Expression of Type 2 Orexin Receptor in Human Endometrium and Its Epigenetic Silencing in Endometrial Cancer

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Context: Orexins A and B are neuropeptides that bind and activate 2 types of receptors. In addition to direct action in the brain, the orexinergic system has broader implications in peripheral organs, and it has been proposed to have a role in the induction of apoptosis. There are very few data on the endometrium.

Objective: The expression and epigenetic regulation of type 2 orexin receptor (OX2R) was investigated in the human endometrium as well as in endometrial endometrioid carcinoma (EEC).

Methods: OX2R localization was studied by immunohistochemistry in normal endometrium (n = 24) and in EEC (n = 32). The DNA methylation status of a CpG island located in the first exon of *OX2R* was analyzed by bisulfite sequencing in normal (n = 18), EEC (n = 34), and 3 endometrial cell lines. On the latter, mRNA expression and Western blotting as well as in vitro induction with orexin were performed.

Results: Expression of the OX2R protein was detected in normal endometrial epithelia, whereas it was frequently lacking in EEC. This loss was associated with hypermethylation of *OX2R* in EEC in comparison with normal endometrium (median CpG methylation percentages of 48.85% and 5.85%, respectively). In cell lines, hypermethylation correlated with weak OX2R expression. Additionally, in vitro treatment of the 3 EEC cell lines with orexins A and B did not result in proliferation change

Conclusions: Altogether our data provide evidence for the epigenetic silencing of *OX2R* in EEC. The implication of the OX2R loss in tumoral progression remains to be elucidated. (*J Clin Endocrinol Metab* **98: 1549–1557, 2013**)

The orexins, also known as hypocretins, are 2 neuropeptides, orexin A (OX A) and B (OX B), processed from a common precursor by proteolytic cleavage (1). Orexins bind to and activate 2 types of serpentine G-coupled receptors, type 1 and type 2 receptors (OX1R and OX2R) with different affinity. OX1R is highly selective for OX A, whereas OX2R is a nonselective receptor that binds both OX A and OX B (2). Although initially described as neuropeptides involved in the regulation of feeding behavior and localized in the hypothalamus, the orexins are now considered to have a broader action in peripheral tissues, and several groups have demonstrated their involvement in a number of physiological processes (reviewed in Reference 3). In the gastrointestinal tract, orexins have been found to regulate not only the gastric, intestinal, and pancreatic secretions but also gut motility (4). In the rat (5, 6) and human (7), the testis holds probably the second highest local orexin concentration after the brain (8), and it is speculated that orexins might act in an autocrine/paracrine manner (7). In the rat ovary, a

Abbreviations: EEC, endometrial endometrioid carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; OX A, orexin A; OX B, orexin B; OX1R, orexin receptor type 1; OX2R, orexin type 2 receptor.

strong up-regulation of both OX1R and OX2R was reported during proestrus together with a similar increase in the hypothalamus and pituitary, suggesting an involvement of orexins in ovulation (9).

The orexinergic system has also been proposed to have implication in the induction of apoptosis (reviewed in Reference 10). Notably the OX1R seems to mediate the apoptosis induced by both OX A and OX B in vitro in colon cancer cell lines (11), leading to the proposal of OX1R as a therapeutic target in cancer therapy (10).

Concerning endometrium and endometrial cancer, very few data exist, and to our knowledge, only the absence of OX A immunohistochemical reactivity has been reported in the human endometrium (12). Endometrial carcinoma is a hormone-related cancer that is the most common gynecological malignancy in the Western world, affecting on average 15–20 women of 100 000 per year. Based on clinical-pathological and molecular characteristics, a clear dichotomy can be drawn along 2 distinct pathways. The first is type I estrogen-dependent endometrial endometrioid carcinoma (EEC), which represents the vast majority of cases, and the second is type II, which includes high-grade papillary serous and clear cell carcinomas. EEC has a general favorable outcome and is often associated with a clinical history of unopposed estrogen exposure. However, although great progress has been recorded recently in the understanding of genetic events during cancer progression (reviewed in Reference 13), the molecular pathogenesis of these cancers remains poorly understood. Deregulation of gene expression is a hallmark of cancer. It is now recognized that in addition to genetics, aberrant epigenetic modifications play a major role in the tumorigenic process (14).

DNA methylation is the most studied epigenetic modification, and hypermethylation of CpG islands within the regulatory region of tumor suppressor genes has been increasingly described for endometrial cancer (reviewed in Reference 15). This event has been shown to contribute in the silencing of the corresponding gene and may confer a growth advantage for tumor cells in many cancers (16, 17). Distinct patterns of DNA methylation among different tumors are now used as signature for diagnosis and prognosis (18). For the determination of methylation intensities, the gold standard is the bisulfite sequencing after bacterial cloning, which allows precise and quantitative mapping of CpG sites in individual alleles (19).

