby buffering the K^+ imbalance[55-57]. Besides ion buffering, Müller cells also contribute to retinal homeostasis by regulation of water clearance via the specialized aquaporin-4 (AQP4) water channels in their cell membrane (see **Figure 5B**)[58,59].

Importantly, Müller cells further support the survival of photoreceptors and other retinal neurons by the secretion of neurotrophic factors, growth factors and cytokines[33]. As for the RPE, one of the most studied growth factors released from Müller cells is VEGF. In response to trauma, Müller cells have also been shown to produce other neuroprotective factors such as basic fibroblast growth factor (bFGF)[60], glial cell line-derived neurotrophic factor (GDNF)[61], PEDF [41], neurotrophins [62,63] and insulin-like growth factor 1 (IGF-1)[64], as discussed further in this chapter. Binding of these factors to their cognate receptors, in an autocrine or paracrine manner, activates downstream signaling pathways which promote cell proliferation, survival or regeneration[65,66].

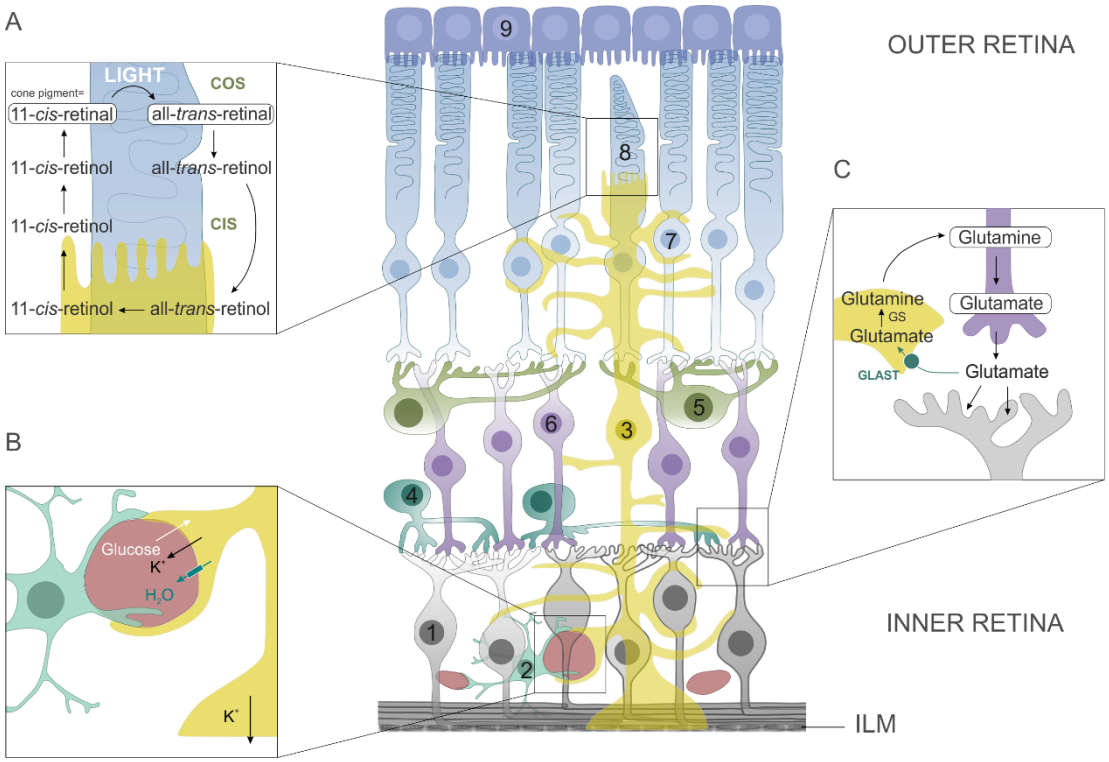


Figure 5 | Schematic representation of some important Müller cell functions. (A) Müller cells play a key role in the cone specific visual cycle: After photolysis, all-*trans* retinal is reduced to all-*trans* retinol in the cone outer segment (COS). All-*trans* retinol is transported to the Müller cells where it is enzymatically converted to 11-*cis* retinol, which in its turn is released for uptake by the cone inner segment (CIS). In the outer segment 11-*cis* retinol is oxidized back to 11-*cis* retinal for pigment regeneration. (B) Müller cells maintain retinal homeostasis by mediating K^+ and water transport: neuron-derived K^+ is taken up by Müller cells in the plexiform layers and redistributed into the blood, the vitreous and the subretinal space, to avoid prolonged K^+ accumulation. Osmotically coupled to this K^+ transport, water from the inner retinal tissue is removed into the vasculature. (C) Müller cells are crucially involved in neurotransmitter recycling: during neurotransmission, they remove excess glutamate in the synaptic spaces via their glutamate aspartate transporter (GLAST) and enzymatically transform it into the non-neuroactive substance glutamine by means of glutamine synthetase (GS). Glutamine is then transported back to the neurons for the re-synthesis of glutamate. ILM: inner limiting membrane; 1:ganglion cell; 2: astrocyte; 3: Muller cell; 4: amacrine cell; 5: horizontal cell; 6: bipolar cell; 7: rod; 8: cone; 9: retinal pigment epithelium cell

Remarkably, most of the current knowledge about Müller cell functions was only obtained during the last 20 years and it is likely that not all roles played by Müller cells have yet been identified. It is important to note that the many features described here are merely a selection, since an extensive overview can be found elsewhere[29].

Müller cell gliosis as response to retinal imbalance

Besides their physiological roles, Müller cells have the unique capacity to respond to many types of retinal injury and diseases in a process often referred to as gliosis[33,67]. Reactive gliosis is a complex response to any pathological alteration, including retinal detachment, photic damage, glaucoma, diabetic retinopathy and retinal degeneration, considered to protect the retinal tissue from further damage and stimulate its repair. During

this process Müller cells undergo changes on a morphological, biochemical and physiological level, which will be determined by the nature and intensity of the insult[67]. Müller cell gliosis is typically characterized by changes in gene and protein expression, and cellular hypertrophy. Especially the upregulation of the intermediate filaments (glial fibrillary acidic protein (GFAP), vimentin and nestin) is a key feature in the gliotic response. This rise in intermediate filaments results in an increase in Müller cell stiffness, which most likely aims to prevent mechanical lesions in the retina[68]. Rapid upregulation of GFAP was found in response to retinal detachment[69], hypoxia[70], ischemia[71], and experimental glaucoma[72,73] and was shown to be highly variable and disease dependent[74]. Another prominent gliotic manifestation is the altered expression of glutamine synthetase (GS), a Müller cell-specific enzyme involved in neurotransmitter recycling (see **Figure 5C**). While the expression of GS is reduced following loss of photoreceptors which normally produce lots of glutamate, GS levels are increased during hepatic retinopathy which requires GS for ammonia detoxification[28]. In diabetic retinopathy and optic nerve crush, on the other hand, no difference is observed in the amount of GS[75]. In addition, it is important to note that individual Müller cells can respond to a harmful stimulus in a different way. Indeed Fischer *et al.* demonstrated such a heterogeneity between adjacent Müller cells in the chick retina, where ~65% of the Müller cells started to proliferate following N-methyl-D-aspartate (NMDA)-induced damage, whereas ~35% did not[76]. Differences in Müller cell response are also observed between species. While mammalian Müller cells mostly respond to retinal injury by increased GFAP expression, hypertrophy and proliferation[28], Müller cells of teleost fish (such as zebrafish) undergo a reprogramming event that allows them to regenerate all major retinal cell types and repair retinal damage[77] (see later in this chapter). Taken together, it is clear that the type of pathological injury and species will strongly influence the glial response.

As a rapid response to injury, gliosis is a beneficial process activating different protective mechanisms aiming to buffer extra K^+ levels, take up excess glutamate and release a variety of factors that protect neurons from degeneration[33]. In response to harmful stimuli, Müller cells are furthermore capable to dedifferentiate to cells reminiscent of stem cells and in some species redifferentiate to new retinal neurons, as is discussed in more detail further in this chapter[78]. This Müller cell dedifferentiation can be seen as an endeavor to regenerate the injured retina.

Unfortunately, in some severe cases and/or longer periods after injury this Müller cell response can be excessive and long-lasting, thereby disturbing the regular Müller cell functions, including their homeostatic mechanisms and their ability to support retinal neurons. Hence, a status of persistent Müller cell gliosis can be detrimental for neuronal function and survival, and often results in neuronal cell death[33]. Long-lasting hypoxia, for

example, results in persistent induction of GFAP and reduced levels of GS, thereby impairing glutamate detoxification and contributing to neovascularization (NV) and neuronal degeneration[79]. Chronic retinal ischemia, inflammation and diabetic retinopathy lead to a downregulation of specialized K^+ channels in Müller cells, which normally release K^+ in the blood, without changes in the K^+ uptake. This increases the osmotic pressure within the Müller cell causing Müller cell swelling and contributing to retinal edema[80,81].

Finally, persistent gliosis can lead to massive Müller cell proliferation throughout the entire retinal tissue, forming glial scars that fill the spaces left by degenerated neurons, RPE and blood vessels[82]. This scar-like tissue prevents the renewing of photoreceptor outer segments, inhibits tissue repair, increases retinal stiffness and exerts tractional forces - all of which can impede normal retinal function[28,83]. The formation of glial scars is thought to be one of the reasons for the very limited degree of regeneration in the mammalian retina and can impede integration of donor cells in the host retina following transplantation[28,84]. From the above it is clear that gliosis is a complex process that can influence the therapeutic outcome of many retinal treatments, including the strategies discussed in this chapter. For a more detailed overview of the gliotic response in the diseased retina readers are referred to[28].

Taken together, it is evident that Müller cells are an elemental part of the healthy retina and that any imbalance sensed by these cells can influence proper retinal functioning. The existing body of research on Müller cells' involvement in the healthy and diseased retina illustrates that Müller cells are vital for retinal homeostasis and integrity on the one hand, yet can also accelerate the progress of neuronal degeneration on the other hand. This plethora of functions makes them an ideal target for therapeutic approaches to slow-down, prevent, or even cure various retinal diseases. More specifically, if one could stimulate the neuron-supportive Müller cell functions and avert the destructive mechanisms of gliosis, it might be possible to use Müller cells in the development of new therapeutic strategies such as neuroprotection or retinal regeneration.

In this chapter, we will mainly address diseases accompanied by photoreceptor degeneration and therefore impaired light detection, such as RP[86] and AMD[87]; diseases with RGC loss, in which photoreceptors may be able to detect light, but visual information is not transmitted to the brain, such as glaucoma[88] and optic nerve damage[89]; and diabetic retinopathy in which abnormalities occur in both photoreceptor and RGCs as well as in amacrine cells and bipolar cells[83] (cfr. **Figure 6**). While neuroprotective and regenerative strategies can be applied for a wide range of retinal diseases, it is the nature of the disease that will define which cell type requires protection or regeneration and which is the preferred therapeutic strategy.

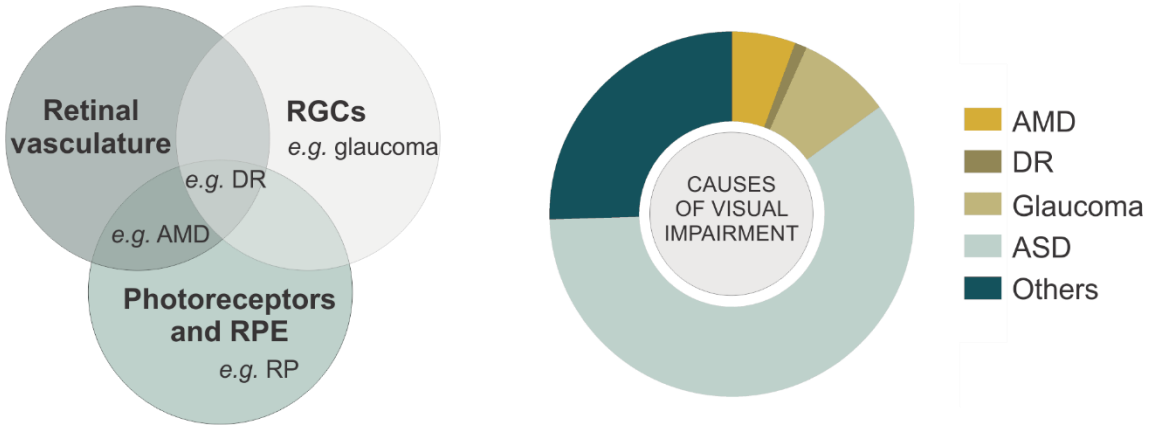


Figure 6 | Classification and prevalence of the main causes of visual impairment. Classification (left) was made based on the primary affected tissue by the corresponding diseases. Prevalence of main causes of global visual impairment (right) representing the latest estimates for the year 2015 as produced by the Vision Loss Expert Group and published in The Lancet[85]. AMD, age-related macular degeneration; ASD, anterior segment disorders (such as cataract); DR, diabetic retinopathy; RP, retinitis pigmentosa

Selective Müller cell targeting

Although Müller cell type-specific targeting is not a necessity for strategies such as retinal neuroprotection or regeneration, it can definitely increase the success of a therapy and minimize unnecessary uptake of drugs and/or their carriers by other retinal cells. In this way, lower doses could be applied and unwanted side effects could be prevented. To restrict transgene expression to a specific retinal cell type, gene therapy is an attractive and promising approach. Especially for neuroprotective interventions, Müller cell specific targeting has a great advantage since Müller cells naturally participate in this process by the synthesis and release of neurotrophic factors to their surrounding neurons. Moreover, as ultimate survivors, they can continue to supply neurotrophic factors until the latest phases of the disease, when retinal neurons are lost to degeneration. Restricting the expression to Müller cells allows the use of fewer (viral) particles and reduces the possibility for ectopic transfection and immune responses. In case of regenerative strategies, factors affecting Müller cell reprogramming can be delivered as such to the entire retina. Targeting Müller cells directly, however, eliminates the risk of undesirable side effects potentially caused by reaching untargeted cells. An overview of possible Müller cell target strategies is given in **Table 1**.

Over the past 20 years, a myriad of therapeutic vectors have been developed for retinal gene delivery. Despite the focus of this thesis on non-viral delivery, we first highlight the use of recombinant viruses as they currently present the leading players in this field[90]. The specificity of viral gene delivery greatly depends on four factors: the injection route, the virus type, the promoter and the viral envelope or capsid. As such, reports indicate that SR

injection of viral vectors mainly transduces cells that border the subretinal space, while IVTR injections are preferable for transduction of the inner retina[91] (illustrated in **Figure 4**). Nevertheless, because Müller cells span the entire retina, they can be targeted via both injection routes. Another factor influencing the cellular tropism is the choice of the virus type. While the most widely used adenoviral vector Ad-5 has been shown to transduce Müller cells after both SR[92] and IVTR[93-96] injection, lentiviral vectors show more tendency toward transfection of photoreceptors and RPE rather than glia[97]. Similarly, most natural adeno-associated viruses (AAV) have a strong tropism for neurons with minimal transduction of glial cells[98,99]. Regardless, efficient Müller cell transgene expression can be driven by the use of glia specific promoters, such as the cluster of differentiation 44 (CD44), GFAP and vimentin promoter. For example, Greenberg *et al.* demonstrated that lentiviral vectors containing one of these three promotors yielded strong eGFP expression in Müller cells after SR injection in adult rodents, whereas the use of strong ubiquitous promoters (such as the human cytomegalovirus (CMV), human ubiquitin-C and hybrid chicken β-actin promoter) drove transgene expression mainly in the RPE[100]. However, IVTR injection of lentiviral vectors commonly fails in transducing retinal cells, as these vectors are largely neutralized in the vitreous humor, are relatively instable and particularly large in size[101]. This is one of the reasons why AAVs have emerged as a favored tool for gene delivery to the retina. AAVs have been shown to lack pathogenicity, elicit a very mild immune response and mediate long-term transgene expression in retinal cells[102]. In addition, their very small size (25 nm) is expected to facilitate diffusion across retinal barriers and therefore enhance delivery of genes into the inner retina following IVTR injection[103]. For these vectors as well, it is possible to restrict transgene expression to Müller cells using Müller glia-specific promoters[104,105]. For instance, it was shown by Dorrell *et al.* that a GFAP promotor-driven AAV strictly transduced activated Müller glia after IVTR injection in mice, while an ubiquitously CMV enhancer/chicken β-actin (CAG) promoter-driven AAV demonstrated nonspecific expression of GFP, mainly localized to RGCs[104]. It should be noted, however, that the use of a CAG promoter still evoked stronger GFP expression compared to the use of the GFAP Müller cell-specific promoter. In addition, the research group of Flannery also reported possible cytotoxicity associated with the use of the GFAP promoter, as they noticed strong autofluorescence in fundus images when comparing SR injection of an AAV9 carrying a GFAP and chicken β-actin (CBA) promoter[106]. Therefore substantial research has been conducted to develop strategies that modify the viral envelope or capsid for improved targeting. For AAVs for instance, engineering the capsid based on rational insertion of defined amino acid sequences has shown some success in increasing cell-specific targeting[107]. Nevertheless, this method of rational design requires prior knowledge on which capsid modifications to use for improved targeting, which is often unavailable. The development of a technique called “directed evolution”, using mutant AAV capsid libraries in

combination with high-throughput screening methods, therefore drastically improved vector design, without the need for mechanistic knowledge of capsid properties. In principle this approach involves the synthesis of AAVs with random capsid mutations which are tested *in vitro* or *in vivo* followed by positive selection of their ability to transduce the desired cell type[108]. Using this strategy, Klimczak *et al.* identified a new AAV variant (ShH10) with enhanced and specific IVTR Müller cell transduction *in vivo*[109]. Specific Müller cell targeting with this vector diminishes the loss of vector genomes to neighboring cells unable of expressing the transgene, while maintaining the use of a strong promoter and therefore high transgene expression[106,110,111]. It is important to note, however, that the ShH10 vector is substantially more selective in transducing Müller glia in rat retinas compared to mice retinas[112]. A potential explanation for this observation might be the difference in barrier function of the ILM, which forms an important drug delivery hurdle as discussed in **Chapter 4**. In mice, a thinner ILM could facilitate penetration and non-specific transduction of the retina, while the thicker ILM in rats favors transduction of Müller cells. Nevertheless, the specificity of the ShH10 vector using a strong ubiquitous promoter remains controversial as other groups found that a Müller glia specific promoter (such as GFAP) is necessary to bring about ShH10-mediated Müller glia specificity[113].

Table 1 (following pages) | Targeting expression to Müller cells *in vivo.* Abbreviations: AMD, age-related macular degeneration; BDNF, brain-derived neurotrophic factor; BRB, blood-retinal barrier; CAG, CMV-enhancer/chicken β-actin; CD44, cluster of differentiation 44; cKO, conditional knockout; CMV, cytomegalovirus; Crb, Crumbs homologue; Crx, cone-rod homeobox; EGFR, epidermal growth factor receptor; GDNF, glial cell-line derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HRE, hypoxia-responsive element; IVT, intravitreal; LCA, Leber's congenital amaurosis; MG; Müller glia; NP, neuroprotection; Nrl, neural retina leucine zipper; NT-4, neutrophin-4; NV, neovascularization; OIR, oxygen-induced retinopathy; Otx2, orthodenticle homeobox2; PR, photoreceptor; RGC, retinal ganglion cells; RLBP, retinaldehyde-binding protein; RP, retinitis pigmentosa; RPE, retinal pigment epithelium; SR; subretinal; VIM, vimentin; WT, wild type.

	Vector type	Promoter	Injection route	Gene	Specificity	Suggested mechanism	Animal model	Suggested application	Refs
Adeno	Ad-5	CMV	IVTR	BDNF	Selective transgene expression in MG	basal end-feet of MG provide a large surface for adsorption of viral particles from the vitreous chamber	WT rats	NP of RGCs; NP of PRs (for macular degeneration and RP)	[93]; [95]
Lentivirus	HIV	CD44 GFAP VIM	SR	eGFP	MG were transduced with high efficiency, leaky expression in adjacent RPE cells	proteins that are localized in MG of which the promoter could therefore confer specific glial expression	Rat model of RP	Retinal NP (for AMD, glaucoma and RP)	[100]
		CMV Ubiquitin	SR	eGFP	Expression restricted to RPE	promoters strongly active in a wide range of cells			
		CD44	IVTR	eGFP	No eGFP expression	proteins at the vitreal surface that bind and inactivate LV vectors; vectors too large for ILM penetration; polarized receptor profile on MG			
Adeno-associated virus	AAV-2	CAG	IVTR	eGFP	Nonspecific expression in the inner retina	promoter strongly active in a wide range of cells	Mouse model of subretinal NV; WT rats	NP; LCA and severe forms of RP	[104]; [109]
		GFAP	IVTR	eGFP, NT-4	Expression in MG surrounding retinal blood vessels	glial specific promoter upregulated in MG located adjacent to subretinal NV	Mouse model of subretinal NV; Crb1 cKO mice		[104]; [123]
		CD44	IVTR	eGFP	Subset of eGFP positive RGCs	natural tropism of AAV2 for RGCs	Rat model of RP	NP	[100]

	Vector type	Promoter	Injection route	Gene	Specificity	Suggested mechanism	Animal model	Suggested application	Refs
Adeno-associated virus		HRE-GFAP hybrid	IVTR	eGFP	eGFP expression predominantly localized in MG	Combination of MG specific promoter and several hypoxia-responsive and aerobically silenced elements	Mouse model of OIR	Diabetic retinopathy and AMD (retinal hypoxia)	[105]
	ShH10	CAG	IVTR	eGFP; GDNF	Highly specific MG transduction; Subset of RGCs and amacrine cells as well (Yao et al.)	Directed evolution for the selection of IVTR transduction of MG Suggested to better bind and transverse ILM and improved binding to the EGFR on the MG	WT rats; Rat model of RP; Mouse model with permeable BRB; WT mice	NP; gene therapy; retinal regeneration	[124,109,106,111,110,113]
		GFAP	IVTR	eGFP, β-catenin, Lin28; Otx2, Crx, Nrl	Highly specific MG expression	Vector selected by directed evolution (see previous) + MG specific promoter	WT mice, cKO mice; Mouse model of congenital blindness	Retinal regeneration	[113; 125]
		CD44	IVTR	eGFP	Week eGFP expression primarily in MG, but not specific		Crb1 cKO mice	CRB1 inherited retinal dystrophies	[112]
			SR	eGFP	Week eGFP expression in MG, RPE and PRs				
		RLBP1	IVTR	eGFP	eGFP expression restricted to MG	Vector selected by directed evolution (see previous) + MG specific promoter			
			SR	eGFP	eGFP expression restricted to RPE and MG				
		CMV	IVTR	eGFP; CRB2	Expression only at the subapical region of MG		Crb1 cKO mice; Crb2 cKO mice	RP	[112; 126]

Although viral vectors are currently dominating ocular gene therapy trials, concerns regarding their immune response and high production cost have encouraged development of non-viral alternatives. Especially lipid-[114,115], polymer-[116,117] and/or protein-[118,119] based nanoparticles have been widely investigated for their ability to deliver drugs and genes to the retina. Their lower transduction efficacy and short-lived gene expression, however, are considered as major drawbacks of the non-viral approach[91]. Nevertheless, their use might be very beneficial in situations where short-term transgene expression is favorable, for example to boost neuronal survival. As for their viral counterparts, cell-type specific delivery of non-viral vectors can also be influenced by the delivery method and the use of cell type-specific promoters[120]. In addition, specificity could be increased by rationally modifying the surface of the particle to target receptors on the cell membrane[121]. Although it has been suggested in literature that some nanoparticles, such as human serum albumin particles are spontaneously taken up by Müller cells after IVTR injection in rats[122], active Müller cell targeting of non-viral vectors has yet to be investigated.

Muller cells for neuroprotection

Müller cells are endowed with the ability to synthesize and secrete a wealth of neurotrophic factors and hence represent a natural target for expression of these proteins. Neurotrophic factors are a family of growth factors known to promote the growth, survival and differentiation of neurons. In many retinal diseases, deprivation of these factors contributes to neuronal cell death, promoting extensive research to neurotrophic factor supplementation therapy. Indeed, administration of neurotrophic factors could halt or slow down neuronal degeneration and help to maintain visual function. Most neurotrophic factors belong to one of three classic families: (1) neurotrophins, including the nerve growth factor (NGF), the brain-derived growth factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4)[127]; (2) GDNF family ligands, including GDNF, neurturin, artemin and persephin and (3) interleukin-6 (IL-6) family of cytokines, including ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and IL-6[128]. Other proteins that have been identified as neurotrophic factors include bFGF, IGF-1, VEGF, transforming growth factor-β (TGF-β) and PEDF[129].

Of all neurotrophic factors, CNTF is the most widely studied for therapeutic neuroprotection of the retina. Extensive research has shown that CNTF supports the survival of rod photoreceptors in almost all animal models of retinal degeneration[130]. In addition, CNTF has also been shown to significantly protect RGCs and inner retinal neurons from death in several disease models[131-133] as well as to promote axonal regeneration[134]. Despite these promising results, some concerns have been raised in using CNTF as a retinal

neuroprotective agent. This ambiguity is caused by the reported dose-dependent suppression of retinal function and decreased electroretinogram (ERG) amplitudes in response to delivery of the CNTF protein or transgene as such[135-138]. In addition to CNTF, the neurotrophin BDNF stands out owing to its powerful neuroprotective effect, particularly on RGCs[139]. Exogenous BDNF protein delivery as well as vector-mediated upregulation of BDNF expression promotes RGC survival after optic nerve axotomy, as described earlier[93,140,141]. Still, in most studies, neuroprotection of RCGs was only short-term an did not permanently rescue RCGs from cell death[93,142]. This transient survival effect of BDNF was attributed to a downregulation of the BDNF receptor (TrkB) on the RGC cell surface, triggered by prolonged BDNF exposure or the axotomy itself. In response to this observation, the group of Cheng *et al*. successfully tested a combination approach of BDNF protein delivery and virus-mediated TrkB gene therapy which greatly increased the extent and level of RGCs survival[143]. Furthermore, molecules that selectively activate TrkB agonists were reported to enhance RGC survival following acute and chronic models of glaucoma[144,145]. Besides RGCs, BDNF delivery also results in photoreceptor survival[95], which is surprising considering their poor expression of the TrkB receptor. Research suggests that BDNF induces photoreceptor survival indirectly by activating Müller cells in an autocrine or paracrine fashion to secrete other neurotrophic factors, such as CNTF and bFGF, which in turn stimulate photoreceptors[95,146].

Other factors of interest include PEDF and GDNF. Increased expression of the latter has been reported to protect the retina from oxidative stress without altering normal retinal function[147] and to protect photoreceptors and RGCs from death in animal models of retinal degeneration[148-150]. A study investigating the long-term safety of AAV-mediated GDNF expression demonstrated no abnormalities in morphology or function of the retina after 1 year[151]. The neurotrophic factor PEDF was initially discovered to be secreted by RPE cells and was shown to exhibit anti-apoptotic, anti-oxidative and anti-inflammatory effects[152]. Surely, intraocular injection of PEDF delayed photoreceptor cell degeneration and apoptosis in genetic and light-induced damage retinal models[22,153,154] and protected RGCs from ischemia-induced cell death[155]. Moreover, human clinical trials have been initiated with PEDF for the treatment of neovascular AMD, since it has proven activity against VEGF[156].

Although it is possible to achieve neurotrophic factor supplementation by delivering recombinant proteins, their short *in vivo* half-lives necessitates frequent ocular injections, which would hamper patient compliance[157-160]. On the contrary, local and sustained delivery of neurotrophic factors within the retina by transfection of retinal cells with neurotrophic factor-encoding genes could avoid some of these limitations. Since, in contrast to neurons, Müller cells are able to survive until the latest stages of retinal degeneration, they serve as

ideal candidates for this strategy. In addition, as they contact all classes of retinal neurons, Müller cells can be directed to express the desired neurotrophic factors throughout the entire retina. The use of Müller cells as a secretion platform for neurotrophic factors was already investigated two decades ago, when Di Polo *et al.* demonstrated that the delivery of viral vectors containing a BDNF transgene resulted in the secretion of the BDNF protein and subsequent survival of injured RGCs in axotomized rats. More importantly, via IVTR injection of Ad-5, it was possible to preferentially transduce Müller cells[93]. Using the same strategy, the research group of Di Polo demonstrated that Müller cell mediated BDNF secretion also markedly increased the survival of photoreceptors following ten days of light-induced photoreceptor degeneration[95]. However, the use of an adenoviral vector in both studies necessitated co-treatment with an immunosuppressant to prevent Ad-mediated inflammation and allow for sustained neurotrophic factor expression[93,95]. In 2001, Liang *et al.* successfully tested IVTR injection of AAVs encoding CNTF for protection of photoreceptors in animal models of RP[135]. The use of a CMV promotor, however, did not restrict CNTF expression to Müller cells, as RGCs were transduced as well[135]. On the contrary, IVTR injection of an AAV vector containing transgenes driven by a GFAP promotor more specifically transduced Müller glia, as demonstrated by Dorrell *et al*. Their GFAP-driven gene delivery of NT-4 resulted in NT-4 production in activated Müller cells and protected photoreceptors from oxidative stress in a mouse model of NV[104]. To further enhance the therapeutic effect of this approach Dalkara *et al.* used the ShH10 AAV vector to overexpress GDNF which resulted in strong, selective transduction of Müller cells after IVTR injection. Moreover, the authors showed that Müller cell-mediated GDNF expression significantly slowed the rate of retinal degeneration in a rat model of RP. By targeting Müller glia, retinal degeneration was postponed for a longer period compared to previous reports using GDNF delivery without Müller cell targeting[106].

Taken together, the use of growth factors is an emerging strategy for retinal neuroprotection. Given the enormous variation in underlying genetic causes of retinal diseases, a mutation-independent strategy like neuroprotection, can serve as a universal approach to halt or slow down the loss of retinal cells (further discussed in **Chapter 6**). Since this approach is based on secreted proteins, neurotrophic gene therapy does not require cell type-specific delivery. Yet, targeting the ultimately surviving Müller cells, rather than losing transgenes to dying retinal neurons, can markedly increase retinal survival. Interestingly, supplementing a combination of different neurotrophic factors may provide even more benefit, as was demonstrated by Koeberle *et al.* for the survival of RGCs[161]. In addition, in diseases where the underlying genetic cause is identified, neurotrophic factor delivery could have an added value to gene replacement strategies. An example of this combination is given by Buch e*t al.* who observed enhanced photoreceptor survival when AAV-mediated

GDNF expression was coupled to Prph2 gene replacement in *Prph2*$^{Rd2/Rd2}$ animal models of inherited retinal degeneration[136]. This strategy where two viral vectors are simultaneously applied, one for gene replacement in the photoreceptors and one for neurotrophic factor secretion in Müller cells, underscores the value of selective Müller cell targeting for neurotrophic factor secretion. Indeed, by transferring the GDNF-secretion task to the Müller cells, no photoreceptor machinery nor energy would be wasted on neurotrophic factor secretion, thus facilitating photoreceptor regeneration[106].

It should be noted, however, that some challenges arise before vector-mediated neuroprotection can be translated into the clinic. First of all, each distinct factor should be optimally dosed to achieve optimal protection from degeneration. In this regard, it remains a question whether the data related to the duration of rescue from rodent models can be extrapolated to human diseases. Another important issue relates to the timing of the therapy as it remains unclear at what time point during the progression of retinal disease neuroprotection would be the most beneficial. Finally, possible (side) effects of sustained, high-level neurotrophic factor expression in the eye should be taken into account, since some factors have shown to cause long-term detrimental effects. The use of viral vectors containing inducible promoters or the use of non-viral pDNA or mRNA strategies to induce protein expression during a limited time frame, might however resolve some of these issues.

Muller cells for regeneration

One of the most intriguing Müller cell functions, revealed during the last decade, is their essential role in retinal regeneration. The observation that Müller cells possess stem cell properties has positioned them as a primary target for endogenous retinal repair[162].

Unlike most cell types of the human body, differentiated retinal neurons do not have the ability to re-enter the cell cycle and divide. When traumatic injuries and diseases result in retinal cell death, lost neurons in the damaged area are not spontaneously replaced and apoptosis ultimately leads to vision loss. This is, however, not the case for all species. Teleost fish, such as zebrafish, possess the remarkable innate ability to regenerate retinal tissue and restore impaired sight[163-165]. The source of this regeneration was discovered in early studies, using goldfish, as a group of proliferating cells migrating from the inner (INL) to the outer nuclear layer (ONL) (cfr. **Figure 2**), where they continue to divide and generate new rod photoreceptors[166,167]. Initially these clusters were assumed to originate from rod precursors[168,169], but the observation that INL cell proliferation preceded regeneration and that Müller nuclei, which reside in the INL, also proliferated and migrated into the empty spaces of lost photoreceptors following injury, raised the possibility that Müller glia were an

alternative source of regenerative neuronal progenitors[170]. Finally, several studies using transgenic zebrafish in which the fate of GFP-tagged Müller cells was followed shortly after retinal injury, were able to confirm Müller glia as the source of these progenitors[171-173]. Using a conditional expression system that allowed permanent labeling of Müller glia-derived progenitors, Ramachandran *et al.* demonstrated that these progenitors are responsible for regenerating all retinal cell types and that these cells are stably integrated into the retinal structure[174]. Despite considerable similarity between the mammal and the zebrafish retina, mammalian Müller glia do not respond to injury by means of dedifferentiation. Instead they experience reactive gliosis, which is often associated with cell proliferation and scar formation (see previous), failing to initiate regeneration[33]. Nevertheless, this response to injury together with their capacity to upregulate genes associated with retinal stem cells indicate that mammalian Müller cells might be stimulated to adopt a stem cell-like state and generate retinal neurons under appropriate circumstances. Surely, rodent as well as human Müller cells have been shown to generate both glial cells and neurons *in vitro*[175-178]. How mammalian Müller cells lost the capability to use this neurogenic potential after injury *in vivo* still remains a pressing question. However, if one could unlock this restricted regenerative potential of mammalian Müller glia and mimic the self-healing capacity of their zebrafish counterparts, it might be possible to restore human retina after injury and regain vision in retinal degeneration diseases. In addition, the use of endogenous Müller cells to promote neuronal regeneration circumvents many risks associated with exogenous cell transplantation and prosthetic devices, such as immune rejection, potential ethical objections and tumor formation[179]. To this end, several research groups have used the zebrafish as a model to gain a better understanding of the factors that control retinal regeneration.

In zebrafish Müller glia reprogramming has shown to be responsible for the regeneration of the retina in various cases of retinal damage, such as light-exposure[173], chemicals[180] and mechanical injury[171]. Following retinal injury, Müller glia dedifferentiate, migrate towards the ONL and undergo a single, asymmetric self-renewing division that preserves the Müller glia cell on the one hand and produces a multipotent progenitor cell on the other hand[181]. This progenitor cell proliferates to form a cluster of neural progenitors, which migrate along the radial process of the daughter Müller cell to the damaged retinal layer, where they withdraw from the cell cycle and can differentiate into all major retinal cell types[78,162] (**Figure 7**).

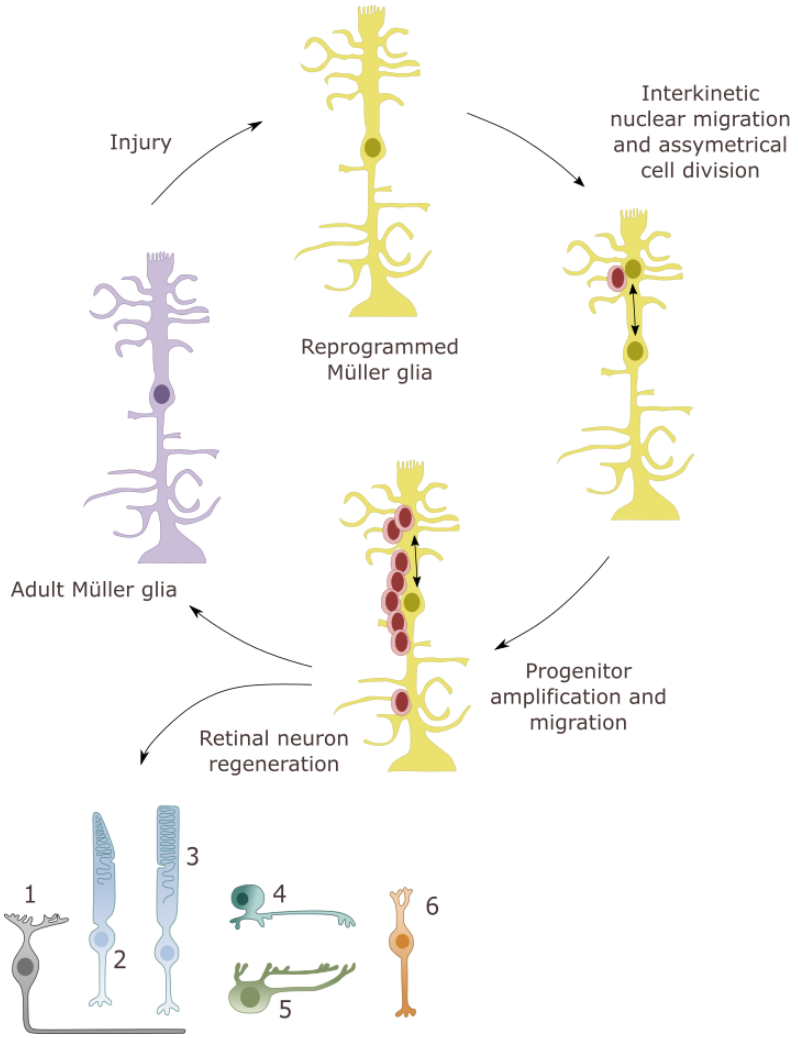


Figure 7 | Retinal regeneration in zebrafish. Adult Müller glia (purple) respond to retinal injury with a gliotic response, accompanied by a reprogramming event in which they adopt retinal stem cell properties (yellow). The nuclei of the reprogrammed Müller glia migrate from the INL to the ONL where they divide asymmetrically and subsequently return to the INL, a process called interkinetic nuclear migration. This asymmetric division results in the formation of a multipotent progenitor which amplifies to create a small population of progenitors capable of regeneration all major retinal neuron types. 1: ganglion cell; 2: rod; 3: cone; 4: amacrine cell; 5: horizontal cell; 6: bipolar cell. Adjusted from [78] and [77].

Although further work is needed to shed more light on the molecular mechanisms driving Müller glia reprogramming in zebrafish, several proteins and signaling pathways involved in this process have been identified (for an excellent overview of the factors affecting Müller glial cell reprogramming and proliferation readers are referred to[78]). Further studies now focus on how these proteins can be used to enable the mammalian retina to regenerate *in vivo* (**Table 2**). The first studies, however, tried to stimulate the proliferative and neurogenic properties of mammalian Müller cells by simply inducing retinal damage. In 2004, Ooto and colleagues demonstrated that NMDA neurotoxicity of RGCs in the adult rat retina induced a small amount of Müller glia to proliferate and produce new cells with markers of early bipolar and rod photoreceptor cell differentiation[182]. Similarly, N-methyl-N-nitrosourea (MNU)-induced photoreceptor degeneration was reported to initiate Müller glia

proliferation in rodents, associated with rare events of rhodopsin expression, indicating photoreceptor regeneration[183]. However, this concept has recently been challenged by Kugler *et al*. which demonstrated that NMDA-induced excitotoxic damage of the rodent retina does not induce Müller cell dedifferentiation[184]. Similar observations were reported by another research group using light exposure to induce neuronal damage without noticeable Müller cell proliferation[185]. These authors found that bromodeoxyuridine (BrdU) was co-localized with DNA Ligase IV, a marker for DNA repair. This suggests that BrdU, a nucleoside commonly used for the detection of proliferation, can be incorporated in cells due to ongoing DNA repair as well, without the occurrence of cellular proliferation. Nevertheless, NMDA- or MNU-induced damage combined with the increased levels of key signaling factors, such as sonic hedgehog (Shh)[186], Wingless/Integrated (Wnt)[187] and achaete-scute homologue 1 (Ascl1)[188,189] or combined with growth factor treatment[190] was indicated to provide Müller glia with the ability to proliferate and regenerate neurons in the rodent retina. Indeed, the intraocular injection of NMDA followed by a single injection of epidermal growth factor (EGF) was shown to be a very promising method for Müller glia regeneration[190]. The EGF receptor expression in Müller glia gradually declines as the retina matures, but seems to be upregulated after damage. Stimulation of this receptor by EGF treatment after injury promoted Müller cell proliferation and activated the expression of progenitor genes, similar to what has been reported for retinal regeneration in non-mammalian vertebrates. This, in contrast to injury or EGF injection alone, which failed to induce a proliferative response in the mouse retina[190].

A factor that was not upregulated after NMDA-induced damage in this study, but has previously been shown to be required for reprogramming and proliferation in zebrafish, is the proneural transcription Ascl1[191-193]. In zebrafish, Ascl1 is upregulated in proliferating Müller glia within 6 hours following retinal injury[191,192] and its inhibition greatly limits Müller glia dedifferentiation[192]. One mechanism by which Ascl1 affects retinal regeneration was reported by Ramachandran *et al.*, who demonstrated that Ascl1 is necessary for expression of Lin28, a pluripotency mRNA binding protein, highly expressed in embryonic stem cells. In their work, the authors demonstrate that Ascl1-dependent induction of Lin-28 supports Müller glia dedifferentiation partially by lowering let-7 microRNA levels, therefore removing repression of mRNAs critical for Müller glia dedifferentiation[191]. The lack of Ascl1 upregulation in the mammalian retina following injury, led to the hypothesis that the Ascl1/Lin28/Let7 pathway might dictate the differences in the regeneration potential between mammalian Müller glia and Müller glia of other species. Indeed, Pollak *et al.* showed that virus-mediated overexpression of Ascl1 activates a neurogenic program in injured mouse Müller glia cultures and postnatal retinal explants, and stimulated generation of cells expressing retinal subtype-specific markers and displaying neuron-like physiological

responses. Although Ascl1 drove expression of early markers of many retinal neurons, later markers were more restricted to bipolar neurons[194]. The same research group subsequently demonstrated that forced expression of Ascl1 in mouse Müller glia *in vivo* promoted proliferation and provided amacrine cells, bipolar cells and photoreceptors after retinal injury[188]. However, this regeneration potential was shown to depend on the age of the animal[188,195]. The reprogramming of Müller glia by Ascl1 involves remodeling of the chromatin at the promoters of progenitor genes from a repressive to an active configuration[194]. Since the accessibility of chromatin is limited in adults, mature mouse Müller glia lose their neurogenic capacity, despite Ascl1 overexpression[188]. Nevertheless, the research group of Reh recently reported that Ascl1 overexpression coupled with a histone deacetylase inhibitor treatment can circumvent this limitation and enables adult mice to generate inner retinal neurons from Müller glia after retinal injury[189]. In addition, these regenerated neurons express markers of inner retinal neurons, integrate into the neuronal circuit and were shown to respond to light. This indicates that, although neurogenesis is still limited, the newly formed neurons are functional and could be able to restore vision.

Besides activating Lin-28 expression, Ascl1 also contributes to Müller cell reprogramming by regulating Wnt signaling and its downstream target β-catenin, another major pathway in the regenerative response in zebrafish[196]. After injury of the zebrafish retina, this pathway was shown to be active in Müller cell-derived progenitors and to control their proliferation[193]. It was found that Asc1 suppresses the expression of Dkk, a Wnt signaling inhibitor, thereby increasing expression of Wnt genes, while Wnt in its turn induces Asc1 expression in activated Müller cells[193]. Also in the murine retina, Osakada *et al.* previously provided evidence that Wnt/β-catenin signaling takes part in the small fraction of Müller cell proliferation that occurs following injury, which can further be enhanced by addition of Wnt receptor agonists[187].

While injuring the mammalian retina, whether or not in combination with growth or signaling factors, stimulates Müller glia proliferation and has led to limited neurogenesis, this injury inevitably causes cell death, which is unfavorable and counterproductive for regeneration. A strategy free of injury, that would not require further damage to an affected retina, would therefore be highly preferable. Remarkably, SR delivery of subtoxic doses of glutamate also stimulates adult murine Müller glia to re-enter the cell cycle and induce a rare population to regenerate without causing retinal damage[197]. In the same line, Del Debbio *et al.* demonstrated that stimulation of Wnt and Notch signaling in a rat model of rod photoreceptor degeneration led to the activation of Müller glia without neurotoxin-mediated retinal injury. Moreover, a small subset of activated Müller glia was observed to express rod photoreceptor-specific markers in degenerated outer nuclear layers and a significant

temporal improvement in light perception was demonstrated[198]. Although there was a strong correlation between the improvement in light perception and the number of activated Müller glia expressing opsin, the authors cannot rule out that this functional improvement is (partly) due to the survival of host photoreceptors. More direct evidence would be necessary to confirm the functionality of these cells. In this study a two-step reprogramming method was used to first activate Müller glia by IVTR injection of Wntb2 (activating Wnt) and Jag1 (activating Notch signaling) followed by injection of Shh and DAPT (inhibiting Notch) to promote differentiation along the rod photoreceptor line. Very recently, a similar approach was published by the group of Bo Chen, which provides evidence that, without injury, selective Müller glia gene transfer of β-catenin (using the previously discussed ShH10 AAV-variant) activates Wnt signaling and a single round of Müller cell division in a first step of their reprogramming method. Two weeks later, these activated Müller glia could subsequently be reprogrammed to generate rod photoreceptors by a second gene transfer of transcription factors essential for rod cell fate determination, namely Crx, Otx2 and Nrl. To prove that the new rod photoreceptors were indeed created from Müller glia, a tdTomato gene driven by a rhodopsin promoter was included in the first injection to label all transduced Müller glia. Finally, these Müller glia derived rods were shown to integrate into retinal circuits and restore visual responses in a mouse model of congenital blindness throughout the visual pathway (from the retina to the part of the brain which receives visual signals)[125].

Table 2 (following pages) | Müller cell regeneration in the mammalian retina *in vivo*. Abbreviations: ANT, Ascl1+NMDA+Tamoxifen; Cabp5, Calcium-binding protein 5; Crx, cone-rod homeobox; Dkk1, dickkopf WNT signaling pathway inhibitor 1; DLL1, delta like canonical Notch ligand 1; EGF, epidermal growth factor; FGF, fibroblast growth factor; GAD67, glutamate decarboxylase 1; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; GNAT1, guanine nucleotide binding protein alpha transducing 1; HuC/D, ELAV like RNA binding protein 3/4; i.p., intraperitoneal; INL, inner nuclear layer; IPL, inner plexiform layer; IVT, intravitreal; MG, Muller glia; NeuN, Neuronal Nuclei (Hexaribonucleotide Binding Protein-3); NMDA, *N*-methyl-D-aspartate; NMU, N-methyl-N-nitrosourea; Nrl, neural retina leucine zipper; NSE, neuron-specific enolase; Otx2, orthodenticle homeobox 2; PAX6, paired box 6; PKC, protein kinase C; PR, photoreceptor cell; Prox1, prospero homeobox 1; RA, retinoic acid; RD, retinal degeneration; RGC, retinal ganglion cell; Shh, Sonic hedgehog; SR, subretinal; TSA, trichostatin-A; Wnt, *Wingless*/Integrated; α-AA, α-aminoadipate.

	Animal	Procedure	Effective factor(s)	Effect?	Newly generated cells	Refs
WITH IINJURY	Adult WT rats	IVTR injection of NMDA followed by 3 injections of growth factors every other day, 2 days after injury	RA	• NMDA causes cell death of RGCs and reduces thickness of the IPL • MG proliferation 2 days post-injury • Dedifferentiation of a small number of MG • RA treatment promotes the number of regenerated bipolar cells	• **Bipolar cells** Marker: PKC, NSE • **Rod PRs** Marker: rhodopsin, recoverin	182
	Wnt/β-catenin reporter mice	IVTR injection of NMDA and Wnta3	Wnta3 (Wnt agonist)	• Cell death is observed in the INL and GCL • Wnt3 treatment markedly increases MG proliferation • Inhibition of Wnt signaling by Dkk1 prevents neuronal regeneration after injury	- *Treatment with RA promoted differentiation into rhodopsin-positive PRs (ex vivo)*	187
	Adult WT rat	I.p. injection of MNU followed by a daily IVTR injection of Shh (7 consecutive days)	Shh	• MNU induce PR degeneration • Treating with Shh markedly increased proliferation of MG-derived progenitors • MG-derived progeny preferentially differentiate in rod-like cells	**Rod PRs** Marker: rhodopsin	186
	GAD67-GFP and Grm6-GFP transgenic mice	Intraocular NMDA injection followed by a single injection of growth factors 2 days after injury	• FGF1 • EGF • FGF1+insulin	• NMDA causes cell death of RGCs and amacrine cells • MG re-enter the cell cycle after injury and upregulate progenitor markers (Pax6, Notch and DLL1) • MG subset differentiates into amacrine cells	**Amacrine cells** Markers: Calretinin, NeuN, Prox1 and GAD67	190
	Adult WT rat Newborn Z/EG mice	I.p. injection of MNU	-	• MNU is known to specifically damage PRs • MG undergo gliosis and proliferation • MG subset differentiates in rod PRs and express synaptophysin, indicating possible synapse formation • Transplantation into damaged retina leads to MG migration and rhodopsin expression	**Rod PRs** Markers: rhodopsin, synaptophysin	183

	Animal	Procedure	Effective factor(s)	Effect?	Newly generated cells	Refs
WITH INJURY	Transgenic adult mice with an increased sensitivity to Wnt through the removal of the negative regulator Axin2	Laser induced injury with 2 laser burns per eye	Wnt	• After injury MG proliferate more, but amount of proliferating MG is small • MG adopt the expression patterns of retinal progenitor cells (PAX6) • Some of them express the rod PR marker, rhodopsin	**Rod PRs** Marker: rhodopsin	207
	Transgenic adult and young mice that overexpress Ascl1 in presence of tamoxifen	I.p. injections of tamoxifen (5 consecutive days) to induce expression of Aslc1, followed by IVTR injection of NMDA or continuous exposure to 10,000 lux light for 8 hours	Ascl1	• NMDA induces death of amacrine and RGCs • Ascl1 overexpression alone does not stimulate neurogenesis in MG • After injury Ascl1 promotes dedifferentiation of MG (loss of Sox9) and initiates neurogenic response (proliferation, Otx2 expression), mature markers of neural/PRs are not expressed in adult mice • After injury Ascl1 gives MG the potential to regenerate neurons (amacrine, bipolar and PRs) in young mice	• **Amacrine cells** Markers: HuC/D, Pax6 • **Bipolar cells** Markers: Otx2, Cabp5 • **Rod PRs** Markers: Otx2, recoverin	188
	Transgenic adult mice that overexpress Ascl1 in presence of tamoxifen	I.p. injections of tamoxifen (5 consecutive days) to induce expression of Ascl1, followed by an IVTR injection of NMDA (2 days later) and TSA (4 days later)	Ascl1 + TSA	• NMDA leads to loss of RGCs and reduction in IPL thickness • Administration of tamoxifen + NMDA promotes dedifferentiation of MG (loss of Sox9) and initiates neurogenic response (Otx2 expression) • MG-derived progeny preferentially differentiate in bipolar cells • MG-derived neurons synapse with host retinal neurons and respond to light	• **Bipolar cells** Marker: Cabp5 • **Amacrine cells** Markers: HUC/D, Pax6	189

	Animal	Procedure	Effective factor(s)	Effect?	Newly generated cells	Refs
WITHOUT INJURY	Adult WT mice	SR injection of glutamate or its analogue α-AA	• Glutamate • α-AA	• Subtoxic levels of glutamate and α-AA directly stimulate MG to re-enter the cell cycle and induce neurogenesis • Single injection of α-AA causes upregulation of progenitor markers (Chx10 and nestin) • MG subset differentiates into rod PRs	**Rod PRs** Markers: rhodopsin and recoverin	197
	Rat model of rod PR degeneration (S334ter rats)	2 step method (IVTR) • Wnt2b and Jag1 for Müller cell activation • Shh and DAPT to promote differentiation	• Wnt2b (Wnt agonist) • Jag1 (Notch agonist) • Shh • DAPT (Notch antagonist)	• MG are activated in response to Notch and Wnt signaling stimulation • A rare population of actived MG differentiates along the rod PR lineage • Differentiation is correlated with the improvement in the perception of light	**Rod PRs** Marker: Opsin	198
	Adult WT; Lin28aflox/flox; Lin28bflox/flox mice	Shh10-GFAP-mediated gene transfer of wild type β-catenin, Lin28a or Lin28b	• B-catenin • Lin28	• β-catenin gene transfer activates MG proliferation in the injured retina • β-catenin gene transfer upregulates RNA levels for both Lin28a and Lin28b and downregulates let-7 miRNA levels • Lin28 regulates MG proliferation in both injured and uninjured retinas • A small number of MG differentiates to amacrine cell in all three treatment groups (β-catenin, Lin28a or Lin28b gene transfer)	**Amacrine cells** Markers: PAX6, Syntaxin1, NeuN	113
	$Gnat1^{rd17}Gnat2^{c\ pfl3}$ double mutant mice, a model of congenital blindness (lack PR mediated light responses)	2 step method (IVTR) Shh10-GFAP-mediated gene transfer of • β-catenin for Müller cell proliferation • Otx2, Crx and Nrl for PR differentiation	• B-catenin • Otx2 • Crx • Nrl	• β-catenin gene transfer activates MG proliferation (mostly one cell division) • After the second injection asymmetric cell division occurs with the production of a rod photoreceptor and a daughter MG • Newly formed rod PRs integrate into the retinal circuits and rescue light response in a mice model of congenital blindness, from the retina to the visual cortex	**Rod PRs** Markers: rhodopsin, peripherin-2, GNAT1, recoverin, ribeye	125

A number of important considerations must be taken into account when studying the neurogenic potential of Müller glia. Indeed, it is important to note that so far most research in this field made use of BrdU injection and cell-type specific labeling for Müller cell lineage tracing. In these studies, the presence of BrdU+ in the neurons was generally seen as evidence that they derived from dedifferentiated Müller glia. Since BrdU can be incorporated into newly synthesized DNA of replicating cells, BrdU+ Müller glia in these studies clearly demonstrate that retinal injury and/or signaling pathway alterations stimulate Müller glia to actively replicate their DNA and enter the cell cycle[199]. However, there is no direct evidence that BrdU+ neurons are generated from Müller glia, since BrdU can also be incorporated in cells repairing their DNA, as stated above[185,200]. In addition, also the use of promoter-inducible fluorescent labels (such as GFP, β-gal and tdTomato) can lead to confounding results due to the process of "material transfer", which was demonstrated in recent reports of four different research groups studying photoreceptor transplantation (Pearson *et al.* 2016[201], Ortin-Martinez *et al.* 2017[202], Santos-Ferreira *et al.* 2016 and Singh *et al.* 2016[203]). Using various techniques, the authors demonstrate that following transplantation most of labeled cells detected in the host retina do not represent integrated photoreceptors cells, but are in fact host cells that have exchanged RNA and/or proteins with the transplanted cells. Therefore, also the results of studies transplanting Müller cell-derived neurons into partially degenerated retinas should be interpreted with caution, since material transfer could lead to assumed retinal integration[176,178,204,205]. The fundamental mechanism of this process is currently unknown, but does not seem to be mediated by sustained donor-host cell contact or release of free protein or nucleic acid in the extracellular space[201]. In contrast, the material might be transferred by immune cells or via vesicle release, which should be investigated in future studies[206].

Although many lessons have been learned by studying the zebrafish model, the mechanisms underlying retinal regeneration are complex and many questions remain unanswered. Understanding why zebrafish Müller glia effectively initiate retinal regeneration and why their mammalian counterparts do not, will be off crucial importance. To identify factors that can unlock the mammalian regeneration stimulating potential, gene expression in Müller cells of healthy and injured zebrafish could be compared to the transcriptome of mammalian Müller cells. Also, although Müller cells appear to obtain the first activating signals from injured cells, it cannot yet be excluded that other cells, such as microglia, might also influence the reprogramming process[34]. Examining the contribution of these cells to retinal regeneration and its capacity to activate Müller glia to a state of neuronal differentiation will therefore be crucial for advancing mammalian regeneration.

Inducing the expression of transcription factors, which are known to be important during cell fate determination, make it possible to direct the cell fate of Müller glia derived progenitors and therefore replace the lost cells. At present, most research is focusing on two key factors that play an integral role in the regenerative response in zebrafish, namely Ascl1 and Wnt. Induction or overexpression of these reprogramming factors and their downstream signaling pathways has shown promising results for sight restoration in two recently published studies[125,189]. However, the study of Yao *et al.* stimulating Wnt signaling highlights some important advantages over the studies addressing Ascl1[125]. Indeed, gene transfer of β-catenin alone is sufficient to activate the Wnt pathway and induce Müller glia proliferation in adult mice, whereas overexpression of Ascl1 requires the presence of injury to yield significant Müller cell proliferation. In addition, the authors demonstrate that stimulation of Wnt/β-catenin signaling combined with ectopic expression of photoreceptor transcription factors (Crx, Otx2 and Nrl) led to the generation of new rod photoreceptors. This in contrast to Ascl1-mediated induction of Müller glia dedifferentiation, which mainly results in the production of inner retinal neurons, such as bipolar and amacrine cells. However, it is currently unknown whether the same combination of photoreceptor fate guiding transcription factors could also induce rod photoreceptors production, when coupled with Ascl1 stimulation. A more detailed understanding of the molecular mechanisms that drive retinal precursors to a particular cell type will be essential for the development of efficient therapeutic strategies. So far, for example, more is known about the commitment towards rod photoreceptors than about cone photoreceptor fate. Nevertheless, given their significance in human vision, identifying factors that will direct cone photoreceptor fate will be of crucial importance in the future.

Finally, as most studies on the regenerative potential of Müller cells have been performed in combination with acute injury, the question remains whether this potential will be maintained in chronic diseases, such as RP and AMD, in which Müller cell responses might differ. Since the gliotic response of Müller cells strongly depends on the type and duration of the disease (see earlier), it is likely that chronic injury will also influence the regeneration process. Indeed, Osakada *et al.* reported that the progression of the disease might restrict the regenerative capacity of the retina, because BrdU incorporation in Müller cells seems to be lost as degeneration proceeds[187]. The study of Yao *et al.* offers much hope for Müller cell-dependent regeneration therapies in mammals, but the use of this strategy in more advanced diseases in which larger numbers of cells are lost or which lack proper synaptic connection, still needs to be analyzed. Surely, the ultimate aim is to rescue vision in the latest stages of degeneration. Precise analysis of how new neurons rewire themselves into the retina after Müller cell regeneration, especially in the absence of host photoreceptor cells, will be necessary to generate a healthy new circuitry and visual function.

CONCLUDING REMARKS

The Müller cell is one of the most multifunctional cells in the retina, which arouses growing interest due to its myriad of functions related to the healthy and diseased retina. As described in this chapter, Müller cells strongly monitor retinal homeostasis and are of vital importance for the proper functioning of the retina. However, in response to retinal imbalance, activated Müller cells can also contribute to retinal degeneration and impede regenerative processes by the formation of glial scars. Though the past couple of years have witnessed an enormous growth in our knowledge regarding the functional roles of Müller cells and their gliotic response, their use as targets for new therapeutic approaches is a nascent field. In particular, gene therapy could be an interesting method to selectively address Müller cells and modulate some of their cellular mechanisms in our advantage for medical treatment. In this chapter we focused on two therapeutic fields in which Müller cells are becoming the central subject of many studies. First, their unique morphology which allows them to interact with all neuronal cell types renders them ideally located for interventions that aim to inhibit neuronal cell death, a strategy called neuroprotection. Second, their latent stem cell potential posits Müller cells as an excellent target for regenerative therapies which aim to stimulate endogenous replacement of injured retinal neurons.

It is worth mentioning that the use of Müller cells for medical treatment is not restricted to these two applications. Their active participation in innumerous retinal processes make them a possible intervention point for multiple ocular therapies. For instance, some strategies are looking into the suppression of VEGF secretion specifically secreted by Müller cells in order to control NV in diseases such as retinopathy of prematurity, AMD and diabetic retinopathy[208-210]. In addition, Müller glial cells are also of interest for gene replacement therapy, since some recessive genetic mutations of genes expressed in Müller cells, such as CRALBP and CRB1, have been reported to cause retinal diseases[126,211,212]. Overall, it is clear that Müller cells are an intriguing retinal cell type, with high versatility for therapeutic interventions. Finally, as our knowledge on the functional roles of Müller cells continues to increase, together with improved targeting and the development of more potent and controllable viral- and non-viral delivery systems, Müller cells are expected to take a prominent place in the development of future therapeutic approaches to treat retinal diseases[33].

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REFERENCES

1 MacDonald, R. B., Charlton-Perkins, M. & Harris, W. A. Mechanisms of Muller glial cell morphogenesis. Current opinion in neurobiology 47, 31-37, doi:10.1016/j.conb.2017.08.005 (2017).

2 Oliveira, A. V., Rosa da Costa, A. M. & Silva, G. A. Non-viral strategies for ocular gene delivery. Materials science & engineering. C, Materials for biological applications 77, 1275-1289, doi:10.1016/j.msec.2017.04.068 (2017).

3 Forrester J. V., D. A. D., McMenamin P. G., Roberts F. PE. The Eye: Basic Sciences in Practice. Elsevier, 1-661 p. (2016).

4 Hoon, M., Okawa, H., Della Santina, L. & Wong, R. O. L. Functional architecture of the retina: Development and disease. Progress in retinal and eye research 42, 44-84, doi:10.1016/j.preteyeres.2014.06.003 (2014).

5 Sung, C. H. & Chuang, J. Z. The cell biology of vision. Journal of Cell Biology 190, 953-963, doi:10.1083/jcb.201006020 (2010).

6 Masland, R. H. The neuronal organization of the retina. Neuron 76, 266-280, doi:10.1016/j.neuron.2012.10.002 (2012).

7 Schnitzer, J. Immunocytochemical studies on the development of astrocytes, Muller (glial) cells, and oligodendrocytes in the rabbit retina. Brain research. Developmental brain research 44, 59-72 (1988).

8 Vecino, E., Rodriguez, F. D., Ruzafa, N., Pereiro, X. & Sharma, S. C. Glia-neuron interactions in the mammalian retina. Progress in retinal and eye research 51, 1-40, doi:10.1016/j.preteyeres.2015.06.003 (2016).

