ESTROGEN RECEPTOR-MEDIATED EFFECTS IN THE GONADOTROPIC LβT2 AND αT3-1 CELL LINES

Frank Eertmans, Apr.

MMVII

ESTROGEN RECEPTOR-MEDIATED EFFECTS IN THE GONADOTROPIC LβT2 AND αT3-1 CELL LINES

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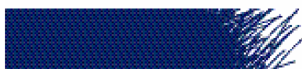

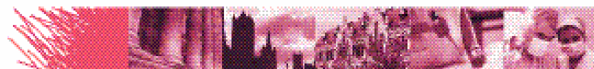

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Ik draag deze thesis op ter nagedachtenis van mijn zus, Veroniek (†17/12/2002).

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"Kracht is het doorzettingsvermogen om moed te behouden. Doorzettingsvermogen is de moed om kracht te behouden. Moed is de kracht om doorzettingsvermogen te behouden."

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Table of contents

Table of contents **1**

List of abbreviations **3**

Summary **6**

Samenvatting **9**

Chapter 1 Introduction **14**

1.1 The Hypothalamo-Pituitary-Gonadal axis 14
1.1.1 Introduction 14
1.1.2 The Hypothalamus 14
1.1.3 The Adenohypophysis 15
1.2 Estrogens 21
1.2.1 Physiology 21
1.2.2 Estrogen receptors 22
1.2.3 Estrogens: mechanisms of action 24
1.2.4 Cross-talk of estrogen receptors with growth factor receptors 33
1.2.5 Xenoestrogens and phytoestrogens 37

Chapter 2 The Study of Estrogenic Effects in Pituitary Gonadotrophs **41**

2.1 Cell cultures 41
2.1.1 Tissue explants and primary cell cultures 41
2.1.2 The pituitary 42
2.1.3 Immortalised cell lines 42

Chapter 3 Methodology **48**

3.1 Introduction 48
3.2 Receptor expression 48
3.2.1 Reverse-transcription polymerase chain reaction (RT-PCR) 48
3.2.2 Western Blot 49
3.3 LH synthesis and secretion in the LβT2 cell line 51
3.3.1 Transient transfections 51
3.3.2 LHß-subunit mRNA expression in the LßT2 cell line 52
3.3.3 Luteinising hormone secretion in the LßT2 cell line 53
3.4 Estrogenic responses in the αT3-1 cell line 54
3.4.1 Transient transfection studies 54
3.4.2 Proliferation experiments 54

Chapter 4 Experimental work 56

4.1 Estrogen Receptor Signaling is an Unstable Feature of the Gonadotropic LβT2 cell line 56

4.2 Estrogen Receptor α (ERα) and Insulin-Like Growth Factor I Receptor (IGF-IR) Cross-Talk in the gonadotropic αT3-1 cell line 66

Chapter 5 Summary and Discussion 75

5.1 Background 75

5.2 The LßT2 cell line: a mature, gonadotropic cell line of murine origin 76

5.3 GnRH-enhanced LH secretion in the LßT2 cell line 77

5.4 Absence of estrogenic effects in the mature, gonadotropic LßT2 cell line 80

5.5 Estrogen receptor-mediated effects in the murine, gonadotropic αT3-1 cell line 83

5.6 Growth factor receptor and estrogen receptor cross-talk in the αT3-1 cell line 88

5.7 Physiological relevance of growth factor and estrogen receptor cross-talk in gonadotrophs? 90

5.8 The αT3-1 cell line: an *in vitro* model for the assessment of potential estrogenic compounds at the level of pituitary gonadotrophs..................... 91

5.9 Advantages and disadvantages of gonadotroph cell lineages 92

References 95

List of publications.. 129

Abstracts & poster presentations 130

Curriculum Vitae 131

Awards 131

Postgraduate Training 131

List of abbreviations

4-OH-Tam	4-Hydroxy-Tamoxifen
8-PN	8-Prenylnaringenin
α-GSU	α-Glycoprotein Hormone Subunit
ACTH	Adrenocorticotropic Hormone
ActR	Activin Receptor
AF-1	Activation Function 1
AF-2	Activation Function 2
AP-1	Activating Protein 1
AR	Androgen Receptor
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
Cg	Chromogranin
CgA	Chromogranin A
Ct	Cycle Threshold
CYP	Cytochrome P450
DAG	Diacyl Glycerol
DBD	DNA Binding Domain
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic Acid
E_1	Estrone
E_3	Estriol
E_2	17-ß-Estradiol
EGF	Epidermal Growth Factor
EGF-R	Epidermal Growth Factor Receptor
EGR-1	Early Growth Response Protein 1
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
ERK	Extracellular Signal-Regulated Kinase
FRET	Fluorescence Resonance Energy Transfer
FSH	Follicle Stimulating Hormone
FSHß-su	FSHß-Subunit

GABA	Gamma Amino Butyric Acid
GH	Growth Hormone
GnRH	Gonadotropin-Releasing Hormone
GnRH-R	Gonadotropin-Releasing Hormone Receptor
GPCR	G-protein-Coupled Receptors
GR	Glucocorticoid Receptor
HB-EGF	Heparin Binding EGF
hCG	Human Chorionic Hormone
HDAC	Histone Acetylase
hERα	Human Estrogen Receptor α
hERß	Human Estrogen Receptor ß
HPG	Hypothalamo-Pituitary-Gonadal
IGF	Insulin-like Growth Factor
IGF-IR	Insulin-like Growth Factor I Receptor
IP3	1, 4, 5-Inositol Triphosphate
LBD	Ligand Binding Domain
LH	Luteinising Hormone
LHß-su	LHß-Subunit
LHRH	Luteinising Hormone-Releasing Hormone
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MTT	Tetrazolium Dye 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide
NCBI	National Centre of Biotechnology Information
NCoR	Nuclear Receptor Corepressor
NR	Nuclear Receptor
PCB	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
PKC	Protein Kinase C
PLC	Phospholipase C
PTX-1	Pituitary Homeobox 1
Q	Quencher
R	Reporter
Ral	Raloxifen
RAR	Retinoic Acid Receptor
REA	ER-selective Repressor of ER Activity
RIA	Radio Immuno Assay

RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
Ser-118	Serine Residue 118
SF-1	Steroidogenic Factor 1
SERM	Selective Estrogen Receptor Modulators
SgII	Secretogranin II
SMRT	Silencing Mediator of RAR and TR
Sp1	Stimulating Protein 1
SRB	Sulphorodamine Β
SRC	Steroid Receptor Co-activator
SV40	Tag Simian Virus 40 T antigen
T	Testosterone
TCA	Trichloro Acetic Acid
TERP-1	Truncated Estrogen Receptor Product 1
TGFß	Transforming Growth Factor ß
TR	Thyroid Hormone Receptor
TSH	Thyroid-Stimulating Hormone
TSHß-su	Thyroid Stimulating Hormone ß Subunit
US-EPA US	Environmental Protection Agency
YES	Yeast Estrogen Screen

Summary

In both the male and the female, normal reproductive function depends on the optimal function of the hypothalamo-pituitary-gonadal axis. The hypothalamic gonadotropin-releasing hormone (GnRH) elicits the release of the gonadotropins luteinising hormone (LH) and follicle stimulating hormone (FSH) by the pituitary gonadotrophs. LH is responsible for sex steroid production while FSH regulates gametogenesis. In turn, gonadal sex steroids such as 17-ß-estradiol (E_2) alter gonadotropin secretion through a feedback loop at the level of the hypothalamus and the pituitary.

In the past, many *in vitro* studies have been conducted using primary pituitary cell cultures and tissue explants to study regulatory effects of E_2 at the pituitary level. However, data interpretation is complex, as gonadotrophs interact with other pituitary cell types. Furthermore, these cells only represent 10% of the total pituitary cell population.

In the beginning of the nineties, two immortalised gonadotropic cell lineages of murine origin, namely the LßT2 and the αT3-1 cell lines, were developed by the group of P. Mellon using targeted oncogenesis to the pituitary of transgenic mice. Although both cell lines express a functional GnRH receptor and the common α-gonadotropin subunit (α-GSU), they differ in their degree of differentiation. The LßT2 cell line has been shown to exhibit structural and functional characteristics of normal pituitary gonadotrophs, including expression of both LHß- and FSHß-subunits, together with the corresponding gonadotropin secretion. In contrast, the αT3-1 cell line is less differentiated as gonadotropin ß-subunit expression appeared to be absent, hereby lacking ability to produce LH and FSH.

Since its development, the LßT2 cell line served as a golden standard for the *in vitro* analysis of regulatory mechanisms involved in gonadotropin synthesis.

In the present project, we have analysed the effects of estrogens and (anti-) estrogenic-active compounds at the level of the pituitary gonadotrophs using the LßT2 and αT3-1 cell lineages.

The first chapter of our experimental work (chapter 4.1) describes the analysis of the regulatory effects of E_2 on GnRH-enhanced LH synthesis and secretion in the LßT2 cells. We have demonstrated that the LH secretory response was dose-dependently enhanced by GnRH, except on the first treatment day. This may indicate that the well-described self priming effect of GnRH on gonadotrophs *in vivo* remained preserved in the LßT2 cell line. However, LH secretion diminished following daily repeated exposure to high concentrations of GnRH. This was not be explained by GnRH receptor down regulation or desensitisation, as LHß-su gene expression was dose-dependently enhanced by GnRH, even at high concentrations of GnRH. Other factors such as alteration of post-

translational processes or vesicle storage may be responsible for this diminished LH secretory response following exposure to high concentrations of GnRH.

In contrast to limited literature data, E_2 was not able to alter GnRH-enhanced LH release in the LßT2 cell line. Analysis of receptor expression revealed that LßT2 cells lack a functional estrogen receptor α (ERα). Even following ERα overexpression, we were not able to demonstrate any ER-mediated effect, hereby indicating disturbed ER signalling. This is further emphasized by the relative strong expression of an ERα messenger RNA splicing variant in comparison to the full length ERα message. This splicing variant (theoretically) encodes for a receptor protein which lacks almost the complete ligand binding domain and may possibly interfere with normal ER signalling in LßT2 cells.

According to our findings, the gonadotropic LßT2 cell line was not a suitable *in vitro* model for the study of estrogenic regulatory effects at the level of the pituitary gonadotrophs. For this reason, we have chosen for the less differentiated αT3-1 cell line. These data are presented in chapter 4.2. Proliferation experiments and transient transfection studies with an estrogen-responsive reporter plasmid have demonstrated the presence of a functional ERα in αT3-1 cells. Although E_2 showed rather modest effects on cell proliferation, the selective estrogen receptor modulator (SERM) 4-hydroxy-tamoxifen and the pure anti-estrogen ICI 182,780 (fulvestrant) strongly repressed basal cell proliferation and ERα-mediated gene transcription. This unexpected result may be explained by cross-talk between ERα and the insulin-like growth factor I (IGF-I) receptor in our αT3-1 cell line. IGF-I enhanced ERα-mediated gene expression dose-dependently. Furthermore, exposure to raising concentrations of this growth factor reversed anti-estrogen-induced inhibition of basal cell proliferation and ERα-mediated gene expression. Another interesting observation was the fact that both IGF-I and E_2 stimulated ERα phosphorylation at serine residue 118, a prerequisite for the ERα transcriptional activity. Our data suggest the involvement of IGF-I in the regulation of ERα-mediated effects at the level of the pituitary gonadotrophs. These findings are supported by limited literature data, showing modulatory effects of IGF-I on LH secretion by the pituitary gonadotrophs. Nevertheless, more research is required to further elucidate this complex mechanism of action.

In contrast to the LßT2 cell line, showing lack of any estrogenic response, the less differentiated αT3-1 cell line appears to be an interesting *in vitro* model for the study of estrogenic effects at the level of the pituitary gonadotrophs. This offers interesting opportunities to screen and unravel the mechanisms of action of estrogenic-active compounds in gonadotrophs. The importance of such studies is emphasized by the use of phytoestrogens as an alternative for hormone replacement therapy. Accumulating evidence also suggests that exposure to xenoestrogens (estrogenic-active chemicals

present in the environment) may be responsible for reported increased prevalence of fertility disorders. In addition, the pharmaceutical industry is interested in the development of SERMs for the treatment of estrogen-dependent diseases such as osteoporosis and breast cancer.

For these reasons, it is important to analyse the effects of phytoestrogens, xenoestrogens and (anti-)estrogenic pharmaceutical drugs at the level of the pituitary gonadotrophs, as these cells may be an important target of these compounds.

At present, ongoing research in our laboratory focuses on the effects of phytoestrogens in the αT3-1 cell line. An example of such experiments has been presented in the discussion chapter (chapter 5.9).

In conclusion, the mature gonadotropic LβT2 cell line, being the golden standard for molecular endocrinological research in pituitary gonadotrophs, was not a suitable model for our purposes because of the demonstrated lack of estrogenic response. In contrast, the less-differentiated αT3-1 cell line showed expression of a functional ERα, as demonstrated using cell proliferation assays and transient transfection assays. In addition, the growth inhibitory effects of SERMs and pure anti-estrogens may be explained by interactions between ERα and the IGF-IR. These data suggest that IGF-I may play an important role in the regulation of ER-mediated effects at the level of the pituitary gonadotrophs. According to our results, the αT3-1 cell line is a promising *in vitro* tool for the assessment of phytoestrogens, xenoestrogens and SERMs in order to unravel their complex mechanisms of action at the level of the pituitary gonadotrophs.

Samenvatting

Bij man en vrouw wordt het fysiologische proces dat verantwoordelijk is voor de vruchtbaarheid gereguleerd door de hypothalamo-hypofysaire gonadale as. Het gonadotropine-releasing hormoon (GnRH) stimuleert de productie van de gonadotropines follikel stimulerend hormoon (FSH) en luteïniserend hormoon (LH) door de hypofysaire gonadotropen. LH is verantwoordelijk voor de synthese van de geslachtshormonen ter hoogte van de gonaden, terwijl FSH de gametogenese moduleert. Via een feedback terugkoppeling naar de hypothalamus en de hypofyse, controleren de gonadale geslachtshormonen, waaronder het 17-ß-estradiol (E_2), op hun beurt de gonadotropine secretie.

Voor de studie van oestrogene effecten ter hoogte van de hypofyse werd vroeger gebruik gemaakt van primaire hypofysaire celculturen en weefselexplanten. De interpretatie van de bekomen data wordt echter bemoeilijkt omdat gonadotropen kunnen interageren met andere hypofysaire celtypes. Een andere beperking is het feit dat gonadotropen slechts 10% uitmaken van de totale hypofysaire celpopulatie.

In het begin van de jaren negentig heeft de groep van P. Mellon twee geïmmortaliseerde, gonadotrope cellijnen ontwikkeld door middel van gerichte oncogenese in transgene muizen. Deze LßT2 en αT3-1 gonadotrope cellijnen brengen de gemeenschappelijke α-gonadotropine subunit en een functionele GnRH receptor tot expressie, maar verschillen in differentiatiegraad. De LßT2 cellijn vertoont structurele en functionele karakteristieken van volwassen hypofysaire gonadotropen. De αT3-1 cellijn daarentegen is minder gedifferentieerd door de afwezigheid van gonadotropine ß-subunits. Dit heeft als gevolg dat αT3-1 cellen niet in staat zijn om LH en FSH te secreteren.

Sinds zijn ontwikkeling diende de LßT2 cellijn als gouden standaard in het onderzoek naar de moleculaire mechanismen die betrokken zijn bij gonadotropine productie.

In het huidige project hebben we gebruik gemaakt van de αT3-1 en LßT2 cellijnen om de effecten van oestrogenen en (anti-)oestrogene componenten ter hoogte van de hypofysaire gonadotropen te bestuderen.

In het eerste luik van ons experimenteel onderzoek (hoofdstuk 4.1) beschrijven we de effecten van E_2 op GnRH-gemedieerde LH productie in de LßT2 cellijn. GnRH stimuleerde LH secretie op een dosisafhankelijke manier, behalve op de eerste behandelingsdag. Dit suggereert dat het “self-priming” effect van GnRH in gonadotropen bewaard is gebleven in de LßT2 cellijn. Dagelijks herhaalde blootstelling aan hoge concentraties GnRH zorgde echter voor een gedaalde LH vrijstelling. Dit kon niet verklaard worden door GnRH receptor downregulatie of desensitisatie, aangezien de expressie van het LHß-subunit gen dosis-afhankelijk werd gestimuleerd, ook door

hogere concentraties van GnRH. Andere factoren, zoals post-translationele processen of opslag in de vesikels kunnen verstoord zijn in de LßT2 cellijn.

In tegenstelling tot beperkte literatuurgegevens, bleek E_2 geen invloed te hebben op GnRH-gestimuleerde LH secretie. Dit kan verklaard worden door de afwezigheid van een functionele oestrogeen receptor α (ERα) in de LßT2 cellijn. Bovendien blijkt ERα-gestuurde signaaltransductie verstoord te zijn, aangezien E_2-gemedieerde effecten afwezig bleven na overexpressie van de oestrogeen receptor. Dit wordt verder bevestigd door de relatief sterke expressie van een ERα messenger RNA splicing variant in vergelijking met normale ERα expressie. Theoretisch gezien codeert deze splice variant voor een receptoreiwit waarvan het ligand bindende domein quasi volledig ontbreekt. Een mogelijke hypothese is het feit dat deze variant kan interfereren met normale ER-gestuurde signaaltransductie in de LßT2 cellijn.

Uit bovenstaande bevindingen kunnen we afleiden dat de gonadotrope LßT2 cellijn niet geschikt is voor *in vitro* studies naar oestrogene effecten ter hoogte van de hypofysaire gonadotropen. Daarom ging de voorkeur uit naar de minder gedifferentieerde αT3-1 cellijn. Deze data zijn voorgesteld in chapter 4.2. Proliferatiestudies en transfecties met een oestrogeen-responsief reporter plasmide hebben aangetoond dat αT3-1 cellen een functionele ERα tot expressie brengen. Ondanks de eerder matige effecten van E_2 op αT3-1 celgroei zorgden de selectieve oestrogeenreceptor modulator (SERM) 4-hydroxy-tamoxifen en het pure anti-oestrogeen ICI 182,780 (fulvestrant) voor een sterke inhibitie van zowel basale celgroei als van ERα-gestuurde gentranscriptie. Dit eerder onverwachte resultaat kan verklaard worden door interacties tussen ERα en de insulin-like growth factor I (IGF-I) receptor in de αT3-1 cellijn. Dit wordt bevestigd door het feit dat IGF-I in staat was om ERα-gestuurde genexpressie dosis-afhankelijk te stimuleren. De anti-oestrogeen-geïnduceerde inhibitie van basale celgroei en ERα-gemedieerde genexpressie werd bovendien geblokkeerd door het toevoegen van stijgende concentraties IGF-I. Een andere belangrijke factor is het feit dat IGF-I in staat was om ERα fosforylatie ter hoogte van serine residue 118 te stimuleren. Deze fosforylatie is namelijk één van de sleutelvoorwaarden voor een optimale ERα transcriptionele activiteit. Uit deze data kunnen we afleiden dat IGF-I een invloed kan hebben op ERα-gemedieerde signaaltransductie in hypofysaire gonadotropen. Dit wordt bevestigd door een beperkt aantal rapporten in de literatuur, waarin beschreven wordt dat IGF-I LH secretie kan beïnvloeden. Extra onderzoek is dus noodzakelijk om dit complexe mechanisme verder uit te pluizen.

Uit bovenstaande resultaten is gebleken dat de minder gedifferentieerde αT3-1 cellijn kan aangewend worden als een *in vitro* model voor de studie van oestrogene effecten in hypofysaire gonadotropen. In de gedifferentieerde LßT2 cellijn daarentegen bleken oestrogenen geen invloed te vertonen op LH en FSH productie. Het bestaan van een

oestrogeen-responsieve gonadotrope cellijn opent veel mogelijkheden voor het onderzoek naar de mechanismen van oestrogeen-actieve componenten ter hoogte van de gonadotropen. De laatste jaren is er namelijk verhoogde interesse naar het gebruik van fyto-oestrogenen (plantaardige oestrogene stoffen) als alternatief voor de klassieke hormonale substitutietherapie. Meer en meer studies tonen ook aan dat blootstelling aan xeno-oestrogenen (oestrogeen-actieve chemicaliën aanwezig in het milieu) de vruchtbaarheid negatief kunnen beïnvloeden. Vanuit de farmaceutische industrie wordt bovendien sterk geïnvesteerd in de ontwikkeling van SERMs voor de behandeling van oestrogeen-afhankelijke aandoeningen zoals osteoporose en borstkanker.

Aangezien gonadotropen een sleutelrol spelen in de regulatie van de vruchtbaarheid, vormen ze dus een mogelijk doelwit voor dergelijke stoffen. Momenteel wordt in ons laboratorium de αT3-1 cellijn aangewend voor de analyse van fyto-oestrogeen geïnduceerde effecten ter hoogte van gonadotropen. In hoofdstuk 5.9 tonen we een voorbeeld van een dergelijk experiment.

Uit onze studie kunnen we concluderen dat de gedifferentieerde gonadotrope LβT2 cellijn niet geschikt is voor ons onderzoek omwille van de aangetoonde afwezigheid van enige oestrogene respons. Daarentegen blijkt de minder gedifferentieerde αT3-1 cellijn een bruikbaar *in vitro* model te zijn door de aanwezigheid van een functionele ERα, zoals aangetoond door middel van proliferatie- en transfectiestudies. De geobserveerde inhibitie van groei- en ERα-gestuurde genexpressie kan mogelijk verklaard worden door interacties tussen ERα en de IGF-I receptor. Deze data suggereren dat IGF-I een belangrijke rol kan spelen in de regulatie van ERα-gestuurde effecten ter hoogte van hypofysaire gonadotropen. Vanuit dit standpunt kunnen we concluderen dat de αT3-1 een geschikt *in vitro* model is voor het onderzoeken van oestrogeen-actieve componenten zoals fyto-oestrogenen, xeno-oestrogenen en SERMs met als finaal doel het moleculaire werkingsmechanisme van deze stoffen ter hoogte van de gonadotropen verder uit te spitten.

Research objectives

The endogenous sex steroid 17-ß-estradiol (E_2) is a key mediator of different physiologic processes, including reproduction, bone metabolism, cardiovascular function and neuroprotection. Its action is strictly regulated in a complex manner, whereby multiple genetic and environmental factors determine the final outcome following receptor activation.

Reproduction is under control of the hypothalamo-pituitary-gonadal axis, whereby estrogens regulate gonadotropin secretion through a negative feedback system at the level of the hypothalamus and/or the pituitary. The gonadotropins luteinising hormone (LH) and follicle stimulating hormone (FSH) are secreted by the pituitary gonadotrophs following stimulus by the hypothalamic gonadotropin-releasing hormone (GnRH) and are key mediators of reproduction in both the male and the female. We were interested in the potential effects of compounds with (anti)estrogenic properties on gonadotropin regulation in the pituitary gonadotrophs. Indeed, the latter may not only be the target of endogenous estrogens.

During recent years, there has been growing interest for the use of phytoestrogens (plant-derived estrogenic compounds) as an alternative for postmenopausal hormone replacement therapy. However, such compounds may have both beneficial as well as adverse clinical effects, including the potential for increased as well as reduced risk for malignancies.

The pharmaceutical industry also focuses on the development of drugs with a selective profile of biological activity, such as selective estrogen modulators (SERMs) to use as postmenopausal substitution therapy or for the treatment of hormone-related diseases such as hormone-responsive cancers and osteoporosis.

In addition, there has been growing public and scientific concern about the raising number of fertility disorders, including decreased sperm quality, urogenital tract abnormalities and hormone-dependent cancers. Many studies suggest that exposure to xenoestrogens (being estrogenic-active chemicals present in the environment) may contribute to the development of these disorders.

In other words, gonadotrophs, which are a major player in the regulation of reproduction, are a potential target for a variety of estrogenic compounds. Nevertheless, little information is available on this topic in the literature. Therefore, the aim of the present study was to analyse estrogen receptor-mediated effects in immortalised gonadotropic cell lines to gain insight in their potential as models for the study of the effects on gonadotrophs of substances with (anti)estrogenic properties.

In the past, studies of estrogenic effects on gonadotropin regulation has been extensively studied *in vivo* in primates, rodents and a variety of other mammalian species such as sheep. These studies allow for the study of estrogenic effects on gonadotropin secretion in a physiological context. There are also obvious practical limitations inherent to *in vivo* animal studies and interpretation of findings is always complex, which makes them less suitable for screening of effects on gonadotropin regulation of the numerous natural and synthetic compounds with (anti)estrogenic properties. Other approaches are the *in vitro* study in pituitary explants or primary cell cultures. However, gonadotrophs represent 10% of the total pituitary cell population, and although presence of other cell types in the culture may offer the advantage of allowing for potentially important cell-cell interactions, this also complicates data analysis. Moreover, standardisation of primary cell cultures is difficult.

The study of gonadotroph-specific biochemical and molecular processes was facilitated by the development of immortalised, gonadotropic cell lines. In the present study, the murine LßT2 cell line was our primary choice because of its characteristics of mature gonadotrophs *in vivo*, including gonadotropin-releasing hormone receptor (GnRH-R) expression and gonadotropin secretion. Furthermore, a limited number of publications reported estrogen-responsiveness of the LßT2 cell line.
The murine αT3-1 cell line reflects an early stage of gonadotroph differentiation as these cells express gonadotroph-specific markers but lack gonadotropin ß-subunit expression and corresponding gonadotropin secretion. In addition, several reports describe the presence of a functional estrogen receptor.
As both LßT2 and αT3-1 cell lines differ in their stage of differentiation, analysis of estrogenic effects in these cells might provide useful information concerning the role of estrogens during embryonic development of pituitary gonadotrophs

Following the introductory chapter and a methodology section, we describe our experimental work on the effects of E_2 on LH synthesis and secretion in the LßT2 cell line. In view of the selective lack of functional estrogen receptor (ER) expression in these cells, the ER-positive αT3-1 cell line served as an alternative *in vitro* model to study the response to estrogens, SERMs and growth factors on gonadotroph function; The ER-mediated effects in the αT3-1 cells are, at least in part, modulated by growth factors such as insulin-like growth factor I (IGF-1).The observations are reported in chapter 4.2. We conclude with a summary of our findings, general discussion and perspectives.

Chapter 1 Introduction

1.1 The Hypothalamo-Pituitary-Gonadal axis

1.1.1 Introduction

In mammals, reproductive competence depends on the normal functioning of the hypothalamic-pituitary-gonadal axis. The episodic release of the hypothalamic gonadotropin-releasing hormone (GnRH) results in the pulsatile secretion of the gonadotropins luteinising hormone (LH) and follicle stimulating hormone (FSH) by the pituitary gonadotrophs (Belchetz *et al.*, 1978; Clarke and Cummins, 1982; Moenter *et al.*, 2003). In the gonads, these gonadotropins regulate gametogenesis and sex steroid synthesis. In turn, sex steroids and peptidergic gonadal secretory products can interact with the hypothalamus and/or the pituitary through a feedback loop to modulate gonadotropin secretion (Wildt *et al.*, 1981; Bousfield *et al.*, 1994; Haisenleder *et al.*, 1994; Burger *et al.*, 2004; Bilezikjian *et al.*, 2006).

In the present work, we have focused on the effects of estrogens at the level of the pituitary gonadotrophs. In this chapter, we describe the hypothalamic-pituitary interactions which are responsible for the regulation of gonadotropin secretion.

1.1.2 The Hypothalamus

1.1.2.1 Physiology

The hypothalamus is located below the thalamus, forming the major portion of the ventral region of the diencephalon and functioning to regulate certain metabolic processes and other autonomic activities (McClellan *et al.*, 2006). As a consequence of neonatal steroid exposure, the hypothalamus in rodents is sexually dimorphic, showing clear differences in both structure and function between males and females (Beyer *et al.*, 1994; Arai *et al.*, 1996; Tobet, 2002).

The hypothalamus links the nervous system to the endocrine system through the pituitary gland by synthesizing and secreting neurohormones, among which the so-called releasing hormones, controlling synthesis and secretion of hormones from the anterior pituitary gland, including GnRH (Sherwood *et al.*, 1993), on which we will focus in this chapter.

1.1.2.2 Gonadotropin-releasing hormone (GnRH)

Mammalian GnRH (GnRH-I), also called luteinising hormone-releasing hormone (LHRH), belongs to a group of seven distinct decapeptide hormones and is responsible for the release of the gonadotropins LH and FSH from the anterior pituitary. GnRH is synthesised as a larger precursor in specific, hypothalamic neurosecretory cells.

Following intracellular processing of this prohormone, GnRH is stored in neurosecretory granules, present in the nerve terminals of the median eminence. GnRH is then released into the hypophyseal portal circulation and transferred to the pituitary gonadotrophs, where it activates its own receptor (GnRH-R), located in the gonadotroph cell membrane (Sherwood *et al.*, 1993; Hadley, 1996c).

In all vertebrates, GnRH secretion shows an intermittent pulsatile pattern, entrained by a yet to be fully elucidated neural oscillator, often referred to as the “GnRH pulse generator”. The intermittent character of GnRH release is essential for the maintenance of its hypophysiotropic action. Adequate functioning of the “GnRH pulse generator” and GnRH secretion is a prerequisite for normal reproductive function, including gametogenesis and sex steroid production in both the male and the female.

The regulation of GnRH secretion is complex and involves direct and indirect influences on GnRH-neurons. Neurotransmitters originating from the adrenergic system, excitatory amino acids (e.g. glutamate), various hypothalamic neuropeptides (e.g. the opioidergic system, GnRH degradation products) and feedback signals from systemic sex steroids and peptide hormones (e.g. inhibin B) may affect GnRH release. Furthermore, stress, metabolic factors and exteroceptive stimuli (e.g. season variation) may also alter the GnRH secretion pattern. For review, see for example Brann and Mahesh, 1994; Goodman *et al.*, 1995; Clarkson and Herbison, 2006; Jawor *et al.*, 2006; Maeda and Tsukamura, 2006.

1.1.3 The Adenohypophysis

1.1.3.1 Physiology

The pituitary consists of an anterior lobe, the adenohypophysis which accounts for 80% of the pituitary’s gland weight, an intermediary lobe and a posterior lobe (neurohypophysis). The adenohypophysis is functionally connected to the hypothalamus by the portal circulation in the stalk that also contains nerve cell projections.

The anterior lobe of the pituitary produces six main hormones through specific secretory cells. The somatotrophs are responsible for growth hormone (GH) production, which regulates growth and physical development. Thyrotrophs secrete thyroid-stimulating hormone (TSH), which regulates thyroid gland function. Corticotrophs secrete adrenocorticotropic hormone (ACTH), which controls glucocorticoid and succeeded sex steroid production by the adrenal cortex. The gonadotrophs are responsible for gonadotropin (FSH and LH) secretion, which regulate gonadal function. The lactotrophs produce prolactin, which is involved in lactation. The anterior and intermediary lobes secrete melanocyte-stimulating hormone and other secretory products such as endorphins (Hadley, 1996b).

As mentioned above, gonadotropin secretion is episodic and depends on the frequency

and amplitude of the hypothalamic GnRH pulses (Wildt *et al.*, 1981).

1.1.3.2 Gonadotrophs

In this chapter, we discuss the regulation of gonadotropin synthesis and secretion by GnRH. First, we look at receptor signalling and the corresponding impact on gonadotropin gene production, together with gonadotropin packaging and secretion.

1.1.3.2.1 The GnRH-receptor

GnRH exerts its effects through specific high-affinity receptors on the membrane of the pituitary gonadotrophs. The GnRH-R belongs to the super family of the rhodopsin-like heptahelical G-protein-coupled receptors (GPCRs) and has been cloned from a wide range of vertebrate species. Other variants of the GnRH-R have also been identified but their role in reproduction remains to be determined for any species (Ruf *et al.*, 2003).
The mammalian GnRH-R is unique among rhodopsin-family receptors in lacking a carboxyl-terminal domain, which results in a relatively slow receptor internalisation, the lack of G-protein receptor kinase phosphorylation, and lack of rapid desensitisation (Chi *et al.*, 1993; McArdle *et al.*, 1995; McArdle *et al.*, 2002). Many studies have demonstrated that the GnRH-R activates phospholipase C (PLC) through $G_{q/11}$ family G-proteins in gonadotropic cell lines and in rodent pituitary primary cultures (Ruf *et al.*, 2003). In either $G_{\alpha q}$ or $G_{\alpha 11}$ knockout mice, GnRH injection enhances LH release, hereby suggesting that both G-proteins can substitute each other in GnRH receptor signalling (Stanislaus *et al.*, 1998). The GnRH-R has also been proposed to activate G_i and G_s subtype G-proteins (Hawes *et al.*, 1993; Imai *et al.*, 1996).
Agonist-binding results in the activation of PLC through the recruitment of the $G_{\alpha q/11}$ G-proteins. PLC catalyzes the hydrolysis of phosphatidylinositol 4, 5-bisphosphate to (1, 4, 5) inositol trisphosphate (IP3) and diacylglycerol (DAG); IP3 mobilizes intracellular calcium which activates conventional protein kinase C (PKC) isoforms, of which α and ßII have been identified in gonadotropic cell lines. The generation of DAG is likely to lead to the activation of novel PKC isoforms (McArdle *et al.*, 1992; Hadley, 1996a; McArdle *et al.*, 2002). The initial phase of calcium mobilisation originates from intracellular calcium stores, whereas the plateau phase depends on external calcium influx through L-type voltage-sensitive Ca^{2+} channels.

1.1.3.2.2 Gonadotropin synthesis and secretion

Gonadotropins are heterodimeric glycoprotein hormones, consisting of two subunits, namely an α glycoprotein hormone subunit (α-GSU), which is common for LH, FSH, human chorionic hormone (hCG) and TSH and a hormone-specific ß-subunit, which determines the biological activity of the hormone (Gharib *et al.*, 1990).

Gonadotropin production strongly depends on the amplitude and frequency of the GnRH pulses and is further regulated by feedback from androgens and estrogens, as well as peptidergic gonadal secretion products, in particular inhibin B.