In the current study, the loss of OX2R protein in a cohort of 34 EECs as compared with 18 normal endometrium samples was shown to be associated with the hypermethylation of the first exon of *OX2R*. To shed light on the implications of the OX2R silencing in the context of endometrial carcinogenesis, 3 endometrial cell lines were induced with graded concentrations of orexins.

Materials and Methods

Endometrial tissues

Endometrial cancer samples were collected from 34 patients who underwent hysterectomy. The study was approved by the Ethical Committee of University of Liege. Each cancer sample was staged and graded by routine pathology analysis according to usual methods and type I endometrioid cancer (EEC) were selected for subsequent analysis. EEC lesions were subdivided into grade 1, 2, and 3. Grading was done independently by 2 pathologists, and discordances were discussed until consensus was reached. Cancer tissue was obtained by macroscopic dissection of the lesion and homogenous cancer tissue was selected, avoiding necrotic regions that may be present in some cases. Normal endometrial glands were obtained from premenopausal (n = 8) or menopausal (n = 10) women (mean age 48 years, range 32–75 years) by curettage with Novak curette. For premenopausal women, normal endometrial tissues were collected during routine exploratory biopsy, regardless of the menstrual cycle. Additionally, a panel of 24 control endometrium representative of the complete menstrual cycle and menopausal status was used to establish the distribution of OX2R.

Immunohistochemical staining for the OX2R antigen on formalin-fixed, paraffin-embedded sections

After dewaxing in xylene, sections were pressure cooked for 1 minute in citrate buffer (pH 6.0) and incubated overnight at 4°C with 1:100 dilution of antibody MAB 52461 (R&D Systems, Europe, Ltd, Abington, United Kingdom). Detection was performed with biotinylated goat antimouse IgG (E0433, 1:400; DakoCytomation, Glostrup, Denmark) followed by streptavidin/horseradish peroxidase (P0397, 1: 500; DakoCytomation). Staining was done with 3–3′-diaminobenzidine hydrochloride (K3468, DakoCytomation) as chromogen and hematoxylin as counterstain. Negative controls were performed by both omitting the primary antibody and incubating the sections with nonspecific IgG. The specificity of MAB 52461 in this protocol was verified on control human testis tissue on which a labeling limited to Leydig cells was obtained as previously described (7).

Cancer cell lines and DNA extraction

Human cell lines, ECC-1, Ishikawa, and MFE-280 were from the European Collection of Cell Cultures and were purchased from Sigma-Aldrich (Health Protection Agency Culture Collections, Salisbury UK) and cultured at low passages. ECC-1 and Ishikawa cell lines are derived from well-differentiated EEC and are often chosen as models of endometrial epithelium in vitro (20). By contrast, the MFE-280 cell line is derived from a recurrent poorly differentiated endometrial carcinoma and, although being nearly diploid, represents a more advanced cancer grade (21). All cell lines were grown in MEM (Gibco, Carlsbad, California) supplemented by 5% or 10% fetal calf serum as recommended by the supplier. DNA from frozen endometrial tissue and cell lines was isolated using a Puregene DNA purification kit (QIAGEN, Hilden, Germany) according to the supplier's rec-

ommendation. The DNA concentration was determined with a spectrophotometer (NanoDrop Technologies Inc, Wilmington, Delaware), and the quality was assessed by agarose gel electrophoresis.

Methylation data validation by bisulfite sequencing

A bisulfite modification-based genomic sequencing was used to establish a detailed mapping of the DNA methylation pattern. Control and cancer DNA (1 μg per modification) was treated by bisulfite conversion (Methyl Easy kit; Human Genetic Signature, Sydney, Australia). Following PCR amplifications using 2 sets of primers for the proximal (RCTCAATACTACAAACTC-CTCTCC and GTGTTGGAATGAGGAGTAATTGAG) or distal regions (CTCTTTAAAACCTTCTCAACC and GGAGTT-GAATGAAATTTAAGAGTTTTTT), fragments were cloned into pJET 1.2 vector (Fermentas, Burlington, Canada), and random colonies were screened for the correct amplicon via colony PCR. A minimum of 6 colonies per samples were sequenced (Big Dye terminator cycle sequencing; Applied Biosystems, Foster City, California) and analyzed using the BISMA web site (22)

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cells pellets using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Five micrograms of total RNA was reverse transcribed and oligo (deoxythymidine) primers and reverse transcriptase (Amersham, Little Chalford, Buckinghamshire, United Kingdom). Five percent of the cDNA mixture were amplified using OX2R sense primer (GTCGCAACTGGT-CATCTGCT) and OX2R antisense primer (CGTCCTCAT-GTGGTGGTTCT). Each of the 35 cycles of amplification consisted of 1 minute at 94°C, 30 seconds at 65°C, and 45 seconds at 72°C. Amplicons were separated by electrophoresis in 1.8% agarose gel and viewed under UV illumination. Similarly, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was amplified using specific primers (sense, TGATGACATCAAGAAGGTGGTGAAG, and reverse, TCCTTGGAGGCCATGTGGGCCAT).

