BioMed Research International

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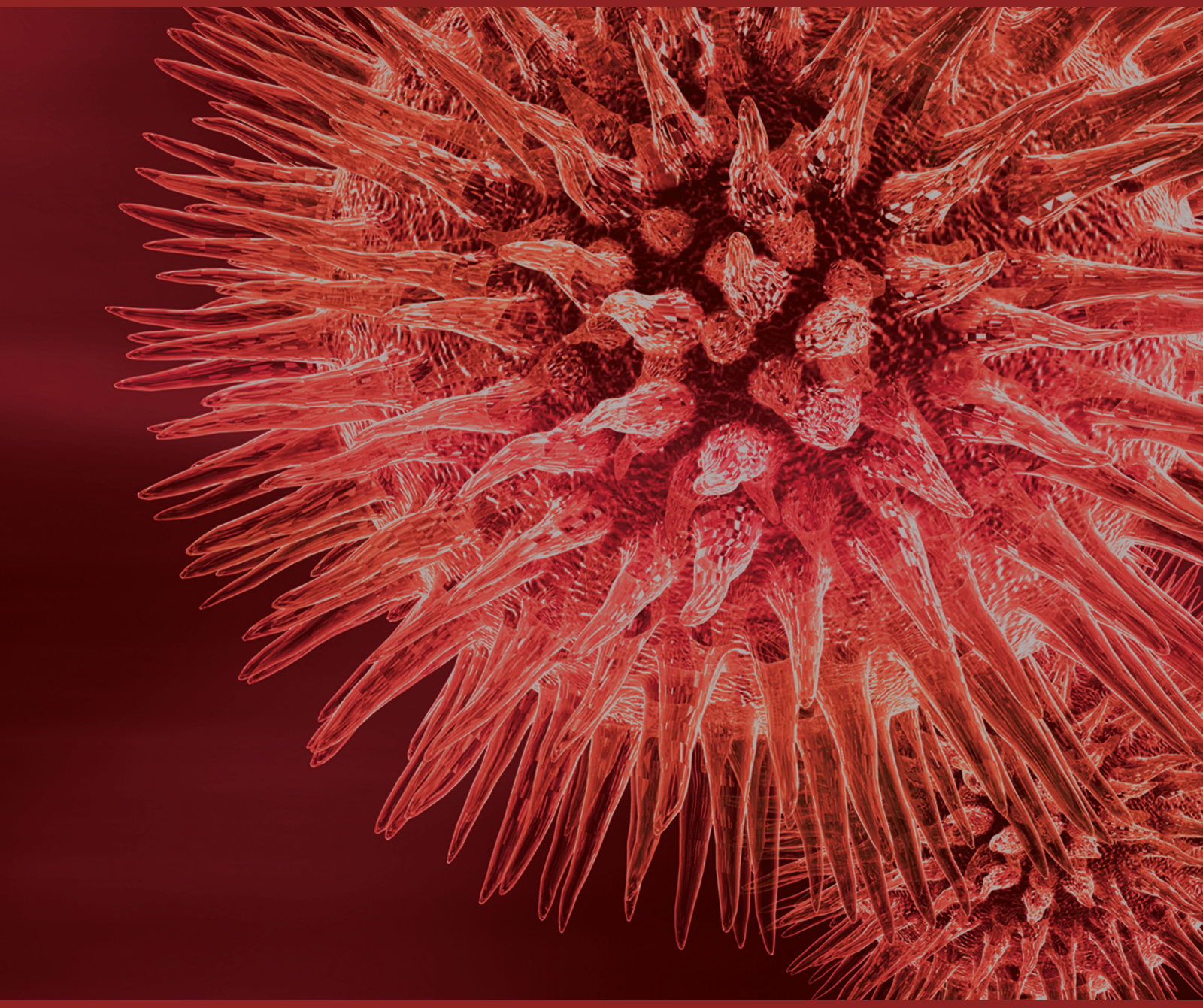
Guest Editors: Shi-Wen Jiang, Brian Brost, Dan Zhang, and Chun-E Ren



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Editorial

Aberrant Gene Regulation in Obstetric, Gynecologic, and Reproductive Diseases

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Abnormal gene expression is often detected in various tissues/organs of patients with gynecologic and reproductive diseases. Research efforts on the regulatory mechanisms and molecular pathways will lead to a better understanding of the pathophysiological process of these diseases. Identification of the key factors and their roles in gynecologic diseases may also facilitate the development of novel diagnostic markers and therapeutic models. This special issue compiles excellent original and review articles covering gene expression and function in the obstetric, gynecologic, and reproductive disorders.