In general, the α-GSU responds to constant exposure or high pulse frequency (10 min), whereas the LHß subunit (LHß-su) promoter responds best to lower frequency pulses (30–60 min) and the FSHß-subunit (FSHß-su) to even lower frequencies. Furthermore, *in vivo* production of the LHß-su and FSHß-su is down regulated by continuous GnRH exposure (Wildt *et al.*, 1981; Crowley *et al.*, 1985; Haisenleder *et al.*, 1991; Burger *et al.*, 2002).

The connections between the signalling network and each gonadotropin subunit promoter are unique. The common α-GSU functions as a primary gene, whereby its GnRH-induced transcription is mediated by the activation of preformed transcription factors. In contrast, the LHß-su gene has a complex promoter that requires simultaneous activation by existing and *de novo* synthesised transcription factors for activation. The differences between both promoters within the signalling network of the gonadotrophs suggest mechanisms for their differential sensitivity to patterns of receptor stimulation. Furthermore, the need for newly synthesised and preformed transcription factors and their modulation by phosphorylation, might contribute to the known frequency-decoding behaviour of the LHß-su (Ruf *et al.*, 2003).

The mechanisms underlying the effects of GnRH on the induction of FSHß are less well defined than those of the common α-GSU or the LHß su genes. Although the role of GnRH in stimulating expression of FSHß expression is well known, the *in vivo* effects are mainly indirect and result from the induction of transforming growth factor ß (TGFß) family members (Burger *et al.*, 2004).

Gonadotropin subunit expression results in the formation of precursor polypeptides, which undergo complex cotranslational processes at the level of the endoplasmic reticulum. Finally, post-translational changes at the level of the Golgi apparatus result in the final production of mature gonadotropins, which are stored in specific secretory granules (Ulloa-Aguirre *et al.*, 2001).

Gonadotrophs are reported to be bihormonal but under certain physiological and experimental conditions, monohormonal cells have been identified. Despite the bihormonal nature of gonadotrophs, these cells are able to differentially regulate gonadotropin secretion. LH is stored intracellularly and released mainly in response to pulses of GnRH through a regulated secretory pathway, although minimal release does occur constitutively. FSH is largely secreted through a constitutive pathway; however it can also be released by an alternative regulated pathway. At the sub-cellular level, specialised secretory granules have been identified in the murine LßT2 cell line (Nicol *et al.*, 2004).

The differential secretion of LH and FSH has been attributed to changes in the total number of granules and in the proportion of each type of granule. Furthermore, these granules also contain acidic soluble proteins from the granin family. At low pH and high calcium concentrations, conditions which are found in the Golgi apparatus, these proteins aggregate and are involved in the packaging of the gonadotropins into the secretory granules. In the rodent pituitary, three different granin proteins have been identified, namely, secretogranin II (SgII) and chromogranin (Cg) A and B. Several studies have demonstrated that LH is associated to SgII while FSH is linked to CgA. It has been hypothesised that the differences in the gonadotropin secretion pattern may be related to their different storage into specific secretory granules (Nicol *et al.*, 2002; Nicol *et al.*, 2004).

1.1.3.2.3 Feedback regulation of gonadotropin secretion

Gonadotropin secretion by pituitary gonadotrophs is regulated through a complex feedback mechanism, whereby sex steroids, activins, inhibins, follistatin and several neuropeptides will determine the final response to GnRH.

In mammals, LH is responsible for sex steroid production at the gonadal level. In turn, androgens and estrogens regulate gonadotropin secretion at the hypothalamic and/or the pituitary level. In the male, testicular steroids alter the GnRH pulse frequency at the level of the hypothalamus, being the major site of negative feedback action of androgens. However, there may be species-specific differences in the pathways that mediate feedback responses and how steroids regulate GnRH gene expression. The actions of testosterone (T) can be mediated either directly or following conversion to E_2 or dihydrotestosterone (DHT), but the relative importance of each remains to be determined. In the human male, estrogens do play a major role in the negative feedback regulation of LH secretion. Indirect evidence indicates that circulating estrogen levels, rather than local aromatisation in the hypothalamic and pituitary tissues, are responsible for this action (Raven et al., 2006; T'Sjoen et al., 2005).

Testicular steroids act to regulate GnRH neurons through neuronal systems that are largely unknown but include endogenous opioid peptides and gamma amino butyric acid (GABA) (Hadley, 1996d; Kaufman, 1996).

In the female, estrogens exert a negative feedback on LH release for the greater part of the ovarian cycle, at least in part through direct actions at the pituitary level. This inhibitory action is transiently reversed into marked facilitation of pituitary LH secretion during the mid-cycle gonadotropin surge (Messinis, 2006). The exact mechanisms at the pituitary level of this remarkable shift in responsiveness are yet to be fully elucidated. In rodents, but not in higher primates, the occurrence of a GnRH surge is a prerequisite for a normal preovulatory gonadotropin surge.

The feedback regulation of FSH is controlled by the actions of inhibin and activin, which belong to the transforming growth factors β (TGF-β) family. Activins are homodimers of inhibin βα and/or βB-subunits. Three different forms have been described according to the combination of these β subunits: activin (βA-βB), activin A (βA-βA) and activin B (βB-βB).

Two forms of inhibin have been identified, namely inhibin A and B, which consist of a common α subunit and the βA (inhibin A) or βB (inhibin B) subunit. Inhibin is produced by both the testis (Sertoli cells) and the ovaries (granulosa cells) and is responsible for feedback suppression of FSH secretion. Inhibin binds to and inactivates activin receptors in a competitive manner. Inhibin B is the physiologically relevant form of inhibin in this context and is a key mediator of FSH feedback regulation (Bilezikjian et al., 2006).

The various components essential for activin signalling are present in the anterior pituitary and activins have been reported to exert effects on multiple pituitary cell types, the best-characterised of which are the gonadotrophs. Bilezikjian et al (2004) demonstrated expression of inhibin/activin α and β-subunit mRNAs in anterior pituitary cells, together with the corresponding secretion of activins A and B. In the rat, activin B produced by rat pituitary cells has been reported to serve as a positive signal locally driving the expression of FSH-β and the secretion of FSH *in vitro*, and to mediate the hypersecretory FSH response to ovariectomy, *in vivo* (Corrigan et al. 1991, DePaolo et al. 1992). Through a paracrine mechanism, activin A originating from other pituitary cell types, including folliculostellate cells, may also participate in modulating the responses of gonadotrophs (Bilezikjian et al. 2003). Activins are permissive for the actions of GnRH on FSH production. In turn, GnRH pulse frequency can alter activin B production by modulating inhibin/activin βB mRNA levels (Weiss et al. 1992, Burger et al. 2002). Transcriptional studies of FSH-β and GnRH receptor promoters in αT3-1 and LβT2 cell lines demonstrated that both are modulated by activin (Fernandez-Vazquez et al. 1996, Duval et al. 1999, Pernasetti et al. 2001, Norwitz et al. 2002, Suszko et al. 2003, Bernard 2004) and involves the Smad2/3 pathway used by activin (Pernasetti et al. 2001, Norwitz et al. 2002, Suszko et al. 2003, Bernard 2004). Activin modulates gonadotroph sensitivity to GnRH by facilitating the action of GnRH to promote FSH-β and GnRH-R gene transcription (Pernasetti et al. 2001, Gregory et al. 2005).

Raising evidence suggests stimulatory effects, rather than inhibitory, of androgens on FSHβ gene transcription in rodents. Previous studies demonstrated that T increases FSHβ mRNA levels in GnRH antagonist-treated rats, whereas α-GSU and LHβ-subunit mRNA levels are decreased (Paul et al., 1990; Dalkin et al., 1992). Furthermore, T selectively induces FSHβ mRNA in both male and female primary rat pituitary cell cultures (Garhib et al., 1990; Winters et al., 1992; Leal et al., 2003). Furthermore,

GnRH-R levels in rodent pituitaries are modulated by androgens which in turn may alter responsiveness of FSHß to GnRH (Marchetti et al., 1982; Jegou et al., 1985; Kaiser et al., 1993).

Follistatins are activin-binding proteins that irreversibly bind to activins and prevent them from binding to their receptors (Michel et al. 1993, Phillips and deKretser 1998, Balemans and Van Hul 2002, Shimasaki et al. 2004). Although first identified as FSH-inhibitory components of gonadal fluids, follistatin is also expressed in many other tissues, including most cell types of the anterior pituitary (Bilezikjian et al. 2004).

Androgens have been shown to modulate levels of follistatin in the rat pituitary *in vivo* and both activin and follistatin in cultured rat pituitary cells (Kaiser et al., 1993; Bilezikjian et al., 1996; Leal et al., 2003). The above described data suggest that the mechanism of androgen action on FSHß-su might be indirect, through modulation of the activin/follistatin system in the pituitary.

Interestingly, activins were also able to enhance LHß-su gene expression in mouse primary pituitary cells and LßT2 cells. This effect was mediated through binding of smad proteins, being cellular mediators of the activin receptor signalling pathway, with specific smad binding elements (SBE), present in the promoter region of the LHß-su gene (Yamada et al., 2004). Furthermore, these proteins were able to interact with transcription factors such as Otx-1 and Ptx1, both being important mediators of LHß-su gene transcription (Coss et al., 2005).

Recently, it has been demonstrated that glucocorticoids and activin were able to synergistically enhance FSH-ßsu gene expression in LßT2 cells (McGillivray et al., 2007).

Other factors, which are not discussed in this work, that may influence gonadotropin secretion through modulation of GnRH release by hypothalamic neurons include kisspeptin, neuropeptide Y, galanin-like peptide (GALP) and proopiomelanocortine (Crown et al., 2007).

1.2 Estrogens

This chapter describes the physiology of the estrogens and their general mechanisms of action. We also discuss the cross-talk between ERs and growth factors and their importance at the level of reproductive tissues. In the last part of this chapter, a short overview is given of compounds, present in the environment, which may exert or interfere with estrogenic action in both humans and animals.

1.2.1 Physiology

Estrogens are naturally occurring cyclopentanophenanthrene compounds, derived from the precursor cholesterol. The most potent and dominant estrogen in humans is 17-ß-estradiol (E_2), but the weaker estrogens estrone (E_1) and Estriol (E_3) are also present.

In premenopausal women, E_2 is the primary sex hormone and is responsible for growth of the breast and reproductive epithelia, bone maturation and the development of the secondary sexual characteristics. The ovaries are the main source of E_2 with secondary production by the adrenal glands and conversion of steroid precursors into estrogens in peripheral tissues, in particular fat tissue (Hadley, 1996e). Both in the ovaries and the peripheral tissues, estrogens are the result of aromatisation of androgen precursors by the CYP19 enzyme aromatase.

Estrogens have always been considered as female sex hormones but their role in the development and the maintenance of the reproductive system in males is emerging (Carreau *et al.*, 2006). In the male, the testes account for 15% of circulating estrogens (Lew *et al.*, 2003); the remaining is produced in the peripheral tissues, mainly at the level of the fat tissue. Both intratesticular and peripheral estrogen production occurs through the conversion of androgens into estrogens by CYP19 enzyme aromatase (Simpson *et al.*, 1994). In the reproductive system of males, aromatase expression has been demonstrated in spermatocytes and spermatids. Furthermore, a role for estrogens in the regulation of epididymal function has been suggested (Lambard *et al.*, 2005). Estrogen production, either intratesticular or extragonadal, is of physiological significance throughout adult life.

Estrogens are not only critical for both male and female reproduction; they also play an important role in the regulation of other physiological processes. In bone, estrogen production appears to be as vital for the maintenance of bone mineralisation and prevention of osteoporosis in men as it is in women (Simpson and Davis, 2001; Compston, 2002). Individuals with a mutation of the gene encoding for ERα or a mutation in CYP19 display failure of epiphysial fusion, osteopenia and delayed bone age (Gennari *et al.*, 2004). Furthermore, in humans, estrogens may also exert a protective role in the cardiovascular system and against neurodegenerative diseases

such as stroke, Alzheimer's disease and Parkinson's disease, although reports on these effects have not been univocal (Nilsson and Gustafsson, 2002).

1.2.2 Estrogen receptors

Estrogens act through binding to specific estrogen receptors (ERs) localised intracellularly at the cytosolic and nuclear level. There are two functional ER isoforms, namely ERα and ERß, which are encoded by the genes ESR1 and ESR2, respectively. Both genes are located on distinct chromosomes (locus 6q25.1 and locus 14q22-24, respectively) and comprise eight exons which are separated by seven intronic sequences. The human ERα gene ESR1 encodes a protein of 595 amino acids with a molecular weight of about 66 kDa, while ERß has 530 amino acids and an estimated molecular mass of about 54 kDa (Walter *et al.*, 1985; Green *et al.*, 1986; Kuiper *et al.*, 1996; Mosselman *et al.*, 1996).

ERα and ERß are expressed in a variety of tissues such as reproductive tissues (e.g. breast, uterus, ovary, prostate, bladder, seminal vesicles and in the testis), the cardiovascular system, bone, liver (only ERα), the urogenital tract and the gastrointestinal tract (only ERß). The highest expression of ERß was seen in the prostate, the ovary and the lungs (Enmark and Gustafsson, 1999; Nilsson *et al.*, 2001; Nilsson and Gustafsson, 2002).

Both receptors consist of six functional domains sharing the common structure of steroid/thyroid hormone nuclear receptors (Kumar *et al.*, 1987; Truss and Beato, 1993). Between the two receptors, the N-terminal domain (A/B) is the less conserved with only 18% homology. The C domain is strongly conserved (97% homology) between both receptor isoforms and consists of two zinc fingers forming a helix-loop-helix motif to bind DNA hormone responsive elements. The D domain functions as a hinge region and contains signals for nuclear localisation of ER, while the E domain is responsible for ligand binding. Both ER isoforms bind E_2 with high affinity, although they share only 58% of homology in this domain (Tsai and O'Malley, 1994; Mangelsdorf *et al.*, 1995; Kuiper *et al.*, 1998c; Osborne and Schiff, 2005). The structure of both human ER isoforms is illustrated in figure 1.1.

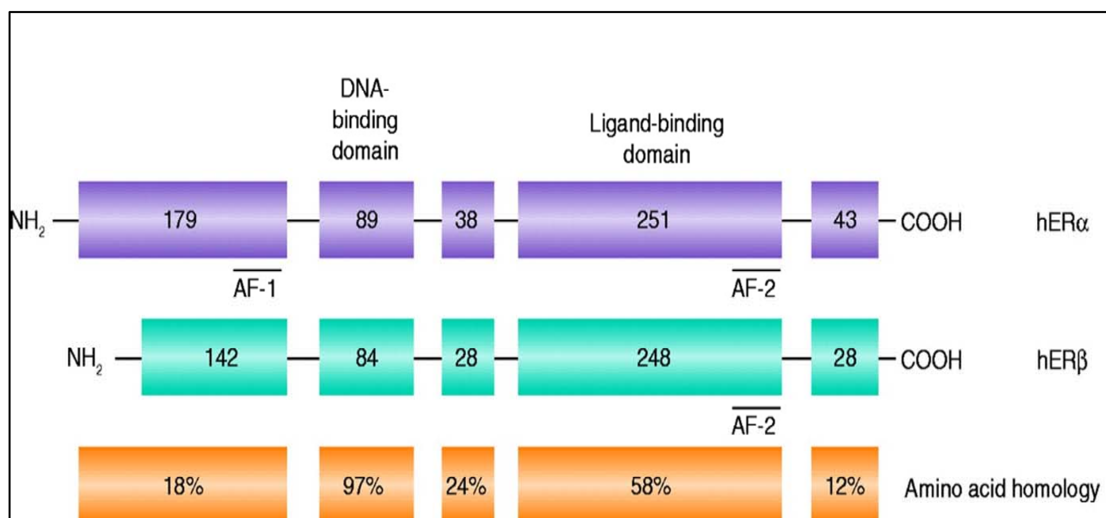

Figure 1.1: Structure of human estrogen receptor α (hERα) and β (hERβ).

Human ERα and ERβ share common structural domains. The A/B domain contains activation function 1 (AF-1), which constitutively contributes to ER transcriptional activity. This domain is one of the least conserved domains between ERα and ERβ, showing only a 30% identity. The DNA binding domain, or C domain, is the most highly conserved region between ERα and ERβ, with 97% identity. For this reason, both receptors can bind to similar target sites. The D domain, or hinge region, is not well conserved (24%) between the receptors and it contains the nuclear localisation signal. Finally, the E/F region encompasses the ligand binding domain (LBD), a coregulator binding surface, the dimerisation domain, a second nuclear localisation signal, and activation function 2 (AF-2). In contrast to AF-1, AF-2 is a ligand-dependent activation function. The E/F domains of ERα and ERβ exhibit a sequence identity of 58% (adapted from Hall and McDonnell, 2005).

Analysis of the binding affinities of a variety of synthetic or naturally occurring estrogenic compounds (including phytoestrogens and environmental estrogenic compounds) has shown that the relative binding affinities for both differ for only some compounds. Genistein and other phytoestrogens have a significantly higher binding affinity for ERβ when compared to ERα (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998a).

In contrast, Schaefer and colleagues demonstrated that 8-prenyl-naringenin (8-PN), isolated from hops, shows higher affinity for ERα in comparison to ERβ. These data were confirmed by experiments performed in our laboratory, whereby human, cervical Hela cells were transiently transfected with either human ERα or ERβ, in the presence of the estrogen-driven ERE-Luc reporter plasmid (data not shown).

Many messenger RNAs (mRNAs) have been identified for ERβ, whereby two splicing variants (ERβ1 and ERβ2) have been characterised (PriceJr *et al.*, 2000; Price *et al.*, 2001; Poola *et al.*, 2002). ERβ2 shows a lower affinity for E_2, presumably due to the 18 amino acids insertion in the ligand binding domain (LBD). The ERβ2 isoform seems to act as a suppressor of ERα and ERβ1 transactivating activity (Zhao *et al.*, 2005).

1.2.3 Estrogens: mechanisms of action

The main mechanism of action of estrogens is through estrogen receptors, which act as signal transducers and ligand-activated transcription factors. Following ligand-binding, the ligand-ER complex interacts with hormone response elements, present in the regulatory region of target genes and alters target gene transcription in a positive or negative manner. ERs are also able to regulate target gene expression through protein-protein interactions with other transcription factors. Furthermore, raising evidence demonstrates rapid, non-genomic actions of estrogens. In the next chapters, both genomic and non-genomic actions of estrogens are described in detail.

1.2.3.1 "Classical" mechanism of action of estrogens

The classical mechanism of action of estrogens involves the ER, its ligands and its coregulatory proteins. In the absence of estrogens, the ER is associated with heat-shock proteins (including hsp70 and hsp90) in a transcriptionally inactive state. Binding of E_2, its natural endogenous ligand, or another agonist induces conformational changes, hereby promoting homo- or heterodimerisation and subsequent nuclear translocation (Truss and Beato, 1993; Mangelsdorf et al., 1995).

The N-terminal activating function 1 (AF-1) domain and the C-terminal AF-2 domain are essential for the transcriptional activity of both estrogen receptors. The ligand-independent AF-1 domain is the least conserved region among nuclear receptors, both in size and sequence. Consequently, the activation capacity of AF-1 domains has been shown to vary considerably between different nuclear receptors. The AF-1 domain is a site-specific phosphorylation region, containing serine (Ser) residues at specific positions, which can be phosphorylated by cellular kinases. Following binding of E_2, ERα becomes predominantly phosphorylated on Ser-118 and to a lesser extent on Ser-104 and Ser-106. Activation of the mitogen-activated protein kinase (MAPK) pathway, for example by growth factor receptor signalling, stimulates phosphorylation of Ser-118 and Ser-167. Phosphorylation of Ser-118 leads to an increased association with known ERα co-activators. The mechanism by which Ser-104, Ser-106 and Ser-167 phosphorylation enhances ERα-mediated transcription remains unknown (Lannigan et al., 2003).

The ligand-dependent AF-2 domain is important for the interaction with co-regulators in a ligand-dependent manner (Kumar et al., 1987; Krauset al., 1995; McInerney and Katzenellenbogen, 1996; McInerney et al., 1996).

The AF-1 domain in ERα is very active in the stimulation of reporter-gene expression from various estrogen response elements (ERE)-reporter constructs in different cell lines (Walker et al., 1984; Klein-Hitpa et al., 1986). In contrast, the activity of the ERß AF-1domain is negligible (Cowley and Parker, 199). For example, both ER isoforms show

a distinctive response to synthetic estrogenic ligands such as the selective estrogen receptor modulators 4-hydroxytamoxifen (4-OH-Tam) and raloxifene. Differences in the N-terminal regions of ERα and ERß is one possible explanation for the difference between the two receptors in their response to various ligands. In ERα, two different parts of the AF-1 domain are required for the agonism of E_2 and the partial agonism of 4-OH-Tam, respectively. In ERß, this dual function of the AF-1 domain is missing. For this reason, the role of the AF-1 domain in ERß transcriptional activity remains to be clarified (McDonnell et al., 1995; McInerney and Katzenellenbogen, 1996; Paech et al.,1997).

Ligand-activated ERs interact with an ERE-sequence, present in the regulatory regions of target genes.

The consensus palindromic element ERE (5'-GGTCAnnnTGACC-3') is based on the estrogen-responsive sequence of the vitellogenin A2 promoter of the *Xenopus laevis* and functions in an orientation and distance-independent manner. This consensus sequence was established by comparing all known ERE-sequences, whereby the most common nucleotide at each position within these sequences was chosen and placed at the corresponding position in the consensus sequence. (Klein-Hitpa et al., 1986; Ponglikitmongkol et al., 1990). Furthermore, several ERE half sites have been described but ER DNA binding remains controversial. It is important to mention that only a fraction of the known mammalian estrogen-responsive palindromic EREs reflect this consensus. Many target genes contain response elements that share little similarity to consensus EREs (Kato *et al.*, 1992; Porter *et al.*, 1996; Porter *et al.*, 1997). It has been demonstrated that the sequence of the response element affects the affinity of the receptor for binding DNA. This explains, at least in part, how the sequence of the response element is an important determinant of the transactivation capacity of ERs. Furthermore, consensus and imperfect EREs also influence the relative ability of ERs to bind to cofactors (Klinge, 2000; Loven *et al.*, 2001a; Loven *et al.*, 2001b).

The genomic mechanism of action of estrogen receptors has been illustrated in figure 1.2.

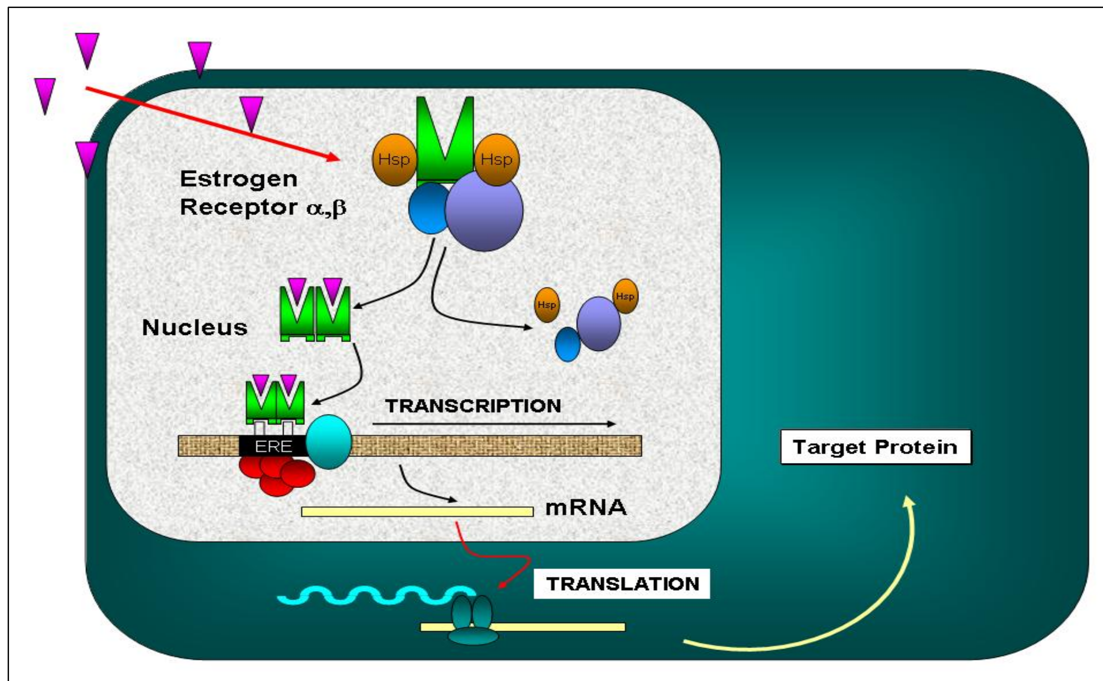

Figure 1.2: Classical, genomic action of estrogen receptors.

In the absence of estrogens, the estrogen receptor (ER) is transcriptionally inactive and forms complexes with heat shock proteins (HSP). Following ligand-binding, ER dimerises (either homo- or heterodimers), bind to an estrogen response element (ERE) in estrogen-responsive target genes and modulate target gene transcription either positively or negatively following interaction with compounds of the transcription machinery.

Despite ERs bind E_2 and SERMs with similar affinity and interact with the same EREs, the transcriptional activity of ERs is distinct. Because of the homology in their AF-2 domains, one should expect that both ERα and ERß recruit co-activators in a similar manner; however, differences have been reported. For instance, E_2 generally stimulates greater transcriptional activity via ERα than through ERß (McInerney *et al.*, 1998; Cowley and Parker, 1999; Hall and McDonnell, 1999). More marked differences are observed with SERM-bound ERs. For ERE-dependent gene expression, 4-OH-Tam is a partial agonist of ERα but is generally not capable to stimulate ERß transcriptional activity (Barkhem *et al.*, 1998; McInerney *et al.*, 1998; Hall and McDonnell, 1999). Conversely, when assessing ER activity on activating protein 1 (AP-1) containing reporter genes, 4-OH-Tam will stimulate ERα and ERß transcriptional activity in a cell-dependent fashion (Paech *et al.*, 1997).

Following binding to an ERE, the ligand-ER complex interacts with basal transcription factors and with coregulators to regulate gene expression. These coregulators can affect the magnitude of gene stimulation or repression and can alter the dose-response profile to a specific ligand. The magnitude of stimulation or repression of receptor transcriptional activity is determined by the nature of the ligand, which controls the

recruitment of coregulators to the ligand-receptor complex. Coregulators can be divided in coactivators and corepressors. These proteins do not bind to DNA, but interact indirectly through association with other DNA-binding proteins (Horwitz *et al.*, 1996; McKenna et al., 1999; McKenna and O'Malley, 2002).

Agonist-bound ERs recruit co-activators. In the last years, more than 50 co-activators have been identified. The first identified co-activator family, also called steroid receptor co-activator (SRC) consists of three related members:

- SRC-1 (p160-1, N-CoA1)
- SRC-2 (TIF-2, GRIP-1, N-CoA2)
- SRC-3 (named ACTR, AIB, P/CIP, PRIP)

These transcription factors are recruited in a ligand- and AF-2-dependent manner through a distinctive common signature motif termed the nuclear receptor (NR)-box which contains the core consensus sequence LxxLL (where L is leucine and x is any amino acid) (Leo and Chen, 2000; Xu and Li, 2003).Several reports have postulated that differences in the activities between ERα and ERβ could be due to differences in their ability to interact with co-regulatory proteins.

SRC not only interact with nuclear receptors, such as the ER, progesterone receptor and thyroid receptor (Takeshita et al., 1997; Han et al., 2006; Ying et al., 2005), but also with other transcription factors, including activator protein-1 (1 (AP-1) nuclear factor-κB (NFκB) and signal transducer and activator of transcription (STAT) (Lee et al., 1998; Werbahj et al., 2000; Arimura et al., 2004). Binding of SRC to transcription factors results in the recruitment of other chromatin modification factors, such as acetyltransferases and methyltransferases. Furthermore, SRCs can modify the chromatin structure and activate transcription of their target genes. In other words, intracellular changes of SRC concentrations may greatly affect the expression levels of many genes and, as a consequence, influence a variety of cellular processes (Chen et al., 1999; McKenna et al., 2002)

There are differences between the affinity of ERα and ERβ for members of the SRC co-activator family. In the presence of E_2, ERα was shown to bind with greater affinity when compared to ERβ, to NR-box regions of the SRC family co-activators (Wong *et al.*, 2001). There exists also a different affinity of full-length SRC family members for ERs (SRC-3 > SRC-1 > SRC-2) (Bramlett *et al.*, 2001; Bramlett and Burris, 2002). In addition, the nature of the ligand influences the relative affinity of ER for particular NR-boxes. For example, the interaction of the E_2-bound ERβ is approximately two times greater than genistein-bound ERβ to SRC-2 NR-box1, whereas genistein-bound ERβ binds approximately five times better to SRC-2 NR-box-3 than the same receptor bound to E_2.

In conclusion, the preferential binding of certain co-activators to ERα or ERβ is an important determinant for E_2 signalling (Routledge *et al.*, 2000).
While the role of coactivators for ERα is well recognised, the importance of corepressors remains unclear at this moment, as the main mechanism of ERα differs from that of other NRs, such as the thyroid hormone (TR) and the retinoic acid receptor (RAR). The latter can bind to DNA in the absence of ligand and actively repress transcription. In the presence of ligand, corepressors are released from TR/RAR, coactivators are recruited, and transcription is initiated (Taggart *et al.*, 1992). In contrast, it is generally believed that ERα only binds to DNA in the presence of ligand, eliminating the requirement of corepressors. However, several studies have demonstrated that the antagonist-ER complex recruits corepressors including REA (ER-selective repressor of ER activity), together with Nuclear Receptor Corepressor (NCoR) and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT: silencing mediator of RAR and TR) (Montano *et al.*, 1999; Delage-Mourroux *et al.*, 2000; Perissi *et al.*, 2004). Figure 1.3 illustrates the effects of coregulatory proteins on ER-functioning in the absence or presence of different ER ligands. In figure 1.4, an overview is given of all coregulators which are responsible for the regulation of ER transcriptional activity.
It is important to mention that the balance between coactivators and corepressors is an important determinant of the agonist/antagonist activity of for example SERMs in a variety of tissues such as the breast (Dobrzycka *et al.*, 2003).
In conclusion, the regulation of the ER transcriptional activity is complex and depends on different factors, including the type of ligand, the tissue-specific expression of both ER isoforms and the presence of coregulatory proteins.

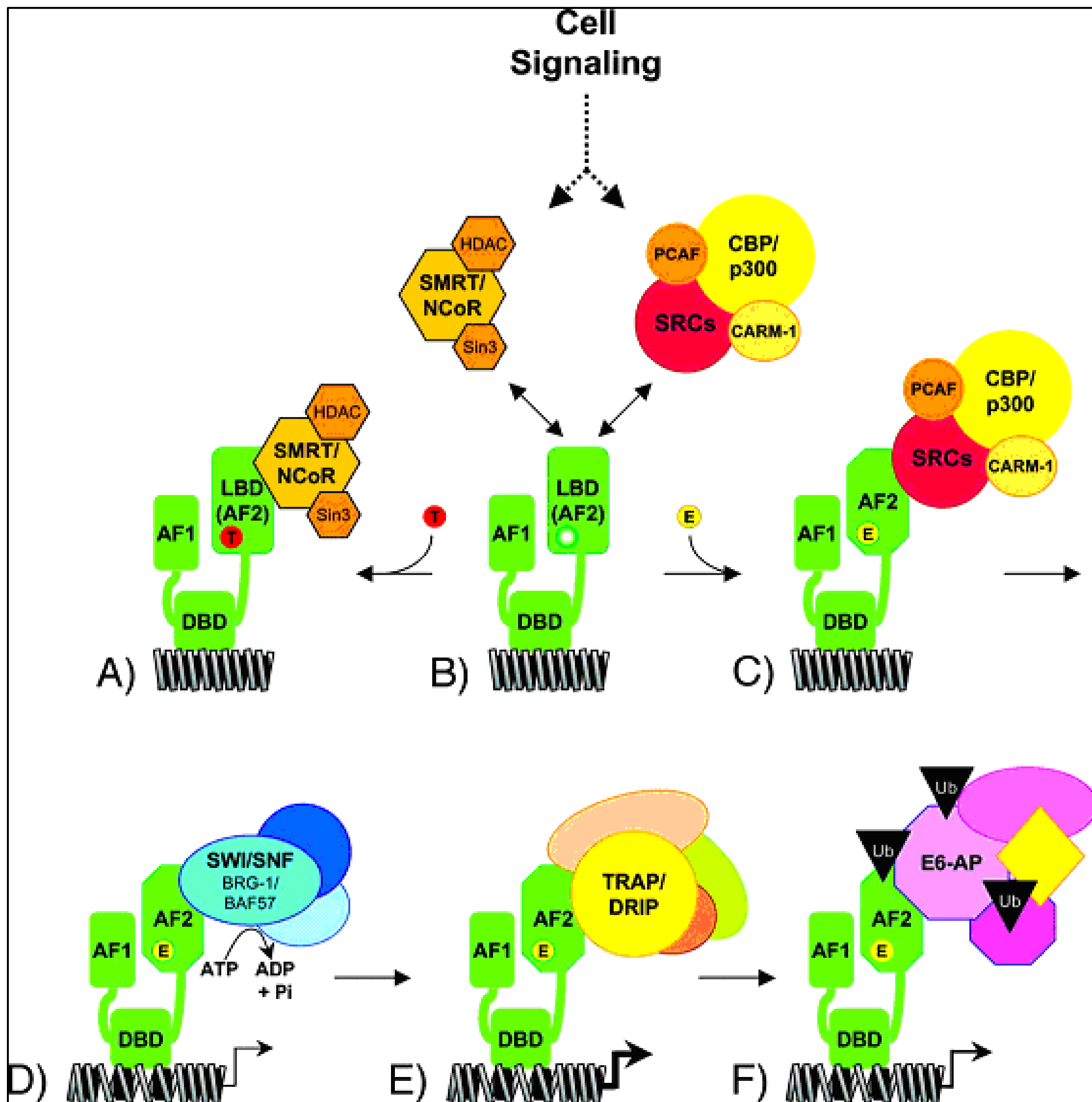

Figure 1.3: Estrogen receptor interactions with coregulatory proteins.

