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GPER and ERα expression in abnormal endometrial proliferations

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Abstract

G-protein coupled estrogen receptor 1 (GPER), a particular extranuclear estrogen receptor (ER), seems not to be significantly involved in normal female phenotype development but especially associated with severe genital malignancies. This study investigated the GPER expression in different types of normal and abnormal proliferative endometrium, and the correlation with the presence of ERα. GPER was much highly expressed in cytoplasm (than onto cell membrane), contrary to ERα, which was almost exclusively located in the nucleus. Both ERs' densities were higher in columnar epithelial then in stromal cells, according with higher estrogen-sensitivity of epithelial cells. GPER and ERα density decreased as follows: complex endometrial hyperplasia (CEH) > simple endometrial hyperplasia (SHE) > normal proliferative endometrium (NPE) > atypical endometrial hyperplasia (AEH), ERα' density being constantly higher. In endometrial adenocarcinomas, both ERs were significant lower expressed, and widely varied, but GPER/ERα ratio was significantly increased in high-grade lesions. *Conclusions*: The nuclear ERα is responsible for the genomic (the most important) mechanism of action of estrogens, involved in cell growth and multiplication. In normal and benign proliferations, ERα expression is increased as an evidence of its effects on cells with conserved architecture, in atypical and especially in malignant cells ERα's (and GPER's) density being much lower. Cytoplasmic GPER probably interfere with different tyrosine/protein kinases signaling pathways, also involved in cell growth and proliferation. In benign endometrial lesions, GPER's presence is, at least partially, the result of an inductor effect of ERα on GPER gene transcription. In high-grade lesions, GPER/ERα ratio was increased, demonstrating that GPER is involved *per se* in malignant endometrial proliferations.

Keywords: GPER, estradiol, ERα, endometrium, benign and malignant proliferations.

Introduction

G-protein coupled estrogen receptor 1 (GPER) was first cloned by Owman *et al.* in 1996 [1], who named it GPR30. Although initially considered as an "orphan" receptor, Filardo *et al.* in 2000 [2] proved its affinity for estradiol and the fact that it acts as a "rapid" estrogen receptor (ER). Its structure is totally different compared with that of the "classic" ERs, being a member of receptors coupled with G-proteins. After estrogen binding, beside intracellular calcium mobilization [3] and adenylyl cyclase activation [4, 5], GPER induces activation of MAP kinases ERK 1/2 pathway through trans-activation of epidermal growth factor receptor tyrosine kinase (EGFR) [2, 6], activation of Src-related tyrosine-kinases and phosphatidylinositol 3-kinase (PI3K)/Akt pathways [6], all these involved in cell growth, differentiation and proliferation.

Supporting this data is also the clinical observation that the overexpression of GPER associate with high-grade and poor prognosis endometrial [7, 8] and breast [9] cancer.

GPER expression is increased by estrogens (mainly estradiol – E2) through ERα [10, 11] and decreased by progesterone, because of progesterone receptor A (PGR-A) activation [11].

This study is focused on the GPER's density quantification in different benign and malignant endometrial proliferations, and to correlate the GPER expression with the ERα presence.

Materials and Methods

The normal proliferative endometrium was obtained from hysterectomies (for leiomyomatosis) performed in proliferative endometrial phase.

The abnormal endometrium was obtained during uterine curettage for menometrorrhagia or for endometrial hypertrophy (discovered by ultrasound) and from hysterectomies performed for different uterine diseases. All gynecological maneuvers took place in the Department of Obstetrics and Gynecology, Emergency County Hospital of Craiova, Romania.

The diagnosis of normal and/or abnormal endometrial proliferation was established by classical Hematoxylin–Eosin (HE) staining in the Department of Pathology of the same Hospital.

From the tissue fragments, there were selected 32 samples, revealing normal proliferative endometrium (n=5), simple (n=4), complex (n=6) and atypical (n=5) endometrial hyperplasia, respectively low (n=7) and high (n=5) grade endometrial adenocarcinomas.

The study continued only after obtaining the patient's informed consent and the approval from the Ethical Committee of the University of Medicine and Pharmacy of Craiova.