9 Rathnasamy, G., Foulds, W. S., Ling, E. A. & Kaur, C. Retinal microglia - A key player in healthy and diseased retina. Progress in neurobiology 173, 18-40, doi:10.1016/j.pneurobio.2018.05.006 (2019).

10 Li, L., Eter, N. & Heiduschka, P. The microglia in healthy and diseased retina. Experimental eye research 136, 116-130, doi:10.1016/j.exer.2015.04.020 (2015).

11 Selvam, S., Kumar, T. & Fruttiger, M. Retinal vasculature development in health and disease. Progress in retinal and eye research 63, 1-19, doi:10.1016/j.preteyeres.2017.11.001 (2018).

12 Horng, S. et al. Astrocytic tight junctions control inflammatory CNS lesion pathogenesis. J Clin Invest 127, 3136-3151, doi:10.1172/JCI91301 (2017).

13 Bok, D. The Retinal-Pigment Epithelium - a Versatile Partner in Vision. J Cell Sci, 189-195 (1993).

14 Guymer, R., Luthert, P. & Bird, A. Changes in Bruch's membrane and related structures with age. Progress in retinal and eye research 18, 59-90, doi:Doi 10.1016/S1350-9462(98)00012-3 (1999).

15 Steinberg, R. H. Interactions between the Retinal-Pigment Epithelium and the Neural Retina. Doc Ophthalmol 60, 327-346, doi:Doi 10.1007/Bf00158922 (1985).

16 Sparrrow, J. R., Hicks, D. & Hamel, C. P. The Retinal Pigment Epithelium in Health and Disease. Curr Mol Med 11, 802-823 (2011).

17 Yildirim, Z., Ucgun, N. I. & Yildirim, F. The role of oxidative stress and antioxidants in the pathogenesis of age-related macular degeneration. Clinics 66, 743-746, doi:10.1590/S1807-59322011000500006 (2011).

18 Strauss, O. The role of retinal pigment epithelium in visual functions. Ophthalmologe 106, 299-304, doi:10.1007/s00347-008-1869-x (2009).

19 Cunha-Vaz, J., Bernardes, R. & Lobo, C. Blood-retinal barrier. European journal of ophthalmology 21 Suppl 6, S3-9, doi:10.5301/EJO.2010.6049 (2011).

20 Young, R. W. Renewal systems in rods and cones. Annals of ophthalmology 5, 843-854 (1973).

21 Grierson, I. et al. Development, Repair and Regeneration of the Retinal-Pigment Epithelium. Eye 8, 255-262, doi:Doi 10.1038/Eye.1994.54 (1994).

22 Cayouette, M., Smith, S. B., Becerra, S. P. & Gravel, C. Pigment epithelium-derived factor delays the death of photoreceptors in mouse models of inherited retinal degenerations. Neurobiology of disease 6, 523-532, doi:10.1006/nbdi.1999.0263 (1999).

23 Lacour, M. The Retinal-Pigment Epithelium Controls the Potassium Activity in the Subretinal Space. Acta Ophthalmol 63, 9-10 (1985).

24 Shimura, M. et al. Expression and permeation properties of the K(+) channel Kir7.1 in the retinal pigment epithelium. The Journal of physiology 531, 329-346, doi:10.1111/j.1469-7793.2001.0329i.x (2001).

25 Kenyon, E., Maminishkis, A., Joseph, D. P. & Miller, S. S. Apical and basolateral membrane mechanisms that regulate pH(i) in bovine retinal pigment epithelium. Am J Physiol-Cell Ph 273, C456-C472 (1997).

26 Strauss, O. The retinal pigment epithelium in visual function. Physiological reviews 85, 845-881, doi:10.1152/physrev.00021.2004 (2005).

27 H., M. Zur Histologie der Netzhaut. Zeitschrift für Wiss Zool 3, 234–237 (1851).

28 Bringmann, A. et al. Muller cells in the healthy and diseased retina. Progress in retinal and eye research 25, 397-424, doi:10.1016/j.preteyeres.2006.05.003 (2006).

29 Reichenbach, A. & Bringmann, A. New functions of Muller cells. Glia 61, 651-678, doi:10.1002/glia.22477 (2013).

30 Reichenbach, A. et al. Muller glial cells of the tree shrew retina. The Journal of comparative neurology 360, 257-270, doi:10.1002/cne.903600205 (1995).

31 Vecino, E., Rodriguez, F. D., Ruzafa, N., Pereiro, X. & Sharma, S. C. Glia-neuron interactions in the mammalian retina. Progress in retinal and eye research 51, 1-40, doi:10.1016/j.preteyeres.2015.06.003 (2016).

32 Winkler, B. S., Arnold, M. J., Brassell, M. A. & Puro, D. G. Energy metabolism in human retinal Muller cells. Investigative ophthalmology & visual science 41, 3183-3190 (2000).

33 Bringmann, A. et al. Cellular signaling and factors involved in Muller cell gliosis: Neuroprotective and detrimental effects. Progress in retinal and eye research 28, 423-451, doi:10.1016/j.preteyeres.2009.07.001 (2009).

34 Xia, X. & Ahmad, I. Unlocking the Neurogenic Potential of Mammalian Muller Glia. International journal of stem cells 9, 169-175, doi:10.15283/ijsc16020 (2016).

35 Chohan, A., Singh, U., Kumar, A. & Kaur, J. Muller stem cell dependent retinal regeneration. Clinica chimica acta; international journal of clinical chemistry 464, 160-164, doi:10.1016/j.cca.2016.11.030 (2017).

36 Madelaine, R. & Mourrain, P. Endogenous retinal neural stem cell reprogramming for neuronal regeneration. Neural regeneration research 12, 1765-1767, doi:10.4103/1673-5374.219028 (2017).

37 Campbell, M. & Humphries, P. The blood-retina barrier: tight junctions and barrier modulation. Advances in experimental medicine and biology 763, 70-84 (2012).

38 Tout, S., Chan-Ling, T., Hollander, H. & Stone, J. The role of Muller cells in the formation of the blood-retinal barrier. Neuroscience 55, 291-301 (1993).

39 Shen, W. et al. Conditional Mullercell ablation causes independent neuronal and vascular pathologies in a novel transgenic model. The Journal of neuroscience : the official journal of the Society for Neuroscience 32, 15715-15727, doi:10.1523/JNEUROSCI.2841-12.2012 (2012).

40 Eichler, W., Yafai, Y., Wiedemann, P. & Reichenbach, A. Angiogenesis-related factors derived from retinal glial (Muller) cells in hypoxia. Neuroreport 15, 1633-1637 (2004).

41 Eichler, W., Yafai, Y., Keller, T., Wiedemann, P. & Reichenbach, A. PEDF derived from glial Muller cells: a possible regulator of retinal angiogenesis. Experimental cell research 299, 68-78, doi:10.1016/j.yexcr.2004.05.020 (2004).

42 Franze, K. et al. Muller cells are living optical fibers in the vertebrate retina. Proceedings of the National Academy of Sciences of the United States of America 104, 8287-8292, doi:10.1073/pnas.0611180104 (2007).

43 Young, R. W. The renewal of photoreceptor cell outer segments. The Journal of cell biology 33, 61-72 (1967).

44 Long, K. O., Fisher, S. K., Fariss, R. N. & Anderson, D. H. Disc shedding and autophagy in the cone-dominant ground squirrel retina. Experimental eye research 43, 193-205 (1986).

45 Bejarano-Escobar, R., Sanchez-Calderon, H., Otero-Arenas, J., Martin-Partido, G. & Francisco-Morcillo, J. Muller glia and phagocytosis of cell debris in retinal tissue. Journal of anatomy 231, 471-483, doi:10.1111/joa.12653 (2017).

46 Jablonski, M. M. & Iannaccone, A. Targeted disruption of Muller cell metabolism induces photoreceptor dysmorphogenesis. Glia 32, 192-204 (2000).

47 Wang, X., Iannaccone, A. & Jablonski, M. M. Contribution of Muller cells toward the regulation of photoreceptor outer segment assembly. Neuron glia biology 1, 1-6, doi:10.1017/s1740925x05000049 (2005).

48 Wang, J. S. & Kefalov, V. J. The cone-specific visual cycle. Progress in retinal and eye research 30, 115-128, doi:10.1016/j.preteyeres.2010.11.001 (2011).

49 Brew, H. & Attwell, D. Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells. Nature 327, 707-709, doi:10.1038/327707a0 (1987).

50 Bringmann, A., Grosche, A., Pannicke, T. & Reichenbach, A. GABA and Glutamate Uptake and Metabolism in Retinal Glial (Muller) Cells. Frontiers in endocrinology 4, 48, doi:10.3389/fendo.2013.00048 (2013).

51 Huster, D., Reichenbach, A. & Reichelt, W. The glutathione content of retinal Muller (glial) cells: effect of pathological conditions. Neurochem Int 36, 461-469, doi:Doi 10.1016/S0197-0186(99)00149-7 (2000).

52 Hurley, J. B., Lindsay, K. J. & Du, J. Glucose, lactate, and shuttling of metabolites in vertebrate retinas. Journal of neuroscience research 93, 1079-1092, doi:10.1002/jnr.23583 (2015).

53 Poitry, S., Poitry-Yamate, C., Ueberfeld, J., MacLeish, P. R. & Tsacopoulos, M. Mechanisms of glutamate metabolic signaling in retinal glial (Muller) cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 20, 1809-1821 (2000).

54 Poitryyamate, C. L., Poitry, S. & Tsacopoulos, M. Lactate Released by Muller Glial-Cells Is Metabolized by Photoreceptors from Mammalian Retina. Journal of Neuroscience 15, 5179-5191 (1995).

55 Reichenbach, A., Henke, A., Eberhardt, W., Reichelt, W. & Dettmer, D. K+ ion regulation in retina. Canadian journal of physiology and pharmacology 70 Suppl, S239-247 (1992).

56 Newman, E. & Reichenbach, A. The Muller cell: a functional element of the retina. Trends in neurosciences 19, 307-312 (1996).

57 Newman, E. A. Inward-rectifying potassium channels in retinal glial (Muller) cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 13, 3333-3345 (1993).

58 Nagelhus, E. A. et al. Aquaporin-4 water channel protein in the rat retina and optic nerve: polarized expression in Muller cells and fibrous astrocytes. The Journal of neuroscience : the official journal of the Society for Neuroscience 18, 2506-2519 (1998).

59 Bringmann, A. et al. Neuronal versus glial cell swelling in the ischaemic retina. Acta ophthalmologica Scandinavica 83, 528-538, doi:10.1111/j.1600-0420.2005.00565.x (2005).

60 Cao, W., Wen, R., Li, F., Cheng, T. & Steinberg, R. H. Induction of basic fibroblast growth factor mRNA by basic fibroblast growth factor in Muller cells. Investigative ophthalmology & visual science 38, 1358-1366 (1997).

61 Zhu, X. et al. Expression of glial cell line-derived neurotrophic factor and its receptors in cultured retinal Muller cells under high glucose circumstance. Anatomical record 295, 532-539, doi:10.1002/ar.22404 (2012).

62 Taylor, S., Srinivasan, B., Wordinger, R. J. & Roque, R. S. Glutamate stimulates neurotrophin expression in cultured Muller cells. Brain research. Molecular brain research 111, 189-197 (2003).

63 Seki, M. et al. Muller Cells as a source of brain-derived neurotrophic factor in the retina: noradrenaline upregulates brain-derived neurotrophic factor levels in cultured rat Muller cells. Neurochemical research 30, 1163-1170, doi:10.1007/s11064-005-7936-7 (2005).

64 Morimoto, T. et al. Transcorneal electrical stimulation rescues axotomized retinal ganglion cells by activating endogenous retinal IGF-1 system. Investigative ophthalmology & visual science 46, 2147-2155, doi:10.1167/iovs.04-1339 (2005).

65 Paulus, Y. M. & Campbell, J. P. Neuroprotection and Retinal Diseases. Developments in ophthalmology 55, 322-329, doi:10.1159/000434703 (2016).

66 Harada, T. et al. Microglia-Muller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 22, 9228-9236 (2002).

67 Bringmann, A. & Wiedemann, P. Muller glial cells in retinal disease. Ophthalmologica. Journal international d'ophtalmologie. International journal of ophthalmology. Zeitschrift fur Augenheilkunde 227, 1-19, doi:10.1159/000328979 (2012).

68 Lu, Y. B. et al. Reactive glial cells: increased stiffness correlates with increased intermediate filament expression. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 25, 624-631, doi:10.1096/fj.10-163790 (2011).

69 Lewis, G. P. & Fisher, S. K. Up-regulation of glial fibrillary acidic protein in response to retinal injury: its potential role in glial remodeling and a comparison to vimentin expression. International review of cytology 230, 263-290 (2003).

70 Kaur, C. et al. Blood-retinal barrier disruption and ultrastructural changes in the hypoxic retina in adult rats: the beneficial effect of melatonin administration. The Journal of pathology 212, 429-439, doi:10.1002/path.2195 (2007).

71 Kim, I. B. et al. Reaction of Muller cells after increased intraocular pressure in the rat retina. Experimental brain research 121, 419-424 (1998).

72 Layton, C. J., Becker, S. & Osborne, N. N. The effect of insulin and glucose levels on retinal glial cell activation and pigment epithelium-derived fibroblast growth factor-2. Molecular vision 12, 43-54 (2006).

73 Xue, L. P. et al. Muller glial cells express nestin coupled with glial fibrillary acidic protein in experimentally induced glaucoma in the rat retina. Neuroscience 139, 723-732, doi:10.1016/j.neuroscience.2005.12.032 (2006).

74 Hippert, C. et al. Muller glia activation in response to inherited retinal degeneration is highly varied and disease-specific. PloS one 10, e0120415, doi:10.1371/journal.pone.0120415 (2015).

75 Chen, H. & Weber, A. J. Expression of glial fibrillary acidic protein and glutamine synthetase by Muller cells after optic nerve damage and intravitreal application of brain-derived neurotrophic factor. Glia 38, 115-125 (2002).

76 Fischer, A. J. & Reh, T. A. Potential of Muller glia to become neurogenic retinal progenitor cells. Glia 43, 70-76, doi:10.1002/glia.10218 (2003).

77 Wan, J. & Goldman, D. Retina regeneration in zebrafish. Current opinion in genetics & development 40, 41-47, doi:10.1016/j.gde.2016.05.009 (2016).

78 Goldman, D. Muller glial cell reprogramming and retina regeneration. Nature reviews. Neuroscience 15, 431-442, doi:10.1038/nrn3723 (2014).

79 Ridano, M. E. et al. Galectin-1 expression imprints a neurovascular phenotype in proliferative retinopathies and delineates responses to anti-VEGF. Oncotarget 8, 32505-32522, doi:10.18632/oncotarget.17129 (2017).

80 Pannicke, T. et al. A potassium channel-linked mechanism of glial cell swelling in the postischemic retina. Molecular and cellular neurosciences 26, 493-502, doi:10.1016/j.mcn.2004.04.005 (2004).

81 Bringmann, A., Reichenbach, A. & Wiedemann, P. Pathomechanisms of cystoid macular edema. Ophthalmic research 36, 241-249, doi:10.1159/000081203 (2004).

82 Bringmann, A. & Wiedemann, P. Involvement of Muller glial cells in epiretinal membrane formation. Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie 247, 865-883, doi:10.1007/s00417-009-1082-x (2009).

83 Subirada, P. V. et al. A journey into the retina: Muller glia commanding survival and death. The European journal of neuroscience 47, 1429-1443, doi:10.1111/ejn.13965 (2018).

84 Hippert, C., Graca, A. B. & Pearson, R. A. Gliosis Can Impede Integration Following Photoreceptor Transplantation into the Diseased Retina. Advances in experimental medicine and biology 854, 579-585, doi:10.1007/978-3-319-17121-0_77 (2016).

85 Flaxman, S. R. et al. Global causes of blindness and distance vision impairment 1990-2020: a systematic review and meta-analysis. The Lancet. Global health 5, e1221-e1234, doi:10.1016/S2214-109X(17)30393-5 (2017).

86 Parmeggiani, F. Clinics, epidemiology and genetics of retinitis pigmentosa. Current genomics 12, 236-237, doi:10.2174/138920211795860080 (2011).

87 Bowes Rickman, C., Farsiu, S., Toth, C. A. & Klingeborn, M. Dry age-related macular degeneration: mechanisms, therapeutic targets, and imaging. Investigative ophthalmology & visual science 54, ORSF68-80, doi:10.1167/iovs.13-12757 (2013).

88 Qu, J., Wang, D. & Grosskreutz, C. L. Mechanisms of retinal ganglion cell injury and defense in glaucoma. Experimental eye research 91, 48-53, doi:10.1016/j.exer.2010.04.002 (2010).

89 Bernstein, S. L., Johnson, M. A. & Miller, N. R. Nonarteritic anterior ischemic optic neuropathy (NAION) and its experimental models. Progress in retinal and eye research 30, 167-187, doi:10.1016/j.preteyeres.2011.02.003 (2011).

90 Bennett, J. Taking Stock of Retinal Gene Therapy: Looking Back and Moving Forward. Molecular therapy : the journal of the American Society of Gene Therapy 25, 1076-1094, doi:10.1016/j.ymthe.2017.03.008 (2017).

91 Planul, A. & Dalkara, D. Vectors and Gene Delivery to the Retina. Annual review of vision science 3, 121-140, doi:10.1146/annurev-vision-102016-061413 (2017).

92 Bennett, J., Wilson, J., Sun, D., Forbes, B. & Maguire, A. Adenovirus vector-mediated in vivo gene transfer into adult murine retina. Investigative ophthalmology & visual science 35, 2535-2542 (1994).

93 Di Polo, A., Aigner, L. J., Dunn, R. J., Bray, G. M. & Aguayo, A. J. Prolonged delivery of brain-derived neurotrophic factor by adenovirus-infected Muller cells temporarily rescues injured retinal ganglion cells. Proceedings of the National Academy of Sciences of the United States of America 95, 3978-3983, doi:DOI 10.1073/pnas.95.7.3978 (1998).

94 Isenmann, S., Klocker, N., Gravel, C. & Bahr, M. Short communication: protection of axotomized retinal ganglion cells by adenovirally delivered BDNF in vivo. The European journal of neuroscience 10, 2751-2756 (1998).

95 Gauthier, R., Joly, S., Pernet, V., Lachapelle, P. & Di Polo, A. Brain-derived neurotrophic factor gene delivery to muller glia preserves structure and function of light-damaged

photoreceptors. Investigative ophthalmology & visual science 46, 3383-3392, doi:10.1167/iovs.05-0362 (2005).

96 Lamartina, S. et al. Helper-dependent adenovirus for the gene therapy of proliferative retinopathies: stable gene transfer, regulated gene expression and therapeutic efficacy. The journal of gene medicine 9, 862-874, doi:10.1002/jgm.1083 (2007).

97 Balaggan, K. S. & Ali, R. R. Ocular gene delivery using lentiviral vectors. Gene therapy 19, 145-153, doi:10.1038/gt.2011.153 (2012).

98 Rolling, F. Recombinant AAV-mediated gene transfer to the retina: gene therapy perspectives. Gene therapy 11, S26-S32, doi:10.1038/sj.gt.3302366 (2004).

99 Lebherz, C., Maguire, A., Tang, W., Bennett, J. & Wilson, J. M. Novel AAV serotypes for improved ocular gene transfer. J Gene Med 10, 375-382, doi:10.1002/jgm.1126 (2008).

100 Greenberg, K. P., Geller, S. F., Schaffer, D. V. & Flannery, J. G. Targeted transgene expression in muller glia of normal and diseased retinas using lentiviral vectors. Investigative ophthalmology & visual science 48, 1844-1852, doi:10.1167/iovs.05-1570 (2007).

101 Cavalieri, V., Baiamonte, E. & Lo Iacono, M. Non-Primate Lentiviral Vectors and Their Applications in Gene Therapy for Ocular Disorders. Viruses-Basel 10, doi:Artn 31610.3390/V10060316 (2018).

102 Schon, C., Biel, M. & Michalakis, S. Retinal gene delivery by adeno-associated virus (AAV) vectors: Strategies and applications. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V 95, 343-352, doi:10.1016/j.ejpb.2015.01.009 (2015).

103 Dalkara, D., Goureau, O., Marazova, K. & Sahel, J. A. Let There Be Light: Gene and Cell Therapy for Blindness. Human gene therapy 27, 134-147, doi:10.1089/hum.2015.147 (2016).

104 Dorrell, M. I. et al. Antioxidant or neurotrophic factor treatment preserves function in a mouse model of neovascularization-associated oxidative stress. J Clin Invest 119, 611-623, doi:10.1172/JCI35977 (2009).

105 Prentice, H. M., Biswal, M. R., Dorey, C. K. & Blanks, J. C. Hypoxia-Regulated Retinal Glial Cell-Specific Promoter for Potential Gene Therapy in Disease. Investigative ophthalmology & visual science 52, 8562-8570, doi:10.1167/iovs.10-6835 (2011).

106 Dalkara, D. et al. AAV mediated GDNF secretion from retinal glia slows down retinal degeneration in a rat model of retinitis pigmentosa. Molecular therapy : the journal of the American Society of Gene Therapy 19, 1602-1608, doi:10.1038/mt.2011.62 (2011).

107 Hickey, D. G. et al. Tropism of engineered and evolved recombinant AAV serotypes in the rd1 mouse and ex vivo primate retina. Gene therapy 24, 787-800, doi:10.1038/gt.2017.85 (2017).

108 Maheshri, N., Koerber, J. T., Kaspar, B. K. & Schaffer, D. V. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. Nature biotechnology 24, 198-204, doi:10.1038/nbt1182 (2006).

109 Klimczak, R. R., Koerber, J. T., Dalkara, D., Flannery, J. G. & Schaffer, D. V. A novel adeno-associated viral variant for efficient and selective intravitreal transduction of rat Muller cells. PloS one 4, e7467, doi:10.1371/journal.pone.0007467 (2009).

110 Vacca, O. et al. AAV-mediated gene delivery in Dp71-null mouse model with compromised barriers. Glia 62, 468-476, doi:10.1002/glia.22617 (2014).

111 Byrne, L. C. et al. Retinoschisin gene therapy in photoreceptors, Muller glia or all retinal cells in the Rs1h(-/-) mouse. Gene therapy 21, 585-592, doi:10.1038/gt.2014.31 (2014).

112 Pellissier, L. P. et al. Specific tools for targeting and expression in Muller glial cells. Molecular therapy. Methods & clinical development 1, 14009, doi:10.1038/mtm.2014.9 (2014).

113 Yao, K. et al. Wnt Regulates Proliferation and Neurogenic Potential of Muller Glial Cells via a Lin28/let-7 miRNA-Dependent Pathway in Adult Mammalian Retinas. Cell reports 17, 165-178, doi:10.1016/j.celrep.2016.08.078 (2016).

114 Apaolaza, P. S. et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. Biomaterials 90, 40-49, doi:10.1016/j.biomaterials.2016.03.004 (2016).

115 del Pozo-Rodriguez, A., Delgado, D., Solinis, M. A., Gascon, A. R. & Pedraz, J. L. Solid lipid nanoparticles for retinal gene therapy: transfection and intracellular trafficking in RPE cells. International journal of pharmaceutics 360, 177-183, doi:10.1016/j.ijpharm.2008.04.023 (2008).

116 Andrieu-Soler, C. et al. Intravitreous injection of PLGA microspheres encapsulating GDNF promotes the survival of photoreceptors in the rd1/rd1 mouse. Molecular vision 11, 1002-1011 (2005).

117 Peters, T. et al. Evaluation of polyesteramide (PEA) and polyester (PLGA) microspheres as intravitreal drug delivery systems in albino rats. Biomaterials 124, 157-168, doi:10.1016/j.biomaterials.2017.02.006 (2017).

118 Kim, H., Robinson, S. B. & Csaky, K. G. Investigating the movement of intravitreal human serum albumin nanoparticles in the vitreous and retina. Pharm Res 26, 329-337, doi:10.1007/s11095-008-9745-6 (2009).

119 Huang, D., Chen, Y. S. & Rupenthal, I. D. Hyaluronic Acid Coated Albumin Nanoparticles for Targeted Peptide Delivery to the Retina. Molecular pharmaceutics 14, 533-545, doi:10.1021/acs.molpharmaceut.6b01029 (2017).

120 Miller, A. M. & Dean, D. A. Tissue-specific and transcription factor-mediated nuclear entry of DNA. Advanced drug delivery reviews 61, 603-613, doi:10.1016/j.addr.2009.02.008 (2009).

121 Huang, D., Chen, Y. S., Green, C. R. & Rupenthal, I. D. Hyaluronic acid coated albumin nanoparticles for targeted peptide delivery in the treatment of retinal ischaemia. Biomaterials 168, 10-23, doi:10.1016/j.biomaterials.2018.03.034 (2018).

122 Koo, H. et al. The movement of self-assembled amphiphilic polymeric nanoparticles in the vitreous and retina after intravitreal injection. Biomaterials 33, 3485-3493, doi:10.1016/j.biomaterials.2012.01.030 (2012).

123 Aartsen, W. M. et al. GFAP-Driven GFP Expression in Activated Mouse Muller Glial Cells Aligning Retinal Blood Vessels Following Intravitreal Injection of AAV2/6 Vectors. PloS one 5, doi:ARTN e1238710.1371/journal.pone.0012387 (2010).

124 Koerber, J. T. et al. Molecular evolution of adeno-associated virus for enhanced glial gene delivery. Molecular therapy : the journal of the American Society of Gene Therapy 17, 2088-2095, doi:10.1038/mt.2009.184 (2009).

125 Yao, K. et al. Restoration of vision after de novo genesis of rod photoreceptors in mammalian retinas. Nature 560, 484-488, doi:10.1038/s41586-018-0425-3 (2018).

126 Pellissier, L. P. et al. Gene therapy into photoreceptors and Muller glial cells restores retinal structure and function in CRB1 retinitis pigmentosa mouse models. Hum Mol Genet 24, 3104-3118, doi:10.1093/hmg/ddv062 (2015).

127 Dawbarn, D. & Allen, S. J. Neurotrophins and neurodegeneration. Neuropathology and applied neurobiology 29, 211-230 (2003).

128 Kolomeyer, A. M. & Zarbin, M. A. Trophic factors in the pathogenesis and therapy for retinal degenerative diseases. Surv Ophthalmol 59, 134-165, doi:10.1016/j.survophthal.2013.09.004 (2014).

129 Pardue, M. T. & Allen, R. S. Neuroprotective strategies for retinal disease. Progress in retinal and eye research 65, 50-76, doi:10.1016/j.preteyeres.2018.02.002 (2018).

130 Wen, R., Tao, W., Li, Y. & Sieving, P. A. CNTF and retina. Progress in retinal and eye research 31, 136-151, doi:10.1016/j.preteyeres.2011.11.005 (2012).

131 van Adel, B. A., Arnold, J. M., Phipps, J., Doering, L. C. & Ball, A. K. Ciliary neurotrophic factor protects retinal ganglion cells from axotomy-induced apoptosis via modulation of retinal glia in vivo. Journal of neurobiology 63, 215-234, doi:10.1002/neu.20117 (2005).

132 Pease, M. E. et al. Effect of CNTF on retinal ganglion cell survival in experimental glaucoma. Investigative ophthalmology & visual science 50, 2194-2200, doi:10.1167/iovs.08-3013 (2009).

133 Takahata, K. et al. Retinal neuronal death induced by intraocular administration of a nitric oxide donor and its rescue by neurotrophic factors in rats. Investigative ophthalmology & visual science 44, 1760-1766 (2003).

134 Hellstrom, M., Pollett, M. A. & Harvey, A. R. Post-injury delivery of rAAV2-CNTF combined with short-term pharmacotherapy is neuroprotective and promotes extensive axonal regeneration after optic nerve trauma. Journal of neurotrauma 28, 2475-2483, doi:10.1089/neu.2011.1928 (2011).

135 Liang, F. Q. et al. Long-term protection of retinal structure but not function using RAAV.CNTF in animal models of retinitis pigmentosa. Molecular therapy : the journal of the American Society of Gene Therapy 4, 461-472, doi:10.1006/mthe.2001.0473 (2001).

136 Buch, P. K. et al. In contrast to AAV-mediated Cntf expression, AAV-mediated Gdnf expression enhances gene replacement therapy in rodent models of retinal degeneration. Molecular therapy : the journal of the American Society of Gene Therapy 14, 700-709, doi:10.1016/j.ymthe.2006.05.019 (2006).

137 McGill, T. J. et al. Intraocular CNTF reduces vision in normal rats in a dose-dependent manner. Investigative ophthalmology & visual science 48, 5756-5766, doi:10.1167/iovs.07-0054 (2007).

138 Wen, R. et al. Regulation of rod phototransduction machinery by ciliary neurotrophic factor. The Journal of neuroscience : the official journal of the Society for Neuroscience 26, 13523-13530, doi:10.1523/JNEUROSCI.4021-06.2006 (2006).

139 Kimura, A., Namekata, K., Guo, X., Harada, C. & Harada, T. Neuroprotection, Growth Factors and BDNF-TrkB Signalling in Retinal Degeneration. International journal of molecular sciences 17, doi:10.3390/ijms17091584 (2016).

140 Chen, H. & Weber, A. J. BDNF enhances retinal ganglion cell survival in cats with optic nerve damage. Investigative ophthalmology & visual science 42, 966-974 (2001).

141 Leaver, S. G. et al. AAV-mediated expression of CNTF promotes long-term survival and regeneration of adult rat retinal ganglion cells. Gene therapy 13, 1328-1341, doi:10.1038/sj.gt.3302791 (2006).

142 Mansour-Robaey, S., Clarke, D. B., Wang, Y. C., Bray, G. M. & Aguayo, A. J. Effects of ocular injury and administration of brain-derived neurotrophic factor on survival and regrowth of axotomized retinal ganglion cells. Proceedings of the National Academy of Sciences of the United States of America 91, 1632-1636 (1994).

143 Cheng, L., Sapieha, P., Kittlerova, P., Hauswirth, W. W. & Di Polo, A. TrkB gene transfer protects retinal ganglion cells from axotomy-induced death in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience 22, 3977-3986, doi:20026382 (2002).

144 Hu, Y., Cho, S. & Goldberg, J. L. Neurotrophic effect of a novel TrkB agonist on retinal ganglion cells. Investigative ophthalmology & visual science 51, 1747-1754, doi:10.1167/iovs.09-4450 (2010).

145 Bai, Y. et al. An agonistic TrkB mAb causes sustained TrkB activation, delays RGC death, and protects the retinal structure in optic nerve axotomy and in glaucoma. Investigative ophthalmology & visual science 51, 4722-4731, doi:10.1167/iovs.09-5032 (2010).

146 Wahlin, K. J., Campochiaro, P. A., Zack, D. J. & Adler, R. Neurotrophic factors cause activation of intracellular signaling pathways in Muller cells and other cells of the inner retina, but not photoreceptors. Investigative ophthalmology & visual science 41, 927-936 (2000).

147 Dong, A., Shen, J., Krause, M., Hackett, S. F. & Campochiaro, P. A. Increased expression of glial cell line-derived neurotrophic factor protects against oxidative damage-induced retinal degeneration. Journal of neurochemistry 103, 1041-1052, doi:10.1111/j.1471-4159.2007.04839.x (2007).

148 Schlichtenbrede, F. C. et al. Intraocular gene delivery of ciliary neurotrophic factor results in significant loss of retinal function in normal mice and in the Prph2Rd2/Rd2 model of retinal degeneration. Gene therapy 10, 523-527, doi:10.1038/sj.gt.3301929 (2003).

149 Tschernutter, M. et al. Long-term preservation of retinal function in the RCS rat model of retinitis pigmentosa following lentivirus-mediated gene therapy. Gene therapy 12, 694-701, doi:10.1038/sj.gt.3302460 (2005).

150 Ishikawa, H. et al. Effect of GDNF gene transfer into axotomized retinal ganglion cells using in vivo electroporation with a contact lens-type electrode. Gene therapy 12, 289-298, doi:10.1038/sj.gt.3302277 (2005).

151 Wu, W. C. et al. Long-term safety of GDNF gene delivery in the retina. Current eye research 30, 715-722, doi:10.1080/02713680591005922 (2005).

152 Polato, F. & Becerra, S. P. Pigment Epithelium-Derived Factor, a Protective Factor for Photoreceptors in Vivo. Advances in experimental medicine and biology 854, 699-706, doi:10.1007/978-3-319-17121-0_93 (2016).

153 Akiyama, G. et al. Photoreceptor rescue of pigment epithelium-derived factor-impregnated nanoparticles in Royal College of Surgeons rats. Molecular vision 18, 3079-3086 (2012).

154 Imai, D., Yoneya, S., Gehlbach, P. L., Wei, L. L. & Mori, K. Intraocular gene transfer of pigment epithelium-derived factor rescues photoreceptors from light-induced cell death. Journal of cellular physiology 202, 570-578, doi:10.1002/jcp.20155 (2005).

155 Takita, H. et al. Retinal neuroprotection against ischemic injury mediated by intraocular gene transfer of pigment epithelium-derived factor. Investigative ophthalmology & visual science 44, 4497-4504 (2003).

156 Campochiaro, P. A. et al. Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. Human gene therapy 17, 167-176, doi:10.1089/hum.2006.17.167 (2006).

157 Thorne, R. G. & Frey, W. H., 2nd. Delivery of neurotrophic factors to the central nervous system: pharmacokinetic considerations. Clinical pharmacokinetics 40, 907-946, doi:10.2165/00003088-200140120-00003 (2001).

158 Tria, M. A., Fusco, M., Vantini, G. & Mariot, R. Pharmacokinetics of nerve growth factor (NGF) following different routes of administration to adult rats. Experimental neurology 127, 178-183, doi:10.1006/exnr.1994.1093 (1994).

159 Dittrich, F., Thoenen, H. & Sendtner, M. Ciliary neurotrophic factor: pharmacokinetics and acute-phase response in rat. Annals of neurology 35, 151-163, doi:10.1002/ana.410350206 (1994).

160 Ejstrup, R. et al. Pharmacokinetics of intravitreal glial cell line-derived neurotrophic factor: experimental studies in pigs. Experimental eye research 91, 890-895, doi:10.1016/j.exer.2010.09.016 (2010).

161 Koeberle, P. D. & Ball, A. K. Neurturin enhances the survival of axotomized retinal ganglion cells in vivo: combined effects with glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor. Neuroscience 110, 555-567 (2002).

162 Ahmad, I., Del Debbio, C. B., Das, A. V. & Parameswaran, S. Muller glia: a promising target for therapeutic regeneration. Investigative ophthalmology & visual science 52, 5758-5764, doi:10.1167/iovs.11-7308 (2011).

163 Mensinger, A. F. & Powers, M. K. Visual function in regenerating teleost retina following cytotoxic lesioning. Visual neuroscience 16, 241-251 (1999).

164 Lindsey, A. E. & Powers, M. K. Visual behavior of adult goldfish with regenerating retina. Visual neuroscience 24, 247-255, doi:10.1017/S0952523806230207 (2007).

165 Sherpa, T. et al. Ganglion cell regeneration following whole-retina destruction in zebrafish. Developmental neurobiology 68, 166-181, doi:10.1002/dneu.20568 (2008).

166 Johns, P. R. Formation of photoreceptors in larval and adult goldfish. The Journal of neuroscience : the official journal of the Society for Neuroscience 2, 178-198 (1982).

167 Raymond, P. A. & Rivlin, P. K. Germinal cells in the goldfish retina that produce rod photoreceptors. Developmental biology 122, 120-138 (1987).

168 Hitchcock, P. F., Lindsey Myhr, K. J., Easter, S. S., Jr., Mangione-Smith, R. & Jones, D. D. Local regeneration in the retina of the goldfish. Journal of neurobiology 23, 187-203, doi:10.1002/neu.480230209 (1992).

169 Raymond, P. A., Reifler, M. J. & Rivlin, P. K. Regeneration of goldfish retina: rod precursors are a likely source of regenerated cells. Journal of neurobiology 19, 431-463, doi:10.1002/neu.480190504 (1988).

170 Braisted, J. E., Essman, T. F. & Raymond, P. A. Selective regeneration of photoreceptors in goldfish retina. Development 120, 2409-2419 (1994).

171 Fausett, B. V. & Goldman, D. A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. The Journal of neuroscience : the official journal of the Society for Neuroscience 26, 6303-6313, doi:10.1523/JNEUROSCI.0332-06.2006 (2006).

172 Kassen, S. C. et al. Time course analysis of gene expression during light-induced photoreceptor cell death and regeneration in albino zebrafish. Developmental neurobiology 67, 1009-1031, doi:10.1002/dneu.20362 (2007).

173 Bernardos, R. L., Barthel, L. K., Meyers, J. R. & Raymond, P. A. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 27, 7028-7040, doi:10.1523/JNEUROSCI.1624-07.2007 (2007).

174 Ramachandran, R., Reifler, A., Parent, J. M. & Goldman, D. Conditional gene expression and lineage tracing of tuba1a expressing cells during zebrafish development and retina regeneration. The Journal of comparative neurology 518, 4196-4212, doi:10.1002/cne.22448 (2010).

175 Das, A. V. et al. Neural stem cell properties of Muller glia in the mammalian retina: regulation by Notch and Wnt signaling. Developmental biology 299, 283-302, doi:10.1016/j.ydbio.2006.07.029 (2006).

176 Giannelli, S. G., Demontis, G. C., Pertile, G., Rama, P. & Broccoli, V. Adult human Muller glia cells are a highly efficient source of rod photoreceptors. Stem Cells 29, 344-356, doi:10.1002/stem.579 (2011).

177 Lawrence, J. M. et al. MIO-M1 cells and similar muller glial cell lines derived from adult human retina exhibit neural stem cell characteristics. Stem Cells 25, 2033-2043, doi:10.1634/stemcells.2006-0724 (2007).

178 Zhao, J. J. et al. Induction of retinal progenitors and neurons from mammalian Muller glia under defined conditions. The Journal of biological chemistry 289, 11945-11951, doi:10.1074/jbc.M113.532671 (2014).

179 Wright, L. S., Phillips, M. J., Pinilla, I., Hei, D. & Gamm, D. M. Induced pluripotent stem cells as custom therapeutics for retinal repair: progress and rationale. Experimental eye research 123, 161-172, doi:10.1016/j.exer.2013.12.001 (2014).

180 Fimbel, S. M., Montgomery, J. E., Burket, C. T. & Hyde, D. R. Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. The Journal of neuroscience : the official journal of the Society for Neuroscience 27, 1712-1724, doi:10.1523/JNEUROSCI.5317-06.2007 (2007).

181 Nagashima, M., Barthel, L. K. & Raymond, P. A. A self-renewing division of zebrafish Muller glial cells generates neuronal progenitors that require N-cadherin to regenerate retinal neurons. Development 140, 4510-4521, doi:10.1242/dev.090738 (2013).

182 Ooto, S. et al. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. Proceedings of the National Academy of Sciences of the United States of America 101, 13654-13659, doi:10.1073/pnas.0402129101 (2004).

183 Wan, J. et al. Preferential regeneration of photoreceptor from Muller glia after retinal degeneration in adult rat. Vision research 48, 223-234, doi:10.1016/j.visres.2007.11.002 (2008).

184 Kugler, M. et al. Heterozygous modulation of TGF-beta signaling does not influence Muller glia cell reactivity or proliferation following NMDA-induced damage. Histochemistry and cell biology 144, 443-455, doi:10.1007/s00418-015-1354-y (2015).

185 Joly, S., Pernet, V., Samardzija, M. & Grimm, C. Pax6-positive Muller glia cells express cell cycle markers but do not proliferate after photoreceptor injury in the mouse retina. Glia 59, 1033-1046, doi:10.1002/glia.21174 (2011).

186 Wan, J., Zheng, H., Xiao, H. L., She, Z. J. & Zhou, G. M. Sonic hedgehog promotes stem-cell potential of Muller glia in the mammalian retina. Biochemical and biophysical research communications 363, 347-354, doi:10.1016/j.bbrc.2007.08.178 (2007).

187 Osakada, F. et al. Wnt signaling promotes regeneration in the retina of adult mammals. The Journal of neuroscience : the official journal of the Society for Neuroscience 27, 4210-4219, doi:10.1523/JNEUROSCI.4193-06.2007 (2007).

188 Ueki, Y. et al. Transgenic expression of the proneural transcription factor Ascl1 in Muller glia stimulates retinal regeneration in young mice. Proceedings of the National Academy of Sciences of the United States of America 112, 13717-13722, doi:10.1073/pnas.1510595112 (2015).

189 Jorstad, N. L. et al. Stimulation of functional neuronal regeneration from Muller glia in adult mice. Nature 548, 103-107, doi:10.1038/nature23283 (2017).

190 Karl, M. O. et al. Stimulation of neural regeneration in the mouse retina. Proceedings of the National Academy of Sciences of the United States of America 105, 19508-19513, doi:10.1073/pnas.0807453105 (2008).

191 Ramachandran, R., Fausett, B. V. & Goldman, D. Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. Nature cell biology 12, 1101-1107, doi:10.1038/ncb2115 (2010).

192 Fausett, B. V., Gumerson, J. D. & Goldman, D. The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 28, 1109-1117, doi:10.1523/JNEUROSCI.4853-07.2008 (2008).

193 Ramachandran, R., Zhao, X. F. & Goldman, D. Ascl1a/Dkk/beta-catenin signaling pathway is necessary and glycogen synthase kinase-3beta inhibition is sufficient for zebrafish retina regeneration. Proceedings of the National Academy of Sciences of the United States of America 108, 15858-15863, doi:10.1073/pnas.1107220108 (2011).

194 Pollak, J. et al. ASCL1 reprograms mouse Muller glia into neurogenic retinal progenitors. Development 140, 2619-2631, doi:10.1242/dev.091355 (2013).

195 Loffler, K., Schafer, P., Volkner, M., Holdt, T. & Karl, M. O. Age-dependent Muller glia neurogenic competence in the mouse retina. Glia 63, 1809-1824, doi:10.1002/glia.22846 (2015).

196 Wan, J., Zhao, X. F., Vojtek, A. & Goldman, D. Retinal injury, growth factors, and cytokines converge on beta-catenin and pStat3 signaling to stimulate retina regeneration. Cell reports 9, 285-297, doi:10.1016/j.celrep.2014.08.048 (2014).

197 Takeda, M. et al. alpha-Aminoadipate induces progenitor cell properties of Muller glia in adult mice. Investigative ophthalmology & visual science 49, 1142-1150, doi:10.1167/iovs.07-0434 (2008).

198 Del Debbio, C. B. et al. Notch and Wnt signaling mediated rod photoreceptor regeneration by Muller cells in adult mammalian retina. PloS one 5, e12425, doi:10.1371/journal.pone.0012425 (2010).

199 Meyn, R. E., Hewitt, R. R., Humphrey, R. M. & Humphrey, R. M. Evaluation of S Phase Synchronization by Analysis of DNA-Replication in 5-Bromodeoxyuridine. Exp Cell Res 82, 137-142, doi:Doi 10.1016/0014-4827(73)90255-3 (1973).

200 Kuan, C. Y. et al. Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. Journal of Neuroscience 24, 10763-10772, doi:10.1523/Jneurosci.3883-04.2004 (2004).

201 Pearson, R. A. et al. Donor and host photoreceptors engage in material transfer following transplantation of post-mitotic photoreceptor precursors. Nature communications 7, 13029, doi:10.1038/ncomms13029 (2016).

202 Ortin-Martinez, A. et al. A Reinterpretation of Cell Transplantation: GFP Transfer From Donor to Host Photoreceptors. Stem Cells 35, 932-939, doi:10.1002/stem.2552 (2017).

203 Singh, M. S. et al. Transplanted photoreceptor precursors transfer proteins to host photoreceptors by a mechanism of cytoplasmic fusion. Nat Commun 7, doi:Artn 1353710.1038/Ncomms13537 (2016).

204 Singhal, S. et al. Human Muller glia with stem cell characteristics differentiate into retinal ganglion cell (RGC) precursors in vitro and partially restore RGC function in vivo following transplantation. Stem cells translational medicine 1, 188-199, doi:10.5966/sctm.2011-0005 (2012).

205 Jayaram, H. et al. Transplantation of photoreceptors derived from human Muller glia restore rod function in the P23H rat. Stem cells translational medicine 3, 323-333, doi:10.5966/sctm.2013-0112 (2014).

206 Boudreau-Pinsonneault, C. & Cayouette, M. Cell lineage tracing in the retina: Could material transfer distort conclusions? Developmental dynamics : an official publication of the American Association of Anatomists 247, 10-17, doi:10.1002/dvdy.24535 (2018).

207 Liu, B. et al. Wnt signaling promotes Muller cell proliferation and survival after injury. Investigative ophthalmology & visual science 54, 444-453, doi:10.1167/iovs.12-10774 (2013).

208 Craft, C. M. Targeting Endostatin to Potentially Prevent Retinal Neovascularization Using a Hypoxia-Responsive Muller Glia Cell-Specific Gene Therapy. Investigative ophthalmology & visual science 55, 8054-8054, doi:10.1167/iovs.14-15996 (2014).

209 Jiang, Y. et al. Targeting Muller cell-derived VEGF164 to reduce intravitreal neovascularization in the rat model of retinopathy of prematurity. Investigative ophthalmology & visual science 55, 824-831, doi:10.1167/iovs.13-13755 (2014).

210 Becker, S. et al. Targeted Knockdown of Overexpressed VEGFA or VEGF164 in Muller cells maintains retinal function by triggering different signaling mechanisms. Scientific reports 8, 2003, doi:10.1038/s41598-018-20278-4 (2018).

211 Zhao, M. et al. A New CRB1 Rat Mutation Links Muller Glial Cells to Retinal Telangiectasia. Journal of Neuroscience 35, 6093-6106, doi:10.1523/Jneurosci.3412-14.2015 (2015).

212 Xue, Y. et al. CRALBP supports the mammalian retinal visual cycle and cone vision. J Clin Invest 125, 727-738, doi:10.1172/JCI79651 (2015).

Chapter 3

mRNA for retinal gene delivery: the obstacle course to the inner retina

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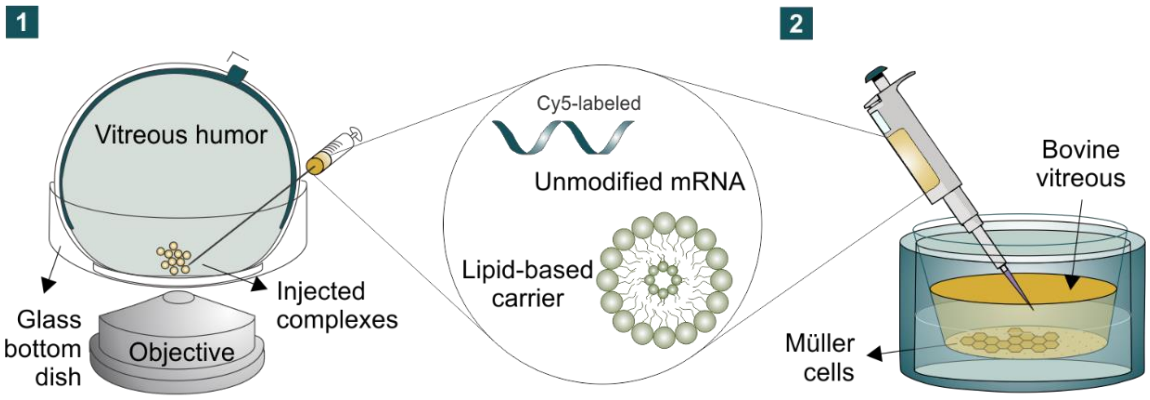
ABSTRACT

Considerable research over the last few years has revealed dysregulation of growth factors in various retinal diseases, such as glaucoma, diabetic retinopathy and photoreceptor degenerations. The use of messenger RNA (mRNA) to transiently overexpress a specific factor could compensate for this imbalance. Indeed, the fact that mRNA can be instantly translated in the cytoplasm without the need for nuclear entry is an important advantage for the transfection of post-mitotic retinal cells. However, a critical challenge of this approach lies in the ability to efficiently deliver mRNA molecules to the retinal target cells. In this chapter, we demonstrate that although intravitreal (IVTR) injection is an attractive administration route, the vitreous forms a major hurdle in the delivery of the cationic mRNA-complexes to retinal cells, both in terms of vitreal mobility and cellular uptake. To improve their intravitreal mobility and avoid unwanted extracellular interactions, we evaluate the use of hyaluronic acid (HA) as an electrostatic coating strategy. This HA-coating provided the complexes with a negative surface charge, markedly enhancing their mobility in the vitreous humor, without reducing their cellular internalization and transfection efficiency. However, although this coating strategy allows the mRNA-complexes to successfully overcome the vitreal barrier, protein expression levels achieved with this carrier are likely not sufficient for future *in vivo* translation.



INTRODUCTION

Interest in nucleic acid (NA)-based therapeutics has increased exponentially over the past decade. The many inherited and acquired diseases of the eye, its easy access and relatively immune-privileged status (as discussed in **Chapter 2**) resulted in a plethora of clinical trials for ocular gene therapy, currently dominated by viral vectors[1,2]. Although viral vectors are known for their efficient gene delivery, concerns regarding their safety and high production costs are still pending. As an alternative, non-viral nanoparticles offer advantages in their large transport capacity, ease of manipulation and low immunogenicity[3,4]. Over the past few years, a large number of non-viral vectors has been developed and tested for gene delivery to the retina[3]. Yet, so far, they fail to compete with the efficiency of their viral counterparts to transfect retinal cells[5,6]. One of the biggest obstacles for effective non-viral gene therapy in the retina is the delivery of the transgene across the nuclear membrane, into the nucleus. In actively dividing cells, this is possible due to a temporary disruption of the nuclear membrane during mitosis, allowing plasmid DNA (pDNA) to enter the nucleus. However, since most retinal cells are post-mitotic, the nuclear region can only be reached through the nuclear pores, which can be problematic for large pDNA molecules, as nuclear pore complexes only allow the passive diffusion of molecules <10 nm[7]. In this regard, the delivery of *in vitro* transcribed (IVT) messenger RNA (mRNA) to the retina has a major advantage over pDNA; as mRNA is completely functional in the cytoplasm and therefore does not require nuclear entry. In addition, since mRNA is unable to integrate into the genome, it entails no risk of insertional mutagenesis. Furthermore, the transient nature of mRNA confers an important benefit when gene expression is only required during a limited time window. Yet, in contrast to non-ocular applications such as anti-cancer immunotherapies and generation of pluripotent stem cells, where IVT mRNA-based therapeutics already play a major role[8], the use of mRNA for ocular gene delivery has never been evaluated before.

As for all strategies aiming to deliver transgenes to the retina, the *in vivo* success of IVT mRNA will greatly depend on its ability to overcome the extracellular barriers that precede mRNA delivery to the retinal target cells. In this regard, intravitreal (IVTR) administration provides an attractive administration route, bypassing several anterior barriers and delivering the mRNA in close proximity to the target site. In the clinic, IVTR injections are routinely performed for the administration of a wide range of drugs, in particular monoclonal antibodies, which are injected on a daily base for the treatment of age-related macular degeneration (AMD). Also for gene delivery, IVTR injections are a widely investigated injection route, especially when (widespread) delivery to the inner retina is desired (to reach targets such as Müller cells or retinal ganglion cells (RGCs))[9,10]. Even

though IVTR administration delivers drugs directly into the vitreous chamber of the eye, several extracellular barriers still need to be considered before effectively reaching the target site. Firstly, following intravitreal injection naked nucleic acids will be exposed to nucleases present in the vitreous humor[11,12]. In order to circumvent this issue, research has been conducted to package NAs into vectors that protect them from IVTR degradation and that facilitate uptake in the retinal cells[3,6]. A second barrier to keep in mind is the vitreous itself, that can interfere with the diffusion of NA delivery carriers and prevent their migration to the retina[10,13-15]. In case of non-viral vectors the surface characteristics of the particles seemed to be key in overcoming the vitreal barrier, since especially a positive charge hindered the particles' diffusion through the anionic vitreal environment[13,16-19]. In order to shield the surface of cationic particles and increase migration towards the retina, surface coating with polyethylene glycol (PEG) has been previously suggested[17]. However, PEG is known to pose steric hindrance, thereby impeding interactions of the complexes with cell membranes and subsequently reducing uptake by the target cells[20,21]. In the present study, an alternative coating strategy by means of hyaluronic acid (HA) was tested. HA is an anionic, biodegradable biopolymer, which is abundantly present in the vitreous humor and widely distributed throughout the retina[22,23].

In this chapter we evaluate the potential of the cationic polymer/lipid formulation *Trans*IT™ for mRNA transfection of retinal cells and modify its surface characteristics by electrostatic coating with HA. In particular, we study the effect of HA-coating on the capacity of these complexes to encapsulate mRNA and on their mobility in the vitreous of an *ex vivo* bovine model. Furthermore, we aim to investigate the ability of the coated *Trans*IT-mRNA particles to transfect retinal cells in the presence of bovine vitreous. As retinal target, Müller cells were chosen. Among multiple other advantages explained in **Chapter 2**, their endfeet are in close contact to the vitreous, which makes them one of the first cell types encountered following IVTR injection[24].

MATERIALS AND METHODS

mRNA

Unmodified eGFP-encoding mRNA was produced by *in vitro* mRNA transcription from pGEM4Z-GFP-A64 plasmids. eGFP was chosen as reporter protein to allow quantitative determination of gene expression by flow cytometry (yielding bright green fluorescence with an emission peak at 509 nm). The plasmids were purified using a QIAquick PCR purification kit (Qiagen, Venlo, The Netherlands) and linearized using the Spe I restriction enzyme (Promega, Leiden, The Netherlands). Linearized plasmids were used as templates for the *in*

vitro transcription reaction using the mMESSAGE mMACHINE T7 transfection kit (Ambion, Life Technologies, Ghent, Belgium), including a 7-methylGpppG cap analog. Subsequently, mRNAs were treated with DNase I and purified using the RNeasy Mini Kit (Qiagen). The mRNA concentration was determined by measuring the absorbance at 260 nm. mRNA was stored in small aliquots at -80°C at a concentration of 1 µg µl^{-1}. To label the mRNA for FCS measurements, a 10 µM solution of YOYO-1 iodide (Molecular Probes, Merelbeke, Belgium) was added to the eGFP encoding mRNA in a 10:1 bp to dye ratio, corresponding with a mixing ratio of 15:1 dye to mRNA (v/w). The mixture was incubated for 4 h at room temperature (RT) and the labeled mRNA was purified by addition of 2.5 volumes ice-cold ethanol and 0.1 volume of 5 M NaCl. Following incubation at -80°C the sample was centrifuged (30 min at 17 000*g*) and washed with 70% ethanol. Finally the pellet was resuspended in RNase free water and the concentration of the fluorescently labeled mRNA was again measured by UV absorption at 260 nm. For microscopy experiments, the mRNA was fluorescently labeled with Cy®5 using the Label IT® Nucleic Acid Labeling kit of Mirus Bio (Madison, WI). Cy®5 was added to the mRNA in a ratio of 1:1 (v:w). The mixture was incubated for 1h at RT and the labeled mRNA was purified according to the manufacturer's instructions by means of G50 microspin purification columns.

mRNA complexation

mRNA, as well as pDNA nanoplexes (i.e. nanocarriers containing NAs) were prepared using the commercially available transfection agents *Trans*IT (Mirus Bio, Madison, WI) and Lipofectamine™2000 (Thermo Fisher Scientific, Merelbeke, Belgium), according to the manufacturer's instructions. All nanoplexes were prepared in a final volume of 50 µl Opti-MEM™ (Thermo Scientific). The obtained nanoplexes were incubated at RT during 5 min for Lipofectamine and 4 min for *Trans*IT. To determine the mRNA complexation efficiency of the transfection agents by gel electrophoresis, mRNA was mixed with the carriers at different volume (µl) to weight (µg) (i.e. cationic transfection reagent-to-mRNA) ratios. An optimal v/w ratio per carrier was chosen for all further experiments, *e.g.,* 3:1 for Lipofectamine and 2:2:1 *Trans*IT-mRNA reagent : Boost reagent : mRNA for *Trans*IT. When complexing different amounts of mRNA (µg) the finale volume and the volume of the reagents were scaled proportionally.

For electrostatic HA-coating of the *Trans*IT complexes, the required amount of HA (Lifecore Biomedical, Minnesota, USA) to achieve a certain HA/*Trans*IT/mRNA ratio (v/v/w) was diluted in RNase free water and added to an equal volume of *Trans*IT-mRNA complexes. In this ratio HA refers to the number of negative charges originating from the carboxyl groups of the HA-monomers. Following 10 s of vortexing, the suspension was

incubated during 10 min at RT to stabilize. HA was purchased with three different molecular weights (MWs) of 20 kDa, 200 kDa and >1.8 MDa according to the manufacturer. These samples were analyzed with gel permeation chromatography and are referred to as HA22, HA137 and HA2700 respectively, based on their weight-averaged MW distributions as reported earlier[25].

Gel electrophoresis

To examine the capacity of the carriers to complex mRNA, nanoplexes were diluted and incubated in Opti-MEM™. For each carrier, different cationic transfection reagent-to-mRNA v/w ratios were assessed. After 30 min incubation at 37 °C in Opti-MEM™, fetal bovine serum (FBS) or adult bovine vitreous (ABV), 5 µl Ambion loading buffer was added. The mixtures were loaded into a 1.2% agarose gel in TRIS/Borate/EDTA (TBE) buffer, to which GelRed (Biotium, Hayward, CA) was supplied for visualization of the mRNA. The gel was run for 40 min at 100 V and imaged by UV illumination and gel photography. A 0.5 to 10 kb RNA ladder (Thermo Scientific) was included. Samples containing free mRNA in Opti-MEM™ were run as controls. Gel analysis was performed using ImageJ software (NIH) to determine the complexation efficiency.

Physical characterization of the complexes

Size distribution and zeta potential of the complexes were measured by dynamic light scattering (DLS) using a Malvern Zetasizer nano-ZS (Malvern Instruments, Worcestershire, UK). All samples were diluted in 20 mM HEPES buffer pH 7.4 (Sigma-Aldrich). Empty carriers (i.e. without mRNA) were measured for comparison of the zeta potential. Size measurements were done in triplicate, with three runs per replicate and presented as number averaged hydrodynamic diameter. Zeta potential measurements were done in triplicate with two runs per replicate.

Cell culture and transfections

The human Müller cell line Moorfields/Institute of Ophthalmology-Müller 1 (MIO-M1) was obtained from the UCL Institute of Ophthalmology, London, UK[26]. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) GlutaMax®pyruvate 1g l-1 glucose (Gibco-Invitrogen) supplemented with 1% L-glutamin, 2% penicillin/streptomycin and 10% FBS (Hyclone). Cells were passaged at 90% confluency and incubated at 37°C with 5% CO_2. Five days prior to transfection, $1x10^4$ cells were plated per well in 24-well

plates. Retinal pigment epithelial cells (ARPE-19, ATCC® CRL-2302TM) were purchased from ATCC and cultured according to the recommended conditions. Two days prior to transfection, $5x10^4$ cells were plated per well in 24-well plates.

In the most simple *in vitro* setup, the cells were seeded at the bottom of a 24-well plate. Nanoplexes were added directly to the cells in complete cell culture medium, followed by 24 h of incubation at 37°C. Unless indicated otherwise, the cells were transfected with 0.5 µg mRNA or pDNA per $1x10^5$ cells.

A second *in vitro* setup served as model to evaluate the effect of vitreous on uptake, transfection and toxicity. In this setup, MIO-M1 cells were seeded on 0.4 µm pore membrane inserts (Greiner Bio-One, Vilvoorde, Belgium) at $6x10^3$ cells/insert in a 12 well plate. Culture medium was added below the insert to assure optimal cell viability. Either culture medium or bovine vitreous was applied on top of the cells. For the latter, fresh bovine eyes were obtained from a local abattoir, cleaned from extra-cellular tissue, washed with 20% ethanol and bisected. The vitreous was sonicated using a tip sonicator (Branson, Swedesboro, NJ) for 3 min with short intervals every 30 s to allow fluent pipetting. Naked or complexed mRNA was added to the vitreous, followed by 3 or 24 h incubation at 37°C, for uptake or transfection experiments respectively.

Flow cytometry

Flow cytometric analysis was conducted on MIO-M1 and ARPE-19 cells that were transfected with eGFP mRNA/pDNA as described above. For uptake experiments eGFP mRNA was labeled with Cy®5. Cells treated with 50 µl Opti-MEM™ alone or cells treated with the same amount of nanoplexes containing firefly Luciferase (fLuc)-encoding mRNA were used as negative controls. The cells were detached from the plate surface with 0.25% trypsin-EDTA (Gibco, Paisly, UK), washed with cell culture medium and resuspended in phosphate-buffered saline (PBS) supplemented with 0.1% sodium azide and 1% bovine serum albumin. To allow identification of dead and apoptotic cells during the transfection experiments, respectively 4',6-diamidino-2-fenylindool (DAPI) and MitoProbe™ $DiIC_1(5)$ (ThermoFischer) stainings were added to the cell suspension according to the manufacturer's instructions. After 30 min incubation at 37°C, cells were analyzed using the CytoFLEX™ Flow Cytometer (Beckman Coulter, Krefeld, Germany) and data analysis was performed using FlowJo software (FlowJo, OR, USA). A minimum of 7000 gated cells was counted per tube. Negative controls were set as max 1% eGFP expressing cells. Mean fluorescence intensity (MFI) was calculated for the entire viable cell population.

Fluorescence single particle tracking (fSPT) microscopy

To determine the diffusion of the complexes in an aqueous environment, nanoplexes containing Cy®5-labeled mRNA were diluted in RNase free HEPES buffer (20 mM, pH 7.4, Sigma-Aldrich) to a concentration of 10^8 to 10^9 complexes per ml. Fifty µl of the samples was transferred into a 96-well microplate (Greiner Bio-One, Vilvoorde, Belgium) and the mobility of the complexes was measured with fluorescence single particle tracking (fSPT) microscopy. fSPT is based on microscopic imaging of fluorescently labeled single molecules to characterize their diffusion. Real-time confocal tracking of individually moving nanoplexes allows to calculate their motion trajectories and diffusion coefficient. All fluorescence video imaging of diffusing nanoplexes was performed on a swept-field confocal microscope (LiveScan Swept Field Confocal Microscope System; Nikon, Brussels, Belgium) equipped with a Plan Apo 100× 1.4 NA oil immersion objective lens (Nikon) and a fast and sensitive EMCCD camera (Ixon Ultra 897, Andor Technology, CT, USA). The microscope was focused at 5-10 µm above the bottom of the well plate and the Cy®5- labeled mRNA nanoplexes were excited with a solid-state 125 mW 640 nm (Agilent Technologies, CA, USA) laser. For each sample, typically 25 movies of about 100 frames each were recorded at different random locations within the sample.