This represents a hypothetical schematic of the exchange of coregulators involved in ER-mediated gene activation. Following interactions of coregulatory proteins with ERα may occur: A. In the presence of a SERM such as tamoxifen (T), the receptor interacts with a complex of corepressor proteins, including SMRT and/or NCoR, which maintains the gene in an inactive state. B. In the absence of ligand, ERα may bind to either corepressor or coactivator complexes. Intracellular signalling can influence the extent of interaction with these complexes and therefore the relative magnitude of basal receptor activity: less active when bound to corepressor complexes and more active when the equilibrium is shifted to coactivator complex interaction. C–E, estrogen (E) binding activates the receptor and initiates coactivator complex formation, which is necessary for target gene transcription. This process involves histone acetylation, which is modulated by histone acetylases (HDAC) such as CBP/p300 and SRCs). Next, a complex consisting of BRG-1/BAF57, unwinds DNA and remodels the chromatin in order to initiate gene transcription by interactions with cofactor complexes. The latter may include SRC-1 or other members of the SRC-1 family. After initiation, transcription is further carried out by

other protein complexes, which, in turn, interact with RNA polymerase II. Finally, coactivator complexes and the receptor itself are turned over at the promoter by ubiquitin-proteasome-dependent processes. This turnover leads to down-regulation of receptor/coactivator levels, and is also required for efficient continued transcription of the gene (adapted from Smith and O'Malley, 2004).

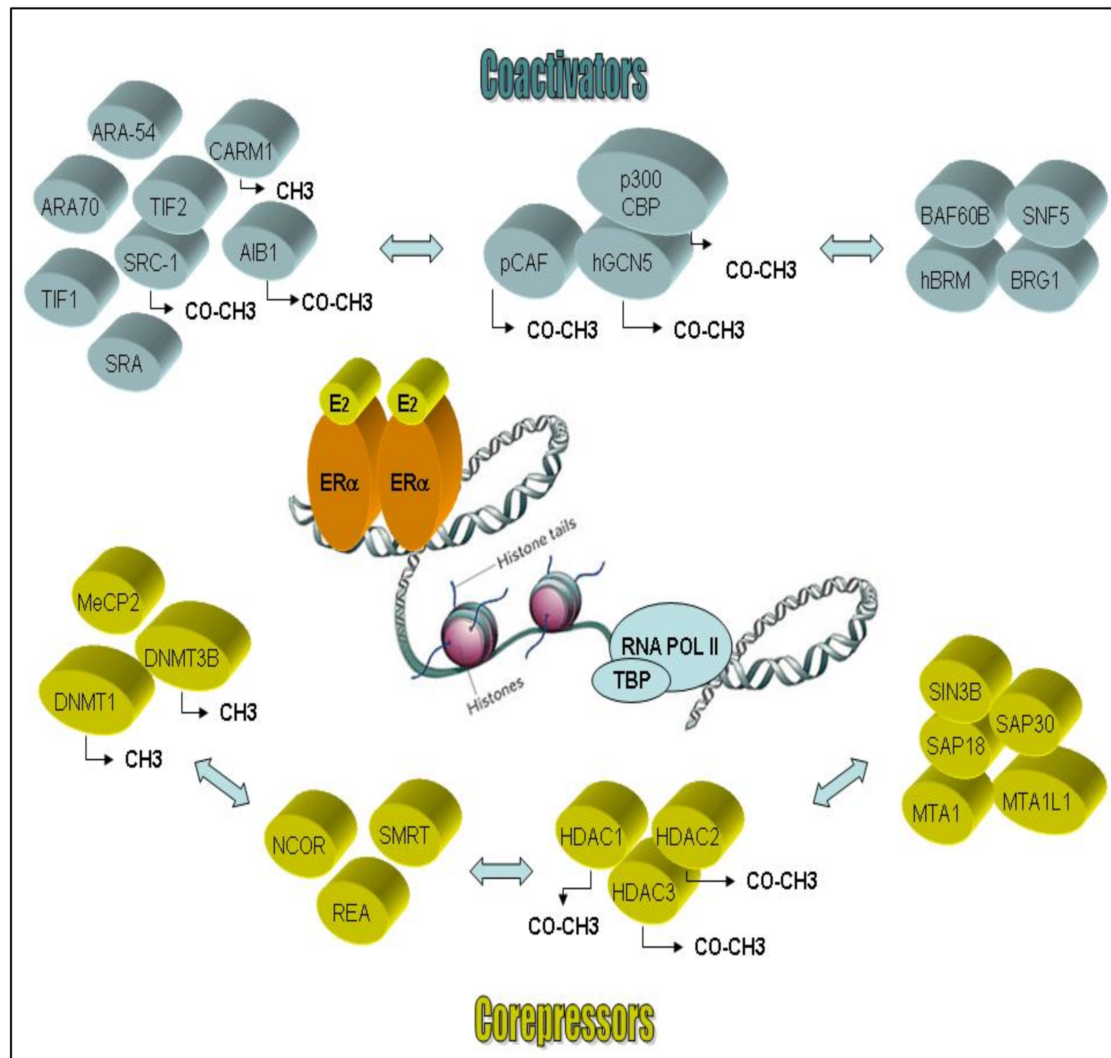

Figure 1.4: Regulation of transcriptional activity of ER target genes by coactivators and corepressors (adapted from: Girault et al., 2006).

1.2.3.2 Protein-protein interactions with transcription factors

The regulatory regions of several estrogen-responsive target genes do not contain ERE-sequences and are therefore not able to directly interact with ERs. The promoters that lack any ERE-like sequences require a second DNA-binding transcription factor to mediate ER association with the DNA, whereby ERs stabilize the DNA binding of that transcription factor. Of the human genes in which indirect binding of ER can result in estrogen regulation, stimulating protein 1 (Sp1) is the predominant mediator response

to estrogenic stimulation. Sp1 binds to the estrogen-responsive DNA-regulatory region, with ER enhancing the binding of Sp1 to the DNA and contributing to co-activator recruitment (Porter *et al.*, 1997; Duan *et al.*, 1998; Sun *et al.*, 1998). Other intermediary factors through which ER can associate with promoter/enhancers include activating transcription factor (ATF)-2/c-jun, ATF-2/CREB and nuclear transcription factor-Y (Karin, 1995; Wang *et al.*, 1999). Both ERα and ERß can interact with the fos/jun transcription factor complex on activator protein 1 (AP-1) sites to stimulate gene expression (Umayahara *et al.*, 1994; Webb *et al.*, 1999; Kushner *et al.*, 2000).

It has been suggested that AP-1-mediated estrogen-responsive genes contribute to the tissue-specific responses to estrogen analogs, via differential activation of ERα and ERß. In the presence of ERα, E_2 and Tam acts as full agonists in the AP-1 pathway, whereas raloxifene only partially activated ERα at AP-1. In contrast, in the presence of ERß, 4-OH-Tam and raloxifene behave as fully competent agonists in the AP-1 pathway, while E_2 acts as an antagonist, inhibiting the activity of both 4-OH-Tam and ral (Paech *et al.*, 1997).

Another important example of the indirect effects of estrogen receptors on gene transcription is the modulation of cyclin D1 gene expression. Cyclin D1 is important for progression of cells through the G1 phase of the cell cycle and is a well-defined target for E_2-bound ERα action in mammary carcinoma cells (Arnold and Papanikolaou, 2005). No ERE-like sequences have been identified in the cyclin D1 promoter (Herber *et al.*, 1994). However, deletion of AP-1 and Sp1 responsive elements resulted in a decrease of promoter responsiveness to E_2. Unlike ERα, E_2-bound ERß represses cyclin D1 expression and blocks ERα– E_2-mediated induction when both receptors are present (Liu *et al.*, 2002).

In conclusion, the protein-protein interactions of ERs with other transcription factors play an important role in the regulation of target genes, of which the promoter region does not contain ERE-like sequences.

1.2.3.3 Non-genomic effects of estrogens

The genomic effects of estrogens, described above, occur after a time-interval of at least two hours following E_2 exposure and explain a variety of hormone functions in physiological and pathological situations. However, rapid estrogenic actions have also been described in several reproductive tissues, including the breast, the uterus and the ovary (Aronica *et al.*, 1994; Tesarik and Mendoza, 1997; Lobenhofer *et al.*, 2000; Song *et al.*, 2002). These effects could not be contributed to genomic actions, as demonstrated by the inability of transcription and translation inhibitors to repress these effects (Losel *et al.*, 2003). Rapid non-genomic actions have been observed at concentrations varying from the picomolar to high micromolar range and are mediated

by membrane-located ERs. The exact structure of these receptors is yet to be fully elucidated. Some reports suggested that these effects are mediated through completely different receptors. In particular, the ability of E_2 to activate G-proteins through an orphan G-protein-coupled receptor-30 (GPR30) has been reported (Ahola *et al.*, 2002; Maggiolini *et al.*, 2004). GPR30 shows low capacity of E_2, with a modest generation of cyclic adenosine monophosphate (cAMP) (Filardo *et al.*, 2002). However, raising evidence indicates that the membrane-localised ER is the same protein as the NR, transported to the plasma membrane by unclear mechanisms (Pappas *et al.*, 1995; Razandi *et al.*, 2004). ERα appears to be the primary endogenous mediator of rapid E_2 actions. Less information is available concerning the role of the E_2-ERß complex to activate rapid non-genomic mechanisms. A limited number of studies suggested a possible role for ERß in the regulation of rapid non-genomic effects. For example, in Chinese hamster ovary cells, ERß is capable of activating IP3 production, extracellular-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and c-Jun kinase phosphorylation (Norfleet *et al.*, 1999; Razandi *et al.*, 1999). Another study demonstrated that the ERß- E_2 complex stimulated p38/MAPK signalling in human colon cells (Acconcia *et al.*, 2005). These data strongly indicate that also ERß could initiate signal transduction cascades through non-genomic actions.

Non-genomic effects of estrogens include calcium channel opening and calcium influx or mobilisation within seconds of binding receptors expressed in target tissues (Thomas *et al.*, 2006). E_2 rapidly generates cAMP, phospholipase C, and inositol triphosphate (IP3). These effects are mediated through G-protein activation, and these early signals result into the rapid stimulation of protein kinase C, protein kinase A, MAPK, and phosphatidyl inositol 3-kinase (PI3K) (Morley *et al.*, 1992; Lobenhofer *et al.*, 2000; Perret *et al.*, 2001). Membrane ER physically associate with and activate various G-protein α-subunits, including Gαs and Gαq (Hermans, 2003). G-protein activation explains how ER generates cAMP ($G_{\alpha s}$ function) or inositol 1, 4, 5-triphosphate and calcium ($G_{\alpha q}$ function).

G-protein coupling enables membrane ERs to initiate signal transduction at the cell surface in different cell types. For example, a rapid activation of the cAMP/PKA pathway has been demonstrated in hippocampal neurons (Gu and Moss, 1996) as well as in duodenal cells (Picotto *et al.*, 1996), pulmonary vascular smooth muscle cells (Farhat *et al.*, 1996) and rat adrenal gland cells (Chen *et al.*, 1998). Other rapid estrogenic effects have been reported, including nitric oxide synthase in endothelial cells (Chambliss *et al.*, 2005), phospholipase (PL) C-dependent inositol triphosphate (IP3) production, calcium influx, and PKCα activation in cultured endometrial cells (Perret *et al.*, 2001), liver-derived HepG2 cells (Marino *et al.*, 1998) and aortic smooth muscle cells (Incerpi *et al.*, 2003).

Estrogens can also interact with MAPK signalling pathways, either in a direct or indirect manner. For example, E_2 rapidly stimulates the activation of MAPK pathways in neuroblastoma cells (Watters *et al.*, 1997), mammary tumor-derived MCF-7 cells (Castoria *et al.*, 2001) and bone cells (Jessop *et al.*, 2001). Furthermore, MAPK signalling pathways are activated by many growth factor receptors. An example of indirect action of ERα is the activation of the insulin-like growth factor 1 (IGF-1) receptor by E_2-activated ERα, hereby leading to activation of the receptor and the corresponding activation of the MAPK signalling pathway (Kahlert *et al.*, 2000). This will be further discussed in the next chapter.

In conclusion, emerging data indicate the importance of the non-genomic, rapid actions of estrogens in different types of cells. However, the responses may vary in a cell-specific manner, as the set of signal transduction molecules and downstream targets, present in the target cells, will determine the final outcome following estrogen exposure.

1.2.4 Cross-talk of estrogen receptors with growth factor receptors

Growth factors are important mediators of cellular proliferation and/or differentiation. These proteins bind to specific high-affinity, low-capacity receptors on the cell surface of responsive cells. Many growth factors are quite versatile, stimulating cellular division in different cell types, while others are specific to a particular cell-type. An intrinsic characteristic of growth factor receptors is their tyrosine kinase activity. Ligand-binding induces receptor dimerisation and conformational changes, hereby inducing transphosphorylation of discrete tyrosine residues, present in the kinase domain of the receptor. Following receptor activation, proteins of signalling transduction pathways are phosphorylated and activated as well. Such proteins include nonreceptor tyrosine kinases such as Src family members, or Grb and SOS family proteins (Herbst, 2004; Scaltriti and Baselga, 2006). In figure 1.5, an example of the insulin signalling pathway is illustrated.

Two important examples of growth factors, which are expressed in reproductive tissues are insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF). In the uterus, E_2 stimulates the production of EGF and activates the EGF receptor (EGFR), hereby enhancing cell growth (Mukku and Stancel, 1985). Upregulation of EGF may explain the strong growth effect of E_2 on uterine epithelium, an action which can be prevented by the administration of an EGF antibody (Nelson *et al.*, 1991). Increased synthesis of EGF by E_2 confirmed the observation that the latter hormone induces EGF secretion from breast cancer cells and associates this interaction in the proliferation of hormonally responsive cancer (Dickson *et al.*, 1986).

Interactions between growth factors and ERs have been extensively investigated in

reproductive tissues. Ligand-activated growth factor receptors stimulate phosphorylation and activation of the nuclear ER through MAPK. Growth factor receptor-activated MAPK (ERK) phosphorylates serine residue 118 (Ser-118) in the A/B domain of the nuclear ERα. This results in an increased ER-related transactivation of genes that are up-regulated by growth factors (Ali *et al.*, 1993; Kato *et al.*, 1995).

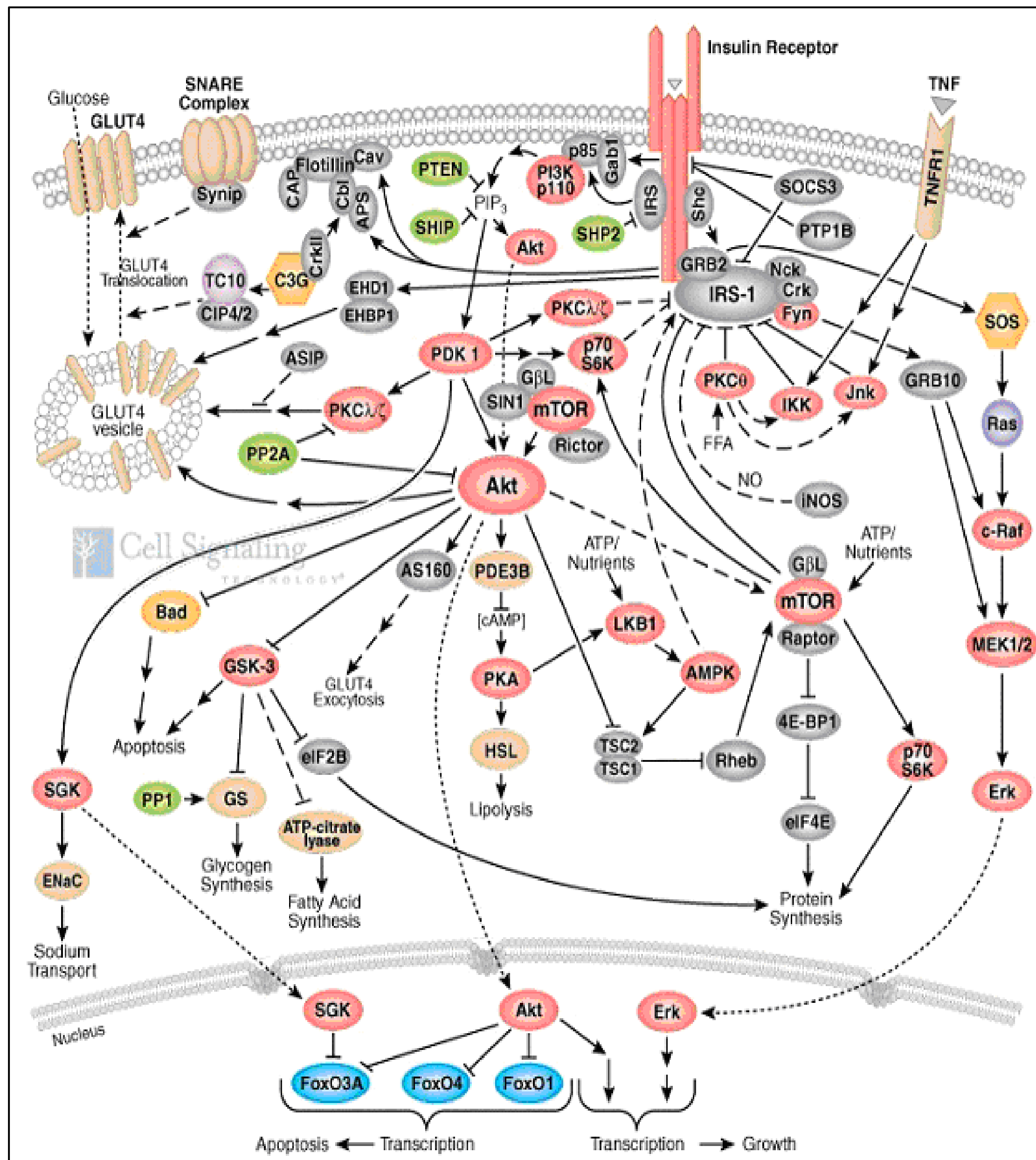

Figure 1.5: Insulin receptor signalling pathway.

Schematic overview of the signal transduction pathways, which are activated following binding of insulin to its receptor (Adapted from Cell Signalling Technology, www.cellsignal.com).

Cross-talk between growth factor receptors and ERα requires the A/B domain (Ignar-Trowbridge *et al.*, 1996). In addition, several kinases can phosphorylate additional amino acid residues within ERα, resulting in increased transcriptional activity of the nuclear receptor (Joel *et al.*, 1998; Chen *et al.*, 2002; Lee and Bai, 2002). Thus, the effects of growth factor receptor-ER interactions depend upon the signalling environment within a particular cell that differentially phosphorylates several residues in the nuclear ER. An example of cross-talk between ERα and the IGF-IR is shown in figure 1.6.

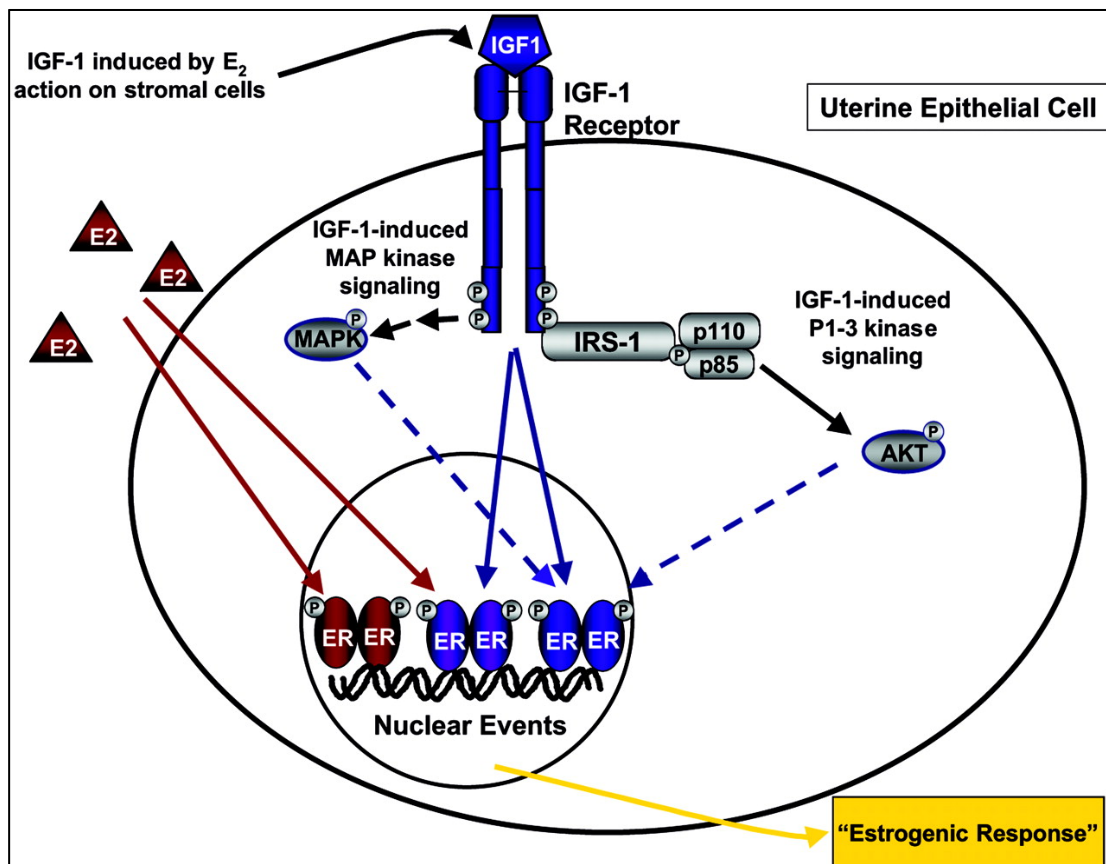

Figure 1.6: Cross-talk between IGF-1R and ERα in the uterus (Adapted from: Klotz *et al.*, 2000).

Another mechanism through which growth factor-induced signalling modulates ER transcriptional activity is via coregulator protein phosphorylation. For example, EGF induces extracellular signal-regulated kinase (ERK) phosphorylates serine 736 of glucocorticoid receptor interacting protein (GRIP) 1, resulting in increased activity of this nuclear receptor nonspecific coactivator protein (Lopez *et al.*, 2001). EGF-enhanced Src and Jnk activation may have a similar function for the cAMP response element-binding protein (Feng *et al.*, 2001). Other coactivator proteins that are

important and specific to ER function could be similarly activated or recruited through signalling-induced post-translational modifications.

In addition, growth factor receptors can also modulate cyclin D1 production, hereby promoting G1/S phase cell cycling (Wiepz *et al.*, 1997). Cyclin D1 activates ER transcriptional function and interacts with the coactivator proteins, SRC-1 and cAMP response element-binding protein/p300, as an additional mechanism to amplify nuclear ER action (Zwijsen *et al.*, 1997).

Several G-protein-coupled receptors (GPCRs) signal to ERK through the transactivation of the EGFR (Daub *et al.*, 1996). In the chapter on non-genomic, rapid effects of estrogens, we have discussed the activation of G-proteins via the membrane ER. The latter may also interact with the membrane EGFR. For example, in breast cancer cells, estrogens rapidly stimulate the EGFR, leading to cAMP and ERK up-regulation. E_2 induces the activation of matrix metalloproteinases (MMPs) 2 and 9, which enhance the release of heparin binding EGF (HB-EGF). In turn, the latter binds and activates the EGFR (Prenzel *et al.*, 2001).

The full extent of membrane-initiated signalling by E_2/ER and its dependence on growth factor receptors remains to be defined, and the *in vivo* significance remains unclear. However, EGF antibodies were able to prevent E_2-induced vaginal and uterine growth, implying that cross-talk from ER to the EGFR at the membrane may be physiologically important (Nelson *et al.*, 1991). For example, ERα directly associates with the membrane-tethered p85 subunit of PI3K. E_2 rapidly activates the latter, leading to the generation of nitrous oxide (NO) and the rescue of rats from ischemia-reperfusion injury of the muscles (Simoncini *et al.*, 2000).

The bidirectional cross-talk between ER and growth factor receptors indicates a potent method for augmenting E_2 or growth factor action. In a specific cell type, there may be an important contribution from one of these pathways, essential to the cell biology. For example, in breast cancer, tamoxifen is effective in preventing the reoccurrence of ER-positive breast cancer, in part because it inhibits aspects of E_2 and EGFR signalling (Guvakova and Surmacz, 1997). In ER-negative breast cancer, there is possibly less control on EGFR signalling to cell proliferation or survival in the absence of ER antagonism, thereby contributing to a more aggressive phenotype. In human breast cancer, ER and EGFR concentrations are inversely correlated, and ER seems to repress the EGFR gene (deFazio *et al.*, 2000; Wilson and Chrysogelos, 2002). Increased EGFRs in ER-negative breast cancer may also contribute to the more active growth and invasive behavior of these tumors (Bucci *et al.*, 1997).

Little information is available concerning the effects of growth factors such as IGF-I on gonadotropin production. Several *in vivo* studies in rats have demonstrated the enhancement of GnRH and LH release following IGF-1 treatment in animals. For

example, intraventricular administration of small doses of IGF-1 to immature juvenile or peripubertal rats increases plasma LH levels. Furthermore, immunoneutralisation of hypothalamic GnRH inhibited the increase in LH released after the third ventricular injection of IGF-1, thus demonstrating *in vivo* that IGF-1 acts, via a centrally mediated mechanism, to stimulate LH release (Hiney *et al.*, 2004).

Other groups reported the presence of IGF-1 immunoreactivity specifically in GnRH neurons of rats and mice, showing co-expression of IGF-1 in GnRH perikarya of adult female rats, hereby indicating that IGF-1 may directly regulate GnRH neuronal function (Longo *et al.*, 1998; Miller and Gore, 2001; Daftary and Gore, 2004).

IGF-1 can also exert actions on the hypothalamo-pituitary-gonadal (HPG) axis through the pituitary gonadotrophs. Expression of IGF-1, its receptor and IGF-1 binding proteins was demonstrated in the anterior pituitary gland. The main site of action of growth factors are the somatotrophs (Tannenbaum *et al.*, 1983; Bach and Bondy, 1992), nevertheless, there is also evidence for effects on gonadotrophs (Lackey *et al.*, 1999). For example, in a primary culture of anterior pituitary cells, IGF-1 significantly increases LH, FSH, and GnRH-stimulated gonadotropin secretion (Kanematsu *et al.*, 1991; Pazos *et al.*, 2004). Furthermore, the gonadotropin-stimulating effects of IGF-1 are suppressed by administration of anti–IGF-1 and anti–IGF-1R antibodies, indicating that IGF-1 also acts at the level of the anterior pituitary to stimulate gonadotropin secretion (Pazos *et al.*, 1999).

In conclusion, pituitary gonadotrophs are also a site at which IGF-1 may influence HPG function, whereby this growth factor reaches the gonadotrophs through the portal capillary vasculature or via the general circulation after being released from the liver.

1.2.5 Xenoestrogens and phytoestrogens

Via the food chain, the air and the water as well as during fetal development, man and wildlife are exposed to many agents of anthropogenic or natural origin, which can interact with the endocrine system. The largest body of evidence exists for compounds that are estrogenic in nature, but the amount of experimental data on other types of interactions, especially anti-androgenic, steadily increases (Akingbemi and Hardy, 2001). Many animal studies provided evidence that several of these chemicals can disturb sexual development and differentiation (Skakkebaek *et al.*, 2001). Because of the growing public and scientific concern, epidemiological studies have been initiated to analyse the short and long-term effects of endocrine disruptors (Eertmans *et al.*, 2003).

The US Environmental Protection Agency (US-EPA) has defined endocrine disruptors as "exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body for the

maintenance of homeostasis and the regulation of developmental processes." (Kavlock *et al.*, 1996). An increasing number of chemical compounds in the environment have been identified as endocrine disruptors using *in vitro* and *in vivo* bioassays. These include pesticides, industrial chemicals, pharmaceuticals and natural hormones acting as ligands for the estrogen-, androgen- or arylhydrocarbon receptor or exerting a combined action (Norgil *et al.*, 2002).

Much research has been performed concerning the effects of endocrine disrupting chemicals with estrogenic effects on male fertility.

Epidemiological data suggest that worldwide, since the 1940s a decrease in sperm quality may have occurred with ensuring regional differences in sperm quality and/or concentrations (Carlsen *et al.*, 1992; Ohlson and Hardell, 2000). The latter differences indicate the involvement of environmental factors. A decrease of ejaculate volume, of sperm concentration and of the percentage of normal motile spermatozoa was reported in sons of women, who were treated with diethylstilbestrol (DES) during pregnancy (Bibbo *et al.*, 1978). Other reports described an inverse correlation between the concentration of polychlorinated biphenyls (PCB) metabolites in blood and seminal plasma and sperm motility (Dallinga *et al.*, 2002). It became obvious from these studies that, in a number of cases, negative effects could be linked to exposure to environmental contaminants (beside genetic predisposition), when the latter occurred during a well-defined sensitive life stage, the so-called "critical window of exposure" (Andersen *et al.*, 2000; Anderson *et al.*, 2000).

Beside decreased sperm quality, the incidence of testicular cancer, one of the most common malignant tumors in young males, increased during the last decades (Dearnaley *et al.*, 2001). The obvious regional differences in incidence and the association with birth cohorts has suggested possible involvement of environmental factors in the development of testicular cancer (McKiernan *et al.*, 1999). Furthermore, an increase in cryptorchidism and hypospadias cases was reported by several groups (Weidner *et al.*, 1998; Hosie *et al.*, 2000). It is important to mention that the prevalence values of these disorders are sometimes difficult to interpret, due to differences in screening techniques. Although the latter differences hamper cross-study comparison, there are clear indications of a rise in incidence in a number of European countries, the United States and Japan (Jensen *et al.*, 1995; Paulozzi *et al.*, 1997; Dearnaley *et al.*, 2001).

1.2.5.1 Xenoestrogens

Among the endocrine disruptors, xenoestrogens have received most of researchers' attention until now. Xenoestrogens are synthetic substances that differ chemically from estrogens produced by living organisms but mimic or enhance the estrogen

effects. The estrogenic stimulation is an unintended side-effect of these agents or their metabolites.

They differ from phytoestrogens (estrogenic substances from plants), mycestrogens (estrogenic substances from fungi) (Branham *et al.*, 2002), and pharmacological estrogens (estrogenic action is intended). Exogenous estrogen substances from a variety of sources may have a cumulative effect upon living organisms, and xenoestrogens may be part of a larger picture of a process of estrogenisation of the environment. Xenoestrogens have only recently been introduced into the environment, as produced by industrial, agricultural, and chemical companies. These compounds show a high structural diversity and can be categorised by usage (herbicides, fungicides, insecticides) or chemical structure (PCBs, dioxins, organochlorines and alkylphenols) (Dhooge *et al.*, 2001; Eertmans *et al.*, 2003).

1.2.5.2 Phytoestrogens

Phytoestrogens are a group of biologically active plant substances which are estrogen-like compounds that occur naturally in many plants and fungi, with a chemical structure showing similarities to that of E_2 (Ibarreta *et al.*, 2001). This structural similarity accounts for the ability of these compounds to bind to ERs and exert various estrogenic or antiestrogenic effects. There are three main classes of phytoestrogens: isoflavones, coumestans, and lignans, which occur in either plants or their seeds (Kurzer and Xu, 1997; Duncan *et al.*, 2003). The isoflavones genistein, daidzein and their precursor's biochanin A and formononetin, are present in high amounts in soy seeds and have received most attention in the literature (Reinli and Block, 1996). Together with coumestrol, present in a number of Cruciferae, these compounds were the most potent phytoestrogens known so far (Kuiper *et al.*, 1998b). However, recently a newly identified phytestrogen has been isolated from hops (*Humulus lupulus*), namely 8-prenylnaringenin (8-PN) (Rong *et al.*, 2000). This compound belongs, together with 6-prenylnaringenin and isoxanthohumol to the group of prenylated flavanones (Milligan *et al.*, 1999). Several *in vitro* studies have identified 8-PN as one the most potent phytoestrogens, with a potency equivalent to, or greater than that of other established phytoestrogens, including genistein and coumestrol (Milligan *et al.*, 2000; Milligan *et al.*, 2002; Matsumura *et al.*, 2005). Currently, there is little data available concerning the *in vivo* effects of 8-PN. Daily beer intake can result in levels which fall within the range of biological activity (Possemiers *et al.*, 2006). Another study demonstrated that 8-PN was able to inhibit angiogenesis both *in vitro* and *in vivo* (Pepper *et al.*, 2004). However, a recent study demonstrated that 8-PN affects mammalian sperm function much more than E_2, hereby suggesting the involvement of mechanisms other than ER binding (Fraser *et al.*, 2006).

Several phytoestrogens including for example genistein, are claimed to help prevent or exert beneficial effects on a multitude of human disorders, including cancers, cardiovascular diseases, osteoporosis, and postmenopausal symptoms (Goldwyn *et al.*, 2000; Morton *et al.*, 2002). However more studies are required to confirm their possible health promoting potential and to trace possible adverse effects.

Phytoestrogens are able to interact with ERs, showing higher affinity to ERß than ERα (Kuiper *et al.*, 1998a). Variable effects may be observed, as ER distribution is cell- and tissue dependent (reviewed in 2.2). Other mechanisms have also been described, including the inhibition of protein tyrosine kinases (Akiyama *et al.*, 1987; Uckun *et al.*, 1995), repression of angiogenesis (Fotsis *et al.*, 1998; Pepper *et al.*, 2004; Kiriakidis *et al.*, 2005) and inhibition of cell cycle progression (Hewitt and Singletary, 2003; Shenouda *et al.*, 2004; Handayani *et al.*, 2006). They are also able to alter ER cellular levels (Cappelletti *et al.*, 2006) and sex steroid hormone binding globulin (SHBG) concentrations (Mousavi and Adlercreutz, 1993; Pino *et al.*, 2000).

Chapter 2 The Study of Estrogenic Effects in Pituitary Gonadotrophs

In this chapter, we describe the potential *in vitro* models for the study of estrogenic effects at the level of the pituitary, together with their advantages and disadvantages. For a summary, we refer to table 2.1 at the end of this chapter.