Two articles are from the field of reproductive endocrinology. Using a cell coculture model, X. Liu et al. characterized the paracrine regulation of steroidogenesis in theca cells by granulosa cells derived from mouse preantral follicles. It was found that granulosa cells were able to promote steroidogenesis and responsiveness to luteinizing hormone in theca cells. M. Rahnama et al. investigated the changes of osteocalcin gene expression in postmenopausal women treated with Hormone Replacement Therapy (HRT) and observed a correlation between osteocalcin gene expression in areas of oral cavity and bone metabolism in these women.

H. Ge et al. report that mitochondrial uncoupling protein 2 (UCP2) is expressed in cultured human cumulus cells and may contribute to the process of ROS production, apoptosis, and steroidogenesis, suggesting that UCP2 may be involved in the regulation of follicle development and oocyte maturation. Y. Zou et al. present data showing that Decorin, a decidua-derived TGF-binding proteoglycan, inhibits the proliferation, migration, and invasion of human trophoblast cells. In the same in vitro culture, Decorin can also promote cell apoptosis.

Noncoding RNAs and their epigenetic actions on gene expression are a hot research topic. P. Laudanski et al. report their results on the profiling of selected microRNAs in proliferative eutopic endometrium of women with ovarian endometriosis. In a parallel study, J. Chen et al. identified several regulatory target genes of miR-183, including those for integrin β1, AMIGO2, VAV3, and PSEN2, in endometrial stromal cell culture. They went on to show that knockdown of miR-183 expression induced the invasiveness and inhibition of apoptosis of endometrial stromal cells.

Lysophosphatidic acid (LPA) level has been found to be significantly increased in the serum of patients with ovarian, cervical, and colon cancers. Y. Sui et al. investigated the effect of LPA on the apoptosis induced by cisplatin (DDP) in cervical cancer cell lines and the underlying changes in signaling pathways.

K. T. Woolery et al. determined whether expression of the BRCA1 185delAG mutant, BRAT, in human ovarian surface

epithelial cells could promote an inflammatory phenotype. Increased cellular and secreted levels of Interleukin-1β (IL-1β) were observed following BRAT expression, providing a novel mechanism by which BRAT may be involved in ovarian cancer development.

This special issue also contains meticulously prepared reviews on the recent findings. Accumulating evidence indicates that the epithelial-mesenchymal transition (EMT) is related to the metastasis and relapse of cancer. L. Campo et al. reviewed the expression and potential roles of EMT-inducing factors in various types of gynecological cancers. M. Yuan et al. review the relationship between preimplantation exposure to two endocrine disrupting chemicals bisphenol A (BPA)/triclosan (TCS) and implantation failure. Unsolved questions and possible future studies are also discussed. WNT/b-catenin pathway participates in the morphogenesis and angiogenesis of endometrium. J. Kiewisz et al. review the involvement of this pathway in normal function as well as the carcinogenic process in human endometrium.

With these articles, we hope that the special issue would provide new and insightful research information to investigators in the field of obstetrics, gynecology, and reproductive medicine.