To measure the diffusion of uncoated nanoplexes and the HA-coated *Trans*IT-mRNA complexes in intact vitreous, fSPT was performed in an *ex vivo* model as previously described by Martens *et al.*[27]. In summary, fresh bovine eyes were obtained from a local abattoir, disposed of extra-ocular material, disinfected in 20% ethanol and washed in sterile CO_2 independent medium. Subsequently, cornea and lens were removed, exposing the hyaloid membrane that holds the vitreous. A volume of 30 µl with a concentration of 50 ng μl^{-1} Cy®5- labeled mRNA was injected at four different places in the vitreous as close as possible to the hyaloid membrane. Next, the complete eye was transferred to a glass bottom dish (*In Vitro* Scientific, Mountain View, CA) with the hyaloid membrane positioned against the glass bottom allowing visualization of the vitreous by fluorescence microscopy. Finally, the samples were stored at RT for 24 h permitting the diffusion of particles through the vitreous. Particle mobility was determined by fSPT. Diffusion analysis of all videos was performed using in-house developed software, as described before[28], providing a distribution of apparent diffusion coefficients.

Fluorescence correlation spectroscopy (FCS)

To confirm coating of the *Trans*IT complexes with HA, fluorescence correlation spectroscopy (FCS) was used. Green *Trans*IT-mRNA complexes were prepared using YOYO- labeled eGFP mRNA and coated with red labeled HA (Hyaluronate-DyLight® 650, 20 kDa, Creative PEGWorks, Chapel Hill, NC). The samples were measured with a confocal microscope equipped with a PicoHarp 300 FCS Unit (Picoquant, Berlin, Germany). A 60x water lens (Nikon, Brussel, Belgium) was used and the samples were measured for 60s. A green and red laser transmitting at 488 nm and 640 nm respectively, with a laser intensity of 5% were used. The excited fluorescent particles were processed by a dual detector unit (Picoquant). The obtained photon count distributions were then analysed with the software symphotime (Picoquant).

Statistical analysis

All data are presented as mean ± standard deviation and are representative for at least 3 independent experiments conducted on 3 different days, unless stated otherwise. Experiments were analyzed for statistical significance with a one or two-way ANOVA followed by the Bonferroni post hoc test for significant differences between treated groups, or the Dunnett post hoc test when compared with a single control group. An unpaired t-test was performed to determine statistically significant differences between the amount of eGFP positive primary MIO-M1 cells after mRNA versus pDNA transfection. Statistical analysis was performed using Graphpad Prism 6 software (La Jolla, CA, USA). Asterisks indicate statistical significance (* $p < 0.05$; ** $p < 0.01$;*** $p < 0.001$).

RESULTS

Physicochemical characterization of the nanoplexes

To guarantee complete protection of the mRNA against enzymatic degradation, we performed gel electrophoresis to examine mRNA complexation of *Trans*IT-mRNA complexes with a varying v/w ratio (i.e. *Trans*IT/Boost reagent (µl) to mRNA (µg) ratio) in biologically relevant media: Opti-MEM™, fetal bovine serum (FBS) and adult bovine vitreous (ABV) (**Figure 1A**). As we made use of the universally applied commercial carrier Lipofectamine™2000 as a positive control for the pDNA *in vitro* experiments, we included this carrier in our characterization studies (**Figure 1B**). From the gel electrophoresis experiments, we quantified the percentage of complexed mRNA using Image J software

(**Figure 1C**). As can be seen from **Figure1A** and **B**, naked mRNA stays intact when incubated with Opti-MEM™ (lane 4), but degrades in both FBS and bovine vitreous (lane 5 and 6). Lipofectamine™2000 showed full complexation (**Figure 1C**) and protection of the mRNA (**Figure 1A** lane 13-14 and 17-18) starting from a v/w ratio (i.e. cationic transfection reagent (µl) to mRNA (µg)) of 3. In contrast, all *Trans*IT formulations kept the mRNA co-localized in the slots of the agarose gel, indicating that the mRNA did not dissociate after incubation in Opti-MEM™ (**Figure 1B** lane 7-10), serum (lane 11-14) or vitreous (lane 15-18) and hence full protection was offered against mRNA degradation. Further evaluation of the cytotoxicity of the nanoplexes in MIO-M1 Müller cells (data not shown) let us determine the v/w ratios offering the highest mRNA complexation with minimal toxicity, being a v/w ratio 3 for Lipofectamine and v/w ratio 2 for *Trans*IT, which were used in all further experiments.

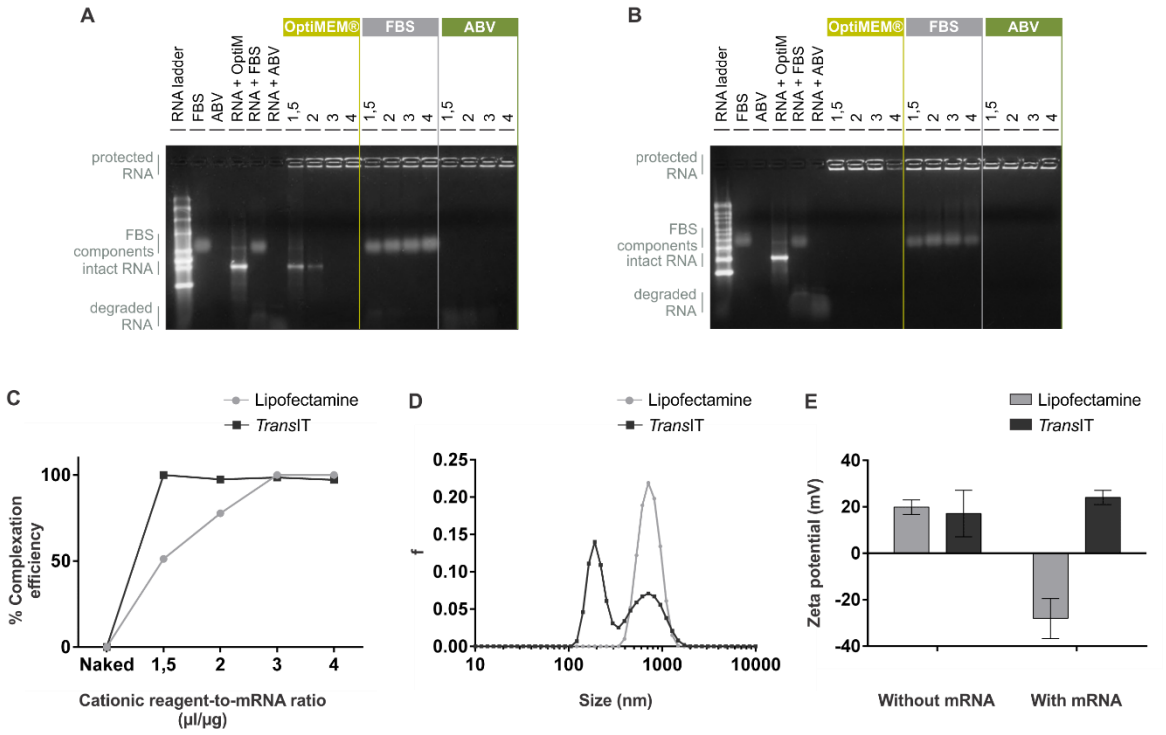


Figure 1 | Characterization of the nanoplexes. Gel electrophoresis on free mRNA and mRNA nanoplexes prepared with (A) Lipofectamine and (B) *Trans*IT at different v/w ratios in Opti-MEM™, fetal bovine serum (FBS) and adult bovine vitreous (ABV). A 0.5 to 10 kb molecular weight marker was included. (C) The percentage complexation efficiency in Opti-MEM™ as quantified from the gel electrophoresis using ImageJ software. (D) Size determination of the nanoplexes by dynamic light scattering (DLS) in HEPES buffer (v/w ratio 3 for Lipofectamine and v/w ratio 2 for *Trans*IT). (E) Zeta potential analysis of the nanocarriers (i.e. without mRNA) and nanoplexes (i.e. containing mRNA) (v/w ratio 3 for Lipofectamine and v/w ratio 2 for *Trans*IT) in HEPES buffer. Data represent mean ± standard deviation (SD) (*n*=2x3).

To determine the size and charge of the complexes used in this study, we measured the hydrodynamic diameter (d_h) and zeta potential of the particles prepared at the optimal as determined above. Lipofectamine resulted in formation of the largest mRNA-containing particles, with a peak size of 712 nm (**Figure 1D**). By contrast, *Trans*IT showed a bimodal

size distribution (PDI = 0,374), with the highest frequency of particles at 190 nm. The second peak in the DLS data suggests that a fraction of the *Trans*IT particles (~43,7%) tend to aggregate, although to a lesser extent than Lipofectamine. The zeta potential was determined respectively before and after addition of mRNA to the transfection reagents. **Figure 1E** shows that both formulations are positively charged before mRNA complexation (+20 mV and +17 mV for Lipofectamine and *Trans*IT respectively), allowing an efficient electrostatic interaction with the anionic mRNA. Addition of mRNA completely inverted the positive zeta potential of Lipofectamine to ~-28 mV, whereas for *Trans*IT mRNA complexation had no significant effect on the zeta potential of the formulation.

mRNA outperforms pDNA for transfecting retinal cells

To test the capacity of the *Trans*IT-complexed mRNA to transfect different retinal cell types, MIO-M1 Müller cells (inner retina) and ARPE-19 cells (outer retina) were treated with complexes containing 0.5 µg mRNA. In addition, we compared the use of mRNA with the classical pDNA-based transfections. As *Trans*IT is specifically designed for mRNA delivery, Lipofectamine^{TM}2000 was included as a widely used transfection reagent for pDNA transfections. Importantly, for all combinations of transfection agents and cell types, the percentage of eGFP positive cells and mean fluorescence intensity per cell was significantly higher for mRNA when compared to pDNA (**Figure 2,** black bars). These results were confirmed in a primary cell culture of Müller cells, which were isolated from a bovine retina (**Supplementary Figure S1**). For pDNA transfections, the highest expression was found in ARPE-19 cells transfected with Lipofectamine (**Figure 2B**, grey bars). Also for mRNA delivery, transfection efficiency was higher when complexed to Lipofectamine compared to *Trans*IT, however with respect to toxicity, *Trans*IT showed the most favorable safety profile, with ~90% viable cells in all situations. Taken together, mRNA seems to be superior over the well-established pDNA, with *Trans*IT is a safe and suitable carrier to transfect retinal cells types.

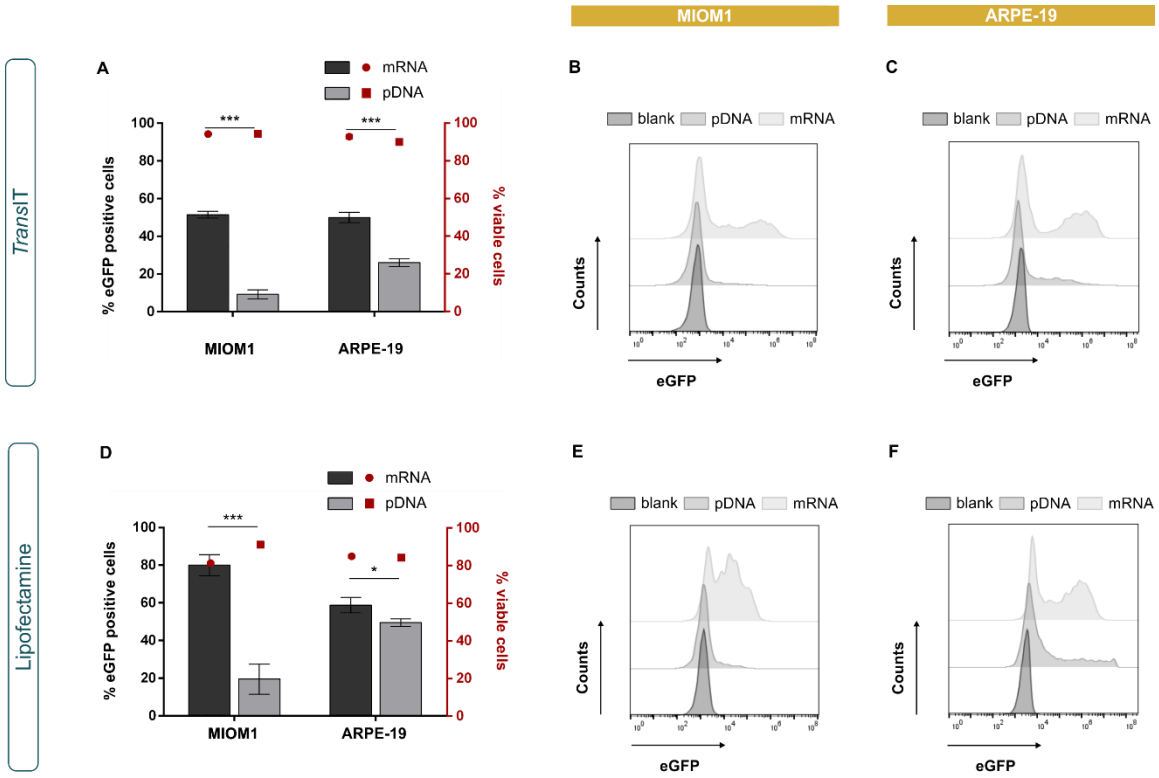


Figure 2 | Transfection efficiency of mRNA versus pDNA containing nanoplexes. Percentages of eGFP transfected MIO-M1 Müller cells and ARPE-19 cells (left y-axis) and cell viability (right y-axis) 24h after incubation with *Trans*IT (A) and Lipofectamine (D) containing unmodified mRNA or pDNA. % viable cells was gated as $DiIC_1(5)^-$/$DAPI^-$. % eGFP positive cells was calculated from the amount of viable cells. Data represent mean ± SD (n=3x3). *, $p < 0.05$; ***, $p < 0.001$ mRNA versus pDNA by two-way ANOVA. Representative flow cytometry histograms (after 24h) of the complete MIO-M1 cell population transfected with *Trans*IT complexes (B) and Lipofectamine complexes (E) and fof the complete ARPE-19 cell population transfected with *Trans*IT complexes (C) and Lipofectamine complexes (F).

The vitreous as a barrier for intravitreal mRNA delivery

When evaluating complexes for IVTR injection, it is essential to assure optimal mobility of the complexes throughout the vitreous and to minimize unspecific interactions with the vitreal components. Therefore we determined whether or not *Trans*IT-mRNA complexes remain mobile in the vitreous, and to what extent the presence of vitreous could affect the transfection efficiency of these mRNA complexes. To measure the movement of the *Trans*IT-mRNA complexes in the vitreous humor, we used an *ex vivo* model previously developed by our group[17]. In this model, nanoplexes with Cy®5-labeled mRNA are injected in the intact vitreous of a cow eye, after which their diffusion is visualized with fluorescence single particle tracking microscopy (fSPT). Motion trajectories of single particles allow to calculate their size distribution and thus their diffusion coefficients. Using this model, maximum vitreous integrity and rigidity can be ensured. From the distribution of the diffusion coefficients **Figure 3A**) we can derive that the complexes are substantially slowed down in vitreous (solid line) relative to their free diffusion in HEPES (dotted line). After IVTR injection the complexes show a

bimodal diffusion distribution with a high fraction of immobilized particles, which is in line with previously reported data for cationic lipid and polymer-based particles[17,19,29,30].

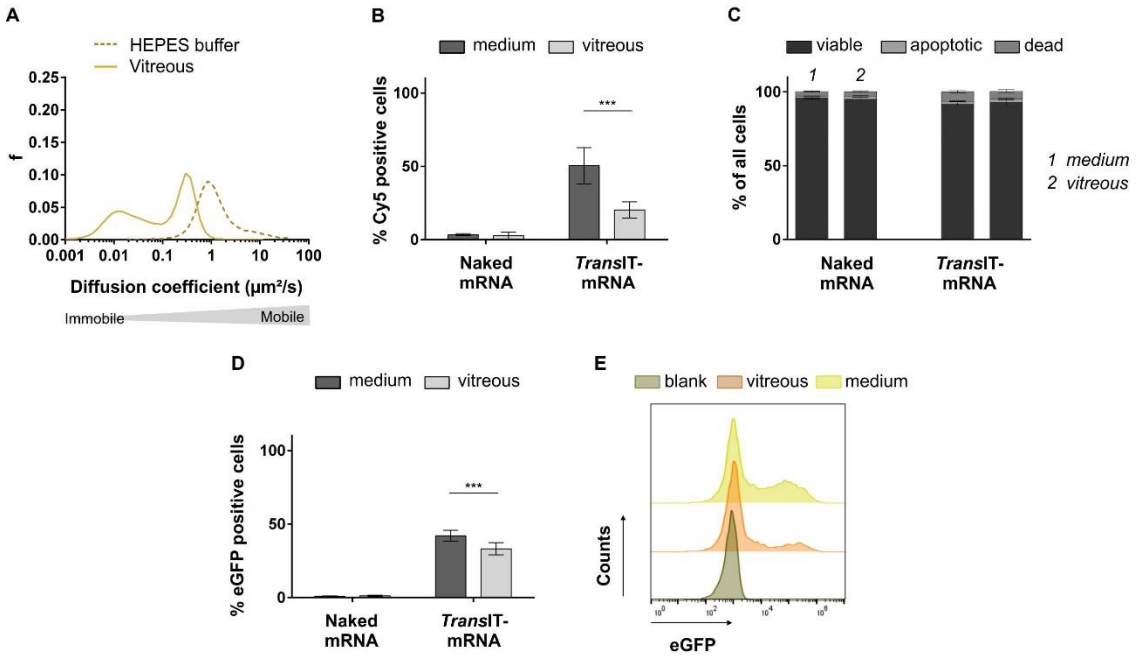


Figure 3 | Influence of bovine vitreous on the mobility, uptake, toxicity and transfection efficiency of naked mRNA and *Trans*IT-complexed mRNA. (A) Diffusion distributions of Cy®5-labeled mRNA-*Trans*IT complexes in intact bovine vitreous humor (solid line), 24 h after IVTR injection compared to their diffusion in HEPES buffer (dotted line). (B) Percentage of MIO-M1 cells that display uptake after 3 h incubation with Cy®5-labeled naked or complexed mRNA. (C) Cell viability 24 h after mRNA incubation with as quantified by flow cytometry. Untreated cells served as negative control. (D) Percentage of eGFP transfected MIO-M1 Müller cells 24 h after incubation with naked and complexed mRNA. Representative flow cytometry histograms are shown in (E). Data is shown as mean ± SD (n=3x3). ***, $p < 0.001$ medium versus vitreous by two-way ANOVA.

Next, an *in vitro* experimental setup was used to assess the effect of the vitreous on the uptake and transfection efficiency of the *Trans*IT-mRNA complexes. In this setup MIO-M1 Müller cells were seeded on the permeable membrane of a Transwell® membrane to which mRNA-containing medium or sonicated vitreous (SV) was added. By allowing the cells to contact culture medium through the bottom of the transwell insert at all time, adequate nutrient supply and optimal cell viability can be ensured. It is important to note that in both uptake and transfection studies SV was used. While retaining all of its components, the collagen network in this vitreous is dismantled by sonication, resulting in a higher mobility of the particles compared to intact vitreous (**Supplementary Figure S2**). As expected from the rapid mRNA degradation shown in **Figure 1A** and **B**, Cy®5-labeled naked mRNA failed to be taken up when added to either medium or vitreous (**Figure 3B**). Subsequently no eGFP expression was induced in either culture condition (**Figure 3D**). *Trans*IT-mRNA complexes, however, were taken up by nearly 50% of the cells when applied in culture medium (**Figure 2B**). The presence of SV significantly decreased uptake of the complexes to 20% and

resulted in a lower transgene expression compared to medium (**Figure 3D,E**). Finally, cytotoxicity of the *Trans*IT-mRNA complexes was evaluated by quantifying the percentage of apoptotic and dead cells via $DiIC_1(5)$ and DAPI staining. From the data presented in **Figure 3C**, we can conclude that *Trans*IT-mRNA complexes are well tolerated by the MIO-M1 Müller cells in both culture conditions. Taken together, even though the vitreal network is mechanically broken up, the vitreal content itself seems to form a significant barrier for *Trans*IT mediated mRNA delivery.

Optimization of HA-coating

Based on our observation that the mobility of cationic particles through the vitreous is hindered due to their interaction with the negatively charged components of the vitreous humor, we evaluated the use of HA to coat the complexes in order to shield their cationic surface. Additionally, since it is well-known that native HA has many different biological functions depending on its molecular weight (MW), we investigated the possible effect of MW on the electrostatically-coated HA-complexes by using HA with MWs of 22 kDa, 137 kDa and 2700 kDa. Size and zeta potential of the *Trans*IT-complexes were determined by dynamic light scattering (DLS) (**Figure 4A** and **B**). Uncoated complexes were prepared by spontaneous complexation of the *Trans*IT reagent to the mRNA at a v/w ratio of 2:1, resulting in particles with a net positive surface charge. Electrostatic coating with increasing amounts of HA decreased the zeta potential, which inverted to a negative charge starting from a HA/*Trans*IT/mRNA ratio of 16:2:1 (v/v/w) (**Figure 4B**). These results correlated well with the particle size: particles with a close to neutral surface charge tended to aggregate, resulting in a mean size of ~1000 nm, whereas a particle size of ~125 nm was maintained at a ratio of 16:2:1 (v/v/w) due to electrostatic repulsion between the negative charges (**Figure 4A**). When comparing the different MWs of HA, no substantial differences were seen in the size of the coated complexes. Interestingly, the higher the MW of the HA, the lower the zeta potential becomes when the same amount of HA monomers was added to the *Trans*IT-complexes (**Figure 4B**). This indicates that addition of more HA monomers is necessary to provide stable negative complexes with HA22 or HA137 compared to HA2700. Since a HA/*Trans*IT/mRNA ratio of 16:2:1 (v/v/w) yields stable anionic particles and requires the least amount of HA monomers, this ratio was chosen for all following experiments.

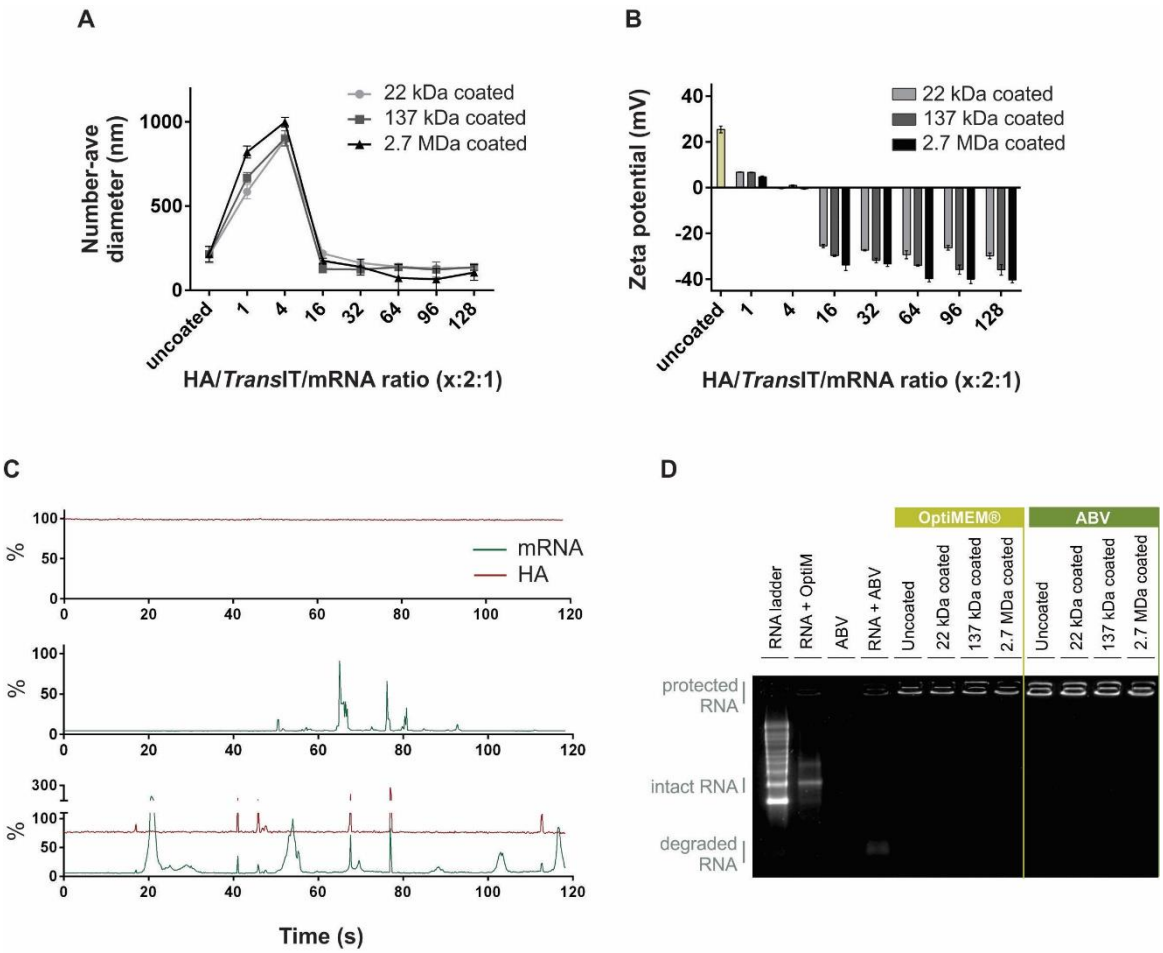


Figure 4 | Physical characterization of HA-coated *Trans*IT-mRNA complexes. Changes in size (number-averaged hydrodynamic diameter) (A) and zeta potential (B) after coating of *Trans*IT-mRNA complexes with HA in different ratios (ratio presents the number of negative charges originating from the carboxyl-group of the HA-monomer) and different molecular weights as measured by DLS in HEPES buffer. Data represent mean ± SD (*n*=3x3). (C) Representative fluorescence fluctuations of free HA (top row), uncoated *Trans*IT-complexed mRNA (middle row) and HA-coated *Trans*IT-complexed mRNA (16:2:1 v/v/w) (bottom row) as measured by fluorescence correlation spectroscopy (FCS) in HEPES buffer. (D) Gel electrophoresis on uncoated and HA-coated *Trans*IT-mRNA complexes demonstrates successful mRNA complexation after coating with different molecular weights of HA in both Opti-MEM™ and ABV. For (C) and (D) a charge ratio HA/*Trans*IT/mRNA of 16:2:1 (v/v/w) was used.

To further confirm successful HA coating of the particles, the association of HA and mRNA to *Trans*IT was followed by fluorescence correlation spectroscopy (FCS). To this end the mRNA was labeled with YOYO-1 (green) and DyLight® 650- labeled HA22 (red) was used. The fluorescence of free HA, representing the negative control, was set at 100% (**Figure 4C**, top row). **Figure 4C** middle row shows intense green fluorescence peaks in the fluctuation profiles of uncoated *Trans*IT-mRNA complexes, indicating dense packing of the fluorescent mRNA which corresponds to the presence of complexes. Following addition of red labeled HA to the complexes in a ratio of 16:2:1, the fluorescence baseline of HA dropped from 100% to 78%. As the baseline fluorescence correlates with the fraction of remaining free HA, this drop confirms binding of 22% of the HA to the complexes. In addition,

peaks appeared in the red HA channel as well, co-occurring with the green mRNA fluorescence peaks (**Figure 4C**, bottom row), demonstrating successful coating of the complexes with HA. It is important to note that some fluorescence peaks in the fluctuation profile of mRNA are not accompanied by a peak in the HA levels. These singular mRNA peaks may indicate that also a fraction of non-coated particles remains.

To assess whether the HA-coating influences the mRNA complexation capacity of the *Trans*IT, HA-coated complexes (ratio 16:2:1 (v/v/w)) were loaded on a 1% agarose gel. As can be seen from the results in **Figure 4D**, HA-coating did not trigger mRNA release from the complexes at any MW. Hence, full encapsulation and protection of the mRNA is offered in both Opti-MEM™ and ABV at all MWs. Taken together, electrostatic coating of *Trans*IT-mRNA complexes results in stable, negatively charged particles, still able to fully protect the mRNA against degradation in bovine vitreous.

Improved vitreal mobility and transfection efficiency after coating with HA

After optimization we assessed whether HA-coating effectively improved the migration of *Trans*IT-mRNA complexes through the vitreous humor. Therefore, HA-coated (22 kDa, 137 kDa and 2700 kDa) complexes were injected in an *ex vivo* model containing intact vitreous and their mobility was compared to that of the uncoated complexes (**Figure 5A**, black line) by fSPT microscopy. Diffusion coefficients of the different particles, established from their movement tracks, are displayed in **Figure 5A**. Electrostatic coating of the *Trans*IT-mRNA complexes with HA clearly improved their mobility, with the highest increase in mobility seen for HA137. This outcome is likely attributed to the negative surface charge of the coated particles along with their smaller particle size (**Figure 5B**). Indeed a substantial decrease in the amount of aggregated particles was seen following HA-coating, as demonstrated by the monodisperse (PDI<0.3) size distribution.

The use of HA as an electrostatic surface coating of the *Trans*IT-mRNA complexes was further evaluated in its capacity to transfect MIO-M1 Müller cells in the presence of sonicated bovine vitreous. **Figure 5C** shows that, despite a negative surface charge, HA-coated complexes are still taken up by the MIO-M1 cells. Although no significant difference is seen in uptake, HA-coating with various MWs does slightly but significantly increase the transfection efficiency compared to the uncoated complexes (**Figure 5D,E**). This modest increase was also noted when transfections were performed in culture medium rather than SV (**Supplementary Figure S3**). Additionally, we examined the cytotoxicity of the coated and uncoated complexes in MIO-M1 Müller cells by flow cytometry. As shown in **Figure 5F**,

no important cytotoxicity was observed for any of the complexes at the concentrations used for uptake and transfection analysis.

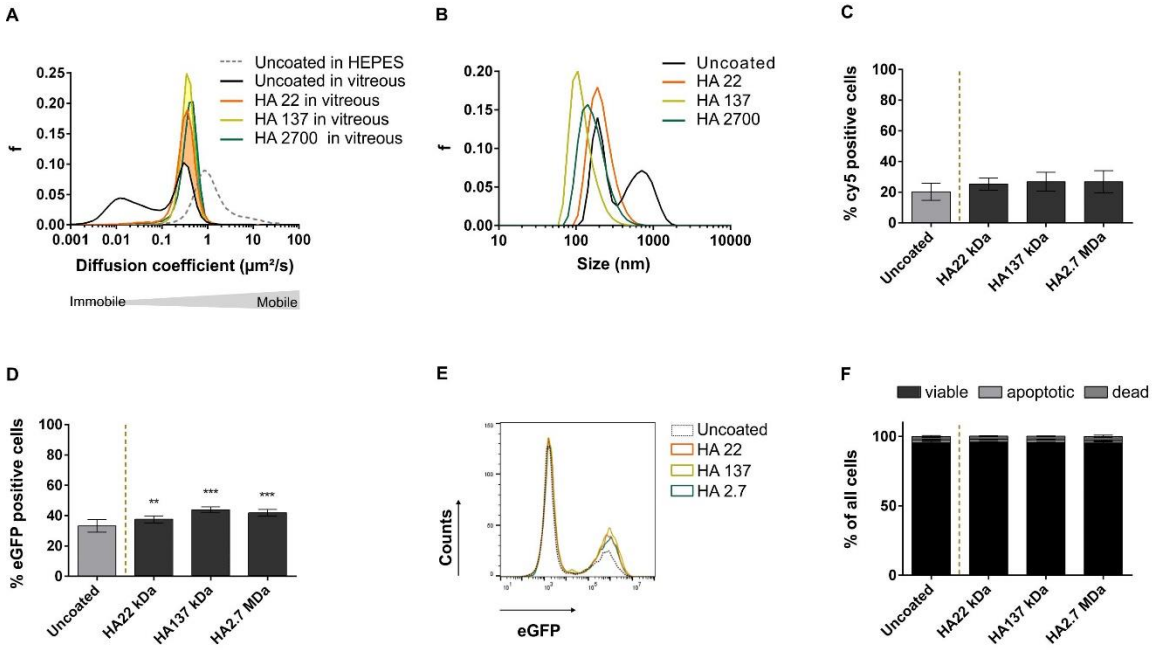


Figure 5 | Influence of HA-coating on vitreal mobility, size, uptake, transfection efficiency and toxicity of *Trans*IT-complexed mRNA. All measurements were done using a HA/*Trans*IT/mRNA ratio of 16. (A) Single particle tracking analysis of the IVTR mobility of Cy®5-labeled mRNA-*Trans*IT complexes before and after HA-coating with different molecular weights in intact bovine vitreous humor (solid lines), compared to the mobility of uncoated complexes in HEPES buffer (dotted line). Coating with 137 kDa HA resulted in the highest mobility increase. (B) Size (number average) of the coated *Trans*IT-mRNA complexes dispersed in HEPES buffer as measured by DLS. The size distribution of the uncoated complexes was added to the graph for comparison (solid black line). (C) Percentage of MIO-M1 Müller cells that have taken up Cy®5-labeled *Trans*IT-complexed mRNA 3 h after transfection. (D) Percentage of eGFP transfected MIO-M1 Müller cells 24 h after incubation with *Trans*IT-complexed mRNA. Representative flow cytometry histograms are shown in (E). Cell viability after transfection evaluated by flow cytometry can be seen in F. Data is shown as mean ± SD (n=3x3). **, $p < 0.01$; ***, $p < 0.001$ coated versus uncoated by one-way ANOVA.

DISCUSSION

Although the potential of IVT mRNA for the production of proteins was already demonstrated about 30 years ago, mRNA was not addressed any further at that time[31]. As discussed in **Chapter 1**, the instability and inherent immunogenicity associated with mRNA prompted researchers to focus on viral and non-viral DNA-based technologies instead. This was reflected in the ocular field as well, since to date no studies have been published using mRNA for the treatment of retinal disorders. Nevertheless, for ocular therapy, one of the main advantages of mRNA over pDNA is its functionality in the cytoplasmic compartment. pDNA requires transfer into the nucleus, hence depends on nuclear envelop breakdown during cell division to exert its function[32]. However, most retinal cells are post-mitotic which forms a major hurdle for pDNA transfections. Indeed, Hansson et *al.* have demonstrated that

both the percentage of eGFP-transfected (hESC-derived) retinal pigment epithelium (RPE) cells, as well as the amount of eGFP expressed per cell, were substantially higher when transfected with mRNA instead of the corresponding plasmid[33]. In agreement with these results, we observed that mRNA is superior in expressing proteins in retinal cell cultures (Müller cells and RPE cells) (**Figure 2**) as well as in primary bovine Müller cells (**Supplementary Figure S1**). Remarkably where mRNA and pDNA resulted in comparable transfection efficiency in fast dividing RPE cells, *Trans*IT- as well as Lipofectamine-based mRNA transfection was much more efficient in the rather slowly dividing Müller cells (**Figure 2**). This most likely results from the fact that pDNA has to reach the nuclear compartment what occurs more efficient during cell division. Nevertheless, most cells in the adult retina are usually non-dividing, which does not play in favor of pDNA transfection. Even more, it should be noted that while RPE cells in culture are fast dividing, RPE cells in the adult retina are usually in a post-mitotic state[34]. In contrast, mRNA delivery is beneficial to non- or slowly dividing cells, as it can be instantly translated once it reaches the cytoplasm. These findings clearly indicate that mRNA is an attractive candidate for ocular applications.

For this purpose, IVTR injection is a powerful administration route, since it is considered safe and circumvents several anterior barriers of the eye. Especially for the delivery of genetic information to the inner retina it is preferable to subretinal injection, since it provokes less retinal trauma and pathologic gliosis and could potentially generate a more wide-spread gene expression[9,10]. Yet, from the limited retinal transfection upon direct injection of these non-viral drug delivery systems into the vitreous humor, it is clear that specific barriers need to be overcome before achieving retinal transgene expression. Indeed, complexes need to efficiently diffuse through the vitreous before reaching the retina[35]. Despite the success of many non-viral vectors to cross this barrier after IVTR injection in small animals, such as rodents, there is a lack of follow-up delivery studies in larger species. This is likely because most carriers fail to overcome the more complex barriers present in larger species and these unfavourable results are not reported on[36]. As such, it has been shown that rodents have a more liquid vitreous when compared to the human situation[37-39]. We therefore made use of an *ex vivo* bovine model previously developed by our research group, which is more representative for human physiology. This model comprises a bovine eye, removed of its anterior parts, which is designed to visualize the diffusion of vectors in the intact vitreous body by means of high-resolution microscopy[17].

The vitreous humor is composed of water (98-99%), salts, proteins (*e.g.,* nucleases) and a complex network of randomly spaced collagen fibers and stabilizing glycosaminoglycans (GAGs), of which HA is the most abundant[12,22,40]. The presence of nucleases in the vitreous limits the applicability of naked mRNA injection due to enzymatic

degradation of the mRNA, as seen in **Figure 1A,B**. Therefore, we formulated the mRNA into particles by electrostatic complexation to a positively charged polymer/lipid formulation *Trans*IT, which was shown to protect mRNA against IVTR hydrolysis (**Figure 1A**). Unfortunately, mere protection of its cargo is not sufficient to ensure successful mRNA delivery to the retina. To exert its function in retinal cells *Trans*IT-complexed mRNA has to migrate through the vitreous humor, which can strongly interfere with its mobility. Our data show that at least a fraction of the *Trans*IT-mRNA complexes encounters difficulties while maneuvering through bovine vitreous (**Figure 3A**). This observation could be attributed to three factors. For one, there is a size limit for particle mobility through the vitreal network, which is estimated to be <550 nm[13,19]. While the single particle fraction with sizes around 190 nm should be sufficiently small to move through the vitreous mesh, the aggregated fraction is likely too large (**Figure 1D**). Secondly, the anionic nature of the vitreous, created by the presence of GAGs, can immobilize positively charged particles due to electrostatic adherence. This effect is even further enhanced by hydrophobic interactions which cause the particles to stick to the collagen fibrils[17,19]. Thirdly, reports have suggested the presence of an ocular "biomolecular corona". This is a layer of absorbed biomolecules, such as vitreal proteins that electrostatically interact with the particles[41]. This coating could increase the size of the complexes, thereby lowering their intravitreal mobility.

In order to look into the interactions of our particles with the vitreous components, while canceling out the immobilizing effect of the meshwork itself, we sonicated the vitreous prior to addition of the complexes. As expected, the immobile fraction, which is withheld in the intact vitreous due to their large size and/or network adherence, was absent in the SV (**Supplementary Figure S2**). Nevertheless, we did observe retardation in particle diffusion compared to HEPES buffer. This is likely attributed to the higher viscosity of SV and/or the formation of a biomolecular corona. Although we did not perform an in depth analysis to determine the presence of such a biomolecular corona, its formation is suggested by our data. When *Trans*IT-complexed mRNA were added to MIO-M1 Müller cells in the presence of sonicated vitreous, we observed a highly reduced uptake and transfection of MIO-M1 cells in SV compared to culture medium (**Figure 3B,D-E**), which is clearly not attributable to changes in cell viability (**Figure 3C**). Therefore, we hypothesize that, although the vitreal network is mechanically broken up, the complexes interact with free GAGs, proteins and/or collagen remnants, which consequently limits intracellular uptake.

To prevent these electrostatic interactions with the vitreal constitutes, complexes can be coated with PEG, a commonly used approach to avoid unwanted interactions with extracellular compounds[42]. Surely, Martens *et al.* demonstrated that PEGylation of cationic polyplexes minimized interactions with the vitreal components and enhanced their diffusivity

through the collagen matrix[17]. However, as PEGylation was also shown to reduce cellular uptake and thus transfection efficiency, the authors suggested to use HA as suitable alternative to PEG. HA, is an anionic, non-sulfated GAG, widely distributed throughout the extracellular matrix, connective tissue, synovial fluid and the vitreous humor of the eye[43]. *In vivo* HA usually occurs as a linear, high MW (up to 10^7 kDa) polymer, yet enzymatic degradation can result in shorter fragments[44-46]. Extensive studies in mammals indicate that its physicochemical properties and functions are dependent on its MW, presumably due to the varying nature and affinity of its interactions with binding proteins and receptors (*e.g.,* CD44)[46-48].

In the present study, we therefore made use of HA with three different MWs (22 kDa, 137 kDa and 2700 kDa), which were previously evaluated for surface coating of cationic polymeric pDNA complexes[25]. Cationic *Trans*IT-mRNA complexes where electrostatically coated by addition of increasing amounts of HA, until stable, negatively charged complexes were obtained (**Figure 4A,B**). Subsequently, the lowest amount of HA required for successful surface coating (HA/*Trans*IT/mRNA ratio of 16:2:1 (v/v/w)) was chosen to carry out further experiments. Strikingly, the use of higher MW HA yielded complexes with lower zeta potentials (**Figure 4B**), suggestive of a difference in packing density depending on the MW of the HA polymer. As we added the same number of negative charges to the *Trans*IT-complexes, independent of the MW (based on the amount of monomers for each polymer), we expected the overall zeta potential to be the same when HA22, HA137 or HA2700 is used. However, although fewer polymers of HA2700 are added, particles do obtain a lower zeta potential. Based on this observation, we believe that there is a difference in structural arrangement of these different-length polymers to the complexes. For example, when HA22 is used, more polymers compete for surface binding, resulting in a rapidly saturated particle surface and less affective adherence of the remaining polymers. Successful surface coating was subsequently confirmed by FCS analysis, where we demonstrate the presence of red-labeled HA on the surface of green-labeled mRNA complexes. As mRNA is complexed to the *Trans*IT reagent by spontaneous electrostatic interactions, addition of the anionic HA could cause a disruption of the complexes, leading to dissociation of the mRNA and subsequent degradation by vitreal RNases. Indeed, HA has been previously suggested to facilitate the release of DNA from nanoparticles consisting on solid lipid nanoparticles (SLNs) and protamine in HEK-293 cells[49]. However, gel electrophoresis experiments revealed that *Trans*IT-mRNA complexes remain stable in both Opti-MEM™ and bovine vitreous after coating with HA of all MWs (**Figure 4D**).

Next we determined the influence of HA-coating on the IVTR mobility of the *Trans*IT-mRNA complexes. When compared to the mobility of the uncoated complexes, a substantial

increase in diffusion efficiency was achieved by all HA polymers. Clearly, HA-coating stimulates movement of the immobile fraction as can be seen in **Figure 5A**. This could be ascribed to two phenomena. Firstly, addition of HA prevents particle aggregation, which is supported by the more monodisperse size distributions of HA-coated *versus* uncoated particles in pure HEPES buffer (**Figure 5B**). Indeed, all HA-coated formulations have an average size <200 nm, which is lower than the estimated mesh size of the vitreal collagen network. Secondly, as the negative HA layer shields the cationic particles form electrostatic and hydrophobic interactions, attachment to the collagen fibrils as well as absorption of vitreal proteins (forming an 'unwanted' corona) could be prevented. Notably, the fraction of particles which was already mobile without any coating, was not altered in their diffusion. Therefore we hypothesize that this portion of uncoated particles is actually mobilized due to spontaneous absorption of native HA on their cationic surface. Of all HA sizes, HA137 gave rise to the smallest HA-coated particles, and the highest frequency of mobile particles, in line with what has been reported by Martens *et al.* for the coating of polymer-based complexes[25]. Also Koo *et al.* investigated the movement of different nanoparticle types through the vitreous and demonstrated migration of self-assembled negatively charged HA nanoparticles through the collagen matrix and in the neural retina[30]. However, it is important to note that this last study was performed in rodents, of which the vitreous has a smaller volume and a more liquid composition[35,50,51]. Therefore, the impact of the vitreous barrier could be underestimated and care should be taken when extrapolating these data to larger animals and humans.

Although electrostatic coating with HA of various MWs clearly improves the particles' IVTR mobility, its negative surface charge could impede interactions with the anionic cell membrane and thus limit cellular uptake, as is the case for PEGylation. Nevertheless, uptake of HA-coated pDNA complexes, such as SLNs and cationic polyplexes, has been previously shown to be successful in ARPE-19 cells in presence of Opti-MEM™ [25,49]. In this study, we verified uptake of the polymer/lipid-based *Trans*IT-mRNA complexes in MIO-M1 Müller cells, taking into account possible interactions with vitreal components by means of sonicated bovine vitreous. Remarkably, despite their negative surface charge, and hence reduced affinity to the cell membrane, no difference in uptake was observed compared to uptake of uncoated complexes (**Figure 5C**). This indicates that, although positively charged particles are generally expected to be endocytosed more efficiently due electrostatic binding to the cell membrane, other factors besides particle charge clearly influence cellular uptake. In the same line, Hornof and de la Fuente demonstrated efficient uptake of HA-coated DNA/PEI polyplexes into human corneal epithelia cells, which was similar to the uncoated control[48]. As HA is an established ligand for the CD44-receptor, internalization of the coated complexes could be mediated by receptor-ligand interactions, as suggested by Martens *et*

al. for RPE cells[25]. Indeed, the authors showed that saturation of the CD44 receptor by pre-incubation with free HA significantly reduced uptake of HA137-coated particles. Also Hornof *et al.* reported the uptake of their HA-coated complexes to be a receptor-mediated process, by antibody-mediated blockage of CD44[48]. As the CD44 receptor is also present on the surface of Müller glia[52], receptor-mediated uptake could be a possible explanation for the observed results in this study. It can be argued however, whether this uptake mechanisms will still hold true following IVTR injection *in vivo.* Indeed, CD44 has been shown to be exclusively expressed on the apical side of Müller glia, facing towards the subretinal space[53]. *In vitro* situations, in which particles can come into contact with the entire Müller cell and not only the 'vitreal' side, should therefore be interpreted with necessary caution.

As previous studies by Mizrahi *et al.* and Wolny *et al.* reported a stronger binding of high MW HA (≥ 130 kDa) to the CD44 receptor[47,54], we expected a higher affinity and therefore increased uptake of the complexes with increasing HA size. However no significant difference was observed in the uptake between the various MWs of HA in the present study. As the results of both previous reports were based on the immobilization of CD44 on a cell-free, planar support (at a 10-100 times higher density of CD44 receptors) caution should be taken, when comparing these results to our experiments performed on CD44-expressing cells. Nevertheless, also studies based on cell cultures showed a correlation between nanoparticle internalization and the size of the grafted HA chains, where uptake increased with increasing HA MW[55,56]. Differences in our results might be attributed to differences in CD44-receptor properties, as different cell types can have a different CD44-receptor density, clustering and turn-over rate, which can all lead to variations in HA-receptor interactions[25,45].

In contrast to carrier uptake, electrostatic HA-coating did slightly improve the transfection efficiency of the complexes in MIO-M1 Müller cells, with HA137-coating producing the highest eGFP expression levels (**Figure 5C,D**). This observation might be due to variations in intracellular trafficking and subsequent endosomal escape, as some studies claim that CD44 receptor-mediated uptake is able to avoid lysosomal degradation leading to higher transfection efficiencies[57,58]. Also, it might be possible that HA coating favors the dissociation of mRNA from the complexes, leading to more efficient intracellular release of mRNA into the cytoplasm.

CONCLUSIONS

Although there are multiple benefits in the use of mRNA for the expression of therapeutic proteins in the retina, this study clearly shows the critical challenge of delivering mRNA-based therapeutics to their target site. Especially when IVTR injection is desired in order to reach the inner retina, the vitreous represents a major gene delivery barrier. As demonstrated in the present study, increasing knowledge concerning the physiological mechanisms that hinder the delivery process allows for smart adjustments, such as coating strategies, to predefined gene carriers in order to obtain the desired physicochemical characteristics to overcome extracellular barriers. As such, we demonstrated that electrostatical coating of the commercial available mRNA carrier *Trans*IT^{TM} with HA provided particles with a negative surface charge and a monodisperse size distribution, which drastically increased their ability to diffuse through the vitreous humor without compromising their transfection efficiency. Unfortunately, these modifications were not sufficient to markedly increase the protein expression levels in an *in vitro* setting. Before the potential of mRNA can be evaluated on a preclinical level, it is therefore important to develop a more efficient carrier, of which the surface charge after mRNA complexation should be negatively charged, as indicated by the present study.

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REFERENCES

1 Bennett, J. Taking Stock of Retinal Gene Therapy: Looking Back and Moving Forward. *Molecular therapy : the journal of the American Society of Gene Therapy* **25**, 1076-1094, doi:10.1016/j.ymthe.2017.03.008 (2017).

2 Gupta, P. R. & Huckfeldt, R. M. Gene therapy for inherited retinal degenerations: initial successes and future challenges. *Journal of neural engineering* **14**, 051002, doi:10.1088/1741-2552/aa7a27 (2017).

3 Adijanto, J. & Naash, M. I. Nanoparticle-based technologies for retinal gene therapy. *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V* **95**, 353-367, doi:10.1016/j.ejpb.2014.12.028 (2015).

4 Planul, A. & Dalkara, D. Vectors and Gene Delivery to the Retina. *Annual review of vision science* **3**, 121-140, doi:10.1146/annurev-vision-102016-061413 (2017).

5 Al-Dosari, M. S. & Gao, X. Nonviral gene delivery: principle, limitations, and recent progress. *Aaps J* **11**, 671-681, doi:10.1208/s12248-009-9143-y (2009).

6 Zulliger, R., Conley, S. M. & Naash, M. I. Non-viral therapeutic approaches to ocular diseases: An overview and future directions. *Journal of controlled release : official journal of the Controlled Release Society* **219**, 471-487, doi:10.1016/j.jconrel.2015.10.007 (2015).

7 Gu, Y. The nuclear pore complex: a strategic platform for regulating cell signaling. *The New phytologist*, doi:10.1111/nph.14756 (2017).

8 Devoldere, J., Dewitte, H., De Smedt, S. C. & Remaut, K. Evading innate immunity in nonviral mRNA delivery: don't shoot the messenger. *Drug discovery today* **21**, 11-25, doi:10.1016/j.drudis.2015.07.009 (2016).

9 Dalkara, D. *et al.* AAV mediated GDNF secretion from retinal glia slows down retinal degeneration in a rat model of retinitis pigmentosa. *Molecular therapy : the journal of the American Society of Gene Therapy* **19**, 1602-1608, doi:10.1038/mt.2011.62 (2011).

10 Da Costa, R. *et al.* A Novel Method Combining Vitreous Aspiration and Intravitreal AAV2/8 Injection Results in Retina-Wide Transduction in Adult Mice. *Investigative ophthalmology & visual science* **57**, 5326-5334, doi:10.1167/iovs.16-19701 (2016).

11 Fattal, E. & Bochot, A. Ocular delivery of nucleic acids: antisense oligonucleotides, aptamers and siRNA. *Advanced drug delivery reviews* **58**, 1203-1223, doi:10.1016/j.addr.2006.07.020 (2006).

12 Ahmad, M. T., Zhang, P., Dufresne, C., Ferrucci, L. & Semba, R. D. The Human Eye Proteome Project: Updates on an Emerging Proteome. *Proteomics* **18**, e1700394, doi:10.1002/pmic.201700394 (2018).

13 Peeters, L. *et al.* Vitreous: a barrier to nonviral ocular gene therapy. *Investigative ophthalmology & visual science* **46**, 3553-3561, doi:10.1167/iovs.05-0165 (2005).

14 Mains, J. & Wilson, C. G. The Vitreous Humor As a Barrier to Nanoparticle Distribution. *J Ocul Pharmacol Th* **29**, 143-150, doi:10.1089/jop.2012.0138 (2013).

15 Rowe-Rendleman, C. L. *et al.* Drug and gene delivery to the back of the eye: from bench to bedside. *Investigative ophthalmology & visual science* **55**, 2714-2730, doi:10.1167/iovs.13-13707 (2014).

16 Kim, H., Robinson, S. B. & Csaky, K. G. Investigating the Movement of Intravitreal Human Serum Albumin Nanoparticles in the Vitreous and Retina. *Pharmaceut Res* **26**, 329-337, doi:10.1007/s11095-008-9745-6 (2009).

17 Martens, T. F. *et al.* Measuring the intravitreal mobility of nanomedicines with single-particle tracking microscopy. *Nanomedicine (Lond)* **8**, 1955-1968, doi:10.2217/nnm.12.202 (2013).

18 Pitkanen, L., Ruponen, M., Nieminen, J. & Urtti, A. Vitreous is a barrier in nonviral gene transfer by cationic lipids and polymers. *Pharmaceut Res* **20**, 576-583, doi:Doi 10.1023/A:1023238530504 (2003).

19 Xu, Q. *et al.* Nanoparticle diffusion in, and microrheology of, the bovine vitreous ex vivo. *Journal of controlled release : official journal of the Controlled Release Society* **167**, 76-84, doi:10.1016/j.jconrel.2013.01.018 (2013).

20 Sanders, N. N., Peeters, L., Lentacker, I., Demeester, J. & De Smedt, S. C. Wanted and unwanted properties of surface PEGylated nucleic acid nanoparticles in ocular gene transfer. *Journal of controlled release : official journal of the Controlled Release Society* **122**, 226-235, doi:10.1016/j.jconrel.2007.05.004 (2007).

21 Mishra, S., Webster, P. & Davis, M. E. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur J Cell Biol* **83**, 97-111, doi:Doi 10.1078/0171-9335-00363 (2004).

22 Bishop, P. N. Structural macromolecules and supramolecular organisation of the vitreous gel. *Progress in retinal and eye research* **19**, 323-344, doi:Doi 10.1016/S1350-9462(99)00016-6 (2000).

23 Clark, S. J. *et al.* Mapping the differential distribution of glycosaminoglycans in the adult human retina, choroid, and sclera. *Investigative ophthalmology & visual science* **52**, 6511-6521, doi:10.1167/iovs.11-7909 (2011).

24 Devoldere, J., Peynshaert, K., De Smedt, S. C. & Remaut, K. Muller cells as a target for retinal therapy. *Drug discovery today*, doi:10.1016/j.drudis.2019.01.023 (2019).

25 Martens, T. F. *et al.* Coating nanocarriers with hyaluronic acid facilitates intravitreal drug delivery for retinal gene therapy. *Journal of Controlled Release* **202**, 83-92, doi:10.1016/j.jconrel.2015.01.030 (2015).

26 Limb, G. A., Salt, T. E., Munro, P. M., Moss, S. E. & Khaw, P. T. In vitro characterization of a spontaneously immortalized human Muller cell line (MIO-M1). *Investigative ophthalmology & visual science* **43**, 864-869 (2002).

27 Martens, T. F. *et al.* Measuring the intravitreal mobility of nanomedicines with single-particle tracking microscopy. *Nanomedicine : nanotechnology, biology, and medicine* **8**, 1955-1968, doi:10.2217/nnm.12.202 (2013).

28 Braeckmans, K. *et al.* Sizing nanomatter in biological fluids by fluorescence single particle tracking. *Nano letters* **10**, 4435-4442, doi:10.1021/nl103264u (2010).

29 Pitkanen, L., Ruponen, M., Nieminen, J. & Urtti, A. Vitreous is a barrier in nonviral gene transfer by cationic lipids and polymers. *Pharmaceutical research* **20**, 576-583 (2003).

30 Koo, H. *et al.* The movement of self-assembled amphiphilic polymeric nanoparticles in the vitreous and retina after intravitreal injection. *Biomaterials* **33**, 3485-3493, doi:10.1016/j.biomaterials.2012.01.030 (2012).

31 Wolff, J. A. *et al.* Direct Gene-Transfer into Mouse Muscle Invivo. *Science* **247**, 1465-1468, doi:DOI 10.1126/science.1690918 (1990).

32 Leonhardt, C. *et al.* Single-cell mRNA transfection studies: delivery, kinetics and statistics by numbers. *Nanomedicine : nanotechnology, biology, and medicine* **10**, 679-688, doi:10.1016/j.nano.2013.11.008 (2014).

33 Hansson, M. L. *et al.* Efficient delivery and functional expression of transfected modified mRNA in human embryonic stem cell-derived retinal pigmented epithelial cells. *The Journal of biological chemistry* **290**, 5661-5672, doi:10.1074/jbc.M114.618835 (2015).

34 Chen, M. *et al.* Retinal pigment epithelial cell multinucleation in the aging eye - a mechanism to repair damage and maintain homoeostasis. *Aging Cell* **15**, 436-445, doi:10.1111/acel.12447 (2016).

35 Peynshaert, K., Devoldere, J., De Smedt, S. C. & Remaut, K. In vitro and ex vivo models to study drug delivery barriers in the posterior segment of the eye. *Advanced drug delivery reviews*, doi:10.1016/j.addr.2017.09.007 (2017).

36 Trapani, I., Banfi, S., Simonelli, F., Surace, E. M. & Auricchio, A. Gene therapy of inherited retinal degenerations: prospects and challenges. *Human gene therapy* **26**, 193-200, doi:10.1089/hum.2015.030 (2015).

37 Lebrun-Julien, F. *et al.* Excitotoxic death of retinal neurons in vivo occurs via a non-cell-autonomous mechanism. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 5536-5545, doi:10.1523/JNEUROSCI.0831-09.2009 (2009).

38 Skeie, J. M. & Mahajan, V. B. Proteomic interactions in the mouse vitreous-retina complex. *PloS one* **8**, e82140, doi:10.1371/journal.pone.0082140 (2013).

39 Peynshaert, K., Devoldere, J., Minnaert, A. K., De Smedt, S. C. & Remaut, K. Morphology and Composition of the Inner Limiting Membrane: Species-Specific Variations and Relevance toward Drug Delivery Research. *Current eye research*, 1-11, doi:10.1080/02713683.2019.1565890 (2019).

40 Le Goff, M. M. & Bishop, P. N. Adult vitreous structure and postnatal changes. *Eye* **22**, 1214-1222, doi:10.1038/eye.2008.21 (2008).

41 Jo, D. H. *et al.* Nanoparticle-protein complexes mimicking corona formation in ocular environment. *Biomaterials* **109**, 23-31, doi:10.1016/j.biomaterials.2016.09.008 (2016).

42 Suk, J. S., Xu, Q. G., Kim, N., Hanes, J. & Ensign, L. M. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Advanced drug delivery reviews* **99**, 28-51, doi:10.1016/j.addr.2015.09.012 (2016).

43 Fraser, J. R., Laurent, T. C. & Laurent, U. B. Hyaluronan: its nature, distribution, functions and turnover. *Journal of internal medicine* **242**, 27-33 (1997).

44 Liao, Y. H., Jones, S. A., Forbes, B., Martin, G. P. & Brown, M. B. Hyaluronan: Pharmaceutical characterization and drug delivery. *Drug delivery* **12**, 327-342, doi:10.1080/10717540590952555 (2005).

45 Guter, M. & Breunig, M. Hyaluronan as a promising excipient for ocular drug delivery. *European Journal of Pharmaceutics and Biopharmaceutics* **113**, 34-49, doi:10.1016/j.ejpb.2016.11.035 (2017).

46 Stern, R., Asari, A. A. & Sugahara, K. N. Hyaluronan fragments: an information-rich system. *European journal of cell biology* **85**, 699-715, doi:10.1016/j.ejcb.2006.05.009 (2006).

47 Mizrahy, S. *et al.* Hyaluronan-coated nanoparticles: the influence of the molecular weight on CD44-hyaluronan interactions and on the immune response. *Journal of controlled release : official journal of the Controlled Release Society* **156**, 231-238, doi:10.1016/j.jconrel.2011.06.031 (2011).

48 Hornof, M., de la Fuente, M., Hallikainen, M., Tammi, R. H. & Urtti, A. Low molecular weight hyaluronan shielding of DNA/PEI polyplexes facilitates CD44 receptor mediated uptake in human corneal epithelial cells. *J Gene Med* **10**, 70-80, doi:10.1002/jgm.1125 (2008).

49 Apaolaza, P. S., Delgado, D., del Pozo-Rodriguez, A., Gascon, A. R. & Solinis, M. A. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. *International journal of pharmaceutics* **465**, 413-426, doi:10.1016/j.ijpharm.2014.02.038 (2014).

50 Skeie, J. M., Tsang, S. H. & Mahajan, V. B. Evisceration of Mouse Vitreous and Retina for Proteomic Analyses. *Jove-J Vis Exp*, doi:Artn E2795 10.3791/2795 (2011).

51 Del Amo, E. M. *et al.* Pharmacokinetic aspects of retinal drug delivery. *Progress in retinal and eye research* **57**, 134-185, doi:10.1016/j.preteyeres.2016.12.001 (2017).

52 Shinoe, T. *et al.* Identification of CD44 as a cell surface marker for Muller glia precursor cells. *J Neurochem* **115**, 1633-1642, doi:10.1111/j.1471-4159.2010.07072.x (2010).

53 Too, L. K., Gracie, G., Hasic, E., Iwakura, J. H. & Cherepanoff, S. Adult human retinal Muller glia display distinct peripheral and macular expression of CD117 and CD44 stem cell-associated proteins. *Acta Histochem* **119**, 142-149, doi:10.1016/j.acthis.2016.12.003 (2017).

54 Wolny, P. M. *et al.* Analysis of CD44-hyaluronan interactions in an artificial membrane system: insights into the distinct binding properties of high and low molecular weight hyaluronan. *The Journal of biological chemistry* **285**, 30170-30180, doi:10.1074/jbc.M110.137562 (2010).

55 Qhattal, H. S. S. & Liu, X. L. Characterization of CD44-Mediated Cancer Cell Uptake and Intracellular Distribution of Hyaluronan-Grafted Liposomes. *Mol Pharmaceut* **8**, 1233-1246, doi:10.1021/mp2000428 (2011).

56 Gan, L. *et al.* Hyaluronan-modified core-shell liponanoparticles targeting CD44-positive retinal pigment epithelium cells via intravitreal injection. *Biomaterials* **34**, 5978-5987, doi:10.1016/j.biomaterials.2013.04.035 (2013).

57 Contreras-Ruiz, L. *et al.* Intracellular trafficking of hyaluronic acid-chitosan oligomer-based nanoparticles in cultured human ocular surface cells. *Molecular vision* **17**, 279-290 (2011).