We utilized single and double sequential immunostaining protocols based on the recommendations of the anti-human antibodies' producers. Briefly, for single immunohistochemistry, 4 μm-thick tissue sections were first processed for antigen retrieval by microwaving for 20 minutes in citrate buffer pH 6 for GPER immunostaining, respectively in EDTA pH 8 for ERα immunostaining. Endogenous peroxidase was next blocked utilizing 0.1% hydrogen peroxide for 30 minutes, and the false antigenic sites were further blocked by incubating the slides in 5% skimmed milk (Bio-Rad, München, Germany). The primary antibody (either polyclonal rabbit anti-human GPER antibody, Novus Biologicals, USA or monoclonal mouse anti-human ERα antibody, Dako, Denmark, both diluted as 1:50) was next incubated on the slides overnight at 4^0C. Next day, the sections were washed in phosphate-buffered saline, signal amplified with a human-adsorbed species-specific polymeric HRP system (Nichirei Biosciences Inc., Tokyo, Japan), and finally estrogenic signals were visualized by adding 3,3'-diaminobenzidine hydrochloride substrate (DAB, Dako).

For double immunohistochemistry, the slides were prepared for sequential double-color enzymatic immunostaining. First, the slides were processed as described above for single immunostaining and DAB-based detection of rabbit anti-human GPER, and after thorough washing in phosphate-buffered saline, the monoclonal mouse anti-human ERα antibody (diluted also as 1:50) was brought on the slides for another overnight incubation. Next day, the sections were washed, signal amplified with a human-adsorbed anti-mouse polymeric alkaline phosphatase (AP) system (Nichirei), and finally estrogen signals were visualized by adding Fast Red substrate (Dako).

After Hematoxylin counterstain, the single immunostained slides were coverslipped in a xylene-based medium (Dako), while the double stained slides were coverslipped with a glycerol-based mounting medium (Dako).

For quantifying the GPER and/or ERα immunostaining, we used semiquantitative histological scoring (HSCORE) method.

For cytoplasmic localization, the signal was scored as negative, weak (tiny cytoplasmic or granular staining – score 1), moderate (diffuse granular cytoplasmic staining – score 2) and strong (diffuse intense cytoplasmic staining – score 3).

For nuclear receptors, the immunostaining was also scored as negative, weak, moderate and strong.

Weak, moderate and strong were considered positive.

H-SCORE = (% of cells stained at intensity 1×1) + (% of cells stained at intensity 2×2) + (% of cells stained at intensity 3×3) [12].

Each scoring was performed by two independent team members for more accurate results. No significant difference was found in the end between the investigators.

H-SCORE was evaluated both for columnar epithelial and stromal cells in simple and atypical hyperplasias and only for epithelial cells in malignant lesion (due to the modification of tissular architecture).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20.0 (IBM Corporation, Armonk, NY, USA) for processing the data. To compare GPER or ERα values in columnar *vs.* stromal cells, we used Wilcoxon's signed rank T-test, with a level of significance α=0.05 (a result p<0.05 is considered statistically significant). To compare GPER or ERα values among all types of endometrium, we used the Kruskal–Wallis (K–W) test, with the same level of significance.

Results

On the endometrium, we found GPER to be much better expressed in the cytoplasm (probably associated with endoplasmic reticulum) than on the membrane, either in columnar or in stromal cells (Figures 1–6).

Opposite to GEPR, ERα was noticed almost exclusively in the nuclei in both cell types (Figures 5–10).

All ERs revealed a significant higher expression on columnar (epithelial) than in stromal (connective) cells (Figures 1–10 and Table 1 for Wilcoxon's p-values).

In benign proliferations, where the tissue architecture was conserved, both GPER and ERα immunoreactivity decreased as follow, either in columnar or stromal cells: complex endometrial hyperplasia (CEH) > simple endometrial hyperplasia (SHE) > normal proliferative endometrium (NPE) > atypical endometrial hyperplasia (AEH) (Table 1, Figure 11) and some of the observed differences were statistical significant (CEH *vs.* AEH for GC and EC and CEH+SEH *vs.* AEH for GS).

The GPER/ERα ratio was approximately constant in all above-mentioned tissues (0.77–0.83) (Table 1).