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Review Article

Participation of WNT and β-Catenin in Physiological and Pathological Endometrial Changes: Association with Angiogenesis

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WNT proteins are involved in embryonic development, sex determination, stem cell recruitment, angiogenesis, and cancer. They take part in morphological changes in the endometrium during development, regulate processes of endometrial proliferation and differentiation. This review presents current knowledge about implication of WNT proteins and β-catenin in physiological endometrial functions as well as their involvement in uterine carcinogenesis. Influence of WNT proteins on the formation of blood vessel, taking place both under healthy and pathological conditions, is also considered. Participation of WNT proteins, β-catenin, and inhibitors and inducers of WNT signaling in the process of endometrial angiogenesis is largely unknown. Thus, confirmation of their local and systemic participation in the process of endometrial angiogenesis may in the long term help to establish new diagnostic and therapeutic approaches in conditions associated with the pathology of the female reproductive system.

1. Introduction

WNT are proteins involved in physiological and pathological processes such as embryonic development, sex determination, malignant transformation, endothelial cell differentiation, and angiogenesis [1, 2]. Angiogenesis is required for formation and remodeling of the endometrial vascular system in the course of menstrual cycle. Impaired process of differentiation of endometrial epithelium may lead to cancer, usually accompanied by pathological angiogenesis.

In this review, we present data about implication of WNT proteins and β-catenin in physiological endometrial functions as well as their involvement in uterine carcinogenesis. We also present current knowledge about influence of WNT proteins on the formation of blood vessel, both under healthy and pathological conditions. The proposed hypothesis about participation of WNT proteins in the regulation of the formation of microvessels might provide a conceptual framework for the design of future experiments.

2. General Characteristics of *WNT* Genes and Proteins

Wingless gene is responsible for segmentation during embryogenesis and legs formation during transformation of *Drosophila melanogaster*. Homologous gene *int-1*, detected in mammals, becomes activated upon cell integration of the MMTV virus (*mouse mammary tumor virus*), which causes mammary tumors in mice. Comparison of the amino acid sequences of the proteins encoded by these two genes showed their high homology, while combination of the abbreviations of gene names gives the name for the whole gene family, *WNT* (*WNT* = *W*g + I*NT*). To date, the largest number (19) of *WNT* genes was found in mice and humans. Their existence was also confirmed in the nematode *Caenorhabditis elegans*, zebrafish (*Danio rerio*), amphibians of the *Xenopus* genus, and chicken (*Gallus gallus domesticus*). Based on their capability to induce the malignant transformation in cell lines derived from murine mammary gland epithelial cells

(C57MG), *WNT* genes were divided into two groups. The first group includes genes encoding cysteine-rich, secretory glycoproteins with oncogenic characteristics: WNT1, WNT3, WNT3A, WNT7, WNT8, and WNT8B, while the other encodes proteins lacking the properties to induce malignant transformation: WNT2, WNT4, WNT5A, WNT5B, WNT6, WNT7B, and WNT11 [2]. Hydrophobic nature and activity of these proteins were associated with binding of cysteine residues with palmitic acid.

WNT proteins activate the canonical (genes involved in the malignant transformation) and noncanonical (genes not involved in the malignant transformation) cell signaling pathways. Canonical signaling pathway known also as WNT/β-catenin signaling pathway is activated by WNT proteins joined with the complex of Frizzled (FZD)/low-density-lipoprotein receptor-related protein (LRP) on the cell surface. Activated cytoplasmic protein called Dishevelled (DSH) inhibits the activity of a protein APC/GSK3β complex (Axin/Adenomatous Polyposis Coli/Glycogen Synthase Kinase 3β) responsible for the degradation of β-catenin. After stimulation with WNT, cytoplasmic β-catenin is translocated to the nucleus, where it activates transcription factors: T cell factor (TCF) and lymphoid enhancer factor (LEF). These transcription factors change the expression level of target genes encoding proteins implicated in cell proliferation and survival (cyclin D1, c-Myc), cellular migration (CD44), cell adhesion (CDH1), digestion of extracellular matrix (MMP7), and many others [3]. Because β-catenin binds to α-catenin and cytoplasmic domain of E-cadherin, one of the methods of inhibiting cell signal induced by WNT is to increase the level of E-cadherin. In mammals, the activation of the WNT/β-catenin pathway leads to the enhanced recruitment of stem cells and amplification of their pluripotency.