58 Hayward, S. L., Wilson, C. L. & Kidambi, S. Hyaluronic acid-conjugated liposome nanoparticles for targeted delivery to CD44 overexpressing glioblastoma cells. *Oncotarget* **7**, 34158-34171, doi:10.18632/oncotarget.8926 (2016).

SUPPORTING INFORMATION

Chapter 3

mRNA for retinal gene delivery: the obstacle course to the inner retina

SUPPORTING INFORMATION:

Supporting Information consist of additional material and methods for the isolation and culture of primary bovine Müller cells and 3 additional figures.

SUPPLEMENTARY MATERIALS AND METHODS

Isolation and culture of primary bovine Müller cells

To obtain primary bovine Müller cells (pMC), fresh bovine eyes were obtained from the local abattoir and kept in 4°C CO_2 independent medium until dissection. Extra-ocular tissue was removed, followed by eye disinfection using 20% ethanol. Using sharp curved scissors the eye was bisected, vitreous was carefully removed and the posterior eye cup was transferred to a culture dish containing PBS buffer with 5% penicillin-streptomycin mixture. Subsequently, the eye cup was cut into 4 flaps, of which the retinal tissue was removed and transferred to a tissue grinder containing separation medium, consisting of advanced D-MEM medium (Gibco-Invitrogen, Merelbeke, Belgium), supplemented with 1% Penicillin-Streptopmycin and 1% Glutamax (Gibco-Invitrogen). After thoroughly grinding of the retinal tissue the cell suspensions were poured into a 40 µm cell strainer mounted on a 50 ml falcon tube and spun down at 300 g for 5min at RT. The supernatant of each falcon tube was discarded and the cell pellets were washed with separation medium. After 3 washing steps, the cell pellets were re-suspended in separation medium supplemented with 10% heat-inactivated FBS (Hyclone, Cramilton, UK) and 4 ng ml^{-1} epidermal growth factor (Sigma-Aldrich, Bronem, Belgium). The retinal tissue of two eye flaps was transferred into one T75 Cellbind flask (Corning®). The cells were cultured in a 5% CO_2 incubator at 37°C and the medium was renewed once a week. After 2-3 weeks the cells were passaged and at passage 3 they were seeded. Five days prior to transfection, $2x10^4$ cells were plated per well in 24-well plates.

SUPPLEMENTARY FIGURES

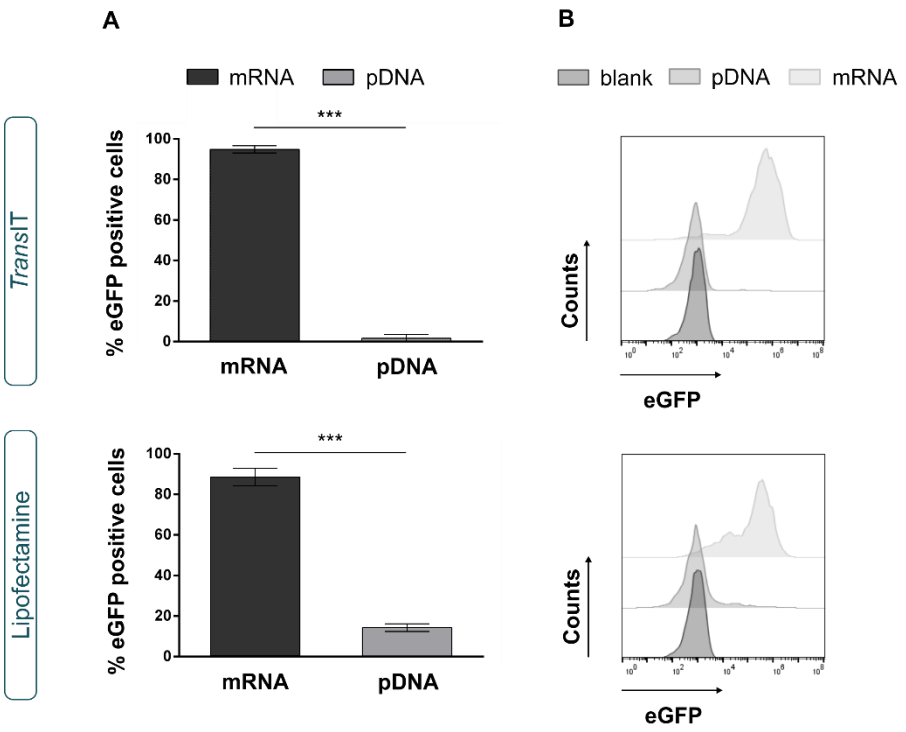

Figure S1 | Transfection efficiency of mRNA versus pDNA containing nanoplexes in primary bovine Müller cells. (A) Percentages of eGFP transfected Müller cells, 24 h after incubation with *Trans*IT and Lipofectamine containing unmodified mRNA or pDNA. Data represent mean ± SD (*n*=1x3). Representative flow cytometry histograms are shown in (B). ***, $p < 0.001$ mRNA versus pDNA by an unpaired t-test.

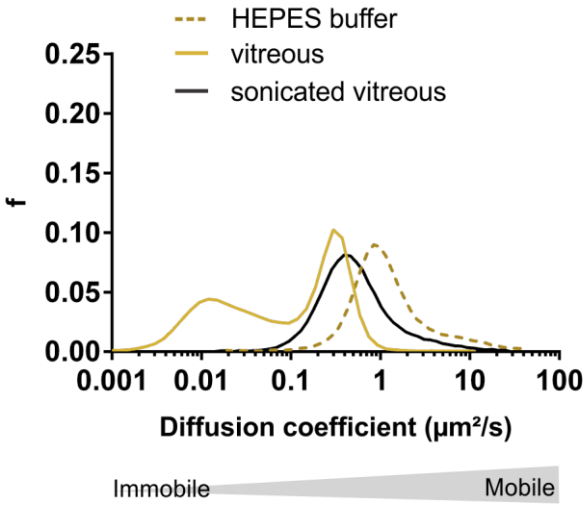

Figure S2 | Diffusive properties of Cy®5-labeled *Trans*IT-complexed mRNA in SV (black solid line) compared to their diffusion in HEPES buffer and intact vitreous. Diffusion distributions are obtained by SPT-analysis, 24 h after IVTR injection or administration of the particles to the corresponding media.

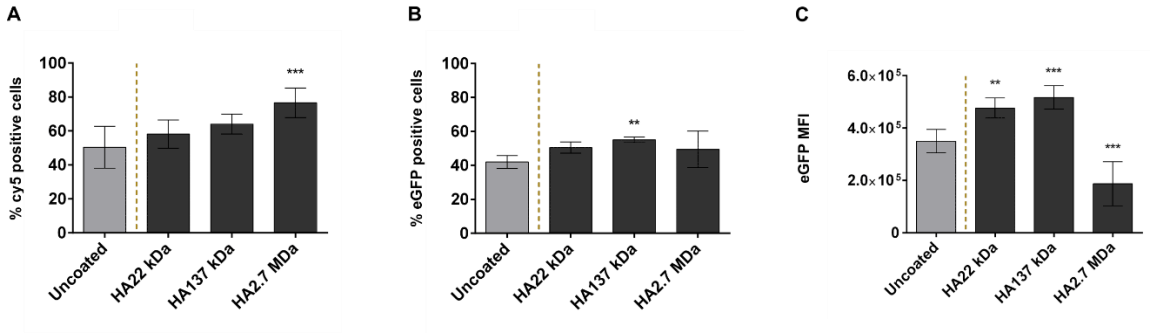

Figure S3 | Influence of HA-coating on the uptake and transfection efficiency of *Trans*IT-complexed mRNA in culture medium. All measurements were done using a HA/*Trans*IT/mRNA ratio of 16. (A) Percentage of MIOM Müller cells which are positive for Cy®5-labeled mRNA after 3 h incubation with the *Trans*IT complexes. (B) Percentage of eGFP transfected MIO-M1 Müller cells and (C) mean fluorescence intensity (MFI) 24 h after incubation with *Trans*IT-complexed mRNA. Data is shown as mean ± SD (n=1x3). **, $p < 0.01$; ***, $p < 0.001$ coated versus uncoated by one-way ANOVA.

Chapter 4

The potential of chemically modified mRNA for retinal protein expression: subretinal *vs.* intravitreal administration

Part of this chapter is under revision for publication as:

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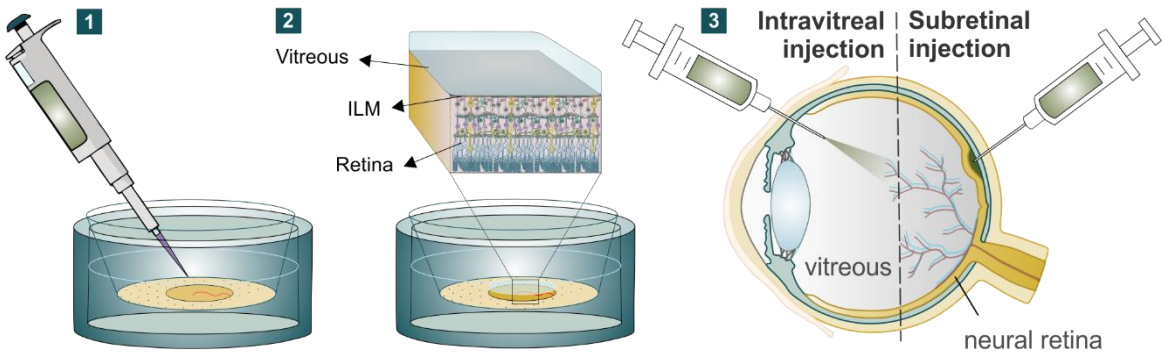
ABSTRACT

Messenger RNA (mRNA) therapeutics have recently experienced a new wave of interest, mainly due to the discovery that chemical modifications to mRNA's molecular structure could drastically reduce its inherent immunogenicity and perceived instability. On this basis, we aimed to explore the potential of chemically stabilized mRNA for ocular applications. More specifically, we investigated the behavior of mRNA-loaded lipid-based carriers in human retinal cells (*in vitro*), in bovine retinal explants (*ex vivo*) and in mouse retinas (*in vivo*). We demonstrate a clear superiority of chemically modified mRNA to induce protein expression in MIO-M1 Müller cells, providing up to ~25-fold higher reporter gene expression compared to unmodified mRNA. Moreover, eGFP expression could be detected for at least 20 days after a single administration of chemically modified mRNA *in vitro*. We furthermore determined the localization and extent of mRNA expression depending on the administration route. After subretinal (SR) administration, mRNA expression was observed *in vivo* and *ex vivo*. By contrast, intravitreal (IVTR) administration resulted in limited expression *in vivo*. Using *ex vivo* bovine explants with an intact vitreoretinal (VR) interface we could attribute this to the inner limiting membrane (ILM), which presents a large barrier for non-viral delivery of mRNA, trapping mRNA complexes at the vitreal side. When the vitreous was removed, which compromises the ILM, mRNA expression was apparent and seemed to colocalize with Müller cells or photoreceptors after respectively IVTR or SR administration. Taken together, this study represents a first step towards mRNA-mediated therapy for retinal diseases.



INTRODUCTION

The development of messenger RNA (mRNA)-based medicine was long deemed inferior to the creation of gene therapeutics based on DNA. Nowadays, *in vitro*-transcribed (IVT) mRNA is reviving as a promising candidate for the delivery of genetic information. Recent investments in improving mRNA synthesis and stability have enabled a wide range of applications, thereby even surpassing the potential that was once envisioned for DNA-based medicine[1]. With regard to ocular delivery, mRNA offers several key advantages in comparison to DNA-based gene therapeutics. Since it can be instantly translated in the cytoplasm without the need for nuclear entry[2], mRNA is an ideal candidate for the transfection of post-mitotic retinal cells. In view of safety concerns, mRNA does not integrate into the host genome, which reduces the risk of insertional mutagenesis. In addition, complete physiological degradation provides mRNA with a transient activity, providing a more controlled temporal expression[3]. Together, these advantages could allow mRNA to safely induce the local expression of various substances in the retina, such as anti-apoptotic proteins[4, 5], reactive oxygen species (ROS) inhibitors or neurotrophic factors[6-10] that could slow down retinal degeneration of many ocular diseases.

This work aims to uncover, for the first time, the potential of non-viral delivery of mRNA for ocular applications. The *in vitro* and *ex vivo* models used in this study are based on the two routinely used injection routes to deliver substances to the retina: intravitreal (IVTR) and subretinal (SR) injection. Therapeutics delivered via IVTR injections end up in the vitreous and therefore require sufficient IVTR mobility and minimal interactions with the vitreal components, as outlined in **Chapter 3**. SR injections on the other hand deliver their cargo directly below the neural retina, in the subretinal space, free of vitreous. Since cationic lipids have been shown to result in an overall better mRNA transfection efficiency than cationic polymers[11-13], we made use of a lipid-based carrier, Lipofectamine™ MessengerMAX, specifically designed for mRNA delivery. In particular, we examine the physicochemical properties of this formulation and study its capacity to transfect MIO-M1 Müller cells with and without the presence of vitreous. As described in **Chapter 2** Müller cells are in close contact with the vitreous as well as the subretinal space making them an ideal target cell accessible to both intravitreally and subretinally injected drugs (see **Figure 1** for a detailed overview of the retinal cell types). The *in vitro* transfection efficiency of the mRNA complexes is further maximized by implementing several chemical modifications to the mRNA. Next, we correlate our *in vitro* findings to experiments in retinal bovine explants and mice and as such further elucidate the transfection potential of non-viral mRNA delivery to the retina following SR and IVTR injection. Finally, we determine the impact of potential delivery obstacles and highlight the individual barrier role of the retina itself, the vitreous and inner limiting membrane (ILM)

for retinal non-viral mRNA delivery. This study underscores the crucial barrier function of the ILM following IVTR delivery and the potential of mRNA transfection following SR delivery. Taken together, this study represents a first step towards mRNA-mediated therapy for retinal diseases.

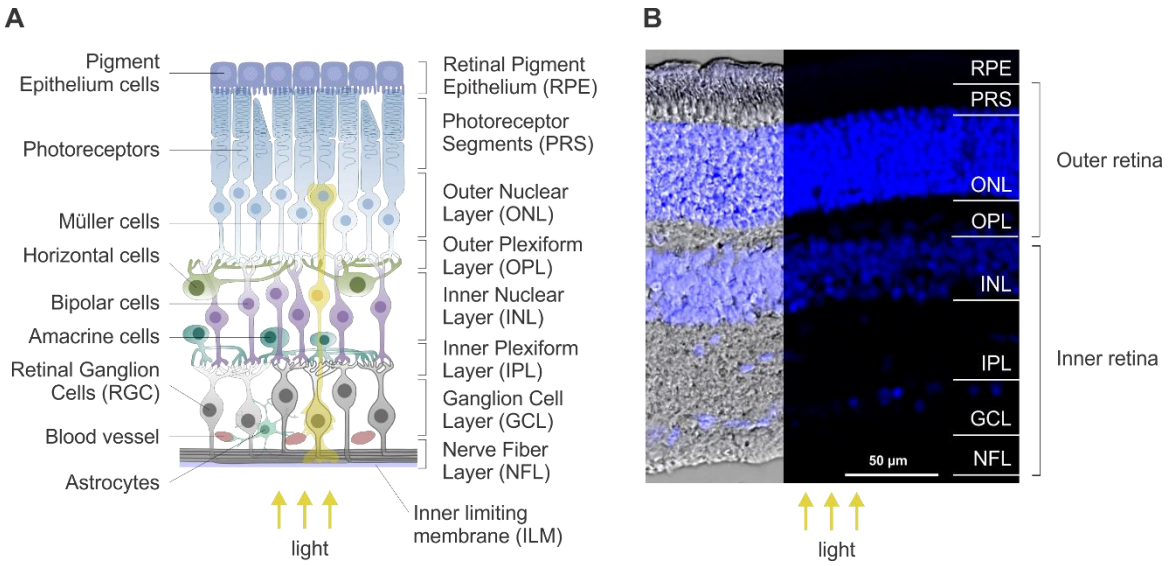


Figure 1 | The retinal structure and its cellular layers. (A) Schematic illustration with indication of the different cell types. (B) Confocal microscope images of vertical frozen sections through a bovine retinal explant. Nuclei were stained with Hoechst (blue), scale bar: 50 µm.

MATERIALS AND METHODS

Plasmids

eGFP and luciferase encoding mRNA were produced by *in vitro* transcription from pGEM4Z-GFP-A64 and pBlue-Luc-A50 plasmids, respectively. The pDNA transfections were carried out with the gwiz-GFP plasmid (Promega, Leiden, The Netherlands). All plasmids were amplified in *E.coli* bacteria (Genlantis, San Diego, CA, USA) and purified from the bacterial suspension using the QIAfilter plasmid purification kit (Qiagen, Venlo, The Netherlands). Purity and concentration were measured by UV absorption at 260 nm and 280 nm. Finally, plasmids were resuspended in 25 mM HEPES, pH 7,2 at a concentration of 1µg μl^{-1} and stored at -20°C.

(modified) mRNA constructs

To generate templates for *in vitro* transcription, pGEM4Z-GFP-A64 and pBlue-Luc-A50 plasmids were linearized using the SpeI and DraI restriction enzymes (Promega),

respectively. Linearized plasmids were purified with the QIAquick PCR purification kit (Qiagen) and quantified spectrophotometrically. *In vitro* transcription reactions for the production of unmodified mRNAs were carried out with the mMESSAGE mMACHINE T7 Transcription kit (Ambion, Life Technologies, Ghent, Belgium), including a 7-methylGpppG cap analog. To generate mRNA modifications, the MEGAscript T7 Transcription Kit (Ambion) was used and conventional nucleotides were partially (25%) or entirely (100%) replaced by modified nucleotides: 5-methylcytidine(m5C)-triphosphate, pseudouridine(ψU)-triphosphate, 2-thiouridine(s2U)-triphosphate and N^1-methyl pseudouridine(m1ψU)-triphosphate (all purchased from from TriLink BioTechnologies, San Diego, CA). All modified mRNAs were capped with the Anti-Reverse Cap Analog (ARCA) (TriLink BioTechnologies) and enzymatically polyadenylated using the poly(A) tail kit provided by Ambion. Following IVTR, mRNAs were treated with DNase I and purified using the RNeasy Mini Kit (Qiagen). The mRNA concentration was determined by measuring the absorbance at 260 nm. mRNA was stored in small aliquots at -80°C at a concentration of 1 µg $µl^{-1}$. For fluorescent labeling of the mRNA with Cy®5, the Label IT® nucleic acid (NA) Labeling kit of Mirus Bio (Madison, WI) was used. Cy®5 was added to the mRNA in a ratio of 1:1 (v:w). The mixture was incubated at room temperature (RT) for 1 h and the labeled mRNA was purified according to the manufacturer's instructions by means of G50 microspin purification columns.

Lipoplex preparation

mRNA, as well as pDNA was complexed to the Lipofectamine™MessengerMAX transfection reagent (Thermo Scientific, Merelbeke, Belgium) according to the manufacturer's instructions. To determine the mRNA complexation efficiency of MessengerMAX by gel electrophoresis, mRNA was mixed with the MessengerMAX reagent in different volume (µl) to weight (µg) (i.e. cationic transfection reagent-to-mRNA) ratios. Lipoplexes (i.e. carriers containing mRNA) were prepared in a final volume of 50 µl Opti-MEM™ (Thermo Scientific) and incubated at RT during 5 min. An optimal v/w ratio of 3:1 was chosen for all further experiments. When complexing different amounts of mRNA (µg) the finale volume and the volume of the reagent were scaled proportionally.

Gel electrophoresis

MessengerMAX-mRNA complexes were prepared as described above. For each desired v/w ratio an appropriate amount corresponding to 0.5 µg mRNA was incubated in Opti-MEM™, fetal bovine serum (FBS) or adult bovine vitreous (ABV). After 30 min incubation at 37°C, 5 µl Ambion loading buffer was added and mixtures were loaded into a

1.2% agarose gel in TRIS/Borate/EDTA (TBE) buffer, to which GelRed (Biotium, Hayward, CA) was added for visualization of the mRNA. The gel was run for 40 min at 100 V and imaged by UV illumination and gel photography. A 0.5 to 10 kb RNA ladder (Thermo Scientific) was included. Samples containing free mRNA in Opti-MEM™, FBS or ABV were run as controls. To determine the complexation efficiency gel analysis was performed using the ImageJ software (NIH).

Particle size and zeta potential

Size distribution and zeta potential of the MessengerMAX lipoplexes were measured by dynamic light scattering (DLS) using a Malvern Zetasizer nano-ZS (Malvern Instruments, Worcestershire, UK). All samples were diluted in 20 mM HEPES buffer pH 7.4 (Sigma-Aldrich). Size measurements were done in triplicate, with three runs per replicate and presented as number averaged hydrodynamic diameter. Zeta potential measurements were done in triplicate with two runs per replicate.

Evaluation of intravitreal lipoplex mobility

To determine the diffusion of the lipoplexes in intact vitreous, single particle tracking was performed in an *ex vivo* model as previously described by Martens *et al.*[14]. In short, fresh bovine eyes were obtained from a local abattoir, cleaned of extra-ocular tissue, disinfected in 20% ethanol and washed in sterile CO_2 independent medium. Hereafter the cornea and lens were removed, leaving the hyaloid membrane that holds the vitreous. Lipoplexes were injected in the vitreous via the posterior side of the eye with a 25GA needle (BD Microlance, Erembodegem, Belgium). A volume of 30 µl with a concentration of 50 ng μL^{-1} Cy®5-labeled mRNA was injected at four injection spots. Next, the complete eye was transferred to a glass bottom dish (In Vitro Scientific, Mountain View, CA) with the hyaloid membrane positioned against the glass bottom and stored for 24 h at RT to allow the diffusion of particles through the vitreous. Then, particle mobility was determined by Fluorescence single particle tracking (fSPT) microscopy.

For the determination of the diffusion in an aqueous environment lipoplexes containing Cy®5-labeled mRNA were diluted in RNase free HEPES buffer (20 mM, pH 7.4, Sigma-Aldrich) to a concentration of 10^8 to 10^9 particles per ml. The samples were transferred into a 96-well microplate (Greiner Bio-One, Vilvoorde, Belgium) before measuring their mobility with fSPT.

Fluorescence single particle tracking (fSPT) microscopy

fSPT is based on microscopic imaging of fluorescently labeled single molecules to characterize their diffusion. Real-time confocal tracking of individually moving lipoplexes allows to calculate their motion trajectories and diffusion coefficient. All fluorescence video imaging of diffusing lipoplexes was performed on a swept-field confocal microscope (LiveScan Swept Field Confocal Microscope System; Nikon, Brussels, Belgium) equipped with a Plan Apo 100× 1.4 NA oil immersion objective lens (Nikon) and a fast and sensitive EMCCD camera (Ixon Ultra 897, Andor Technology, CT, USA). The microscope was focused at 5-10 µm above the bottom of the well plate and the Cy®5-labeled mRNA lipoplexes were excited with a solid-state 125 mW 640 nm (Agilent Technologies, CA, USA) laser. For each sample, typically 25 movies of about 100 frames each were recorded at different random locations within the sample. Diffusion analysis of the videos was performed using in-house developed software, as described before[15], providing a distribution of apparent diffusion coefficients.

Cell culture and transfections

Moorfields/Institute of Ophthalmology-Müller 1 (MIO-M1), spontaneously immortalised human Müller glial cells, were kindly provided by Astrid Limb (Institute of Ophthalmology, University College London, UK)[16]. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) GlutaMax®pyruvate 1g l^{-1} glucose (Gibco-Invitrogen) supplemented with 1% L-glutamin, 2% penicillin/streptomycin and 10% FBS (Hyclone). Cells were passaged at 90% confluency and incubated at 37°C with 5% CO_2. Five days prior to transfection, $1x10^4$ cells were plated per well in 24-well plates.

In the most simple *in vitro* setup, the cells were seeded at the bottom of a 24-well plate. Lipoplexes were added directly to the cells in complete cell culture medium, followed by 24 h of incubation at 37°C. For long-term expression analyses, cells were subcultured at 90% confluency.

A second *in vitro* setup served as model to evaluate the effect of vitreous on uptake, transfection and toxicity. In this setup, cells were seeded on 0.4 µm pore membrane inserts (Greiner Bio-One, Vilvoorde, Belgium) at $6x10^3$ cells/insert in a 12 well plate. Fresh bovine vitreous, obtained from a local abattoir, was applied on top of the cells. To this end, the vitreous was sonicated using a tip sonicator (Branson, Swedesboro, NJ) for 3 min with short intervals every 30 s to allow fluent pipetting. Culture medium was added below the insert to assure optimal cell viability. Naked or complexed mRNA was added to the vitreous, followed

by 3 or 24 h incubation at 37°C, for uptake or transfection experiments respectively. Unless indicated otherwise, the cells were transfected with 0.5 µg mRNA or pDNA per $1x10^5$ cells.

Flow cytometry

Flow cytometric analysis was conducted on MIO-M1 that were transfected with naked or MessengerMAX-complexed eGFP mRNA as described above. Cells treated with 50 µl Opti-MEM™ alone or cells treated with the same amount of complexes containing firefly Luciferase (fLuc)-encoding mRNA were used as negative controls. The cells were detached from the plate surface with 0.25% trypsin-EDTA (Gibco, Paisly, UK), washed with cell culture medium and resuspended in phosphate-buffered saline (PBS) supplemented with 0.1% sodium azide and 1% bovine serum albumin. To allow identification of dead and apoptotic cells, respectively 4',6-diamidino-2-fenylindool (DAPI) and MitoProbe™ $DiIC_1(5)$ (ThermoFischer) stainings were added to the cell suspension according to the manufacturer's instructions. After 30 min incubation at 37°C, cells were analyzed using the CytoFLEX™ Flow Cytometer (Beckman Coulter, Krefeld, Germany) and data analysis was performed using FlowJo software (FlowJo, OR, USA). A minimum of 7000 gated cells was counted per tube. Percentages of eGFP-expressing cells were calculated from the percentage of the cell population that exceeded the fluorescence intensity of the control cells. Mean fluorescence intensity (MFI) was calculated for the entire viable cell population.

Intraocular injections

All experiments with live animals were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by local ethics committees. Before the injections, 20 C57BL6/J mice were anesthetized by intraperitoneal injection of Ketamine and Xylazine and injected intravitreally or subretinally with 0.8 µg Cy®5-labeled m1ψU-eGFP mRNA either in its naked form or complexed to MessengerMAX, prepared as described before. The sclera was incised at the pars plana with a 21G-needle, followed by insertion of the blunt end injection needle (32G) (Hamilton, Switzerland). After the injection procedure, eyes were rinsed with antibiotic eyedrops to avoid any ocular inflammation and covered by 2% Methocel (Omni Vision) to avoid over-drying of the cornea. 24 h and 7 days after the injection, animals were humanely euthanized by CO_2 overdose and cervical dislocation.

Dissection and culture of a conventional bovine explant

This explant is called “conventional” as the protocol is based on a dissection method used by most research groups to prepare explants of larger species[17-19]. In summary, fresh bovine eyes were obtained from a local abattoir within 15 min after sacrificing the animal, eyes were cleaned, disinfected and washed. The eye was bisected and the anterior segment lens and vitreous body were removed, exposing the neural retina. 10 mm trephine blades (Beaver Visitec) were used to cut circular explants out of the posterior segment, which was submerged in CO_2 independent medium. Subsequently, the retina was removed from the choroid by pipetting medium below. Two of these explants were transported to a 75 mm Transwell® explant filter (Corning) with either the photoreceptor side (mimicking SR administration) or the vitreal side (mimicking IVTR administration) facing up. Next, the explant filter was moistened with culture medium (Neurobasal®-A, 1% B-27® supplement, 1% Penicillin-streptomycin, 0.5% L-glutamin) and 20 ml of the same medium was added below the explant filter. 25 µg of Cy®5-labeled m1ψU-fLuc mRNA, naked or complexed with MessengerMAX, was administered on top of the bovine explants. Finally, explants were incubated for 24 h at 37°C and 5% CO_2.

Dissection and culture of a vitreoretinal bovine explant

Bovine retinal explants with an intact vitreoretinal (VR) interface, so-called “VR explants”, were prepared according to a protocol, recently developed and validated in our lab [20]. This explant model differs from conventional explants by the preservation of vitreous and intact ILM during dissection. In short, an incubation period of 20-30 min in CO_2 independent medium at RT allowed gently warming of the bovine eye. Subsequently, the eye was bisected, the anterior segment was removed and a posterior eye cup filled with vitreous gel remained. Next, the retina was gently detached from the choroid at the rim of the eyecup and the vitreous was gently pulled down during which the attached retina came along. The whole tissue was transported with vitreous side upwards into a culture dish of 10 cm (Corning) filled with cold CO_2 independent medium and cut into three pieces of VR explant. A plastic Pasteur pipette was used to gently aspire one of the VR explant and transfer it to a dry 75 mm Transwell® explant filter (Corning). Excess amounts of vitreous was removed by aspiration and cutting and 20 ml of supplemented NeurobasalR-A medium is added below the explant filter. 25 µg of Cy®5-labeled m1ψU-fLuc mRNA, naked or complexed with MessengerMAX, was injected in the vitreous of the VR explants. Finally, explants were incubated for 24 h at 37°C and 5% CO_2.

Cryosections

Mice eyes were enucleated and immersion fixed in 4% formaldehyde during 1 h. Cornea and lens were removed and the resulting eye-cups were cryoprotected in 10% sucrose for 1 h at 4°C, followed by an overnight incubation in 30% sucrose before embedding in Tissue-Tek® optimal cutting temperature compound (OTC) (Sakura Finetek, Berchem, Belgium).

Bovine explants were fixed for 2 h at 4°C in 4% paraformaldehyde and cryoprotected in 30% sucrose overnight at 4°C. Next, tissues were embedded in OTC before snap freezing with liquid nitrogen. 10–12 µm sections of the frozen samples were cut at -21°C using a cryostat (Leica Biosystems, Diegem, Belgium).

Immunostaining

Before staining, tissue sections were dried at RT, washed with PBS and permeabilized with a PBS-Triton 0.1% solution. Retinal sections were blocked in 1% normal goat serum and 0.05% Tween20 in PBS for 1 h at RT. For immunohistochemical staining of eGFP, *in vivo* sections were treated with anti-GFP rabbit polyclonal anti-body (Abcam, Cambridge, UK) at a 1:1000 dilution in blocking solution overnight at 4°C. After washing with PBS, the sections were incubated for 2 h at RT with a 1:500 dilution of goat anti-rabbit Alexa Fluor®488 (Abcam). Bovine *ex vivo* sections were stained with 1:200 rabbit antibody against Collagen IV for ILM staining or 1:500 mouse anti-glutamine synthetase antibody (Abcam) for Müller cell staining. Goat anti-rabbit (Abcam) and goat anti-mouse Alexa Fluor®488 (Thermofisher) 1:500 dilutions were respectively used as secondary antibody. Slices were rinsed in PBS and counterstained with 1 µg ml^{-1} Hoechst for 30 min at RT. Finally, samples were mounted in 1% propyl gallate mounting media and examined by confocal microscopy (C1-si, Nikon Belux, Brussels, Belgium) using a 60x oil objective (NIR Apo, Nikon).

Bioluminescence imaging

For the *ex vivo* transfection experiments, 25 µg of m1ψU-fLuc mRNA was applied on top of the bovine explants, either naked or complexed with MessengerMAX. 24 h after transfection, 100 µl VivoGlo™ Luciferin (Promega) was added to the samples. After 5 min of incubation, bioluminescence images were acquired by the IVIS lumina II system (PerkinElmer, Waltham, MA) with an acquisition time of 4 min. Images were quantified using the Living Image software (PerkinElmer).

Statistical analysis

All data are presented as mean ± standard deviation and are representative for at least 3 independent experiments conducted on 3 different days, unless stated otherwise. Experiments were analyzed for statistical significance with a one or two-way ANOVA followed by the Bonferroni post hoc test for significant differences between treated groups, or the Dunnett post hoc test when compared with a single control group. An unpaired t-test was performed to determine statistically significant differences between expression levels in the *ex vivo* explant models. Statistical analysis was performed using Graphpad Prism 6 software (La Jolla, CA, USA). Asterisks indicate statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

RESULTS

Characterization of the MessengerMAX lipoplexes

As the aim of this study is to look into the potential of mRNA as a therapeutic cargo for retinal delivery, rather than developing a new delivery system, we made use of the commercial available lipid-based vector Lipofectamine™MessengerMAX, which already showed promising results for mRNA transfection *in vitro*[21, 22]. Similar to what was done in **Chapter 3** for *Trans*IT, the percentage of complexed mRNA for varying v/w ratios (i.e. cationic transfection reagent (µl) to mRNA (µg) ratio) was determined by gel electrophoresis and subsequently quantified by Image J software (**Figure 2A,B**). Interestingly, MessengerMAX resulted in only partial complexation at all studied ratios, with maximally ~70% of mRNA complexed at the highest v/w ratio of 4. Further evaluation of the cytotoxicity however (**Supplementary Figure S1**), demonstrated that a v/w ratio of 4 is not optimal due to toxicity reasons. Therefore, a v/w ratio of 3 was chosen to complex mRNA with MessengerMAX in further experiments. At this v/w ratio the MessengerMAX-mRNA complexes display a bimodal size distribution, with the highest frequency of complexes at 190 nm (**Figure 2C**). The second peak in the DLS data suggests that MessengerMAX particles tend to aggregate, although to a lesser extent than *Trans*IT and Lipofectamine (cfr. **Chapter 3**). Upon mRNA complexation the MessengerMAX particles exhibit a negative zeta potential of -33 mV, which was shown to be necessary for efficient migration through the vitreous (crf. **Chapter 3**).

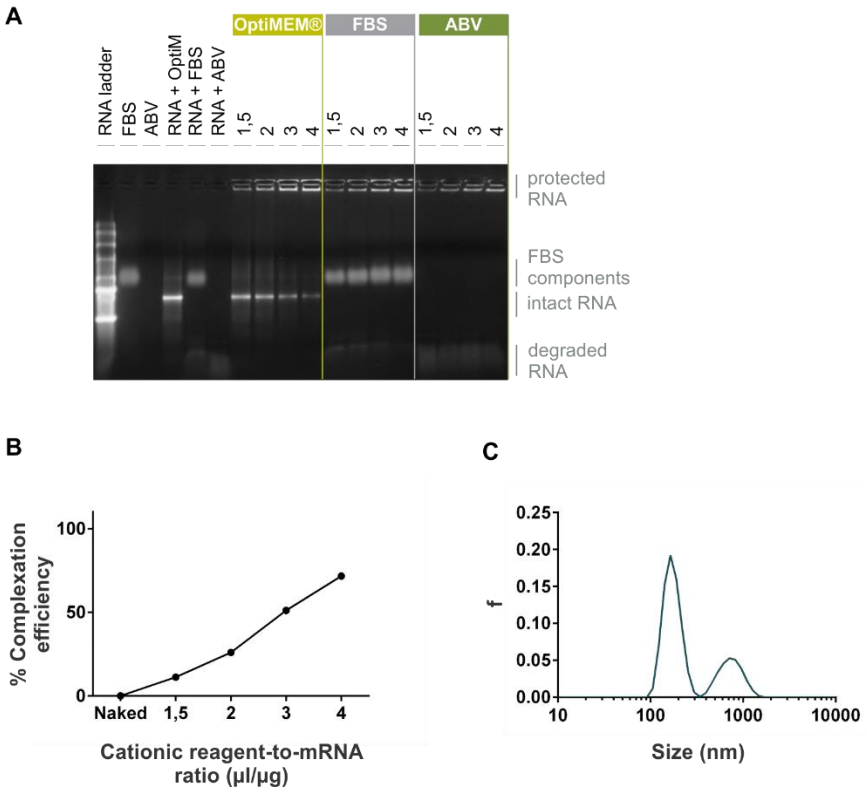


Figure 2 | Characterization of the MessengerMAX lipoplexes. (A) Gel electrophoresis on free mRNA and MessengerMAX-complexed mRNA demonstrates only partial complexation at all studied v/w ratios in Opti-MEM™, FBS and ABV. A 0.5 to 10 kb molecular weight marker was included. (B) The percentage complexation efficiency in Opti-MEM™ as quantified from gel electrophoresis using ImageJ software. (C) Size (number average) of MessengerMAX-mRNA complexes dispersed in HEPES buffer by dynamic light scattering (DLS) at v/w ratio 3.

MessengerMAX-mRNA complexes retain their mobility and transfection efficiency in the presence of vitreous

After intraocular injection, the particles need to migrate from the injection site and depending on the administration route, different barriers will be encountered before reaching the retina. When IVTR administration is the method of choice, for example when delivery to the inner retina is desired, we have previously shown that the vitreous can seriously hinder mRNA delivery to the retina (cfr. **Chapter 3**). In order to evaluate the mobility of the MessengerMAX complexes in the vitreous humor, Cy®5-labeled MessengerMAX-complexed mRNA was injected in the previously described *ex vivo* model that contains intact bovine vitreous[23]. As for *Trans*IT (cfr. **Chapter 3**), the movement of the complexes was visualized by fSPT microscopy 24 h after injection and compared to their diffusion in HEPES buffer. It can be derived from **Figure 3A** that the lipoplexes retained sufficient mobility in the vitreous humor (solid line, 0.42 µm² s^{-1}), even though they were slowed down ~5,5 times when compared to HEPES (dotted line, 2.28 µm² s-1).

Next, we evaluated the capacity of MessengerMAX-complexed mRNA to transfect MIO-M1 Müller cells (one of the cell types encountered after IVTR injection) and analyzed the uptake and transfection efficiency of the complexes in the presence of bovine vitreous compared to culture medium. To this end we made use of a newly developed *in vitro* setup, which is extensively described in **Chapter 3**. As expected**,** naked mRNA resulted in a lack of cellular uptake (**Figure 3B**), and consequently eGFP expression (**Figure 3D**) in medium as well as vitreous. This limited uptake of naked mRNA is likely attributed to the presence of extracellular nucleases in both media. Indeed, when incubated with vitreous, naked mRNA as well as the uncomplexed fraction of MessengerMAX-mRNA were degraded within 30 min as seen by gel electrophoresis (**Figure 2A** lane 6 and lane 15-18). In contrast, mRNA lipoplexes were taken up to a similar extend in the presence of vitreous than in culture medium (**Figure 3B**). These results were nicely correlated to the eGFP expression levels, with >95% eGFP positive cells in both media (**Figure 3D,E**).

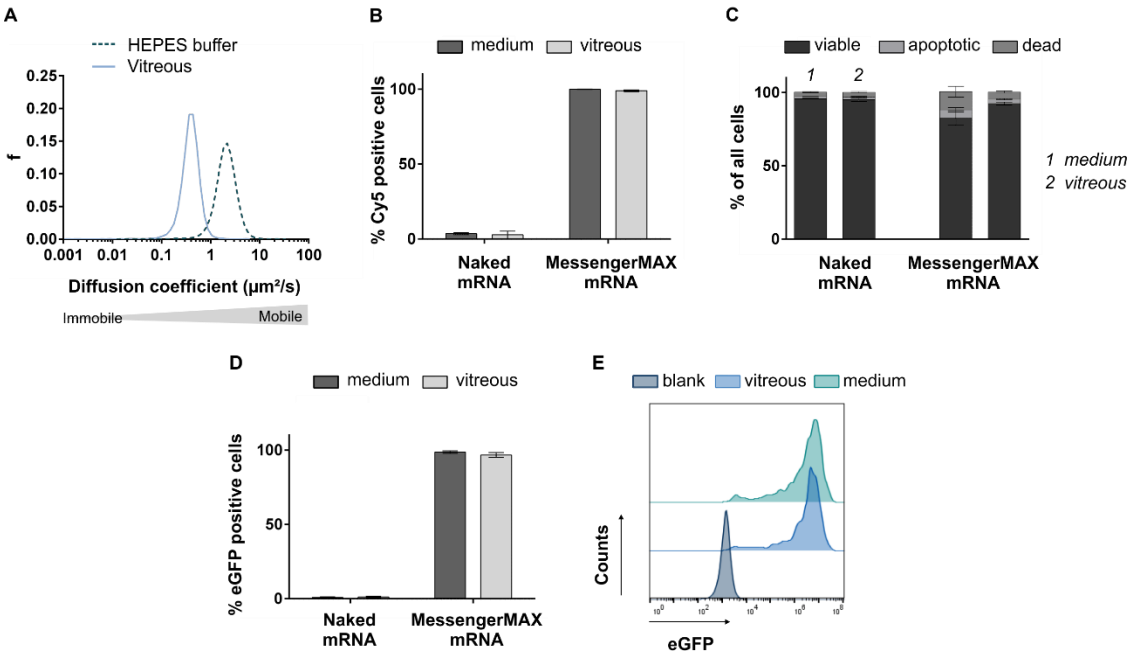


Figure 3 | Influence of bovine vitreous on the mobility, uptake, toxicity and transfection efficiency of naked mRNA and MessengerMAX-mRNA lipoplexes. (A) Diffusion distributions of lipoplexes in intact bovine vitreous humor (solid line), 24 h after IVTR injection compared to their diffusion in HEPES buffer (dotted line). (B) Percentage of MIO-M1 cells that display uptake after 3h incubation with Cy®5-labeled naked or complexed mRNA. (C) Cell viability 24h after lipoplex incubation as quantified by flow cytometry. Untreated cells served as negative control. (D) Percentage of eGFP transfected MIO-M1 Müller cells 24 h after incubation with naked and complexed mRNA. Representative flow cytometry are shown in (E). Data is shown as mean ± standard deviation (SD) (*n*=3x3).

We additionally tested the toxicity of the lipoplexes by measuring the percentage apoptotic and dead cells via $DiIC_1(5)$ and DAPI staining respectively. As shown in **Figure 3C** the cytotoxicity observed in cells transfected with MessengerMAX lipoplexes in culture

medium was canceled out in the presence of vitreous. In conclusion, MessengerMAX complexes showed promising mobility in the intact vitreous of a bovine eye, achieved remarkably high eGFP expression levels in culture medium and retained this transfection potential in the presence of bovine vitreous. Therefore MessengerMAX was a safe and attractive candidate for the evaluation of mRNA-based protein expression in the retina.

m1ψU mRNA modification significantly improves and prolongs transfection efficiency

As discussed in **Chapter 1**, recent research has demonstrated that the incorporation of naturally occurring modified nucleosides into the mRNA backbone can successfully enhance mRNA stability[24]. In this regard, we synthesized 16 differently modified mRNAs and investigated whether the mRNA expression levels obtained with MessengerMAX as carrier could be further improved. All modified mRNA's were, in addition to the incorporation of modified nucleosides, ARCA capped and the plasmid-encoded 64 nt-long poly(A)-tail was extended to ~200 nt-long, using poly(A) polymerase. In contrast, unmodified mRNA contained a classical (7-methylGpppG) cap and the poly(A)-tail was not extended. The expression levels of all 16 mRNA variants are displayed in **Supplementary Figure S2**.

Figure 4 shows the results of the three best performing mRNA modifications, in comparison with unmodified mRNA. We found that all three mRNA transcripts achieved over 95% eGFP positive cells when complexed with MessengerMAX with a slight but significant improvement when compared to unmodified mRNA (**Figure 4A**). Importantly, all modified mRNAs showed a significant higher MFI when compared to the unmodified transcript (**Figure 4B,C**). In particular, 100% replacement of uridine with N^1-methylpseudouridine (m1ψU) resulted in the highest eGFP expression with a ~25-fold increase of the MFI relative to unmodified mRNA. Lowering the amount of m1ψU to 25% of the uridine nucleosides, however, increased the MFI only 3.5 times compared to unmodified mRNA. In the same line, replacement of uridine with pseudouridine (ψU), long regarded as the state-of-the art in mRNA modification, increased the MFI only ~5 times (**Figure 4B,C**). As shown in **Chapter 5**, MessengerMAX-mRNA was also able to transfect RPE cells (being a major cell type exposed to drugs upon SR injection). Interestingly, in this cell type, the same trends in expression efficiency of the modified mRNA variants were observed, indicating that the effect of mRNA modification on the translation capacity was independent of the retinal cell type.

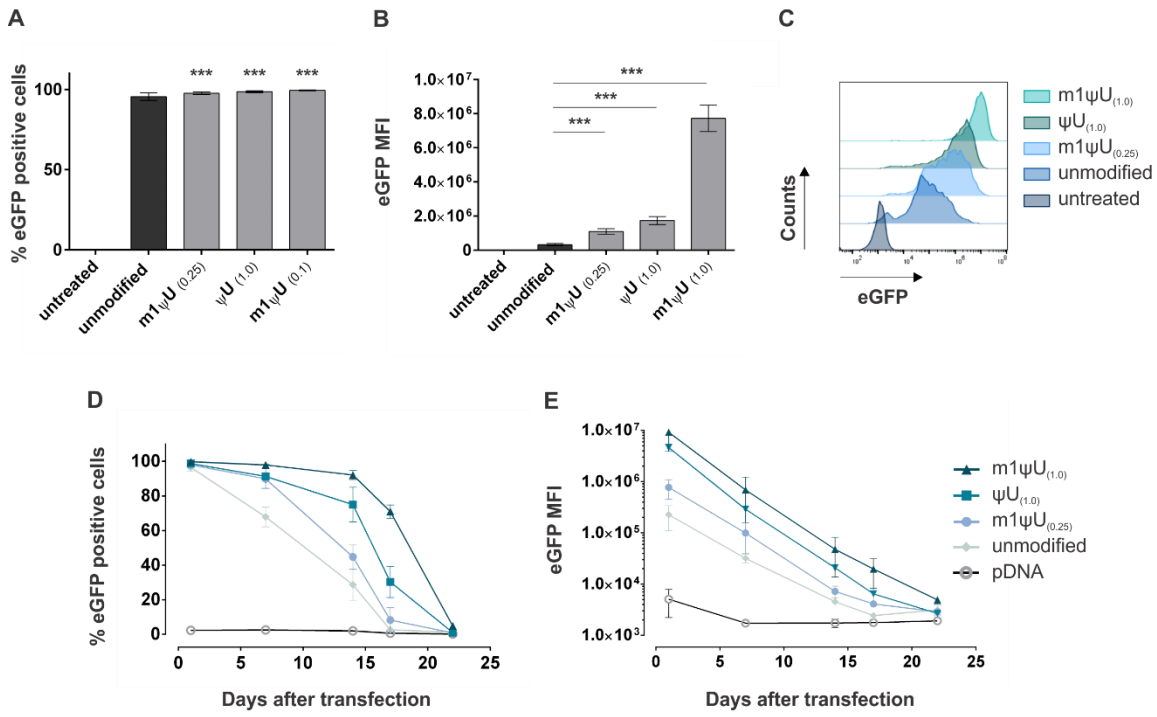


Figure 4 | Comparison of eGFP expression in MIO-M1 Müller cells after transfection with either unmodified or modified (ψU and m1ψU) mRNAs using MessengerMAX as transfection reagent. Percentage eGFP positive cells (A) and mean fluorescence intensity (MFI) (B) 24h after incubation of the cells with the lipoplexes in serum-containing medium. $m1\psi U_{(0.25)}$ represents modified mRNA with replacement of 25% of total uridine by N^1-methylpseudouridine; $\psi U_{(1.0)}$ and $m1\psi U_{(1.0)}$ represents complete replacement of total uridine by pseudouridine and N^1-methylpseudouridine, respectively. Data represent mean ± SD (n≥3x3). ***, $p < 0.001$ versus unmodified eGFP mRNA by one-way ANOVA. (C) Representative flow cytometry histograms (after 24h) of transfected MIO-M1 cells. (D) and (E) show respectively % eGFP positive cells and MFI for each mRNA variant and pDNA as a function of time. Data represent mean ± SD (n=2x3)

Subsequently, we investigated whether the use of chemically modified mRNA could also prolong the duration of eGFP expression and compared the results to the duration of pDNA-based expression. As shown in **Figure 4D**, a single administration of $m1\psi U_{(1,0)}$ allowed to maintain > 90% of eGFP positive cells up to 14 days post transfection, after which transfection levels drop to ~70% after 17 days. In comparison, 17 days after a single administration of unmodified mRNA only 8% of the cells still show expression of eGFP. Also, mRNA with full incorporation of m1ψU excels in eGFP expression levels at each time point (**Figure 4E**); 7 days after administration of $m1\psi U_{(1,0)}$ the MFI of the cells is still ~20 times and ~480 times higher when compared to unmodified mRNA and pDNA, respectively. Taken together, although protein expression using pDNA is generally known to start later and last longer compared to mRNA[12], the levels of protein expression induced by pDNA never surpassed those obtained by mRNA. Modifying the mRNA transcript with $m1\psi U_{(1,0)}$ further improved the mRNA translation capacity and provided eGFP expression for at least 20 days after a single administration. Finally, it is relevant to mention that $m1\psi U_{(1,0)}$ modified mRNA could not result in protein expression when delivered to MIO-M1 Müller cells in its naked form (**Supplementary Figure S3**).

Onset of in vivo mRNA expression is delayed when compared to in vitro or ex vivo mRNA expression upon SR administration

As many retinal disease therapies are focused on treatment of the outer retina (i.e. photoreceptors and RPE), SR injections are widely investigated, delivering their cargo right at the target site[25]. To assess the ability of the m1ψU-mRNA in transducing the outer retina *in vivo*, we delivered Cy®5-labeled (red) eGFP encoding mRNA either naked or complexed with MessengerMAX to the subretinal space of the eye of 6-7-week-old C57BL6/J mice. The localization and expression of the mRNA was examined in retinal cross sections by confocal microscopy (**Figure 5A**). PBS-injected eyes were used to exclude retinal autofluorescence. 24h post injection, the presence of (red) mRNA was detected in the photoreceptor segments (PRS) and the outer nuclear layer (ONL), demonstrating uptake by the photoreceptor cells for both naked and particle-formulated mRNA. In addition, the mRNA seemed to accumulate around the nuclei (blue), inside the cytoplasm. Although mRNA was taken up in both its naked and complexed forms, only in eyes where the mRNA was formulated into lipoplexes, a low amount of eGFP expression could be observed in the PRS (**Figure 5A**, white arrows). After 7 days, the eGFP expression level of lipoplex-injected eyes increased but remained restricted to the RPE cell layer and the photoreceptor cells at the injection site. Surprisingly, at that time point, the fluorescence of Cy®5-labeled mRNA was largely lost. For naked mRNA, no eGFP expression was observed, although a small amount of the naked mRNA is still present at the injection site.

As briefly touched upon in **Chapter 3**, it is generally known that rodents, although widely used as a mammalian model for biomedical eye research, exhibit different anatomical features (e.g., more liquid vitreous and thinner ILM) which are therefore not representative of the setting in larger animals and humans[26, 27]. In the next set of experiments we therefore determined the localization and expression of mRNA in a conventional bovine retinal explant. To this end, the vitreous was removed prior to retina isolation and fLuc encoding m1ψU-mRNA was chemically labeled with Cy®5. To mimic SR injection, the mRNA was administered either naked or complexed with MessengerMAX to the photoreceptor side of the retinal explant (see **Figure 5B**). In agreement with the *in vivo* data, both naked and MessengerMAX-complexed mRNA were observed at the PRS and the ONL, however a less efficient penetration was observed compared to the rodent eyes. When we assessed the fLuc expression 24h after administration, significant expression levels were detected from both formulations, with a slightly higher expression achieved with MessengerMAX-complexed mRNA compared to the naked form (**Figure 5B**, right panel).

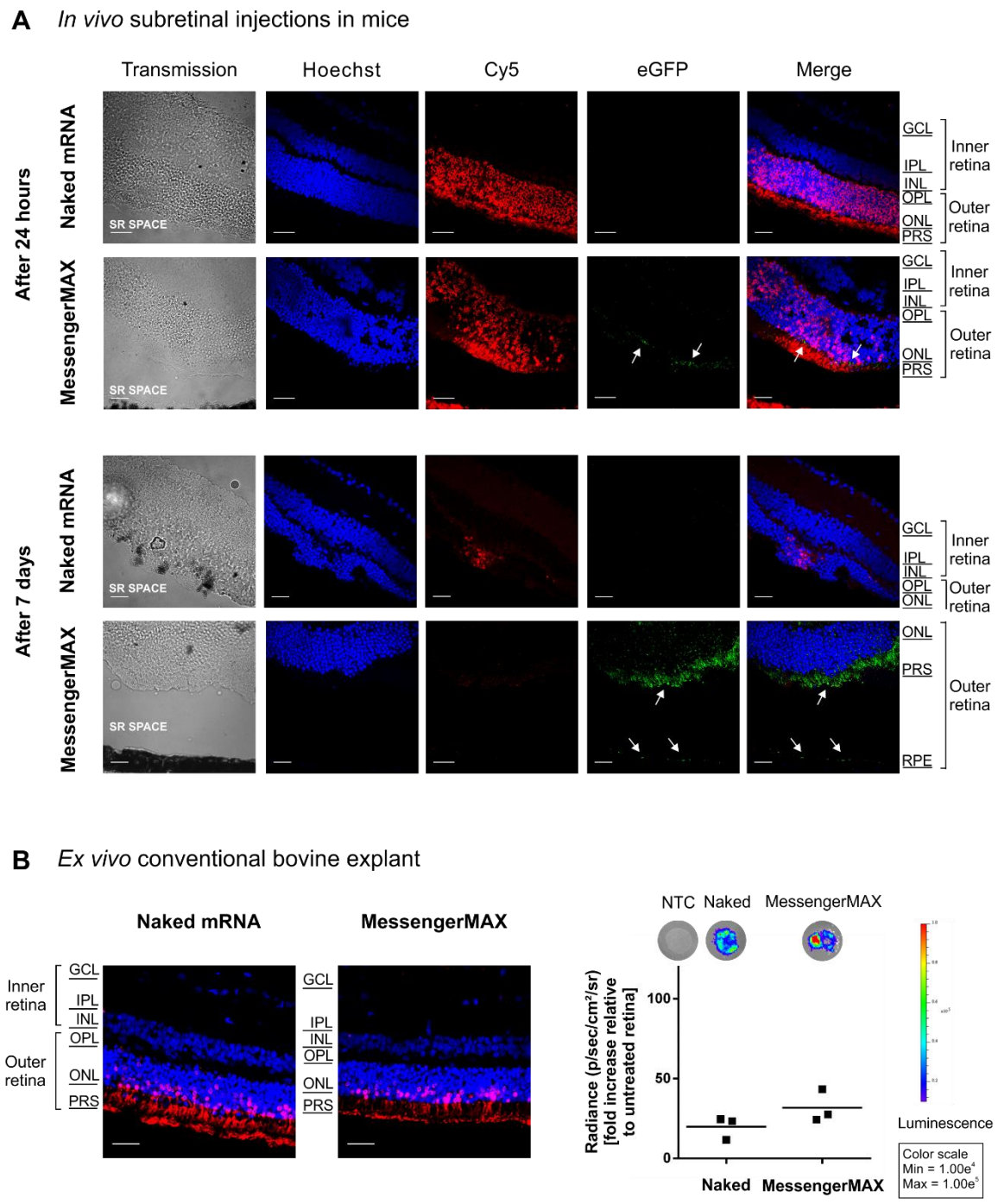


Figure 5 | Retinal distribution and expression of Cy®5-labeled m1ψU-mRNA after SR delivery *in vivo* and mimicked SR administration *ex vivo* and *in vitro*. (A) Representative confocal microscope images of vertical frozen sections displaying the localization (red) and the expression (green, highlighted with white arrows) of eGFP encoding mRNA in the retina 24h and 7 days after SR injection in mice of either naked or MessengerMAX-complexed mRNA. PBS-injected eyes were used as controls. (B) Retinal cross sections of a bovine explant after administration of naked or MessengerMAX-complexed fLuc encoding mRNA to the photoreceptor segment of the explant (mimicking SR administration). Graph displays bioluminescence obtained 24h after transfection with fLuc mRNA. Individual values represent different retinal explants. Explants treated with Opti-MEM™ were used as non-treated control (NTC). Results were obtained from 3 independent experiments. $p < 0.05$ by an unpaired t-test. Corresponding representative bioluminescence images are displayed above the graph. All nuclei are stained with Hoechst (blue), scale bar: 30 µm.

The inner limiting membrane is a barrier for mRNA delivery upon IVTR administration

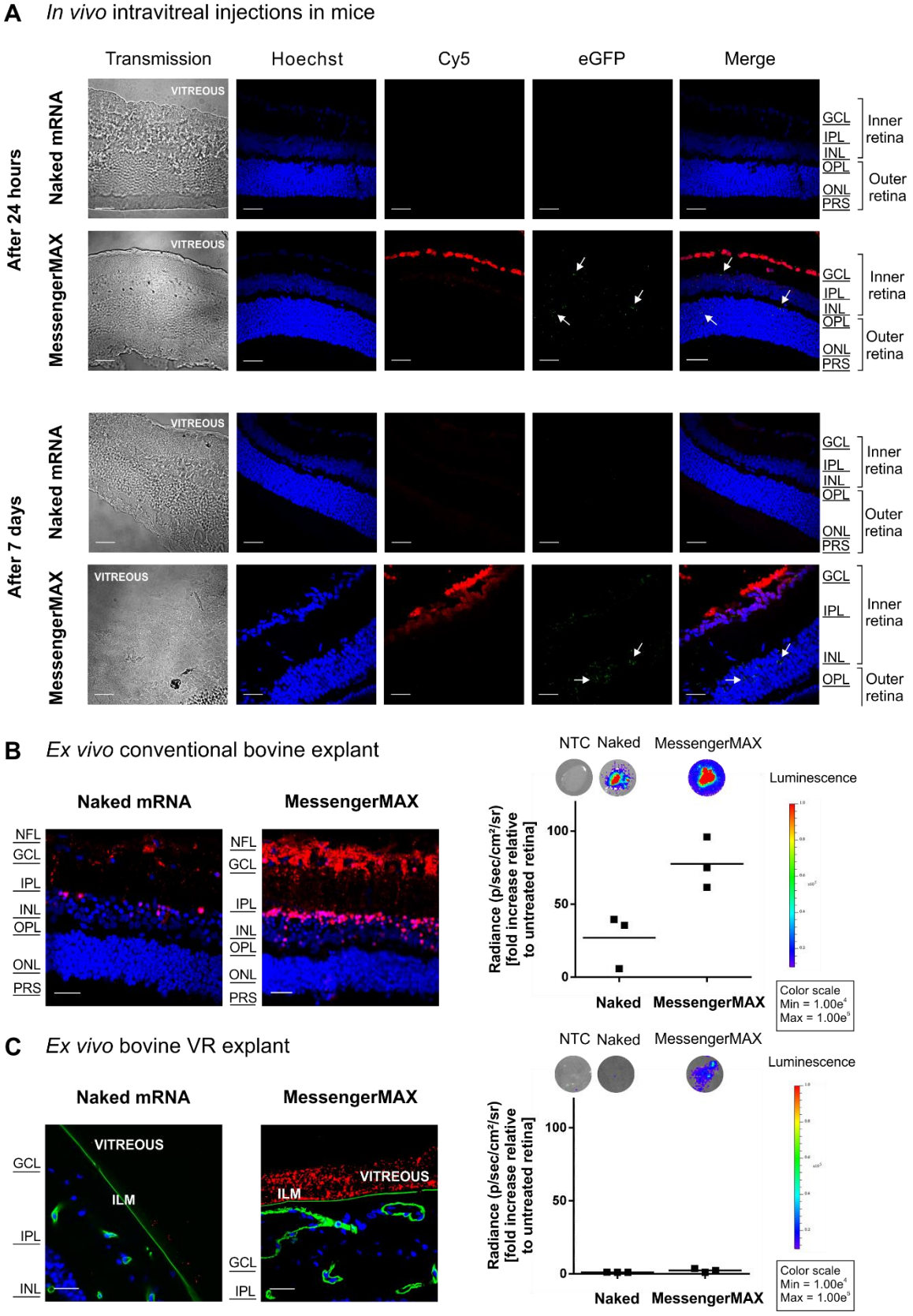
Since practically all barriers in the posterior segment of the eye are circumvented, SR injections are a very efficient retinal drug delivery route. However, when retinal cells in the inner retina are targeted (such as Müller cells or retinal ganglion cells (RGCs)) SR injections are less suitable[28]. As mentioned earlier, a less invasive, safer method to reach the inner retina is IVTR injection. In the next set of experiments, the capacity of mRNA to transfect the inner retina was tested by injecting Cy®5-labeled (red) eGFP encoding m1ψ$U_{(1,0)}$-mRNA either in its naked or complexed form in the vitreous of mice. 24 h and 7 days post IVTR injection ocular cryosections were prepared. As **Figure 6A** demonstrates, no uptake of naked mRNA was observed at any time point. Subsequently, mRNA in its naked form did not result in eGFP expression after IVTR injection. In contrast, a strong Cy®5-signal could be detected in the lipoplex-injected eyes, which is confined to the ganglion cell layer (GCL) and to a lesser extent to the inner plexiform layer (IPL) and upper layers of the inner nuclear layer (INL). A similar distribution of the mRNA was observed 7 days post injection. Except for a faint signal in a few cells (white arrows), almost no eGFP expression was observed after IVTR injection of the MessengerMAX complexed mRNA.

Given the promising mobility of the MessengerMAX-mRNA complexes in the bovine vitreous, as demonstrated above, we assumed that the vitreous itself was not the main hurdle for retinal transfection following IVTR injection. To identify which other factors limit transfection efficiency of IVTR injected mRNA, the penetration and expression of mRNA was determined in bovine retinal explants. First, conventional bovine retinal explants were used, in which the vitreous was separated from the retina and naked or MessengerMAX-mRNA was administered to the vitreal side of the explant. As can be seen from **Figure 6B**, naked mRNA only reached the inner layers of the INL, while MessengerMAX-mRNA penetrated to the most outer layers and even to the outer nuclear layer (ONL). Also, considerably more cells have taken up messengerMAX-mRNA compared to naked mRNA, which is clearly demonstrated by the amount of red cells in the GCL as well as the INL. This observation is also reflected in the fLuc expression levels, which were ~3 times higher after administration of MessengerMAX-mRNA compared to naked mRNA.