The H-SCOREs for stromal cells varied non-concordant with those for epithelial cells (Table 1).

In malignant lesions, ERs' expression was significantly lower than for normal endometrium or all types of hyperplasia (K–W p=0.00138 <0.01 for GPER, K–W p=0.000169 <0.001 for ERα), and widely varied, being specific for

each tissue (Table 1, Figure 11). GPER/ERα ratio was constantly increased compared to benign lesion: 0.91 in low grade and 1.24 in high-grade cancers.

One probe from low (*n*=7 – 14%) and one from high (*n*=5 – 20%) grade adenocarcinomas were GPER and ERα-free (14%), and one tissue from high-grade adenocarcinomas (*n*=5 – 20%) was and ERα-free and GPER weak immunoreactive.

Due to local invasion, the H-SCOREs for stromal cells in endometrial adenocarcinoma could not be calculated.

The detailed H-SCOREs regarding GPER and ERα's immunoreactivity in normal proliferative endometrium, in simple, complex and atypical endometrial hyperplasias (both in columnar and stromal cells), respectively in low and high-grade endometrial adenocarcinomas (solely in epithelial cells) are detailed in Table 1 and Figure 11.

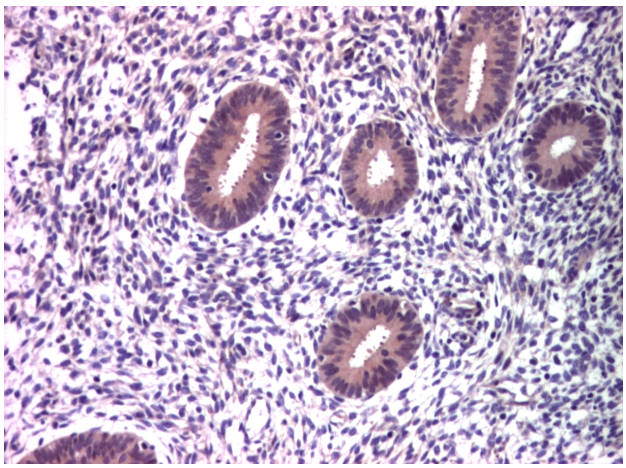

Figure 1 – ***GPER's immunoreactivity (in brown) in normal endometrium in proliferative phase, ×100.***

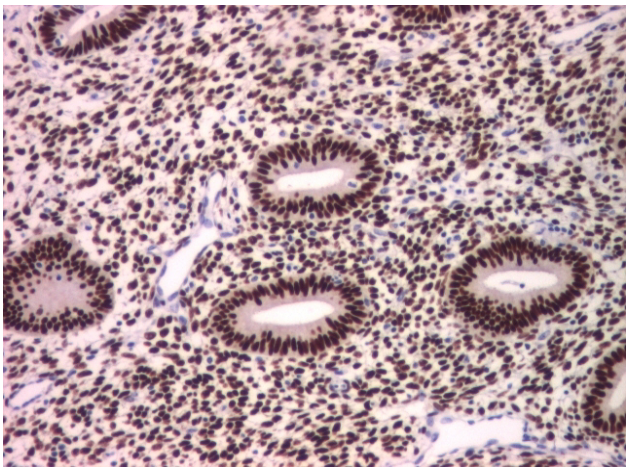

Figure 2 – ***ER's immunoreactivity (in brown) in normal endometrium in proliferative phase, ×100.***

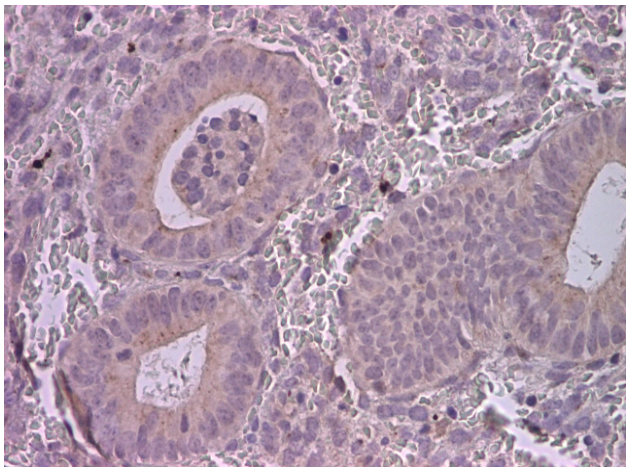

Figure 3 – ***GPER's immunoreactivity (in brown) in simple endometrial hyperplasia, ×200.***