The other group of WNT which act through β-catenin-independent pathway can activate FZD receptor family. The initiation of signal cascades results in the release of calcium ions, activation of protein kinase C (PKC) and calcium-calmodulin dependent protein kinase II (CAMKII). It has been established that the WNT/Ca^{2+} signal transduction pathway antagonizes the action of WNT/β-catenin signaling [4] and may be associated with cell proliferation and migration [5]. In the second type of signaling pathway, the planar cell polarity pathway and FZD receptors through DSH protein activate small G proteins, Rac and Rho kinases, and c-Jun N-terminal kinase (JNK). This leads to the restructuring of the cytoskeleton proteins, migration of the cell, and the acquisition of cell polarity [2].

WNT-mediated signaling pathways can be modulated through secreted Frizzled-related proteins (sFRP) and Dickkopf (DKK) proteins. sFRP proteins bind directly to WNT proteins, while DKK proteins block LRP5/6 coreceptors. In both cases, WNT ability for signal transduction is blocked [2].

3. *WNT* Genes and Proteins in the Endometrial Physiology

3.1. WNT Proteins in the Female Reproductive Tract Development. Developmental changes of the endometrium are mainly associated with the expression of *WNT4*, *WNT5A*, and *WNT7A* genes as demonstrated in mouse [6–8] and pig [9, 10]. However, *WNT4*, *WNT5A*, and *WNT7A* genes expression was presented also in developed uterus in humans [11, 12], sheep [13], horse [14], and pig [9, 10, 15, 16].

Wnt4 gene is expressed in the primordial gonads of mouse embryos [17] and Wnt4 protein influences the process of gametogenesis [18]. During mouse embryonic development, *Wnt4* gene is expressed in stromal cells of the forming endometrium [19]. In mice lacking *Wnt4* gene, sex reversion, partial atrophy of the Müllerian ducts, masculinization, and morphological and functional changes of the gonads were described [18]. Moreover, mutation of *Wnt4* gene in mouse causes ectopic expression of Leydig cells markers (e.g., 17-alpha-hydroxylase and 17-beta-hydroxysteroid dehydrogenase) [18]. Increased amounts of testosterone were also secreted [18].

The key role of the *WNT5A* gene has been documented by finding that *Wnt5a* knockout mice have no reproductive organs and live no longer than 24 hours [20]. Constitutively expressed *Wnt5a* gene was observed in mice gonadal ridges [21]. Wnt5a protein is present in the mouse endometrial stromal cells and its amount decreased in the area of myometrium formation what was established with the use of ribonuclease protection analysis and RNA *in situ* hybridization [20, 22, 23].

Female mice lacking *Wnt7a* genes expression have deformed wall of the uterus and undeveloped ovaries [6]. Moreover, it was shown that the expression of the Wnt7a may have impact on the transformation of Müllerian ducts [6].

3.2. Role of WNT Proteins in Endometrial Physiology. In physiological condition (Figure 1), *WNT4* gene expression is higher in endometrial stroma in comparison to its expression in epithelial cells [12]. Injection of estradiol (E_2) into ovariectomized mice upregulated, while administration of progesterone (P_4) had no effect on, *Wnt4* gene expression in stromal cells of endometrium [24]. Similar pattern of expression as those of *Wnt4* was presented by *Wnt5a* during the luteal phase of the estrous cycle in mice [23] but expression of *Wnt5a* was observed only in stromal cells just before and soon after estrus occurrence [23]. However, other authors could not confirm these findings [24]. Treatment of cyclic ewes with P_4 and antagonist of P_4 receptor at the same time (RU 486) increased endometrial *WNT5A* mRNA level at day 12 of pregnancy [25].