Next, IVTR administration was performed in a recently developed explant model of our group, which is bovine-derived and keeps the vitreous attached to the retina during dissection and explant culture[20]. This vitreoretinal (VR) explant is more related to the actual *in vivo* situation, as it keeps the ILM intact and allows to intravitreally inject naked or complexed fLuc-encoding m1ψU-mRNA *ex vivo* and examine retinal penetration. Consistent with the *in vivo* data, no uptake of Cy®5-labeled naked mRNA could be observed in the

bovine explant after IVTR injection (**Figure 6C**, left). Also when delivered in its complexed form, IVTR injected mRNA did not migrate into the bovine retina (**Figure 6C**, middle panel). Remarkably, messengerMAX-complexed mRNA clearly remained present in the vitreous (red dots) and did not penetrate through the ILM (green). As a result, both naked and complexed mRNA failed to induce fLuc expression in the *ex vivo* retinal explant when the VR interface (vitreous and ILM) remains intact. Interestingly, locations where the ILM was slightly compromised, however, clearly allowed an enhanced penetration of messengerMAX-mRNA into the retina (see **Supplementary Figure S4**), confirming the barrier function of the ILM. In contrast to the messengerMAX lipoplexes, the *Trans*IT complexes, optimized with HA137 coating in **Chapter 3**, showed a monodisperse size distribution centered around 125 nm. Therefore we wondered whether their smaller size would allow them to penetrate the ILM. As shown in **Supplementary Figure S5**, 24 h after IVTR injection, the HA137*Trans*IT-complexed mRNA (red) was able to migrate through the vitreous and a small fraction was present in the GCL. However, the majority of the mRNA also accumulated at the ILM (green) and could not penetrate into the neural retina.

Figure 6 (following page) │ Retinal distribution and expression of Cy®5-labeled m1ψU-mRNA after IVTR delivery *in vivo* and mimicked IVTR administration *ex vivo*. (A) Representative confocal microscope images of vertical frozen sections displaying the localization (red) and the expression (green, highlighted with white arrows) of eGFP encoding mRNA in the retina 24 h and 7 days after IVTR injection in mice of either naked or MessengerMAX-complexed mRNA. PBS-injected eyes were used as controls. (B) Retinal cross sections of a bovine explant after administration of naked or MessengerMAX-complexed fLuc encoding mRNA to the vitreal side of a conventional explant without vitreous. Graph displays bioluminescence obtained 24 h after transfection with fLuc mRNA. Individual values represent different retinal explants. Explants treated with Opti-MEM™ were used as non-treated control (NTC). Results were obtained from 3 independent experiments. $p < 0.05$ by an unpaired t-test. Corresponding representative bioluminescence images are displayed above the graph. (C) Representative cryosection images showing the transport of naked and complexed mRNA through the vitreoretinal (VR) interface, 24 h after IVTR injection in the VR explant. ILM is stained by anti-collagen antibodies (green), which also stains blood vessels. Graph on the right: idem as for B. All nuclei are stained with Hoechst (blue), scale bar: 30 µm.


A
In vivo intravitreal injections in mice
Transmission
Hoechst
Cy5
eGFP
Merge
After 24 hours
Naked mRNA
MessengerMAX
After 7 days
VITREOUS
GCL
IPL
INL
OPL
ONL
PRS
Inner retina
Outer retina
B
Ex vivo conventional bovine explant
Naked mRNA
MessengerMAX
NFL
GCL
IPL
INL
OPL
ONL
PRS
NTC
Naked
MessengerMAX
Luminescence
Radiance (p/sec/cm²/sr) [fold increase relative to untreated retina]
100
50
0
Color scale
Min = 1.00e⁴
Max = 1.00e⁹
C
Ex vivo bovine VR explant
ILM

Müller cells as gateway for mRNA distribution to the outer retina?

Although uptake of the particles seemed to be restricted to the upper layers of the inner retina after IVTR injection in mice, a faint signal of expression was observed in the deeper layers (**Figure 6A**). Also in the conventional explants, in which the vitreous was removed, MessengerMAX-complexed mRNA penetrated towards the outer retina. Upon IVTR administration, it is hypothesized that particles are taken up by the Müller cells (**Figure 1A**), which could act as a gateway and transport the mRNA towards the photoreceptors[29]. In response to the weak eGFP signal after IVTR injection *in vivo* (**Figure 6A**, white arrows) in the deeper layers of the retina, we wanted to challenge this hypothesis by selective Müller cell staining. To avoid hindrance of particle penetration by the vitreous and ILM, a conventional explant was used to determine co-localization of the mRNA delivered by MessengerMAX with Müller cells 4h after transfection of the vitreal side of the explant (**Figure 7**).

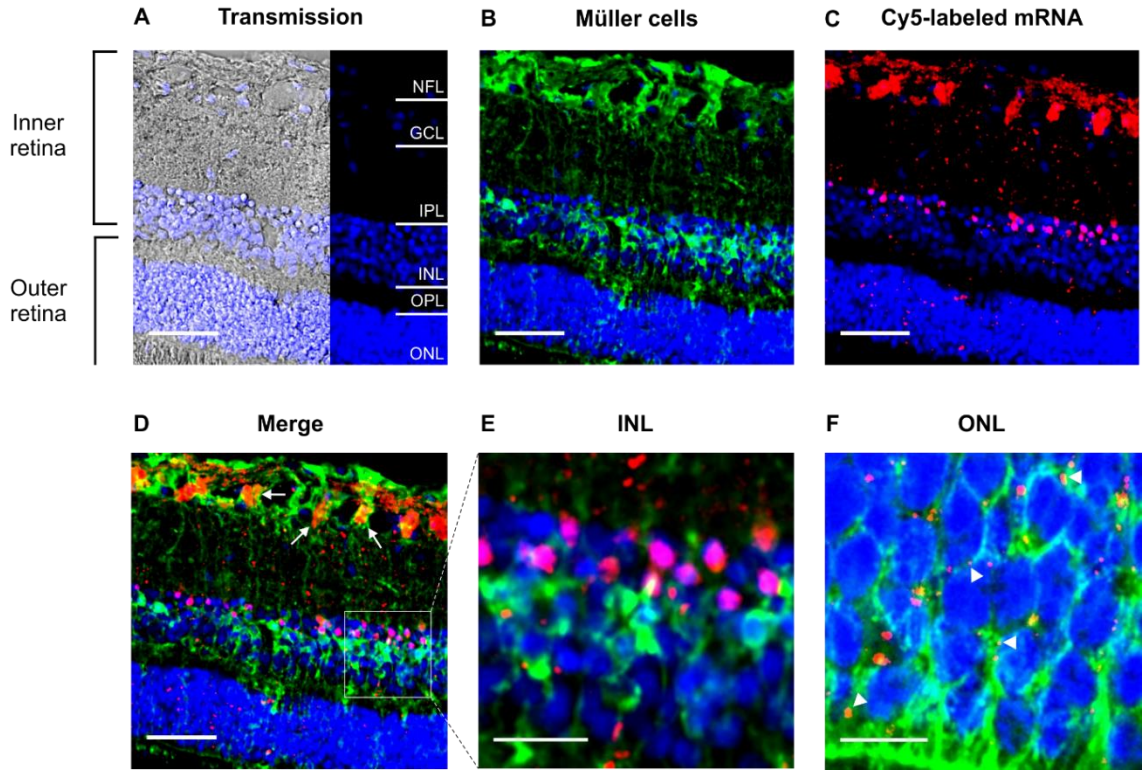


Figure 7 | Co-localization of the mRNA (red) and the Müller cells (green), after administration of the MessengerMAX/mRNA complexes to the vitreal side of the retina. Representative confocal microscope images of vertical frozen sections showing (A) a transmission image of the retinal explant, (B) Müller cell immunostaining with antibodies against glutamine synthetase, (C) MessengerMAX/mRNA distribution 4 h after transfection and (D) co-localization. A detailed zoom of the IPL is displayed in (E). (F) represents a detailed co-localization in the ONL within the same retinal explant (F). Nuclei are stained with Hoechst (blue), scale bar is 50 µm, 20 µm and 10 µm for images (A-D), (E) and (F), respectively.

We observed that MessengerMAX-mRNA (red) moderately co-localizes with the Müller cells (green) in the NFL and the GCL, suggesting that they might be endocytosed by the Müller cell endfeet (**Figure 7D**, white arrows). A detailed confocal image within the same retinal explant shows mRNA and Müller cell co-localization in the ONL as well (**Figure 7F**, arrow heads). However, when we focus on the IPL and INL (**Figure 7E**), most of the MessengerMAX-mRNA is not co-localized with the Müller cells. Taken together, it is likely that the MessengerMAX-mRNA is taken up by multiple cell types in the inner retina, including the Müller cells, providing the ILM can be overcome.

DISCUSSION

Visual perception is one of our most valued senses and vision impairment requires an immense personal and economic toll on both patients and societies[30]. Over the past few years, considerable efforts have been made to understand the molecular mechanisms underlying ocular degenerative diseases and develop therapeutic approaches to stall or treat these disorders. As most blinding diseases originate in the cellular components of the retina, delivery of therapeutics (*e.g.,* antibodies, steroids, genes) to the back of the eye has been at the forefront of ocular therapy research[31]. For the delivery of genetic material both strategies to induce the expression of desired proteins in the retina (*e.g.,* by plasmid DNA delivery) or to mediate gene silencing (*e.g.,* by small interfering RNA or splice-correction oligonucleotides) are being evaluated as therapeutic applications. While RNA-based therapeutics have been used to interfere with gene expression in the retina, the use of mRNA for the expression of proteins in the intact retina has not been evaluated before. Most likely, ocular application of mRNA has been hindered due to its inherent immunogenicity and perceived instability (as explained in **Chapter 1**). However, recent advances in overcoming these challenges have re-established mRNA as a tool for gene delivery with higher expression efficiencies and lower immunogenicity[1]. This study therefore examined the potential of non-viral mRNA delivery to induce protein expression in the retina. As discussed below, the administration route (SR versus IVTR) is a great determinant of the eventual success in clinical applications.

Lipid-based mRNA delivery in retinal cell culture

When using vectors for gene delivery, an ideal carrier system should possess some characteristic features: (i) it should protect NAs against extra- and intracellular degradation, (ii) it should aid in their cellular entry and endosomal escape, and (iii) it should induce sufficient protein expression without noticeable toxicity. In this study, the commercially

available carrier, Lipofectamine™MessengerMAX was evaluated in its capacity to efficiently deliver mRNA. A lipid-based carrier was chosen, as it has been demonstrated before that polymers are less suitable for mRNA delivery due to their strong binding affinity which can impede mRNA release from the complexes[12, 13]. In addition, it was previously shown that following intraocular delivery, the movement of particles from the injection site towards the retina is greatly determined by their physical characteristics[20, 23]. For instance, the mesh size of bovine vitreous is estimated to be ~550 nm and was shown to hinder diffusion of cationic particles, due to their interaction with the anionic glycosaminoglycans (GAGs) (cfr. **Chapter 3**)[14, 29, 32, 33]. In this regard, the MessengerMAX-mRNA complexes used in this study exhibit some favorable physical properties as the negative surface charge of the lipoplexes prevents binding to polyanions in the vitreous, and the size of the majority of the particles is approximately 190 nm which favors migration through the vitreal meshwork (**Figure 2**). This most likely explains why the vitreous itself did not severely lower the mobility and transfection efficiency of MessengerMAX-mRNA complexes (**Figure 3A**).

Interestingly, when evaluating the mRNA complexation efficiency, MessengerMAX showed only partial binding of mRNA at all tested v/w ratios (**Figure 2A**). As both serum and bovine vitreous display nuclease activity, this fraction of unbound mRNA was rapidly degraded (**Figure 2A** lane 11-18). Nevertheless, as shown in **Figure 3D,E**, MessengerMAX-mRNA complexes induced very high protein expression in MIO-M1 Müller cells and retained this ability in presence of bovine vitreous. In this regard, it should be noted that, when using gel electrophoresis, mRNA that is loosely attached to the outside of the lipoplexes could dissociate under influence of the applied electrical field. Hence, the actual amount of complexed mRNA might be higher than observed by this technique. In addition, previous reports postulated that a loose association of mRNA to the surface of the MessengerMAX could facilitate intracellular dissociation, contributing to the high transfection efficiencies[22].

Besides the many advantages listed in **Chapter 1** and **3**, mRNA has the additional benefit that its structure can by modified to increase the level and duration of protein expression. As described in **Chapter 1**, IVT mRNA is recognized by several endosomal and cytoplasmic pattern recognition receptors (PRRs) of the innate immune system, which might result in mRNA degradation and inhibition of its translation[1]. Modifying the mRNA transcript with naturally occurring nucleosides can reduce or even eliminate this intracellular innate immune response while simultaneously enhancing the general resistance of the mRNA molecule to extra- and intracellular degradation.[34, 35] This could have dual effects: on the one hand, a more stable, degradation-resistant mRNA molecule is expected to have a longer half-life and could therefore provoke more durable protein expression; on the other hand, by circumventing an anti-mRNA, antiviral-like immune response, cellular apoptosis can be

avoided, thus improving the viability of the mRNA-transfected cells. As several studies have identified both Müller cells and RPE cells to play a pivotal role in the innate immune system, this also holds true in the retina. Both cell types were reported to express Toll-like receptors (TLRs) 3, 7 and 8, and are therefore intrinsically able to recognize unmodified mRNA[36-38]. These findings are clearly confirmed by our results, as all modifications to the mRNA transcript resulted in an increase of viable MIO-M1 cells (**Supplementary Figure S2B**). In addition, unmodified mRNA exhibited a significantly lower eGFP expression relative to the modified transcripts in both cell types (**Figure 4A-C**), which is in line with most literature data reporting a higher translation capacity for modified mRNA (in for example long epithelial cells, fibroblasts, dendritic cells, macrophage derived cells and mesenchymal stem cells[39-41]). Moreover, we found that a complete replacement of uridine by m1ψU outperformed all other mRNA variants in its translation capacity, both in Müller cells and RPE cells (cfr. **Chapter 5**). This was also documented by Andries *et al*., who ascribed this observation to an increased capacity of the m1ψU-mRNA to evade immune activation[40]. Remarkably, further incorporation of m5C into the mRNA transcript, m1ψU/m5C$_{(1.0)}$, which has previously been shown to be superior to other modifications in Hela cells[40], drastically lowered the expression efficiency in our hands (**Supplementary Figure S2**). This discrepancy can likely be attributed to a difference in coding sequences or a difference in cell type, which have both been shown to play a role in protein expression by different chemically modified mRNAs[42]. Kormann *et al.* indicated that replacement of only 25% uridine and cytidine with respectively 2-thiouridine (s2U) and 5-methylcytidine (m5C) results in the best combination of reduced immunogenicity and increased translation in mice[43]. However, this observation did not seem to apply for m1ψU, since in case of only 25% replacement of uridine eGFP expression was much lower compared to a complete replacement of uridine (**Figure 4A-C**).

Also with regard to protein expression kinetics, m1ψU$_{(1,0)}$ prolonged eGFP expression more than the other mRNA variants, maintaining more than 70% of eGFP positive cells up to 17 days after single administration (**Figure 4D,E**). However, it is important to note that, as a function of time eGFP expression decreased similarly for all mRNA variants. Therefore, we have no direct evidence that modified mRNA shows increased stability in the cytoplasm. Also, although the onset of protein expression using pDNA is generally slower and is hypothesized to last longer when compared to mRNA[12], at any point of time the amount of eGFP expression induced by pDNA was lower than the amount observed with mRNA (**Figure 4E**). Interestingly, the transient expression pattern of mRNA and the ability to fine-tune the mRNA expression levels by differential mRNA modification might be beneficial for therapies in which the long-term safety of increased protein expression is still unknown. As an example, mRNA-based therapy could be promising for the delivery of neurotrophic factors

to the retina, in order to preserve the viability of neurons, decrease inflammation and prevent neovascularization regardless of the underlying pathogenic cause[44-47]. Indeed the transient nature of mRNA might avoid the known detrimental effects caused by the long-term expression of some neurotrophic factors[8, 48-50]. In addition, the short term expression levels obtained with mRNA might also be beneficial in gene editing applications like CRISPR/Cas9, which only require a short period of Cas9 nuclease expression to induce targeted gene knockouts or stimulate site-specific transgene insertion[51, 52]. A more detailed description of the potential clinical applications for mRNA-based ocular gene delivery is provided in **Chapter 6**.

Subretinal vs. intravitreal administration and the importance of relevant models

The choice of administration route is of major importance for the eventual success and specificity of drug delivery to the retina. In general, preference is given to the route of administration that delivers the drugs and/or their carriers in closest proximity to the target cell type. Although in general, the topical route (*e.g.,* eye drops) is the most favorable and patient compliant mode of drug administration to the eye, it is less suited to reach structures in the posterior segment, like the retina, as penetration of the cornea is very inefficient.[53] Instead, when retinal drug delivery is desired, IVTR or SR administration is necessary to bring the therapeutic closer to its target. For viral vectors it is already broadly demonstrated that subretinally delivered vectors will mainly transduce photoreceptors and RPE cells, which surround the subretinal space, whereas IVTR injection primarily leads to transduction of the RGCs and Müller cells, that border the retina at the vitreal side[54]. Also for non-viral systems we found that the eventual distribution of the genetic material and the gene expression levels largely depend on the administration route.

Following SR administration, messengerMAX/m1ψU mRNA complexes as well as the corresponding naked m1ψU mRNA were efficiently taken up in the photoreceptors, both *in vivo* and in conventional *ex vivo* bovine retinal explants (**Figure 5A,B**). For naked mRNA, this was somewhat unexpected as naked mRNA showed no uptake whatsoever *in vitro* (**Supplementary Figure S3**). These results are, however, in line with numerous studies that report uptake and transfection of naked mRNA in various target cell types *in vivo* (such as muscle cells and dendritic cells) [55-57]. A very recent report by Bhosle *et al.* attribute the discrepancy between *in vivo* and *in vitro* transfection of cells with naked mRNA to the tissue stiffness. Culturing human skeletal muscle cells on softer substrate hydrogels (compared to untreated glass surface) changed endosomal uptake entry and release and resulted in upregulation of translation related genes and increased fLuc expression[58]. In addition, photoreceptors are strong phagocytic cells, which could favor naked mRNA uptake.

Remarkably, we noticed that mRNA uptake was often restricted to the site of injection, which suggests that the retina itself is a hard-to-cross barrier, impeding broad mRNA distribution following SR injection. Furthermore, we found that cellular uptake of mRNA is not automatically followed by expression of the encoded protein. Indeed, mRNA translation was only observed when delivered via complexes and not when delivered in its naked form. This might be attributed to differences in cellular uptake mechanism, intracellular trafficking and degradation of the naked versus complexed mRNA. These are, however merely hypothesis, as a thorough investigation of the difference in intracellular trafficking of mRNA after delivery as such or via non-viral carriers, was beyond the scope of this study.

We also observed a discrepancy in the onset of mRNA expression in the different models used. While mRNA expression occurred quite fast in *ex vivo* bovine retinal explants, the expression levels *in vivo* were still low after 24 h and only increased after longer incubation times. This *ex vivo* – *in vivo* discrepancy is not completely unexpected as *ex vivo* studies take place in a highly controlled artificial environment and cells or tissues are removed from their natural environment. Therefore the results obtained *ex vivo* might not accurately predict the conditions in a living organism. One explanation for the low expression levels *in vivo* could be the defined injection volume required for SR injection in small animals, limiting the administrable dose of mRNA. In addition different intracellular processes *in vivo* such as inefficient endosomal escape and fast degradation of the mRNA, might also contribute to the observations in mice. Furthermore, a substantial increase in eGFP expression after 24 h, as seen after SR injection *in vivo*, cannot be excluded in the *ex vivo* bovine model. However, as the cell viability in this model decreased after 48 h of culture, we were limited in the timeframe during which expression could be studied. Therefore it is possible that the expression occurs equally fast *in vivo*, yet is simply higher *in vitro* and *ex vivo*. Finally, it is remarkable that the eGFP expression *in vivo* increases towards day 7, while the mRNA fluorescence seems to disappear. This is presumably due to the presence of extracellular clearance mechanisms, a well-established shortcoming of most *ex vivo* models. It should be noted that while the red fluorescent signal is immediately lost when mRNA is cleared from the subretinal space, the fluorescence signal of the expressed proteins can persist longer due to the 24 hours half-life of the eGFP protein.

After IVTR injection *in vivo,* naked mRNA was not observed in the mouse retina, neither did we found eGFP expression, indicating that naked mRNA is rapidly degraded and/or cleared from the vitreous. When complexed with MessengerMAX, mRNA was observed in the GCL after 24 h and 7 days, but was not productive as almost no eGFP expression was seen. After 7 days, some expression was found in the deeper INL, although very limited. By means of a conventional *ex vivo* bovine retinal explant, we demonstrated

that trafficking of the complexed mRNA is most likely attributed to uptake and distribution by Müller cells (**Figure 6B** and **Figure 7**). This transretinal distribution of complexed mRNA is comparable to the results of Koo *et al*. who demonstrated co-localization of human serum albumin (HSA) particles with Müller cells[29]. In addition, the pattern of Müller cell co-localization in our study was similar to the pattern observed with HSA particles in a previous study by Kim *et al.* as co-localization was observed in the NFL, GCL and ONL, but was missing in the IPL. They concluded that the particles were endocytosed by the Müller cells at the ILM, transported via the Müller cells to the ONL and subsequently released in the interphotoreceptor matrix[59]. Our results suggest that the mRNA is also taken up by other cell types (especially at the level of the IPL and INL), which are most probably amacrine and/or bipolar cells, both known to interact with the Müller cells at the IPL[60].

It should be noted that mice are generally not considered the best model as the delivery barriers encountered after IVTR administration are not representative for that of larger species[27, 33, 61]. This is in particular true when looking into the barrier role of the ILM. The ILM serves as the structural interface between the vitreous body and the retina and mainly consists of collagen type IV, laminin and heparin sulfate proteoglycans which form a complex sheet-like network by specific crosslink interactions[27]. Several reports have demonstrated that the ILM is a critical barrier impeding both viral and non-viral vectors to reach the retina after IVTR injection[29, 62-66]. Based on previous studies trying to elucidate the physicochemical characteristics that determine nanoparticle behavior through the vitreoretinal interface, we expected our negatively charged MessengerMAX-lipoplexes to penetrate into the retina[29, 65, 66]. Indeed, it is generally accepted in both rodent as well as bovine eyes, that predominantly positively charged particles are hindered by the ILM, while neutral to negatively charged are able to pass[61]. In addition, also their particle size of ~190 nm (**Figure 2C**) suggested successful transport through the ILM, as larger particles (up to 350 nm) were previously shown able to enter the retina in rodents[29, 67, 68]. Similar to those studies, we found successful penetration of our MessengerMAX-mRNA complexes through the ILM in mice (**Figure 6A**) and by using conventional bovine retinal explants (**Figure 6B**). However, it should be noted that conventional retinal explants are cultured by removing the vitreous before isolating the retina[17, 18, 69], which can severely compromise the integrity of the ILM[70] and result in an overestimation of the particle uptake in retinal tissue[20]. This finding was again confirmed in the present study by means of the recently developed VR explant, which keeps the vitreous attached to the retina during culture, assuring an intact ILM[20]. Following injection in the vitreous of the VR explant, mRNA complexes were clearly trapped at the ILM and failed to reach the GCL layer (**Figure 6C**). The barrier role of the ILM was also reflected in the large decrease in fLuc expression in the VR explant when compared to a conventional retinal explant. Therefore, in analogy with others, we demonstrate that the ILM is a crucial barrier

for drug delivery after IVTR injections[62, 64, 71-73]. Moreover, our data show that the choice of a model which takes the ILM into account as a hard-to-cross barrier is crucial to clearly identify the potential of a drug delivery system to transport material from the vitreous into retina cells. This underscores the large interspecies differences in the build-up of the retina and its barriers, and therefore prompts the evaluation of new drug and gene delivery systems in more relevant models (*e.g.* larger animal *ex vivo* and *in vivo* models such as cow, pig, non-human primates or even the use of post-mortem isolated human eyes) to provide results that are more predictable for the human situation.

In this regard, it is important to mention that several methods to improve transport across the ILM have been proposed in literature. Dalkara *et al.*, for example, demonstrated that enzymatic lysis of the ILM induced by protease treatment substantially increased retinal transduction of various intravitreally injected adeno-associated virus (AAV) serotypes[74]. Similarly, recent studies in non-human primates demonstrated the power of surgical ILM peeling on retina penetration of AAV2 vectors, which clearly resulted in a larger area and higher intensity of retinal GFP expression[71, 72]. Finally, also laser photocoagulation pretreatment was shown to improve viral transduction of the mice retina, presumably caused by cell stress response and upregulation of capsid receptors[75]. Whether or not the use of these ILM manipulation techniques will be necessary in the diseased retina, in which the ILM might be breached during retinal degeneration, remains to be seen[76-78].

CONCLUSION

Our data demonstrate the clear benefit of chemically modified mRNA to induce protein expression in non-dividing retinal cell types, where m1ψU-mRNA is the best modification to obtain high and sustained (up to 20 days) protein expression in cell culture. Using an *ex vivo* bovine retinal explant, we demonstrate for the first time the potential of mRNA to reach retinal cells after administering mRNA to respectively the photoreceptor side (mimicking SR administration) and the vitreal side of the retina (mimicking IVTR administration). There is a clear benefit of packaging the mRNA in lipid based vectors, as, when compared to naked mRNA, an enhanced expression is obtained in cells of the retinal explant using MessengerMAX-mRNA complexes. *In vivo*, SR injections induce eGFP expression of complexed mRNA after 7 days, at the photoreceptors and RPE cells around the injection side. IVTR injections are less productive, although some expression was seen after 7 days, most likely induced by Müller cell involved trafficking to deeper retinal cell layers. Using a VR bovine retinal explant, we found that not the vitreous, but mainly the ILM is a large barrier to particle mediated mRNA delivery to the retina following IVTR injections. This work therefore encourages systematic studies into particle properties for successful retinal entry in larger species such as cows, pigs or even humans. As demonstrated by the present study, reducing the size of non-viral carriers without compromising their ability to efficiently encapsulate mRNA will be of major importance to overcome the ILM and therefore improve future ocular mRNA delivery.

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REFERENCES

1. Devoldere, J., Dewitte, H., De Smedt, S.C. & Remaut, K. Evading innate immunity in nonviral mRNA delivery: don't shoot the messenger. *Drug Discov Today* **21**, 11-25 (2016).
2. Leonhardt, C. et al. Single-cell mRNA transfection studies: delivery, kinetics and statistics by numbers. *Nanomedicine* **10**, 679-88 (2014).
3. Sahin, U., Kariko, K. & Tureci, O. mRNA-based therapeutics--developing a new class of drugs. *Nat Rev Drug Discov* **13**, 759-80 (2014).
4. Hare, W.A. et al. Efficacy and safety of memantine treatment for reduction of changes associated with experimental glaucoma in monkey, I: Functional measures. *Investigative Ophthalmology & Visual Science* **45**, 2625-2639 (2004).
5. Shan, H. et al. AAV-mediated gene transfer of human X-linked inhibitor of apoptosis protects against oxidative cell death in human RPE cells. *Invest Ophthalmol Vis Sci* **52**, 9591-7 (2011).
6. Dalkara, D. et al. AAV mediated GDNF secretion from retinal glia slows down retinal degeneration in a rat model of retinitis pigmentosa. *Mol Ther* **19**, 1602-8 (2011).
7. Byrne, L.C. et al. Viral-mediated RdCVF and RdCVFL expression protects cone and rod photoreceptors in retinal degeneration. *J Clin Invest* **125**, 105-16 (2015).
8. MacLaren, R.E. et al. CNTF gene transfer protects ganglion cells in rat retinae undergoing focal injury and branch vessel occlusion. *Exp Eye Res* **83**, 1118-27 (2006).
9. Haurigot, V. et al. Long-term retinal PEDF overexpression prevents neovascularization in a murine adult model of retinopathy. *PLoS One* **7**, e41511 (2012).
10. Lipinski, D.M. et al. CNTF Gene Therapy Confers Lifelong Neuroprotection in a Mouse Model of Human Retinitis Pigmentosa. *Mol Ther* **23**, 1308-1319 (2015).
11. Rejman, J., Tavernier, G., Bavarsad, N., Demeester, J. & De Smedt, S.C. mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers. *Journal of Controlled Release* **147**, 385-391 (2010).
12. Tavernier, G. et al. mRNA as gene therapeutic: How to control protein expression. *Journal of Controlled Release* **150**, 238-247 (2011).
13. Zhang, H., De Smedt, S.C. & Remaut, K. Fluorescence Correlation Spectroscopy to find the critical balance between extracellular association and intracellular dissociation of mRNA-complexes. *Acta Biomater* (2018).
14. Martens, T.F. et al. Measuring the intravitreal mobility of nanomedicines with single-particle tracking microscopy. *Nanomedicine* **8**, 1955-1968 (2013).
15. Braeckmans, K. et al. Sizing nanomatter in biological fluids by fluorescence single particle tracking. *Nano Lett* **10**, 4435-42 (2010).
16. Limb, G.A., Salt, T.E., Munro, P.M., Moss, S.E. & Khaw, P.T. In vitro characterization of a spontaneously immortalized human Muller cell line (MIO-M1). *Invest Ophthalmol Vis Sci* **43**, 864-9 (2002).
17. Fradot, M. et al. Gene therapy in ophthalmology: validation on cultured retinal cells and explants from postmortem human eyes. *Hum Gene Ther* **22**, 587-93 (2011).

18. Wang, J., Kolomeyer, A.M., Zarbin, M.A. & Townes-Anderson, E. Organotypic culture of full-thickness adult porcine retina. *J Vis Exp* (2011).
19. Rettinger, C.L. & Wang, H.C. Quantitative Assessment of Retina Explant Viability in a Porcine Ex Vivo Neuroretina Model. *J Ocul Pharmacol Ther* **34**, 521-530 (2018).
20. Peynshaert, K. et al. Toward smart design of retinal drug carriers: a novel bovine retinal explant model to study the barrier role of the vitreoretinal interface. *Drug Deliv* **24**, 1384-1394 (2017).
21. Yoshioka, N. & Dowdy, S.F. Enhanced generation of iPSCs from older adult human cells by a synthetic five-factor self-replicative RNA. *PLoS One* **12**, e0182018 (2017).
22. Zhang, H., De Smedt, S.C. & Remaut, K. Fluorescence Correlation Spectroscopy to find the critical balance between extracellular association and intracellular dissociation of mRNA complexes. *Acta Biomater* (2018).
23. Martens, T.F. et al. Measuring the intravitreal mobility of nanomedicines with single-particle tracking microscopy. *Nanomedicine (Lond)* **8**, 1955-68 (2013).
24. Kariko, K., Buckstein, M., Ni, H. & Weissman, D. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* **23**, 165-75 (2005).
25. Peng, Y., Tang, L. & Zhou, Y. Subretinal Injection: A Review on the Novel Route of Therapeutic Delivery for Vitreoretinal Diseases. *Ophthalmic Res* **58**, 217-226 (2017).
26. Slijkerman, R.W.N. et al. The pros and cons of vertebrate animal models for functional and therapeutic research on inherited retinal dystrophies. *Progress in Retinal and Eye Research* **48**, 137-159 (2015).
27. Peynshaert, K., Devoldere, J., Minnaert, A.K., De Smedt, S.C. & Remaut, K. Morphology and Composition of the Inner Limiting Membrane: Species-Specific Variations and Relevance toward Drug Delivery Research. *Curr Eye Res*, 1-11 (2019).
28. Igarashi, T. et al. Direct comparison of administration routes for AAV8-mediated ocular gene therapy. *Curr Eye Res* **38**, 569-77 (2013).
29. Koo, H. et al. The movement of self-assembled amphiphilic polymeric nanoparticles in the vitreous and retina after intravitreal injection. *Biomaterials* **33**, 3485-93 (2012).
30. Bourne, R.R.A. et al. Magnitude, temporal trends, and projections of the global prevalence of blindness and distance and near vision impairment: a systematic review and meta-analysis. *Lancet Glob Health* **5**, e888-e897 (2017).
31. Trapani, I. & Auricchio, A. Seeing the Light after 25 Years of Retinal Gene Therapy. *Trends in Molecular Medicine* **24**, 669-681 (2018).
32. Pitkanen, L., Ruponen, M., Nieminen, J. & Urtti, A. Vitreous is a barrier in nonviral gene transfer by cationic lipids and polymers. *Pharmaceutical Research* **20**, 576-583 (2003).
33. Xu, Q. et al. Nanoparticle diffusion in, and microrheology of, the bovine vitreous ex vivo. *J Control Release* **167**, 76-84 (2013).
34. Anderson, B.R. et al. Nucleoside modifications in RNA limit activation of 2'-5'-oligoadenylate synthetase and increase resistance to cleavage by RNase L. *Nucleic Acids Research* **39**, 9329-9338 (2011).

35. Anderson, B.R. et al. Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. *Nucleic Acids Research* **38**, 5884-5892 (2010).
36. Kumar, A. & Shamsuddin, N. Retinal Muller Glia Initiate Innate Response to Infectious Stimuli via Toll-Like Receptor Signaling. *Plos One* **7** (2012).
37. Kumar, M.V., Nagineni, C.N., Chin, M.S., Hooks, J.J. & Detrick, B. Innate immunity in the retina: Toll-like receptor (TLR) signaling in human retinal pigment epithelial cells. *Journal of Neuroimmunology* **153**, 7-15 (2004).
38. Moses, S., Jambulingam, M. & Madhavan, H.N. A pilot study on expression of toll like receptors (TLRs) in response to herpes simplex virus (HSV) infection in acute retinal pigment epithelial cells (ARPE) cells. *Journal of Postgraduate Medicine* **60**, 243-247 (2014).
39. Verbeke, R. et al. Co-delivery of nucleoside-modified mRNA and TLR agonists for cancer immunotherapy: Restoring the immunogenicity of immunosilent mRNA. *J Control Release* **266**, 287-300 (2017).
40. Andries, O. et al. N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J Control Release* **217**, 337-44 (2015).
41. Uchida, S., Kataoka, K. & Itaka, K. Screening of mRNA Chemical Modification to Maximize Protein Expression with Reduced Immunogenicity. *Pharmaceutics* **7**, 137-51 (2015).
42. Li, B., Luo, X. & Dong, Y. Effects of Chemically Modified Messenger RNA on Protein Expression. *Bioconjug Chem* **27**, 849-53 (2016).
43. Kormann, M.S. et al. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat Biotechnol* **29**, 154-7 (2011).
44. Paulus, Y.M. & Campbell, J.P. Neuroprotection and Retinal Diseases. *Dev Ophthalmol* **55**, 322-9 (2016).
45. Dalkara, D., Goureau, O., Marazova, K. & Sahel, J.A. Let There Be Light: Gene and Cell Therapy for Blindness. *Hum Gene Ther* **27**, 134-47 (2016).
46. Buch, P.K., MacLaren, R.E. & Ali, R.R. Neuroprotective gene therapy for the treatment of inherited retinal degeneration. *Curr Gene Ther* **7**, 434-45 (2007).
47. Pardue, M.T. & Allen, R.S. Neuroprotective strategies for retinal disease. *Prog Retin Eye Res* **65**, 50-76 (2018).
48. Wen, R. et al. Regulation of rod phototransduction machinery by ciliary neurotrophic factor. *J Neurosci* **26**, 13523-30 (2006).
49. Gerwins, P., Skoldenberg, E. & Claesson-Welsh, L. Function of fibroblast growth factors and vascular endothelial growth factors and their receptors in angiogenesis. *Critical Reviews in Oncology Hematology* **34**, 185-194 (2000).
50. Rousseau, B. et al. Neural and angiogenic defects in eyes of transgenic mice expressing a dominant-negative FGF receptor in the pigmented cells. *Exp Eye Res* **71**, 395-404 (2000).
51. Zhang, X. et al. Biodegradable Amino-Ester Nanomaterials for Cas9 mRNA Delivery in Vitro and in Vivo. *ACS Appl Mater Interfaces* **9**, 25481-25487 (2017).

52. Miller, J.B. et al. Non-Viral CRISPR/Cas Gene Editing In Vitro and In Vivo Enabled by Synthetic Nanoparticle Co-Delivery of Cas9 mRNA and sgRNA. *Angewandte Chemie-International Edition* **56**, 1059-1063 (2017).
53. Patel, A., Cholkar, K., Agrahari, V. & Mitra, A.K. Ocular drug delivery systems: An overview. *World J Pharmacol* **2**, 47-64 (2013).
54. Planul, A. & Dalkara, D. Vectors and Gene Delivery to the Retina. *Annu Rev Vis Sci* **3**, 121-140 (2017).
55. Wolff, J.A. et al. Direct Gene-Transfer into Mouse Muscle Invivo. *Science* **247**, 1465-1468 (1990).
56. Carralot, J.P. et al. Polarization of immunity induced by direct injection of naked sequence-stabilized mRNA vaccines. *Cellular and Molecular Life Sciences* **61**, 2418-2424 (2004).
57. Kreiter, S. et al. Intranodal Vaccination with Naked Antigen-Encoding RNA Elicits Potent Prophylactic and Therapeutic Antitumoral Immunity. *Cancer Research* **70**, 9031-9040 (2010).
58. Bhosle, S.M. et al. Unifying in vitro and in vivo IVT mRNA expression discrepancies in skeletal muscle via mechanotransduction. *Biomaterials* **159**, 189-203 (2018).
59. Kim, H., Robinson, S.B. & Csaky, K.G. Investigating the Movement of Intravitreal Human Serum Albumin Nanoparticles in the Vitreous and Retina. *Pharmaceutical Research* **26**, 329-337 (2009).
60. Vecino, E., Rodriguez, F.D., Ruzafa, N., Pereiro, X. & Sharma, S.C. Glia-neuron interactions in the mammalian retina. *Progress in Retinal and Eye Research* **51**, 1-40 (2016).
61. Peynshaert, K., Devoldere, J., De Smedt, S.C. & Remaut, K. In vitro and ex vivo models to study drug delivery barriers in the posterior segment of the eye. *Adv Drug Deliv Rev* (2017).
62. Hellstrom, M. et al. Cellular tropism and transduction properties of seven adeno-associated viral vector serotypes in adult retina after intravitreal injection. *Gene Ther* **16**, 521-32 (2009).
63. Boyd, R.E. et al. Photoreceptor-targeted gene delivery using intravitreally administered AAV vectors in dogs. *Gene Therapy* **23**, 223-230 (2016).
64. Mowat, F.M. et al. Tyrosine capsid-mutant AAV vectors for gene delivery to the canine retina from a subretinal or intravitreal approach. *Gene Ther* **21**, 96-105 (2014).
65. Pitkanen, L., Pelkonen, J., Ruponen, M., Ronkko, S. & Urtti, A. Neural retina limits the nonviral gene transfer to retinal pigment epithelium in an in vitro bovine eye model. *AAPS J* **6**, e25 (2004).
66. Lee, J. et al. Effective Retinal Penetration of Lipophilic and Lipid-Conjugated Hydrophilic Agents Delivered by Engineered Liposomes. *Mol Pharm* **14**, 423-430 (2017).
67. Bourges, J.L. et al. Ocular drug delivery targeting the retina and retinal pigment epithelium using polylactide nanoparticles. *Investigative Ophthalmology & Visual Science* **44**, 3562-3569 (2003).
68. Apaolaza, P.S. et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. *Biomaterials* **90**, 40-49 (2016).
69. Orlans, H.O., Edwards, T.L., De Silva, S.R., Patricio, M.I. & MacLaren, R.E. Human Retinal Explant Culture for Ex Vivo Validation of AAV Gene Therapy. *Methods Mol Biol* **1715**, 289-303 (2018).

70. Russell, S.R. What we know (and do not know) about vitreoretinal adhesion. *Retina* **32 Suppl 2**, S181-6 (2012).
71. Takahashi, K. et al. Improved Intravitreal AAV-Mediated Inner Retinal Gene Transduction after Surgical Internal Limiting Membrane Peeling in Cynomolgus Monkeys. *Mol Ther* **25**, 296-302 (2017).
72. Teo, K.Y.C. et al. Surgical Removal of Internal Limiting Membrane and Layering of AAV Vector on the Retina Under Air Enhances Gene Transfection in a Nonhuman Primate. *Investigative Ophthalmology & Visual Science* **59**, 3574-3583 (2018).
73. Boyd, R.F. et al. Photoreceptor-targeted gene delivery using intravitreally administered AAV vectors in dogs (vol 23, pg 223, 2016). *Gene Therapy* **23**, 400-400 (2016).
74. Dalkara, D. et al. Inner limiting membrane barriers to AAV-mediated retinal transduction from the vitreous. *Mol Ther* **17**, 2096-102 (2009).
75. Lee, S.H. et al. Laser Photocoagulation Enhances Adeno-Associated Viral Vector Transduction of Mouse Retina. *Human Gene Therapy Methods* **25**, 83-91 (2014).
76. Gan, L. et al. Hyaluronan-modified core-shell liponanoparticles targeting CD44-positive retinal pigment epithelium cells via intravitreal injection. *Biomaterials* **34**, 5978-5987 (2013).
77. Kolstad, K.D. et al. Changes in Adeno-Associated Virus-Mediated Gene Delivery in Retinal Degeneration. *Human Gene Therapy* **21**, 571-578 (2010).
78. Vacca, O. et al. AAV-mediated gene delivery in Dp71-null mouse model with compromised barriers. *Glia* **62**, 468-76 (2014).

SUPPORTING INFORMATION

Chapter 4

The potential of chemically modified mRNA for retinal protein expression: subretinal *vs.* intravitreal administration

SUPPORTING INFORMATION:

Supporting Information consist of an additional material and methods section describing the performed MTT-assay and the *in vitro* evaluation of uptake and expression using confocal microscopy of which the results are displayed in the corresponding additional figures. 5 figures are added as supplementary information.

SUPPLEMENTARY MATERIALS AND METHODS

MTT-assay

The viability of MIO-M1 Müller cells was evaluated 24 h after addition of the MessengerMAX lipoplexes, which were prepared as described above at different v/w cationic lipid-to-mRNA ratios. After removal of the lipoplexes, fresh cell medium containing 5 mg/ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent (Sigma-Aldrich, USA) was added to the cells. After 3 h incubation at 37°C, cells were washed with PBS and the newly formed formazan crystals were dissolved by addition of 100% DMSO. The plates were covered in aluminum foil and placed on an orbital shaker (Rotamax 120, Heidolph, Germany) for 45 min at 1200 rpm. Finally, the absorbance was measured at 590 nm and 690 (background) with an Envision plate reader (Perkin Elmer, Zaventem, Belgium). Cells treated with 50 µl Opti-MEM™ alone were used as positive controls, representing 100% viability.

In vitro evaluation of uptake and expression using confocal microscopy

Five days prior to transfection, MIO-M1 Müller cells were seeded in 35 mm CELLview microscopy dishes with glass bottom (Greiner Bio-One, Vilvoorde, Belgium) at a density of $5x10^4$cells in 1.5 ml. Cells were transfected with naked or MessengerMAX-complexed Cy®5-labeld $m1\Psi U_{(1.0)}$ mRNA as described before. After 24 h incubation at 37°C, cell nuclei were stained with Hoechst 33342 staining (1 mg/ml in PBS; 1000x diluted) and incubated for 15 min at 37°C. Next, cells were washed with PBS and provided with fresh cell culture medium. Live-cell imaging was performed using a confocal laser scanning microscope (C1si, Nikon, Japan) with a Plan Apo VS 60x 1.4 NA oil immersion objective lens (Nikon, Japan). Image processing was performed using ImageJ software.

SUPPLEMENTARY FIGURES

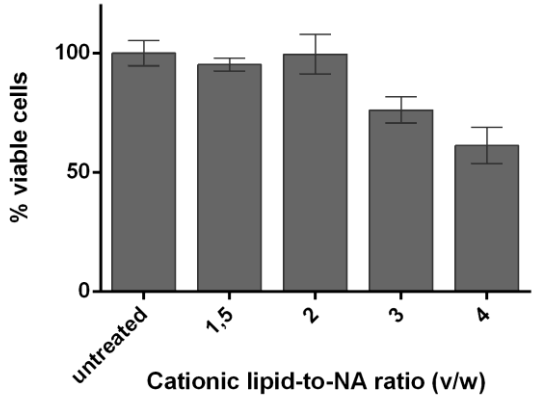

Figure S1 | Cytotoxicity of different v/w ratios of MessengerMAX lipoplexes. Cell viability of MIO-M1 cells 24h after incubation with MessengerMAX-complexed mRNA at different v/w ratios as determined by MTT assay. Cells treated with Opti-MEM™ alone served as a blank. Data reflect mean ± SD (*n*=1x3).

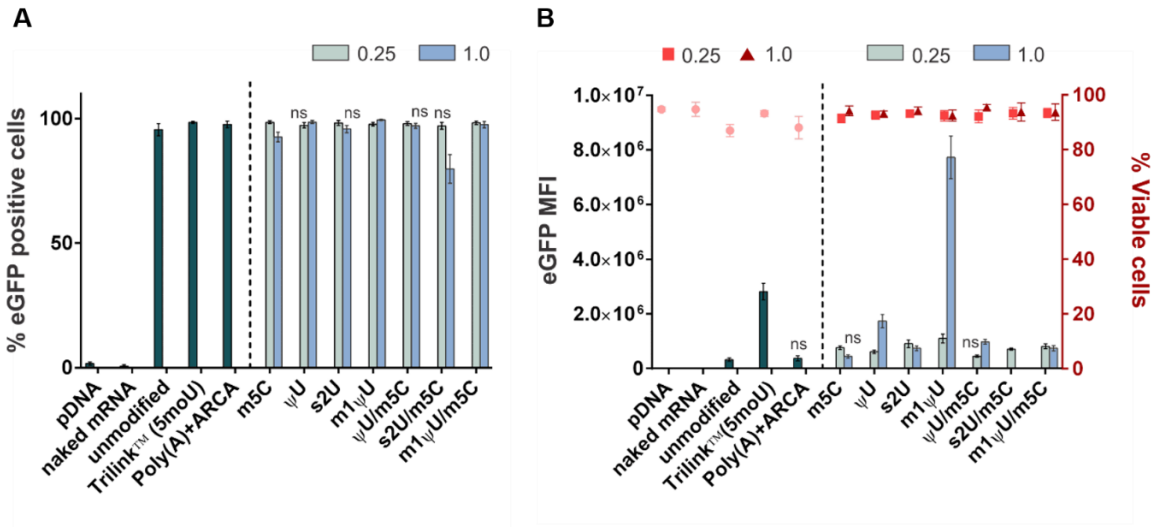

Figure S2 | eGFP expression after transfection of chemically modified mRNAs with MessengerMAX in MIO-M1 cells. (A) Percentage eGFP positive cells 24 h after incubation with the lipoplexes in serum-containing medium. Mean fluorescence intensity (MFI) and corresponding percentage of viable cells as determined by flow cytometry are shown in (B). % viable cells was gated as $DiIC_1(5)^-$/$DAPI^-$. m5C: 5-methylcytidine; ψU: pseudouridine; s2U: 2-thiouridine and m1ψU: N^1-methylpseudouridine; 0.25 symbolizes mRNA with replacement of 25% of total uridine or cytidine by the corresponding modified nucleoside; 1.0 symbolizes mRNA with complete replacement of uridine or cytidine by the corresponding modified nucleoside. Cells treated with eGFP encoding pDNA, naked (i.e. unpackaged) mRNA, unmodified mRNA, CleanCap™ Cyanine 5 EGFP mRNA (5moU) purchased from Trilink (San Diego, CA) and mRNA only modified by polyadenylation and ARCA capping were used as control transcripts. Data represent mean ± SD (*n*≥2*x*3). ns: not significant, *p* > 0.05 versus unmodified eGFP mRNA by one-way ANOVA.

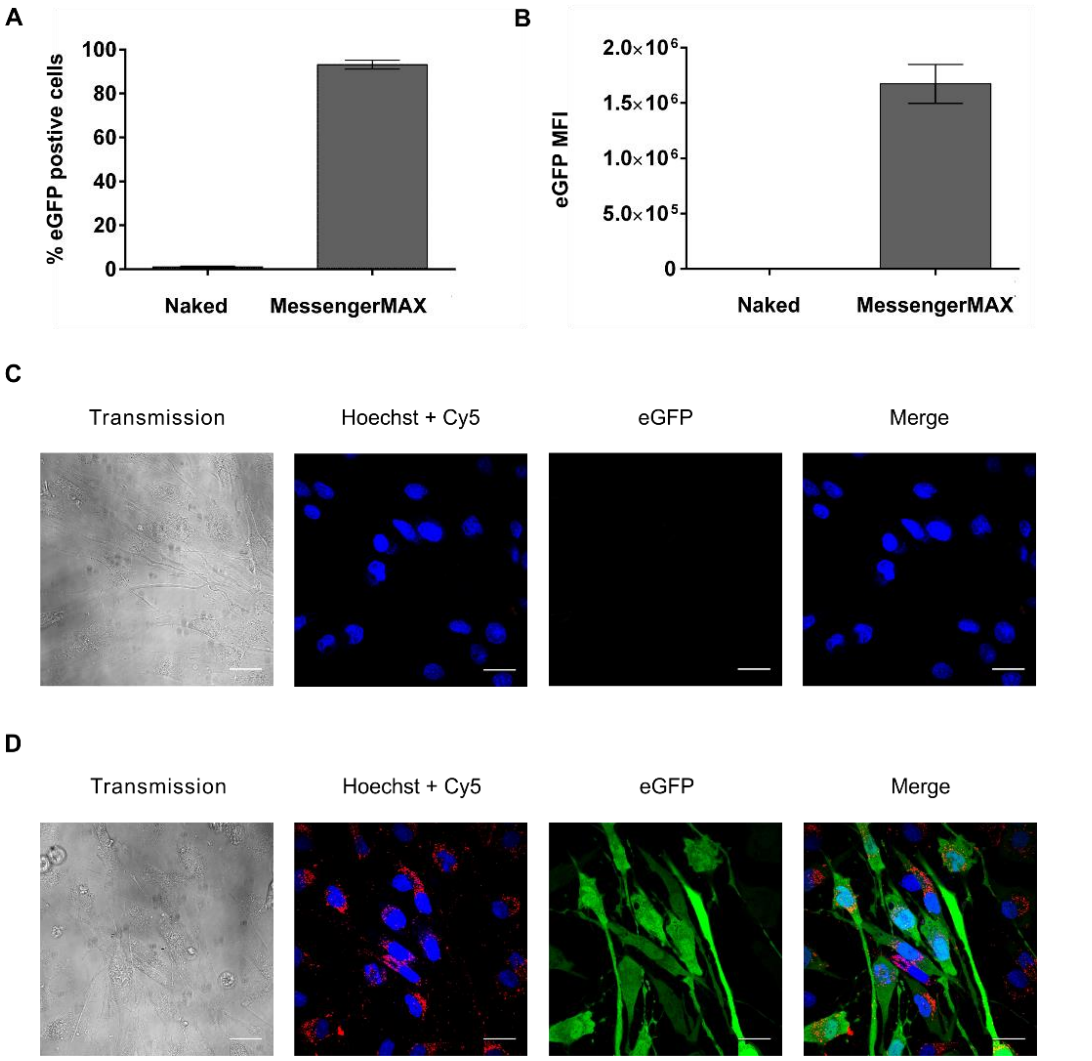

Figure S3 | Transfection efficiency and uptake of m1ΨU$_{(1.0)}$-mRNA in its naked versus MessengerMAX-complexed form. Percentage of eGFP positive cells (A) and MFI (B) 24h after incubation of MIO-M1 cells with naked or MessengerMAX-complexed mRNA in serum-containing medium. Data represent mean ± SD (*n*=1x3). Representative confocal images showing uptake (red) and eGFP expression (green) of Cy5-labeled m1ΨU(1.0)-mRNA administered as such (C) or complexed with the MessengerMAX carrier (D). All nuclei are stained with Hoechst (blue), scale bar: 30 µm.

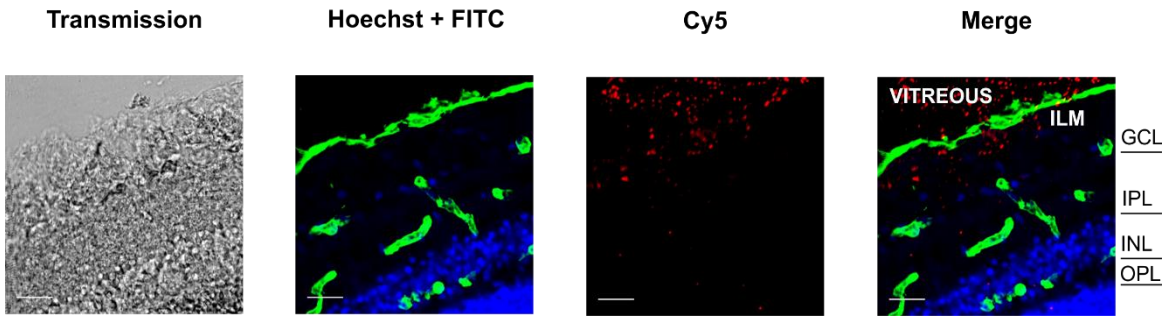

Figure S4 | Retinal distribution of Cy®5-labeled m1ψU$_{(1.0)}$-mRNA after IVTR injection in the VR bovine explant. Figure represents representative confocal microscope images of vertical frozen sections showing the transport of messengerMAX-complexed mRNA through the VR interface 24 h after IVTR injection. Locations with comprised ILM show penetration of lipoplexes in the retina. ILM is stained by anticollagen antibodies (green), which also stains blood vessels. All nuclei are stained with Hoechst (blue), scale bar: 30 µm.

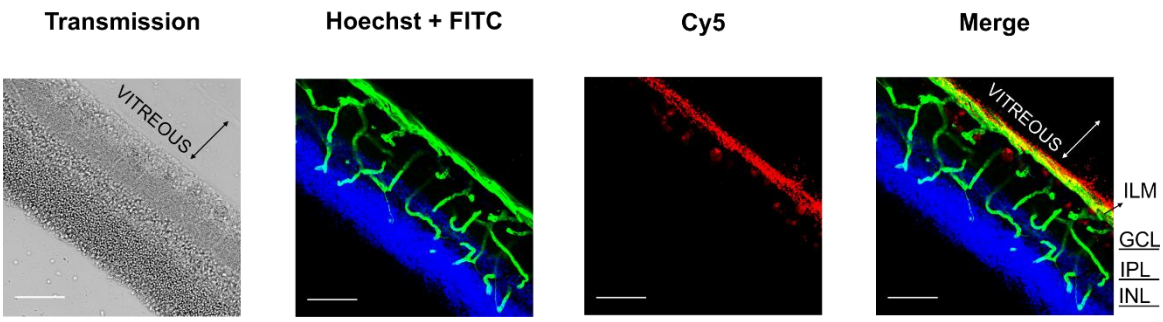

Figure S5 | Confocal microscopy images of transverse retinal sections 24 h after IVTR injection of Cy®5-labeled *Trans*IT-complexed mRNA in the VR bovine explant. The vitreous layer and ILM are indicated. ILM and retinal blood vessels are stained with anti-collagen antibodies (green), nuclei are stained with Hoechst (blue).

Chapter 5

Small molecule innate immune inhibitors to suppress the inherent immunogenicity of synthetic mRNA

This chapter contains unpublished data

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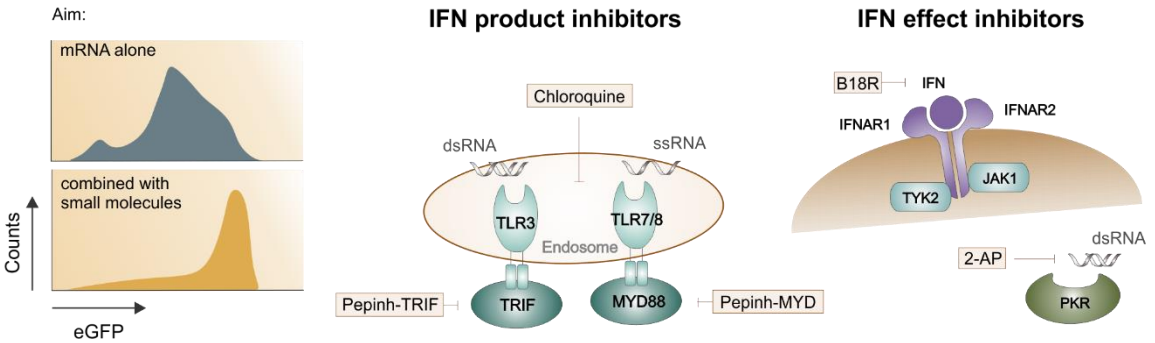
ABSTRACT

As outlined before, messenger RNA (mRNA)-based gene transfer could indeed be a promising new strategy for the treatment of congenital and acquired retinal disorders. The ability to transfect post-mitotic cells, the absent risk of integration into genomic DNA and the strong and adjustable protein expression are just a few promising benefits of mRNA therapeutics. However, one of the main reasons the use of mRNA has long been subjected to controversy, especially for protein replacement strategies, is its strong inherent immunogenicity, which is linked to toxicity and limits mRNA translation. In **Chapter 4** we have shown that the inflammatory responses can be suppressed by de-immunizing the mRNA molecule itself. This chapter focuses on the investigation of an alternative and less expensive method to counteract mRNA-evoked innate immune responses: the use of small molecule inhibitors of type I IFN signaling. In this chapter, we therefore screened five innate immune inhibitors for their potential to not only increase the safety of mRNA therapeutics, but also enhance the mRNA's transfection efficiency. Within the suggested working concentrations, only B18R was able to enhance the total protein expression in ARPE-19 cells. Although the other four small molecules also silenced the IFN-β response, none of them increased the translation of mRNA. By contrast, an unexpected inhibition of the mRNA expression was observed after pretreatment with these molecules.



INTRODUCTION

As discussed in **Chapter 1**, *in vitro* transcribed (IVT) messenger RNA (mRNA) holds considerable promise for the delivery of genetic information, avoiding several limitations associated with conventional DNA-based medicines. Most notably, mRNA induces only transient protein expression, which grands a broad therapeutic utility, without the likelihood of genomic integration[1].

Although significant efforts have been made to develop safe and efficient mRNA delivery vehicles, these carriers are not able to shield the foreign mRNA from the immune system[2]. Indeed, as outlined in **Chapter 1**, both immune and non-immune cells feature so-called pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and RIG-I-like receptors (RLR), which detect double or single-stranded RNA molecules. Binding of mRNA these PRRs evoke a signaling cascade, which results in anti-viral type of innate immune response, coinciding with the production and secretion of proinflammatory cytokines and type I interferons (IFNs). This eventually forces the transfected and adjacent cells into an overall anti-viral state, compromising the mRNA translation efficiency. Several intracellular pathways have been identified that play a key role in this anti-RNA response, including 2'-5'-oligoadenylate synthetase (OAS) and dsRNA-dependent protein kinase (PKR), which respectively stimulate mRNA degradation and inhibit mRNA translation (see **Figure 1** for a summary of the immune response upon mRNA recognition)[3, 4].

As previously discussed, this strong immunostimulatory effect of IVT mRNA presents an added benefit for vaccination strategies, because it can contribute to the desired cellular and humoral immune response[5-9]. For applications such as protein or growth factor supplementation therapies, however, mRNA-induced immune stimulation can be a major disadvantage. As shown in **Chapter 4**, one of the most promising strategies to de-immunize IVT mRNA is the incorporation of naturally occurring modified nucleosides, such as 5-methylcytidine, 2-thiouridine, pseudouridine and N_1-methylpseudouridine into the mRNA backbone. Such modifications appear to passively avoid PRR detection, thereby significantly reducing IFN production, leading to a higher production of the desired mRNA-encoded protein.

In **Chapter 1**, we hypothesized that the active inhibition of immune-related proteins, as is done by RNA-viruses, could be an alternative approach to increase the mRNA transfection. Indeed, viruses are able to circumvent innate immunity by interfering at different levels in the signaling cascades, thereby downregulating the induced immune responses[10, 11]. In this study we aim to mimic this viral immune evasion, by means of small molecules with antagonistic activity against specific elements of the immune pathways. Given the innate

immune response to mRNA is bimodal, evasion of the response can be divided in two aspects as well, namely (i) prevention of the initial type I IFN production and (ii) inhibition of the IFN-induced effects. Because of the abundance of possible interfering small molecules, we limited our screening to five molecules that were discussed in **Chapter 1**. Three molecules that intervene with the IFN production were chosen, namely chloroquine (CLQ), known to hamper mRNA detection in the endosomal compartment[12], and Pepinh-TRIF and Pepinh-MYD, which inhibit specific adaptor molecules in the mRNA-induced signal transmission[13, 14]. A second approach to avoid innate immune activation is to inhibit the effects induced by IFNs. This can be achieved by inhibiting binding of IFNs with their receptor, which does not only avert autocrine IFN stimulation, but also inhibits IFN-induced signaling in neighboring cells. As an example, we evaluate the use of B18R, an IFN-binding protein which has been shown to increase cell viability during mRNA-based reprogramming protocols[15, 16]. Finally, it is also possible to interfere with the IFN-induced anti-RNA response. 2-aminopurine (2-AP), for instance, is a potent inhibitor of PKR, preventing its phosphorylation and thus avoiding inhibition of the mRNA translation[17, 18].

In this work, we explored the use of these five small molecules to enhance mRNA translation. Bearing in mind future ocular applications, this small molecule strategy was tested on retinal cells. As MIO-M1 Müller cells did not produce type I IFNs in response to the introduction of foreign mRNA (data not shown), we made use of another important retinal target cell type, being RPE cells. We evaluated the capacity of the small molecules to inhibit innate immune responses of these cells by quantifying the IFN-β production during transfection. Using eGFP mRNA as a reporter gene, transfection efficiency was quantified *via* flow cytometry and directly compared with the IFN inhibition. Finally, this small molecule approach was compared to the well-established use of de-immunized IVT mRNA.