Western blotting

Protein lysates were run on a Laemmli 10% bis tris gel under reducing conditions for 90 minutes at 100 V. Separated proteins were blotted onto polyvinyl difluoride membranes (GE Healthcare, Uppsala, Sweden) for 60 minutes (100 V) and blocked 1 hour in 5% milk/PBS. Primary antibody against OX2R (ab3094, 1:500, overnight at 4°C; Millipore, Bellerica, Massachusetts) was followed by antirabbit secondary antibody (Amersham). Blots were developed using an ECL Plus Western blotting detection system (GE Healthcare) and detected with ECL hyperfilm. After a stripping step, the same blot was assayed with anti-GAPDH antibody (Amersham) using the same method.

Cell proliferation and apoptosis assays

Cells were plated in a 96-well tissue culture plate (625, 1250, or 2500 cells/well) containing OX A or OX B (Polypeptide Laboratories, Strasbourg, France) at concentrations of 10^{-6}, 10^{-7}, or 10^{-8} M. A cell proliferation test was performed after 24, 48, and 72 hours using the WST1 kit as described by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). The

experiment was repeated at least 3 times in duplicate. To monitor the apoptosis in the same culture conditions, a caspase-3/7 assay was used according to the supplier's recommendation (ApoTox-Glo; Promega BioSciences, Madison, Wisconsin).

Statistical analysis

We assessed the statistical differences between different experimental groups using a Mann-Whitney test. A $P < .05$ (*) was considered as significant. The statistical analyses were carried out using the Prism 5.0 software (GraphPad, San Diego, California) (*$P < .05$; **$P < .01$).

Results

The expression pattern of OX2R protein was first investigated in normal cycling endometrium by immunohistochemistry (IHC) using an anti-OX2R monoclonal antibody. We tested a panel of 24 normal endometrial tissues from untreated women covering the entire menstrual cycle as well as postmenopausal endometrium. An intense to moderate immunoreactivity with an OX2R antibody was found in glandular cells. The most intense staining was detected in the premenopausal secretory phase as well as in the menopausal condition (Figure 1A and Table 1). By contrast, when the OX2R antibody was applied on the 32 EEC cases collected for this study (Table 2), 21 samples (65.6% of the total) obtained an IHC score 0 corresponding to an absence of staining either complete either with minor area of labeling, which often localized close to the interface with the myometrium (Figure 1, B and C). For the remaining 11 cases of EEC (34.4% of the total), a weak or moderate immunostaining was recorded on the tumoral tissue (IHC scores 1 or 2, Figure 1D).

To explain the frequent loss of OX2R immunostaining, the methylation status of the CpG island located in exon 1 of the *OX2R* gene was evaluated on 32 EEC samples tested on IHC and on 18 controls endometria using bisulfite sequencing. Figure 2A shows the sequence of *OX2R* exon 1 locus with the localization of the 27 CpG sites investigated (14 in the proximal portion and 13 in the distal portion). Two different PCRs were used to cover the whole exon 1 locus (proximal and distal portion) with the exception of 90 nucleotides comprising 6 CpG sites. Those 90 nucleotides were excluded for technical reasons (inability to successfully amplify these regions by PCR).

Representative bisulfite sequencing charts of individual CpG sites located on OX2R exon 1 are displayed in Figure 2, B and C, for patient 119 (grade 1) and control 15. The complete bisulfite sequencing data for EEC and controls samples are available in Supplemental Figures 1 and 2, respectively, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. Table 2 and Figure 3A display the percentages of methylated
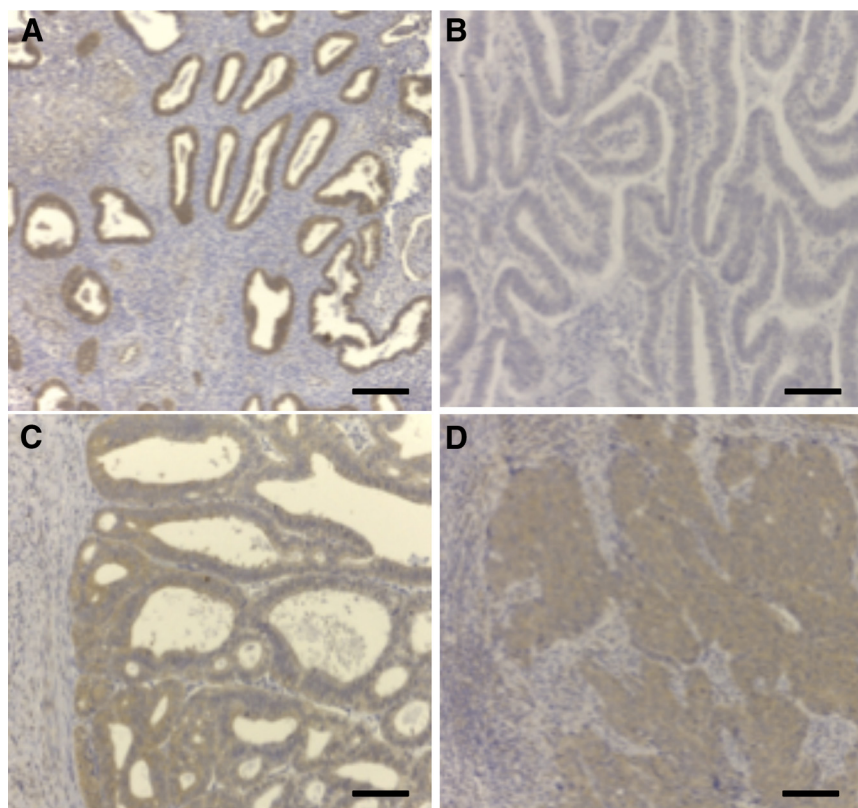
Figure 1. Representative IHC of OX2R staining in control endometrium (A) and EEC (B–D). A, In normal secretory endometrium, glands are intensely stained. B, No staining was detected in grade 1 EEC (case 104, IHC score 0). C, Grade 2 EEC in which a focal zone localized close to the myometrium is showing weak diffuse staining, whereas most the carcinoma was characterized by an absence of staining (case 130, IHC score 0). D, Moderate staining in grade 2 EEC (case 124, IHC score 2). Scale bar is 100 μm.