Fan and coworkers [26] showed that *WNT7A* mRNA levels in the female endometrial tissue were higher in the proliferative phase in comparison to secretory phase of the menstrual cycle. However, other authors did not find correlation of the *WNT7A* gene expression with phases of the menstrual cycle with the use of real-time PCR, digoxigenin-labeled cRNA probes, and *in situ* hybridization technique [11, 27, 28]. Presumably, *WNT7A* expression is stimulated by estradiol [29] which coordinates WNT7A-mediated process of postmenstrual reepithelialization and regeneration of the endometrium [26]. The influence of estradiol on *WNT7A* gene expression was presented in *in vitro* culture of luminal epithelial cells of human endometrium [30] or neonatal piglets [10]. Presence of WNT7A was marked in regenerating newly formed surface epithelium and upper endometrial

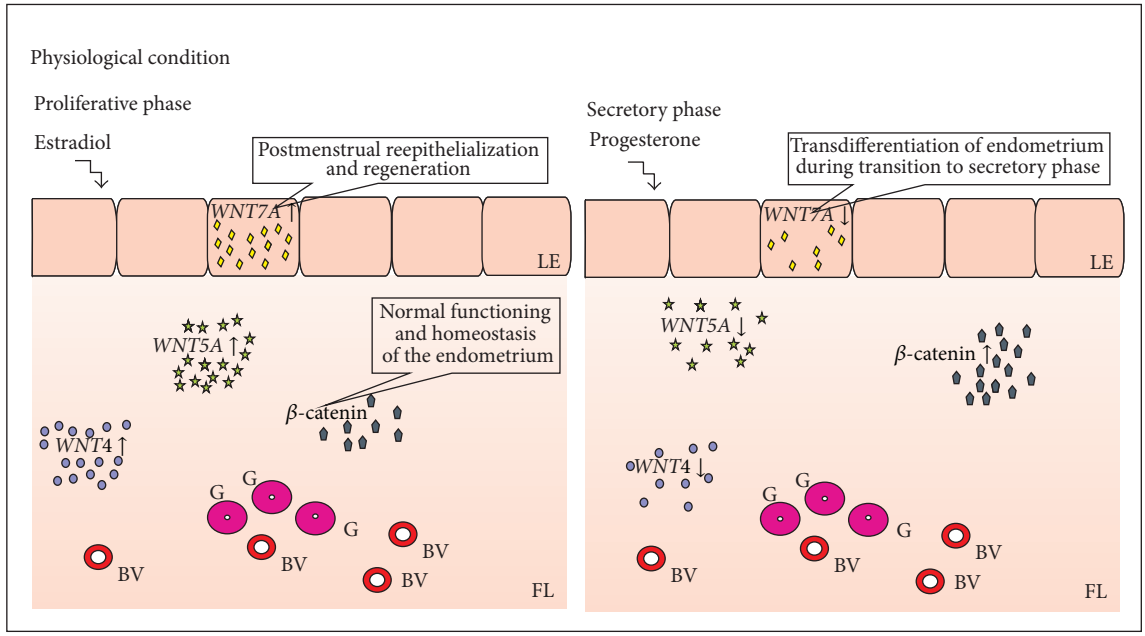

Figure 1: Endometrial expression of *WNT* genes. LE: luminal epithelium; FL: functional layer; BV: blood vessel; G: glands; ↓: decreased gene expression; ↑: increased gene expression.

glands [11, 26–28] but not in the lower glands and stroma of human endometrium [26, 28]. These observations support the view that luminal epithelium secretes factors that are important for glandular function and stromal transformation [11]. Moreover, progesterone-mediated downregulation of *WNT7A* gene expression may be essential for the transdifferentiation of endometrium during its transition to the secretory phase [26]. In mice, *Wnt7a* gene expression was completely suppressed in the surface epithelium and was undetectable in glandular epithelium and endometrial stroma after seven days of progesterone treatment [26].

β-catenin, the mediator of Wnt/β-catenin signaling, was first isolated as an intracellular protein constituting the binding domain of E-cadherin with cell's cytoskeleton [31]. The available data suggest that β-catenin is essential for normal functioning of the uterus and seems to be responsible for establishing of the endometrial homeostasis [32]. In human endometrial tissue, the immunoreactivity of β-catenin was observed in intercellular borders of luminal and glandular epithelial cells as well as in stroma and endothelial cells [33]. Examples of β-catenin positive staining of physiological endometrium are presented in Figure 2.