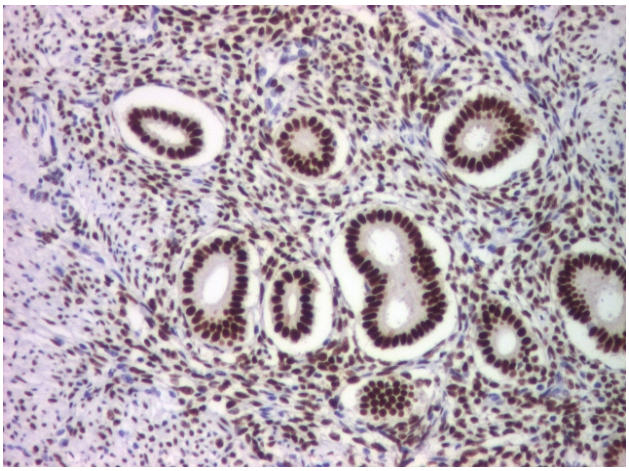

Figure 4 – ***ERα's immunoreactivity (in brown) in simple endometrial hyperplasia, ×100.***

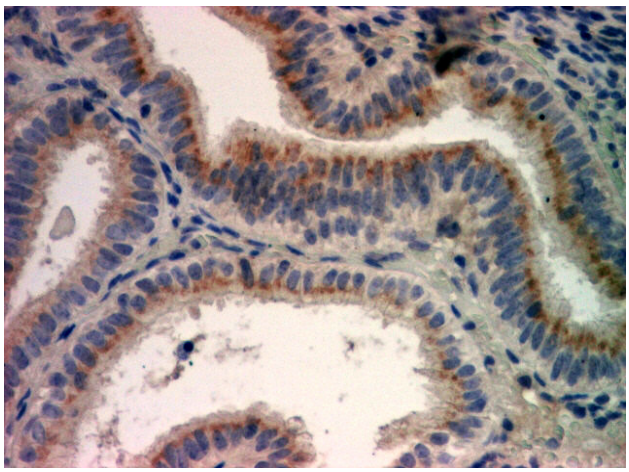

Figure 5 – ***GPER's immunoreactivity (in brown) in complex endometrial hyperplasia, ×200.***

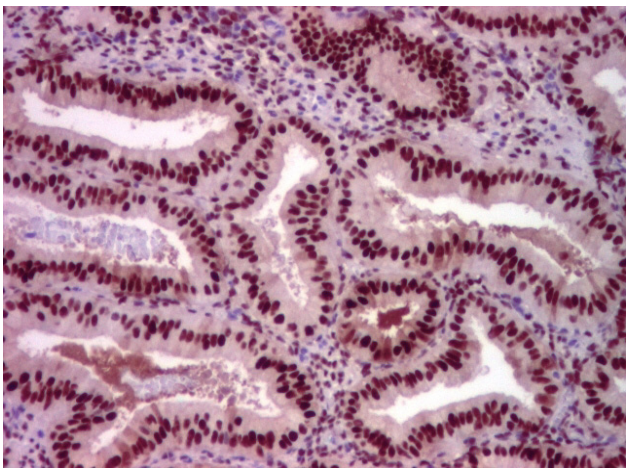

Figure 6 – ***ERα's immunoreactivity (in brown) in complex endometrial hyperplasia, ×100.***