ylation categories for the controls and EEC, showing again significant differences ($P = .0001$). Figure 3, C–G, show bisulfite sequencing data allocated into the 3 methylation categories for each control woman (3C) and each EEC patient (grades 1–3, Figure 3, D–F). The results summarized as mean values in Figure 3G show comparable values for the 3 methylations categories for grades 1 and 2 EEC and a tendency toward less hypermethylated clones in grade 3, although not statistically significant because of a low inclusion of grade 3 EEC (n = 3).

Next, the methylation of the first exon of *OX2R* gene was assayed in 3 endometrial cell lines of epithelial lineage (Figure 2D). In Ishikawa cells, bisulfite sequencing revealed sparse methylation thorough the exon 1 except on CpG sites 11 and 12 from the proximal region, which were heavily methylated. By contrast, clones from the ECC-1 cell line were hypermethylated. In MFE280, both completely methylated and unmethylated clones were recorded, but the analysis of the sequence of these clones (data not shown) did not allow the distinction between the paternal and the maternal alleles by the means of any nucleotide polymorphism. The transcription of the *OX2R* gene was evaluated by RT-PCR and the presence of OX2R protein by Western blotting in the 3 cell lines (Figure 2, E and F). By RT-PCR, MFE280 cells displayed the highest mRNA expression levels, whereas OX2R mRNA was weak in Ishikawa cells and virtually absent in ECC-1 cells. The Western blotting analysis confirmed the presence of OX2R protein in MFE280 cells by revealing a band at 38 kDa as reported (23) and a weaker signal for Ishikawa and ECC-1 cells.

The proliferation of the 3 cell lines upon OX A or OX B induction was assessed with the WST1 assay. Using various experimental conditions for cell culture and induction (data not shown), the supplementation of the cell culture medium with OX A or OX B had no effect on the proliferation of the 3 cell lines (concentrations of 10^{-8}, 10^{-7}, and 10^{-6} M for an incubation period of 24, 48, or 72 hours). A representative set of data generated with the WST1 assay is shown in Figure 2G in which the 3 cell lines were induced for 48 h with OX A and OX B. Similar data showing no difference

CpG sites. Cancer and control samples were significantly different using this parameter, with median values of 48.85% and 5.85%, respectively ($P = .0001$, Table 3). In Figure 3B, methylation percentages are plotted against IHC scores, showing the apparent lack of direct correlation between these parameters (Spearman r = $-0.3169, P = .0772$).

Additionally, to improve the reporting of bisulfite sequencing data, each clone was classified into 1 of the 3 methylation categories as defined by the Human Epigenome Project criteria (19): unmethylated (<20% methylated CpGs), partially methylated (20%–80% methylation), and hypermethylated (>80% methylation). Table 3 summarizes the median values of frequency by the meth-

Table 1.   IHC of OX2R in Normal Endometrium

	Glands	Stroma
Premenopausal proliferative	+	−
Premenopausal secretory	++	− or focally +
Menopausal	++	−

−, negative; +, moderate; ++, intense.