2.1 Cell cultures

2.1.1 Tissue explants and primary cell cultures

Currently, several culture methods are available to evaluate the effects of compounds in a cell- or tissue-specific manner. The oldest method is the usage of tissue explants, whereby the target tissue or organ is isolated from laboratory animals or humans. Primary cell cultures can be prepared from these target tissues or organs, whereby cells are cultured directly following isolation. However, these cultures can contain mixed cell types or consist predominantly of a single cell type. Given the complex interrelationships of cells in any organ or tissue, primary cultures rarely consist exclusively of a single cell type. The cell type of interest can be further purified using enzymatic methods or by physical dissociation. Alternatively, primary cultures can be maintained in conditions chosen to positively select for the survival of only one cell type.

Primary cell and organ cultures have the advantage in that they are recently removed from the *in vivo* situation and might therefore be expected to more closely resemble the function of that cell or tissue *in vivo*. The disadvantage is that these cultures are reacting to a constantly changing environment over the first days or weeks *in vitro*, including the changes in the mix of cell types in the culture, changes in cell shape, changes in cell associations, and changes in the factors secreted from the cells and the receptors and other cell surface proteins present on the cells. In addition, when different cell types are present in the culture, cellular interactions can complicate the interpretation of the observed effects. Other drawbacks are the requirement of the use of living animals or the availability of fresh tissues and the fact that preparation is mostly time consuming. Furthermore, there can be considerable variation from one preparation to another. Other disadvantages are a limited lifespan, potential contamination problems and a limited growth potential. This is important to take into consideration in determining how long primary cultures can be studied and in interpreting the results obtained (Merchant *et al.*, 1964; Freshney, 1983).

2.1.2 The pituitary

The aim of the present work is to study estrogenic effects at the level of the pituitary gonadotrophs. As the adenohypophysis consists of 5 different hormone secreting cell types namely gonadotrophs, lactotrophs, thyrotrophs, somatotrophs and corticothrops (reviewed in 1.3.1), interpretation of gonadotroph-specific manner is complicated by several factors. First of all, the percentage of gonadotrophs in the pituitary is quite low, varying between 10-15% (Wang, 1988). Furthermore, interactions between gonadotrophs and lactotrophs or somatotrophs are described in the literature and, albeit at least in part of these interactions are physiologically relevant; they also may also complicate the interpretation of the obtained data (Cheung, 1983; Denef and Andries, 1983; Andries *et al.*, 1995).

The ultimate goal of our project is to study potential interfering effects of estrogenic-active compounds such as xenoestrogens and phytoestrogens on reproductive function, especially through alterations of gonadotropin secretion. Therefore, we have preferentially studied models which reflect as closely as possible characteristics of mature pituitary gonadotrophs. In this particular context, the strategic choice was made to use immortalised, gonadotropic cell lines. Other potential alternatives for the analysis of estrogenic effects at the level of the pituitary, such as the use of lactotroph-related models, were not considered in this work.

2.1.3 Immortalised cell lines

The second type of cell culture is the culture of established or immortalised cell lines. A vast majority of these are derived from tumors (e.g., HeLa) or from cells transformed *in vitro*, although some cell lines were established from normal embryonic tissue (e.g., 3T3, CHO). These cell lines have been developed with the aim of maintaining a representative phenotype combined with the ability to grow the cell, or its precursor, indefinitely in culture. This can be accomplished using conditional transformation or by establishing the cell line from stem cell or precursor cells, which can then be induced to differentiate into a terminally differentiated cell type in culture.

More recently, transgenic animals have been produced that widely express transforming genes such as the Simian Virus (SV) 40 T antigen (Tag) (Ali and DeCaprio, 2001). Tissues from these animals can be used to more easily establish cell lines *in vitro*. Other transgenic animals have been created with targeted expression of transforming genes that predictably form tumors in specific tissues or cell types or with gene deletions that predictably lead to tumor formation in specific tissues (Neufeld *et al.*, 1987; Heath *et al.*, 1989).

Immortalised or transformed cell lines are extremely valuable but it is important to keep in mind that they are transformed and will therefore have properties significantly

different from those of the normal tissue from which they are derived. These differences will frequently involve changes in the growth regulation of these cells types.

In the literature, several immortalised cell lines showing gonadotropic properties have been described, including the LßT2, αT3-1, LH2, HP75 and RC-4B cell lines.

The murine, gonadotropic LßT2 and αT3-1 cell lines differ in their stage of differentiation and are derived from pituitary tumors which were isolated from male, transgenic mice. An 1800 bp fragment of the regulatory region of the rat LHß-subunit (LßT2 cell line) respectively the rat common α-subunit gene (αT3-1 cell line), was linked to the coding region of the oncogene SV40 T antigen (Windle et al., 1990; Alarid et al., 1996). This initiated a transformation process that immortalised pituitary cells at the stage when specific regulatory regions become active. During embryonic development, the earliest marker of anterior lobe differentiation is the glycoprotein hormone α-subunit, which is expressed at embryonic day 11.5 (E11.5) in mice. In the days between E11.5 and E16.5, other cell types of the anterior pituitary will differentiate, including corticotrophs, thyrotrophs and somatotrophs. The gonadotrophs are the last cells which differentiate, showing two stages, marked by expression of the ß-subunits of either LH (E16.5) or FSH (17.5) (Windle et al., 1990; Alarid et al., 1996).

According to the literature, the LßT2 cell line shows characteristics of mature gonadotrophs i*in vivo*, including the expression of a functional GnRH-R, the expression of both gonadotropin subunits and GnRH-enhanced LH secretion (Turgeon et al., 1996). Initially, it was assumed that these cells were not able to secrete FSH. However, Graham and colleagues (1999) reported that FSHß-subunit expression and the corresponding FSH release is induced by activin A treatment. Additional studies have demonstrated stimulatory actions of activin and steroids (including androgens and glucocorticoids) on FSHß-expression in LßT2 cells (Pernasetti et al., 2001; Bailey et al., 2004; Spady et al., 2004; Thackray et al., 2006; McGillivray et al., 2007).

As androgens and estrogens are important regulators of gonadotropin secretion, the LßT2 cell line served as an *in vitro* model for the study of their regulatory effects. LßT2 cells show expression of a functional androgen receptor (AR) (Curtin et al., 2001; Lawson et al., 2001; Okada et al., 2003; Spady et al., 2004). Furthermore, a limited number of publications described the presence of ER expression and estrogenic regulatory effects on LH synthesis, storage and secretion (Turgeon et al., 1996; Schreihofer et al., 2000; Nicol et al., 2002; Kowase et al., 2007). Other reports describe the importance of transcription factors such as steroidogenic factor 1 (SF-1), early growth response protein (Egr1) and pituitary homeobox 1 (Ptx1) in the regulation of LHß-subunit gene expression (Dorn et al., 1999; Rosenberg and Mellon, 2002; Buggs

et al., 2006; Lawson et al., 2007).

The αT3-1 cell line shows expression of the GnRH-R and the common α-subunit but lacks gonadotropin hormone ß-subunit expression (Alarid et al., 1996). Many groups used this cell line to analyse the GnRH-R signalling pathway (McArdle et al., 1996; Garrel et al., 1997; Kakar et al., 1997; Reiss et al., 1997; Brinkmeier et al., 1998; Poulin et al., 1998; Rose et al., 2004). The regulation of the common α-subunit gene expression and the involvement of transcription factors including SF-1 and Ptx1, has been extensively studied in αT3-1 cells (Tsujii et al., 1995; Attardi et al., 1998; Burin et al., 1998; Tremblay et al., 1998; Wood et al., 1998; Bryan et al., 1999; Fowkes et al., 2002; Harris et al., 2003; Xie et al., 2005; Aikawa et al., 2006). Several reports described the presence of the AR (Okada et al., 2003; Burger et al., 2007) and ER (Schreihofer et al., 1999; Schreihofer et al., 2000; Williams et al., 2000; Demay et al., 2001; Schreihofer et al., 2001).

The LH2 cell line was generated by stably transfecting αT3-1 cells with the complementary DNA (cDNA) encoding for the rat LHß-subunit. LH2 cells expressed the rat LHß subunit and were shown to secrete LH following GnRH challenge. The authors used this cell line to investigate the effects of secretagogues on LH secretion (Yonahara et al., 2003).

The human HP-75 cell line was derived from gonadotroph tumour cells from a clinically non-functioning human pituitary adenoma. HP-75 cells expressed chromogranin A, both gonadotropins and enzymes (proconvertases) which are involved in chromogranin processing, hereby reflecting properties of differentiated gonadotrophs *in vivo*. HP-75 cells have been mostly used to analyse the effects of growth factors and leptin at the level of the pituitary (Tsumanuma et al., 2000; Onguru et al., 2004).

The rat RC-4B cell line was derived from a pituitary adenoma and consists of all five pituitary cell types (Berault et al., 1990; Polkowska et al., 1991). These cells show morphological characteristics of differentiated anterior pituitary cells. Following immunocytochemical analysis, a part of these cells (20% and 8.6% respectively) appeared to be positive for both gonadotropin subunits and the GnRH-R. Other present cell types expressed prolactine, growth hormone, the thyroid stimulating hormone ß (TSHß) subunit and adrenocorticotropic hormone (ACTH), respectively (Berault et al., 1990). Because of the presence of all these hormone-secreting cell types, interpretation of obtained data is more complex.

In summary, we have retained the LßT2 and αT3-1 cell lines for the further investigation of estrogenic effects at the level of the pituitary gonadotrophs because 1) for both cell lines indications of estrogen responsiveness were available in the literature and 2) they were the best established gonadotroph-related cell lines in the literature. A summary of the characteristics of both cell lines in relation to mature

gonadotrophs *in vivo*, based on the available literature is shown in table 2.2. Finally, the properties of the LH2 and HP-75 cell lines make them potentially interesting alternative models for the LβT2 and αT3-1 cell lines. However, at the time that our work was already in a more advanced stage, we did not have access to the LH2 cell line. Furthermore, to the best of our knowledge, as to date neither for these cells nor for HP-75 cells are literature data available showing estrogenic responsiveness.
In conclusion, we have chosen to use the immortalised LβT2 and αT3-1 cell lines for our experiments. In view of the ultimate goal of this project, being the development of an approach for analysis of estrogenic actions of compounds specifically at the level of in a gonadotrophs, gonadotropic cell lineages appeared to be the most appropriate *in vitro* study model. Nevertheless, it should be pointed out that this is a strategic choice in a particular context and that for other purposes, such as the study of some aspects of the physiological regulation of gonadotropin secretion and gonadotroph function, primary cell cultures and/or tissue explants can be preferable *in vitro* models.

Table 2.1: Advantages and disadvantages of different culture models.

	Advantages	*Disadvantages*
Organ culture	• Maintained tissue architecture • Normal physiological functions and interactions are maintained • Cells remain fully differentiated	• Limited growth potential & viability • Fresh explantation is required for every experiment • Rather large number of animals needed • Potential for variability depending on animal status at sacrifice(e.g. stress, health status)
Tissue culture	• Some normal functions are maintained • Cells remain fully differentiated	• Variable & limited duration of viability • Labour intensive and relatively expensive • Rather large number of animals needed
Primary cells	• Differentiated characteristics of cell *in vivo* • Normal physiological functions are maintained to various degree according to type of culture (e.g. 3D reaggregation allows analysis of interactions between different pituitary cell types)	• Loss of normal tissue architecture (partially compensated in 3D aggregates) • Initally heterogeneous but later dominated by fibroblasts • Variable & limited duration of viability • Rather large number of animals needed
Immortalised cell lines	• Continuous use / longer viability • No interference of other cell types • Reduction of animal use • Standard procedures facilitating interlaboratory reproducibility	• Absence of normal physiological, intercellular interactions • Potential loss of cell-specific characteristics / dedifferentiation • Physiological relevancy of observed responses?

Table 2.2: Properties of the gonadotropic LβT2 and αT3-1 cell lines as compared to mature gonadotrophs, according to literature data (see text; NK: not known).

Mature Gonadotrophs	*LβT2*	*αT3-1*
• Gonadotropin-releasing hormone receptor	+	+
• α-glycoprotein hormone subunit	+	+
• Luteinizing hormone β subunit (LHβ-su)	+	-
• Follicle Stimulating Hormone β-subunit (FSHβ-su)	+	-
• Luteinizing hormone (LH) secretion	+	-
• Follicle stimulating hormone (FSH) secretion	+	-
• Estrogen receptor α (ERα) mRNA	+	+
• Estrogen receptor β (ERβ) mRNA	+	+
• Truncated estrogen receptor product 1 (TERP-1) mRNA	+	+
• TERP-1 protein	Variable	Variable
• ERα protein	+	+
• ERβ protein	NK	NK
• Androgen receptor expression (mRNA/protein)	+	+
• Glucocorticoid receptor (mRNA/protein)	+	NK
• Pituitary homeobox factor 1 (Ptx1)	+	+
• Steroidogenic factor I (SF-I)	+	+
• Early growth response protein 1 (Egr1)	+	+
• Activin receptors (ActR IA, IB, IIA, IIB)	+	+
• Smad proteins	+	+
• Chromogranin A (CgA)expression (mRNA/protein)	+	NK
• Secretogranin II (SgII; mRNA/protein)	+	NK

Chapter 3 Methodology

3.1 Introduction

In this section, we describe the general methodology of our studies on estrogenic effects in the murine, gonadotropic αT3-1 and LβT2 cell lines.

In the first part of the experimental section, we have evaluated the effects of estrogens on LH synthesis and secretion in the differentiated LβT2 cell line using transient transfection studies, the real time (or quantitative) polymerase chain reaction (PCR) technique and a commercial radioimmunoassay (RIA).

In the second part of our work, we have analysed the interactions between the IGF-IR and ER in the immature αT3-1 cell line. Proliferation experiments, transient transfection studies and Western blot analysis were performed to test our hypothesis.

In the final part of the discussed experimental section, we present some preliminary data on the growth modulatory effects of 8-prenylnaringenin (8-PN) and naringenin derivatives on αT3-1 cell proliferation. ER selectivity of 8-PN and these derivatives were tested in a human, cervical carcinoma cell line (Hela), transiently transfected with an estrogen-responsive reporter plasmid, in the presence of an expression vector encoding for either hERα or hERβ.

All applied techniques will be discussed in detail in the next sections. The choice of these procedures is based on standard techniques, as described in the literature.

3.2 Receptor expression

3.2.1 Reverse-transcription polymerase chain reaction (RT-PCR)

Two different techniques have been used to determine receptor expression in our gonadotropic cell lines, i.e. reverse transcriptase PCR (RT-PCR) and Western Blot analysis. First, the expression of the messenger RNA (mRNA), which encodes for a specific protein, has been investigated using the reverse transcriptase PCR (RT-PCR) technique. Total RNA is extracted from a fixed number of cells, using a commercial RNA extraction kit (RNeasy mini kit), according to the instructions of the manufacturer (Qiagen Benelux, Venlo, The Netherlands). RNA concentration is then determined following spectrophotometrical analysis at 260 nm (A260) using the following formula: dilution factor * A260 * 40 μg/mL (50 μg/mL for DNA). However, RNA (or DNA) extracts may be contaminated with proteins or phenol during the extraction protocol. To check for purity, for each sample the ratio of A260 and A280 is assessed. Values above 1.8 (or 1.9 for DNA) suggest that the samples are free of these contaminants. Next, extracts are diluted using RNase and DNase free water to obtain a stock concentration of 1 μg/μL. Total RNA is then reverse transcribed to its complementary DNA (cDNA) using a

commercial kit (RevertAid First Strand cDNA Synthesis Kit), according to the manufacturers instructions (Fermentas GMBH, St. Leon-Rot, Germany).
Next, the obtained cDNA is amplified using specific primer sets for each amplicon of interest. These primers are developed using primer design software, taking into account parameters such as primer length, melting temperature, GC-content and amplicon length. The reference sequences, encoding for the protein of interest, are described in the Nucleotide database of the National Centre of Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and the gene databases of Ensembl (www.ensembl.org).
Preliminary experiments are performed to determine the optimal PCR conditions for each primer set, including RNA concentration, annealing temperature and primer concentration. In the final PCR run, both positive and negative controls have been included beside the RNA extracts of interest. RNA extracted from cells, showing expression of the protein of interest, served as a positive control, while cells, lacking the presence of this protein were used as an alleged negative control. PCR efficiency was controlled by amplifying a housekeeping gene (e.g. ß-actin).
Following PCR amplification, electrophoresis was performed whereby amplicons are separated on a 2% agarose gel according to their size and electric charges. A DNA size marker, consisting of several amplicons of different lengths, served as a reference to determine the length of each amplicon. Finally, amplicons were visualised under ultraviolet light following ethidium bromide staining (Sambrook J, 1989).

3.2.2 Western Blot

RT-PCR is an excellent tool to determine mRNA expression. However, mRNA has to be translated in order to produce the corresponding protein. In addition, post-translational processes can modify the final protein structure. In order to analyse protein expression, we have used the Western blot analysis technique (Burnette, 1981).
First, cell lysates are prepared using Laemmli buffer, which consists of a buffer (mostly Tris base), a sulfhydryl compound (typically ß-mercapto-ethanol), an anionic lipophilic detergent (sodium dodecyl sulphate (SDS) and glycerol. Then, samples are boiled, which allows denaturation and complete unfolding of proteins. SDS, present in the Laemmli buffer, will surround the protein with a negative charge, while ß-mercapto-ethanol prevents the reformation of disulfide bonds. The glycerol is necessary to increase the density of the sample in order to facilitate sample loading.
The proteins of the sample are separated according to molecular weight using gel electrophoresis. Following electrophoresis, proteins are transferred from the gels onto nitrocellulose or polyvinylidene fluoride (PVDF) membranes by electroblotting. The charged proteins move from within the gel onto the membrane while maintaining the

organisation they had within the gel. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Next, the membrane is incubated in a solution of bovine serum albumin (BSA) or non-fat dry milk, containing a small percentage of detergent (e.g. Tween 20) in order to prevent non-specific protein interactions between the membrane and the antibody used for detection of the target protein.

After blocking, a primary antibody is incubated with the membrane under gentle shaking. Typically, the solution is comprised of buffered saline solution with a small percentage of detergent and with powdered milk or BSA. The antibody can be incubated for a period varying from 30 minutes to overnight. After rinsing the membrane to remove unbound primary antibody, it is exposed to another antibody, directed to a species-specific portion of the primary antibody. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to just about any mouse-sourced primary antibody.

The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This step confers an advantage in that several secondary antibodies will bind to one primary antibody, providing enhanced signal.

Most commonly, a horseradish peroxidase-linked secondary is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein (Constantine *et al.*, 1994; Lischke *et al.*, 1996; Liu *et al.*, 1997). A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot.

In the second part of the results section, phosphorylation of the serine residue at position 118 (Ser118) in the activating function 1 (AF-1) domain of ERα was investigated following exposure to E_2 and growth factors. Cells were treated for 4h with test compounds and lysates were prepared as described above. A primary antibody was used that only detects ERα when the receptor is phosphorylated at Ser118, and not at serine residues, located at other positions in the AF-1 domain. Furthermore, this antibody does not cross-react with phospho- ERα. Next, blots were stripped and stained for total ERα, using a primary antibody that recognizes both phosphorylated and non-phosphorylated ERα (total ERα). Following densitometric analysis using specialised image analysis software (Digimizer, Mariakerke, Belgium), the ratio of phospho-ERα over total ERα was made for each treatment group and corrected for ß-tubulin, serving as a loading control.

3.3 LH synthesis and secretion in the LβT2 cell line

The differentiated gonadotropic LßT2 cell line is able to produce both gonadotropins LH and FSH (Thomas *et al.*, 1996; Turgeon *et al.*, 1996). In our work, the effects of GnRH alone or in combination with E_2 on LH biosynthesis were investigated at three different levels. LH consists of a common α-subunit and a specific ß-subunit which determines the biological activity of the hormone (Gharib *et al.*, 1990). As LHß-subunit production is the rate-limiting parameter of LH synthesis, we have focused on LHß -su gene expression.

3.3.1 Transient transfections

We have performed transient transfections to analyse the promoter activity of the rat LHß-subunit gene following test compound treatment. In transfections, foreign DNA is introduced into eukaryotic cells. Experimentally, this is most often done as a transient transfection, in which the transfected gene is expressed only transiently, only for a short period of time. This process is usually used to test how various genetic modifications affect the functioning of particular genes. In transient transfection, the introduced gene can be lost from the cell at any time depending on environmental factors. At the latest, the transient gene will be lost during cell mitosis; neither daughter cell will retain the transient gene. In order to retain the introduced gene in the cell's genetic material, stable transfections must occur.

Cells can be transfected using different methods. The foreign DNA can be introduced into the cells through microinjection or using chemical or biological reagents such as a calcium ion or liposome that creates a "gate" in the cell allowing the uptake of the foreign DNA.

In our experiments, all transient transfections were performed using the commercial Lipofectamine 2000 reagent, which consists of cationic lipids. These lipids will complex DNA and allow a better uptake into the cells, as these lipids can easily merge with the cell membrane (Dalby *et al.*, 2004).

In the present work, LßT2 cells were transiently transfected with a reporter plasmid, consisting of an 1800 bp fragment of the regulatory region of the rat LHß-su gene, coupled to a gene encoding for the enzyme luciferase (Rosenberg and Mellon, 2002). When transfected cells are exposed to GnRH, the GnRH-R signalling transduction cascade will be activated in the cells, resulting in enhanced transcription of GnRH-target genes, including the LHß-subunit gene. As our reporter construct consists of the latter's regulatory region, luciferase expression will be stimulated by GnRH. Following exposure to test compounds, cells are lysed using a specific lysis buffer by adding luciferin. The latter is the enzyme substrate, which produces light at a wavelength of

562 nm when it reacts with the enzyme luciferase in the presence of oxygen and ATP. This enzyme catalyzes a light-emitting (chemiluminescent) reaction whereby the intensity of the response depends on the amount of luciferase produced in the cells. This reaction is measured using a luminometer (Gould and Subramani, 1988). This reaction is measured using a luminometer.

It is important to mention that transfection efficiency may vary between experiments. For this reason, the cellular protein content, which is an indication for the number of cells, can be determined using standard protein assays, such as the bicinchoninic acid (BCA) (Walker, 1994) or Lowry assay (Waterborg and Matthews, 1994). Luciferase values are then corrected for protein content. Another method is the co-transfection of a reporter plasmid, encoding for a gene that is constitutively expressed such as the house-keeping genes ß-galactosidase (ß-gal) and glyceraldehyde-3-phosphate dehydrogenase (GADPH). These enzymes also catalyze light-emitting reactions in the presence of a specific substrate. Luciferase values are then normalised for ß-gal or GADPH values, hereby by-passing the differences in transfection efficiency.

3.3.2 LHß-subunit mRNA expression in the LßT2 cell line

Activation of the promoter region of a target gene results in enhanced gene transcription and corresponding mRNA expression. In the LßT2 cells, we have evaluated LHß-subunit mRNA expression following test compound exposure. For this purpose, we have used real-time PCR analysis based on the Taqman® assay (Watson and Li, 2005). This assay is based on a classic PCR reaction; however, the reaction mix also contains a specific probe that binds to the amplicon of interest. This probe is designed in parallel with the choice of the primer set, using specific primer design software for real-time PCR analysis. It consists of two types of fluorescent labels. While the probe is attached or unattached to the template DNA and before the polymerase acts, the quencher (Q) fluorophore (usually a long-wavelength colored dye, such as red) reduces the fluorescence from the reporter (R) fluorophore (usually a short-wavelength colored dye, such as green). It does this by the use of Fluorescence Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. The reporter dye is found on the 5' end of the probe and the quencher at the 3' end. Once the TaqMan® probe has bound to its specific piece of the template DNA after denaturation (high temperature) and the reaction cools, the primers anneal to the DNA. Taq polymerase then adds nucleotides and removes the Taqman® probe from the template DNA. This separates the quencher from the reporter, and allows the reporter to emit its energy. The more times the denaturing and annealing takes place, the more opportunities there are for the Taqman® probe to bind and, in turn, the more emitted light is detected. The light emitted from the dye in the excited state is

received by a computer and shown on a graph display showing PCR cycles on the X-axis and a logarithmic indication of intensity on the Y-axis.

In theory, DNA of RNA can be amplified infinitely. However, it is important to mention that, in practice, this does not occur. PCR amplification shows a sigmoid pattern, reaching a plateau when the number of PCR cycles rise. The more RNA or DNA is present in a sample, the faster the amplification curve will reach its plateau phase.

To analyse the obtained real-time PCR data, a predetermined threshold value is chosen in such a manner that it intersects with all PCR amplification curves during their exponential phases. When the threshold value is set, CT-values can be determined for all samples. This value corresponds to the intersection of the threshold and the PCR amplification curve. This means that the more mRNA of interest is present in a sample, the lower the number of PCR cycles is necessary to achieve a plateau and the lower the CT-value will be.

Finally, CT-values for each sample are quantified against a standard curve, prepared by a serial dilution (1/10) of a fixed amount of total RNA. Furthermore, the CT-values for the housekeeping gene ß-actin, which was amplified in parallel with the amplicon of interest, were quantified against a separate standard curve, identically prepared as described above. For all samples, the corresponding dilutions were determined from both standard curves and the dilution ratio for LHß-su and ß-actin was then calculated.

3.3.3 Luteinising hormone secretion in the LßT2 cell line

Two different stimulation protocols, varying in the time of exposure, were applied to evaluate the effects of GnRH, alone or in the presence of E_2, on LH secretion in the LßT2 cell line. Following stimulation, medium samples were collected and stored at -20°C until analysis. Rat LH concentrations were determined using a commercial radio-immunoassay (RIA) according to the instructions of the manufacturer (Amersham Pharmacia (GE Healthcare, Diegem, Belgium). The detection limit of the assay was 0.8 ng/mL. The concentration range of the kit was between 0.8 and 50 ng/mL.

3.4 Estrogenic responses in the αT3-1 cell line

The response to estrogens was evaluated in the αT3-1 cell line using two different techniques, namely transient transfection studies and proliferation experiments. Both techniques are described in the next two sections.

3.4.1 Transient transfection studies

In the second and final part of the results section, αT3-1 cells were transiently transfected with two different estrogen-responsive reporter constructs. The ERE-TK-Luc vector consists of the vitellogenin ERE sequence coupled to the thymidine kinase promoter. The 3X-ERE-TATA-Luc reporter plasmid contains three copies of the vitellogenin ERE in the pGL2-TATA-Inr plasmid (Kalkhoven *et al.*, 1998). It has been demonstrated that estrogenic effects not only depends on the type of ERE-sequence, but also in a promoter-dependent manner. Despite the fact that both reporter constructs contain a different type of promoter, the luciferase response to E_2 was identical when transfected into the αT3-1 cell line.

Exposure of transfected αT3-1 cells to estrogens results in the activation of the estrogen receptor, which will dimerises and interact with the ERE-sequence, present in our reporter constructs. This results in enhanced luciferase gene transcription, dependent on the type and concentration of the estrogenic ligand.

The transfections in the αT3-1 cell line were performed under identical conditions as for the LβT2 cell line, as described in paragraph 3.3.1.

3.4.2 Proliferation experiments

In the present work, we have used the sulforodamine B (SRB) assay to assess growth modulatory responses following treatment with test compounds. The SRB-assay was developed in 1990 and is a rapid, colorimetric screening assay (Skehan *et al.*, 1990). It relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA). SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass. The strong intensity of SRB staining allows the assay to be carried out in a 96-well format. The assay can detect densities as low as 1,000–2,000 cells per well, and with a signal-to-noise ratio of 4.83 at a density of 5,000 cells per well. This level of sensitivity is comparable to those of fluorescent dye-staining methods, and is superior to those of other protein-staining methods using conventional

visible dyes (Skehan *et al.*, 1990). In addition, the SRB method has proven to be practical, because after the TCA-fixed and SRB-stained cell monolayers are dried they can be stored indefinitely. Color extracted from SRB-stained cells is also stable. With its high level of sensitivity, adaptability to the 96-well format and endpoint stability, the SRB assay is well suited to large-scale screening applications, as well as research.

The effectiveness of the SRB assay is frequently compared to that of another method using the tetrazolium dye 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). The MTT assay requires cellular metabolic activity to convert the colorless tetrazolium to the purple-colored formazan dye; therefore, it detects only viable cells, whereas the SRB method does not distinguish between viable and dead cells. This difference, however, does not compromise the ability of the SRB assay to detect cytotoxic effects of a drug. Studies undertaken by several groups showed that results from the SRB assay correlated well with those of the MTT assay, although the IC50 values of compounds tested using the SRB method were slightly higher. However, the SRB assay has several advantages over the MTT assay. For example, some compounds can directly interfere with MTT reduction without having any effects on cell viability, while SRB staining is rarely affected by this type of interference. Furthermore, SRB staining is independent of cell metabolic activity; therefore, fewer steps are required to optimize assay conditions for specific cell lines than in the MTT assay (Rubinstein *et al.*, 1990; Keepers *et al.*, 1991; Griffon *et al.*, 1995; Haselsberger *et al.*, 1996).

In conclusion, the SRB-assay is an efficient and sensitive tool to assess cell proliferation.

Chapter 4 Experimental work

4.1 Estrogen Receptor Signaling is an Unstable Feature of the Gonadotropic LβT2 cell line

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Estrogen receptor signaling is an unstable feature of the gonadotropic LβT2 cell line

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Abstract

The murine, gonadotropic LβT2 cell line was assessed as a potential *in vitro* model to analyze estrogen receptor (ER)-mediated regulation of luteinizing hormone (LH) synthesis and secretion. In agreement with limited literature data, repeated exposure to (sub) physiological concentrations of gonadotropin-releasing hormone enhanced LHβ-subunit gene expression, being the rate-limiting step of LH synthesis, and the corresponding LH secretory response. However, in the same subclone of the LβT2 cell line, we observed that LH production was not affected following exposure to E_2, which is in contrast to previously reported weak or modest effects. One explanation may be the absence of measurable ERα protein expression on the one hand and impaired ER signal transduction on the other. Furthermore, an alternative ERα mRNA splicing variant was detected in the LβT2 cell line, which (theoretically) encodes for a protein that may alter ERα transcriptional activity, depending on the cellular context.

The studied LβT2 subclone did not show a generalized impairment of nuclear receptor function, as we observed androgen- and glucocorticoid-induced gene transcription, together with enhanced LH secretory response following dexamethasone treatment.

Since its development, the gonadotropic LβT2 cell line served as a reference model to study gonadotroph-specific effects because of its mature properties. Nevertheless, this cell line does not seem to be a suitable *in vitro* model for the study of estrogenic regulatory effects at the level of the pituitary gonadotrophs in view of the unstable nature of ER signaling in LβT2 cells.
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1. Introduction

Reproductive competence depends on the interplay between the different compartments of the hypothalamic–pituitary–gonadal axis. The episodic release of hypothalamic gonadotropin-releasing hormone (GnRH) results in a pulsatile pattern of luteinizing hormone (LH) secretion by the pituitary gonadotrophs (Wildt et al., 1981). Luteinizing hormone is responsible for the synthesis and the release of gonadal steroids, which in turn alter gonadotropin secretion through feedback interactions at the level of the hypothalamus and the pituitary (Belchetz et al., 1978; Haisenleder et al., 1994; Bousfield et al., 1994). In males, both testosterone (T) and its aromatization product 17-β-estradiol (E_2) contribute to the regulation of LH secretion (Hayes et al., 2000; Tilbrook and Clarke, 2001). In females, E_2 exerts a negative feedback on LH release for the greater part of the ovarian cycle, at least in part through direct actions at the pituitary level. This inhibitory action is transiently reversed into marked facilitation of pituitary LH secretion during the mid-cycle LH surge (Knobil, 1988; Herbison, 1998; Kerdelhué et al., 2002; Moenter et al., 2003).

The role of E_2 in the GnRH-mediated regulation of LH release by pituitary gonadotrophs has been extensively investigated using primary pituitary cell cultures and isolated perifused pituitaries (Emons et al., 1989; Ortmann et al., 1992a, 1992b) However, gonadotrophs represent only 10–15% of the anterior pituitary cell population (Wang, 1988) It has been established that paracrine interactions between gonadotrophs and other pituitary cell types, including lactotrophs and somatotrophs, may influence basal or GnRH-induced LH secretion (Cheung, 1983;

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Denef and Andries, 1983; Andries et al., 1995; Gregory et al., 2004), hereby complicating data interpretation. The need for *in vitro* models to study estrogenic effects at the pituitary level is further emphasized by the increasing interest for the therapeutic use of phytoestrogens and the development of selective estrogen receptor modulators on the one hand and growing concern for potentially disrupting effects of chemical pollutants with estrogenic actions on reproductive function on the other hand (Eertmans et al., 2003).

The murine, gonadotropic LβT2 cell line shows important similarities with mature gonadotrophs *in vivo*, including the expression of the GnRH receptor (GnRH-R) and the production of both gonadotropins LH and follicle stimulating hormone (FSH) (Mellon et al., 1991; Alarid et al., 1996; Thomas et al., 1996; Graham et al., 1999) Therefore, this cell line is a useful *in vitro* model to unravel cellular mechanisms involved in LH synthesis and secretion and thus potentially also for the study of estrogen-specific regulation at the level of the pituitary gonadotrophs.

In the present study, LH secretion and the corresponding LHβ-subunit (LHβ-su) gene expression were assessed in the LβT2 cells following exposure to GnRH alone or in combination with E_2.

2. Materials and methods

2.1. Plasmids, chemicals and materials

The reporter plasmid rLHβ-Luc (Rosenberg and Mellon, 2002) was kindly provided by Dr. D. Coss (University of San Diego, La Jolla, CA, USA). The ERE-tk-Luc vector (Harnish et al., 2000) was a kind gift of Dr. W. Vanden Berghe (Ghent University, Ghent, Belgium). The pSG-hERα66 (HEGO) vector (Green et al., 1994), encoding for the human estrogen receptor α (hERα) was a kind gift of Dr. J.A. Gustafsson (Karolinska Institute, Stockholm, Sweden). The MMTV-Luc reporter plasmid (De Vos et al., 1993) was kindly provided by Dr. F. Claessens (Faculty of Medicine, University of Leuven, Leuven, Belgium). The pSV-β-Gal vector, encoding for the enzyme β-galactosidase, was purchased from Promega (Leiden, The Netherlands). Gonadotropin-releasing hormone (GnRH), 17-β-estradiol (E_2), dexamethasone (Dex) and primary antibodies against α-tubulin were obtained from Sigma (Bornem, Belgium). All cell culture products and lipofectamine Plus were purchased from Invitrogen (Merelbeke, Belgium). The rat LH RIA, the anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase and ECL Western Blotting Detection Reagents were from Amersham Biosciences (Roosendaal, The Netherlands). RNAlater solution and the RNeasy mini-kit were purchased from Westburg (Leusden, The Netherlands). The Taqman PCR core reagent kit was obtained from Applied Biosystems (Lennik, Belgium). The RevertAid First Strand cDNA Synthesis Kit was from Fermentas (Sint Leon-Rot, Germany). PCR primers were purchased from Operon (Leiden, The Netherlands). Primary antibody against ERα (NCL-L-ER-6F11) was from Novocastra (Newcastle, United Kingdom).