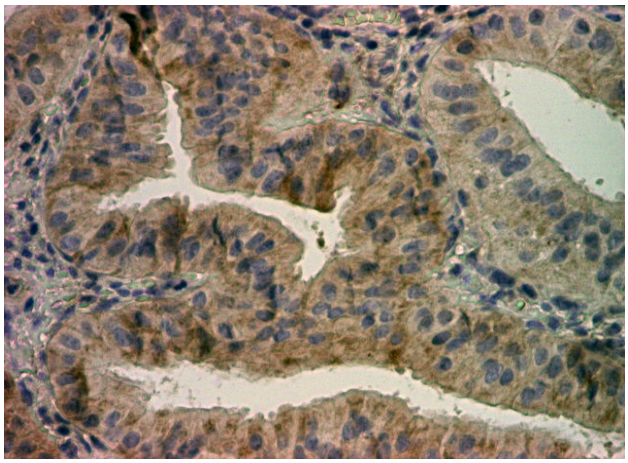

Figure 7 – *GPER's immunoreactivity (in brown) in atypical endometrial hyperplasia, ×200.*

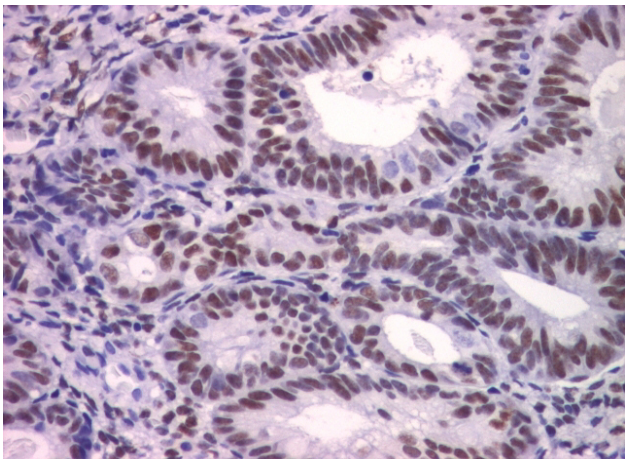

Figure 8 – *ERα's immunoreactivity (in brown) in atypical endometrial hyperplasia, ×200.*

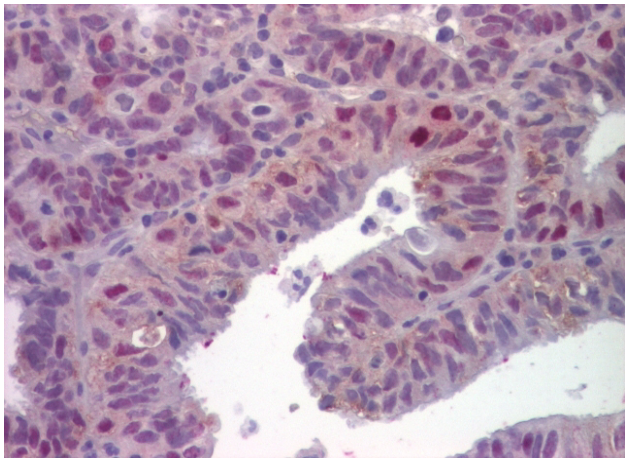

Figure 9 – *GPER (in brown) and ERα (in red) immunoreactivity in low-grade endometrial adenocarcinoma, ×200.*

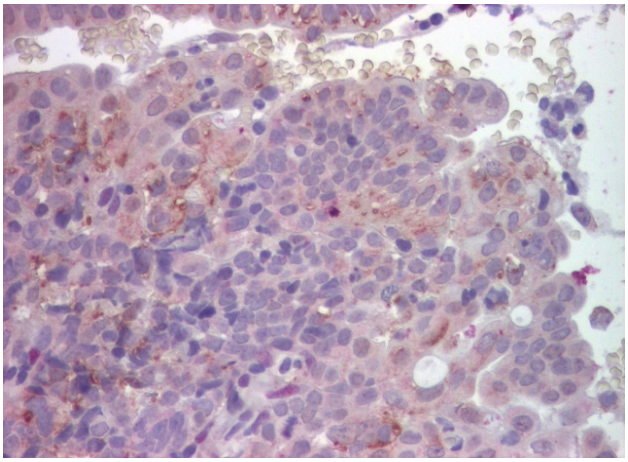

Figure 10 – *GPER (in brown) and ERα (in red) immunoreactivity in high-grade endometrial adenocarcinoma, ×200.*

Table 1 – *H-SCOREs for GPER and ERα immunoreactivity in columnar and stromal cells in different types of endometrial proliferations. It is also mentioned the GPER/ERα ration in columnar cells in benign and malignant endometrial lesions*