Table 2. Clinical Features, Methylation Status of OX2R, and IHC Scores for Controls (n = 18) and EEC Patients Listed Separately After the Pathological Grade: Grade 1 (n = 21); Grade 2 (n = 10); Grade 3 (n = 3)

	Case Number	Menopause	Age, y	Methylation, %	IHC Score
Controls	1	No	40	3.4	ND
	2	No	48	7.9	ND
	3	No	40	4.8	ND
	4	No	37	2.6	ND
	5	No	42	10.9	ND
	6	No	39	2.7	ND
	7	No	38	5.9	ND
	8	No	32	7.2	ND
	9	Yes	52	7.5	ND
	10	Yes	54	5.8	ND
	11	Yes	55	16.7	ND
	12	Yes	50	10.7	ND
	13	Yes	50	5.3	ND
	14	Yes	54	2.2	ND
	15	Yes	54	5.5	ND
	16	Yes	49	5.9	ND
	17	Yes	75	4.7	ND
	18	Yes	54	7.3	ND
EEC grade 1	101	Yes	60	50.0	1
	102	Yes	78	10.9	1
	103	Yes	54	47.7	1
	104	Yes	69	67.1	0
	105	Yes	60	38.7	1
	106	Yes	81	80.9	0
	107	Yes	74	35.2	0
	108	Yes	65	43.2	0
	109	Yes	58	25.4	1
	110	Yes	68	13.4	0
	111	Yes	78	32.2	0
	112	Yes	80	72.4	0
	113	Yes	60	44.0	0
	114	Yes	70	72.1	0
	115	No	39	39.8	0
	116	Yes	58	61.6	0
	117	Yes	75	60.4	0
	118	Yes	74	42.8	0
	119	Yes	65	41.1	1
	120	Yes	69	68.6	0
	121	Yes	72	66.8	ND
EEC grade 2	122	Yes	69	64.5	2
	123	Yes	74	50.7	0
	124	Yes	64	25.2	2
	125	Yes	65	53.8	0
	126	No	47	69.5	1
	127	Yes	55	5.4	0
	128	Yes	67	65.9	0
	129	Yes	61	33.1	0
	130	Yes	81	63.6	0
	131	Yes	84	64.7	1
EEC grade 3	132	Yes	86	36.0	1
	133	Yes	54	23.8	0
	134	Yes	79	50.0	ND

Abbreviation: ND, not determined. IHC scores include the following: 0, general absence of staining; 1, global weak staining; and 2, global moderate staining.

between treated and untreated have been collected at other time points (24 hours, 72 hours, etc). To further evaluate the apoptosis in the same culture conditions, a caspase-3/7 assay was used. No evidence of apoptosis was demonstrated after 48 hours of induction with OXA or OXB (Figure 2H).