2.2. Cell culture

The murine, gonadotropic αT3-1 and LβT2 cell lines were kindly provided by Dr. P. Mellon (University of San Diego, La Jolla, CA, USA). The human cervical Hela cell line and the human breast cancer cell line MCF-7 (subclone AZ) were a gift from Dr. P. Briand (Jack Bell Research Center, British Columbia, Canada). Lung fibroblasts and pituitaries, isolated from C57BL/6 mice, were provided by Dr. K. Vermaelen (Ghent University Hospital, Ghent, Belgium). All cell lines were grown as a monolayer in a 5% CO_2, humidified atmosphere (37 °C) and weekly passaged in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/ml penicillin G and 100 μg/ml streptomycin, except for the murine Sertoli TM4 cell line, which was grown in DMEM: Nutrient Mix F12 supplemented with 5% horse serum, 2.5% FCS, 100 units/ml penicillin G and 100 μg/ml streptomycin.

2.3. LH secretion studies

LβT2 cells (5×10^5 cells/well) were seeded in 24-well plates and incubated for 48 h, after which culture medium was replaced by phenol red-free DMEM: Nutrient Mix F12 supplemented with 5% dextran-coated charcoal (DCC) treated FCS and antibiotics, containing GnRH (0.1–1000 nM), alone or in combination with E_2 (0.2 nM) and Dex (20 nM), either alone or combined. Two stimulation setups were used whereby in a first approach, a 15 min exposure was followed by a 75 min collection period in GnRH-free vehicle. In the second protocol, the cells were incubated continuously for several hours (1–6 h). "Conditioned medium" was collected at the end of each incubation and stored at −80 °C until analysis. Cells were washed and incubated overnight in fresh steroid-free medium or medium containing one or both steroid hormones. Both stimulation protocols were repeated for the next two days. At day 5 (third day of stimulation), following medium collection, cells were counted and then stored at −80 °C in a mixture of 50 μl of phosphate-buffered salines (PBS) and 250 μl of RNAlater solution until total RNA extraction. For all experiments, experimental medium containing 0.1% ethanol, served as vehicle control, indicated by C in the figures. LH was measured using a commercial RIA (Amersham Biosciences, Roosendaal, The Netherlands) according to the manufacturer's protocol.

2.4. RNA isolation, real time PCR analysis and reverse transcription PCR

Total RNA was extracted from LβT2 and TM4 cells, and from lung fibroblasts and total pituitaries, freshly isolated from C57BL/6 mice using the RNeasy mini-kit, treated with DNase and frozen at −20 °C until analysis.

Quantitative expression of LHβ-subunit (LHβ-su) messenger RNA (mRNA) with mouse β-actin as the internal standard was performed using the Taqman® real-time analysis method on a Perkin-Elmer ABI Prism 7700 sequence detection system (Applied Biosystems, Lennik, Belgium). A LHβ-su-specific 120 bp fragment was amplified using forward primer (fp) 5′-CATCACCTTCACCACCAGCAT-3′ and reverse primer (rp) 5′-GAGGCGAAGCGCAGCTC-3′, in combination with the TaqMan® probe 5′-FAM-CCTCCCGTGCCTCAGCCAGTGT-TAMRA-3′. A mouse β-actin specific 138 bp fragment was amplified using fp, 5′-AGAGGGAAATCGTGCGTGAC-3′ and rp, 5′-CAATAGTGATGACCTGGCCGT-3′, in combination with Taqman® probe 5′-FAM-CACTGCCGCATC CTCTTCCTCCC-TAMRA-3′. All primer/probe sets were developed using the Primer Express software (Applied Biosystems, Lennik, Belgium), based on genbank entries NM_012858 (LHβ-su) and NM_007393 (β-actin). PCR conditions were as follows: a reverse transcription step at 48 °C for 30 min and an initial denaturation step at 91 °C for 10 min, followed by 40 cycles consisting of a denaturation step at 91 °C for 1 min, a 1 min annealing step at 65 °C and a 2 min extension step at 72 °C. An elongation step of 10 min at 72 °C finalized the amplification. LHβ-su mRNA levels were quantified against a standard curve, prepared by a serial dilution (1/10) of 50 ng total RNA extracted from LβT2 cells; β-actin mRNA was quantified as a reference gene against a separate standard curve. Threshold cycle (C_T) values for the LHβ-su and β-actin were obtained for each sample, the corresponding dilutions were determined from both standard curves and the dilution ratio for LHβ-su and β-actin was calculated. Values for treated cells are expressed relative to the control (untreated cells), set to 100%.

For qualitative reverse transcription PCR analysis of the androgen receptor (AR), the glucocorticoid receptor (GR) and the ERα pituitary-specific variant truncated estrogen receptor product 1 (TERP-1), total RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit, according to the manufacturer's instructions. The following PCR primers were used (fp = forward primer, rp = reverse primer): a 203 bp-specific amplicon for AR fp, 5′-CTCTTCTTCCTGGCATACTCTCTT-3′, AR rp 5′-CTGGTGGAGTTGTGAACAGAGTAC-3′, a 461 bp-specific amplicon for the GR fp, 5′-GCCTGGTGTGCTCCGATGAA-3′, GR rp, 5′-CACTGCGGCAATCACTTGGC-3′,a 370 bp-specific amplicon for TERP-1, TERP-1 fp, 5’-CCATTTCTTGAGCTTGTTGAACAG-3’, TERP-1 rp, 5’-GTGTCTGTGATCTTGTCCAGGAC-3’ (adapted from Schreihofer

et al., 2000) and a 238 bp-specific amplicon for β-actin fp, 5′-CTGGCACCACACCTTCTA-3′, β-actin rp, 5′-GGGCACAGTGTGGGTGAC-3′. Primers were based on genbank entries NM_013476, NM_008173, NM_007956 and NM_007393 for AR, GR, ERα respectively β-actin. Two microlitres of cDNA were amplified as follows: an initial denaturation step at 92 °C for 10 min was followed by 40 cycles consisting of a denaturation step at 92 °C for 30 s, a 30 s annealing step (58°) and a 45 s extension step at 72 °C. An elongation step of 10 min at 72 °C finalized the amplification. PCR products were analyzed using a 2% agarose gel electrophoresis, followed by ethidium bromide staining.

Expression of full length ERα and possible ERα mRNA splicing variants was analyzed using two primer sets. The first primer set was taken from Swope et al. (2002) and consists of a common FP and two different RPs, which are able to distinguish between full length ERα (516 bp) and a variant message (480 bp). Following RT-PCR analysis and agarose gel electrophoresis, densitometric analysis was performed using Digimizer image analysis software (Medcalc Software, Mariakerke, Belgium) to quantify full length ERα and the variant messages. The ratio of full length ERα to the variant message was calculated for each tested cell line and corrected for β-actin, which was amplified in parallel and served as a control for PCR efficiency on the one hand and as loading control on the other. The murine, gonadotropic αT3-1 cell line and the murine TM4 Sertoli cell line served as a positive control, while mouse lung fibroblasts served as an alleged negative control.

The second primer set (fp, 5′-GTCTGGTCCTGCGAAGGCTGC-3′ and rp 5′-TGACGTAGCCAGCAACATGTCAAAG-3′), adapted from the one described by Pasqualini et al. (1999) in the rat, yields an amplicon of 702 bp for full length ERα, while alternative splicing messages correspond to an amplicon of 585 bp (exon 4 deletion), 366 bp (exon 5 deletion) and 249 bp (exons 4 and 5 deletion), respectively. PCR conditions were similar to the conditions described above, differing only in annealing temperature of the reverse primer (55 °C instead of 45 °C).

2.5. Western blot

Lysates were prepared of MCF-7 cells (untransfected) and of LβT2 and Hela cells, prior to and following transfection with a human estrogen receptor α (hERα) expression vector (50 ng). Cells were washed twice with PBS and lysed in Laemmli buffer (0.25 M Tris, 1.92 M glycine and 1% sodium dodecyl sulphate in aqueous solution). Lysates were centrifuged at 14,000 rpm for 30 min at 4 °C, and protein concentration was determined by Lowry assay. All samples were diluted with sample buffer (62 mM Tris–HCl, pH 6.8, 10% glycerol, 5.0% β-mercaptoethanol, 2.0% SDS, 0.0012% bromophenol blue) and heated at 94 °C for 5 min. A total of 25 μg of protein was loaded for each sample. Electrophoresis was carried out on a 10% separating gel at 100 V for 1 h in a Mini-Protean II electrophoresis system (BioRad). After electrophoresis, proteins were transferred from polyacrylamide gels onto nitrocellulose membranes by electroblotting (0.8 mA/cm^2 constant current, 60 min, room temperature). Following transfer, membranes were blocked with 5.0% dehydrated nonfat milk in TBS-T (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.10% Tween-20) for 60 min at room temperature. Membranes were then washed three times in fresh TBS-T followed by a 1 h incubation with primary antibody. Following washing (3× TBS-T, 5 min), membranes were incubated for 1 h in secondary antibody. Immunodetection was performed with an enhanced chemiluminescence (ECL) system according to manufacturer's specifications. Chemiluminescence was captured on photographic film. Each Western blot was exposed for 15, 30 and 60 s to guarantee linearity of the film.

2.6. Transient transfection studies

Cells were grown in 24-well plates at a density of 1.25×10^5 cells per well (LβT2 cell line) respectively 3×10^4 cells per well (Hela and TM4 cell lines) in identical conditions as for the secretion experiments. Following overnight incubation, cells were transfected with rLHβ-Luc (250 ng) or MMTV-Luc (50 ng) using Lipofectamine Plus according to the instructions of the manufacturer. The ERE-TK-Luc (250 ng) reporter plasmid was transfected in LβT2 cells, alone or in combination with the HEGO expression vector, encoding for the hERα (50 ng). In each experiment, a reporter plasmid, encoding for the enzyme β-galactosidase (β-gal; 20 ng) was co-transfected in order to control for transfection efficiency. Twenty-four hours later, cells were treated for 16 h with test compounds. After cell lysis, luciferase and β-gal activities were determined using a Packard Lumicount Microplate luminometer (Packard Instrument Company Inc., Meriden, CT, USA). Luciferase values were normalized for β-gal values and the obtained results for treated cells were expressed relative to the control (untreated cells), set to 100%.

2.7. Statistical analysis

Values in the figures are presented as the average ± S.D. Curve analysis (four parameter logistic regression) was performed using Sigmaplot 8.0 (SPSS Inc., Chicago, USA). Statistical analysis was carried out by oneway ANOVA using the SPSS 11 statistical package (SPSS Inc., Chicago, USA). Significant differences were determined by the Dunnet's post hoc test; statistical significance was inferred at $P < 0.05$.

3. Results

3.1. LH secretion and LHβ-subunit (LHβ-su) gene expression following GnRH challenge

A short pulse setup was applied to investigate the LH secretory response of LβT2 cells following repeated GnRH treatment. LβT2 cells were exposed to 15 min GnRH, followed by a 75 min collection of GnRH-free medium. On the first treatment day, LH secretion, as measured by RIA, was not induced by raising concentrations of GnRH (0.1–1000 nM). On treatment day 2, LH release was significantly induced, reaching a plateau at 10 nM of GnRH. At day 3, maximal induction of secretion was shown at 10 nM of GnRH followed by a declining LH response at higher GnRH concentrations, the secretion at 1 μM being no longer different from the blank. When investigating the effect of varying the duration of GnRH exposure from 1 h up to 6 h, stimulation for 4 h was found to result in the largest difference between stimulated and unstimulated cells. Despite the fact that LH medium concentrations were approximately 1.55-fold higher for cells treated with GnRH for 4 h in comparison to LβT2 cells exposed for 15 min, no differences were observed in the LH release profile between both stimulation setups (Fig. 1A and B).

In a next series of experiments, the short pulse setup was used to investigate the effects of E_2 (0.2 nM) and Dex (20 nM) on LH secretion as assessed on the third day of repeated exposure to GnRH. 17-β-Estradiol alone did not alter cell number or the LH secretory response. In contrast, a significant ($P < 0.001$) decrease of 20% in cell number was observed in Dex-exposed LβT2 cells, with a significant ($P = 0.002$) increase in LH release, corrected for cell number, when compared to cells exposed to GnRH (10 nM) alone. We observed a comparable 19% reduction in cell number (Fig. 2) and a borderline significant ($P = 0.06$) induction of LH secretion, corrected for cell number, in LβT2 cells, treated with both E_2 and Dex, in comparison to cells treated with GnRH (10 nM) alone. Results are shown in Fig. 3.

LHβ-subunit (LHβ-su) messenger RNA (mRNA) expression, as determined using real-time PCR, was significantly ($P < 0.05$) induced by GnRH in a dose-dependent manner; at 1 μM of GnRH, mRNA levels were approximately three-fold

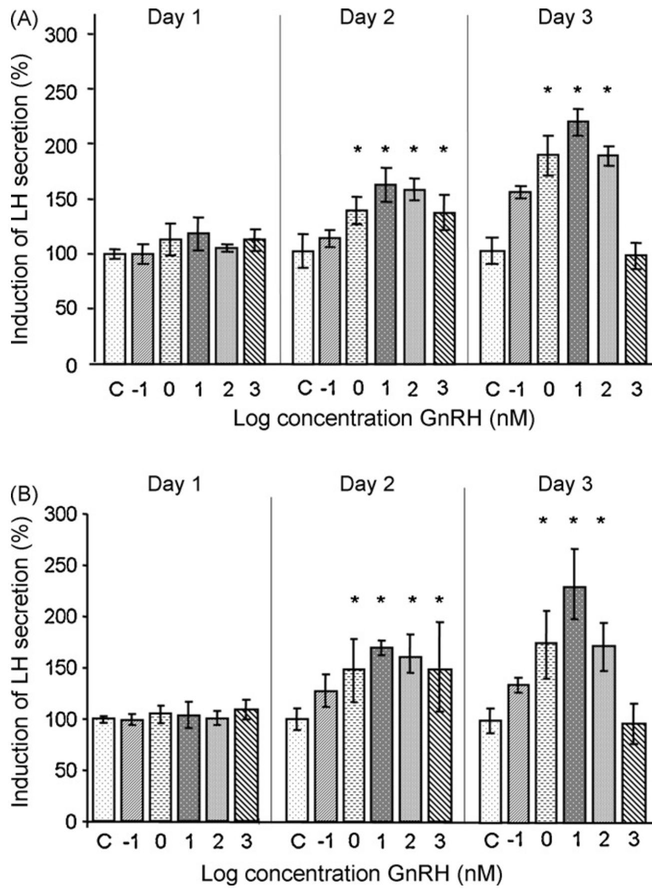

Fig. 1. Gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone (LH)-secretion in the LβT2 cell line. LβT2 cells were exposed daily to GnRH (0.1–1000 nM) during three consecutive days using a short pulse setup (Fig. 1A; 15 min GnRH–75 min collection of GnRH-free medium) or a 4 h exposure setup (Fig. 1B). Values for treated cells were corrected for cell number and are expressed relative to vehicle control (experimental medium containing 0.1% ethanol), set at 100%. Results are presented as the mean ± S.D. from three separate experiments. *$P < 0.05$ vs. vehicle control (C).

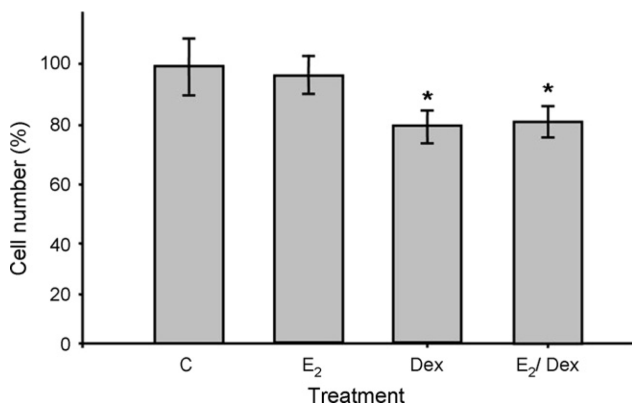

Fig. 2. Effect of E_2 (0.2 nM) and/or Dex (20 nM) treatment on cell proliferation in the LβT2 cell line. At the end of the LH secretion experiments (third day of repeated exposure to GnRH), cell number was determined using the trypan blue exclusion method. Results were expressed relative to vehicle control (C), set at 100%. Results are presented as the mean ± S.D. from three separate experiments. *$P < 0.05$ vs. vehicle control (C).

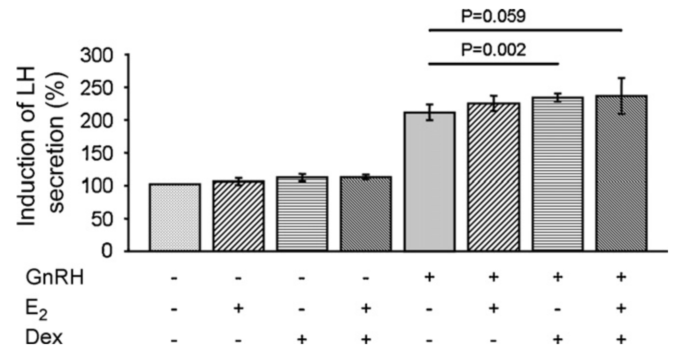

Fig. 3. Effects of different steroid treatments on LH secretion following repeated exposure to 10 nM of GnRH. LβT2 cells were exposed daily to 10 nM of GnRH during three consecutive days using a short pulse setup (15 min GnRH–75 min collection of GnRH-free medium) in the absence or presence of 0.2 nM E_2 and 20 nM Dex, alone or combined. At treatment day 3, LH medium concentrations were determined and corrected for cell number. Shown are also controls (in absence of GnRH). Values are expressed relative to vehicle control (experimental medium containing 0.1% ethanol), set at 100%. Results are presented as the mean ± S.D. from three separate experiments. *$P < 0.05$ vs. vehicle control.

higher compared to the control. In the presence of E_2 and Dex, LHβ-su mRNA levels remained unchanged in comparison to cells treated with GnRH alone (Fig. 4). The expression of mouse β-actin mRNA, which served as an internal standard, was not affected by any treatment (data not shown).

Finally, LHβ-su promoter activity was studied following treatment with GnRH using the reporter plasmid rLHβ-Luc. Luciferase activity was dose-dependently induced by GnRH, while co-incubation with E_2 and Dex did not affect promoter functioning (Fig. 5).

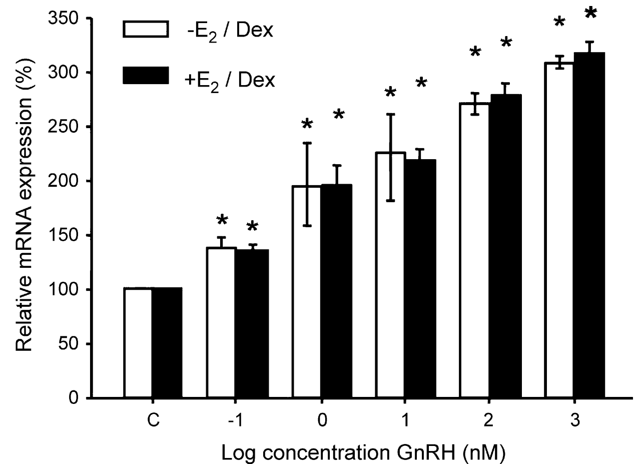

Fig. 4. Dose-dependent stimulation of rat LHβ-subunit (LHβ-su) mRNA expression by GnRH in the LβT2 cells. LβT2 cells were exposed daily to GnRH (0.1–1000 nM), alone or in the presence of E_2 (0.2 nM) and Dex (20 nM) during three consecutive days. At the final day, LβT2 cells were harvested and total RNA was extracted for real-time PCR analysis. Values for treated cells are expressed relative to their respective control (experimental medium containing 0.1% ethanol and experimental medium supplemented with E_2 (0.2 nM) and Dex (20 nM), respectively), set at 100%. Results are presented as the mean ± S.D. from three separate experiments. *$P < 0.05$ vs. respective control (C); GnRH-induced LHβ-su mRNA expression was not significantly altered following treatment E_2/Dex-treatment.

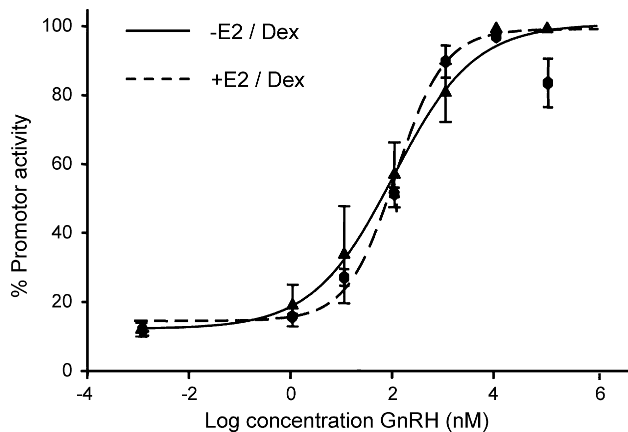

Fig. 5. Modulation of rLHβ-su promoter activity by GnRH. LβT2 cells were transiently transfected with the rLHβ-Luc construct (250 ng), which consists of an 1800 bp fragment of the 5′ flanking region of the rLHβ-su gene, coupled to a gene encoding for the luciferase enzyme. LβT2 cells were exposed to GnRH (0.1–10,000 nM), alone (full line) or in combination (dashed line) with E_2 (0.2 nM) and Dex (20 nM) for 16 h. Results are presented as the mean ± S.D. from three separate transfections.

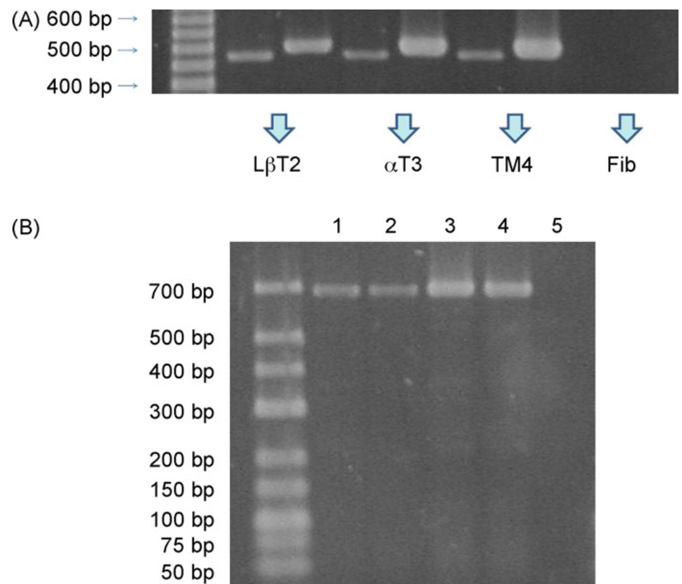

Fig. 6. Reverse-transcription PCR analysis of ERα expression in the LβT2 cell line. Fig. 6A: the expression of ERα and an alternative splice variant was investigated using reverse transcriptase polymerase chain technique (RT-PCR). Full length ERα and the alternative message correspond to an amplicon of 516 and 480 bp, respectively. The estrogen-responsive TM4 Sertoli cell line and the gonadotropic αT3-1 cell line served as a positive control, while lung fibroblasts were used as an alleged negative control. (B) RT-PCR was applied to analyze the expression of alternative ERα mRNA splicing variants, which contains an exon 3 and/or exon 4 deletion. Full length ERα mRNA corresponds to a 702 bp amplicon. Sample 1 and 2 represent two different RNA extracts of the LβT2 cell line. Samples 3 and 4 correspond to the αT3-1 and TM4 cell lines, respectively. Murine, lung fibroblast RNA (lane 5) served as an alleged negative control.

3.2. Gonadotropin subunits and hormone receptor expression

Expression of the mRNAs encoding for the GnRH-receptor and the gonadotropin subunits was demonstrated in our subclone of the LβT2 cell line and in C57BL/6 mouse pituitaries, serving as a positive control (data not shown).

Expression of full length ERα and possible ERα mRNA splicing variants was analyzed in LβT2 cells using different primer sets. The first primer set distinguishes between full length ERα (516 bp) and a variant message (482 bp), which results from inappropriate splicing at the 3′-end of exon 4. The levels of full length ERα were 1.6-fold higher in comparison to the variant message. In contrast, the ratio of full length ERα to the variant message was significantly ($P < 0.05$) higher (3.7- and 3.5-fold, respectively) in the murine, gonadotropic, αT3-1 cell line and in the murine, TM4 Sertoli cell lines, both serving as a positive control. No bands could be detected in the mouse, lung fibroblast. An example of a representative gel electrophoresis is illustrated in Fig. 6A.

In a second series of experiments, a primer set was used which is able to detect both full length ERα mRNA and splicing variants, which result from deletion of exon 4, exon 5 or the combination of both exons. Following RT-PCR analysis, only full length ERα was amplified, while no splicing variants could be detected in the LβT2 cell line. Observations were identical in the αT3-1 and TM4 cell lines, showing higher expression of full length ERα mRNA when compared to the LβT2 cells (Fig. 6B). Furthermore, we were unable to demonstrate expression of the pituitary-specific truncated estrogen receptor product-1 (TERP-1) in the LβT2 cell line, while positive results were obtained using C57BL/6 mouse pituitaries (data not shown).

Following Western analysis, neither ERα protein (Fig. 6) nor the (theoretically) expected protein of 42.3 kDa could be detected. Similar negative results were obtained for the demonstration of full length ERα (66 kDa) in LβT2 cells which were grown in DMEM containing varying concentrations (1–10%) of either non-treated or DCC-treated FCS, respectively (data not shown).

Transfection with an expression vector, encoding for the human ERα (HEGO), strongly induced receptor expression. Lysates from human MCF-7 breast cancer cells and from human cervical Hela cells, transfected with the HEGO expression vector, served as a positive control (Fig. 7). In contrast to the low ERα mRNA abundance, the glucocorticoid receptor (GR) and the androgen receptor (AR) were strongly expressed in the LβT2 cell line. For all amplicons of interest, all primer sets yielded negative results when applied to mouse fibroblast mRNA which served as an alleged negative control. Mouse β-actin expression was used to control for PCR efficiency (data not shown).

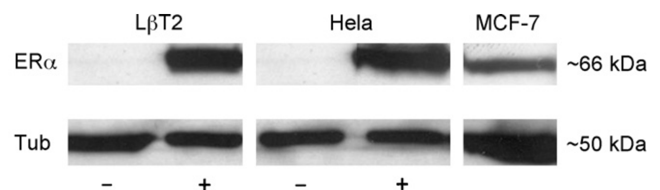

Fig. 7. Western Blot analysis of ERα expression in the LβT2 and the Hela cell line. Lysates were prepared from LβT2 and Hela cells, without (−) and with (+) prior transfection with a human ERα expression vector, and analyzed for the presence of ERα protein (66 kDa fragment). The human MCF-7 cell line served as a positive control. Mouse tubulin (Tub.; 50 kDa fragment) was used as loading control.

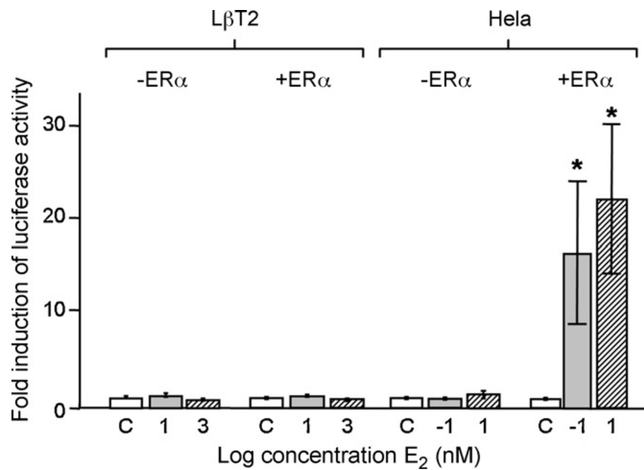

Fig. 8. Assessment of estrogen receptor (ER)-mediated gene transcription in the LβT2 and the Hela cell line. LβT2 and Hela cells were transiently transfected with the ERE-tk-Luc construct alone, or in combination with a human ERα expression vector. The ERE-tk-Luc reporter plasmid consists of two copies of the *Xenopus laevis* vitellogenin A2 ERE (58-GGTCACAGTGACC-38), linked to the thymidine kinase (tk) promoter and coupled to a gene encoding for the luciferase enzyme. Cells were exposed to E_2 (10 and 1000 nM for LβT2 cells; 0.1 and 10 nM for Hela cells) for 16 h. Results are presented as the mean ± S.D. from three separate transfections. $^{*}P < 0.05$ vs. vehicle control (C).

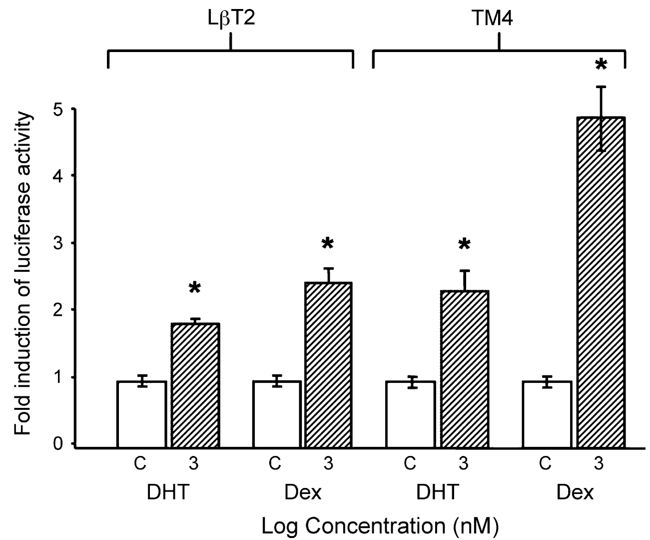

Fig. 9. Differential response of the MMTV-promoter in the LβT2 and TM4 cell lines. Both cell lines were transiently transfected with the AR- and GR-driven MMTV-Luc reporter plasmid and treated with Dex (1 μM) or dihydrotestosterone (DHT; 1 μM) for 16 h. Results are presented as the mean ± S.D. from three separate transfections. $^{*}P < 0.05$ vs. vehicle control (C).

3.3. Assessment of hormone receptor-mediated gene expression

No response could be observed in LβT2 cells, transiently transfected with an ER-driven reporter construct (ERE-TK-Luc), following exposure to E_2 (16 h; 10 pM to 1 μM). In addition, LβT2 cells did not respond to E_2 following co-transfection with an expression vector encoding for the human ERα. As a positive control, the human Hela cell line was transfected in parallel. E_2 was able to stimulate luciferase activity in a dose-dependent manner, with an EC50 of 1.68 pM. At the highest concentration of E_2 (10 nM) tested, ER-mediated luciferase induction was 22.55-fold in comparison to vehicle control. Results are presented in Fig. 8.

AR- and GR-mediated gene transcription was investigated in LβT2 cells using a MMTV-Luc reporter construct. At a concentration of 1 μM, MMTV promoter activity was significantly ($P < 0.05$) stimulated by dihydrotestosterone (DHT) and Dex (1.83- and 2.5-fold, respectively, in comparison to vehicle control) in the LβT2 cell line. As a positive control, the mouse Sertoli TM4 cell line was transfected in parallel. Luciferase activity showed a significant ($P < 0.05$) 2.21- and 4.56-fold increase when compared to vehicle control following treatment with DHT and Dex respectively. Results are shown in Fig. 9.

4. Discussion

Whereas the results of the present study confirm that the murine, gonadotropic LβT2 cell line displays characteristics of mature gonadotrophs *in vivo* with gonadotropin-releasing hormone (GnRH) inducible luteinizing hormone (LH) secretion, in our hands, extensive experiments failed to demonstrate regulatory effects of 17-β-estradiol (E_2).

Using a short pulse setup, daily repeated exposure to (sub) physiological concentrations of GnRH enhanced LH release by LβT2 cells, except on the first treatment day, showing lack of secretory response following GnRH challenge. These findings suggest that the previously well-documented self-priming effect of GnRH (Aiyer et al., 1974; Waring and Turgeon, 1980) was preserved in the LβT2 cell line. Turgeon et al. (1996) and Nicol et al. (2002) also reported increased responsiveness of LβT2 cells, although there was already a significant response on day 1. In our experiment, increasing the exposure time to 4 h, although resulting in higher basal medium LH concentrations, still failed to show a significant response to GnRH challenge on day 1. In addition, the LH secretion pattern in response to GnRH, seen on the following treatment days was identical for the short and longer stimulation setups.

On the third day of exposure, we observed a maximal effect at 10 nM of GnRH, while higher concentrations resulted in a decreased secretory response, which is in agreement with the findings of Nicol et al. (2002). Currently, the mechanism underlying the latter phenomenon remains unknown, but post-transcriptional processes or vesicle storage of LH may be impaired in the LβT2 cell line at these supraphysiological GnRH concentrations. Indeed, in our experiments, a diminished response to higher GnRH concentrations was not seen for LHβ-subunit (LHβ-su) promoter activity and the corresponding messenger RNA (mRNA) expression, which were both dose-dependently stimulated by GnRH, hereby reaching a maximal effect at 1 μM.