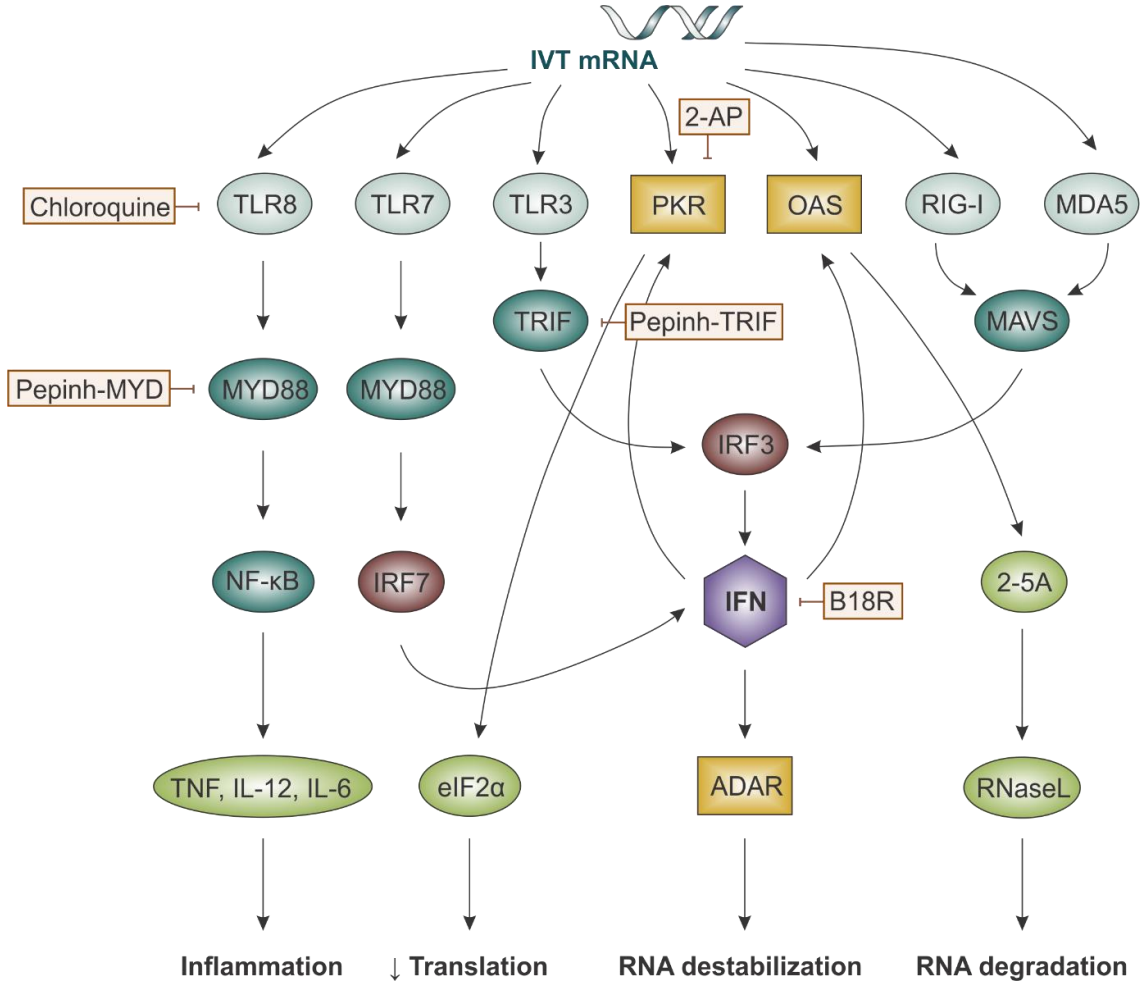


Figure 1 | Innate immune responses to synthetic mRNA. After cellular internalization, *in vitro* transcribed (IVT) mRNA is recognized by various endosomal (Toll-like receptor 3 (TLR3), TLR7 and TLR8) and cytoplasmic (retinoic acid-inducible gene I protein (RIG-I) and melanoma differentiation-associated protein 5 (MDA5)) pattern recognition receptors. Signaling through these different pathways eventually results in an inflammation response producing type 1 interferon (IFNs), tumor necrosis factor (TNF), interleukin-6 (IL-6) and IL-12 and leads to transcription of so-called "anti-RNA" infectors (dsRNA-dependent protein kinase (PKR), 20-50-oligoadenylate synthetase (OAS) and RNA-specific adenosine deaminase (ADAR)). Overall, these create an antiviral microenvironment enhancing RNA degradation, causing RNA destabilization and stalling RNA translation. 2-AP, 2-aminopurine; eIF2α, eukaryotic translation initiation factor 2α; IRF, interferon regulatory factor; MAVS, mitochondrial antiviral signaling protein; MYD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor-κB; RNase L, ribonuclease L; TRIF, Toll-IL-1 receptor domain-containing adapter protein inducing IFN-β. Figure adjusted from [4].

MATERIALS AND METHODS

mRNA

Synthesis of unmodified eGFP-encoding mRNA was performed as described before. Briefly, pGEM4Z-GFP-A64 plasmids, containing a T7 promoter, were linearized with the Spe I restriction enzyme (Promega, Leiden, The Netherlands). After purification, linearized plasmids were used as templates for the *in vitro* transcription reaction using the mMESSAGE mMACHINE T7 transfection kit (Ambion, Life Technologies, Ghent, Belgium), including a 7-

methylGpppG cap analog. Subsequently, mRNAs were treated with DNase I and purified using the RNeasy Mini Kit (Qiagen). The mRNA concentration was quantified by spectrophotometry and small aliquots of 1 µg $µl^{-1}$ were stored at -80°C. For uptake experiments, the mRNA was fluorescently labeled with Cy®5 using the Label IT® Nucleic Acid Labeling kit of Mirus Bio (Madison, WI). Cy®5 was added to the mRNA in a ratio of 1:1 (v:w). The mixture was incubated for 1 h at RT and the labeled mRNA was purified according to the manufacturer's instructions by means of G50 microspin purification columns.

Cell culture and mRNA transfections

Retinal pigment epithelial cells (ARPE-19, ATCC® CRL-2302TM) were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium supplemented with growth factor F12 (DMEM:F12 (1:1), 10% FBS, 2 mM L-glutamine and 50 µg ml^{-1} penicillin/streptomycin). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured at 90% confluency. Two days prior to transfection, $1x10^4$ cells were plated per well in 96-well plates. Cells were treated with small molecules 1 h before eGFP mRNA transfection at concentrations within the working range, as suggested by the manufacturer. To clearly evaluate the influence of small molecules on the mRNA transfection, a suboptimal concentration of mRNA was used. To this end ARPE-19 cells were transfected with 0.1 µg mRNA per $1x10^5$ cells using the Lipofectamine™ MessengerMAX™ transfection reagent (Thermo Fisher Scientific, Merelbeke, Belgium) at a cationic lipid-to-mRNA ratio (µl $µg^{-1}$) of 3:1. Afterwards, cells were incubated at 37°C during 3 or 24 h, for uptake or transfection experiments respectively. Cy®5 and eGFP fluorescence were analysed by flow cytometry. Cells treated with 10 µl Opti-MEM™ alone were used as negative controls.

Table 1 | Overview of the innate immune inhibitors used in this study.

Small molecule	Suggested working concentration	Solvent	Manufacturer
CLQ	10-100 µM	Nuclease-free water	Sigma Aldrich®
Pepinh-TRIF	5-50 µM	Endotoxin-free water	InvivoGen®
Pepinh-MYD	5-50 µM	Endotoxin-free water	InvivoGen®
B18R	100-200 ng ml^{-1}	Phosphate-buffered saline	ThermoFisher®
2-AP	1-10 mM	Phosphate-buffered saline:glacial acetic acid (200:1)	Sigma Aldrich®

Flow cytometry

Cell culture medium was removed and cells were detached from the plate surface with 0.25% trypsin-EDTA (Gibco, Paisly, UK). Next the trypsin was neutralized with cell culture medium and cells were centrifuged at 300*g* during 5 min. The supernatant was removed and cells were resuspended in PBS supplemented with 0.1% sodium azide and 1% bovine serum albumin. Subsequently, samples were analyzed using the CytoFLEX^{TM} Flow Cytometer (Beckman Coulter, Krefeld, Germany) and data analysis was performed using FlowJo software (FlowJo, OR, USA). A minimum of 7000 gated cells was counted per tube. Negative controls were set as max 1% Cy®5 or eGFP fluorescence positive cells. Mean fluorescence intensity (MFI) was calculated for the Cy®5 or eGFP positive cell population.

MTT-assay

The viability of ARPE-19 cells was evaluated 24 h after mRNA transfection. After removal of the lipoplexes, fresh cell medium containing 5 mg ml^{-1} of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent (Sigma-Aldrich, USA) was added to the cells. After 3 h incubation at 37°C, cells were washed with PBS and the newly formed formazan crystals were dissolved by addition of 100% DMSO. The plates were covered in aluminum foil and placed on an orbital shaker (Rotamax 120, Heidolph, Germany) for 45 min at 1200 rpm. Finally, the absorbance was measured at 590 nm and 690 nm (background) with an Envision plate reader (Perkin Elmer, Zaventem, Belgium). Cells treated with 10 µl Opti-MEM^{TM} alone were used as positive controls, representing 100% viability.

Enzyme-linked immunosorbent assay (ELISA)

Supernatant of ARPE-19 cells was collected 24 h after mRNA transfection and samples were stored at -20°C. The production of IFN-β was determined using the Human IFN-β ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Statistical analysis

All data are presented as mean ± standard deviation and are representative for at least 3 independent experiments conducted on 3 different days, unless stated otherwise. An unpaired t-test was performed to determine statistically significant differences between mere mRNA transfection and the untreated control. Experiments with small molecules were analyzed for statistical significance relative to a single control group transfected without small

molecule treatment, using a one-way ANOVA followed by the Dunnett post hoc test. Statistical analysis was performed using Graphpad Prism 6 software (La Jolla, CA, USA). Asterisks indicate statistical significance (* $p < 0.05$; ** $p < 0.01$;*** $p < 0.001$).

RESULTS

Small molecules efficiently inhibit IFN-β production

First, we evaluated whether pretreatment with IFN production or IFN effect inhibitors indeed reduced mRNA-triggered innate immunity in ARPE-19 cells. To this end, cells were incubated during 1 h with increasing concentrations of small molecules (in a range as suggested by the manufacturer), after which they were transfected with a fixed amount of MessengerMAX-complexed mRNA. Twenty-four hours after transfection, the supernatant was analyzed to determine the secretion of IFN-β, a key commander of intracellular innate immune signaling. As expected, transfection with unmodified mRNA alone induced a very high release of IFN-β with an average concentration of ~1.2 ng ml^{-1} (**Figure 2A**).

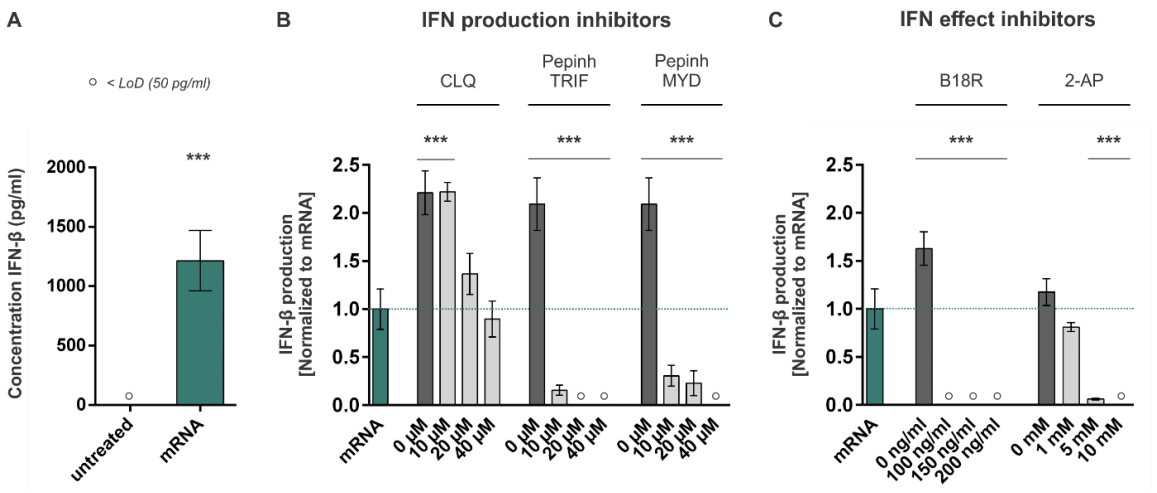


Figure 2 | IFN-β production of ARPE-19 cells transfected with mRNA-lipoplexes alone or combined with small molecule pretreatment as determined by ELISA. (A) Absolute IFN-β concentration in the supernatant of ARPE-19 cells 24 h following incubation with mRNA-lipoplexes. (B) Relative IFN-β production of ARPE-19 cells 24 h after lipoplex-based mRNA transfection, preceded by 1 h incubation with small molecules inhibiting either IFN production or (C) IFN-induced effects. The lowest concentration of IFN-β that could be detected with the used ELISA assay, i.e. the limit of detection (LoD) was 50 pg ml-1. To investigate the impact of the vehicle, ARPE-19 cells were exposed to the highest volume of solvent in which the corresponding inhibitors were dissolved. ELISA results of B and C were normalized to the average values of control groups that were transfected without small molecules treatment. Data reflect mean ± SD (n=1x3) and statistical significance is indicated when appropriate (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

To investigate the impact of the small molecule solvent, ARPE-19 cells were first exposed to the highest volume of corresponding solvents; this is nuclease-free (NF) water

for CLQ, endotoxin-free (EF) water for Pepinh-TRIF and Pepinh-MYD, phosphate-buffered saline (PBS) for B18R and PBS:glacial acetid acid (GAA) for 2-AP (indicated by a small molecule concentration of 0). Strikingly, solvent incubation drastically increased IFN-β production, which even doubled in case of water-based solvents. Pretreatment with small molecules, however, significantly decreased the secreted levels of IFN-β in a concentration-dependent manner, with IFN-β levels dropping below the detection limit (50 pg ml^{-1}) for the highest concentrations of Pepinh TRIF and Pepinh MYD. Chloroquine, by contrast, did not manage to fully eliminate the IFN-β production in the concentrations tested. Ten μM CLQ had no effect when compared to addition of the solvent alone, and higher CLQ levels merely counteracted the solvent-induced IFN-β production (**Figure 2B**). Complete abrogation of IFN-β production was observed with B18R and the highest concentration of 2-AP (**Figure 2C**).

Influence of small molecule treatment on eGFP expression

As we hypothesized that inhibiting IFN-β-mediated innate immunity would enhance the mRNA transfection potential, we next examined the effect of small molecule pretreatment on a suboptimal concentration of eGFP-encoding MessengerMAX-complexed mRNA in ARPE-19 cells. In the absence of small molecule treatment, MessengerMAX-mRNA complexes were efficiently internalized by the ARPE-19 cells (**Figure 3A**) and induced ~70% eGFP expression (**Figure 3B**), with acceptable cell viability (>85%) (**Figure 3C**).

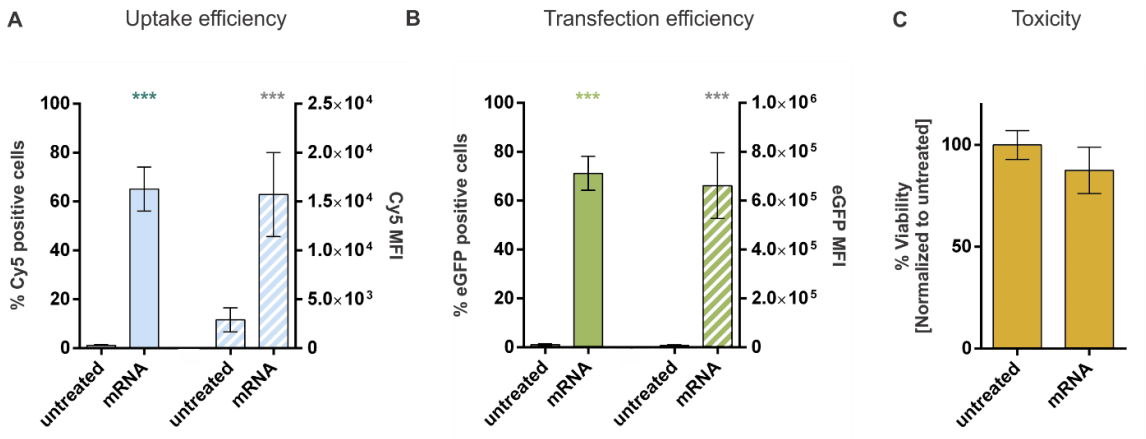


Figure 3 | Uptake, transfection and toxicity measurements 24 h after mere incubation with MessengerMAX-mRNA complexes. (A) Absolute percentage of Cy®5-positive ARPE-19 cells (left y-axis) and Cy®5-MFI (right y-axis) 24 h following incubation with mRNA-lipoplexes compared to untreated cells. (B) Percentage of eGFP positive cells (left y-axis) and eGFP-MFI (right y-axis) induced by mRNA-lipoplexes compared to untreated controls. (C) Cytotoxicity of mRNA-lipoplexes normalized to the untreated control. Data reflect mean ± SD (n=3x3). ***, $p < 0.001$ versus untreated mRNA by an unpaired t-test. The colored asterisks represent significant differences with respect to the percentage cells, while the grey asterisks show significant differences regarding the MFI.

Remarkably, pre-incubation with small molecules did not enhance but even reduced the number of eGFP transfected cells, as shown in **Figure 4**. This effect was even more pronounced with increasing concentrations. Compared to mRNA transfection alone, the highest decrease in percentage of eGFP-expressing cells was obtained with Pepinh-TRIF. Indeed, even 10 μM of this IFN production inhibitor reduced the transfection efficiency by 50%. As shown in **Figure 4B**, the small molecules tested in this study did not interfere with the mean fluorescence intensity (MFI) of the transfected cell population. This implies that they only reduce the overall number of eGFP-expressing cells, but that there is no difference in amount of protein produced per transfected cell. Of note, incubation of the cells with merely the corresponding solvent for the inhibitors, did not influence eGFP expression levels, despite their increase in IFN-β production.

The only exception to these counter-intuitive observations was B18R, which significantly improved the percentage transfection efficiency (~1.5 times) as well as the MFI (~1.3 times) at all used concentrations (**Figure 4A2** and **B2**). Here too, effects seemed dose-dependent, whereas no clear correlation between the impact of B18R on IFN-β levels on the one hand, and transfection efficiency on the other hand, could be detected.

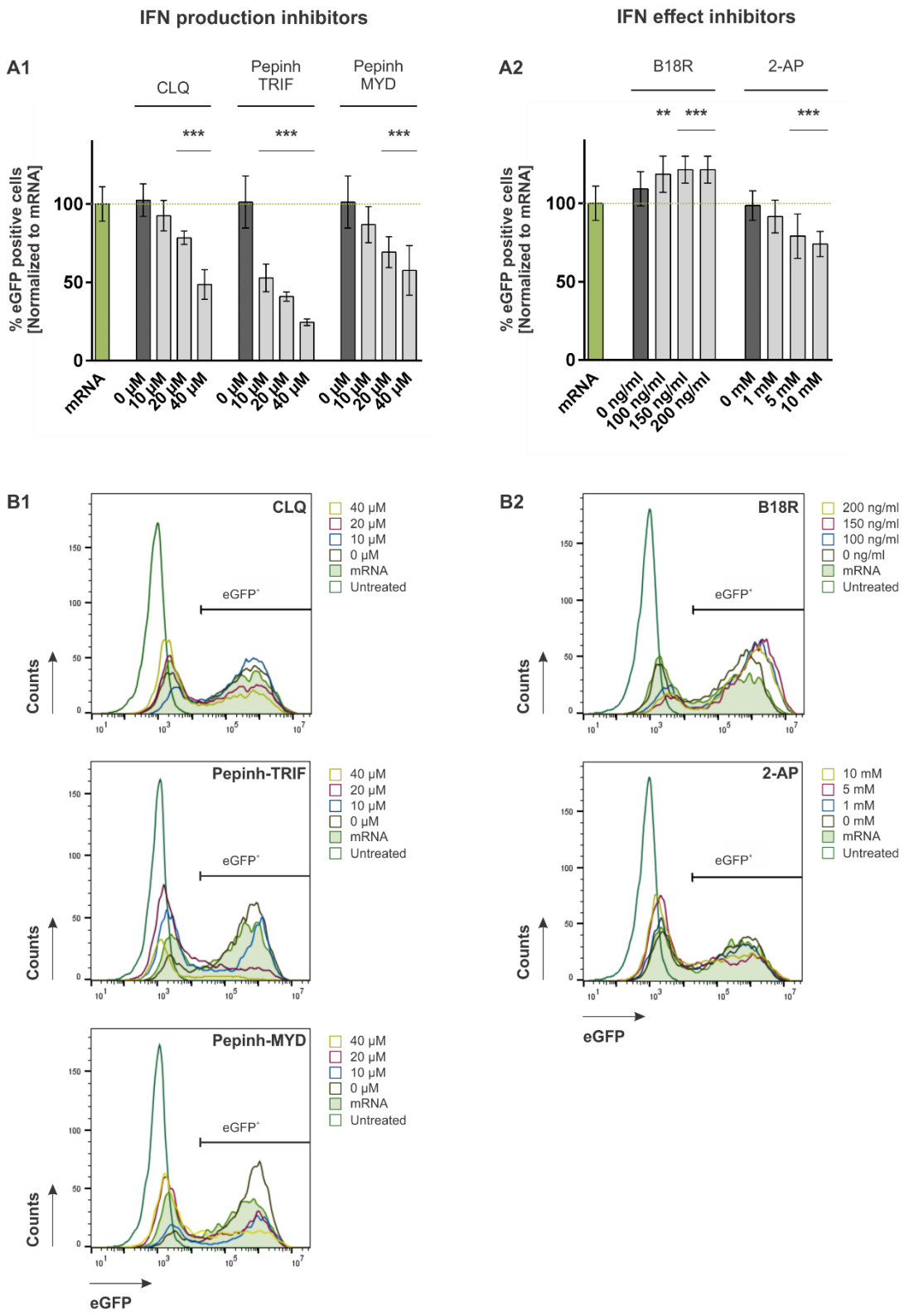


Figure 4 | Transfection efficiency of MessengerMAX-mRNA complexes, when administered alone or in combination with small molecule pretreatment. Relative percentage of eGFP positive cells 24 h after lipoplex-based mRNA transfection, preceded by 1 h incubation with small molecules inhibiting either IFN production (A1) or IFN-induced effects (A2). To investigate the impact of the vehicle, ARPE-19 cells were exposed to the highest volume of solvent in which the corresponding inhibitors were dissolved (which never exceeded 7vol%). Flow cytometry results were normalized to the average values of control groups that transfected without small molecules treatment. Data reflect mean ± SD (n ≥ 3x3) and statistical significance with respect to mRNA alone is indicated when appropriate (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Representative flow cytometry histograms (after 24 h) of transfected ARPE-19 cells are displayed in B.

Role of cytotoxicity

Given the decrease in transfection efficiency was completely against our expectations based on the confirmed IFN-β inhibiting effect of almost all applied small molecules, we next investigated whether a difference in cytotoxicity might explain this observation. In order to quantify the amount of viable cells 24 h after mRNA transfection, an MTT assay was performed. As illustrated in **Figure 5**, only high concentrations of Pepinh-TRIF exerted a significant toxic effect compared to MessengerMAX-mRNA treatment alone. Conversely, neither of the other small molecules, nor lower concentrations of Pepinh-TRIF (≤ 40 μM) or the pure solvents significantly lowered cell viability. Moreover, CLQ and B18R pretreatment even evoked a ~1.3 fold increase in cell metabolism. Taken together, these results indicate that, with the exception of 40 μM Pepinh-TRIF, a difference in cytotoxicity cannot account for the decrease in transfection efficiency.

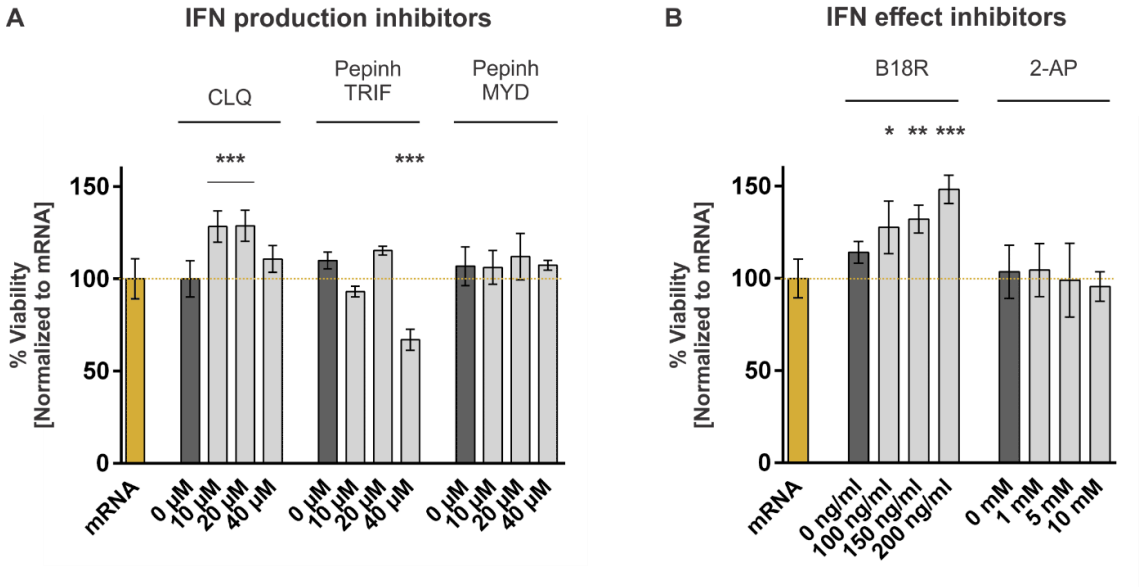


Figure 5 | Cytotoxicity of mRNA-lipoplexes alone or combined with small molecules as determined by MTT assay. Cell viability of ARPE-19 cells 24 h after lipoplex-based mRNA transfection, preceded by 1 h incubation with small molecules inhibiting either (A) IFN production or (B) IFN-induced effects. To investigate the impact of the vehicle, ARPE-19 cells were exposed to the highest volume of solvent in which the corresponding inhibitors were dissolved. Results were normalized to the average values of control groups that were solely treated with mRNA-lipoplexes. Data reflect mean ± SD (n=1x3) and statistical significance is indicated when appropriate (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Effect on the particle uptake

In further pursuit of an explanation concerning the observed decrease in transfection efficiency following small molecule pretreatment of ARPE-19 cells, we investigated to what extent the used small molecules might impact on cellular uptake of the mRNA. To this end,

eGFP-encoding mRNA was labeled with Cy®5 and uptake of MessengerMAX-Cy®5 mRNA was determined by means of flow cytometry. A first striking observation, is the drastically reduced mRNA uptake when ARPE-19 cells were pre-treated with endotoxin-free water, which is the solvent for Pepinh-TRIF and Pepinh-MYD. None of the other solvents (including nuclease-free water) had any significant effect on mRNA uptake. In addition to these solvent effects, a clear difference in Cy®5-mRNA^{+} cells can be seen between the IFN product inhibitors and the effect inhibitors. While pre-incubation with IFN effect inhibitors (**Figure 6A2**) had no significant influence on the internalization of mRNA-lipoplexes, pretreatment with IFN product inhibitors markedly decreased the amount of cells that took up mRNA (**Figure 6A1**). In similarity to the inhibitor's effects on mRNA expression, only lower percentages of mRNA-engulfing cells were observed, whereas the total mRNA uptake per cell (as expressed by the Cy®5 MFI, **Figure 6B**) remained unaltered. Importantly, the reduction in mRNA uptake for both peptide inhibitors was not as pronounced as their overall reduction in % mRNA-transfected cells: a maximal reduction to 60% mRNA-containing cells was observed, whereas percentages of mRNA-expressing cells could drop to merely 25%. Therefore, reduced mRNA uptake can only be partially held responsible for the lower transfection efficiencies for these compounds. Also when evaluating the effects of 2-AP, there is no correlation between the reduced mRNA transfection efficiency and the mRNA uptake, which is largely unaltered for all concentrations tested.

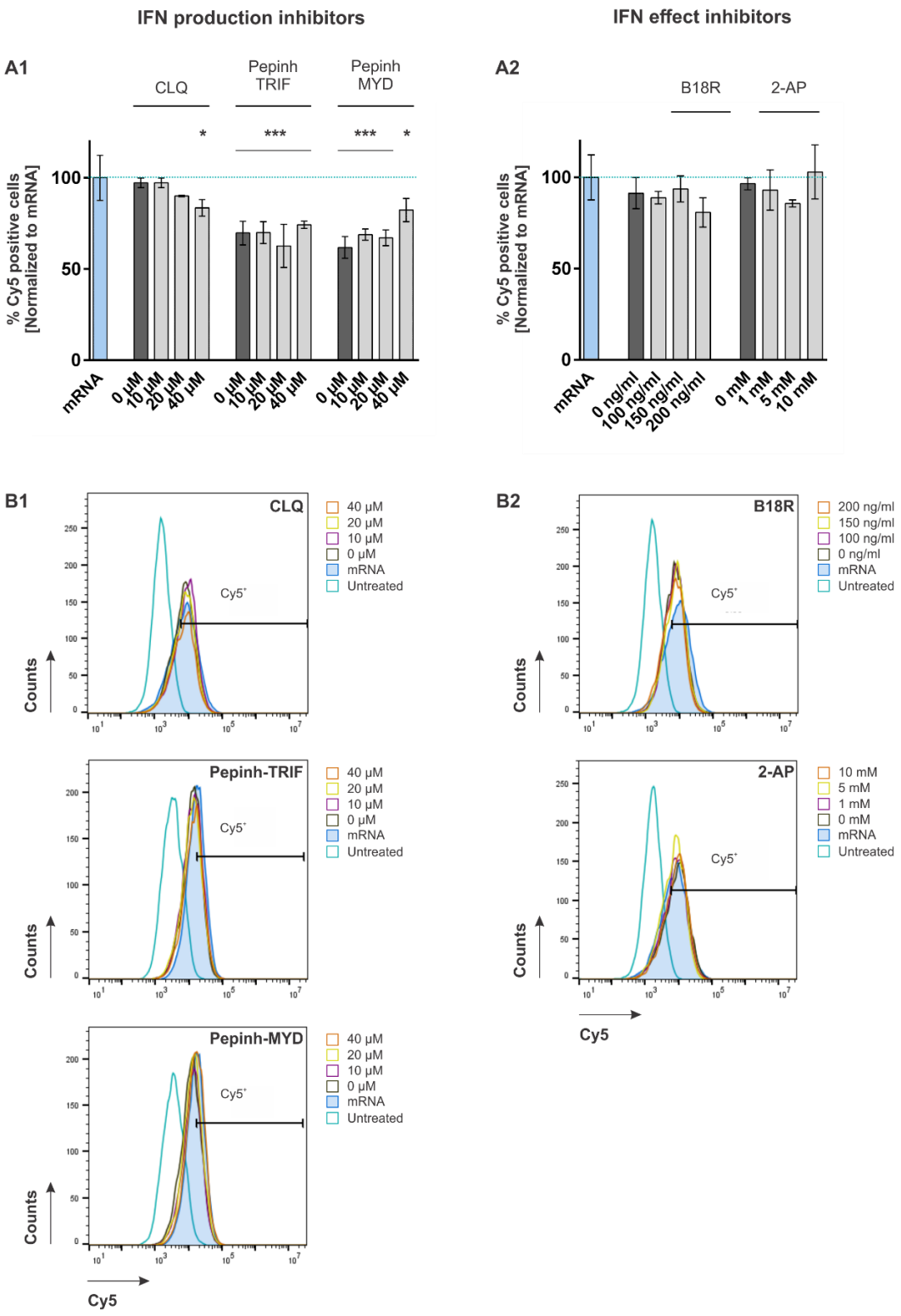


Figure 6 | Cellular uptake of Cy®5-labeled MessengerMAX-complexed mRNA, when administered alone or in combination with small molecule pretreatment. Relative percentage of Cy®5 positive cells 3 h after lipoplex-based mRNA transfection, preceded by 1 h incubation with small molecules inhibiting either (A1) IFN production or (A2) IFN-induced effects. To investigate the impact of the vehicle, ARPE-19 cells were exposed to the highest volume of solvent in which the corresponding inhibitors were dissolved. Flow cytometry results were normalized to the average values of control groups that transfected without small molecules treatment. Data reflect mean ± SD (n=1x3) and statistical significance with respect to mRNA alone is indicated when appropriate. Grey asterisks represent significant differences compared to the influence of the corresponding solvent (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Representative flow cytometry histograms (after 3 h) of transfected ARPE-19 cells are displayed in B.

Comparison with modified mRNA

The counterintuitive results obtained with the innate immune inhibitors, prompted us to test the more established approach to acquire immunosilent mRNA, namely the incorporation of modified nucleotides, and to compare its immune response-evading capacity to our small molecule pretreatment in this experimental setting. Firstly, the effect of mRNAs incorporating different modified nucleotides on the IFN-β secretion levels was evaluated.

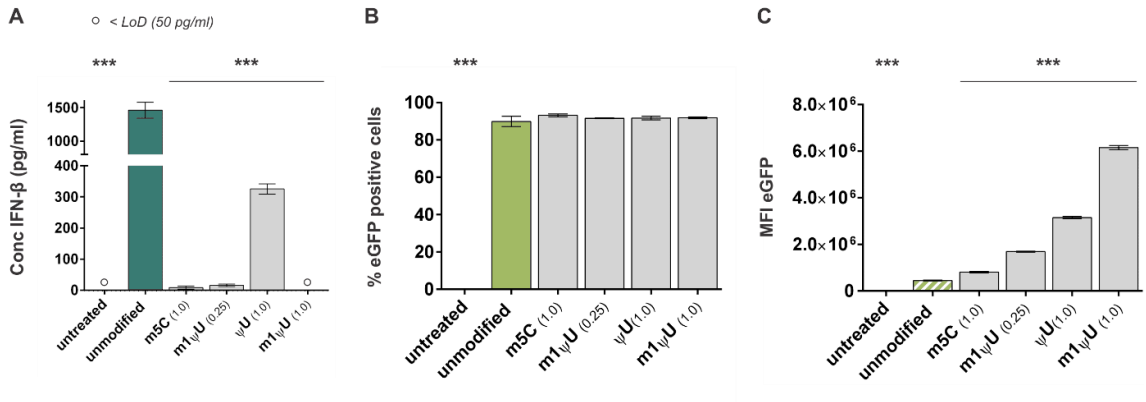


Figure 7 | IFN-β production and eGFP expression results of ARPE-19 cells transfected with mRNA-lipoplexes containing either unmodified or modified (m5C, ψU and m1ψU) mRNAs. No small molecule pretreatment was conducted. It should be noted that for this experiment optimal mRNA concentrations (0.5 µg for 100 000 cells) were used. (A) Absolute IFN-β concentration in the supernatant of ARPE-19 cells 24 h following incubation with mRNA-lipoplexes. Percentage eGFP positive cells (B) and mean fluorescence intensity (MFI) (C) 24 h after incubation of the cells with the lipoplexes in serum-containing medium. m5C(1.0) represents modified mRNA with complete replacement of total cytidine by 5-methylcytidine; m1ψU(0.25) represents modified mRNA with replacement of 25% of total uridine by N1-methylpseudouridine; ψU(1.0) and m1ψU(1.0) represents complete replacement of total uridine by pseudouridine and N1-methylpseudouridine, respectively. Data represent mean ± SD (n=1x3). ***, $p < 0.001$ versus unmodified eGFP mRNA by one-way ANOVA.

From the graph in **Figure 7A**, it is clear that all mRNA variants reduced IFN-β production, albeit not to the same extent. While 100 % replacement of uridine with N^1-methylpseudouridine (m1ψU) completely neutralized the IFN-β innate immune response, replacement with pseudouridine (ψU) still resulted in a considerable IFN-β production. In accordance to the experiments performed for the small molecule approach, we also evaluated the effect of modified mRNA on the eGFP expression. In contrast to the small molecules, mRNA modifications did not affect the number of eGFP-transfected cells, but significantly increased the MFI (**Figure 7B,C**). In particular, complete replacement of uridine with m1ψU extensively enhanced eGFP expression resulting in a ~15-fold increase of the MFI relative to unmodified mRNA. Surprisingly, no correlation was apparent between the IFN-β production levels and the transfection efficiency of the differently modified mRNAs; mRNA in which all uridine was replaced by pseudouridine resulted in an ~8-fold increase in

eGFP MFI, despite the fact that it triggers the production of relatively high levels of IFN-β by the ARPE-19 cells.

DISCUSSION

Although the field of mRNA has made tremendous progress over the last couple of years by addressing its instability and inherent immunogenicity, several challenges still lie ahead. For instance, the current state-of-the-art technique to reduce the anti-viral response triggered by mRNA delivery, namely the incorporation of modified nucleotides, does not yet completely evade innate immune recognition[19]. Therefore we looked into an alternative strategy to temper the innate immune response induced by mRNA delivery. Inspired by viruses, which have developed ingenious strategies to bypass our innate immune system, one novel possibility is the use of innate immune inhibitors, as reviewed in **Chapter 1**. Nevertheless, so far, only a few studies have tested the potential of these IFN-inhibiting small molecules, with very contradictory results. In this study we evaluated five small molecules that where suggested in **Chapter 1** of this dissertation and divided them in two categories: (i) molecules inhibiting IFN production (CLQ, Pepinh-TRIF and Pepinh-MYD) and (ii) molecules suppressing the effects of the produced IFNs (B18R and 2-AP). We expected the small molecules to inhibit primary or secondary IFN production, thereby increasing the mRNA-induced eGFP expression. An ideal molecule would then provide a perfect balance between mRNA translation and innate immune response.

A first small molecule that was tested, was the antimalarial and anti-inflammatory agent CLQ, which has previously been reported to inhibit type I IFNs response to viral and non-viral ssRNA delivery in dendritic cells[20, 21]. As described in **Chapter 1**, the inhibitory activity of CLQ is not completely apparent, but it is generally assumed that CLQ inhibits TLR-mediated signal transduction as it inhibits endosomal acidification, which is thought to be essential for TLR activation. An alternative inhibition-mechanism, suggested by Kuznik *et al.* and Lamphier *et al.*, is the direct binding of CLQ to nucleic acids, which makes them unrecognizable for the TLRs[12, 22]. Both of these suggested mechanisms should avoid mRNA recognition in the endosomes and therefore inhibit primary IFN production. Although a decrease in IFN-β production was indeed observed compared to the solvent control (**Figure 2B**), in the concentrations used, CLQ could not fully down-regulate the IFN-β levels induced by mRNA transfection. Higher concentrations of CLQ might be more immunosuppressing, provided there is no negative impact on cell viability. Drews *et al.*, however, noticed a strong concentration-dependent cytotoxic effect when using concentrations up to 100 μM CLQ. Even though the levels of a few innate immune response-associated genes were slightly reduced, the authors did not see a significant decrease in the produced IFNβ levels caused

by synthetic mRNA delivery to human foreskin fibroblasts (HFFs)[19]. In the same line, in this publication no marked reduction in the expression of immune response-associated genes could be observed when the HFFs were pretreated with Pepinh-TRIF or Pepinh-MYD[19]. This is in contrast to our results, as these two peptides did significantly reduce the IFNβ production at the same suggested concentrations. This shows that the ability of small molecules to inhibit mRNA induced innate immune response is likely as cell type-dependent as the ability to recognize the mRNA in the first place[23].

A second approach to temper the interferon-mediated immune activation is to inhibit the effects induced by IFN production. This could be done by interfering with the IFN-signaling pathway or, as we tested here, by molecules that counter the action of the so-called “IFN-induced effectors” (crf. **Chapter 1**). An example hereof is the purine analogue, 2-AP, a widely used inhibitor of PKR. Binding of 2-AP to PKR results in the suppression of subsequent PKR signaling such as phosphorylation of the eukaryotic initiation factor 2 (eIF2α), which stalls mRNA translation[24]. Since PKR not only functions as an RNA-induced effector, but also acts as an RNA-activated PRR[3], 2-AP binding should inhibit impaired mRNA translation as well as prevent a PKR-mediated secondary IFN production[25]. As shown in **Figure 2C** increasing concentrations of 2-AP indeed gradually suppressed mRNA-induced IFNβ production. Although PKR is also established as a critical mediator of apoptosis, 2-AP pretreatment did not noticeably influence ARPE-19 cell viability (**Figure 5B**).

Despite the potent inhibition of the IFNβ production, none of the above mentioned small molecules was able to enhance the *in vitro* mRNA transfection efficiency in ARPE-19 cells. Moreover, all molecules unexpectedly reduced eGFP expression in a concentration-dependent manner. In case of CLQ this decreasing amount of GFP expressing cells might be attributed to an increased cell proliferation: although CLQ is generally known to be quite cytotoxic, the lowest concentrations used in this study significantly increased cell viably. The relative increase in total number of cells might therefore explain the relative decrease in transfected cells. In case of the peptide inhibitors, the underlying cause of the diminishing transfection efficiency could be a significantly reduced uptake of the mRNA particles when pretreated with the small molecules (**Figure 6A1**). However, as no difference in uptake was observed compared to the solvent control, the drop in mRNA internalization is mainly caused by the solvent itself. Nevertheless, cells solely pretreated with EF water, did not reduce GFP transfection efficiency (**Figure 4A1**) nor IFN-β production levels (**Figure 2B**), meaning that the gradual diminution of transfected cells was not attributed to a decrease in mRNA uptake. Similarly, the detrimental effect of 2-AP on the mRNA transfection efficiency could not be clarified by the uptake results.

Interestingly, the present study is not the only study reporting failure of innate immune inhibitors to enhance *in vitro* mRNA transfection. Very recently, *Liu et al.* performed a detailed screening of 15 different small molecules, which were used for a 1 h pretreatment of BJ fibro-blasts (a HFF cell line) that were subsequently transfected with eGFP mRNA nanoparticles. Although most of the tested molecules were able to reduce mRNA-induced IFN-β production, none of the inhibitors enhanced mRNA transfection efficiency and a third of the tested compounds even inhibited eGFP expression in these cells[26]. As mentioned in **Chapter 1**, it is clear that the intracellular innate immune cascades interact with each other *via* a complex network and are able to replace each other's function when a connected pathway is inhibited. This means that inhibition of only one immune-associated protein might by completely negated, resulting in no net effect on the translation efficiency. Nevertheless, the net effect seen in our study, as well as the study of Liu *et al.,* is a loss in total eGFP expression, which will probably not be prevented by simultaneous inhibition of different pathways. In this regard, it is important to note that most signaling pathways are involved in many other regulatory aspects of the cell as well, meaning that inhibition of other cascades could be at the base of the obtained results. 2-AP for instance is known to inhibit other kinases at these concentrations[18], therefore a decrease in translation might be caused by nonspecific inhibition of other components in the translation machinery. Furthermore, it is possible that pre-incubation with these inhibitors induces alarm signals within the treated cells, which causes them to save energy for other processes, thereby silencing the translation mechanisms.

Finally we also tested the potency of B18R, a Vaccinia virus decoy receptor specific for type I IFNs. B18R captures the secreted IFNs, thereby preventing engagement with their receptor. Consequently, B18R inhibits the IFN-induced effects by averting the autocrine IFN amplification loop as well as preventing the induction of IFN-triggered signaling in the surrounding cells. This small molecule has been widely used to mitigate the mRNA-induced immune response for the generation of induced pluripotent stem cells and although it is suggested to increase the levels of mRNA mediated protein expression in HFF cells[15, 27], only few studies directly correlate this increased transfection efficiency to the reduction of innate immunity[28]. In addition, there is a lot of controversy on the efficiency of B18R. Drews *et al.* for example show that B18R did not induce a measurable decrease in innate immune response upon mRNA delivery in HFF1 cells[19] and no increase in mRNA transfection efficiency was seen by the research group of Byrne in primary human skin cells[29]. In another study on HFF cells, using an eGFP-expressing replicon RNA, IFN-β transcription levels were only reduced when co-transfected with a combination of mRNAs encoding for the vaccinia virus evasion proteins E3, K3 or B18R. Co-lipofection with either B18R mRNA or treatment with recombinant B18R protein alone did not reduce IFN-β induction[30]. Nevertheless, in our

hands, B18R completely abrogated mRNA-induced IFN-β production in ARPE-19 cells (**Figure 2C**), while significantly increasing eGFP expression levels (**Figure 4A2,B2**) and cell viability (**Figure B**), without noticeable change in the mRNA uptake efficiency (**Figure 6A2,B2**). Our results therefore show that the use of B18R could be a potent method to enhance the mRNA based gene-transfer in ARPE-19 cells. The fact that B18R significantly increased the cell viability of mRNA transfections, was also mentioned in the study of Warren *et al.*, although no data were shown to support this claim. As the processes induced by IFN signaling not only hamper mRNA transfection but can eventually also result in apoptosis, the increased cell viability could therefore be an added effect of the IFN-β neutralization capacity of B18R. In contrast to the other small molecules, B18R exerts its function extracellularly.

Owing to the unexpected results obtained with the majority of the small molecule inhibitors we also evaluated the use of modified mRNA on the ARPE-19 cells. In agreement with that data obtained in **Chapter 4** for MIO-M1 cells, all modified mRNA transcripts resulted in over 90% eGFP positive cells and a significant increase in MFI compared to the unmodified molecule was observed. Strikingly, an enhanced eGFP MFI did not necessarily correlate with an equivalent decrease in IFN-β response. In particular $\psi U_{(1.0)}$-mRNA still induced high IFN-β secretion, however, this did not seem to hamper mRNA translation. As modifying the structural elements of IVT mRNA is also known to optimize the intracellular stability of the molecule, this aspect will also contribute to a higher translation outcome. Indeed, superior translation of $\psi U_{(1.0)}$-mRNA has previously been attributed to its increase stability as well as reduced binding to PKR[31, 32]. In contrast the enhanced effect of $m1\psi U_{(1.0)}$-mRNA was shown to be mainly caused by its lower inherent immune stimulation[33]. This clearly shows that the type I IFN response is not the only determinant for the mRNA translation capacity. In fact, it could be possible that the innate immune response should not be completely blocked to increase translation efficiency. As stimulation of PRRs triggers the cell to up-regulate innate immune-related proteins (crf. **Chapter 1**), this might also have beneficial effects on the translation of exogenous mRNA. It could therefore be hypothesized that optimal transfection efficiency could be achieved when a balance is retained between intracellular innate immunity and RNA degradation. Accordingly, a combination approach of modified mRNA and small molecule inhibitors might be worth investigating. Nevertheless, it is clear that the use of modified mRNAs has more potential for *in vivo* applications. Firstly, as mRNA modification also influences the intracellular mRNA stability, one might be able to fine-tune protein expression levels by incorporating differential modified nucleotides. Secondly, while modified mRNA transcripts are already widely evaluated *in vivo* and even reached clinical settings[8], care should be taken when interfering with intracellular signaling cascades as the majority of the pathways is associated with other crucial aspects of the cell as well.

CONCLUSION

Although the immune-stimulatory activity of IVT mRNA is a well-known safety concern for protein replacement therapies, few studies have tested the additional use of immune response targeting inhibitors. Out of the small molecules tested in the present study, only B18R increased the expression of synthetic mRNA in ARPE-19 cells. All other innate immune inhibitors effectively reduced IFN-β production, but also diminished total mRNA expression. When compared with the transfection potential of our previously optimized mRNA constructs, we can conclude that the incorporation of modified bases, especially $m1\psi U_{(1.0)}$, has the most potential to cause a revolution in mRNA-based medicine.

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REFERENCES

1. Zhong, Z.F. et al. mRNA therapeutics deliver a hopeful message. *Nano Today* **23**, 16-39 (2018).
2. Hajj, K.A. & Whitehead, K.A. Tools for translation: non-viral materials for therapeutic mRNA delivery. *Nature Reviews Materials* **2** (2017).
3. Sadler, A.J. & Williams, B.R. Interferon-inducible antiviral effectors. *Nat Rev Immunol* **8**, 559-68 (2008).
4. Sahin, U., Kariko, K. & Tureci, O. mRNA-based therapeutics--developing a new class of drugs. *Nat Rev Drug Discov* **13**, 759-80 (2014).
5. Van Lint, S. et al. The ReNAissanCe of mRNA-based cancer therapy. *Expert Rev Vaccines* **14**, 235-51 (2015).
6. Lutz, J. et al. Unmodified mRNA in LNPs constitutes a competitive technology for prophylactic vaccines. *Npj Vaccines* **2** (2017).
7. Pardi, N., Hogan, M.J., Porter, F.W. & Weissman, D. mRNA vaccines - a new era in vaccinology. *Nature Reviews Drug Discovery* **17**, 261-279 (2018).
8. Bahl, K. et al. Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses. *Molecular Therapy* **25**, 1316-1327 (2017).
9. Iavarone, C., O'hagan, D.T., Yu, D., Delahaye, N.F. & Ulmer, J.B. Mechanism of action of mRNA-based vaccines. *Expert Review of Vaccines* **16**, 871-881 (2017).
10. Smith, G.L. et al. Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. *Journal of General Virology* **94**, 2367-2392 (2013).
11. Schulz, K.S. & Mossman, K.L. Viral Evasion Strategies in Type I IFN Signaling - A Summary of Recent Developments. *Frontiers in Immunology* **7** (2016).
12. Kuznik, A. et al. Mechanism of Endosomal TLR Inhibition by Antimalarial Drugs and Imidazoquinolines. *Journal of Immunology* **186**, 4794-4804 (2011).
13. Loiarro, M. et al. Peptide-mediated interference of TIR domain dimerization in MyD88 inhibits interleukin-1-dependent activation of NF-kappa B. *Journal of Biological Chemistry* **280**, 15809-15814 (2005).
14. Toshchakov, V.U., Basu, S., Fenton, M.J. & Vogel, S.N. Differential involvement of BB loops of Toll-IL-1 resistance (TIR) domain-containing adapter proteins in TLR4-versus TLR2-mediated signal transduction. *Journal of Immunology* **175**, 494-500 (2005).
15. Warren, L. et al. Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. *Cell Stem Cell* **7**, 618-630 (2010).
16. Mandal, P.K. & Rossi, D.J. Reprogramming human fibroblasts to pluripotency using modified mRNA. *Nature Protocols* **8**, 568-582 (2013).
17. Hu, Y.H. & Conway, T.W. 2-Aminopurine Inhibits the Double-Stranded Rna-Dependent Protein-Kinase Both in-Vitro and in-Vivo. *Journal of Interferon Research* **13**, 323-328 (1993).
18. Sugiyama, T. et al. Mechanism of inhibition of lipopolysaccharide-induced interferon-beta production by 2-aminopurine. *Molecular Immunology* **52**, 299-304 (2012).

19. Drews, K. et al. The cytotoxic and immunogenic hurdles associated with non-viral mRNA-mediated reprogramming of human fibroblasts. *Biomaterials* **33**, 4059-68 (2012).
20. Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. & Reis e Sousa, C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**, 1529-31 (2004).
21. Katashiba, Y. et al. Interferon-alpha and interleukin-12 are induced, respectively, by double-stranded DNA and single-stranded RNA in human myeloid dendritic cells. *Immunology* **132**, 165-73 (2011).
22. Lamphier, M. et al. Novel Small Molecule Inhibitors of TLR7 and TLR9: Mechanism of Action and Efficacy In Vivo. *Molecular Pharmacology* **85**, 429-440 (2014).
23. Eigenbrod, T. et al. Recognition of Specified RNA Modifications by the Innate Immune System. *Methods Enzymol* **560**, 73-89 (2015).
24. Jammi, N.V., Whitby, L.R. & Beal, P.A. Small molecule inhibitors of the RNA-dependent protein kinase. *Biochemical and Biophysical Research Communications* **308**, 50-57 (2003).
25. Schulz, O. et al. Protein Kinase R Contributes to Immunity against Specific Viruses by Regulating Interferon mRNA Integrity. *Cell Host & Microbe* **7**, 354-361 (2010).
26. Liu, Y., Krishnan, M.N. & Phua, K.K.L. Suppression of mRNA Nanoparticle Transfection in Human Fibroblasts by Selected Interferon Inhibiting Small Molecule Compounds. *Biomolecules* **7** (2017).
27. Yoshioka, N. et al. Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell Stem Cell* **13**, 246-54 (2013).
28. Beissert, T. et al. Improvement of In Vivo Expression of Genes Delivered by Self-Amplifying RNA Using Vaccinia Virus Immune Evasion Proteins. *Human Gene Therapy* **28**, 1138-1146 (2017).
29. Awe, J.P., Crespo, A.V., Li, Y., Kiledjian, M. & Byrne, J.A. BAY11 enhances OCT4 synthetic mRNA expression in adult human skin cells. *Stem Cell Research & Therapy* **4** (2013).
30. Poleganov, M.A. et al. Efficient Reprogramming of Human Fibroblasts and Blood-Derived Endothelial Progenitor Cells Using Nonmodified RNA for Reprogramming and Immune Evasion. *Human Gene Therapy* **26**, 751-766 (2015).
31. Anderson, B.R. et al. Nucleoside modifications in RNA limit activation of 2'-5'-oligoadenylate synthetase and increase resistance to cleavage by RNase L. *Nucleic Acids Research* **39**, 9329-9338 (2011).
32. Anderson, B.R. et al. Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. *Nucleic Acids Research* **38**, 5884-5892 (2010).
33. Andries, O. et al. N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J Control Release* **217**, 337-44 (2015).

Chapter 6

Broader international context, relevance and future perspectives

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ABSTRACT

After overviewing the potential of messenger RNA (mRNA) as a new emerging drug class for the prevention and treatment of a broad array of diseases in **Chapter 1**, we then focused on its use for retinal gene delivery. Yet, in contrast to non-ocular applications such as cancer immunotherapy, vaccination strategies and cellular reprogramming, where mRNA-based therapeutics are currently in the spotlight, no documentation was found on the evaluation of mRNA to treat retinal diseases. Nevertheless, we found some intriguing benefits of its use for ocular applications and demonstrated the suitability of mRNA as a therapeutic cargo for retinal protein expression in mice. The biggest challenge we encountered in this thesis, however, was the efficient delivery of the mRNA molecule to its target site. Finding the right delivery materials will therefore be of major importance for the future development of mRNA-based retinal therapies. In this final chapter, we will discuss the broader international context of our work and its relevance to the field of gene therapy. To this end, we will focus on the recent progress in ocular gene therapy and the key challenges that need to be the subject of continued research. Furthermore, other new emerging applications are introduced that may revolutionize the field of ocular gene therapy as we know it and finally we attempt to envision how mRNA-based therapeutics could contribute to these new strategies to bring back sight to the blind.

OCULAR GENE THERAPY: VIRAL *VS.* NON-VIRAL

Gene therapy: from idea to reality

Ever since the mystery behind our genetic composition was unraveled, scientists have dreamed of the opportunity to utilize gene expression as an asset in treating human diseases. In a mere few decades, gene therapy has evolved from a fantasy to clinical reality. Indeed, the use of the host's gene expression machinery to transcribe and translate exogenous delivered genetic information has experienced tremendous progress, with three new gene therapeutics approved for market release in the United states in the last two years[1-3]. Nevertheless, the field of gene therapy has also known other historic periods that were less promising. The occurrence of harmful side effects, at a much higher rate than originally anticipated, considerably tempered the excitement for gene therapy[4-7]. Indeed, gene therapy has been subjected to the typical ups and downs of over-enthusiasm, disappointment and revival. The question therefore raises, as gene therapy has survived its previous failures will it be able to meet its expectations in the future?

The road to success with viral vectors

The eye presents some unique advantages as a target tissue and has therefore been leading the edge in translational research of gene therapy. Firstly, the eye is considered immune privileged as separation from the systemic circulation limits foreign material, including vectors used for gene therapy, to elicit a potentially damaging immune response[8, 9]. Secondly, its small size makes the eye a very attractive target organ because only small amounts of agents are required to achieve therapeutic effects. Moreover, many retinal disease animal models and non-invasive *in vivo* imaging techniques allow for a defined evaluation of the effectiveness of a therapy[10-12]. Finally, the possibility to locally administer genetic material into specific ocular compartments and to use the other eye as a simultaneous control, minimizes the risk for clinical trials.

It is therefore not surprising that most successes in the field of gene therapy in the last decade were made for the treatment of monogenetic inherited blinding diseases, in particular one specific form of Leber's Congenital Amaurosis (LCA). These advances marked the beginning of a new era in medicine leading to the first *in vivo* gene therapy approved by the Food and Drug Administration (FDA), i.e. voretigene neparvovec[13]. This vector, with the brand name Luxturna™ (Spark Therapeutics), is intended for the treatment of LCA type 2, which is caused by mutations in retinal pigment epithelium (RPE)65 that result in severely reduced vision at birth and slowly progressive degeneration of retinal photoreceptors[14, 15].

This newly approved gene therapy product is based on an adeno-associated virus (AAV). While initial attempts to transduce retinal cells, more than 25 years ago, made use of adeno- and lentivirus (LV)-mediated gene transfer, AAVs steadily gained ground owing to their small size, high transduction levels, variety of available serotypes and limited immunogenicity.

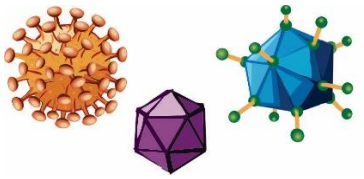
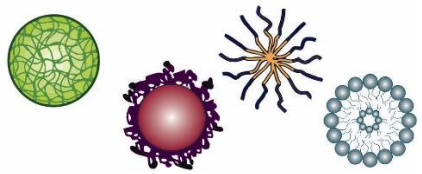
Despite the highly promising results generated with AAVs, there are still a few challenges ahead. A principal shortcoming of AAVs is their limited cargo capacity of max 5 kb[16]. Many genes mutated in inherited retinal diseases are therefore too large to be carried by AAVs. Thus, several strategies are being investigated to expand viral transfer capacity, including lentiviral vectors (accommodating sequences up to 10 kb) and the development of multiple vector systems. Another potential limitation of AAV-mediated gene therapy is the patient's preexisting anti-AAV neutralizing antibodies, which could result from childhood exposure to one or more serotypes or from previous AAV administration[17, 18]. Although the eye is generally considered to be immune privileged, AAV administration has been reported to induce innate as well as adaptive immune responses, which can seriously impact the safety and efficacy of the therapy[19]. These findings were more pronounced after intravitreal (IVTR) delivery, but can also occur after subretinal (SR) injection[19, 20]. A third major challenge to the use of AAVs is the difficulty to create manufacturing capacity for producing clinical quality vectors to scale at a price that will be economically viable[3, 21]. As a result, the first gene-based therapeutic approved in Europe, namely the AAV-based Glybera™ also known as the "million-dollar drug" was withdrawn from the market for financial reasons[22].

Non-viral vectors to the rescue?

An alternative that may solve some of these problems is the use of non-viral vectors, that is, natural or synthetic (nanosized) carriers based on cationic lipids and/or polymers that spontaneously complex negatively charged nucleic acids[23]. Compared to viral delivery systems, non-viral vectors are easily synthesized and usually have much lower production costs[24]. Moreover, their low immunogenicity renders them a favorable safety profile, increasing their potential for repeated administration. Finally, non-viral vectors possess almost four times as much transport capacity compared to AAVs (up to 20 kb), which allows packing of larger genes (*e.g.,* ABCA4, the most common mutated gene in Stargardt disease). Nevertheless, despite these promising advantages, no synthetic vectors have reached ocular clinical trials thus far and only few approaches have been able to achieve therapeutic levels of transgene expression in animal models. For example, PEG-substituted polylysine (CK30PEG) nanoparticles, have been evaluated in various disease models (reviewed in [25]). While many non-viral approaches design particles to encapsulate DNA, CK30-PEG particles are based on the neutralization of DNA's negative charges, so it can fold upon itself and

condense into a compact nanostructure which is easily internalized by endocytosis. Following SR delivery CK30PEG nanoparticles induced structural and functional improvements in several mice disease models, including retinitis pigmentosa[26-28], Stargardt disease[29] and LCA[30, 31] models. In a recent study, these particles were furthermore proven safe and non-toxic in non-human primate eyes[32]. Remarkably their efficacy on the short-term is comparable to that of AAVs, however, over time gene expression induced by CK30PEG becomes a lot lower than those obtained by AAVs[33].

Table 1 | Relative advantages and disadvantages of viral vs. non-viral vectors. Adapted from [3]

	Viral vectors	Non-viral vectors
		
Transfection efficiency	High	Low; key limitation for non-viral vectors
Duration of expression	Years and possibly a lifetime of expression persistence	Generally shorter persistence of expression
Possibility to reverse the effect	In general not possible to reverse the effect	Possible to reverse the effect, depending on the mechanism of action (*e.g.,* protein replacement vs. permanent gene editing)
Ability to titrate dose to effect in patient	Not possible; dose required for effectiveness is difficult to predict; requires applications with a large therapeutic window	Possible, although dose-effect response should be empirically established
Risk at immune response	Immune response to first dosing may limit effectiveness or prohibit repeated administration	Repeated dosing more likely less risky, although immune response to a novel product may still pose a limitation
Risk of insertional mutagenesis	Existing risk (although extremely low for AAV, minimized in newer LVs)	Extremely low (non-existing in case of RNA)
Packaging capacity	Low; up to 5 kb for AAVs and 10 kb for LVs	High; up to 20 kb

In addition to these polymer-based nanoparticles, other nanoparticle formulations have been successfully explored in therapeutic animal models. As such, IVTR injection of hyaluronic acid (HA)-coupled solid lipid nanoparticles carrying RS1-encoding pDNA was shown to efficiently transduce photoreceptors and improve the structural integrity of a mouse model of X-linked juvenile retinoschisis[34]. Also the use of liposome-protamine-DNA

complexes (LPDs) represents a promising approach for ocular gene delivery. Indeed, Rajala *et al*. showed that LPDs carrying RPE65 encoding DNA, led to substantial improvements in electroretinography (ERG) response and photoreceptor integrity when subretinally delivered in RPE65 knock-out mice[35].

Although there is a lot of debate on which strategy, viral or non-viral, is the most promising for the future of retinal gene therapy, it is clear from the above that both approaches are still in development and carry their own set of unique challenges and limitations, which will need to be addressed. Seeing that both types of vectors present advantages and disadvantages (represented in **Table 1**), neither one should be considered superior but rather a tailored approach specific to each disease or application should be pursued.

OCULAR GENE THERAPY: THE PROMISE AND PERILS

Although the concept of gene therapy originated more than 40 years ago, gene therapy has only seen limited success so far. Indeed, despite the overwhelming amount of promising pre-clinical data, technical and fundamental challenges remain and make us wonder whether gene therapy will ever hold place in the medical world of tomorrow.