Discussion

Discovered 15 years ago, the orexinergic system has attracted major interest with now more than 2500 publications recorded in PubMed web site with the keyword orexin. First analyzed in the brain, the orexins and orexin
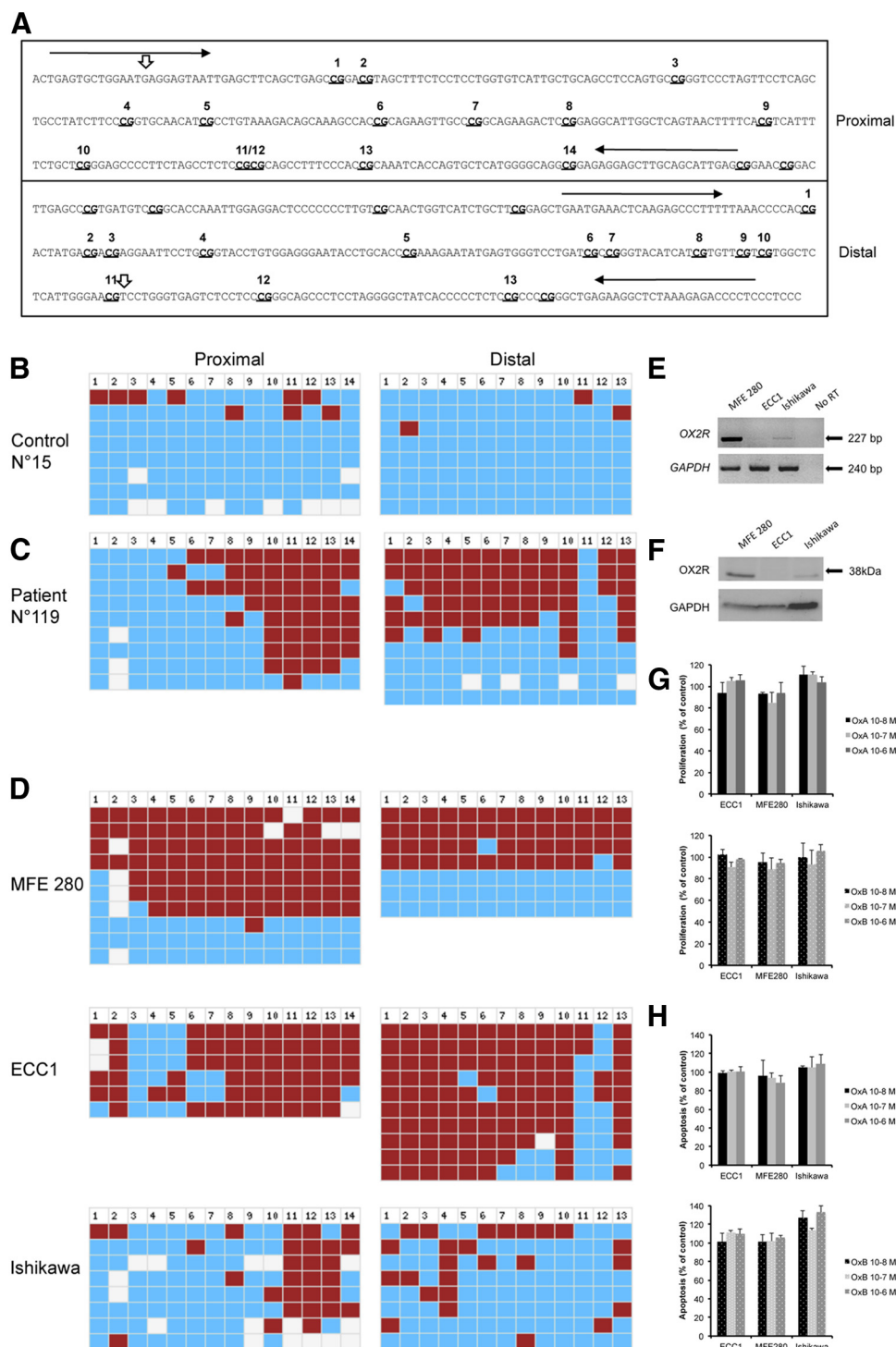
Figure 2. Methylation on *OX2R* locus in normal endometrium, EEC and cell lines. A, The sequence of exon 1 is presented with open arrows to indicate the exon's limits. CpG sites are numbered as appearing in the methylation patterns below. Horizontal arrows indicate the position of primers used for proximal and distal PCR amplification. B and C, Representative methylation patterns of control and EEC cases. Each numbered column represents a CpG site and every row represents a unique allele. Blue, red, and white squares, respectively, represent unmethylated, methylated, or undetermined CpG sites. The pattern in panel B shows mostly unmethylated alleles in control endometrium (control 15), whereas EEC patient 119 in panel C shows intense methylation, which is maximal in CpG sites 10–13 from the proximal portion. D, Methylation pattern of *OX2R* exon 1 in MFE 280, ECC-1, and Ishikawa cells. In MFE 280, hypermethylated as well as completely unmethylated clones are observed. In ECC-1 cells, the intense methylation pattern is recorded. In Ishikawa cells, methylated CpG sites are concentrated at positions 11 and 12 from the proximal portion. RT-PCR for OX2R (E) and Western blot (F) using anti-OX2R antibody for the 3 endometrial cell lines. Only MFE 280 showed strong signals in E and F. For ECC-1 and Ishikawa cells, absent or very weak signals are recorded. Loading controls are given by GAPDH RT-PCR (E) and Western blot (F). G, Representative experiment showing the values (percentage of control) of WST1 tests performed with cell cultures (ECC-1, MFE280, and Ishikawa) induced for 48 hours with graded concentrations of OX A and OX B. Orexins have no effect on cell proliferation. H, Representative caspase-3/7 assays performed with cell cultures (ECC-1, MFE280, and Ishikawa) induced for 48 hours with graded concentrations of OX A and OX B. The values expressed in the percentage of noninduced controls show no effect of both orexins on apoptosis.
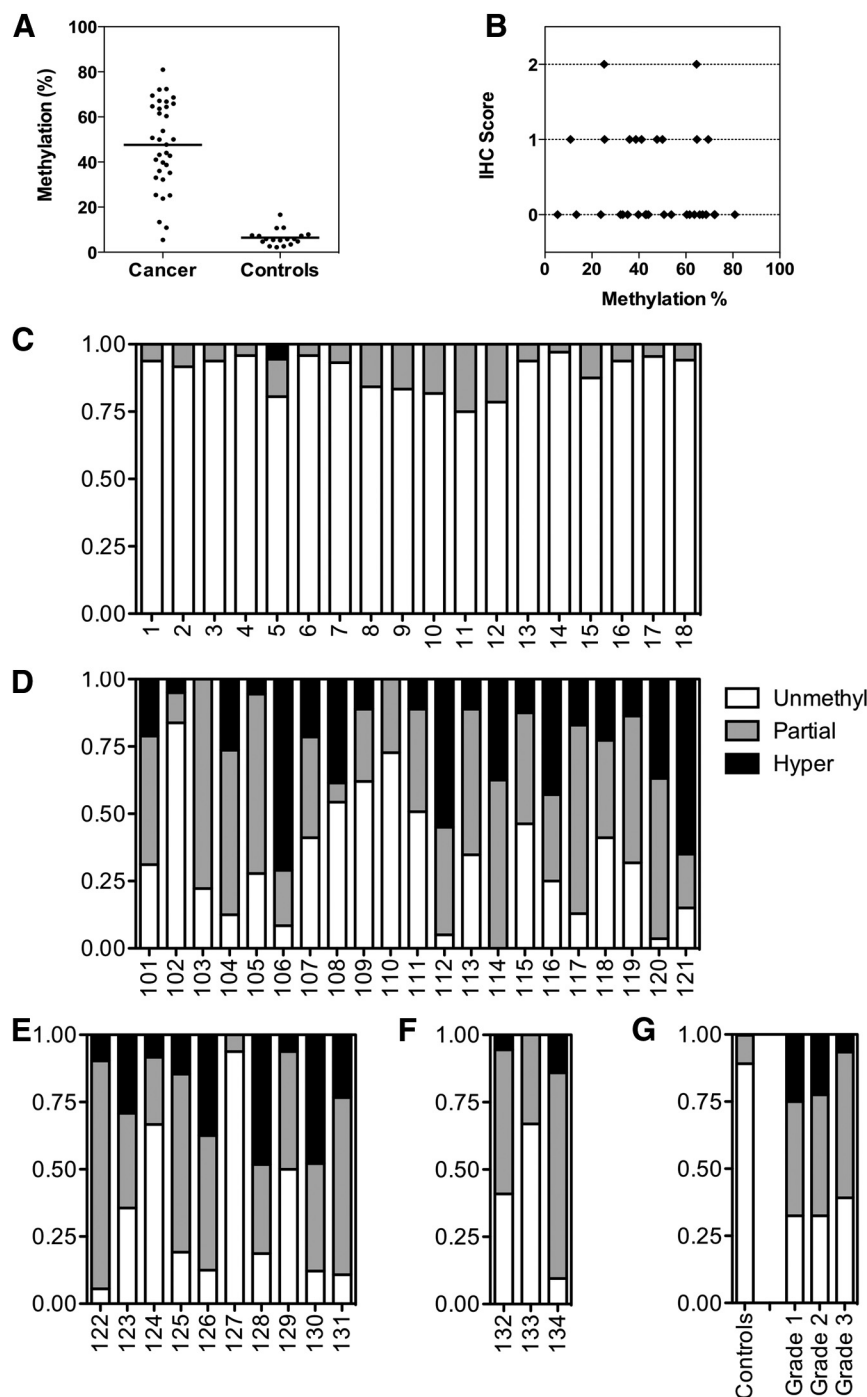
Figure 3. Quantitative methylation of *OX2R* first exon. A, Percentages of methylation for cancer (n = 34) and controls (n = 18) samples. B, EEC patients (n = 34): plotting of methylation percentages and IHC scores showing the absence of correlation between them. C–G, Allocation of *OX2R* bisulfite sequencing clones to the 3 methylations categories in controls (C; n = 18) and EEC grade 1 (D; n = 21), grade 2 (E; n = 10), grade 3 (F; n = 3). G, Mean values for controls and EEC of all 3 grades are displayed. In panel C, clones are mostly unmethylated. In panels D and E, most of the samples show a high proportion of hypermethylated clones, which tends to be lower in panel F for grade 3 EEC.