Type of endometrium	H-SCORE				GPER/ERα ratio (Columnar cells)
	GPER		*ERα*		
	Columnar cells	*Stromal cells*	*Columnar cells*	*Stromal cells*	
Normal proliferative endometrium (*n*=5)	136±13.66 (*p*=0.043 <0.05)	72±12.51	163±21.66 (*p*=0.043 <0.05)	73.2±15.22	0.83
Simple endometrial hyperplasia (*n*=4)	145±20.99 (*p*=0.048 <0.05)	89±12.11	181±25.52 (*p*=0.048 <0.05)	72±13.71	0.81
Complex endometrial hyperplasia (*n*=6)	158±18.79 (*p*=0.028 <0.05)	83±16.89	203.33±24.69 (*p*=0.028 <0.05)	57±12.92	0.77
Atypical endometrial hyperplasia (*n*=5)	121±15.89 (*p*=0.043 <0.05)	57±12.41	154±17.51 (*p*=0.041 <0.05)	61±17.36	0.78
Low-grade adenocarcinoma (*n*=7)	96.14±45.45	–	105±52.48	–	0.91
High-grade adenocarcinoma (*n*=5)	72±43.02	–	58±36.84	–	1.24

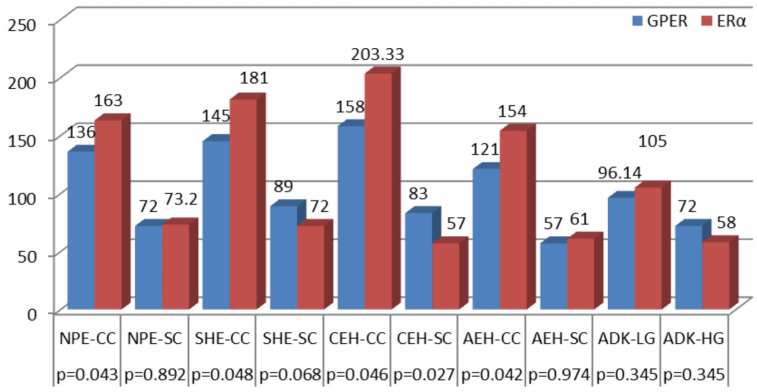

Figure 11 – *H-SCOREs regarding GPER and ERα's immunoreactivity in normal proliferative endometrium (NPE), simple (SEH), complex (CEH) and atypical (AEH) endometrial hyperplasias [both in columnar (CC) and stromal cells (SC)], respectively in low (ADK-LG) and high (ADK-HG) grade endometrial adenocarcinomas (in epithelial cells). n: No. of tests.*

Discussion

The endometrium is the main target of physiological estrogens, estradiol (E2) being the most active. These hormones induce strong hypertrophic and hyperplasic activities on endometrial cells [13], especially through their "slow" genomic mechanism of action, involving specific nuclear receptors (ERs): ERα [14] and ERβ [15]. ERα is the most important nuclear estrogen receptor [6], ERβ having a far lower expression in the female reproductive system [16].

After binding ERs and dimerisation, E2 finally modulates different transcriptional processes.

We found ERα being almost exclusively in the nucleus, a fact correlated with the most important mechanism of action of the estrogens – the genomic one, responsible for all their effect involved in female phenotype.

However, estrogens are able to induce also "rapid", non-genomic effects, by binding GPER and extranuclear ERα.

GPER has a completely different structure compared to "classical" ERs, being a member of the G-protein coupled receptors. Different studies uniquely situated it on cell membrane [5], exclusively intracellular [3], or on both sites [2, 17].

We found GPER in much higher density in the cytoplasm, probably associated with endoplasmic reticulum, which demonstrate its involvement in "rapid" estrogens' effects.

After steroid binding, GPER activates several pathways involved in cell growth and proliferation: Src-tyrosine kinases [6], (MAPK) ERK1/2 kinases [2, 6] and PI3 kinases [3, 6], beside other mechanisms of action as calcium mobilization from internal stores [3] or adenylyl cyclase activation [4, 5].

GPER expression is increased by estrogens secondary to ERα but not to ERβ or GPER activation [11].