Fujimoto and coworkers [34] revealed upregulation of β-catenin (*CTNNB1*) mRNA level during secretory phase in human endometrium. It correlated with steroid hormone profile because progesterone but not estradiol increased *CTNNB1* mRNA level in human endometrial stromal cells cultured *in vitro* [35]. During proliferative phase of the menstrual cycle, the amount of nuclear β-catenin increased. β-catenin was allocated from the nucleus to the cytoplasm and cell membrane during the secretory phase [36]. However, Tulac and coworkers [11] showed no statistically significant difference in *CTNNB1* gene expression in human endometrium between proliferative and secretory phases.

Moreover, usage of LiCl, potential inhibitor of WNT/β-catenin signaling, induced estradiol-mediated proliferation and hyperplasia of endometrial cells in mice [37] and humans [38]. Activation of WNT/β-catenin signaling pathway increased endothelin 1 mRNA level which is target gene of β-catenin action as well as participant of endothelial cells differentiation [39]. Thus, it may be concluded that Wnt/β-catenin signaling regulates processes of endometrial proliferation and differentiation. Specific pattern of *WNT* genes expression and pattern of hormonal regulations are summarized in Table 1.

4. *WNT* Protein and Gene Expression in Endometrial Cancer

4.1. General Characteristics of Endometrial Cancer. Endometrial cell carcinomas (ECCs) are the most common malignancy of the female genital tract in the Western world and the fourth most common one after breast, lung, and colorectal cancer in women. A constant increase of endometrial cancer has been observed in the recent years [40]. ECCs occur mainly in postmenopausal women at the age of 55–65. Factors increasing the probability of the endometrial carcinoma occurrence include long-term estrogen therapy, polycystic ovarian syndrome, a history of nulliparity or infertility, irregular menstrual cycles, obesity, diabetes mellitus, and hypertension [41]. The curability of EECs is as high as five-year survival rate for the G1 and 1st stage is 90%. However, 5-year survival rapidly decreases to 30–50% for the 2nd and to 20% for the 3rd stage [41]. Women with ECCs experience dysfunctional endometrial bleeding which makes tumor detection seemingly an easy task. However, major diagnostic and prognostic problems often arise by histopathological assessment (WHO classification) since seven types of endometrial