On the third day of repeated exposure to 10 nM of GnRH, we did not observe any changes in cell number and the LH secretory response of LβT2 cells in the presence of E_2. In contrast, dexamethasone (Dex) treatment decreased cell number while stimulating the LH secretory response. Results for the combination of both steroids were similar as for Dex alone. Turgeon and

colleagues reported that a significant effect of E_2 was seen only on the fourth day of repeated exposure. Furthermore, in their experiments, the GnRH-induced secretory response to Dex was approximately two-fold higher in comparison to the E_2-treated group and was comparable to the effects they observed in E_2- and Dex-treated (E_2/Dex) cells (Turgeon et al., 1996). In contrast, at day 3 of repeated exposure, Nicol and colleagues reported that LH secretion was significantly stimulated by all steroid treatments, with a stronger induction in the Dex-group in comparison to E_2-treated cells (Nicol et al., 2002). Similar to the observations of Turgeon et al. (1996) the combination of both steroids had no additional effects above the Dex-mediated response.

In our hands, the combination of E_2 and Dex did not influence GnRH-induced LHβ-su gene expression, being the rate-limiting step of LH synthesis (Evans, 1999; Yamada et al., 2004). Our data are in agreement with the findings of Turgeon et al. (1996), but are at variance with the observations of Nicol et al. (2002), showing inconsistent effects on LHβ-su mRNA expression. The lack of estrogenic response in our LβT2 cell line may plausibly be attributed to the rather weak abundance of the mRNA encoding for the ERα and the absence of demonstrable receptor protein. Similar findings were reported by the group of Niswender (2006), who transfected ERα in the LβT2 cell line because of the absence of the receptor (Colorado AES Projects COL00220; ref. 32).

Our data are conflicting with the observations of another group, reporting strong ERα mRNA and protein expression in the LβT2 cells (Schreihofer et al., 2000). In our hands, different culture conditions did not affect ERα mRNA and protein expression. Furthermore, we demonstrated that our LβT2 cell line preserved the main properties of mature gonadotrophs *in vivo*, including mRNA expression of the GnRH receptor (GnRH-R) and the gonadotropin subunits, which is in agreement with other reports (Turgeon et al., 1996; Lawson et al., 2001). LβT2 cells also showed a strong expression of the mRNAs encoding for the androgen receptor (AR) and the glucocorticoid receptor (GR).

Dedifferentiation in function of time and passage number may be a possible explanation for the loss of ER expression in our subclone. This phenomenon was reported by Kim et al. (2000) in immortalized hepatocytes, which were generated using simian virus 40 (SV40) T-antigen oncogenesis. The authors demonstrated that dedifferentiation was caused by chromosomal damages, induced by raising T-antigen levels following continuous passages. As SV40 T-antigen oncogenesis was used to develop the LβT2 cell line (Alarid et al., 1996), we cannot exclude that similar effects occurred in these cells, resulting in the loss of cell characteristics, including ERα expression. Another explanation could have been overexpression of the truncated estrogen receptor product 1 (TERP-1) in our cell line. This pituitary-specific ER-isoform is able to modulate E_2-mediated gene transcription, depending on the ER over TERP-1 concentration ratio (Resnick et al., 2000). However, TERP-1 expression appeared to be also absent in our LβT2 cell line. Furthermore, another group reported that TERP-1 expression in the LβT2 cells was considerably lower when compared to ERα and also varied between experiments (Schreihofer et al., 2000).

Although we were not able to demonstrate TERP-1 expression in the LβT2 cell line, we did show the expression of an alternative ERα mRNA, which results from inappropriate splicing at the 3′-end of exon 4, whereby an intronic sequence is incorporated at this 3′-end and downstream exons are deleted. Theoretically, this mRNA variant encodes for a protein of 42.4 kDa, which lacks most of the ERα ligand binding domain (LBD) (Swope et al., 2002). In the mouse, the exact function of this protein remains currently unknown but a similar variant has already been described in humans, where it was able to enhance or inhibit full length ERα transcriptional activity, depending on the cellular context (Bollig and Miksicek, 2000). Although we were not able to demonstrate the expression of this truncated protein in the LβT2 cell line with the applied primary antibody, one can postulate that the observed lack of estrogenic effects in the LβT2 cell line might be related to the low ratio of full length ERα to the variant message. These findings are in contrast to our observations in the gonadotropic αT3-1 and TM4 Sertoli cell lines, which are both estrogen-responsive. In the latter cell lines, substantially higher levels (3.7- and 3.5-fold) of full length ERα in comparison to the variant message were measured.

In the rat, Pasqualini et al. (2001) have described ERα splicing variants showing deletions of exon 3 and/or exon 4, which can alter ER transcriptional activity either positively or negatively. Furthermore, these variant mRNAs were expressed in a stage- and region-specific manner (Pasqualini et al., 1999) in the presence of full length ERα mRNA. We have analyzed the expression of corresponding ERα splicing variants in the LβT2 cell line. One important difference is the fact that the rat ERα gene contains only 8 exons, which is in contrast to the mouse gene, which consists of nine exons. In other words, the murine ERα gene exons 4 and 5 correspond to rat exons 3 and 4. We have performed RT-PCR analysis using a primer set which was derived from the one described by Pasqualini and colleagues (1999). However, no ERα splicing variants were detected in the LβT2 cell line nor in the other two tested murine cell lines (αT3-1 and TM4 cell line)

In order to by-pass the apparent absence of ERα protein, LβT2 cells were transiently transfected with an expression vector encoding for the human ERα in combination with an ER-driven reporter construct (ERE-tk-Luc). Despite strong expression of ERα protein, as demonstrated using Western blot analysis, E_2 treatment still failed to stimulate estrogen response element (ERE)-mediated luciferase expression. In contrast, Dex and dihydrotestosterone (DHT) were able to stimulate the mouse mammary tumor virus (MMTV) promoter, which is under transcriptional control of the GR and the AR. These observations, together with the demonstrated mRNA expression of both receptors, suggest a specific problem of ER signaling in the LβT2 cell line, rather than a generalized impairment of nuclear receptor functioning.

It should be noticed that we transfected human ERα into a murine cell line, and thus that a species-specific incompatibility might underlie our negative transfection results in the LβT2 cell line. However, similar experiments were performed in the mouse TM4 Sertoli cell line, whereby transfection with a HEGO vector and ERE-LUC resulted in a significant increase in estrogenic

response above cells, transfected with ERE-Luc alone (data not shown).

Recently, Turgeon and Waring (2006) demonstrated that progesterone receptor (PR) A and B expression in LβT2 cells was not affected by E_2, which was in contrast to their observations in rat and mouse pituitary cultures, showing increased expression of both PR isoforms following E_2-exposure. Another group reported a decrease in GnRH-induced aromatase promoter activity in LβT2 cells treated with E_2 (Galmiche et al., 2006), however at a 50,000-fold higher concentration (10 μM) in comparison to our and other experiments (Turgeon et al., 1996; Nicol et al., 2002).

The present results indicate the importance of careful analysis of data, obtained in highly specific cell lines. In the case of the LβT2 cell line, the previously reported estrogenic effects were rather modest or weak (Turgeon et al., 1996; Nicol et al., 2002) Moreover, variable outcome may occasionally be observed even for cell lines yielding much more robust estrogenic responses than those previously described for LβT2 cells, such as in the human MCF-7 breast cancer cell line, which has been widely used in validated *in vitro* assays to assess estrogenic effects (Rasmussen and Nielsen, 2002). One can postulate that ER-mediated signaling is an unstable feature of the LβT2 cell line, which is vulnerable to dedifferentiation.

In conclusion, in our hands, extensive investigations failed to demonstrate any regulatory effect of E_2 on the GnRH-induced LH secretory response and the corresponding LHβ-su gene expression in the LβT2 cell line. Underlying causes appear to be situated at different levels involved in ER signal transduction, including rather weak ERα mRNA expression, the absence of a functional ERα protein and lack of estrogenic response following co-transfection with an ERα expression vector. Furthermore, the relative strong expression of an ERα mRNA splicing variant in comparison to full length ERα mRNA suggests a possible role for this variant in abnormal ER signaling in the LβT2 cell line. In view of the inconsistencies between laboratories or subclones in the expression of ER-mediated signaling, the LβT2 cell line does not seem to be a suitable *in vitro* model for the study of estrogen regulation of gonadotropin synthesis and secretion. It seems relevant to point out this limitation in view of the unique position of the LβT2 cell line as an immortalized cell line reflecting essential properties of mature gonadotrophs and a well established and useful model for *in vitro* studies of gonadotropin regulation.

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4.2 Estrogen Receptor α (ERα) and Insulin-Like Growth Factor I Receptor (IGF-IR) Cross-Talk in the gonadotropic αT3-1 cell line

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Estrogen Receptor Alpha (ERα) and Insulin-like Growth Factor I Receptor (IGF-IR) Cross-talk in the Gonadotropic αT3-1 Cell Line

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In reproductive tissues such as the breast and the uterus, cell proliferation and differentiation is strongly regulated by complex interactions between estrogen receptor α (ERα) and growth factor receptors. In the present study, we investigated the potential occurrence of such cross-talk in the murine, gonadotropic αT3-1 cell line, which expresses ERα and the IGF-I receptor (IGF-IR). Under estrogen-free conditions, basal cell proliferation and ER-mediated gene transcription was strongly inhibited by the selective estrogen receptor modulator (SERM) 4-hydroxy-tamoxifen (4-OH-Tam) and by the pure anti-estrogen ICI 182,780 (ICI). These effects can be reversed by either 17-β-estradiol (E_2) or insulin-like growth factor I (IGF-I), both exerting modest mitogenic effects in the αT3-1 cell line. Furthermore, IGF-I enhanced both basal and E_2-induced ER-driven gene transcription. This may be explained, at least in part, by enhanced phosphorylation of ERα at serine 118, a prerequisite for the transactivation capacity of the receptor. Finally, the IGF-I-induced response on cell growth and ER-mediated transactivation can be inhibited with either ICI or 4-OH-Tam. In conclusion, our data indicate IGF-IR and ER interactions in the αT3-1 cell line, an in vitro model for the pituitary gonadotrophs, hereby suggesting a role of IGF-I in the regulation of gonadotropin synthesis and secretion.

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Estrogens play an important role in the neuroendocrine regulation of gonadotropin synthesis and secretion by modulating the release of the hypothalamic gonadotropin-releasing hormone (GnRH) on the one hand and by direct interaction with the pituitary gonadotrophs on the other (Knobil, 1988; Tilbrook and Clarke, 2001; Burger et al., 2004; Malyala et al., 2005).

Several studies have demonstrated protective effects of 17-β-estradiol (E_2) in the nervous system (Dluzen and McDermott, 2000; Mendez et al., 2005; Manthey and Behl, 2006), an action that appears to involve estrogen receptor (ER) and growth factor receptor interactions in neural cells (Singer et al., 1999; Topalli and Etgen, 2004). Likewise, such cross-talk has been demonstrated in extensive studies in reproductive tissues, for example, in the female breast and the uterus, demonstrating that E_2 and growth factors such as insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) act together in order to regulate cell differentiation and proliferation (Ruan et al., 1995; Richards et al., 1996; Lee et al., 1999; Klotz et al., 2000; Oesterreich et al., 2001).

In the absence of estrogens, agonist-bound growth factor receptors can enhance ER transcriptional activity through kinase signaling pathways which induce phosphorylation of the receptor at specific serine residues (Kato et al., 1995; Kahlert et al., 2000; Chen et al., 2002).

In the central nervous system, IGF receptors (IGF-R) are widely expressed, whereby many brain cells show additional co-expression of the ER (Cardona-Gomez et al., 2001). In particular, several authors report the presence of the IGF-IR not only at the level of the hypothalamus (Daftary and Gore, 2004, 2005) but also in the pituitary (Xia et al., 2001; Weiss et al., 2003; Rose et al., 2004).

A limited number of studies have investigated the effects of IGF-I on gonadotropin secretion by the pituitary gonadotrophs. In ovariectomized female rats, E_2 and IGF-I were shown to interact at the hypothalamic level to modulate luteinizing hormone (LH) secretion (Hiney et al., 2004). Furthermore, IGF-I was not only able to stimulate GnRH release from the median eminence but also to enhance LH release by male rat pituitary cells (Soldani et al., 1995). Another report demonstrated additive effects of E_2 and IGF-I on GnRH-induced LH secretion in female rat pituitary cells (Xia et al., 2001). These observations indicate that ER and IGF-IR interactions at the level of the hypothalamus and the pituitary gonadotrophs may contribute to the regulation of gonadotropin synthesis and secretion. However, little information is available concerning the degree of cross-talk between ER and growth factors at the level of the pituitary gonadotrophs. In the present study, we have investigated possible interactions in the murine, gonadotropic αT3-1 cell line, which expresses the GnRH receptor (GnRH-R) and the gonadotropin common α-subunit, but not their β-subunits (Mellon et al., 1991; Alarid et al., 1996). We report for the first time the existence of ERα interaction with the IGF-IR, but not the EGF-R in αT3-1 cells, as revealed by cell proliferation assays, reporter gene studies and Western analysis.

Materials and Methods

Plasmids, chemicals and materials

The ERE-tk-Luc vector (Harnish et al., 2000) was a kind gift of Dr. W. Vanden Berghe (Ghent University, Ghent, Belgium). The pSV-β-Gal

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vector, encoding for the enzyme β-galactosidase, was purchased from Promega (Leiden, The Netherlands); 17-β-estradiol (E_2), tamoxifen (Tam), 4-hydroxy-tamoxifen (4-OH-Tam), insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), the mitogen-activated protein kinase (MAPK) kinase inhibitor PD 98059, trichloroacetic acid (TCA), sulforodamine B (SRB), trizma base and primary antibodies against α-tubulin were obtained from Sigma (Bornem, Belgium); ICI 182,780 was purchased from AstraZeneca (Macclesfield, UK). All cell culture products and Lipofectamine Plus were from Invitrogen (Merelbeke, Belgium). Activated charcoal was from Merck (Darmstadt, Germany). Dextran T70 was obtained from Pharmacia (Uppsala, Sweden). Antibodies against ERα (NCL-L-ER-6F11) and EGF receptor (EGF-R) were from Novocastra (Newcastle, United Kingdom). The antibody for the detection of phosphorylated ERα (at serine residue 118) was obtained from Cell Signaling Technology (Leiden, The Netherlands). Antibodies against insulin-like growth factor I receptor (anti-IGF-IRα; SC-712) were from Santa Cruz (Heidelberg, Germany). The enhanced chemiluminescence (ECL) Western Blotting Detection Reagents and secondary anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were from Amersham Biosciences (Roosendaal, The Netherlands).

Cell culture

The murine, gonadotropic αT3-1 cell line was kindly provided by Dr. P. Mellon (University of San Diego, La Jolla, CA, USA). The human cervical HeLa cell line and the human breast cancer cell line MCF-7 (subclone AZ) were a gift of Dr. P. Briand (Department of Tumor Endocrinology, Danish Cancer Society, Copenhagen, Denmark). Lung fibroblasts and pituitaries, isolated from C57BL/6 mice, were provided by Dr. K. Vermaelen (Ghent University Hospital, Ghent, Belgium). All cell lines were grown as a monolayer in a 5% CO_2, humidified atmosphere (37°C) and weekly passaged in growth medium (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/ml penicillin G and 100 μg/ml streptomycin. The murine Sertoli TM4 cell line was grown in DMEM: Nutrient Mix F12 supplemented with 5% horse serum, 2.5% FCS, 100 units/ml penicillin G and 100 μg/ml streptomycin.

Removal of sex steroids from fetal calf serum (FCS)

Sex steroids were removed from FCS by dextran-coated charcoal (DCC) treatment. Briefly, a mixture of 10% activated charcoal and 1% Dextran T70 (50/50) was prepared in a volume equal to that of the serum to be treated. The mixture was centrifuged (15 min, 4,000 rpm), the supernatant removed, and the pellet combined with the serum and mixed by rotation overnight at 4°C. The next day, the mixture was centrifuged (15 min, 4,000 rpm), the supernatant was combined with a freshly prepared pellet and rotated for 8 h. Following centrifugation (15 min, 4,000 rpm), the supernatant was ultracentrifuged (20,000 rpm, 30 min, 4°C) and filter-sterilized (0.2 μm) before use.

RNA isolation and reverse transcription PCR

Using the RNeasy mini-kit, total RNA was extracted from αT3-1 cells, TM4 cells and from lung fibroblasts, freshly isolated from C57BL/6 mice, treated with DNase and frozen at −20°C until analysis.

For reverse transcription PCR analysis of the estrogen receptor α (ERα), total RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit, according to the manufacturer's instructions. The following PCR primers were used (fp, forward primer; rp, reverse primer): a 320 bp-specific amplicon for ERα fp, 5′- TGGCGCTCCATGGAACACC-3′, ERα rp, 5′-TGCTGCAGAGTCAGGCCAGAT-3′. Total RNA from TM4 cells was used as a positive control, while mouse fibroblast total RNA served as an alleged negative control. To check for PCR efficiency, a 238 bp-specific amplicon for gene β-actin was amplified using fp, 5′-CTGGCACCACACCTTCTA-3′ and rp, 5′-GGGCACAGTGTGGGTGAC-3′. Primers were based on GenBank entries **NM_007956** and **NM_007393** for ERα and β-actin, respectively. Two microlitres of cDNA were amplified as follows: an initial denaturation step at 92°C for 10 min was followed by 40 cycles consisting of a denaturation step at 92°C for 30 sec, a 30 sec annealing step 50°C, and a 45 sec extension step at 72°C. An elongation step of 10 min at 72°C finalized the amplification. PCR products were analyzed using a 2% agarose gel electrophoresis, followed by ethidium bromide staining.

Proliferation assay, sulforodamine B (SRB) assay

Optimal growth conditions, including cell density, serum concentration and exposure time (3, 5, and 7 days) were extensively investigated resulting in the following protocol: αT3-1 cells (5,000 cells/well) were seeded in growth medium in 96-well plates. After 48 h, culture medium was replaced by 200 μl of experimental medium phenol red free DMEM: Nutrient Mix F12 supplemented with 5% DCC FCS, 100 units/ml penicillin G and 100 μg/ml streptomycin containing the test compounds. Experimental medium containing 0.1% ethanol served as vehicle control. Cell number was assessed using the colorimetric SRB assay, according to the protocol, described by Skehan and colleagues (Skehan et al., 1990) after 5 days, found to be an optimal incubation period when compared to the 3 and 7 days exposure period, respectively.

Briefly, cells were fixed by adding 50 μl of a 50% TCA solution to each well, incubated for 1 h at 4°C, subsequently washed five times with water and air-dried. Next, 100 μl of a 0.4% solution (w/v) SRB in 1% acetic acid solution was added to each well and incubated for 30 min. Plates were washed with 1% acetic acid solution and air-dried. Cell-bound SRB was solubilized with 100 μl Trizma base (10 mM) and the absorbance (492 nm) was determined directly in the 96-well plates using a Multiskan EX microplate reader (Thermo Labsystems, Brussels, Belgium). The absorbance at 620nm was used for background correction. A standard curve, prepared using a serial dilution (1/2) of 16×10^4 cells/well, was used to calculate cell number. Data are expressed as a percentage of vehicle control.

Reporter gene studies

αT3-1 cells and TM4 Sertoli cells were seeded in 24-well plates at a density of 2×10^5, respectively, 3×10^4 cells per well in growth medium. Following overnight incubation, cells were transfected with ERE-tk-Luc (250 ng) using Lipofectamine Plus according to the instructions of the manufacturer. In each experiment, a reporter plasmid, encoding for the enzyme β-galactosidase (β-gal; 20 ng) was co-transfected in order to control for transfection efficiency. Twenty-four hours later, cells were incubated for 16 h in experimental medium containing test compounds. Experimental medium containing 0.1% ethanol served as vehicle control. For the experiments with the selective MAPK kinase (MEK) inhibitor PD98059, αT3-1 cells were preincubated for 2 h with 50 μM of the inhibitor, prior to 16h stimulation with E_2 (100 pM) or IGF-1 (100 ng/ml), in the presence of PD 98059. After cell lysis, luciferase and β-gal activities were determined using a Packard Lumicount Microplate luminometer (Packard Instrument Company Inc., Meriden, CT, USA). Luciferase values were normalized for β-gal values and the obtained results for treated cells were expressed relative to the control (untreated cells), set to 100%.

Western analysis

Lysates were prepared of αT3-1 cells and MCF-7 cells (either vehicle- or test compound-treated for 4 h), using Laemmli buffer (0.25 M Tris, 1.92 M glycine and 1% sodium dodecyl sulphate (SDS) in aqueous solution). Lysates were centrifuged at 14,000 rpm for 30 min at 4°C, and protein concentration was determined by Lowry assay. All samples were diluted with sample buffer (62 mM Tris–HCl, pH 6.8, 10% glycerol, 5.0% β-mercaptoethanol, 2.0% SDS, 0.0012% bromophenol blue) and heated at 94°C for 5 min. A total of 25 μg of protein was loaded for each sample. Electrophoresis was carried out on a 10% separating gel at 100 V for 1 h in a Mini-Protean II electrophoresis system (BioRad, Nazareth-Eke, Belgium). After electrophoresis, proteins were transferred from polyacrylamide gels onto nitrocellulose membranes by electroblotting (0.8 mA/cm^2 constant current, 60 min, room temperature). Following transfer, membranes were blocked with 5.0% dehydrated non-fat milk in TBS-T (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.10% Tween-20) for 60 min at room temperature. Membranes were then washed three times in fresh TBS-T followed by incubation (1 h) with primary antibody. Following washing (3× TBS-T, 5 min), membranes were incubated for 1 h in secondary antibody. Immunodetection was performed with an enhanced chemiluminescence system (Amersham Biosciences, Roosendaal, The Netherlands) according to manufacturer's specifications. Chemiluminescence was captured on photographic film. Each Western blot was exposed for 15 sec, 30 sec and 60 sec to guarantee linearity of the film.

To determine the phosphorylation status of ERα, densitometric analysis was performed using Digimizer image analysis software (Medcalc Software, Mariakerke, Belgium). Briefly, immunoreactive bands at 66 kDa were quantified following incubation with specific antibodies directed to phosphorylated ERα and non-phoshphorylated ERα (total ERα), respectively. Values for phosphorylated ERα were expressed relative to the corresponding values for total ERα and normalized for α-tubulin. The obtained results for test-compound treated cells were expressed relative to the control (vehicle-treated cells), set to 100%.

Statistics

Data are expressed as a mean ± standard deviation of at least three independent experiments. Curve analysis (four parameter logistic regression) was performed using Sigmaplot 8.0 (SPSS Benelux, Brussels, Belgium). Statistical analysis was carried out by one-way ANOVA using the SPSS 12 statistical package (SPSS). Significant differences were determined by the Dunnet's post hoc test.

Results

Hormone receptor expression

The expected RT-PCR product (320 bp) for ERα messenger RNA (mRNA) was detected in the murine, gonadotropic αT3-1 cell line and in murine Sertoli TM4 cells but not in mouse fibroblast mRNA, which was used as an alleged negative control.

In αT3-1 cells, Western analysis showed the presence of a ~66 kDa protein, with immunoreactivity towards an ERα specific antibody. Following incubation with an insulin-like growth factor I receptor (IGF-IR)-specific antibody, two bands were found at ~130 kDa and ~200 kDa. No bands were detected using an antibody directed to the epidermal growth factor receptor (EGFR). In contrast, the human breast cancer MCF-7 cell line, which served as a positive control, expressed all three receptors. Results are presented in Figure 1.

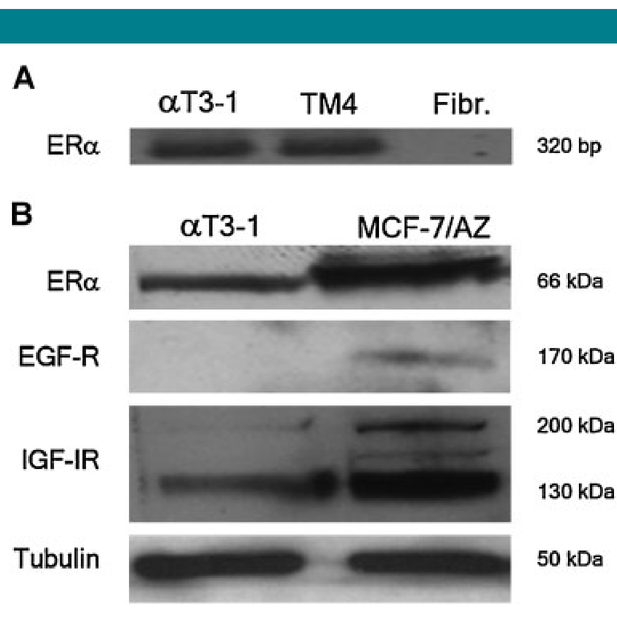

Fig. 1. Hormone receptor expression in the murine, gonadotroph-derived αT3-1 cell line. The expression of estrogen receptor α (ERα) was investigated using the reverse-transcriptase polymerase chain technique (RT-PCR) using a specific primer set, yielding an amplicon of 320 bp. The ER-positive TM4 Sertoli cell line served as a positive control, while lung fibroblasts were used as an alleged negative control. Western blot analysis was performed to confirm the presence of ERα protein and to analyze the expression of the insulin-like growth factor I receptor (IGF-IR) and the epidermal growth factor receptor (EGF-R). Mouse α-tubulin served as a protein loading control. Lysates from the human MCF-7/AZ breast cancer cell line were used as a positive control.

Effects of 17-β-estradiol (E_2), growth factors and anti-estrogens on basal cell growth

Optimal growth conditions were obtained using 5% DCC-FCS, while lower serum concentrations resulted in retarded cell growth (data not shown). E_2 and IGF-I, but not EGF, dose-dependently stimulated cell proliferation in the αT3-1 cell line, showing a maximal induction of 33% (100 pM; Fig. 2A) and 82% (100 ng/ml; Fig. 2B), respectively, relative to vehicle control, set to 100%. IGF-I exerted additive effects on E_2-induced proliferation, reaching a maximum effect of 74% above the estrogen response (Fig. 3A). Conversely, in αT3-1 cells treated with a fixed concentration of IGF-I (100 ng/ml), E_2 dose-dependently enhanced cell proliferation, showing a maximal response of 35% at 100 pM (Fig. 3B).

Next, dose-response experiments were performed with the selective estrogen receptor modulator (SERM) tamoxifen (Tam), its metabolite 4-hydroxy-tamoxifen (4-OH-Tam) and the pure anti-estrogen ICI 182,780 (ICI). ICI and 4-OH-Tam, but not Tam, dose-dependently inhibited basal cell proliferation with a 50% inhibitory concentration (IC50) of 216 pM and 1763 pM for ICI and 4-OH-Tam, respectively. Maximal growth inhibition of αT3-1 cells was larger for treatment with ICI (78% reduction at 100 nM), compared to 4-OH-Tam (55% reduction at 100 nM; Fig. 4A).

Growth inhibition, induced by 10 nM of 4-OH-Tam or ICI, respectively, was dose-dependently reversed by raising concentrations of E_2 with respective EC50 of 56 pM and 1116 pM (Fig. 4B).

IGF-I reversed ICI (10 nM)-induced basal growth inhibition in a dose-dependent manner. At the highest tested concentration (1,000 ng/ml), cell number was 3.83-fold increased in comparison to cells treated with ICI alone (Fig. 5A). IGF-I exerted similar effects in αT3-1 cells exposed to 4-OH-Tam (10 nM; Fig. 5B). Conversely, the growth induction, induced by 100 ng/ml of IGF-I (87%), was dose-dependently inhibited by ICI and 4-OH-Tam, with an IC50 of 166 pM and 1895 pM, respectively. As expected, EGF was not able to alter anti-estrogen-mediated growth inhibition (data not shown).

ER phosphorylation

Following test compound treatment (4 h), the phosphorylation status of ERα at serine residue 118 (Ser-118) was investigated by Western analysis, using site-specific anti-ERα-phosphoserine antibodies. In the αT3-1 cell line, upon E_2-exposure, the level of ERα phosphorylation was 2.6-fold increased ($P < 0.05$) relative to vehicle control. A similar response was seen following treatment with 4-OH-Tam or ICI (2.22-fold and 2.5-fold, respectively). IGF-I stimulated phosphorylation 1.6-fold ($P < 0.05$), while no significant effects could be observed following EGF-treatment. In the human MCF-7 breast cancer cell line, serving as a positive control, E_2 induced Ser-118 phosphorylation 4.4-fold ($P < 0.05$), while exposure to 4-OH-Tam or ICI resulted in a 3.72-fold and 2.44-fold increase when compared to vehicle control. IGF-I and EGF stimulated phosphorylation 2.0-fold and 1.7-fold ($P < 0.05$), respectively. The combination of IGF-1 and E_2 resulted in a synergistic effect on phosphorylation in both cell lines. Similar effects were seen when MCF-7 cells were exposed to the combination of E_2 and EGF. In contrast, the latter growth factor did not alter the estrogen-induced phosphorylation status in the αT3-1 cell line. Results are presented in Figure 6A, B for the αT3-1 and MCF-7 cell line, respectively.

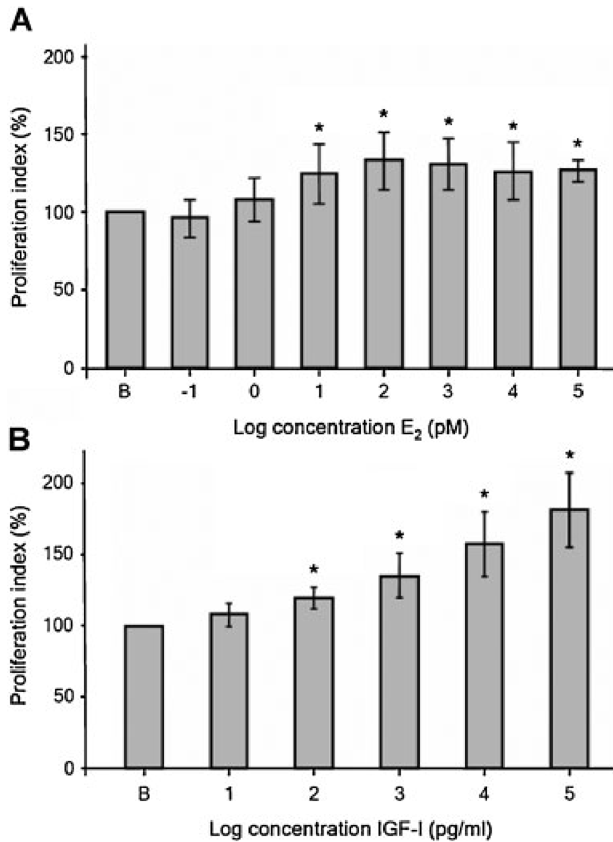

Fig. 2. Influence of 17-β-estradiol (E_2) and IGF-I on basal cell growth in the murine, gonadotroph-derived αT3-1 cell line. αT3-1 cells were treated for 5 days with either E_2 (100 pM; (A)) or IGF-I (100 ng/ml; (B)) and cell proliferation was determined using the SRB assay. Data are calculated as a percentage of vehicle control, set to 100%, and expressed as a mean ± standard deviation (SD) of at least six independent experiments. Asterisk (*) designates significant ($P < 0.05$) differences from vehicle control (B).

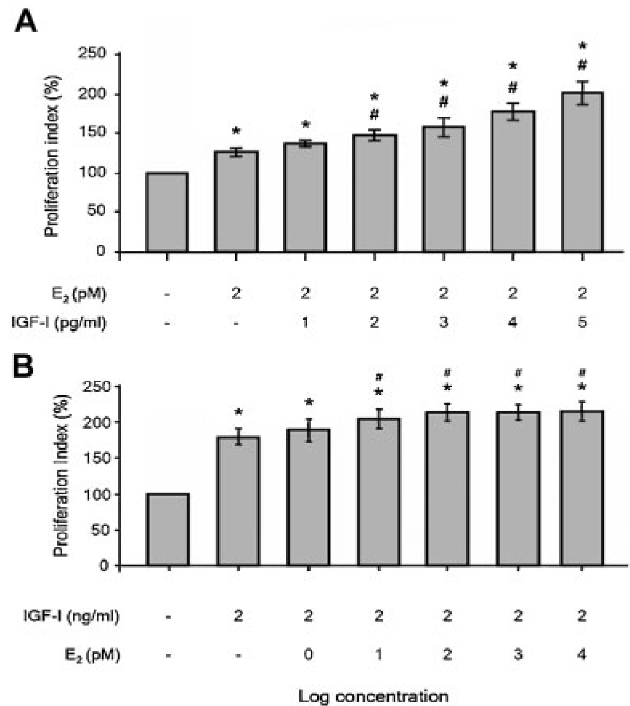

Fig. 3. Influence of combined IGF-I and 17-β-estradiol (E_2) exposure on basal growth in the murine, gonadotroph-derived αT3-1 cell line. αT3-1 cells were treated for 5 days with E_2 (100 pM; (A)) or IGF-1 (100 ng/ml; (B)) alone or in combination with raising concentrations of IGF-I (0.01–100 ng/ml) or E_2 (1 pm–10 nM), respectively. Cell proliferation was determined using the SRB assay. Data are calculated as a percentage of vehicle control, set to 100%, and expressed as a mean ± standard deviation (SD) of at least six independent experiments. Asterisk (*) designates significant ($P < 0.05$) differences from vehicle control (B). Number sign (#) designates significant ($P < 0.05$) differences from E_2-treated cells (A) or IGF-1-treated cells (B), respectively.

Effects of E_2, growth factors and anti-estrogens on ER-mediated gene transcription

In αT3-1 cells, transiently transfected with an ER-driven reporter construct (ERE-tk-Luc), E_2 dose-dependently induced luciferase activity (EC50 = 0.78 pM). At 100 pM, a maximal induction of 58% was achieved. IGF-I, but not EGF, dose-dependently enhanced ER-driven luciferase gene expression, with a 1.9-fold increase at 100 ng/ml relative to vehicle-treated cells.

IGF-I exerted additive effects on E_2 (100 pM)-induced luciferase activity, showing a maximal induction of 1.7-fold at 10 ng/ml relative to E_2-treated cells. At the highest concentration (100 ng/ml) tested, luciferase activity was only 1.2-fold increased when compared to the estrogen-exposed αT3-1 cells. The results are presented in Figure 7.