Despite its ubiquitous presence and its several known induced intracellular pathways, GPER does not seem to be essential for urogenital development and fertility [17], but it is highly-expressed in abnormal endometrial proliferations [11, 18, 19].

Our data show that both ERα and GPER immunoreactivity is significant higher in columnar than in stromal cells, which is in concordance with already known higher estrogen-sensitivity of the epithelial cells.

During menstrual cycle, the maximal density of ERs in endometrium is recorded in the proliferative phase [11]. We found a gradual increase in ERα immunoreactivity from normal proliferative endometrium to simple and to the maximal values in complex endometrial hyperplasia. This is concordant with the growth and hyperplasic effect of activated ERα (by genomic pathway) on normal and benign proliferative endometrium [20]. When the cells begin to present different structural abnormalities, such as in atypical endometrial hyperplasia, the ERα's density slight decrease.

GPER follows the trend of ERα expression, being maximal in complex, higher than in simple endometrial hyperplasia and furthermore higher than in normal proliferative endometrium. This parallelism probably is the result of ERα's inductor effect on GPER gene expression.

The variation of stromal ERs' immunoreactivity in different types of endometrial tissue is less important, highlighting supplementary the lower estrogen-sensitivity of stromal comparing with epithelial cells.

In malignancies, the normal cell's architecture is affected, including ERs' expression, which is constantly lower than in normal or in benign proliferative endometrium [21].

The ERs widely varied for each tissue, with some of the samples expressing no ERs, these tissues being from patients with poor prognosis tumors.

It is very important the fact that the GPER/ERα ratio was increased in all malignancies compared with normal and/or benign proliferations, and high GPER density associated with high-grade and/or poor prognosis adenocarcinomas. It is also the case of the high-grade ERα-free but GPER slight positive uterine cancer.

Furthermore, there is no detectable aromatase activity in normal endometrium [18], but it is high-expressed in endometriotic and malignant endometrial cells [19, 22, 23], demonstrating the intracrine and paracrine role of the estrogens synthesized in abnormal endometrial cells proliferations.

It is very interesting that GPER activation increase the aromatase expression in endometriotic and malignant endometrial cells [19], proving its role in increasing of intracellular estrogen production in abnormal hyperplasic endometrium.

The predominant intracellular localization of GPER can be a part of the mechanism of self up-growth regulation of abnormal endometrial cells: GPER induce aromatase expression, increasing intracellular estrogen synthesis and estrogens, at their turn, by intracrine way, will activate the intracellular GPER, amplifying the abnormal cell proliferation rate.

Nevertheless, intracellular synthesized estrogens can act on GPER situated on adjacent cells, by a paracrine pathway, also inducing cell proliferation.

This hypothesis is consistent with the clinical observation that GPER associates with high-grade endometrial [7, 8] and breast cancer [9] as well as with uterine carcinosarcoma [24], and its presence is a factor of poor prognosis, due to rapid proliferation, invasion and metastasis [7–9, 24].

Conclusions

ERα is almost exclusively situated in nucleus, being involved in the most important mechanism of action of estrogens – the genomic one, mechanism responsible for cell growth and proliferation. The gradually increase of ERα's density in normal proliferative endometrium, and more in simple hyperplasia, being maximal in complex endometrial hyperplasia, proves the growth and proliferative effect of activated ERα. The fact that ERα's density decrease in atypical endometrial hyperplasia and is much smaller in low and even more in high-grade adenocarcinomas, demonstrates that ERα is involved only in growth and proliferation of cells with conserved architecture (normal or benign hyperplasic cells). Atypical, and especially malignant cells, significantly less express ERα, lower expression being a sign of poor prognosis and/or high-grade lesion. In normal and benign proliferations, GPER expression is proportional increased due

to an inductor effect of ERα on GPER gene. In cancer cells, GPER/ERα ratio is constantly increased compared with normal or benign proliferations, highlighting the possibility that in endometrial adenocarcinomas GPER has a malignant effect *per se*. Supporting this idea is also the observation that tissues with higher GPER/ERα ratio or even more in those GPER positive, but ERα negative, were obtained from more severe lesions.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Author contribution

Andrei-Adrian Tica and Maria Bogdan equally contributed to this article.

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