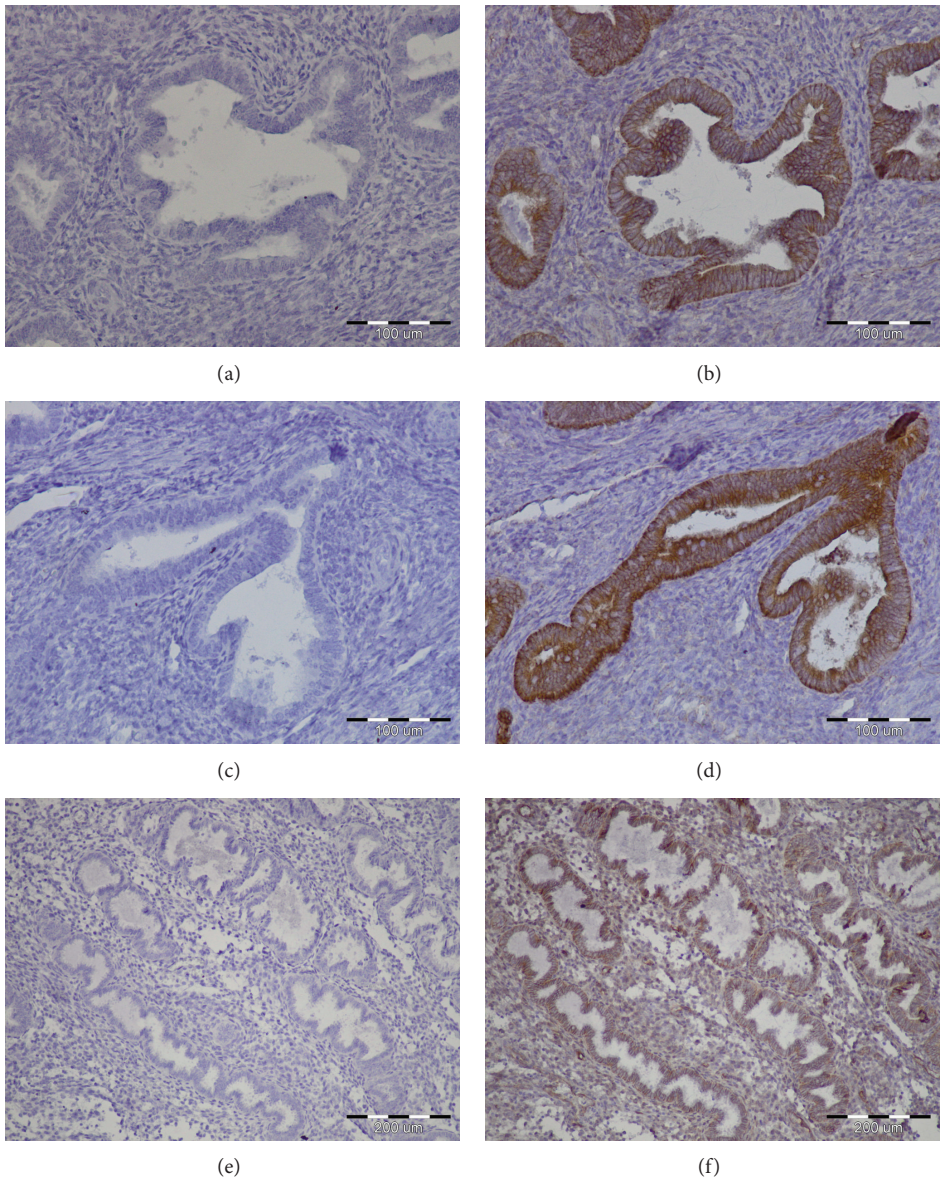

Figure 2: Examples of immunohistochemical staining confirming presence of β-catenin in physiological endometrium. Positive staining (BD Transduction Laboratories Cat. number 610153) of endometrial glands, magnification ×20 (b, d) and ×10 (f). Negative controls magnification ×20 (a, c) and ×10 (e).

carcinoma can be distinguished. The most common subtype of ECC is an endometrial endometrioid adenocarcinoma (EEAC), classified as type I or estrogen-dependent cancer [42]. Approximately 80% of newly diagnosed endometrial carcinomas in the Western world are of the endometrioid (EEAC) type [43]. Any factor that increases exposure to unopposed estrogen (estrogen-replacement therapy, obesity, anovulatory cycles, and estrogen-secreting tumors) increases the risk of these tumors, whereas factors that decrease exposure to E_2 or increase P_4 levels (oral contraceptives, smoking) tend to be protective [44]. Type I endometrial cancer consists of low-grade endometrioid histology, starts with the background of endometrial hyperplasia, and may have better prognosis [41]. Endometrial serous adenocarcinoma (ESC) and clear cell endometrial carcinoma (ccEC) are aggressive neoplasia carrying a poor prognosis [45]. ESC or ccEC is estrogen-independent and is classified as type II [42]. The average age of patients with nonendometrioid cancer is 67 years, and at least half of them had cancer already spread beyond the corpus of the uterus at the time of diagnosis. The 5-year survival is approximately 62% for clear cell carcinomas and 53% for papillary serous cancers [44]. Although the ECCs are highly curable, there are particular morphological variations and histopathological features which do not allow for their clear identification [42]. Each subtype has specific genetic alterations showing microsatellite instability and mutations in *PTEN*, *PIK3CA*, *K-ras*, and *CTNNBI* (*β-catenin*) genes summarized in Table 2. However, their specificity as a biomarker has been widely discussed [42].

Table 1: Function and hormonal regulation of WNT and β-catenin (*CTNNB1* gene) in the endometrium.