ing the whole menstrual cycle and in menopausal conditions. In EEC, 65.6% of 32 tumoral samples investigated were devoid of OX2R staining either completely or with minimal focal labeling. Additionally, hypermethylation of the first exon of *OX2R* was demonstrated in EEC by comparison with normal endometrium (median methylation percentage of 48.85% and 5.85%, respectively). Comparisons between methylation categories for the 3 grades of EEC in Figure 3G showed similar methylation for grades 1 and 2 and a tendency toward lower percentages of the partially methylated and hypermethylated categories for grade 3, although this has to be confirmed on a larger number of clinical samples. Similar observations reporting reduced methylation rates in advanced tumor stages have already been done as, for example, in colorectal cancer, in which O^6-methylguanine DNA methyltransferase (*MGMT*) hypermethylation is more intense in T1/T2 carcinomas than in the more advanced T3/T4 stages (24). The MGMT protein has then been reported to be reexpressed in colon adenocarcinomas (25), whereas it was absent in *MGMT*-methylated adenomas (26) from which carcinomas are supposed to derive.

This is the first time that epigenetic regulation is identified for *OX2R*. Its genomic locus was recently studied and revealed the presence of 3 nontranslated 5′ exons and the use of alternative promoters (27). Here in vitro studies using cell lines allowed the establishment of a correlation between the methylation of the first exon and the lack of gene expression. The methylation of first exons have indeed been recently identified as major gene-silencing events (28). In ECC-1 cells, an intense methylation of exon 1 was correlated with the absence of signal in RT-PCR and in OX2R immunoblotting. In Ishikawa cells, weak OX2R transcription and protein expression was associated with

receptors have been recently shown to have a broader role in peripheral organs and in cancer. Many aspects of their physiological roles are probably still to be uncovered.