Challenges and limitations of gene therapy

Limited number of disease targets

One major challenge of gene therapy is the extensive amount of affected genes in retinal diseases. To date, more than 300 genes have been associated with inherited retinal cell death, with most genes containing a wide variety of pathogenic mutations[36]. This enormous diversity in disease-inducing mutations hampers the development of gene augmentation strategies, as only those people with a specific mutation will benefit from the therapy. In addition, since the therapies are mutation-specific, each treatment targeting a new mutation has to go through all the steps of drug development and regulatory processes. The enormous costs involved in the drug development process (as discussed later in this section) further limit the opportunity to develop new genetic treatments for inherited retinal diseases, as the majority of these diseases are caused by rare mutations and do therefore not represent a large enough group of people to render the research financially viable. Furthermore, current clinical gene therapy trials are limited to diseases caused by so called “loss-of-function” mutations, meaning that the gene product is partially or completely nonfunctional[37]. Diseases based on “gain-of-function” mutations, in which mutations lead to

an altered gene product with detrimental effects, most likely won't benefit from gene augmentation strategies[38]. As these diseases require silencing of the mutant gene to result in therapeutic efficacy, other therapies are being developed, the most prominent technology being gene editing, which we will discuss later in this chapter[39].

Required cell viability

A second and very important challenge of gene therapy is that the cell type expressing the mutated gene must be viable at the time of therapy. Hence, advanced stages of retinal degeneration, in which lots of cells are lost due to the degeneration process, may hinder full realization of gene replacement benefits. In the case of advanced retinal degeneration, strategies such as optogenetics are emerging as a valuable alternative to mutation-specific gene therapy. An overview of the therapeutic window of possible intervention strategies is displayed in **Figure 1**.

Long-term efficacy

Although retinal gene therapy presents an acceptable safety profile in many of the clinical trials, efficacy remains uncertain. For instance, despite the detectable visual gain that lasts at least 3 years after RPE65 gene therapy, two of the three clinical trials reported a decrease in the levels of visual improvements, indicating that the therapy was unable to halt the continuing retinal degeneration[40, 41]. As the follow-up period of the recently accepted RPE65 gene therapeutic Luxturna™ is limited to 3 years, the longevity of gene expression from a one-time delivery is unknown and reduction of improved vision areas might be due to declining transgene expression[42]. In addition, although there is a significant improvement in the navigation capacity of the patients, visual acuity was not significantly improved[43]. Finally, it is unknown whether or not repeated administration of the vector will be necessary and if so, whether or not the eye will be able to tolerate additional treatments. Therefore the clinical significance of the changes in cell-mediated and humoral immune responses, though extremely low, needs to be elucidated.[42]

Commercial viability

Another challenge that lies ahead is the translation of the achieved success in clinical development to success in the market. In contrast to regular drug therapies, which treat disease symptoms continuously over time, gene therapies seek to cure a disease in a single treatment. Although these one-time treatments are very attractive for the patients, questions raise about the commercial viability of gene therapy products for pharmaceutical

companies[3, 44]. Unlike other therapies in which the investment costs can be redeemed either over time or by treating a very high amount of patients, gene therapy must derive its profit from the first and perhaps only treatment of a small group of patients. For the production to be economically viable, very high prices will be required for a single therapy. In order to bring the costs of this technology down, a broader potential market and therefore broadening the spectrum of treatable diseases (as discussed earlier) will be necessary[45].

Alternatives to gene therapy

While the refined strategy of replacing a defective gene with its normal counterpart has proven to be successful, the limited amount of disease targets, the timing of treatment, the uncertain duration of effect, the risk at immunogenicity and the commercial viability are important challenges to which solutions may be as complex as the diseases themselves. Therefore the specific market for gene therapy may soon be occupied by other cutting-edge therapies, including stem cell strategies and retinal prostheses. Indeed, in more advanced stages of retinal diseases, in which a large number of cells is lost or severely degenerated, these therapies present a more appropriate alternative. Stem cell therapies are a promising approach to restore visual function in degenerative retinal diseases such as retinitis pigmentosa (RP), age-related macular degeneration (AMD) and Stargardt macular dystrophy, as stem cells have the potential to differentiate in any mature cell type[46]. The idea underlying this approach is to generate new retinal cells from pluripotent embryonic stem cells (ESCs), patient-derived induced pluripotent stem cells (iPSCs) or retinal progenitor cells, to replace the damaged cells in the diseased retina[47]. In addition, stem cells are able to provide trophic support and temper degeneration by prevention of apoptosis[48]. Nevertheless, retinal neuron replacement is a complex process, as it requires optimal delivery in the human retina and correct integration in the remaining neural network. Also, additional drawbacks can be associated with specific stem cell sources[49]. A growing number of preclinical and clinical trials are addressing these issues and some early-phase clinical studies suggest acceptable safety profiles[50, 51].

Besides advanced biological strategies, technological advancements have also proven to be successful in helping patients suffering from vision loss. An example of a technology-based device approved for use in patients is the Argus II retinal prosthesis, also known as the bionic eye. Argus II is an implantable device, developed to translate phonic information into electrical stimulation of the surviving neurons to induce visual perception in blind individuals[47, 52]. The implant relies on an external camera in the glasses of the patient, which captures visual information and sends it to a video-processing unit attached to the glasses. Here, the information is transformed into stimulation patterns which are send back

to the glasses and are wirelessly transmitted to the retinal implant. The implant then emits small electrical pulses that bypass the damaged photoreceptors and stimulates the remaining retinal cells, such that the signals are transmitted along the optic nerve to the brain[52]. Although devices such as the Argus II offer only rudimentary functional visual perception, the field of artificial vision is rapidly advancing and continuous efforts to reconcile the gap between artificial and natural vision hold great promise for the treatment of blinding conditions in the near future[53].

NOVEL EMERGING APPLICATIONS FOR GENE DELIVERY

Due to the rapid development and fierce competition between the different strategies to provide sight to the blind, gene augmentation strategies might soon be replaced by innovative cell-based therapies or retinal prostheses. Nevertheless, in the recent years, new applications of gene delivery have emerged that broaden the reach of the therapy beyond specific loss-of-function mutations. In my opinion these strategies can cause a revelation in the field of gene therapy, especially in ophthalmology.

Gene editing

Genome editing is the introduction of changes into the genome of a living organism, with the ability to correct a specific genetic defect while preserving natural regulatory sequences. As this enables knockdown of mutated genes and replacement by the correct one, gene editing is especially beneficial for gain-of-function mutations, in which it is important to avoid overproduction of the mutated protein as this can lead to detrimental toxicity[54]. An example of a strategy that is transforming the gene editing field is the CRISPR/Cas9 system, which is based on a bacterial defense system against viruses[55]. This strategy relies on the Cas9 DNA endonuclease complex which is guided to a specific sequence of the genome by a synthetic guide RNA (sgRNA), engineered to be complementary to the sequence of interest[56]. Following recognition the Cas9 nuclease opens both strands of the targeted sequence and introduces a double-stranded cleavage which can be repaired by the cell's machinery at random or by a more precise process in which template DNA pairs up with the cut ends and replaces the original sequence[54, 55]. As such, CRISPR/Cas9 has shown great preclinical successes in ophthalmology, which point towards clinical trials in the near future. Recently, for example, Maeder *et al*. used the CRISPR/Cas9 editing system to successfully remove the major underlying splice mutation causing LCA10 in human cell and retinal explants. Following SR injection in humanized mice and non-human primates, the authors subsequently demonstrated efficient and safe application of CRISPR mediated CEP290 editing *in vivo*[57].

Mutation-independent genetic strategies

As the enormous genetic diversity of retinal diseases challenges the development of mutation-specific therapies, strategies that do not require specific gene targeting have the potential to treat a larger number of patients and represent a more cost-effective approach. To extend the scope of gene therapy, interest increases in neuroprotective strategies. As explained in **Chapter 2**, neuroprotection aims to preserve the viability of neurons and prolong their lifespan regardless of their underlying genetic anomaly or pathogenic cause. In contrast to gene replacement approaches, neuroprotection may provide a general treatment for retinal degeneration, including very rare diseases for which the causative mutation is still unknown. Multiple examples of this approach in diseased animal models are reported in **Chapter 2**. Another approach which has the potential to restore visual function without addressing the causative genetic mutation is optogenetics. Optogenetics is a technique that aims to restore vision by introducing genes encoding for light-sensitive proteins in surviving retinal neurons[58]. Expression of these proteins enables the cells to become light-sensitive and capture visual information when photoreceptors are dysfunctional or degenerated. One prerequisite is the presence of at least some retinal cells that can be targeted, such as remaining light-insensitive cones, bipolar cells or ganglion cells[59-61]. The primary concern is the fact that the high light intensities required to stimulate the opsins are very high and hold the risk of photochemical damage. Nevertheless, effective application of optogenetics was recently demonstrated in cynomolgus macaques in which opsin expressing ganglion cells robustly responded to light intensities below the safety threshold for illumination of the human eye. We are therefore convinced that ongoing generation of opsins with increases photosensitivity, improvement in targeted gene delivery and advances in development of light-delivery devices will pave the way for the clinical development of optogenetics[62]. A final example of a mutation-independent approach in which gene therapy could become of increasing importance is retinal regeneration. As extensively discussed in **Chapter 2**, selective transfer of genes encoding certain transcription or growth factors to Müller glia can activate a reprogramming event which can eventually lead to regeneration of lost retinal cells by a second gene transfer dictating cell fate determination. The ability to use the intrinsic stem cell potential of endogenous Müller cells for retinal repair is very attractive because of the many risks associated with stem cell transplantation and retinal prostheses. Although the idea of a self-healing retina may seem a faraway fantasy, remarkable advances in studies restoring vision in mammals make me believe that retinal regeneration therapies may soon be in sight.

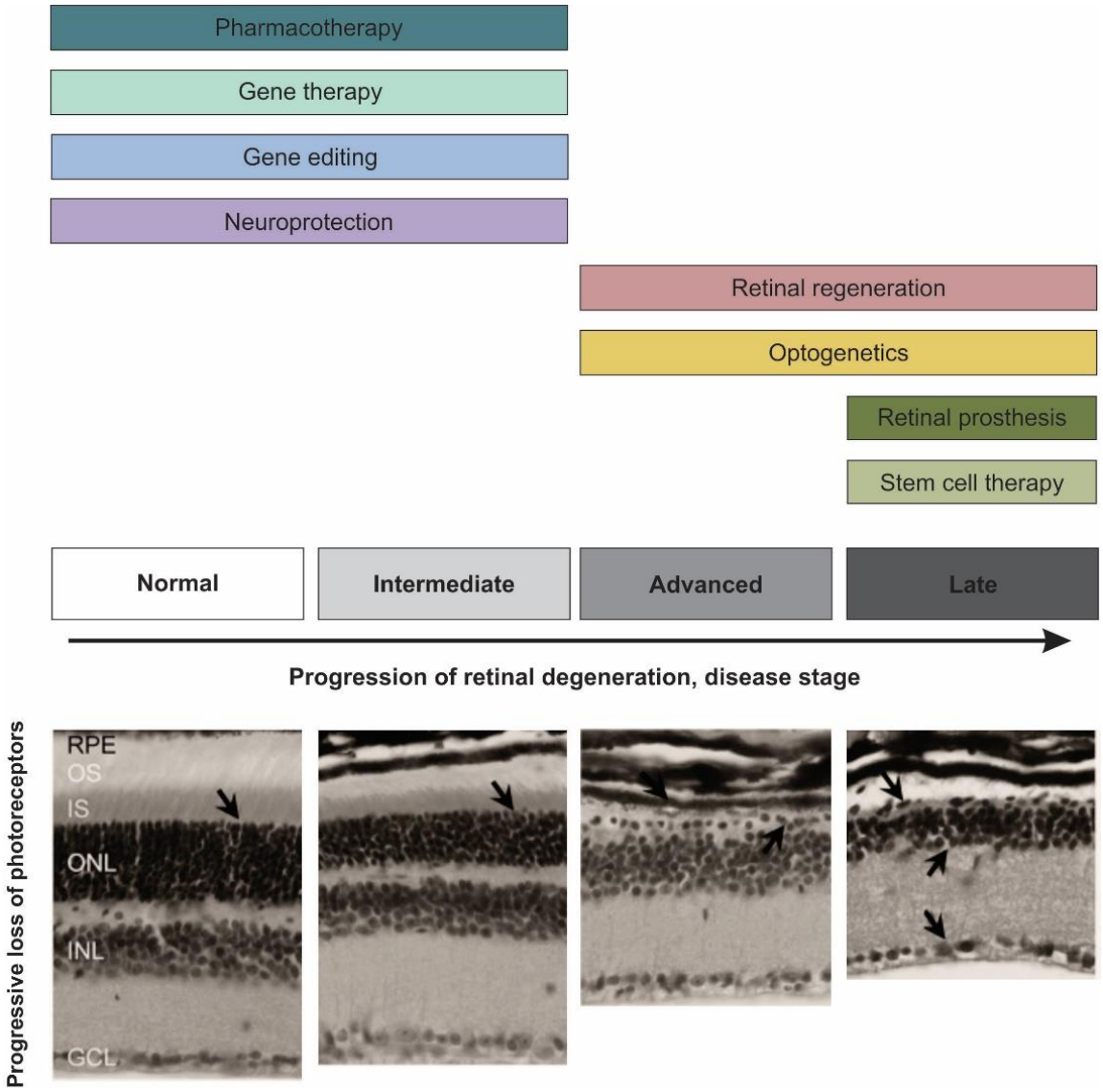


Figure 1 | Therapeutic windows of opportunity for possible interventions. Therapeutic strategies at each stage of the disease are indicated. At the early stages, affected retinal cells can be target using mutation-specific gene therapy (gene augmentation or gene editing therapy). Death of retinal cells can also be prevented or delayed by mutation-independent strategies by pharmacotherapy or neuroprotection. Retinal regeneration, optogenetics, retinal prosthesis and stem cell therapy are needed to restore vision during the later stages of retinal degeneration. Images of retinal section of a mouse model of retinitis pigmentosa are shown as an example of a photoreceptor degeneration process. GCL, ganglion cell layer; INL, inner nuclear layer; IS, inner segments; ONL, outer nuclear layer; OS, outer segments; RPE, retinal pigment epithelium. Figure adjusted from [63] and [64].

THE POTENTIAL AND PITFALLS OF MRNA

The potential of mRNA therapy

As stated above it was long believed that non-viral vectors would revolutionize retinal gene therapy due to their advantageous properties. Despite the wealth of DNA carrying vectors that have been developed over the past several decades, non-viral vectors for gene therapy are still not available in a clinical setting. The shortcoming of non-viral gene

therapeutics is mainly attributed to the requirement that pDNA must cross the nuclear membrane to allow translation[65]. As shown in **Chapter 3**, this is a major disadvantage of retinal gene therapy as most retinal cells normally do not divide. Another drawback responsible for slowing clinical translation, is that many of the plasmids used in pDNA-based gene therapies carry antibiotic resistance genes as selection markers for plasmid production[66]. As gene transfer to the patient's bacteria cannot be excluded, safety concerns have spurred development of antibiotic-free approaches[67]. Finally, pDNA- as well as viral vector-based therapeutics give rise to an unpredictable duration of protein expression, which may persist for many years. Unlike a small molecule drug for which the administration can be stopped or technological devices which can be switched off when unwanted side effect occurs, gene therapy may lead to a lifetime of protein expression (see **Table 1**). This is an added benefit for therapies in which lifelong expression is desired, allowing for a single treatment, however in view of safety, uncontrollable expression can be a primary concern. As discussed in detail in **Chapter 1**, the use of messenger RNA (mRNA) therapeutics has emerged as a promising alternative to overcome these limitations. In addition, recent advances in mRNA synthesis and stability, have enabled a wide range of applications exceeding the potential that was originally anticipated for DNA-based medicine. With this belief in mind, we were the first to evaluate the potential of non-viral mRNA delivery for ocular applications (cfr. **Chapter 3** and **4**).

Similar to pDNA and viral vectors, mRNA is recognized by the immune system as a foreign molecule, thereby inducing an immune response which can be detrimental for mRNA translation (cfr. **Chapter 1**). One way to temper this mRNA triggered-immune reaction is the use of innate immune inhibitors that either prevent mRNA recognition or block downstream mediators in the activated signaling cascades. Indeed, evaluation of five well-known innate immune inhibitors resulted in a decrease of the mRNA related interferon response, as demonstrated in **Chapter 5**. Unfortunately, in contrast to what we expected, suppression of this response did not directly correlate with an increased mRNA transfection efficiency. A more established method to obtain immunosilent synthetic mRNA is the incorporation of modified nucleosides into the mRNA molecule. As proven in **Chapter 4**, synthetic mRNA containing pseudouridine or N^1-methylpseudouridine drastically enhanced the mRNA translation potential, which can be attributed to an increased mRNA stability as well as evasion of the immune activation. Interestingly, our data not only demonstrated an increase in the level and duration of protein expression when using modified mRNA, a clear difference was noticed between the different mRNA modifications which allows fine-tuning of the mRNA expression by adjusting the molecule. This capacity opens up a wide range of potential therapies in which the long-term safety of increased protein expression is still unknown.

With regard to retinal diseases, the use of mRNA is extremely attractive for the expression factors which aim to prolong the life-span of the affected cells, such as neurotropic factors, anti-apoptotic proteins, regulators of oxidative defense or inflammation inhibitors. As documented in **Chapter 2** neuroprotective factors such as ciliary neurotrophic factor (CNTF) or brain-derived neurotrophic factor (BDNF) can protect photoreceptors and retinal ganglion cells (RGCs) from different types of injury, including mechanical damage and degeneration caused by inherited mutations[68-72]. These neuroprotective factors can be delivered to the retina as such, though this has proven ineffective due to the short half-life of the delivered proteins[73-75]. A prolonged neurotrophic effect can be obtained through the delivery of neurotrophic factor encoding transgenes. However, the use of pDNA or viral vectors raises concerns as the long-term effects of high expression levels of some neurotrophic factors (such as CNTF) have been shown to cause inflammation and gliosis and can be detrimental to retinal functioning[76-78]. We believe that the transient nature of mRNA, could make this mutation-independent approach a safe and successful therapy. Compared to the use of proteins, mRNA encoding neurotrophic factors can extend the neurotrophic effect, as a single mRNA molecule gives rise to multiple copies of a protein over the course of hours or days. Compared to the use of pDNA or viral vectors, on the other hand, mRNA generates only short-term protein expression of which the duration will be greatly determined by the half-lives of both the mRNA molecule and the protein product[79]. This mRNA-based neuroprotective approach could be a stand-alone treatment to prevent retinal degeneration, when delivered in the early stages of the degeneration process. Alternatively, it could be used to broaden the therapeutic window for gene-augmentation therapy or to provide a healthier environment for stem cell treatments.

The short-term expression levels obtained with mRNA might also be beneficial in gene editing applications like CRISPR/Cas9 (as illustrated in **Figure 2**), which only require a short period of expression. Because of its ability to specifically ‘cut’ and ‘paste’ into the patients genome, this strategy holds incredible promise for one-time cures of genetic diseases. However, while CRISPR/Cas9 is already widely used for creating genetically modified organism and the study of gene functionality, the high frequency of nuclease activity at undesired locations in the genome (>50%) is a major concern for therapeutic and clinical applications[81]. Especially in post-mitotic tissues, such as the retina, continuous expression of Cas9 nucleases by viral vectors or pDNA can extensively increase the potential for off-target effects. mRNA could therefore be a safe alternative to express the Cas9 protein as it acts much faster compared to pDNA and degrades rapidly, limiting the risk for off-target effects[82]. Moreover, as mRNA and sgRNA are both single stranded molecules, their delivery can often be mediated by the same carrier. For successful genome editing it is, however, important that sgRNA is not degraded by the time the mRNA is translated into the Cas9

protein. Indeed, Jiang *et al.* recently demonstrated that by simply delaying the administration of sgRNA to a point in time at which Cas9 expression levels were the highest, a maximum editing potential could be achieved[83]. Alternatively, the research group of Anderson avoided this timing issue by chemically modifying the sgRNA molecule thereby reducing its susceptibility to nuclease degradation. Using this modified sgRNA the authors show that a single co-delivery of sgRNA and Cas9 RNA resulted in nearly complete editing of the target gene in hepatocytes *in vivo*[84]. This complete RNA-based strategy may enable the expansion of CRISPR/Cas9 editing to clinical settings.

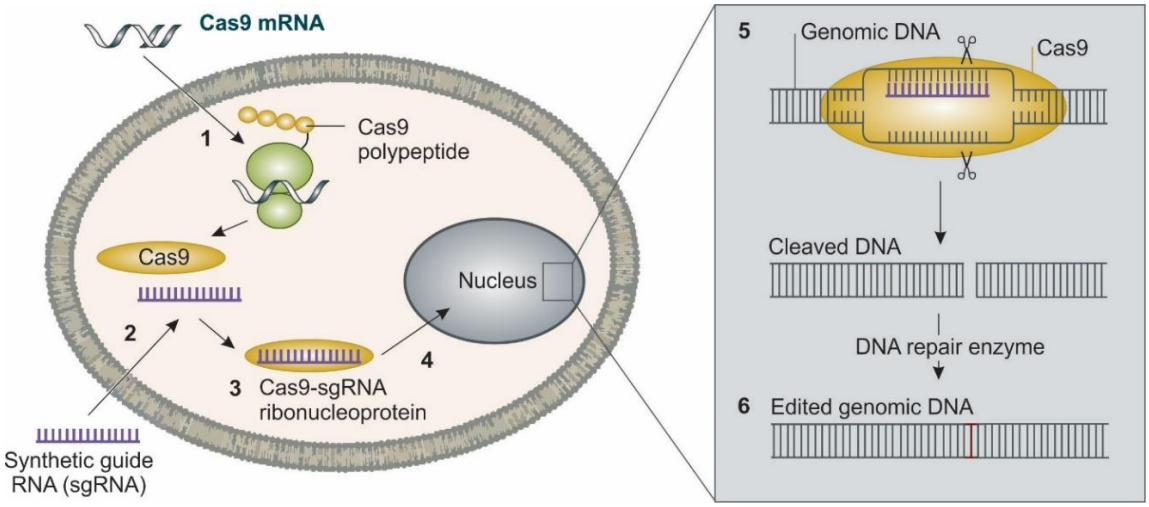


Figure 2 | The mechanism of mRNA-based CRISPR/Cas9 gene editing. (1) mRNA encoding the Cas9 (CRISPR-associated protein 9) nuclease is taken up by the cell and is translated at the ribosomes. (2) The synthetic guide RNA (sgRNA), which has affinity for a specific DNA sequence, is also transfected into the target cell and (3) complexes with the Cas9 protein to form a ribonucleoprotein. (4) This complexes translocates to the nucleus where it binds to a DNA sequence complementary to the sgRNA and (5) introduces a double-stranded DNA break into the genome. (6) This break is then repaired by the endogenous cellular DNA repair machinery that catalyzes an error-prone mechanism called non-homologous end joining which can lead to a gene knockout or catalyzes the replacement of a newly inserted DNA sequence in the presence of a 'repair template', a process known as homology-directed repair.
Figure adjusted from [80].

The pitfalls of mRNA therapy

Despite remarkable advances in controlling mRNA's translation efficacy (determined by its immunogenicity and intrinsic stability), the main challenge for successful mRNA-based therapeutics remains, as with all gene delivery strategies, their efficient intracellular delivery. When delivering mRNA via biological fluids containing nucleases, such as blood, synovial fluid or vitreous, safe and effective drug delivery vehicles are required to protect the mRNA against enzymatic degradation. Inspired by pDNA or short-interfering RNA (siRNA) delivery, a wide range of vectors, varying in physicochemical characteristics and transfection efficiency, have been tested for their ability to deliver mRNA[80]. However, considering the

differences between pDNA, siRNA and mRNA regarding molecular structure, length and application, it is important to note that research into non-viral pDNA or siRNA delivery materials is not always predictive for the efficiency of mRNA delivery and vice versa[85], as is also shown in **Chapter 3**. Nevertheless, insight into which particles are potentially toxic and which are safe and effective can indeed be gained from the neighboring fields of pDNA and siRNA and should be used when designing similar or completely new mRNA delivery particles. As such, formulations carrying a positive charge at physiologic pH after mRNA complexation are not preferable as they are generally associated with *in vivo* toxicity[86]. As an alternative, ionizable carriers such as ionizable lipid nanoparticles (LNPs) have been developed, which are positively charged at low pH (ideal for mRNA complexation) but turn neutral at physiological pH, leading to reduced toxicity when administered *in vivo*[87-89]. Ionizable LNPs are therefore regarded as the ideal platform for *in vivo* mRNA delivery and have shown considerable potential in protein replacement, vaccination strategies and gene editing technologies amongst others[66, 80].

To our knowledge, evaluating mRNA for protein expression in the retina, which was the ultimate aim of this thesis, had never been done before. Two factors likely holding off mRNA-based retinal gene delivery studies are the presumed molecular instability of synthetic mRNA and the lack of sufficient effective delivery systems. Concerns about instability are gradually decreasing as we learn more about the strategies to modify structural elements of the mRNA molecule that can systematically improve its intracellular stability and translation efficiency. Concerns about delivery, however, remain justified, as both viral and non-viral delivery systems are hindered by the very barriers that make the eye an ideal privileged organ for gene therapy. Because of the low bioavailability, potential off-target effects and restricted permeability of the blood-retinal barrier (BRB), intravenous injection is not preferred for the administration of mRNA therapeutics[90, 91]. Likewise, delivery to the retina via topical administration is primarily hindered by the corneal epithelium and poor drug availability due to reflex blinking[92]. Instead, SR injection can overcome these hurdles, bypassing several anterior barriers and delivering the mRNA in close proximity to the target site. Indeed, as shown in **Chapter 4**, SR injection of mRNA, complexed to a lipid-based carrier, successfully transfected photoreceptors and RPE cells in mice. However, SR injection is a very invasive delivery method, requires a pars plana vitrectomy and entail the risk at transient detachment of RPE from the underlying photoreceptors. Therefore, a much debated question is whether SR injections are safe to perform in case of a diseased retina, which is already in a compromised state because of the degenerative process[24]. In addition, SR injections deliver the transgene to a restricted area of the retina and thus transfection is often limited to the cells surrounding the injection spot, an observation that was also made in **Chapter 4**. Although evaluation of a new delivery procedure, namely sub-inner limiting

membrane (ILM) injection is currently under investigation[93], IVTR delivery still represents the safest, least invasive strategy to transfect the retinal tissue. In addition, IVTR injection promotes pan-retinal protein expression as it offers a widespread distribution of the vector through to the retina. Unfortunately, as underscored throughout this thesis, two major physical barriers stand in the way of fluent vector migration towards the retinal cells, namely the vitreous and the ILM. Thanks to continuous efforts to investigate the interactions hindering the delivery process, insight was gained into the ideal particle surface characteristics to overcome the vitreal barrier. A nice example of this is provided in **Chapter 3**, showing that smart adjustments, such as coating strategies for predefined gene carriers can result in the desired physicochemical characteristics for adept delivery of mRNA across the vitreous. Regrettably, the physicochemical requirements to efficiently cross the ILM are less documented. Moreover, drawing conclusions about ILM penetration from literature is challenging, owing to the enormous variety of vectors tested on different species. It is well-established indeed and underscored by our research that the barrier roles of the different ocular layers are highly species-dependent. However, almost all studies focusing on non-viral retinal delivery of nucleic acids are performed in small laboratory animals in which results are very promising. When these same delivery systems are subsequently tested in larger animals results are often less encouraging, presumably due to the more complex physiological barriers present in these species. Despite the amount of information that can be gathered out of these negative results, they are mostly left in the dust as publication of negative results is often not supported. This subsequently leads to a vicious circle of examining new formulations in rodents which are introduced as 'the newest breakthrough' yet are in the end non-applicable for clinical translation. We are therefore convinced that a more detailed study of the biological barriers in the eye, such as the ILM, in larger species can enable the development of a new generation of rationally-designed vectors capable of overcoming the many hurdles for efficient mRNA delivery to the retina. To this end, elegantly designed *ex vivo* experiments, such as the ones used in **Chapter 3** and **4**, can be even more valuable than *in vivo* studies, as they enable the use of larger animals with a physiology more closely resembling the human one. Additionally, *ex vivo* studies allow for a detailed investigation of the different secluded delivery barriers and the capacity of a delivery system to overcome a single barrier[91]. By examining the properties and in particular the potential differences amongst species an appropriate *ex vivo* model for evaluation of a certain barrier closely relating the complex human situation could be developed[94]. We are furthermore convinced that acquiring more knowledge about the exact composition and morphology of certain ocular barriers, such as the ILM, will help in the clever design of new particles or adjust well-established ones in order to surmount the obstacle course to the retina[95]. As shown in this dissertation, our research group has recently developed two of these *ex vivo* models, one to evaluate the interaction of carriers with the vitreous[96] and one to evaluate the

ability of carriers to cross the vitreoretinal interface[94]. Using the latter, our results demonstrated that the commercial lipid-based mRNA-carriers used in this thesis (**Chapter 3** and **4**), do not have the correct physicochemical properties to penetrate the ILM. Reducing the size of non-viral carriers without compromising their ability to efficiently encapsulate mRNA will be of major importance to overcome the ILM and therefore improve future ocular mRNA delivery.

CONCLUSIONS

Only a few decades have passed since the identification of the first mutation causing retinal degeneration and yet treatment of these diseases at their genetic roots has already become a feasible reality. However, issues regarding the delivery, immune rejection and scalability of viral gene therapy and the limited efficiency of non-viral delivery systems, make us wonder whether gene therapy will survive its original enthusiasm or rather soon be replaced by the emerging cell-based and technological strategies. The emergence of gene editing, neuroprotection, optogenetics and retinal regeneration strategies argues against this and opens the door for new applications that continue to expand the therapeutic reach of gene therapy for retinal diseases. In particular the use of mRNA could have an added benefit for some of these new strategies, as its transient nature limits the risk of detrimental side effects caused by unpredictable long-term expression of certain proteins. Similar to other forms of retinal gene therapy, however, the most challenging aspect of mRNA therapy is the efficient delivery to its target site. To enable the design of the suitable delivery materials, it will be necessary to increase our fundamental knowledge regarding the biology of the different ocular barriers and to understand how these obstacles hinder efficient gene transfer. In conclusion, with the use of safe and effective delivery materials, mRNA has the potential to become a big player in the field of ocular gene therapy. Together with the use of other approaches, each with its own therapeutic window for treatment at different stages of retinal degeneration, it is safe to assume that mRNA therapy can help create better visual outcomes for patients in the near future.

REFERENCES

1. Naldini, L. Gene therapy returns to centre stage. *Nature* **526**, 351-360 (2015).
2. Dunbar, C.E. et al. Gene therapy comes of age. *Science* **359**, 175-+ (2018).
3. Kaemmerer, W.F. How will the field of gene therapy survive its success? *Bioengineering & Translational Medicine* **3**, 166-177 (2018).
4. Raper, S.E. et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Molecular Genetics and Metabolism* **80**, 148-158 (2003).
5. Marshall, E. Gene therapy: Second child in French trial is found to have leukemia. *Science* **299**, 320-320 (2003).
6. Howe, S.J. et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *Journal of Clinical Investigation* **118**, 3143-3150 (2008).
7. Hacein-Bey-Abina, S. et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *New England Journal of Medicine* **348**, 255-256 (2003).
8. Anand, V. et al. A deviant immune response to viral proteins and transgene product is generated on subretinal administration of adenovirus and adeno-associated virus. *Molecular Therapy* **5**, 125-132 (2002).
9. Bennett, J. Immune response following intraocular delivery of recombinant viral vectors. *Gene Therapy* **10**, 977-982 (2003).
10. Capozzi, M.E., Gordon, A.Y., Penn, J.S. & Jayagopal, A. Molecular Imaging of Retinal Disease. *Journal of Ocular Pharmacology and Therapeutics* **29**, 275-286 (2013).
11. Baghaie, A., Yu, Z. & D'souza, R.M. State-of-the-art in retinal optical coherence tomography image analysis. *Quantitative Imaging in Medicine and Surgery* **5**, 603-617 (2015).
12. Dysli, C. et al. Fluorescence lifetime imaging ophthalmoscopy. *Progress in Retinal and Eye Research* **60**, 120-143 (2017).
13. FDA approves hereditary blindness gene therapy. *Nature Biotechnology* **36**, 6-6 (2018).
14. Voretigene Neparvovec-rzyl (Luxturna) for Inherited Retinal Dystrophy. *Medical Letter on Drugs and Therapeutics* **60**, 53-55 (2018).
15. Trapani, I. & Auricchio, A. Seeing the Light after 25 Years of Retinal Gene Therapy. *Trends in Molecular Medicine* **24**, 669-681 (2018).
16. Salganik, M., Hirsch, M.L. & Samulski, R.J. Adeno-associated Virus as a Mammalian DNA Vector. *Microbiology Spectrum* **3** (2015).
17. Kotterman, M.A. et al. Antibody neutralization poses a barrier to intravitreal adeno-associated viral vector gene delivery to non-human primates. *Gene Therapy* **22**, 116-126 (2015).
18. Vandamme, C., Adjali, O. & Mingozzi, F. Unraveling the Complex Story of Immune Responses to AAV Vectors Trial After Trial. *Human Gene Therapy* **28**, 1061-1074 (2017).
19. Reichel, F.F. et al. AAV8 Can Induce Innate and Adaptive Immune Response in the Primate Eye. *Molecular Therapy* **25**, 2648-2660 (2017).

20. Heier, J.S. et al. Intravitreous injection of AAV2-sFLT01 in patients with advanced neovascular age-related macular degeneration: a phase 1, open-label trial. *Lancet* **390**, 50-61 (2017).
21. Lundstrom, K. Viral Vectors in Gene Therapy. *Diseases* **6** (2018).
22. Yu, T.T.L., Gupta, P., Ronfard, V., Vertes, A.A. & Bayon, Y. Recent Progress in European Advanced Therapy Medicinal Products and Beyond. *Front Bioeng Biotechnol* **6**, 130 (2018).
23. Zulliger, R., Conley, S.M. & Naash, M.I. Non-viral therapeutic approaches to ocular diseases: An overview and future directions. *J Control Release* **219**, 471-487 (2015).
24. Planul, A. & Dalkara, D. Vectors and Gene Delivery to the Retina. *Annu Rev Vis Sci* **3**, 121-140 (2017).
25. Adijanto, J. & Naash, M.I. Nanoparticle-based technologies for retinal gene therapy. *Eur J Pharm Biopharm* **95**, 353-67 (2015).
26. Cai, X. et al. A Partial Structural and Functional Rescue of a Retinitis Pigmentosa Model with Compacted DNA Nanoparticles. *Plos One* **4** (2009).
27. Cai, X. et al. Gene delivery to mitotic and postmitotic photoreceptors via compacted DNA nanoparticles results in improved phenotype in a mouse model of retinitis pigmentosa. *Faseb Journal* **24**, 1178-1191 (2010).
28. Han, Z.C. et al. Genomic DNA nanoparticles rescue rhodopsin-associated retinitis pigmentosa phenotype. *Faseb Journal* **29**, 2535-2544 (2015).
29. Han, Z.C., Conley, S.M., Makkia, R.S., Cooper, M.J. & Naash, M.I. DNA nanoparticle-mediated ABCA4 delivery rescues Stargardt dystrophy in mice. *Journal of Clinical Investigation* **122**, 3221-3226 (2012).
30. Koirala, A. et al. Persistence of non-viral vector mediated RPE65 expression: Case for viability as a gene transfer therapy for RPE-based diseases. *Journal of Controlled Release* **172**, 745-752 (2013).
31. Koirala, A., Makkia, R.S., Conley, S.M., Cooper, M.J. & Naash, M.I. S/MAR-containing DNA nanoparticles promote persistent RPE gene expression and improvement in RPE65-associated LCA. *Human Molecular Genetics* **22**, 1632-1642 (2013).
32. Kelley, R.A. et al. DNA nanoparticles are safe and nontoxic in non-human primate eyes. *Int J Nanomedicine* **13**, 1361-1379 (2018).
33. Han, Z. et al. Comparative analysis of DNA nanoparticles and AAVs for ocular gene delivery. *PLoS One* **7**, e52189 (2012).
34. Apaolaza, P.S. et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. *Biomaterials* **90**, 40-9 (2016).
35. Rajala, A. et al. Nanoparticle-assisted targeted delivery of eye-specific genes to eyes significantly improves the vision of blind mice in vivo. *Nano Lett* **14**, 5257-63 (2014).
36. Bennett, J. Taking Stock of Retinal Gene Therapy: Looking Back and Moving Forward. *Mol Ther* **25**, 1076-1094 (2017).
37. Gupta, P.R. & Huckfeldt, R.M. Gene therapy for inherited retinal degenerations: initial successes and future challenges. *J Neural Eng* **14**, 051002 (2017).

38. Sahel, J.A., Marazova, K. & Audo, I. Clinical Characteristics and Current Therapies for Inherited Retinal Degenerations. *Cold Spring Harbor Perspectives in Medicine* **5** (2015).
39. Wu, W.Y., Tang, L.S., D'Amore, P.A. & Lei, H.T. Application of CRISPR-Cas9 in eye disease. *Experimental Eye Research* **161**, 116-123 (2017).
40. Jacobson, S.G. et al. Improvement and decline in vision with gene therapy in childhood blindness. *N Engl J Med* **372**, 1920-6 (2015).
41. Bainbridge, J.W. et al. Long-term effect of gene therapy on Leber's congenital amaurosis. *N Engl J Med* **372**, 1887-97 (2015).
42. (ed. Use, C.f.M.P.f.H.) (European Medicines Agency United Kingdom, 2019).
43. Moore, N.A., Morral, N., Ciulla, T.A. & Bracha, P. Gene therapy for inherited retinal and optic nerve degenerations. *Expert Opinion on Biological Therapy* **18**, 37-49 (2018).
44. Ameri, H. Prospect of retinal gene therapy following commercialization of voretigene neparvovec-rzyl for retinal dystrophy mediated by RPE65 mutation. *Journal of Current Ophthalmology* **30**, 1-2 (2018).
45. Campbell, J.P., McFarland, T.J. & Stout, J.T. Ocular Gene Therapy. *Dev Ophthalmol* **55**, 317-21 (2016).
46. Oner, A. Stem Cell Treatment in Retinal Diseases: Recent Developments. *Turk Oftalmoloji Dergisi-Turkish Journal of Ophthalmology* **48**, 33-38 (2018).
47. Barriga-Rivera, A. & Suaning, G.J. Visual prostheses, optogenetics, stem cell and gene therapies: splitting the cake. *Neural Regeneration Research* **13**, 805-806 (2018).
48. Zarbin, M. Cell-Based Therapy for Degenerative Retinal Disease. *Trends Mol Med* **22**, 115-134 (2016).
49. Madelaine, R. & Mourrain, P. Endogenous retinal neural stem cell reprogramming for neuronal regeneration. *Neural Regen Res* **12**, 1765-1767 (2017).
50. Jones, M.K., Lu, B., Girman, S. & Wang, S. Cell-based therapeutic strategies for replacement and preservation in retinal degenerative diseases. *Prog Retin Eye Res* **58**, 1-27 (2017).
51. Rao, R.C., Dedania, V.S. & Johnson, M.W. Stem Cells for Retinal Disease: A Perspective on the Promise and Perils. *Am J Ophthalmol* **179**, 32-38 (2017).
52. Finn, A.P., Grewal, D.S. & Vajzovic, L. Argus II retinal prosthesis system: a review of patient selection criteria, surgical considerations, and post-operative outcomes. *Clinical Ophthalmology* **12**, 1089-1097 (2018).
53. Bloch, E., Luo, Y. & da Cruz, L. Advances in retinal prosthesis systems. *Ther Adv Ophthalmol* **11**, 2515841418817501 (2019).
54. Porteus, M. Genome Editing: A New Approach to Human Therapeutics. *Annu Rev Pharmacol Toxicol* **56**, 163-90 (2016).
55. Eid, A. & Mahfouz, M.M. Genome editing: the road of CRISPR/Cas9 from bench to clinic. *Exp Mol Med* **48**, e265 (2016).
56. Jinek, M. et al. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **337**, 816-821 (2012).
57. Maeder, M.L. et al. Development of a gene-editing approach to restore vision loss in Leber congenital amaurosis type 10. *Nature Medicine* **25**, 229-+ (2019).

58. Duebel, J., Marazova, K. & Sahel, J.A. Optogenetics. *Curr Opin Ophthalmol* **26**, 226-32 (2015).
59. Busskamp, V. et al. Genetic reactivation of cone photoreceptors restores visual responses in retinitis pigmentosa. *Science* **329**, 413-7 (2010).
60. Lagali, P.S. et al. Light-activated channels targeted to ON bipolar cells restore visual function in retinal degeneration. *Nat Neurosci* **11**, 667-75 (2008).
61. Bi, A. et al. Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. *Neuron* **50**, 23-33 (2006).
62. Chaffiol, A. et al. A New Promoter Allows Optogenetic Vision Restoration with Enhanced Sensitivity in Macaque Retina. *Molecular Therapy* **25**, 2546-2560 (2017).
63. Scholl, H.P. et al. Emerging therapies for inherited retinal degeneration. *Sci Transl Med* **8**, 368rv6 (2016).
64. Petit, L., Khanna, H. & Punzo, C. Advances in Gene Therapy for Diseases of the Eye. *Hum Gene Ther* **27**, 563-79 (2016).
65. Tavernier, G. et al. mRNA as gene therapeutic: How to control protein expression. *Journal of Controlled Release* **150**, 238-247 (2011).
66. Zhong, Z.F. et al. mRNA therapeutics deliver a hopeful message. *Nano Today* **23**, 16-39 (2018).
67. Vandermeulen, G., Marie, C., Scherman, D. & Preat, V. New Generation of Plasmid Backbones Devoid of Antibiotic Resistance Marker for Gene Therapy Trials. *Molecular Therapy* **19**, 1942-1949 (2011).
68. van Adel, B.A., Arnold, J.M., Phipps, J., Doering, L.C. & Ball, A.K. Ciliary neurotrophic factor protects retinal ganglion cells from axotomy-induced apoptosis via modulation of retinal glia in vivo. *J Neurobiol* **63**, 215-34 (2005).
69. Marangoni, D. et al. Intravitreal Ciliary Neurotrophic Factor Transiently Improves Cone-Mediated Function in a CNGB3(-/-) Mouse Model of Achromatopsia. *Investigative Ophthalmology & Visual Science* **56**, 6810-6822 (2015).
70. Hellstrom, M., Pollett, M.A. & Harvey, A.R. Post-injury delivery of rAAV2-CNTF combined with short-term pharmacotherapy is neuroprotective and promotes extensive axonal regeneration after optic nerve trauma. *J Neurotrauma* **28**, 2475-83 (2011).
71. Gauthier, R., Joly, S., Pernet, V., Lachapelle, P. & Di Polo, A. Brain-derived neurotrophic factor gene delivery to muller glia preserves structure and function of light-damaged photoreceptors. *Invest Ophthalmol Vis Sci* **46**, 3383-92 (2005).
72. Chen, H. & Weber, A.J. BDNF enhances retinal ganglion cell survival in cats with optic nerve damage. *Invest Ophthalmol Vis Sci* **42**, 966-74 (2001).
73. Tria, M.A., Fusco, M., Vantini, G. & Mariot, R. Pharmacokinetics of nerve growth factor (NGF) following different routes of administration to adult rats. *Exp Neurol* **127**, 178-83 (1994).
74. Dittrich, F., Thoenen, H. & Sendtner, M. Ciliary neurotrophic factor: pharmacokinetics and acute-phase response in rat. *Ann Neurol* **35**, 151-63 (1994).
75. Ejstrup, R. et al. Pharmacokinetics of intravitreal glial cell line-derived neurotrophic factor: experimental studies in pigs. *Exp Eye Res* **91**, 890-5 (2010).

76. McGill, T.J. et al. Intraocular CNTF reduces vision in normal rats in a dose-dependent manner. *Invest Ophthalmol Vis Sci* **48**, 5756-66 (2007).
77. Schlichtenbrede, F.C. et al. Intraocular gene delivery of ciliary neurotrophic factor results in significant loss of retinal function in normal mice and in the Prph2Rd2/Rd2 model of retinal degeneration. *Gene Ther* **10**, 523-7 (2003).
78. Xue, W. et al. Ciliary Neurotrophic Factor Induces Genes Associated with Inflammation and Gliosis in the Retina: A Gene Profiling Study of Flow-Sorted, Muller Cells. *Plos One* **6** (2011).
79. Sahin, U., Kariko, K. & Tureci, O. mRNA-based therapeutics--developing a new class of drugs. *Nat Rev Drug Discov* **13**, 759-80 (2014).
80. Hajj, K.A. & Whitehead, K.A. Tools for translation: non-viral materials for therapeutic mRNA delivery. *Nature Reviews Materials* **2** (2017).
81. Zhang, X.H., Tee, L.Y., Wang, X.G., Huang, Q.S. & Yang, S.H. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Molecular Therapy-Nucleic Acids* **4** (2015).
82. Liang, X. et al. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol* **208**, 44-53 (2015).
83. Jiang, C. et al. A non-viral CRISPR/Cas9 delivery system for therapeutically targeting HBV DNA and pcsk9 in vivo. *Cell Res* **27**, 440-443 (2017).
84. Yin, H. et al. Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing. *Nat Biotechnol* **35**, 1179-1187 (2017).
85. Meng, Z. et al. A new developing class of gene delivery: messenger RNA-based therapeutics. *Biomater Sci* **5**, 2381-2392 (2017).
86. Lv, H., Zhang, S., Wang, B., Cui, S. & Yan, J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J Control Release* **114**, 100-9 (2006).
87. Walsh, C.L., Nguyen, J., Tiffany, M.R. & Szoka, F.C. Synthesis, characterization, and evaluation of ionizable lysine-based lipids for siRNA delivery. *Bioconjug Chem* **24**, 36-43 (2013).
88. Hassett, K.J. et al. Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines. *Mol Ther Nucleic Acids* **15**, 1-11 (2019).
89. Pardi, N. et al. Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. *J Control Release* **217**, 345-51 (2015).
90. Campbell, M. et al. An experimental platform for systemic drug delivery to the retina. *Proc Natl Acad Sci U S A* **106**, 17817-22 (2009).
91. Peynshaert, K., Devoldere, J., De Smedt, S.C. & Remaut, K. In vitro and ex vivo models to study drug delivery barriers in the posterior segment of the eye. *Adv Drug Deliv Rev* (2017).
92. Bachu, R.D., Chowdhury, P., Al-Saedi, Z.H.F., Karla, P.K. & Boddu, S.H.S. Ocular Drug Delivery Barriers-Role of Nanocarriers in the Treatment of Anterior Segment Ocular Diseases. *Pharmaceutics* **10** (2018).
93. Gamlin, P.D., Alexander, J.J., Boye, S.L., Witherspoon, C.D. & Boye, S.E. SubILM Injection of AAV for Gene Delivery to the Retina. *Methods Mol Biol* **1950**, 249-262 (2019).

94. Peynshaert, K. et al. Toward smart design of retinal drug carriers: a novel bovine retinal explant model to study the barrier role of the vitreoretinal interface. *Drug Deliv* **24**, 1384-1394 (2017).
95. Peynshaert, K., Devoldere, J., Minnaert, A.K., De Smedt, S.C. & Remaut, K. Morphology and Composition of the Inner Limiting Membrane: Species-Specific Variations and Relevance toward Drug Delivery Research. *Curr Eye Res*, 1-11 (2019).
96. Martens, T.F. et al. Measuring the intravitreal mobility of nanomedicines with single-particle tracking microscopy. *Nanomedicine (Lond)* **8**, 1955-68 (2013).

Summary and conclusions

In May 1961, two papers appeared in Nature, authored by Brenner, Crick and Meselson on the one hand, and Watson's team on the other, revealing the existence of an unstable, relatively simple molecule, carrying information from genes to ribosomes for protein synthesis. Although no Nobel Prize was awarded for its discovery because of to the large number of researchers involved, the identification of messenger RNA (mRNA) was of decisive importance for our understanding of gene function. Indeed, mRNA was the missing link in the molecular path from DNA to protein, the mediator in the central dogma of biology. Despite its importance for genetic translation, mRNA's significance for the therapeutic field was not immediately evident: mRNA is neither a permanent genetic solution, nor is it a functional end product. Nevertheless, almost 60 years later, it is clear that its transient nature renders mRNA suitable for a broader range of therapeutic applications than most other drug classes.

In **Chapter 1** we described the many advantages that made mRNA a central player in the field of cancer immunotherapy. Unfortunately, the main asset by which mRNA ushered a new era in immunotherapy, namely its inherent immunogenicity, is at the same time a serious drawback for non-immunotherapy-related applications, such as protein replacement and reprogramming strategies. For these applications, the intrinsic immune-stimulatory activity of mRNA directly interferes with the aimed therapeutic outcome, as it can drastically compromise the expression of the desired protein. Hence, tremendous efforts were invested in modifying structural elements of the mRNA molecule to reduce its immunogenicity, improve its intracellular stability and ameliorate its translational efficiency. In this chapter we presented an overview of the most important signalling pathways involved in the intracellular innate immune response following synthetic mRNA delivery and discussed some promising methods to silence this 'unwanted' response in case of non-immunological applications. Especially the realization that structural alterations to the mRNA backbone itself, by modifying the nucleobases could tremendously de-immunize synthetic mRNA has been pivotal for the revival of mRNA for therapeutic applications. Indeed, mRNA has been widely investigated in the field of regenerative medicine for the reprogramming of cell fate. Yet, its use for upregulation of proteins or gene editing strategies is merely at the beginning of development.

Also in the ocular field, it is striking how mRNA-based therapy is still in its infancy: so far no research group looked into the possibility of using mRNA for the treatment of retinal

disorders. Therefore the aim of this dissertation was to make use of the intriguing advantages of mRNA and discover its potential for protein expression in the retina. Hence, we provided a general introduction of the anatomy of the retina and highlighted the reasons for its attractive position as target tissue in **Chapter 2**. Considering our interest in the use of mRNA for the production of growth factors or regenerative applications, we furthermore focused on the Müller cells as a target cell type, as they are naturally involved in neuroprotection and retinal regeneration. We emphasized the importance of Müller cells in maintaining a healthy and functioning retina and discussed their ambivalent role in various pathological events. A proper understanding of their protective *vs.* detrimental mechanisms and how to modulate these to our advantage, will be essential for the development of efficient therapeutic strategies. Indeed, stimulating their neuron-supportive effects, while preventing their destructive functions lies at the base of strategies aiming to protect retinal neurons or to stimulate retinal self-repair.

In **Chapter 3** we report on a first delivery tool which we tested for mRNA transfection of retinal cells, namely the cationic polymer/lipid formulation *Trans*IT™. Using this carrier, we confirmed our hypothesis that mRNA has a higher transfection efficiency compared to pDNA in the post-mitotic retinal Müller cells, as mRNA avoids the necessity for nuclear entry. Furthermore, we investigated the potential of this carrier to deliver mRNA *via* intravitreal (IVTR) injections, a widely used and relative safe method for retinal drug delivery. To this end, we explored the mobility of the mRNA particles in the vitreous humor by means of an *ex vivo* model that contains intact bovine vitreous. Analysis on a single-particle level revealed that a large fraction of the positively charged complexes was completely withheld in the vitreal network, mainly due to electrostatic interactions with the vitreal components. Also when the vitreal network was mechanically broken up, particle association with free collagen remnants and vitreal proteins seriously hampered cellular uptake and transfection. To shield the cationic surface from interactions with the vitreal constitutes, we subsequently investigated the use of hyaluronic acid (HA) to electrostatically coat the *Trans*IT-mRNA complexes. Unlike the well-established polyethylene glycol (PEG)-coating, HA is known to bind the CD44 receptor, present on the surface of many retinal cell types, including Müller cells, and is therefore reported not to reduce cellular uptake in these cell types. In this study we demonstrated that electrostatic HA-coating of the cationic *Trans*IT particles was achievable without compromising the mRNA complexation efficiency. HA-coated particles were negatively charged and their size remained within the same range. Furthermore, HA-coating markedly enhanced IVTR mobility of the particles and even slightly increased transfection efficiency in Müller cells in the presence of vitreous. Nonetheless, the obtained protein expression levels were too low for the evaluation of this delivery tool in an *in vivo* setting.

In order to increase the efficiency of our mRNA-based delivery, we investigated the use of a second transfection reagent which was completely lipid-based, namely Lipofectamine™MessengerMAX. As our previous results pointed out that the physicochemical properties are of vital importance for the migration of particles through the vitreous, their negative surface charge after mRNA complexation favored MessengerMAX particles for IVTR delivery. Indeed, in **Chapter 4** we demonstrated that neither the mobility, nor the transfection efficiency of MessengerMAX-mRNA complexes was markedly reduced in the presence of bovine vitreous. However, following IVTR administration in the eyes of mice, mRNA presence was restricted to the upper layer of the inner retina and only very limited mRNA expression was observed after 7 days. Therefore, we next investigated how the particles behaved in the vitreoretinal (VR) interface (the region where the vitreous passes into the retina) using a newly developed bovine explant with an intact VR. Using this model, it was clear that the particles were trapped at the vitreal side due to the presence of the inner limiting membrane (ILM), which completely blocked penetration of the particles into the retina. When applying the MessengerMAX-complexes on an explant of which the vitreous was removed, also resulting in a compromised ILM, mRNA uptake was apparent in the deeper layers of the inner retina and the retinal mRNA expression levels drastically increased. This study highlighted the crucial barrier function of the ILM for IVTR drug delivery and demonstrated that the results obtained in small laboratory animals following IVTR injection are not representative for the situation in larger species. In addition to this IVTR injection, we evaluated the delivery of MessengerMAX-mRNA particles for subretinal (SR) injection, a frequently used injection route when delivery to the outer retina is desired. Following SR injection *in vivo*, mRNA-induced eGFP expression was observed after 24 h which continued to increase after longer incubation times. Remarkably, mRNA uptake was often restricted to the site of injection, suggesting that the retina itself is a hard-to-cross barrier, impeding broad mRNA distribution. Taken together, the results in this chapter show that mRNA has substantial potential to induce the expression of proteins, such growth factors, in the retina. However, the development of smaller particles which retain their capacity to efficiently complex mRNA is expected to favor both the migration through the ILM following IVTR injection as well as its diffusion through the different retinal layers following SR injection. This will therefore be an essential next step for the further development of mRNA-based retinal medicines towards clinical application.

As described in **Chapter 1**, considerable efforts have been made to unriddle mRNA recognition pathways and limit the immune-stimulatory activity of synthetic mRNA. Besides the well-known modifications that can be made to the mRNA molecule itself, as evaluated in **Chapter 4**, a number of potential innate immune-inhibitors have been identified and are currently under investigation. **Chapter 5** contains preliminary data on the evaluation of some

of these immune-inhibitors (*i.e.* CLQ, Pepinh-TRIF, Pepinh-MYD, B18R and 2-AP). In this chapter another important retinal cell type was investigated, namely retinal pigment epithelial (RPE) cells, which are a potential target following SR injection. Although all of these small molecules were able to quell the IFN-β response following synthetic mRNA delivery, only B18R was able to enhance *in vitro* mRNA transfection efficiency. Pretreatment with the other inhibitors unexpectedly reduced the amount of eGFP-expressing RPE cells. These results indicate that the type I IFN response is not always a good predictor for the mRNA translation capacity. In fact, it could be possible that a complete blockage of the innate immune response might not be favorable for the translation efficiency in specific cell types, such as RPE cells: perhaps an optimal balance exists between the intracellular innate immunity and the mRNA translation. When comparing the use of small molecules with the current state-of-the art mRNA de-immunizing technique, namely the incorporation of modifying nucleotides, it was clear that the latter approach had the most potential to further develop mRNA therapeutics for ocular therapy. The use of small molecules might interfere with other regulatory aspects of the cell as well and should therefore be investigated with caution.

In **Chapter 7** we provided a critical view on the future of ocular gene therapy and introduced the question whether the field will fulfill its alleged potential. Indeed, other cutting-edge therapies, including stem cell-based strategies and retinal prosthesis, are emerging and might soon present a highly promising alternative, especially in situations where the retinal degeneration is already progressed too far to be amenable for protein addition therapy. Nevertheless, new gene-based therapies are being developed which could drastically extend the therapeutic spectrum of ocular gene therapy, such as neuroprotection strategies and retinal regeneration as discussed in **Chapter 2**. In these strategies, the short-term expression levels obtained by mRNA might be very attractive for translation into practice. The most critical challenge in retinal mRNA-based therapy will without any doubt be its delivery to its target, as demonstrated throughout this dissertation. With the use of small, safe and effective delivery materials, however, mRNA has the potential revolutionize non-viral ocular gene therapy.

Samenvatting en conclusies

In mei 1961 verschenen twee artikels in het wetenschappelijk tijdschrift *Nature*, geschreven door Brenner, Crick en Meselson enerzijds en de onderzoeksgroep van Watson anderzijds, die het bestaan onthulden van een onstabiele, relatief eenvoudige molecule, die een centrale rol speelt in het vertalen van genetische informatie naar eiwitten. Hoewel er geen Nobelprijs werd toegekend voor de ontdekking van messenger-RNA (mRNA), vanwege het grote aantal betrokken onderzoekers, was de identificatie ervan van doorslaggevend belang voor ons inzicht in het genexpressieproces. Sterker nog, mRNA bleek de ontbrekende schakel te zijn in het moleculaire pad van DNA naar eiwitten, de bemiddelaar in het centrale dogma van de biologie. Ondanks het belang voor genetische translatie, was de betekenis van mRNA voor het therapeutische veld niet meteen duidelijk: mRNA is geen permanente genetische oplossing en het is ook geen functioneel eindproduct. Toch is het, bijna 60 jaar later, duidelijk dat mRNA door zijn tijdelijke werkzaamheid geschikt is voor een breder scala aan therapeutische toepassingen dan de meeste andere geneesmiddelenklassen.

In **Hoofdstuk 1** beschreven we de vele voordelen die van mRNA een belangrijke speler maken in de kankerimmuuntherapie. Helaas is de belangrijkste troef van mRNA in immuuntherapie, namelijk zijn vermogen om een immuunrespons op te wekken, tegelijkertijd een groot nadeel voor toepassingen buiten de immuuntherapie, zoals het toevoegen van een ‘gezond’ gen of het herprogrammeren van cellen tot stamcellen. Voor deze toepassingen heeft de intrinsieke immuunstimulerende werking van mRNA een directe invloed op het beoogde therapeutische resultaat, omdat het de expressie van het gewenste eiwit drastisch in gevaar kan brengen. Daarom werden enorme inspanningen geleverd om de structurele elementen van de mRNA- molecule aan te passen en aldus de immunogeniciteit te verminderen, de intracellulaire stabiliteit te verbeteren en de translatie efficiëntie te verhogen. In dit hoofdstuk gaven we een overzicht van de belangrijkste signaalwegen betrokken bij de intracellulaire aangeboren immuunrespons na de toediening van synthetisch mRNA en bespraken we enkele veelbelovende methoden om deze 'ongewenste' respons te onderdrukken bij niet-immunologische toepassingen. Vooral het besef dat structurele veranderingen aan de mRNA molecule zelf, namelijk het implementeren van gemodificeerde basen, het synthetische mRNA enorm zouden kunnen de-immuniseren, is cruciaal geweest voor de heropleving van mRNA voor therapeutische toepassingen. Dit leidde tot uitgebreid onderzoek naar het toepassen van mRNA in de regeneratieve geneeskunde. Toch staat het gebruik van mRNA voor het aanmaken van

‘gezonde’ eiwitten of voor het gericht bewerken van genetische aandoeningen nog maar aan het begin van de ontwikkeling.

Ook in het oculaire veld is het opvallend hoe mRNA-gebaseerde therapie nog in de kinderschoenen staat: tot nu toe heeft geen enkele onderzoeksgroep zich gebogen over de mogelijkheid om mRNA te gebruiken voor de behandeling van netvliesaandoeningen. Het doel van dit proefschrift was dan ook om gebruik te maken van de intrigerende voordelen van mRNA en de mogelijkheden voor eiwitexpressie in het netvlies te ontdekken. Daarom gaven we in **Hoofdstuk 2** een algemene inleiding over de anatomie van het netvlies en bespraken we waarom het netvlies een aantrekkelijk doelweefsel vormt. Gezien onze interesse in het gebruik van mRNA voor de productie van groeifactoren of regeneratieve toepassingen, hebben we ons verder gefocust op de Müllercellen als doelceltype. Müllercellen zijn namelijk van nature betrokken bij processen zoals neuroprotectie en regeneratie van het netvlies. We benadrukten het belang van Müllercellen in het behoud van een gezond en functionerend netvlies en bespraken hun ambivalente rol in verschillende pathologische gebeurtenissen. Inzicht in zowel hun beschermende als schadelijke mechanismen en hoe deze in ons voordeel te moduleren, zal essentieel zijn voor de ontwikkeling van efficiënte therapeutische strategieën. Het stimuleren van hun neuron-ondersteunende effecten en het voorkomen van hun destructieve functies ligt aan de basis van nieuwe strategieën gericht op het beschermen van retinale neuronen of het stimuleren van een zelfherstellend netvlies.

Hoofdstuk 3 beschrijft het eerste partikel dat we getest hebben voor de aflevering van mRNA aan de netvliescellen, namelijk de positief geladen polymeer/lipide formulering *Trans*IT™. Met behulp van deze vector bevestigden we onze hypothese dat mRNA beter presteert in het transfecteren van de niet-delende Müllercellen dan pDNA, doordat mRNA in tegensteling tot DNA, niet genoodzaakt is zich te verplaatsen naar de nucleus. Verder onderzochten we het potentieel van dit partikel om mRNA af te leveren via intravitreale (IVTR) injecties, een veelgebruikte en relatief veilige methode voor de toediening van geneesmiddelen aan het netvlies. Hiertoe hebben we de mobiliteit van de mRNA-bevattende partikels onderzocht in het vitreum van een *ex vivo* model gebaseerd op een runderoog. Analyse op niveau van het partikel toonde aan dat een groot deel van de positief geladen complexen volledig weerhouden werd in het vitreale netwerk, voornamelijk als gevolg van elektrostatische interacties met de componenten van het vitreum. Ook wanneer het vitreale netwerk eerst mechanisch werd opgebroken, werd de cellulaire opname en transfectie belemmerd door het binden van de partikels met vrije collageenresten en eiwitten. Om het positief geladen oppervlak af te schermen van interacties in het vitreum, onderzochten we het gebruik van hyaluronzuur (HA) om de *Trans*IT-mRNA-complexen elektrostatisch te

coaten. In tegenstelling tot de polyethyleenglycol (PEG)-coating, zal HA de opname in de cellen van het netvlies niet belemmeren, aangezien HA bindt aan de CD44-receptor die aanwezig is op het oppervlak van veel netvliescellen, waaronder ook de Müllercellen. In deze studie toonden we aan dat het mogelijk was de positief geladen *Trans*IT-partikels te coaten zonder afbreuk te doen aan de mRNA-complexatie-efficiëntie. Na HA-coating kregen de partikels een negatieve lading, maar behielden hun grootte. Bovendien verbeterde de coating aanzienlijk de mobiliteit van de deeltjes in het vitreum en werd ook de transfectie-efficiëntie in aanwezigheid van het vitreum licht verhoogd. Desalniettemin waren de verkregen eiwitexpressieniveaus te laag voor de evaluatie van dit partikel in een *in vivo* setting.