Similar to the growth experiments, the anti-estrogens ICI and 4-OH-Tam dose-dependently inhibited basal luciferase-activity, with an IC50 of 349 pM and 802 pM, respectively. Maximal inhibition, observed at 100 nM, was similar in the ICI-treated group (67% reduction) and the 4-OH-Tam-treated group (64% reduction). Under our experimental conditions, Tam was not able to inhibit basal luciferase activity (Fig. 8).

As a control, TM4 Sertoli cells were transfected in parallel with the same ER-driven reporter construct. Although E_2 induced a maximal response of 14.5-fold at 100 pM, with an EC50 of 12 pM, ICI and 4-OH-Tam were not able to alter the basal response in the TM4 cell line (data not shown).

In αT3-1 cells, co-incubation with raising concentrations of E_2 dose-dependently reversed the inhibition of basal luciferase activity, induced by 4-OH-Tam (10 nM) or ICI (10 nM), with EC50 values of 130 pM and 940 pM for cells treated with 4-OH-Tam and ICI, respectively (data not shown). Furthermore, IGF-I dose-dependently reversed ICI-induced inhibition of ER-mediated luciferase activity. At the highest tested concentration (1,000 ng/ml), luciferase activity was 2.8-fold increased in comparison to cells, treated with ICI alone. Similar results were observed for IGF-I in 4-OH-Tam-treated cells (Fig. 9).

Next, we assessed the effects of the mitogen activated protein kinase (MAPK) kinase (MEK) inhibitor PD 98059 on ER-mediated luciferase activity in αT3-1 cells, treated with either E_2 (100 pM) or IGF-1 (100 ng/ml). PD 98059 significantly ($P < 0.001$) inhibited both E_2- and IGF-1-stimulated luciferase gene expression, by 17% and 65%, respectively. The response to IGF-1 in PD 98059-exposed cells did not differ from vehicle control (Fig. 10).

Discussion

In the present study, under estrogen-free conditions, the pure anti-estrogen ICI 182,780 (ICI) and the selective

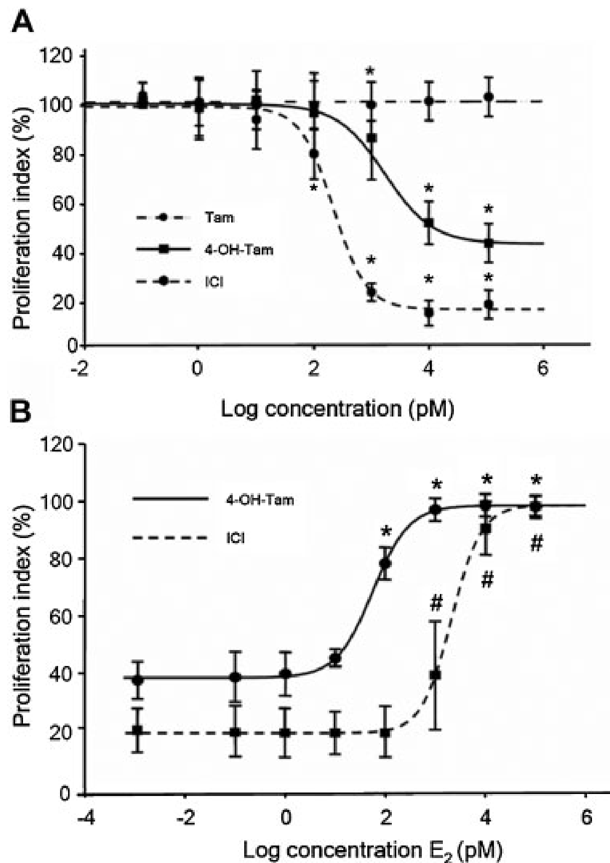

Fig. 4. Anti-estrogen-induced basal cell growth inhibition and reversal by E_2 in the murine, gonadotroph-derived αT3-1 cell line. αT3-1 cells were exposed to tamoxifen (Tam; 1-100,000 pM), 4-hydroxy-tamoxifen (4-OH-Tam; 1-100,000 pM) or ICI 182,780 (ICI; 1-100,000 pM), either alone (A) or in combination with raising concentrations of E_2 (0.1-100,000 pM; (B)) for 5 days. Cell proliferation was determined using the SRB assay. Data are calculated as a percentage of vehicle control (A) or as a percentage of the values obtained with the highest concentration of E_2 and expressed as a mean ± SD of three independent experiments (B). Asterisk (*) designates significant ($P < 0.05$) differences from vehicle control (A) or from 4-OH-Tam-treated cells (B). Number sign (#) designates ($P < 0.05$) significant differences from ICI-treated αT3-1 cells (B).

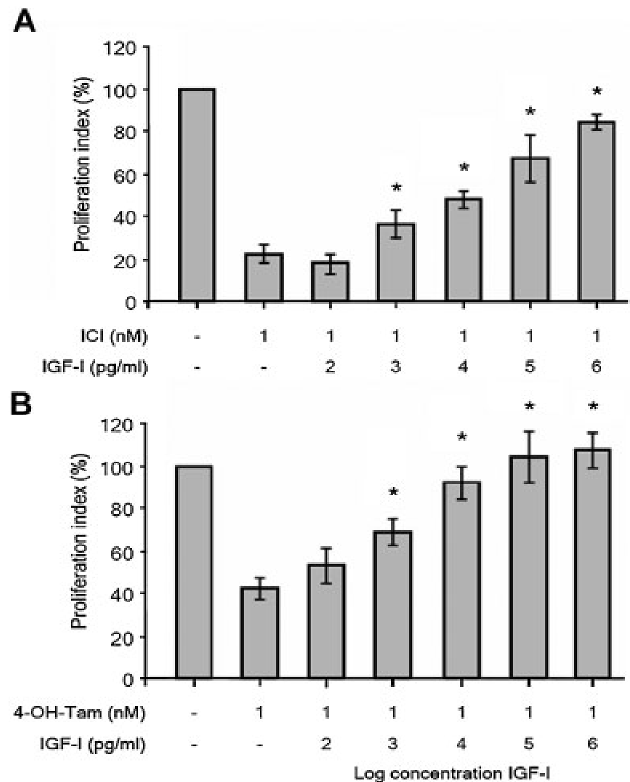

Fig. 5. Effect of IGF-I on anti-estrogen-induced growth inhibition in the murine, gonadotroph-derived αT3-1 cell line. αT3-1 cells were treated for 5 days with ICI (10 nM; (A)) of 4-OH-Tam (10 nM; (B)), alone or in combination with raising concentrations of IGF-I (0.1–1,000 ng/ml). Cell proliferation was determined using the SRB assay. Data are calculated as a percentage of vehicle control and expressed as a mean ± SD of three independent experiments. Asterisk (*) designates significant ($P < 0.05$) differences from anti-estrogen-treated cells.

estrogen receptor modulator (SERM) 4-hydroxy-tamoxifen (4-OH-Tam) elicited a dose-dependent inhibition of basal cell proliferation and estrogen receptor (ER)-mediated gene expression in the murine, gonadotropic αT3-1 cell line. In contrast, no effects were seen following treatment with tamoxifen (Tam), hereby suggesting the lack of cytochrome P450 isoenzyme CYP2D6 expression, which catalyzes the conversion of Tam into its active metabolite 4-OH-Tam (Goetz and Loprinzi, 2003), in the αT3-1 cells. These growth-inhibitory effects could be reversed by either 17-β-estradiol (E_2) or insulin-like growth factor I (IGF-I), both exerting modest mitogenic effects in the αT3-1 cell line. Another group reported a maximal growth induction of 60% following E_2-exposure. Furthermore, growth suppression, induced by the SERM raloxifen, was dose-dependently reversed by E_2 in αT3-1 cells. The authors suggested that raloxifen may block estrogen receptor (ER) binding and its corresponding activation by residual estrogens, present in dextran-coated charcoal (DCC)-treated fetal calf serum (FCS) (Williams et al., 2000). They used 2% stripped FCS, consisting of serum which was DCC-treated for only 25 min. This protocol differs from ours, whereby FCS was DCC-treated twice for at least 8 h. Although this does not exclude the possibility that very low residual estrogen concentrations still induced a cellular response in the αT3-1 cells, this seems unlikely in view of our parallel observations for the TM4 Sertoli cell line. These cells were transiently transfected with the same ER-driven reporter construct under identical experimental conditions (same batches of DCC-FCS) as for the αT3-1 cell line. We observed no effects of ICI and 4-OH-Tam in this highly estrogen-responsive cell line. In any case, even though it is not possible to exclude that minimal residual estrogens may stimulate the highly sensitive αT3-1 cells under our assumed estrogen-free conditions, this does not change the fact that the inhibition of basal cell proliferation and basal ER-driven gene transcription by anti-estrogens was effectively reversed by both E_2 and IGF-1 (Fig. 5A, B).

In reproductive tissues, complex estrogen and growth factor receptor interactions are involved in the regulation of cell proliferation and differentiation (Sato et al., 2002; Levin, 2003; Song and Santen, 2006). Growth factor receptor-activated mitogen-activated protein kinase (MAPK) enhances phosphorylation of serine residue 118 (Ser-118), present in the amino-terminal activation function (AF-1) domain of ERα. We have demonstrated both ERα and insulin-like growth factor-I

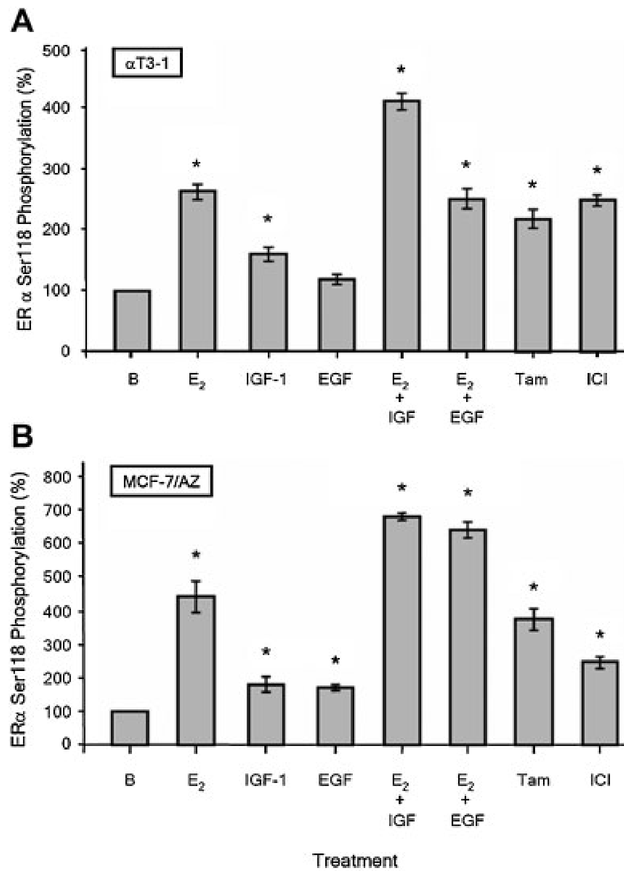

Fig. 6. Phosphorylation of ERα at serine residue 118 (Ser118). Cells were exposed to E_2 (100 pM), IGF-I (I; 10 ng/ml), EGF (10 ng/ml), 4-OH-Tam (Tam; 10 nM) and ICI (10 nM) for 4 h. In parallel, cells were treated with the combination of E_2 (100 pM) and IGF-I (10 ng/ml), or EGF (10 ng/ml), respectively. The phosphorylation status at Ser118 was determined by Western analysis using a site-specific phospho-ERα antibody. Using densitometric analysis, bands were quantified and the obtained results for test-compound treated cells were expressed relative to the control (vehicle-treated cells; (B)), set to 100%. A and B shows the results for the αT3-1 cell line and the human MCF-7 breast cancer cell line (subclone AZ; positive control) respectively. Asterisk (*) designates significant ($P < 0.05$) differences from vehicle control.

receptor (IGF-IR) expression, while the epidermal growth factor receptor (EGF-R) seemed to be absent in the αT3-1 cell line. The latter observation is supported by the lack of any response following EGF-treatment. In our hands, Ser-118 phosphorylation was significantly ($P < 0.05$) stimulated by both E_2 and IGF-I in either αT3-1 or MCF-7 cells, with an additive effect when both hormones were combined. It has been demonstrated that Ser-118 phosphorylation resulted in an increased ER-transcriptional activity (Kato et al., 1995; Bunone et al., 1996). Indeed, in the αT3-1 cell line, both E_2 and IGF-I stimulated ER-mediated transactivation of an estrogen response element (ERE), with a further enhancement of the E_2-mediated response by IGF-I. Furthermore, basal and E_2- or IGF-I-induced gene transcription was dose-dependently inhibited by either ICI or 4-OH-Tam, respectively. It is important to mention that Ser-118 phosphorylation was also induced by both anti-estrogens, in agreement with other experiments, performed in breast cancer cells (Lipfert et al., 2006).

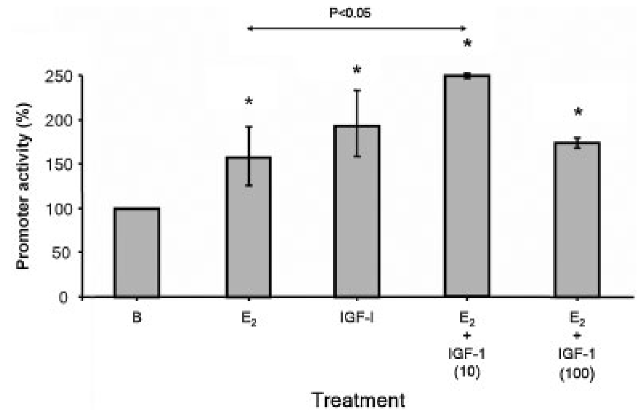

Fig. 7. Influence of E_2 and IGF-I on ER-mediated transcriptional activity αT3-1 cells were transiently transfected with an estrogen-responsive reporter plasmid (ERE-tk-Luc), in combination with a control vector encoding for β-galactosidase (β-gal). Transfected cells were stimulated for 16 h with E_2 (100 pM), IGF-I (100 ng/ml) or the combination of both hormones (10 or 100 ng/ml of IGF-1), respectively. Luciferase values were normalized for β-gal values and expressed relative to vehicle control, set to 100%. Asterisk (*) designates significant ($P < 0.05$) differences from vehicle control.

Our findings indicate a key role for ERα in the regulation of αT3-1 cell proliferation. Furthermore, IGF-I synergistically enhanced ER-mediated phosphorylation, which is in contrast to our proliferation and transfection experiments, showing only additive effects of IGF-I on the E_2-induced responses. The exact mechanisms of action in the αT3-1 cells are yet to be fully elucidated. However, many reports have demonstrated that ligand-activated growth receptors can induce Ser-118 phosphorylation through the mitogen-activated protein kinase (MAPK) pathway (Kahlert et al., 2000; Lannigan, 2003; Levin,

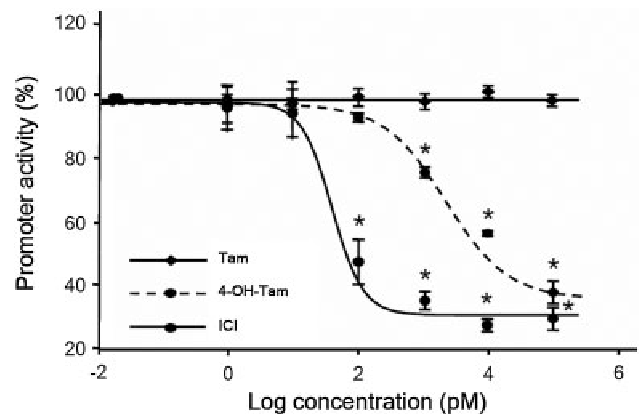

Fig. 8. Influence of anti-estrogens on ER-mediated transcriptional activity in the murine, gonadotroph-derived αT3-1 cell line. αT3-1 cells were transiently transfected with an estrogen-responsive reporter plasmid (ERE-tk-Luc), in combination with a control vector encoding for β-galactosidase (β-gal). Transfected cells were stimulated with Tam (1–10,000 pM), 4-OH-Tam (1–100,000 pM) or ICI (1–10,000 pM) for 16 h. Luciferase values were normalized for β-gal values and expressed relative to vehicle control, set to 100%. Asterisk (*) designates significant ($P < 0.05$) differences from vehicle control.

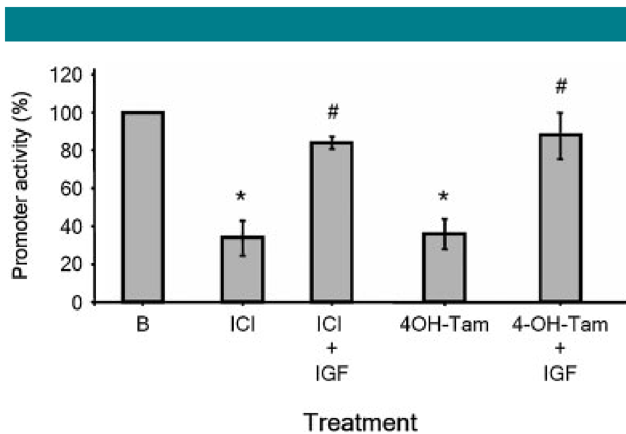

Fig. 9. Influence of IGF-1 on anti-estrogen-induced inhibition of ER-mediated transcriptional activity in the murine, gonadotroph-derived αT3-1 cell line. αT3-1 cells were transiently transfected with an estrogen-responsive reporter plasmid (ERE-tk-Luc), in combination with a control vector encoding for β-galactosidase (β-gal). Transfected cells were stimulated with 4-OH-Tam (10 nM) or ICI (10 nM), alone or in the presence of IGF-1 (1,000 ng/ml) for 16 h. Luciferase values were normalized for β-gal values and expressed relative to vehicle control, set to 100%. Asterisk (*) designates significant ($P < 0.05$) differences from vehicle control. Number sign (#) designates ($P < 0.05$) significant differences from anti-estrogen-treated αT3-1 cells

2003). Indeed, in our experiments, the specific MAPK kinase (MEK) inhibitor PD 98059 was able to significantly inhibit ER-mediated luciferase gene transcription, induced by either E_2 (100 pM) or IGF-I (100 ng/ml). The IGF-1-mediated response was strongly repressed (66%) by the MEK inhibitor and did no longer differ from vehicle control, hereby indicating the involvement of MAPK in the activation of ERα through the IGF-IR signaling pathway. Furthermore, it is interesting that PD 98059 partly inhibited the E_2-mediated response, although, not as strong as observed for IGF-I. In this regard, it can be noticed

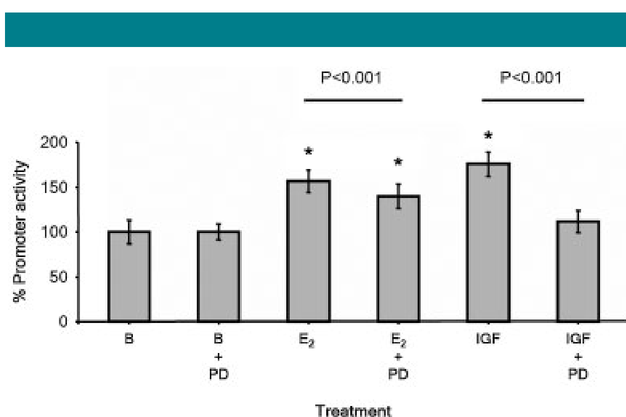

Fig. 10. Influence of the MEK-inhibitor PD 98059 on E_2- and IGF-1-induced ER-mediated transcriptional activity in the murine, gonadotroph-derived αT3-1 cell line. αT3-1 cells were transiently transfected with an estrogen-responsive reporter plasmid (ERE-tk-Luc), in combination with a control vector encoding for β-galactosidase (β-gal). Transfected cells were treated with E_2 (100 pM) or IGF-1 (100 ng/ml), either alone or in the presence of the MEK inhibitor PD 98059 (50 μM) for 16 h. Luciferase values were normalized for β-gal values and expressed relative to vehicle control, set to 100%. Asterisk (*) designates significant ($P < 0.05$) differences from vehicle control (B).

that ER activation in αT3-1 cells may, at least in part, be regulated by MAPK signaling. Alternatively, the limited decrease in the E_2-mediated response following PD 98059 treatment may be explained by the inhibition of effects, induced by low concentrations of IGF-1, present in our experimental medium, although no inhibitory effect was observed in cells, treated with this MEK-inhibitor alone.

Recent studies have demonstrated that rapid, non-genomic effects of estrogens are mediated through the Src/Ras/Raf/MAPK signaling pathway (Wong et al., 2002; Barletta et al., 2004).

Coupling between ERα and this pathway occurs through a newly identified protein, called modulator of non-genomic activity of ER (MNAR), which facilitates ERα interactions with members of the Src family in a ligand-dependent manner. Activation of Src by ERα stimulates the Src/Ras/Raf/MAPK signaling pathway, resulting in increased phosphorylation of Ser-118 in the AF1 domain of ERα.

In the present study, we have only analyzed the effects of E_2 and IGF-1 on the phosphorylation status of Ser-118. However, other serine residues (at positions 104, 106, and 167), present in the AF-1 domain of ERα are also important determinants of ER transcriptional activity. Barletta et al. (2004) demonstrated that MNAR was not able to enhance ERα transcriptional activity when these serine residues (including Ser-118) were mutated to alanine.

However, ERα transcriptional activity not only depends on phosphorylation of the ligand-independent AF-1 domain, but also on interactions of the latter with the hormone-dependent AF-2 domain, located in the ligand-binding domain (LBD) (Moggs and Orphanides, 2001). For instance, 4-OH-Tam blocks AF-2, but allows weak AF-1 activity (Tzukerman et al., 1994; McDonnell et al., 1995) while other anti-estrogens such as ICI allow neither AF-1 nor AF-2 activity (McDonnell et al., 1995; Metzger et al., 1995). Furthermore, cofactor recruitment, their corresponding phosphorylation and other post-transcriptional processes such as ligand-dependent proteasome degradation are parameters which influence ER transcriptional activity (Moggs and Orphanides, 2001; Nawaz and O'Malley, 2004).

In the present study, we describe interactions between ERα and the IGF-IR. However, the physiological significance of this cross-talk at the level of the pituitary gonadotrophs remains to be established. Our findings support the results of other reports, demonstrating modulatory effects of either E_2 or IGF-I on pituitary gonadotrophs, in particular on LH secretion (Soldani et al., 1995; Xia et al., 2001; Hiney et al., 2004). Rose et al. (2004) demonstrated cross-talk between the GnRH-receptor (GnRH-R) and the IGF-IR signaling pathways in the αT3-1 cells. Furthermore, estrogens may interfere with the GnRH-R signaling pathway in a non-genomic manner. Several reports demonstrated physical interaction with and consequent activation of $G_{\alpha s}$- and $G_{\alpha q}$ proteins by membrane-bound ERs (Wyckoff et al., 2001; Levin, 2005; Zhang and Trudeau, 2006). These G proteins play a key role in the cellular regulation of cyclic AMP (cAMP), inositol-1,4,5-triphosphate (IP_3) and calcium production (Hubbard and Hepler, 2006).

It is important to mention that, in pituitary gonadotrophs, these second messengers mediate the effects of gonadotropin-releasing hormone (GnRH) following binding to its receptor (Yuen et al., 2002; Ruf et al., 2003).

All these findings may suggest that estrogenic action on GnRH-induced gonadotropin production and release may, at least in part, be realized through complex interactions between the GnRH-R, the IGF-IR and ERα at the level of the pituitary gonadotrophs.

In conclusion, our data indicate the occurrence of cross-talk between the ER and the IGF-IR in the murine, gonadotropic αT3-1 cell line. Although the physiological significance of this

phenomenon is yet to be established, rising evidence suggest the involvement of IGF-I in the regulation of gonadotropin synthesis and secretion by the pituitary gonadotrophs.

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Chapter 5 Summary and Discussion

5.1 Background

To date, extensive research has been conducted on substances of both natural and anthropogenic origin, which may affect normal function of the endocrine system. Especially in the field of human reproduction, there is growing scientific and public concern, as the number of fertility disorders, including decreased sperm quality, urogenital tract abnormalities and cancers of reproductive tissues appears to have become more prevalent (for review see Eertmans *et al.*, 2003). From this point of view, estrogen and androgen receptors represent two main targets of these endocrine modulating substances.

In the current project, we have focused on the effects on the pituitary gonadotrophs of compounds showing either estrogenic or anti-estrogenic properties. The importance of these effects is emphasized by the widespread occurrence of environmental xenoestrogens, the raising intake of phytoestrogens and the growing number of pharmaceutical estrogenic compounds (human estrogen-replacement therapy and oral contraceptives), selective estrogen receptor modulators (SERMs) used in the management of diseases such as osteoporosis, and compounds with predominant anti-estrogenic action, which are used for the treatment of estrogen-dependent malignancies (Singleton and Kahn, 2001; Safe and Papineni, 2006). In table 5.1, we present an overview of the clinical use of some SERMs, together with their tissue-specific effects.

During recent years, many studies revealed that estrogens are important mediators of cell proliferation and differentiation in reproductive tissues such as the breast and the uterus (Diel, 2002; Ikeda and Inoue, 2004). In contrast, less information is available concerning these regulatory effects at the level of the pituitary gonadotrophs, being key players for reproduction. This relative lack of information is surprising, as gonadotrophs are responsible for gonadotropin synthesis and secretion, driven by the hypothalamic gonadotrophin-releasing hormone (GnRH) (Wildt *et al.*, 1981) and modulated by gonadal hormone feedback. In particular, estrogens can alter luteinising hormone (LH) and follicle stimulating hormone (FSH) release in either a positive or negative manner (for review see Introduction chapter 1.1.3.2.3).

Table 5.1: Overview of the clinical use of SERMs and their tissue specificity.

SERM	*Indication*	*Agonism*	*Antagonism*
Clomifene	Male infertility	• Bone	• Hypothalamus • Breast • Endometrium • Cervix
Raloxifene	Osteoporosis	• Bone • Serum lipids	• Breast • Uterus
Tamoxifene	Breast cancer	• Endometrium • Bone • Cardiovascular markers	• Breast

5.2 The LßT2 cell line: a mature, gonadotropic cell line of murine origin

The original aim of the present work was to develop and validate an *in vitro* bioassay using the murine, gonadotropic LßT2 cell line for the assessment of (anti-)estrogenic effects at the level of the pituitary gonadotrophs. This cell line has been developed by directing the simian virus 40 (SV40) T antigen oncogene to pituitary gonadotrophs of transgene mice using an 1800 bp fragment of the rat LHß-subunit (LHß-su) promoter region. Following characterisation of clones, isolated from the resulting tumour, LßT2 cells appeared to closely reflect characteristics of mature gonadotrophs *in vivo*. They expressed a functional GnRH receptor (GnRH-R), together with the common α glycoprotein hormone subunit (α-GSU) and the rat LHß-su (Alarid *et al.*, 1996; Mellon *et al.*, 1991; Windle *et al.*, 1990). Under basal conditions, LßT2 cells secrete rat LH, while repeated exposure to GnRH enhanced LH release, hereby demonstrating that the well-described self priming effect of gonadotrophs *in vivo* has been preserved (Turgeon *et al.*, 1996). However, it was initially presumed that LßT2 cells were not able to secrete FSH, as expression of its subunit appeared to be absent. Although, Graham and colleagues (1999) demonstrated FSH release following GnRH stimulus in the presence of

activin A.

The mature properties of the LßT2 cell line, together with the scarcity of similar gonadotropic cell lineages, makes it an important tool for researchers who are interested in the regulation of gonadotropin synthesis and secretion. A potential advantage of this cell line is the fact that it might replace the use of primary pituitary cell cultures, which show a limited lifespan, require labour-intensive preparation and are subjected to batch variability. Furthermore, gonadotrophs only represent 10-15% of the total pituitary cell population (Wang, 1988), hereby complicating the analysis of gonadotroph-specific effects when using primary pituitary cell cultures. For these reasons, we have chosen the LßT2 cell line to study estrogenic regulatory mechanisms at the level of pituitary gonadotrophs.

5.3 GnRH-enhanced LH secretion in the LßT2 cell line

In the present work, the first part of the results section describes the characterisation of the LßT2 cell line, followed by the analysis of estrogen-mediated effects on GnRH-induced LH synthesis and secretion

The cell line which was used in our laboratory originates from the laboratory of Dr. Mellon (San Diego, California, USA). This batch was directly obtained from her laboratory with some (negative) findings being checked in a second directly obtained batch. Using the reverse transcriptase polymerase chain reaction (RT-PCR) technique, we confirmed the expression of messenger RNA (mRNA) encoding for the GnRH-R, the α-GSU and both gonadotropin ß subunits (showing variable expression for FSHß). These results indicate that the LßT2 cell line, which was used for our experiments, preserved the characteristics of mature gonadotrophs. In table 5.2, we give a short overview of hormone (receptor) mRNA expression in the LßT2 cell line, as observed in our laboratory.

Table 5.2: Overview of hormone (receptor) mRNA and/or protein expression in the gonadotropic LßT2 cell line according to literature data, compared to our observations.

Hormone (receptor) mRNA expression in LßT2 cells	*Expected*	*Results*
• Gonadotropin-releasing hormone receptor	+	+
• α-glycoprotein hormone subunit expression	+	+
• Luteinizing hormone ß subunit expression	+	+
• Follicle Stimulating Hormone ß-subunit	+	variable
• Estrogen receptor α mRNA	+	+
• Estrogen receptor ß mRNA	+	weak
• Estrogen receptor α protein	+	+
• Estrogen receptor ß protein	not determined	-
• Truncated estrogen receptor product 1 mRNA	+	-
• Truncated ERα mRNA (exon 4 deletion)	not determined	+
• Androgen receptor expression	+	+
• Glucocorticoid receptor expression	+	+

In a next series of experiments, the effects of GnRH alone on LH synthesis and secretion have been evaluated in the LßT2 cell line. In order to allow data comparison, we have adapted the protocol of Turgeon *et al* (1994) and Nicol *et al* (2002). In our hands, daily repeated exposure to short pulses of GnRH (15 min) enhanced LH secretion, except on the first treatment day. Similar findings were reported by the above-mentioned groups. However, in their experiments, GnRH was able to stimulate LH release at the first treatment day. These data indicate that LßT2 cells may be naïve to GnRH and require self-priming in order to upregulate LH release. Nevertheless, at the third treatment day, the maximal secretory response was observed for cells treated with 10 nM of GnRH, while LH release decreased at higher concentrations, in agreement with the observations of Nicol and colleagues (2002). It has been well described that potent GnRH-R agonists and sustained exposure to GnRH may induce GnRH-receptor desensitisation and downregulation. According to our experiments, this seems an unlikely explanation for the decrease of LH secretory response during exposure to high GnRH concentrations. Indeed, rat LHß-su promoter activity and mRNA expression, as determined following repeated GnRH exposure at treatment day three, were dose-dependently stimulated by GnRH, showing maximal induction at 1 μM. In addition, when longer exposure periods (1h-6h) were applied, the LH secretory pattern was identical in comparison to cells treated according to the short pulse protocol (15

min). One possible explanation for the observed decrease in LH release in LßT2 cells which were repeatedly exposed to high concentrations of GnRH may be impairment of post-transcriptional processes and/or vesicle storage.

In this first part of the work, we have described the effects of GnRH alone at three important levels, involved in LH production, namely rat LHß-su promoter activity, the corresponding mRNA expression and LH secretion. This has shown that LßT2 cells do secrete LH in response to GnRH stimulation, display a self priming response to repeated exposure to GnRH that is reminiscent of a similar response *in vivo*, and that impaired response of LH during exposure to high GnRH concentrations does not seem to result from GnRH receptor desensitisation. Table 5.3 illustrates our results concerning LH synthesis and secretion in the LßT2 cells.

In a next step, we have evaluated LH secretory response following co-incubation with 17-ß-estradiol (E_2).

Table 5.3: Overview of the effects of GnRH alone, or in the presence of either E_2 or Dex, on LH synthesis and secretion in the gonadotropic LßT2 cell line according to literature data and compared to our observations.

LH synthesis/secretion in LßT2 cells	*Our results*
• GnRH-enhanced LHß-su mRNA expression	• Dose-dependent activation
• GnRH-enhanced LHß-su gene expression	• Dose-dependent activation
• Dose-dependent LH secretion by GnR	• Day 1: No effect • Day 2: Dose-dependent stimulation • Day 3: U-shaped dose-response curve
• E_2 stimulation of GnRH-enhanced LHß-su gene transcription and corresponding LH secretion	• No effects of E_2
• Dex stimulation of GnRH-enhanced LH-secretion	• Stimulatory effect of Dex

5.4 Absence of estrogenic effects in the mature, gonadotropic LßT2 cell line

Surprisingly, GnRH-mediated LH synthesis remained unaltered, while Turgeon *et al.* (1994) and Nicol *et al.* (2002) described significant albeit rather modest effects of estrogen exposure on LH secretion. Our negative results cannot be explained by the use of a different experimental protocol, as identical culture and experimental conditions were applied in our experiments. Moreover, those negative findings were confirmed by extensive evaluation of alternative experimental conditions (data not shown)

In order to evaluate ER functionality in the LßT2 cell line, we have performed cell proliferation assays and transient transfection studies using an estrogen-responsive plasmid (ERE-tk-Luc). Neither E_2 nor anti-estrogens such as the pure anti-estrogenICI 182,782 (also known as fulvestrant) and 4-hydroxy-tamoxifen (4-OH-Tam), the active metabolite of the SERM tamoxifen were able to elicit any effect on cell growth or estrogen receptor (ER)-mediated gene transcription (data not shown).

Analysis of estrogen receptor expression in our LßT2 cell line demonstrated the presence of estrogen receptor α (ERα) mRNA, while the ERα isoform transcript appeared to be absent. Furthermore, neither ERα nor ERß protein expression could be detected in Western analysis using several specific primary antibodies. The absence of any estrogenic response in our LßT2 cells can, at least in part, be explained by the absence of both ER isoforms. However, when LßT2 cells were transfected with an expression vector, encoding for the human ERα (hERα) in the presence of an estrogen responsive reporter plasmid, E_2 was not able to enhance ER-mediated gene transcription, despite the demonstrated expression of ERα protein using Western blot analysis. One could postulate that factors related to species specificity may be responsible for the latter negative results. However, parallel experiments, performed in the highly estrogen-responsive , murine TM4 Sertoli cell line demonstrated that overexpression of hERα enhanced ER-mediated luciferase expression in comparison to TM4 cells, transiently transfected with the ERE-tk-Luc vector alone (data not shown).