We described here the localization of OX2R protein in the epithelial compartment of normal endometrium dur-

Table 3.   Global and Detailed Methylation Evaluation in Controls and EEC of All Grades

	Controls (n = 18)	All Grades Cancer (n = 34)	P Value
Global methylation, %	5.85 (4.05–7.70)	48.85 (34.15–65.30)	[a]
Detailed distribution of clones			
Unmethylated (<20%)	0.9348 (0.8254–0.9479)	0.2944 (0.1232–0.5040)	[a]
Partially methylated (from 20% to 80%)	0.0652 (0.0521–0.1623)	0.4063 (0.2971–0.6181)	[a]
Hypermethylated (>80%)	0.0 (0.0–0.0)	0.1585 (0.0729–0.3750)	[a]

Median values (25th and 75th percentile) are given. *P* values were calculated using the Mann-Whitney test.

[a] $P < .0001$.

sparse methylation except around CpG sites 11 and 12 in the proximal zone. In the MFE280 cells, for which a moderate OX2R expression is detected, both completely unmethylated and methylated clones were identified making the locus hemizygous and permissive for OX2R expression. Unfortunately, no nucleotide polymorphism could be found in the sequenced fragments, and it was not possible to establish whether the observed heterogeneous methylation would segregate together with paternal and maternal alleles. This observation is interesting because it affects MFE280, a poorly differentiated cell line. This could be related to the tendency to lower methylation density observed in grade 3 EEC. Heterogeneous methylation appears to be frequent in the human genome and is not always connected to gene imprinting (29). The *OX2R* gene has not been reported to be imprinted, although in a recent computational study (30), it was included in a list of imprinted gene candidates.

By contrast, in vivo, no clear correlation could be established between the methylation percentages and the IHC scores evaluated on tumoral tissues (Figure 3B). However, the samples with lower methylation levels (<50%) had a mean IHC score higher than samples with high (>50%) methylation levels (0.50 vs 0.29). This failure to reach statistical significance for the correlation could stem from either the low number of samples of highest tumor grade or the sampling method applied. Indeed, the material used for DNA extraction and the methylation study comes from a different part of the tumor than the one used for OX2R IHC. In agreement with our hypothesis, data from Faquin et al (31) and Feng et al (32) also showed substantial heterogeneity in EEC.

In our study, the bisulfite sequencing after bacterial cloning allowed the quantification of methylation at the level of individual alleles later allocated into 3 methylation categories. This approach is particularly necessary when analyzing heterogeneous material as tumoral samples (33). The identification of *OX2R* methylation in EEC could be prolonged by the establishment of an *OX2R*-based test applicable in clinical diagnostic situations, using a methylation-specific PCR technology on endometrial tissue obtained by curettage as starting material.

WST1 proliferation assays in the presence of OX A and OX B or the caspase-3/7 tests did not show any difference compared with controls, demonstrating the absence of orexin-induced apoptosis. This result was obtained for the 3 cell lines including MFE280 with demonstrated expression of *OX2R*. An alternative explanation could be that the level of expression of OX2R in MFE280 cells would be too low to trigger orexin-induced apoptosis. In other cancer models expressing *OX2R*, such an apoptosis has been reported in a pancreatic rat cell line (34), whereas for adrenocortical adenoma cells, a stimulation of growth and cortisol secretion was described upon orexin induction (35). Thus, in the context of endometrial cancer, the direct implication of OX2R in the control of cell proliferation is not established and the epigenetic *OX2R* down-regulation reported here may impact cancer cells by other means that have yet to be uncovered. De Carvalho et al (36) recently studied the methylation of promoter regions that were necessary for cancer cell survival. Intriguingly, no classical tumor suppressor gene was evidenced by their screening, which identified many cell signaling molecules, notably several G protein-coupled receptors, leading the authors to conclude that the role of GPRCs in tumor progression was probably underestimated. In this study the *OX2R* silencing by DNA methylation might certainly be considered on the same line.

In the normal endometrium, the physiological role of OX2R and the orexinergic system also remains to be better understood. Very recently the presence of OX A and OX B was established in the porcine endometrium (37), which, if confirmed in human tissues, could suggest an autocrine/paracrine interaction of the orexins with OX2R as foreseen in the male genital tract (7).

In conclusion, the OX2R was shown to be present in normal endometrium, whereas in EEC, a frequent OX2R loss is associated with the frequent hypermethylation of its first exon. The methylation intensities are similar in grades 1 and 2 EEC and tend to regress in grade 3 clinical samples, although more grade 3 cases would be required to confirm the tendency. Heterogeneous methylation with the presence of completely methylated and unmethylated alleles was also observed on the MFE280 cell line characterized by poor differentiation.

Acknowledgments

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