Om de efficiëntie van de mRNA-aflevering te verhogen, onderzochten we het gebruik van een tweede transfectiereagens, dat volledig op lipiden gebaseerd is, namelijk Lipofectamine^{TM}MessengerMAX. Zoals onze vorige resultaten aantoonden, zijn de fysico-chemische eigenschappen van vitaal belang voor de migratie van deeltjes door het vitreum. De negatieve oppervlaktelading van de MessengerMAX-partikels na binding van het mRNA vormt dan ook een groot voordeel voor IVTR toediening. In **Hoofdstuk 4** toonden we aan dat noch de mobiliteit, noch de transfectie-efficiëntie van de MessengerMAX-mRNA-complexen sterk verminderd werd door de aanwezigheid van rundervitreum. Wanneer we vervolgens het mRNA toedienden in het vitreum van muizen, zagen we dat het mRNA enkel aanwezig was in de binnenste lagen van het netvlies en werd na 7 dagen slechts een zeer beperkte expressie waargenomen. Daarom onderzochten we hoe de partikels zich gedroegen in de vitreoretinale (VR) interface (*i.e.*de overgang van het vitreum naar het netvlies) met behulp van een nieuw ontwikkelde runderexplant met een intacte VR. Met behulp van dit model zagen we dat de partikels bleven steken aan de vitreale zijde door de aanwezigheid van de 'inner limiting membrane' (ILM), die de migratie van de partikels naar het netvlies volledig blokkeerde. We ontdekten bovendien dat het verwijderen van het vitreum, waardoor de ILM beschadigd wordt, ervoor zorgde dat het mRNA doordrong tot in de diepere lagen van het netvlies en de eiwitexpressie drastisch verhoogde. Deze studie benadrukte de cruciale barrièrefunctie van het ILM na IVTR toediening van geneesmiddelen en toonde aan dat de resultaten die verkregen worden in kleine laboratoriumdieren niet altijd representatief zijn voor de situatie bij grotere diersoorten. Wanneer afgifte aan het buitenste netvlies gewenst is, is subretinale (SR) injectie de meest gebruikte toedieningstechniek. Na SR injectie *in vivo*, werd na 24 uur eGFP-expressie waargenomen, die nog verder toenam na langere incubatietijden. Opmerkelijk is dat de opname van mRNA vaak beperkt bleef tot de plaats van de injectie, wat doet vermoeden dat het netvlies zelf een moeilijk te doorbreken barrière is, die een brede verspreiding van mRNA belemmert. Uit de resultaten in dit hoofdstuk blijkt dat mRNA veelbelovend is voor de toediening van eiwitten, zoals

groeifactoren, aan het netvlies. Het zal echter nodig zijn om kleinere partikels te ontwikkelen die toch hun capaciteit behouden om mRNA te binden, zodat zowel de migratie door het ILM na IVTR injectie als de diffusie door de verschillende netvlieslagen na SR injectie bevorderd wordt. Dit zal daarom een noodzakelijke volgende stap zijn voor de verdere ontwikkeling van mRNA-gebaseerde geneesmiddelen voor het netvlies.

Zoals beschreven in **Hoofdstuk 1**, werden aanzienlijke inspanningen geleverd om de mRNA-herkenningsroutes te ontrafelen en de immuunstimulerende activiteit van synthetisch mRNA te beperken. Naast de gekende modificaties die kunnen aangebracht worden aan de mRNA molecule zelf, zoals geëvalueerd werd in **Hoofdstuk 4**, werden een aantal potentiële inhibitoren van de aangeboren immuunrespons geïdentificeerd, die momenteel onderzocht worden voor hun gebruik in mRNA-afgifte. **Hoofdstuk 5** bevat de voorlopige resultaten van de evaluatie van enkele immuuninhibitoren (CLQ, Pepinh-TRIF, Pepinh-MYD, B18R en 2-AP). In dit hoofdstuk werd een ander belangrijk celtype van het netvlies onderzocht, namelijk de cellen van het retinaal pigment epitheel (de RPE-cellen), aangezien deze een potentieel doelwit zijn na SR injectie. Hoewel al deze moleculen in staat waren om de IFN-β-respons na synthetische mRNA-aflevering te onderdrukken, kon alleen B18R de *in vitro* mRNA-transfectie-efficiëntie verhogen. Behandeling met de andere inhibitoren resulteerde zelfs in een vermindering van het aantal cellen die eGFP tot expressie brachten. Deze resultaten tonen aan dat de type I IFN-respons geen goede voorspeller is van de mRNA-translatiecapaciteit. Het is zelfs mogelijk dat een volledige blokkering van de aangeboren immuunrespons eigenlijk niet gunstig is voor de translatie-efficiëntie. Mogelijks bestaat er een optimale balans tussen de intracellulaire aangeboren immuniteit en de mRNA-translatie. Wanneer we het gebruik van deze inhibitoren vergeleken met de huidig gebruikte techniek om synthetisch mRNA te de-immuniseren, namelijk de integratie van modificerende nucleotiden, werd duidelijk dat deze standaardtechniek het meeste potentieel had voor de verdere ontwikkeling van mRNA voor oculaire therapie. Moleculen die het immuunsysteem inhiberen kunnen namelijk ook interfereren met andere belangrijke aspecten van de cel en moeten daarom met de nodige voorzichtigheid verder worden onderzocht.

In **Hoofdstuk 6** gaven we een kritische blik op de toekomst van oculaire gentherapie en stelden we de vraag of het veld zijn vermeende potentieel zal vervullen. Andere geavanceerde therapieën, waaronder stamcel-gebaseerde strategieën en netvliesprotheses, zijn in opkomst en kunnen binnenkort een veelbelovend alternatief vormen, vooral in situaties waarin de degeneratie van het netvlies al te ver gevorderd is om in aanmerking te komen voor gentherapie. Desalniettemin worden er nieuwe therapieën ontwikkeld die het therapeutische spectrum van oculaire gentherapie drastisch zouden kunnen uitbreiden, zoals neuroprotectieve strategieën en regeneratie van het netvlies

(besproken in **Hoofdstuk 2**). In deze strategieën kan de kortetermijnwerking van mRNA een zeer aantrekkelijk voordeel bieden. De meest kritische uitdaging voor mRNA-gebaseerde therapie ligt onbetwistbaar in de aflevering van het mRNA naar het netvlies, zoals in dit proefschrift werd aangetoond. Wanneer kleine, veilige en effectieve toedieningsmaterialen ontwikkeld worden, heeft mRNA het potentieel om niet-virale oculaire gentherapie te revolutioneren.

Curriculum Vitae

PERSONALIA

Name	Devoldere
First names	Joke Anne Erika
Nationality	Belgian
Place of birth	Ghent
Date of birth	05/10/1990
Private address	Sint-Jansvest 4 9000 Gent
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Professional address	Laboratory for General Biochemistry and Physical Pharmacy, Faculty of pharmaceutical sciences, Ghent university Ottergemsesteenweg 460 9000 Gent Belgium
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Websites	https://www.biofys.ugent.be https://www.researchgate.net/profile/Joke_Devoldere

DEGREES

June 2013	Ghent University, Ghent, Belgium Master of Science in Farmaceutical Care Magna cum laude **Master thesis**: "*Optimization of ultrasound induced transfection and elucidating the sonoporation mechanism with confocal microscopy*" under supervision of Dr. apr. Ine De Cock and the promotorship of Prof.dr.apr. Katrien Remaut of the Laboratory for general biochemistry and physical pharmacy, Ghent University, Belgium
June 2011	Ghent University, Ghent, Belgium Bachelor in Pharmaceutical Sciences Magna cum laude
June 2008	St. Jans College, Sint-Amandsberg, Belgium High school degree (Latin Mathematics) Magna cum laude

LANGUAGES

Dutch:	Native language
English:	Full professional proficiency
French:	Limited proficiency
German:	Elementary proficiency

INTERNATIONAL PEER-REVIEWED A1 PUBLICATIONS

Published

Joke Devoldere, Heleen Dewitte, Stefaan C. De Smedt, Katrien Remaut, "Evading innate immunity in non-viral mRNA delivery: don't shoot the messenger", *Drug Discovery Today* 2016, 21(1), 11-25 (IF 2016 = 6.4)

Karen Peynshaert, Joke Devoldere, Valérie Forster, Serge Picaud, Christian Vanhove, Stefaan C. De Smedt, Katrien Remaut, "Toward smart design of retinal drug carriers: a new bovine retinal explant model to study the barrier role of the vitreoretinal interface", *Drug Delivery* 2017, 24(1), 1384-1394 (IF 2017 = 6.4)

Karen Peynshaert, Joke Devoldere, Stefaan C. De Smedt, Katrien Remaut, "In vitro and ex vivo models to study drug delivery barriers in the posterior segment of the eye", *Advanced Drug Delivery Reviews* 2018, 126, 44-57 (IF 2018 = 13.6)

Laura Vanden Daele, Charlotte Boydens, Joke Devoldere, Katrien Remaut, Johan Van de Voorde. "Search for the source of the retinal relaxing factor" Current Eye Research 2018, 43(11), 1383-1388 (IF 2018 = 2.1)

Karen Peynshaert, Joke Devoldere, An-Katrien Minnaert, Stefaan C. De Smedt, Katrien Remaut, "Morphology and Composition of the Inner Limiting Membrane: Species-Specific Variations and Relevance toward Drug Delivery Research", Current Eye Research 2019, 1-11 (IF 2018 = 2.1)

Joke Devoldere, Karen Peynshaert, Stefaan C. De Smedt, Katrien Remaut, "Muller cells as a target for retinal therapy", *Drug Discovery Today* 2019 (*In Press, published online)* (IF 2018 = 6.8)

Submitted

Joke Devoldere, Karen Peynshaert, Heleen Dewitte, Christian Vanhove, Lies De Groef, Lieve Moons, Sinem Yilmaz Özcan, Deniz Dalkara, Stefaan C. De Smedt, Katrien Remaut, "Non-viral delivery of chemically modified mRNA to the retina: subretinal versus intravitreal administration" *Journal of Controlled Release*

Joke Devoldere, Mike Wels, Karen Peynshaert, Heleen Dewitte, Stefaan C. De Smedt, Katrien Remaut, "The obstacle course to the inner retina: hyaluronic acid-coated lipoplexes cross the vitreous but fail to overcome the inner limiting membrane" *European Journal of Pharmaceutics and Biopharmaceutics*

National & International Conferences with oral presentation

May 28th – 29th 2015	18th Forum of Pharmaceutical Sciences, Blankenberge, Belgium *"Efficiency of mRNA translation compromised by co-delivery with other nucleic acids"*
October 5th-8th 2016	European Association for Vision and Eye Research (EVER) Annual Meeting 2016, Nice, France *"Therapeutic potential of non-viral mRNA delivery to Müller cells for neuroprotection"*
October 10th 2016	European Association for Vision and Eye Research (EVER) Annual Meeting 2016, Nice, France *"Intravitreal injection of mRNA containing nanoparticles to induce sustained expression of neurotrophic factors in Müller cells"*
November 24th 2016	Meeting of the Belgian-Dutch Biopharmaceutical Society, Utrecht, The Netherlands *"mRNA to induce sustained expression of neurotrophic factors in the retina"*
September 19th-20th 2017	F-tales meeting on Nanomaterials in Biomedical Sciences, Ghent, Belgium *"Non-viral delivery of chemically modified mRNA as ocular neuroprotection therapy"* **Awarded for best oral scientific presentation**

National & International Conferences with poster presentation

December 12 2014	Biopharmacy day, Vlaardingen, Netherlands *"Understanding the protein expression kinetics upon non-viral co-delivery of different kinds of nucleic acids"*
July 26th-29th 2015	42nd CRS Annual Meeting & Exposition, Edinburgh, Scotland *"Co-delivery of different kinds of nucleic acids affects the efficiency of mRNA translation"*
November 11th-12th 2015	3rd International mRNA Health Conference, Berlin, Germany *"Therapeutic potential of non-viral mRNA delivery to Müller cells"* **Awarded with "best poster" prize**
November 23th 2015	Biopharmacy day, Leuven, Belgium *"Non-viral mRNA delivery to Müller cells for neuroprotection"*
April 26th 2016	6de Studenten Onderzoek Symposium, Ghent, Belgium *"Therapeutic potential of non-viral mRNA delivery to Müller cells"* **Awarded with "best poster" prize**

May 1st-5th 2016	ARVO 2016 Annual Meeting, Seattle, Washington *"Intravitreal delivery of chemically modified mRNA for neuroprotection through Müller cell transfection"*
March 17th-18th 2017	The annual Dutch Ophthalmology PhD Students (DOPS), Nijmegen, The Netherlands *"mRNA to induce sustained expression of neurotrophic factors in the Retina"*
April 20th 2017	Research Day - Student Research Symposium, Ghent, Belgium *"mRNA to induce sustained expression of neurotrophic factors in the retina"*
September 19th-20th 2017	F-tales meeting on Nanomaterials in Biomedical Sciences, Ghent, Belgium *"Non-viral delivery of chemically modified mRNA as ocular neuroprotection therapy""*
November 1st-2nd 2017	5th International mRNA Health Conference, Berlin, Germany *"Chemically stabilized mRNA as ocular neuroprotection therapy"*
November 22th-24th 2017	Opthalmologica Beglica OB 2017, Brussels, Belgium *"Intravitreal injection of mRNA containing nanoparticles to introduce sustained expression of neurotrophic factors in Müller cells."*

AWARDS AND GRANTS

October 2014 – September 2016	FWO 2 year research grant
November 2015	"Best poster" award 3rd International mRNA Health Conference, Berlin, Germany
April 2016	"Best poster" award 6th Student Research Symposium, Ghent, Belgium
December 2016	Funds for Research in Ophthalmology (FRO) research grant of €10.000
October 2016 – September 2018	FWO 2 year research grant
September 2017	Award for "best oral scientific presentation" at F-tales meeting on Nanomaterials in Biomedical Sciences, Ghent, Belgium

INTERNATIONAL RESEARCH EXPERIENCE

January 30th–31th 2017	Short visit to Paris to perform a pilot *in vivo* experiment in the vision institute "l'Institute de la vision" (Paris, France) – Intravitreal injection of naked mRNA and messengerMAX/mRNA lipoplexes
November 22th–23th 2017	Visit to Paris for bigger *in vivo* experiment in the vision institute "l'Institute de la vision" (Paris, France) – Subretinal injection of naked mRNA and messengerMAX/mRNA lipoplexes

SCIENTIFIC COMMUNICATION

August 10th 2017	Utopia: een proeftuin met kunst en wetenschap voor kinderen, Ghent, Belgium *Participation in science project organized by Ghent University in which children all over the city get to opportunity to get in touch with art and science. I explained my research in an accessible and enthusiastic way by means of scientific ocular experiments in which the children could participate.*

TEACHING ACTIVITIES

2013-2015	Tutor and lab instructor for the Pharmaceutical Bachelor Thesis (FaBaP).
2015-2017	3 years of teaching assistant for the practical courses on the subject of Biochemistry and Physical Pharmacy (Course of Prof. Katrien Remaut and Prof. Stefaan De Smedt)
2014-2018	Supervisor of 5 student's during their Master/Bachelor thesis: Steffie Van Schelvergem, Master dissertation (Master of Industrial Pharmacy, Ghent University). Understanding the protein expression kinetics upon non-viral co-delivery of different kinds of nucleic acids. (2014-2015) Ruth O'Beirne, Bachelor dissertation (Biomolecular Sciences, Dublin Institute of Technology). Therapeutic Potential of non-viral mRNA delivery to Müller cells for neuroprotection.(2015-2016) Nanine Keirse, Master dissertation (Pharmaceutical Sciences – Pharmaceutical Care, Ghent University). Onderzoek naar het

	potentieel van gemodificeerd mRNA in Müller cellen als mogelijke oculaire neuroprotectie therapie. (2016-2017)
	Mike Wels, Master dissertation (Pharmaceutical Sciences – Drug Innovation, Utrecht University). Intravitreal delivery of mRNA targeted towards Müller cells using lipid-based nanoparticles. (2016-2017)
	Lauren Vanderherten, Master Dissertation (Pharmaceutical Sciences – Pharmaceutical Care, Ghent University). Inhibitie van de intracellulaire immuunrespons ter optimalisatie van mRNA-gebaseerde neuroprotective voor retinitis pigmentosa.(2017-2018)
2016-2018	Supervision of 2 students of the Honours Programme in Life Sciences
	Sigrid Deprez, Honour Programme (Pharmaceutical Sciences, Ghent University). Therapeutic potential of non-viral mRNA delivery to Müller cells. (2016-2017)
	Marthe Vandeputte, Honour Programme (Pharmaceutical Sciences, Ghent University). Co-delivery of nucleic acids to MIO-M1 cells: is less really more? (2017-2018)
2014-2018	Tutor Problem-based learning 2nd Bachelor - Unraveling the biochemistry of DNA - Case: Leber Congenital Amaurosis

COURSES

Communication

2013	Practical English 5 – B2 (UCT, Ghent)
2013	Medical and Scientific writing (Doctoral Schools, Ghent)
2014	Advanced Academic English: Writing Skills (Doctoral Schools, Ghent)
2016	Communication skills (True Colours)

Reseach and valorization

2014	FLAMES Summer School in Methodology and Statistics
2015	Speed-Reading (Doctoral Schools, Ghent)

Science

2017	Nanomaterials in Biomedical Sciences (Ghent)
2016	Laboratory Animal Science I & II (by Prof. Katleen Hermans) at Ghent University

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Katrien, hoe kan ik de vijf fantastische jaren met jou hier samenvatten? Dat wij geen enkel probleem gingen hebben om overeen te komen, was snel duidelijk toen je mij in één van de eerste weken het reilen en zeilen van de flow cytometrie uitlegde met tussen het wachten door een leuke sing-along sessie achter de computer. Ik zie ons daar nog steeds zitten luidkeels meezingend met Milky Chance, terwijl we probeerden te achterhalen welke de beste settings voor mijn experimenten waren. Al gauw bleek het financiële prijskaartje van het eerste project enorm hoog en constanteerden we al vrij snel dat het moeilijk concurreren werd met de grote bedrijven. Hierdoor werd mijn eerste jaar vooral een zoektocht naar het juiste project om mee verder te gaan. Over de combinatiestrategieën met verschillende nucleïnezuren hadden we niet steeds dezelfde mening, maar onze gemeenschappelijke passie voor mRNA leidde wel tot een heel mooie review die de basis vormde voor mijn verdere werk. Samen met Karen rolde ik mee de oculaire wereld in en het duurde niet lang voor we onze naam maakten als ‘oculadies’ in de oogwereld. Met jou op

congres gaan was altijd een geweldige ervaring: samen chocomelk drinken op de kerstmarkt in Keulen, vele gezellige uren op de trein naar Düsseldorf en Parijs, jouw verjaardag vieren met cocktails in Berlijn, sushi op ons rooftop terras in Seattle, samen gaan shoppen in Nice, af en toe (en vooral door mijn toedoen) moeten lopen om ons vliegtuig nog te halen, en last but not least een fantastische meidengroep vormen inclusief opblaas gitaar én daardoor de voorpagina van de ARVO nieuwsbrief halen door 'toch eens een kijkje te gaan nemen' op de karaoké avond. Ook al was het vaak wat zoeken op wetenschappelijk vlak, met af en toe wat gebrek aan planning en heel wat tegenslagen met de *in vivo* experimenten gedurende mijn laatste jaar, heeft jouw aanwezigheid en motivatie op persoonlijk vlak mij doorheen heel wat moeilijke periodes geholpen. Katrien, bedankt voor de enorme steun die je mij gaf het afgelopen jaar, jouw blijvende vertrouwen in mijn capaciteiten, hulp en geruststelling wanneer ik het even niet meer zag zitten. Ik kijk met trots terug op wat we samen bereikt hebben en hoop dat ons onderzoek het begin kan zijn van een mooi verhaal.

Stefaan, zoals je waarschijnlijk al meermaals gehoord hebt, zijn jouw lessen een grote bron van inspiratie voor vele studenten. Ook ik was na een semester boeiende woorden over de fysicochemische principes van geneesmiddelen en het kritisch nadenken over hoe bepaalde zaken tot stand komen, volledig overtuigd stage in jouw labo te lopen. Het voltooien van mijn masterproef in deze briljante omgeving nam dan ook mijn twijfel over 'wat nadien' volledig weg, waardoor ik met vol enthousiasme aan een doctoraat onder jouw begeleiding begon. Diezelfde gepassioneerde manier van praten en filosoferen hanteer je ook in het begeleiden van jouw doctoraatsstudenten. Elke meeting met jou gaf mij weer een enorme boost en het zien van 'the bigger picture' was soms exact wat ik nodig had als ik mezelf even aan het verliezen was in de details van mijn onderzoek. Ook op persoonlijk vlak hebben we beiden geen eenvoudig jaar achter de rug en de vraag hoe het met mij en mijn familie ging, gevolgd door een kleine babbel, deed mij telkens enorm veel deugd. Het vertrouwen dat je toonde in mij, wanneer het publiceren wat stroef verliep, hielp mij ook in mezelf en in het verloop van mijn doctoraat geloven. Bedankt ook voor de vele kansen die ik kreeg om op congres te gaan in het buitenland en voor de prachtige reis naar Porto, waar we allen ten volle van genoten hebben.

Jo, wij kennen elkaar al iets langer dan mijn eerste stap in het labo ☺. Helaas, hebben zowel mijn opa als mijn tante dit niet meer meegemaakt. Ze zouden echter beiden heel blij geweest zijn dat ik mijn doctoraat in jouw labo mocht starten en trots geweest zijn op wat we samen verwezenlijkt hebben. Mijn opa zei mij altijd: "leren doe je voor gans je leven" en jij was daar het perfecte bewijs van. Na al die jaren die ongetwijfeld met vele veranderingen gepaard gingen, bleef jij telkens op de hoogte van wat er zich in het labo afspeelde en bood

je nog steeds jouw ervaring aan waar nodig. Bedankt, om mij de kans te geven dit prachtige avontuur te starten.

Kevin, ik heb enorm veel bewondering en respect voor de manier waarop jij mee de werking in het labo helpt realiseren. Jouw steeds kritische en oprecht geïnteresseerde vragen bij mijn presentaties gaven me vaak nieuwe ideeën en extra vertrouwen. Bedankt voor de goede zorgen en koffiekoeken die we kregen wanneer we voor jou examentoezicht deden. Dit maakte het vroege opstaan alleszins een heel stuk aangenamer ☺. Net zoals Stefaan, wil ik ook jou bedanken voor de prachtige reis naar Porto, waar we dankzij jullie de kans toe kregen. Je bent een indrukwekkende begeleider en ik denk dat iedereen die bij jou in het team zit zich heel gelukkig mag prijzen voor wat je samen met hen bereikt. Ik ben ervan overtuigd dat het photoporatie-team het nog heel ver zal schoppen.

Koen, ook al hebben we nooit rechtstreeks met elkaar samengewerkt, jouw deur stond altijd open voor een vraag of een wetenschappelijke babbel. Ik heb enorm veel bewondering voor de passie die jij hebt voor je vak en de hoeveelheid kennis die je bezit, zelfs over onderwerpen die buiten jouw domein liggen. Bedankt voor de gezellige fietstochtjes wanneer we elkaar onderweg eens tegenkwamen en bedankt voor de zeer relevante papers, die jou op één of andere manier steeds sneller bereikten dan mij ;-).

Ine L, als ik mij niet vergis zou ik normaal gezien mijn thesis gestart zijn onder jouw begeleiding. Alleen kwam een klein wondertje deze plannen verstoren en nam Ine DC mij voor haar rekening. Gelukkig heb ik tijdens mijn doctoraat de kans gekregen om jou toch te leren kennen, zowel als een fantastische begeleidster voor jouw studenten, geweldige mama en enorm creatieve madam ! De gesprekken samen met Heleen in ons bureautje om wat frustraties los te laten of juist een gat in de lucht te springen bij het slagen van bepaalde projecten geassocieerd met de nodige zenuwen voor wat volgen zou, kwamen steeds op het juiste moment. Ook in de middag pauze konden we beiden een leuke babbel appreciëren en als de zon scheen wist ik telkens dat er nog iemand heel enthousiast zou zijn om mee buiten te gaan eten. Ine, ik heb jou al meerdere malen gevraagd of je toch niet eens een kledinglijn zou starten voor volwassenen ook, zodat ik ook kan stralen op Instagram in één van jouw prachtige ontwerpen. Ik hoop nog steeds dat je hier in de toekomst werk van maakt ;-).

Toon, jah, wat kan ik over jou zeggen dat je niet al duizend keer gehoord hebt? Bij deze dan een duizend-en-eenste keer: hoe heeft dit labo ooit kunnen draaien zonder jou? Zelfs al heb ik deze tijd nog meegemaakt, ik kan mij eigenlijk niet meer voor ogen halen hoe het er toen aan toeging. Een toppertje van formaat ben jij, zowel door met oplossingen te komen voor al onze microscopie problemen, als door de lat van onze verkleedfeestjes enorm

hoog te leggen met je zalige outfits. Ook jouw muzikale bijdrage op mijn housewarming, waar ik nog steeds met bewondering aan terugdenk, zal ik niet gauw vergeten. Ik ben ervan overtuigd dat jij nog vele studenten enorm veel dankbaarheid zal opleveren, mits er een voldoende grote voorraad koffie voor handen blijft natuurlijk.

Herlinde, wij kennen elkaar nog niet zo heel lang, maar al gauw werd heel duidelijk dat jij volledig binnen dit labo zou passen. Tijdens mijn laatste experimenten had ik het niet al te makkelijk en het uitvallen van de confocale deze zomer was voor mij dan ook een regelrechte ramp. Super erg bedankt om mij te helpen zoeken naar een oplossing en mij te begeleiden op de coupure. Deze laatste hulp was exact wat ik nodig had om er weer volledig voor te gaan toen ik het even niet meer zag goedkomen.

En dan, ons fantastisch klavertje vier, Bart, Hilde, Ilse & Katharine, aan wie ik ontzettend veel geluk te danken heb en zonder wie het lab niet draaiende zou blijven. **Bart**, ik ben compleet de tel kwijt van hoeveel keer ik al aan jouw bureau gestaan heb, jou gemaild heb, of soms zelfs mocht sms'en voor problemen van allerlei aard. Meehelpen zoeken naar materiaal dat weer eens verloren ging in het lab, opzoeken hoe lang het nog zou duren voor iets toekwam vergezeld door een dringende telefoon wanneer dit niet snel genoeg ging en op zoek gaan tussen de oude spullen naar materiaal waarmee we iets konden knutselen om…, waren enkele van jouw specialiteiten. Als je eens niet op het labo was, zaten we vaak met de angst wat te doen als er zich een probleem zou voordoen. Ook voor het begeleiden van het practicum heb jij me meer dan ooit kunnen helpen en was je de rust zelve die we nodig hadden wanneer de dingen in het honderd liepen ☺ Buiten het labo was je steeds van de partij voor een feestje of weekendje weg. Ik herinner mij nog goed mijn eerste laboweekend waarbij Rein, Lotte en ik geplet zaten op de achterbank van de Multipla en toch een fantastische rit beleefden! Bedankt om mijn redder in nood te zijn, die telkens weer een glimlach op mijn gezicht kon toveren. **Hilde**, bedankt om ons labo te komen aanvullen en Bart te ondersteunen in zijn vele taken. Tijdens mijn laatste jaren als practicumbegeleidster ben jij een enorme hulp geweest. Door jouw efficiënte manier van handelen en jouw probleemoplossend denken, liepen de practica enorm vlot en rustig. Ook buiten het lab hebben we ondertussen al heel wat toffe momenten beleefd: een kleine rondleiding in Brugge, aangevuld door een heerlijk diner in t'Zwarthuis en dit jaar het onvergetelijke laboweekend waar je iedereen overtrof met je 'slap cup' talenten ☺ Merci Hilde, voor de vele leuke momenten! **Ilse** en **Katharine,** bedankt voor al de logistieke en administratieve zaken die jullie voor mij regelden, alsook het openen van de bareel als ik weer eens mijn badge vergeten was en de toffe en gezellige babbels aan het koffiemachine of de printer. **Ilse**, het lijkt mij dat ons contact niet zal verdwijnen, aangezien we elkaars toffe momenten, voorzien van de nodige likes en girlstalk, nog goed kunnen volgen op Instagram

;-). **Katharine**, heel erg bedankt om mij te helpen bij de laatste loodjes, de steun in de laatste maanden en met het inbinden van mijn doctoraat. Jullie zijn fantastisch!

Hoe zou ik dit doctoraat ooit hebben kunnen volhouden zonder mijn fantastische bureaugenootjes? Gedurende deze vijf jaar werd 'den bureau' al een aantal keer onderworpen aan een metamorfose, van een complete vrouwenbureau, over een aantal visitors heen, naar 'Laurens en de ladies' tot eigenlijk een volgevuld bakje van 7, zonder dat we aan plezier moesten inboeten.

Eén constante voor mij: **Heleentje.** Ik heb het jou al vaker gezegd: zonder jou was het mij nooit gelukt. Ik denk dat je mij al meerdere malen tranen van geluk in mijn ogen hebt doen krijgen wanneer ik jou op het laatste moment toch nog iets mocht doorsturen en dan weeral eens een super goed nagelezen versie van één van mijn papers terug kreeg. Telkens grondig doorlezen, met commentaren die mijn werk een enorme meerwaarde gaven en steeds vergezeld van een positieve en motiverende noot. Zélfs tijdens je zwangerschapsverlof maakte je tijd vrij om mij te helpen. Hoe kan ik jou ooit voldoende bedanken in dit kleine tekstje? Al vanaf mijn derde jaar Farmacie, waar je samen met Lynn mijn bachelorproef begeleidde, heb ik steeds naar jou opgekeken. Jouw enorme wijsheid, creativiteit en talent voor onderzoek, maakten mij volledig warm om ook het onderzoek in te stappen. Het begin van mijn doctoraat kon dan ook niet meer stuk toen ik bij jou in de bureau terecht kwam. Al gauw werd duidelijk dat we beiden streven naar efficiëntie, graag het initiatief nemen om zaken te organiseren, ordelijk zijn in ons werk maar een nogal rommelige bureau achterlaten, graag het uitschrijven en editten van de filmpjes op ons nemen en veel aan elkaar zouden hebben tijdens onze talloze uren achter de computer. Ook naast het labo, ben ik blij dat ik deel mag blijven uitmaken van jouw leven en vele filmpjes te zien krijg van jouw nieuw aangevulde gezinnetje. Het bezoek in het ziekenhuis toen je net bevallen was van Victor, was voor mij een lichtpuntje op een moeilijke dag.

Lynn, één van de sterkste persoonlijkheden die ik ken. De manier waarop jij in het leven staat heb ik altijd enorm bewonderd. Naast een fantastische mama van een pracht gezinnetje, stond je altijd voor iedereen klaar op het labo en zorgde je in den bureau voor een aantal hilarische momenten. Van achter het hand gefluister tot een aantal reeds bekend geworden gebaren, een aantal lichamelijke trucjes en natuurlijke onze 'top-focus' tijdens de laatste maanden van je doctoraat, ik heb jou al veel gemist in de bureau, zeker wanneer ik graag eens wat wou planken ;-). Ik ben heel blij dat ik jullie mocht vervoegen op de super gezellige WWW-avonden, waardoor we zeker geen contact verliezen en ik op de hoogte blijf van de zottigheden van Sam en Gust!

Liefste **Laura**, wat herken in mezelf zo vaak in jou ☺. Toen ik mijn thesis deed in het labo hadden we nog niet echt veel contact, maar toen ik bij jullie mijn doctoraat startte duurde het nog geen week vooraleer we ontdekten dat we heel wat gemeenschappelijk hadden. Onze zelfde kledingstijl, onze passie voor muziek en dan voornamelijk voor zingen, onze ijverige precisie en klein hartje én onze passie voor persoonlijke ontwikkeling. We hebben samen zoveel lol beleefd zowel op het werk als daarbuiten. Je was altijd in voor een feestje, tot in de late uurtjes en Rihanna gaan aanvragen is ondertussen een niet te ontbreken traditie geworden. Je was mijn vaste fitnesspartner en af en toe eens gaan lopen met jou kon mijn gedachten helemaal verzetten. Babbelen over elkaars dromen, plannen, maar ook twijfels,… soms had ik het gevoel dat je me beter kende dan ik mezelf ;-). Ik ken niet veel personen die zo warmhartig en oprecht zijn als jij. Bedankt voor de vele troostende en geruststellende momenten. Je bent mijn grote voorbeeld en naast een fantastische bureau-maatje ben je dan ook één van mijn beste vriendinnen geworden. Ik heb jou al heel veel gemist in het labo, maar gelukkig maken we dat vaak goed met een WWW-avondje, een heerlijke brunch of een gezellig avondje gewichtheffen. Dat er nog veel mogen volgen ;-).

Laurens, al vanaf dag 1 dat jij ons op de bureau kwam vergezellen, merkte ik dat wij goed met elkaar konden opschieten. In het begin was het waarschijnlijk wat wennen voor jou, zo tussen de vrouwen, maar doordat je altijd wel nieuwsgierig was naar de laatste roddels, was dat niet zo'n probleem, me dunkt ;-). Ook het schuren op de feestjes kreeg je al snel onder de knie en maakte jou direct een passende aanwinst voor labo biochemie. Dat je in de smaak viel bij de vrouwen, maar dat zelf niet altijd even goed doorhad, heeft ons altijd goed doen lachen. En wanneer we ontdekten dat een timer aan of uitduwen makkelijker ging met ons hoofd omdat we daardoor onze handen in de lafkast konden houden, was het hek helemaal van de dam. Ik was maar al te blij om met jou een team te vormen in het practicum wat dan ook van een leiden dakje liep. Ik wil jou bedanken voor jouw Channel doosje, dat mij meerdere malen uit de nood geholpen heeft, je hulp bij mijn verhuis, en de vele praatjes en troostende knuffels. Je bent een super aangenaam persoon en ik hoop dat we ook na mijn doctoraat nog toffe tijden kunnen beleven!

Late night working is much more fun with someone next to you. **Heyang**, thank you for the good company during the final months of my PhD and for the fun conversations. I remember when we were discussing how you can recognize people by the way they knock on our office door and how Chinese people lose more hair in Europe, because we have different water in our showers :D You always make me laugh and I loved that you were my roommate last year at the mRNA conference in Berlin! I had lots of fun talking to you about girly things in our pyjamas. Good luck with everything, I really admire your hard work.

Robi, after saying goodbye to Laura and Lynn, I was so happy that you were joining our office. I will never forget our fantastic roadtrip to the Biopharmacy Day and the good times we had in Porto. Also in the office we always had so much fun choosing outfits and gossiping about Italian models ;-). I hope you don't leave Belgium too soon, cause you have become an amazing friend and I would like to keep you here so we can shake some more on Mura Masa and Justin Bieber together.

My dear **Rita**, yes also you were part of the office once ☺ I was so sad when you had to leave our lab. We had such a nice time together, going to the gym, playing Smurfette in Lynn's movie, doing some sexy dancing on the floor while imitating Justin Bieber's video clip and laughing really hard with the word 'coco' and that time we were looking for a 'slogan' for our Excellerate running team. I will never forget our trip to Scotland, were we really got to know each other and had some deep talks about our past and our dreams. Rita, you were always there for me and I still regret that I wasn't able to join your wedding. I hope we will still see each other a lot, which we are currently doing very successfully.

Mijn nieuwe beste vriendin ;-) **AK**, wat ben ik blij dat ik nog de kans heb gekregen een jaartje met jou te mogen samenwerken. Je bent echt een fantastische meid en een even fantastische vriendin! Door onze gemeenschappelijk liefde voor ogen en vooral voor die van Noah Centineo groeiden we snel naar elkaar toe en maakte ik mij al gauw, door jouw toedoen, belachelijk bij het maken van bril-selfie-filmpjes en bracht jou al graag eens in gêne door jouw naam door te geven aan heel wat vrijgezelle mannen ;-) An-Katrien, merci voor de steun tijdens de laatste maanden van mijn doctoraat, het aanhoren van mijn vele geklaag en het nalezen van de laatste stukjes tekst! Ik heb mij al rot geamuseerd met jou en hoop dit nog lang te kunnen doen. Ik wens je heel veel succes met je verdere onderzoek en ben ervan overtuigd dat jij dit buitengewoon goed gaat doen.

Mike, één van mijn beste thesisstudentjes. Toen je me in de auto op weg naar ons zoveelste bezoek aan het slachthuis vertelde dat je misschien graag wou doctoreren, twijfelde ik er geen seconde aan dat dit een goed idee was. Jouw talent en motivatie was exact wat ik nodig had om erin te slagen twee studenten tegelijk te begeleiden, en dit in een periode dat ik ook nog eens practicum gaf. Jij hebt mijn werk enorm vooruit geholpen en we moeten dringend eens een glaasje heffen op onze paper die recent gepubliceerd werd. Jij hebt een enorme bijdrage geleverd aan mijn doctoraat, niet alleen op experimenteel vlak, maar ook op technisch vlak mij vaak uit de nood te helpen met mijn computerproblemen. Ik ben ervan overtuigd dat diezelfde gedrevenheid jou heel ver gaat brengen in het onderzoek!

Mijn twee rosétjes, of zoals ze zeggen *"Best friends are therapists you can drink with"*. **Elinetje**, jou leerde ik als eerste kennen, en hoe, beiden toevallig wakker worden in dezelfde

crib, toen al fan van eitjes bij het ontbijt ;-). Ik vond jou al van in het begin een toppertje en het feit dat je dan ook mijn toekomstige collega zou worden maakte het alleen nog maar beter. Het eerste jaar leerden we elkaar snel kennen op het werk en een ongelukkig toeval bracht ons nog dichter bij elkaar, letterlijk dezelfde 100 m² delend. Ik ben met mijn gat in de boter gevallen met jou als mijn roomie. We hebben ons samen keihard door een moeilijke periode geslaan, ons volop aan elkaar opgetrokken en ik kan toch zeker voor mezelf spreken dat ik zoveel sterkte aan jou te danken heb. Ook dit jaar heb ik zoveel gehad aan jouw emotiegrafieken, jouw wonderwoman-berichtje en de vele troostgevende knuffels. We hebben samen veel gehuild en gevloekt maar nog meer gelachen en gedanst ! Jouw oneindige enthousiasme en positieve spirit zijn zo aanstekelijk en jouw woordspelingen deden mij telkens weer op de grond liggen van het lachen. Samen gaan brunchen, ons eens goed laten verwennen in de wellness, elkaars kleren aandoen, naar de winkel spurten voor shopadvies, cocktails gaan drinken en een danske placeren zijn slechts een aantal van onze favoriete bezigheden, waar ik ten volle van genoten heb. Ook op wetenschappelijk vlak heb ik enorm veel bewondering voor jouw tomeloze inzet en drive. Naast de vele 'places to be' die je mij in Gent leerde kennen, is de belangrijkste hotspot toch wel zeker de biofilm geweest. Het gaf mij een enorme boost om die laatste maanden schrijven met iemand te kunnen delen en de rustgevende woorden die we niet bij onszelf geloofden, konden we dan wel vol overtuiging aan de ander geven. Onlangs namen we met pijn in het hart afscheid van onze casa palaza en daardoor officieel van één van de beste jaren in mijn leven. Elinetje, you are the gin to my tonic en ik kan maar één ding wensen: dat is dat er nog veel van deze jaren mogen volgen. **Silketje**, aka. celculture, ik herinner me nog goed de dag dat je voor het eerst naar het labo kwam en de reden waarom: jouw prachtige kledingstijl, ik weet nog perfect wat je aanhad ;-) Ik denk dat we al langer dan vandaag weten dat we dezelfde kleding- en interieursmaak hebben, iets waar we elkaar dan ook meer dan vaak advies over vragen ☺ Wij hebben in deze korte tijd dat we elkaar kennen al zoveel samen beleefd, al veel tranen gedeeld en elkaar al vaak opgebeld voor de nodige peptalks en het editten van elkaars berichten, wanneer we zelf niet goed meer wisten 'wat we nu eigenlijk wouden zeggen'. Ook het editten van de vele doctoraatsfilmpjes werd één van onze gezamenlijke specialiteiten. Als fan van Kygo en deep house chill muziek konden we het ook ontzettend goed vinden in de celkelder ;-). Dat resulteerde in uitstapjes naar pukkelpop, het sportpaleis, de AB, de Lokerse feesten en talrijke afterworks en feestjes. Silketje, jij bent een enorm warm persoon met een gouden hart en ik ben zo blij dat wij de laatste jaren nog meer naar elkaar zijn toegegroeid. Onze reisjes samen naar Panama en Sicilië en onze nieuwe culturele uitstapjes, mogen nog vele vervolgen kennen. We kunnen ook officieel een clubje 'miserie met muizen en wachten op papers' oprichten, maar jouw doorzettingsvermogen is inspirerend en de vele tegenslagen hebben van jou een enorm sterke madam gemaakt. Bovendien kan ik niet klagen want deze tegenslagen gaven ons vele redenen om rosétjes

te gaan drinken. Jij staat nu voor de eindspurt en ik ben ervan overtuigd dat jij dit geweldig gaat doen! Lieve poesjes, ook al is het misschien wat eng dat we binnenkort allemaal onze eigen weg gaan, “I hope we're friends until we die. Then I hope we stay ghost friend and walk through walls and scare the shit out of people.”

Lieve lieve **Lotte**, hoe kan ik wat ik jou te zeggen heb hier nu samenvatten op zo’n klein papiertje? We hebben elkaar eigenlijk pas echt leren kennen op de bierbowling in ons laatste jaar farma en ik denk dat we op één avond, na redelijk wat pintjes, ongeveer elkaars volledige leven kenden. Nadien zijn we dan ook samen aan dit avontuur begonnen in dit fantastische labo en werden we alleen maar betere vriendinnen. Ik herinner mij nog goed onze eerste week hier en het feestje in de Charlatan waar jij je handtas kwijt was en ik “subtiel” wat langer was gebleven ;-), ons eerste labweekend waar we al onmiddellijk tot een stuk in de nacht bleven babbelen en ons eerste congres samen met Rein in Blankenberge, “de pareltjes aan de kust”. Elk introvertje heeft zijn extravertje zeg jij soms, en ik denk dat dat in ons geval inderdaad volledig klopt. Wij vullen elkaar fantastisch aan. Jij bent een enorm sterke persoon die niet op haar mondje is gevallen, en I love you for it! Ik durf mijzelf al eens te verliezen en dan ben jij er om mij met mijn voeten op de grond te houden. Bedankt voor alle steun, vertrouwen, inspiratie, kortom bedankt om er te zijn voor mij ! De reis naar Australië kwam voor mij in een moeilijke periode en je hebt er een onvergetelijke 3 weken van gemaakt. Ik kon mij niemand beter inbeelden om kangoeroes te strelen, bij 30°C kerstmutsen te dragen, te snowboarden op het zand en kinderliedjes te zingen, dan jou. Op naar nog meer van die avonturen!

Jokie, Joke nummer 2, wat een levendige en zorgende persoonlijkheid ben jij. Joke en Joke, alvast het begin van een geweldige combinatie. Bedankt voor de vele lieve berichtjes, de persoonlijke praatjes in de bureau, je oprechte bezorgdheid en de uren die je samen naast mij doorbracht aan de microscoop toen ik het heel erg moeilijk had. Jouw passie voor plannen en organiseren zullen er vast en zeker voor zorgen dat wij elkaar nog heel vaak zullen zien. Ik kijk alvast uit naar onze volgende girlsnight met stembriefjes, spannende weetjes en vele roze zakken ;-).

Gaëlle, met jouw komst in het labo had ik mijn nieuwe partner in het organiseren van feestjes gevonden. Laten we één ding onthouden, een single night met vragenkaartjes is niet voor ons weggelegd ;-). Ik zal jou missen als ik van het labo wegga, maar hopelijk compenseren we dat voldoende door lange uren boytalk en aperollekes op de vele feestjes die nog mogen komen, instagramwaardige etentjes en zonnige open airs!

Reintje, wij hebben deze ervaring echt wel samen doorlopen, van onze eerste farma verkleedfeestjes, ik herinner mij iets van “bling bling” en een gestolen discobol en verder iets

van Minnie mouse en Filiberke, over de hilarische momenten samen met Katrien tijdens onze thesis, tot het samen schrijven aan ons doctoraat in deze laatste periode. Ik moet toegeven dat ik in het begin ontzettend mijn best moest doen om jou te kunnen verstaan, maar ondertussen ken ik jouw woordenschat als mijn broekzak. We hebben samen zoveel lol beleefd, ons eerste congres in Blankenberge, waar we tot laat 's avonds nog gesprekken voerden over één of ander egoïstisch gen ;-), culturele bezoekjes in Berlijn, waarna we belandden op een oktoberfest-achtige braspartij op zoek naar Michael Kormann die daar helemaal niet was, onze uitstap naar de Stasi gevangenis, gevolgd door het vastzitten in de metro, vastzitten in de tram en bijgevolg ontzettend hard rennen om onze vlucht nog te kunnen halen, het samen aan de tooghangen, waar jij dan meestal heel de avond verbleef terwijl ik jou tevergeefs probeerde te overtuigen dat het veel toffer vertoeven was op de dansvloer en onze fantastische jaren samen in het practicum. Reintje, merci voor de motiverende woorden, sappige verhalen en hilarische mopjes en woordspelingen. Ik lig nog steeds plat met de soldaat onder de boom, de hond met de bril en natuurlijk, de onvergetelijke pinguinmop. We hebben dit samen tot een heel mooi einde gebracht en we gaan daar zoals gezegd 'nog ne keer goe op gaan klinken'!

Pieterjan, wat een intellectuele topkerel ben jij ! Ik ken weinig mensen die zoveel weten over zoveel. Jouw droge humor als ook jouw komische woordspelingen zijn fenomenaal en hebben mij al meerdere keren enorm doen lachen. Ik bewonder enorm jouw empathisch vermogen, jouw rustgevende woorden en oprechte interesse in anderen. Altijd even vragen hoe het met me ging, of de experimenten goed verliepen en hoe het zat met het schrijven, gevolgd door wat bemoedigende woorden bij een pintje aan de keukentafel of bij het naar huis fietsen van één of ander feestje. Ik hoop dat je "het zwembadje" niet te lang met je meedraagt ;-) maar vooral de mooie momenten en toffe herinneringen die we samen beleefden blijft koesteren! Je bent nu zelf ook bijna aan het einde. Nog even op de tanden bijten. Ik ben ervan overtuigd dat jouw doctoraat een prachtexemplaar zal zijn.

Thijske, onze playboy sinds de zomer van 2018, die houdt van kwissen gebaseerd op IQ, af en toe wat verwondingen oploopt tijdens het uitgaan en al graag eens een koprolleke doet! Bedankt voor de vele speculooskes, het uitwisselen van menige doctoraatsfrustraties, je poging enthousiast te zijn bij het practicum ;-) en het fantastische verhaal over de pollepel. Als nieuwe nabije buur, verwacht ik snel eens een uitnodiging voor dat museumbezoek !

Juanito, from the moment you stepped into the lab you filled every room with your sparkling personality. I really enjoyed ballroom dancing in the hallway, the singing of Disney songs during the walk on labweekend, taking sexy selfies in the sushibar and dancing our ass off on Justin Bieber. Brussels is doing you very good and I hope we can stay friends for a long time so I can follow all your hot stories on Instagram ;-)

Ranhua, I still remember my first labweekend, were you were imitating the guitarsolo of a Belgian popsong, singing very loud with your hands in the air and drinking jenever when you actually thought it was milk – genius ! I really admire your dedication and enthusiasm for science. You are definitely responsible for some memorable moments and nicknames in the lab ☺.

Jelter, ik zal jou altijd blijven linken aan het bezitten van grote visknuffels, het enorm liefhebben van planten, jammer genoeg mee sukkelen met mij en mijn rugproblemen, het schrijven van heel grappige emails en het vertellen van zeer interessante weetjes aan de lunchtafel. Merci voor de goeie sfeer en toffe babbels!

Jingie, you are a very beautiful, genuine and amazingly smart girl. Your first lab weekend was probably a bit overwhelming, but in the meantime you've become an indispensable person in our group. Thanks for always helping out with the PhD movies, brighten up the diners with your presence, thank you for your many compliments, comforting words and for being a fantastic model for my make-up lesson!

Molood, your PhD journey hasn't always been easy, but you've almost reached the end! I really admire your strong personality. You have so much fire in you, I'm not worried at all that you'll go far.

Aranit, een man met het hart op de juiste plaats. Bedankt voor de gezellige gesprekken aan de vriesmicrotoom, het babbeltje in den bureau tijdens de laatste maanden van mijn doctoraat, je advies over mijn future perspectives en je bruisende enthousiasme. Ik apprecieer enorm je bereidheid om iedereen te helpen en bewonder jouw gedrevenheid in het onderzoek. Ik wens jou nog heel veel succes toe en hoop dat je mij nog kent als je die Nobelprijs wint ;-).

Felix, thanks a lot for sharing your knowledge about the fascinating world of the vitreous and helping me with my vitreal mobility problems. I really enjoyed your presence and jokes during lunchtime and your hilarious messages in the LGBPP whatsapp group. I wish you all the best with your floater project!

Bram, Jana, Helena, Christina, to bad I can't spend some more time with you guys in the lab. **Bram**, thanks for your recent help with the moviemaking ;-). I wish you lots of fun in Tell Aviv, lots of luck with your CADs and keep poppin' those bottles, I think I see a future CPO! **Jana**, I remember you telling me how amazing it was that a couple of years ago I was your teacher in the practical sessions and now we were dancing together in the Vooruit. I already had lots of fun with you, doing Halloween make-up and taking snapchatfilterselfies at labweekend. I hope that those nice moments keep coming! **Helena**, welcome to team

Katrien. You are doing so great with your project and I really admire your independency. I guess we don't have to worry about still seeing each other after I leave. See you at the next handball game or housewarming ;-). **Christina**, unfortunately we didn't got the chance to really get to know each other, but when I'm gone you can have all my mRNA ;-). I wish you lots of luck with your research and btw I'm really in love with your hair!

De ancientjes, natuurlijk ben ik jullie niet vergeten. Bedankt voor het super onthaal in mijn eerste jaar, door de vele feestjes voelde ik mij meteen thuis.

Ine DC, bedankt om mij zo fantastisch te begeleiden met mijn masterthesis tijdens jouw eerste jaar. Dat was geen makkelijke opdracht, maar ik denk dat we het er samen met wat zoeken en zwoegen heel goed vanaf gebracht hebben. Merci ook voor de hilarische momenten, om mijn getuige te zijn toen de DJ mijn playlist op at, je geweldige speech over kaas en de uitnodiging voor jullie prachtig trouwfeest. **Freya**, vele uurtjes heb ik naast jou zitten pipetteren in het cel labo en daar begonnen dan ook onze grappige gesprekken. Wat een danstalent ben jij ! Ik was onmiddellijk vol bewondering voor jou, toen je me vertelde dat jij de droom van vele meisjes om in het samsonballet te dansen écht mocht uitvoeren. Dat talent was ook niet weg te stoppen op de dansvloer en jij was altijd te vinden voor een stevig drankje en een feestje. Bedankt voor de leuke tijden tijdens het thrift shoppen, de kerstmarkt in Brussel, de vele labweekends en concertjes en een heel toffe tijd samen in Porto! **George**, did you know you were the first one to talk to me when I started in the lab? You asked me about my trip to Malaysia and told me that it was a very nice idea to travel before starting my PhD and it would have positive implication on all aspects of life ;-). And that was just the start of many wisdom you share during my PhD. Thanks for helping me out especially with the experiments during my first year. Thanks for your help in moving my piano, for the really nice conversations we had in Scottland, the fun moments during ESCDD, the necessary hydration in between the shots on labweekend, for lending me some money when I forgot my wallet at the AH ;-) and thanks for the advice you gave me recently when applying for a job. In other words, thank you for being you ! **Katrien F**, wat ik me van jou altijd al blijven herinneren is het feit dat we jou mochten nabootsen in je gele piranha pak, jouw hilarisch photoshoptalent op vrijdag met George als superman of Thomas op een wrecking ball en jouw super microscopietalent! Ik vond het heel leuk om onlangs ook kennis te maken met kleine Lotte, wat lijkt ze veel op jou.

Mijn maatjes, wat allemaal begon bij het nachtelijk ronddwalen in Egmond aan Zee op zoek naar één of andere "Generation" leidde tot vele gezellige avonden "boy"talk, vuistjes, pintjes drinken, schunnige mopjes, bakken bier proberen vervoeren in mijn go-cartje, saunaplezier leidde tot een sterke vriendschap. **Stephan**, ook al ben je nog steeds aanwezig op het labo, ik vond het toch iets gepaster om jou hier te bespreken. We hadden beslist dat

ik eigenlijk niet veel ging schrijven omdat we het beiden wel “voelen in ons hart” ;-) maar ik ga toch mijn best doen. Merci Stephan voor de late night talks op het werk tijdens het schrijven van mijn review (al dan niet vergezeld van een goed glaasje wijn), voor het meesterlijk opdienen van ons meestal zelf samengestelde shotjes en de heerlijke discussies over onze favoriete tv-programma’s “Are you the one” en “Temptation Island”. Ik ga jou enorm missen als ik het labo hier verlaat, maar ik hoop stiekem toch dat we nog af en toe enen kunnen gaan drinken en dat je die fameuze housewarming/babyborrel geeft en die dan Karen-en-Alain-gewijs jaarlijks herhaalt in uw ongetwijfeld prachtige nieuwe woonst ! Ik wens je ook heel veel succes toe met TrinCE en je weet het, ik ben binnenkort op zoek naar een job ;-). Mijn liefste peter **Koentje**, mijn CPO voorganger, ik geloof dat de eerste woorden die jij, na een aantal pintjes in de Charla, tegen mij zei iets waren in de aard van: “Wil jij mijn kleine zus worden?” In plaats daarvan werd ik jouw “metekindje” en dat ben ik met veel trots geweest. Wij hebben ons uren geamuseerd met het van buiten leren van namen tijdens het practicum, onze schuurskills professioneel verbeterd op de dansvloer, samen met Stephan en Rita getraind voor de Ekiden, niet te vergeten “vet geruled” in onze top labo biochemie band, de keeltjes van onze collega’s nooit droog gelaten (met nefaste gevolgen voor onze portemonnees) en het feestje het liefst vergezeld geweten van een goed fleske Gold Strike. Aangezien mijn favoriete aanvraagnummer dan ook nog eens ons gemeenschappelijk danslied is, gaat er geen feestje voorbij waarop ik niet aan jou moet denken: *“Gyal, me wann fi hold yuh, put me arms right around ya, Gyal, you give me the tightest hold me eva seen in my life”*. Ik vind het jammer dat ik er toch niet zal kunnen bijzijn op jullie trouw, maar we gaan daar zeker nog eens een fleske op opendoen! **Thomas**, aangezien je teveel bbq worsten naar binnen gewerkt had (of was het nog daarvoor?) gedurende ons eerste laboweekend en daardoor ziek in je bed moest vertoeven, wist ik eigenlijk nog niet goed dat jij ook deel uitmaakte van labo biochemie ;-). Het duurde echter niet lang voor we doorhadden dat we veel gemeenschappelijke interesses hadden, ik heel hard moest lachen om jouw Kerstmanskill en jij om mijn “wiped cream”, we veel plezier beleefden op de thrift shopparty in de Oude Vismijn en in de Charla en samen met Koen en Karen een topband oprichtten. Je bent een fantastische muzikant en een heel warm persoon, die dan nog eens iets kent over ogen ook, wat van jouw boekje mijn bijbel maakte tijdens het schrijven van mijn doctoraat!

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mopje met de echo die leidde tot veel verbazing bij mijn vrienden en familie ;-) en ook bedankt om mij in te leiden in de prachtige wereld van Excel.

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Naast mijn lieve collega's heb ik ook een heleboel vriendinnen die ik enorm dankbaar ben. Mijn **"Strekewijfjes"**, zonder jullie had ik nooit de stap gezet ! Bedankt om mij zo te entertainen de voorbije jaren, voor de zalige reisjes, vele times-up en Harry avonden en hilarische kerstfeestjes. **"Tilde en de single ladies"**, ik denk dat het hoog tijd wordt dat we deze groepsnaam eens veranderen ☺ De boog kan niet altijd gespannen staan, merci voor de vele brunchen, heerlijke momenten in Le Beaucet, jaren luisteren naar mijn gezeur, mij opvrolijken door een poging mij te overtuigen dat jullie leven ook niet fantastisch is ;-) de lekkere etentjes, sappige verhalen, vele wijntjes, ontzettende non-judging capaciteiten en heel veel liefde. **"Girls in charge"**, ja Maga, we hebben eindelijk de naam door ;-). Voor redelijk eeuwig zijn wij met elkaar verbonden of we het nu willen of niet ! Bedankt om mij door dik en dun te steunen, voor de aanmoedigende sms'jes, de tonnen afleiding, voor de ontzettend vele momenten die we samen reeds deelden en de vele die nog zullen komen!

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Last but not least, mijn familie. Je dacht dat ik je misschien vergeten was **Karentje**, maar ik heb hier toch een speciaal plaatsje voor jou voorzien. Omdat ik jou, na deze fantastische jaren samen, niet alleen een super collega, geweldige vriendin, maar sinds kort ook echt familie mag noemen ☺. Jij was voor mij een nieuw gezicht toen ik mijn eerste jaar begon, maar onze klik was er geloof ik al vanaf dag 1. Ik werd onmiddellijk ondergedompeld in de verhalen over jouw meest gekke dromen, jouw passie voor al wat onder water leeft, je eindeloze enthousiasme en fantastische dansskills, jouw talent voor het organiseren van de zaligste housewarmings en vooral jouw enorme behulpzaamheid. Ik denk dat ik officieel kan zeggen dat mijn doctoraat niet eens bestaan zou hebben zonder jou. De keren dat jij mij gedurende deze jaren uit de nood geholpen hebt, kan ik niet meer op mijn beide handen tellen. Van het samen brainstormen over een nieuw project, het oprichten van talrijke technieken voor ons prille oculaire team, stinkend naar huis komen na een bezoek aan het

slachthuis, onze hilarische eerste momenten achter de cryotoom, onze eerste wondermooie retinale coupe onder de microscoop, de hulp bij het schrijven van oneindig veel protocols, het nalezen van FRO-projecten en talrijke abstracts tot uiteindelijk de enorme steun bij het insturen van mijn papers gedurende het laatste jaar, meehelpen nadenken over geslijm voor de reviewers, ontzettend veel uren op zoek naar eGFP in de retinale coupes, en het verbeteren van mijn laatste stukjes tekst voor mijn doctoraatsboekje. Karentje, moest ik het niet al duizend keer gezegd hebben, MERCI ! Wij hebben de voorbije jaren zoveel gedeeld, zoveel aan elkaar verteld, zoveel gehuild en zoveel gelachen. Je was er voor mij op de meest moeilijke momenten van mijn leven, maar maakte ook deel uit van de mooiste! Samen concertjes afschuimen, ons volledig laten gaan bij het maken van PhD movies, het volgen van een zeer interessante cursus statistiek, songs schrijven en een fantastische band oprichten, urenlange voicemails achterlaten met onze prachtige London Gramar imitatie, jouw autootje proberen overladen met liefde om maar niet stil te vallen op weg naar het labweekend én dan toch als eerste arriveren, de afterworktraditie eindeloos proberen onderhouden, bergtoppen beklimmen en geen hand voor ons ogen zien in Schotland, onderbroeken shoppen en karaoké̲nummers zingen in Seattle, overdreven zware donuts eten in de regen in Barcelona, gaan ontbijten op de vlasmarkt tijdens de Gentse feesten, stiekem fan zijn van Gossip Girl, geblinddoekt eten en prijzen winnen in Nijmegen, tenten en koeienogen vasthouden tijdens onze wetenschappelijke uitleg op Utopia, talloze slaappartijen en knuffels en ik kan nog lang doorgaan denk ik…Om dan nog maar de zwijgen over het nieuwe wondertje dat sinds kort ook deel uitmaakt van ons leven, babysaurusje Tilda ! Wat ben ik zo blij en trots dat ik haar meter mag zijn ☺ Ik kijk al uit naar de vele babysitavonden en uitstapjes als ze wat groter is! Met zo'n twee ouders, kan het niet anders dan dat Tillie zal uitgroeien tot een pracht persoontje. Karen, je bent er eentje uit het duizend, die zelf niet doorheeft hoe fantastisch ze is, ik ben zo gelukkig dat jij in mijn leven gekomen bent en ik zou het me niet meer kunnen voorstellen zonder jou !

Timmiewimmie en **Nikita**, ook al bevinden jullie je helaas aan de andere kant van de wereld, we zijn maar een skype gesprekje verwijderd en ik kom maar al te graag eens af naar Australië. Bedankt voor de fantastische reis afgelopen kerst, het was voor mij de ideale afwisseling tussen schrijven en met volle teugen genieten. Nikita bedankt voor het nalezen van mijn future perspectives en om mij een heel goede reden te geven terug mee te vliegen naar Australië in September ;-) Ik kijk er al heel hard naar uit om tante Pokie's kleine sloeber **Zita** terug te zien (die ondertussen reeds heel erg bekend is in ons labo door de vele hilarische filmpjes) en kennis te maken met mijn metekindje **Indi** !

Mijn lieve **Tinie**, er is geen betere vriendin dan een zus en er is geen betere zus dan jij ! Ook al zijn wij twee volledig verschillende types, wij komen zo ontzettend goed overeen.

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Merci iedereen ! ♥