The lack of estrogenic response in our LßT2 cell line is not a general nuclear receptor problem because androgens and glucocorticoids were able to enhance AR- respectively GR-mediated gene transcription, as determined using transient transfection studies with the androgen and glucocorticoids responsive MMTV-reporter construct.

A short overview of the nuclear-receptor mediated effects on gene transcription in the LßT2 cell line is presented in table 5.4.

Table 5.4: Overview of the nuclear receptor (NR)-mediated gene transcription in the gonadotropic LβT2 cell line according to literature data and compared to our observations.

NR-mediated gene transcription in LβT2 cells	*Our results*
• Estrogen receptor-mediated gene transcription	• No response
• Androgen receptor-mediated gene transcription (MMTV-promoter)	• Dose-dependent activation
• Glucocorticoid receptor-mediated gene transcription (MMTV-promoter)	• Dose-dependent activation

Furthermore, in our experiments, the glucocorticoid dexamethasone (Dex) enhanced GnRH-induced LH secretion, in accordance to the reports by Turgeon *et al.* (1994) and Nicol *et. al* (2002). The LH secretory response in cells treated with both E_2 and Dex was identical in comparison to cells exposed to Dex alone.

Other factors may be responsible for the lack of estrogenic effects in our LβT2 cell line. In the literature, many researchers have investigated the existence of ERα mRNA splicing variants and their possible effects on full length ERα signalling, especially in humans (Poola and Speirs, 2001; Herynk and Fuqua, 2004). These mRNA variants may encode for proteins which can either enhance or inhibit ER transcriptional activity. In the rat and the mouse pituitary, a specific ERα variant, also called truncated estrogen receptor product 1 or TERP-1, has been identified by the group of Schreihofer. TERP-1 itself is transcriptionally inactive but can interfere with full length receptor activity in a positive or negative manner, depending on the ratio of TERP-1 over ERα. (Schreihofer *et al.*, 1999; Resnick *et al.*, 2000; Schreihofer *et al.*, 2000). However, we were not able to demonstrate TERP-1 expression in the LβT2 cell line, hereby excluding the hypothesis that this truncated isoform could negatively modulate ER activity in our LβT2 cell line. For this reason, we did screening for other possible ERα mRNA variants.

Currently, little information is available concerning ER expression during embryonic differentiation of the mouse pituitary gonadotrophs. In the chicken, Liu and colleagues (2005) demonstrated that ERα expression was already present in an early stage of pituitary development and was predominantly found in gonadotrophs. Furthermore, the authors reported an increase in ERα expression until the day of birth.

In the rat, ERβ expression appeared to be higher than that of ERα during embryonal

development, showing a reversal around day of birth (Nishihara et al., 2000). As mentioned before, ERß mRNA expression was rather low (αT3-1) or undetectable (LßT2), while no receptor protein could be detected in either cell lines following Western analysis (data not shown).

According to our experiments, wild type ERα is already present in an early stage of differentiation (E11.5; αT3-1 cells), hereby suggesting an important role of ERα in gonadotroph differentiation. However, in a later phase of gonadotroph development (E16.5-17.5; LßT2 cells), ERα expression appeared to be completely absent.

Using a primer set adapted from Swope and colleagues (2002), an alternative ERα mRNA splicing variant was detected in both gonadotropic αT3-1 and LßT2 cell lines. According to our knowledge, we describe for the first time the existence of this ERα mRNA splicing variant in pituitary gonadotrophs. This alternative message theoretically encodes for a 42.4 kDa protein, lacking most of the ER ligand binding domain. Although, we were unable to demonstrate the presence of this protein in the αT3-1 and LßT2 cell lines following Western analysis. One hypothesis may be that, following translation of this mRNA variant, the resulting protein folds in a different way in comparison to the wild type ERα. This may result in altered secondary and tertiary structures, whereby specific epitopes, to which the applied antibody is directed, are oriented differently and may not be recognised. Another possibility is the fact that this mRNA splicing variant is highly unstable. It has been demonstrated that unliganded nuclear receptors are stabilised by heat shock protein 90 (HSP90) by binding with the LBD (Fang et al., 2006). As this variant message encodes for a protein, lacking most of the LBD, it is possible that HSP90 is not able to bind this truncated protein, hereby resulting in faster protein degradation.

It is important to mention that the ratio of full length ERα mRNA to this mRNA splicing variant was remarkably lower in LßT2 cells in comparison to the αT3-1 gonadotropic and TM4 Sertoli cell lines, being both estrogen-responsive. As described previously, in our hands ERα expression was completely absent in the LßT2 cell line. Furthermore, following overexpression of ERα in these cells, no ER-mediated gene transcription could be demonstrated. These data suggest that, in view of the higher expression of the truncated message in comparison to the wild type ERα, the former may influence ERα signalling in a negative manner in the LßT2 cell line.

Our findings suggest an important role for this splicing variant in the regulation of ER signalling at the level of the pituitary gonadotrophs. The relative abundance of full length ERα and this variant message depends on the stage of gonadotroph differentiation and may determine the degree of estrogenic responsiveness. Future studies must reveal whether this truncated protein could act as dominant negative or positive transcription factors to modulate the expression of ER target genes and what

may be the regulatory mechanisms that regulate its relative abundance. Elucidation of these points may ultimately contribute to our understanding of the mechanisms underlying the developmental and physiological actions of estrogens in the pituitary gonadotrophs.

5.5 Estrogen receptor-mediated effects in the murine, gonadotropic αT3-1 cell line

As our interest pertains primarily to estrogenic effects at the level of the pituitary gonadotrophs, the LβT2 cell line was not a suitable model for our purposes. For this reason, we turned to the αT3-1 cell line as an alternative gonadotropic cell line for the study of estrogenic effects. The αT3-1 cell lineage was generated using SV40 Tag oncogenesis by the group of Mellon (Mellon *et al.*, 1991; Windle *et al.*, 1990). An 1800 bp fragment of the promoter region of the α-GSU gene was targeted to the pituitary of transgene mice. One of the obtained clones, namely the αT3-1 cell line, corresponded to an earlier stage of embryonic differentiation, showing GnRH-R and α-GSU expression, while gonadotropin β-su expression and corresponding hormone release remained absent. The presence of a functional ER, as described in several reports (Williams *et al.*, 2000; Demay *et al.*, 2001; Schausi *et al.*, 2003; Weiss *et al.*, 2003) made us decide to choose for the αT3-1 cell line as an alternative for LβT2 cells. The cell line, used in our laboratory, expressed mRNAs encoding for ERα and ERβ respectively, as demonstrated by RT-PCR. Furthermore, Western blot revealed the presence of both ERα and ERβ proteins, showing higher expression for the former isoform. Following transient transfection with an estrogen-responsive reporter plasmid (ERE-tk-Luc), E_2 dose-dependently stimulated ER-mediated gene transcription, hereby confirming receptor functionality. In table 5.5, we present an overview of hormone (receptor) expression in the αT3-1 cell line.

Table 5.5: Overview of hormone (receptor) mRNA and/or protein expression in the gonadotropic αT3-1 cell line according to literature data, compared to our observations.

Hormone (receptor) expression in αT3-1 cells	*Expected*	*Results*
• Gonadotropin-releasing hormone receptor mRNA	+	+
• α-glycoprotein hormone subunit mRNA	+	+
• Luteinizing hormone ß subunit expression	-	-
• Follicle Stimulating Hormone ß-subunit	-	-
• Estrogen receptor α mRNA	+	+
• Estrogen receptor ß mRNA	+	Weak
• Estrogen receptor α protein	+	+
• Estrogen receptor ß protein	-	-
• Insulin-like growth factor I receptor mRNA	+	+
• Insulin-like growth factor I receptor protein	Not determined	+
• Epidermal growth factor (EGF)	Not determined	-

As estrogens are important mediators of cell growth and differentiation, we have performed cell proliferation experiments to evaluate possible mitogenic effects of E_2 in the αT3-1 cell line. Several parameters, including serum concentration, start cell density and exposure time were thoroughly investigated in order to obtain optimal culture conditions. Following a five days incubation period, E_2 exhibited rather modest proliferative effects. Interestingly, under presumed estrogen-free conditions, ICI 182,780 and 4-OH-Tam appeared to be strong inhibitors of basal cell proliferation. Similar results were obtained in αT3-1 cells transiently transfected with the ER-responsive reporter plasmid ERE-tk-Luc. One could postulate that the presence of residual estrogens in the stripped fetal calf serum explains the observed growth reduction. For this reason, we have performed parallel experiments in the highly estrogen-sensitive TM4 Sertoli cell line. Incubation with raising concentrations of E_2 resulted in a biphasic growth curve, showing either growth stimulation or repression at low respectively higher concentrations. In contrast to our findings in the αT3-1 cells, ICI 182,780 and 4-OH-Tam were not able to affect basal cell proliferation. Furthermore, E_2 strongly enhanced ER-mediated gene transcription in TM4 cells which were transiently transfected with the ERE-tk-Luc reporter construct. In similarity to the growth experiments, neither of the two anti-estrogens affected basal gene transcriptional activity (data not shown). According to these data, we may conclude

that the observed growth inhibition in αT3-1 cells exposed to compounds with anti-estrogenic action is highly unlikely caused by blockade of residual estrogens in our incubation system but rather suggests involvement of other factors important for cell proliferation and differentiation.

In summary, this part of our experiments confirmed the presence of a functional ER-mediated signalling in the αT3-1 cells and showed that cell proliferation is modestly stimulated by E_2 while, surprisingly, inhibited by anti-estrogens under presumed absence of estrogenic stimulation. A short overview of the proliferation and transfection experiments, performed in αT3-1 cells, is given in tables 5.6 and 5.7.

Table 5.6: Overview of growth modulatory effects of E_2, IGF-I, the pure anti-estrogen faslodex and the SERM 4-OH-Tam in the gonadotropic αT3-1 cell line according to literature data, compared to our observations.

Growth modulatory effects in αT3-1 cells	*Expected*	*Results*
• Stimulatory effects of E_2	+	Dose-dependent stimulation
• Stimulatory effects of IGF-I	+	Dose-dependent stimulation
• Inhibitory effects of faslodex (ICI 182,780)	Not determined	Dose-dependent inhibition
• Inhibitory effects of 4-OH-Tamoxifen	Not determined	Dose-dependent inhibition
• Reversal of faslodex-induced growth inhibition by E_2	Not determined	Dose-dependent reversal by E_2
• Reversal of faslodex-induced growth inhibition by IGF-I	Not determined	Dose-dependent reversal by IGF-I
• Reversal of 4-OH-Tam-induced growth inhibition by E_2	Not determined	Dose-dependent reversal by E_2
• Reversal of 4-OH-Tam-induced growth inhibition by IGF-I	Not determined	Dose-dependent reversal by IGF-I

Table 5.7: Overview of the influence of E_2, IGF-I, the pure anti-estrogen faslodex and the SERM 4-OH-Tam on ER-mediated signaling in the gonadotropic αT3-1 cell line according to literature data, compared to our observations.

ER-mediated signaling in αT3-1 cells	*Expected*	*Results*
• Stimulatory effects of E_2 on ER-mediated gene transcription	+	Dose-dependent stimulation
• Stimulatory effects of IGF-I on ER-mediated gene transcription	Not determined	Dose-dependent stimulation
• Inhibitory effects of faslodex (ICI 182,780) on ER-mediated gene transcription	Not determined	Dose-dependent inhibition
• Inhibitory effects of 4-OH-Tamoxifen on ER-mediated gene transcription	Not determined	Dose-dependent inhibition
• Reversal of faslodex-induced inhibition of ER-mediated gene transcription by E_2	Not determined	Dose-dependent reversal by E_2
• Reversal of faslodex-induced inhibition of ER-mediated gene transcription by IGF-I	Not determined	Dose-dependent reversal by IGF-I
• Reversal of 4-OH-Tam-induced inhibition of ER-mediated gene transcription by E_2	Not determined	Dose-dependent reversal by E_2
• Reversal of 4-OH-Tam-induced inhibition of ER-mediated gene transcription by IGF-I	Not determined	Dose-dependent reversal by IGF-I
• ER-phosphorylation (serine 118) by E_2	Not determined	+
• ER-phosphorylation by IGF-I	Not determined	+

5.6 Growth factor receptor and estrogen receptor cross-talk in the αT3-1 cell line

In reproductive tissues such as the breast and the uterus, several studies revealed that estrogens may elicit their mitogenic effects through complex interactions with growth factors such as insulin-like growth factor (IGF) and epidermal growth factors (EGF) (Garcia-Segura *et al.*, 2006; Silva and Shupnik, 2007). We demonstrated the presence of the transcript encoding for the IGF-I receptor and expression of the corresponding protein in the αT3-1 cell line. In contrast, the EGF receptor (EGF-R) appeared to be absent, as assessed both at mRNA and protein level.

In our hands, IGF-I stimulated basal cell proliferation and appeared to be a stronger mitogen in αT3-1 cells in comparison to E_2. As expected, EGF did not alter αT3-1 cell growth. Interestingly, both E_2 and IGF-I were able to reverse anti-estrogen-induced growth inhibition to a similar extent. In addition, IGF-I stimulated ER-mediated gene transcription in αT3-1 cells which were transiently transfected with ERE-tk-Luc. In similarity to our observations in the proliferation experiments, this growth factor dose-dependently reversed anti-estrogen-induced inhibition of basal luciferase activity. Conversely, both IGF-I-induced effects on cell proliferation and ER-mediated effects were dose-dependently inhibited by the anti-estrogens ICI and 4-OH-Tam. These findings are in agreement with other reports, showing that both anti-estrogens repressed expression of important mediators of the IGF-IR signalling pathway Pollak *et al.*, 1992; Colletta *et al.*, 1994; Chan *et al.*, 2001).

Our data indicate that interactions between IGF-IR and ER may occur in αT3-1 cells. Currently, the mechanisms of action are not completely elucidated, although, there is evidence that the estrogen-mediated and growth factor-mediated pathways of cell proliferation require common downstream signalling pathways. Growth factors act through high affinity tyrosine kinase receptors (Herbst, 2004; Scaltriti and Baselga, 2006) while estrogen receptors belong to the superfamily of nuclear receptors (Mangelsdorf *et al.*, 1995; Truss and Beato, 1993). Many studies have investigated these receptor interactions in the breast and the uterus. The main mechanism of action involves activation of the mitogen-activated protein kinase (MAPK) signalling pathway following ligand binding to the IGF-1R. In turn, activated MAPKs stimulate ERα phosphorylation, which finally results in enhanced receptor transcriptional activity, even in the absence of estrogens (Chen *et al.*, 2002; Joel *et al.*, 1998; Lee and Bai, 2002). Interestingly, activation of MAPK pathway is also induced by E_2 itself and requires mobilisation of intracellular calcium. It appears that a feed-forward system exists where E_2 activates MAPK, an event that in turn enhances ERα transcriptional

activity. This has been extensively described in chapter 1.2.4 of the introduction.
It is important to mention that cross-talk only occurs between the IGF-IR and ERα, and not with ERß. These differences may be in part due to different activating function 1 (AF-1) domains because ERα reveals constitutive AF-1 activity, whereas ERß lacks constitutive AF-1 activity (Mendez *et al.*, 2006).
Several studies showed that ERα phosphorylation occurs at the amino-terminal A/B domain. However, outside of this domain, sites have been identified in the DNA binding domain (DBD), in the hinge region and in the ligand binding domain (LBD). Within the A/B domain, Ser-104, -106, -118, -154, and -167 were characterised as phosphorylation sites in the presence of E_2. However, one of the main targets of phosphorylation is Ser-118 (Ali *et al.*, 1993; Kato *et al.*, 1995). Indeed, in our experiments, using a site-specific phospho-ERα antibody, we demonstrated that both E_2 and IGF-1 induced ERα phosphorylation at Ser-118. However, a higher phosphorylation status was observed for αT3-1 cells treated with E_2 when compared to IGF-I. The combination of both hormones resulted in a synergistic effect on ERα phosphorylation. We already mentioned that increased ERα phosphorylation resulted in enhanced receptor transcriptional activity. However, in our experiments, we observed that both 4-OH-Tam and ICI were able to stimulate ERα phosphorylation at Ser-118, while ER-mediated gene transcription dose-dependently decreased following SERM or pure anti-estrogen exposure. Similar findings were reported in breast cancer cells by Le Goff *et al.* (1994) and Joel *et al.* (1998). However, ERα phosphorylation is mediated by different kinases, depending on agonist or antagonist binding. Furthermore, ER transcriptional activity not only depends on its phosphorylation status. Other factors, including receptor dimerisation, receptor conformation and the binding of coregulators will determine the final outcome following ligand binding (see also Introduction chapter 1.2.3.1 for review).
Our data indicate that MAPK signalling may be involved in cross-talk between ERα and the IGF-IR in the αT3-1 cell line. Indeed, the specific MAPK kinase inhibitor PD-98059 strongly repressed IGF-1-induced luciferase activity in αT3-1 cells transiently transfected with ERE-Luc. Interestingly, the effect of E_2 was only partially inhibited following co-incubation with PD-98059. These data suggest that not only MAPK is involved in ERα activation following E_2-binding, but that other kinases are involved in ERα phosphorylation in the αT3-1 cell line.
It has long been assumed that estrogens only elicit genomic actions through their receptor. However, in recent years, rapid, non-genomic actions of estrogens have been reported, whereby the MAPK signalling pathway plays an important role (Dos Santos *et al.*, 2002; Xiao-Dong *et al.*, 2007).

5.7 Physiological relevance of growth factor and estrogen receptor cross-talk in gonadotrophs?

According to our knowledge, very limited literature data have reported interactions between ERα and the IGF-IR at the level of the pituitary gonadotrophs (Kanematsu *et al.*, 1991; Adam *et al.*, 2000; Pazos *et al.*, 2004; Weiss *et al.*, 2006). Currently, the physiological relevance of this phenomenon remains unclear. However, a limited number of studies demonstrated that IGF-I was able to alter LH secretion. Furthermore, Rose and colleagues (2004) reported interactions between the GnRH-R and the IGF-IR in αT3-1 cells. As extensively described in the introduction (chapter 1.3.2.1), GnRH elicits its effects through its G-protein coupled receptor. Following ligand-binding, the GnRH-R activates second messengers such as cyclic AMP (cAMP), inositol-1, 4, 5-triphosphate (IP3) and calcium production. It has been described that estrogens may also interfere with this signalling pathway in a non-genomic manner via physical interactions between membrane-bounds ERs and Gαs- and Gαq proteins. These G-proteins play a key role in the cellular regulation of above mentioned second messengers of the GnRH signalling pathway. All these findings together suggest that estrogens may, at least in part, elicit their effects on GnRH-mediated LH synthesis and secretion through complex interactions with the IGF-IR. However, more research is required to further elucidate the exact mechanisms of action behind this cross-talk.

In summary, we have demonstrated that cross-talk exists between ERα and the IGF-IR in the αT3-1 cell line. Both receptors interact with each other through the MAPK signalling pathway, which plays a key role in the regulation of cell differentiation and proliferation. As previously mentioned, the physiological importance of this phenomenon remains unclear at the present. However, a limited number of reports in the literature, together with our data, indicate that IGF-I signalling may be an important mediator of estrogenic effects at the level of the pituitary gonadotrophs. According to our experiments in the more differentiated LβT2 cell line, IGF-IR expression remained absent, as determined at mRNA and protein level respectively (data not shown). Mellon and colleagues demonstrated that the latter cell line corresponds to embryonic day 17.5, while αT3-1 cells reflect an earlier stage of differentiation (E11.5) (Mellon *et al.*, 1991; Windle *et al.*, 1990). One could postulate that, in mice, IGF-I and ERα may play a crucial role in the differentiation process of gonadotrophs during embryonic development, while in a later stage of differentiation, IGF-IR and ERα signalling may disappear, as reflected by our observations in LβT2 cells. Nevertheless, this seems highly unlikely, as other groups have reported modulatory effects of IGF-I on LH secretion in rodent. Moreover, E_2 is a well documented regulator of gonadotropin secretion at the pituitary level, as shown *in vivo* in a variety of

mammal species.

A more acceptable hypothesis is the fact that LßT2 cells may not faithfully reflect *in vivo* characteristics of pituitary gonadotrophs, whereby E_2 signalling seems to be a less stable feature of these cells, being sensitive to dedifferentiation due to presently unknown factors.

Despite the lack of gonadotropin ß su expression and the corresponding hormone release in the αT3-1 cells, this cell line seems to be an interesting *in vitro* model for the study of estrogenic regulatory effects in pituitary gonadotrophs.

5.8 The αT3-1 cell line: an *in vitro* model for the assessment of potential estrogenic compounds at the level of pituitary gonadotrophs

In previous parts of this work, we have evaluated estrogen receptor-mediated effects in two immortalised, gonadotropic cell lines of murine origin, differing in stage of differentiation; only the αT3-1 cell line seems to be potentially suited for the *in vitro* study of estrogenic effects on gonadotrophs *in vitro*. Despite some limitations, including the lack of gonadotropin ß-su gene expression, the αT3-1 cell line is a useful *in vitro* tool to assess agents with potential estrogenic properties at the level of the pituitary gonadotrophs.

In ongoing work in our laboratory, potential estrogenic activity of phytoestrogens on pituitary gonadotrophs is assessed using the αT3-1 cell line. These kinds of studies are needed because of the growing interest in the use of phytoestrogens as estrogen replacement therapy in menopausal women. It has been widely demonstrated that estrogen withdrawal may play an important role in the onset of typical menopausal symptoms such as hot flushes, vaginal dryness and tachycardia. Furthermore, these symptoms are correlated with elevated gonadotropin serum levels. For this reason, the intake of phytoestrogens may be an alternative for hormonal replacement therapy, as these compounds mainly elicit their effects through estrogen receptors. Nevertheless, in clinical studies, the observed effects of phytoestrogens, such as isoflavones, on menopausal symptoms remain contradictory and therefore inconclusive (Wuttke *et al.*, 2007).

The target site of these plant-derived compounds in the central compartment of the reproductive axis may be located at the level of the hypothalamus and/or the pituitary. However, at present, rather little information is available concerning gonadotroph-specific effects of these plant-derived compounds. Therefore, results obtained in the above-described study in αT3-1 cells, may provide useful information on possible regulatory effects and the corresponding mechanisms of action of these

plant-derived compounds at the level of the gonadotrophs.

As an example, we show in figure 5.1 an example of preliminary data on growth modulatory effects in αT3-1 cells exposed to derivatives of 8-prenyl-naringenin (8-PN) derivatives. At this moment, 8-PN has been identified as one of the most potent phytoestrogens, together with genisteine (Milligan *et al.*, 2000; Milligan *et al.*, 2002). 8-PN belongs to the group of prenylated flavanones, together with 6-prenylnaringenin and isoxanthohumol. These compounds have been isolated from hops (*Humulus lupulus*), a flower which is used as a flavouring agent in the brewing industry (Milligan *et al.*, 1999). In our hands, 8-PN dose-dependently induced cell proliferation in a similar manner as E_2 in the αT3-1 cell line (data not shown).

Recently, Roelens and colleagues have developed synthetic derivatives of 8-PN by substituting the prenyl group at position 8 by alkyl chains, differing in chain length and branching pattern (Roelens *et al.*, 2006). In our laboratory, we have investigated ERα and ERß transactivation capacities of 8-PN and its derivatives. For this purpose, human, cervical Hela cells were transiently transfected with an estrogen-responsive reporter plasmid in the presence of either a human ERα respectively ERß expression vector. Based on the obtained results, we have selected compounds showing ER antagonistic properties. These derivatives were characterised by the presence of a long alkyl chain (being n-heptyl, n-nonyl respectively n-undecyl) at position 8. Following exposure to these naringenin-derivatives, αT3-1 cell proliferation was dose-dependently inhibited, showing a similar response as observed with the pure anti-estrogenICI 182,780 and 4-OH-Tam.

As mentioned before, these data are preliminary and additional experiments, including transient transfections and analysis of growth factor receptor cross-talk, are required to further elucidate the effects of phytoestrogens such as 8-PN and its derivatives in the αT3-1 cell line.

5.9 Advantages and disadvantages of gonadotroph cell lineages

The effects of GnRH on gonadotropin release by pituitary gonadotrophs have been well described. Nevertheless, the molecular mechanisms of action behind gonadotropin synthesis and release are not fully elucidated. Therefore, gonadotropic cell lines such as the LßT2 cell line, expressing both LH and FSH ß subunits, are welcome *in vitro* tools for this purpose. According to our knowledge, the number of gonadotropic cell lineages is very limited. Less differentiated cell lines (for example the αT3-1 cell line) are also available, but the lack of ß-subunit expression is a limitation for the study of gonadotropin regulation. For this reason, Yonehara and colleagues (2003) stably

transfected the αT3-1 cell line with the complementary DNA (cDNA) encoding for the LHß-subunit. The obtained clone, the LH2 cell line, was found able to synthesize and secrete LH.

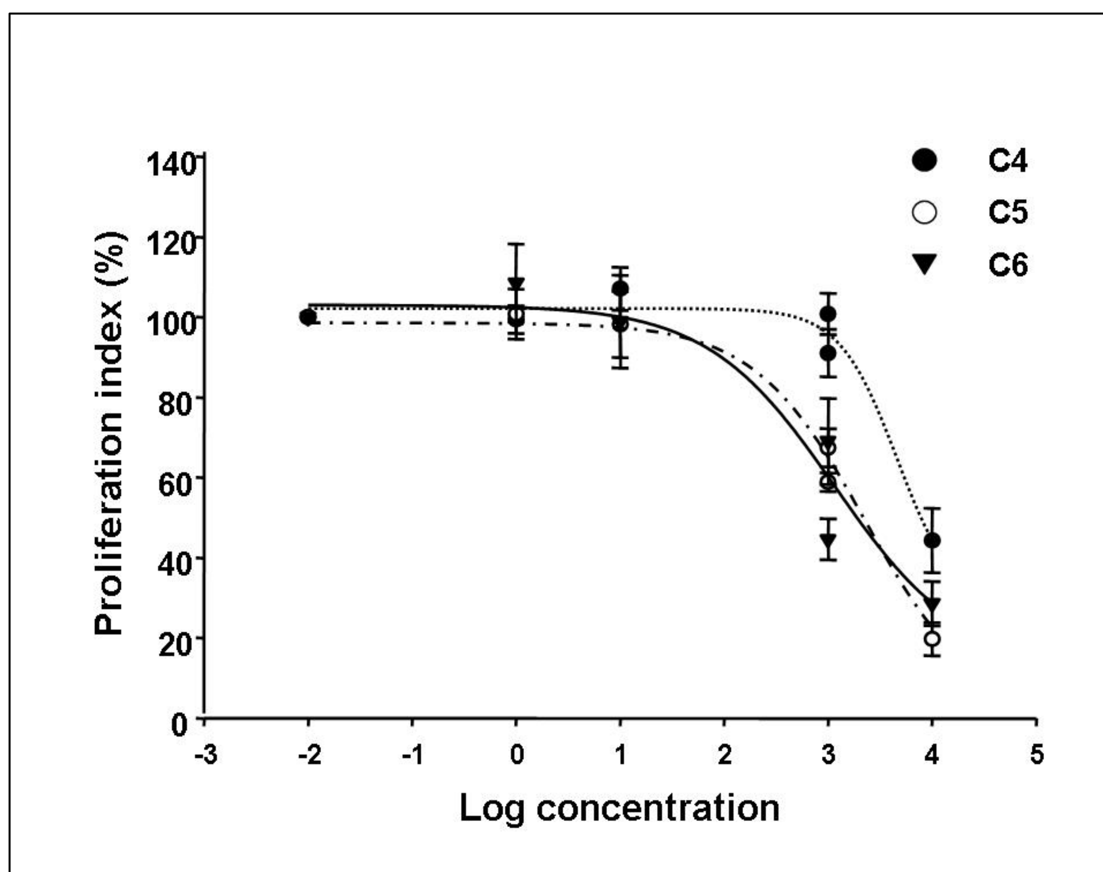

Figure 5.1: Growth inhibitory effects of 8-prenyl-naringenin derivatives in the gonadotropic αT3-1 cell line.

Gonadotropic αT3-1 cells were exposed to derivatives of 8-prenyl-naringenin, whereby the prenyl group at position 8 was substituted by a long alkyl chain, varying in chain length (C4: n-heptyl; C5: n-nonyl; C6: n-undecyl). αT3-1 cells were treated for 5 days with raising concentrations (1-10000 nM) of these compounds. Cell proliferation was determined using the SRB assay. Data are calculated as a percentage of vehicle control and expressed as a mean ± SD of 3 independent experiments (Unpublished results by Eertmans F., Roelens F., De Keukeleire D. and Kaufman J.-M.)

However, since its development, the LßT2 cell line served as the golden standard for *in vitro* analysis of gonadotroph-specific effects because of its mature characteristics, reflecting physiological properties *in vivo*. During recent years, a large body of research data described different aspects of gonadotropin synthesis, secretion and storage in LßT2 cells. However, caution is required when interpreting data, obtained

with immortalised cell lines. This is nicely illustrated by the absence of estrogenic response in the LβT2 cell line in our hands. Moreover, this finding being at variance with prior reports of estrogenic responses in LβT2 cells, although the latter reports are rather limited and the reported effects rather weak. Several factors, including dedifferentiation and culture conditions may contribute to these observed discrepancies.

Another limitation of studies such as ours in isolated cell lines is the fact that only gonadotroph-specific responses have been evaluated. It has been demonstrated that, *in vivo*, gonadotrophs interact with other (secretory and non secretory) pituitary cell types, including lactotrophs and somatotrophs (Andries *et al.*, 1995; Cheung, 1983; Denef and Andries, 1983). Nevertheless, these interactions may complicate data analysis when using tissue explants or primary pituitary cell cultures, which are also difficult to standardize. Obviously the choice of experimental model will depend on the nature of the questions asked (e.g. screening for modulatory effects of pharmacological compounds versus study of complex physiological regulatory mechanism) and different models for *in vitro* study of gonadotropin regulation should be regarded as complementary, *in vitro* study being in turn complementary to *in vivo* approaches.

In conclusion, notwithstanding the described limitations and the necessity of careful data analysis, gonadotropic cell lines such as the αT3-1 and LβT2 cell lines are useful tools for the study of gonadotropin synthesis and secretion. In our hands, only the αT3-1 cell line was found suitable for the purpose of analysing estrogenic effects in gonadotrophs. In particular, we expect that in ongoing and future work, this model can prove useful to assess potential (anti-)estrogenic effects of phytoestrogens and xenoestrogens the gonadotrophs...

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Eertmans F, Dhooge W, De Wever O, Vanden Berghe W, Bogaert V, Bracke M, Haegeman G, Comhaire F, Kaufman JM. 2007. Estrogen receptor signalling is an unstable feature of the gonadotropic LßT2 cell line. *Molecular and Cellular Endocrinology* **273** (1-2): 16-24.

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Abstracts & poster presentations

"Development of a bioassay to determine the estrogenic endocrine disrupting effects of pure agents and effluents."; Eertmans F, Dhooge W and Kaufman JM; *Symposium Milieu en Gezondheid*, Leuven. January 2001.

"The LβT2 cell line as a model to investigate the action mechanisms of endocrine disruptors."; Eertmans F, Dhooge W, Comhaire FH, Kaufman JM; *15th symposium of the Journal of Steroid Biochemistry and Molecular Biology,* Munich, Germany. June 2002.

"The LβT2 cell line as a potential model for the evaluation of endocrine modulating substances at the level of the gonadotroph."; Eertmans F, Dhooge W and Kaufman JM; *16th symposium of the Journal of Steroid Biochemistry and Molecular Biology*, Seefeld, Austria. June 2004.

"Differential effects of (anti-)estrogens on cell proliferation in the αT3-1 and LβT2 gonadotropic cell line."; Eertmans F, Dhooge W and Kaufman JM; *European Congress of Endocrinology*, Göteborg, Sweden. September 2005.

"Insulin-like growth factor I receptor and estrogen receptor cross-talk in the murine αT3-1 gonadotropic cell line."; F.Eertmans, W. Dhooge and J.-M. Kaufman; *17th symposium of the Journal of Steroid Biochemistry and Molecular Biology*, Seefeld, Austria. June 2006.

Curriculum Vitae

Frank Eertmans (° Moeskroen, 1976) behaalde in juni 1999 het diploma van Apotheker aan de Universiteit Gent met onderscheiding. Na een jaar forensisch onderzoek op het laboratorium van Prof. Dr. Apr. E. Van Den Eeckhout (Faculteit Farmaceutische wetenschappen) startte hij op 1 januari 2000 zijn doctoraatsstudie op de dienst Endocrinologie onder leiding van zijn promoter Professor Kaufman. De eerste twee jaar waren gericht op het analyseren van estrogene activiteit van een reeks geneesmiddelen op vraag van een farmaceutische firma. Vervolgens behaalde hij een vierjarige beurs bij het Bijzonder Onderzoeks Fonds (BOF). Eind 2006 liep dit BOF-mandaat af en werkte hij als apotheker in het Psychiatrisch Centrum Sint Jan de Deo te Gent. Tijdens deze periode is hij ook gestart met zijn postdoctoraal mandaat bij Professor Depypere, dienst Gynaecologie en vrouwenziekten, UZ Gent.

Frank Eertmans is (co)-auteur van 5 internationale en 2 nationale publicaties. Hij won 1 prijs op het symposium van de Belgian Endocrine Society (BES) in de categorie "Fundamental Research".

Awards

Young Investigators Award. Belgian Endocrine Society. 24 november 2006.

Postgraduate Training

Course in Bio-Informatics. Ghent. 2004.

<u>Oral presentations</u>:

"Endocrine disruptors: effects on male fertility and screening tools for their assessment"; Invited speaker; Invitox 2002, Formia, Italy. October 2002.

"Ecologische invloeden op de menselijke vruchtbaarheid"; Invited speaker; WHO dag van de vruchtbaarheid, Leuven, Belgium. April 2005.

"Insulin-like growth factor I receptor and estrogen receptor cross-talk in the murine αT3-1 gonadotropic cell line."; Belgian Endocrine Society meeting, Brussels, Belgium. November 2006.