Gene		Description	References
WNT4	Function	Formation of the primordial gonads	[18]
		Gametogenesis	[17]
		Uterine wall morphogenesis	[19]
		Decidua formation	[24, 60, 88]
	Hormonal regulation	E_2 ↑	[13]
		E_2 ↓	[9, 10]
		P_4	[13]
		E_2 ↓ + P_4	[13]
WNT5A	Function	Uterine wall morphogenesis	[19]
	Hormonal regulation	E_2 ↓	[9, 10]
		P_4 + RU486 ↑	[25]
WNT7A	Function	Development and functioning of the gonads	[6]
		Postnatal uterine gland morphogenesis and function	[89]
	Hormonal regulation	E_2 ↓	[9, 10]
		E_2 + P_4 ↓	[24]
CTNNB1	Function	Normal functioning of the uterus Endometrial homeostasis	[32]
	Hormonal regulation	P_4 ↑	[35]
		E_2	[35]

↓: decreased gene expression; ↑: increased gene expression; E_2: estradiol; P_4: progesterone, RU486: antagonist of progesterone receptor.

Table 2: Immunohistochemical and molecular markers for ECCs classification.

Method	Type of EEC				References
	Normal	EEAC	ESC	CcEC	
IHC	PTEN	PTEN ↓	PTEN −	PTEN −	[90]
	Active β-catenin	Active β-catenin +	Active β-catenin −	Active β-catenin −	[91]
	p53	p53 −/+	p53 +	p53 +	[92]
Real-time PCR	PTEN	PTEN ↓	PTEN	PTEN	[90]
	Survivin	Survivin ↑			[92]
	K-ras	K-ras ↑	K-ras ↓	K-ras ↓	[93]
	p27	p27 ↓			[92]

+: protein is present; −: protein is absent; −/+: protein is expressed moderately.
↓: decrease of gene expression; ↑: increase of gene expression.

4.2. Wnt Gene Expression in Endometrial Cancer. The expression of genes encoding WNT proteins and proteins involved in WNT signaling pathways was found to be changed also in endometrial cancer [12] (Figure 3). The *WNT4* mRNA level was lower while *WNT2*, *WNT3*, and *WNT5A* mRNA levels were higher in endometrial carcinoma in comparison to normal endometrium [12]. Also *WNT2*, *WNT3*, *WNT4*, and *WNT5A* genes expression was higher in normal human primary epithelial and stromal endometrial cultures compared to endometrial carcinoma cell lines, what suggest their participation in endometrial neoplasia [12].

Most of the studies concentrated on *WNT7A* gene expression. In 63% patients of one series of endometrial carcinoma, *WNT7A* gene expression was absent or reduced and negatively correlated with FIGO stage, grade, lymph node metastasis, depth of myometrial invasion, lymph vascular space involvement, and peritoneal cytology [28]. In large-scale population study on 244 EEC patients, *WNT7A* overexpression was found in most cases of endometrial cancer in comparison with normal endometrium and benign endometrial lesion [46]. However, negative expression of *WNT7A* gene correlated positively with overall survival and disease-free survival of endometrial cancer [46].

In the Ishikawa cell line model of endometrial adenocarcinoma, estrogen receptors were probably involved [47] in the downregulation of *WNT7A* expression mediated by estradiol [48]. Moreover, *WNT7A* and *WNT7B* genes expression was increased in endometrial carcinoma cell lines and normal endometrial tissues as compared with primary cultures of human endometrial cells [12].

WNT10A and WNT10B proteins have been implicated in estrogen-related carcinogenesis of endometrial cancer. The amount of WNT10B protein was higher in endometrial cancer than in hyperplastic and normal endometrium as determined by Western blot technique [49]. In early stages of endometrial cancer, the expression of *WNT10B* was higher than in later stages. WNT10B proteins were mainly detected in patients with the cancer of endometrioid type, who had high graded and advanced-staged tumor without lymph node metastasis [49]. This clinical study was partially confirmed by

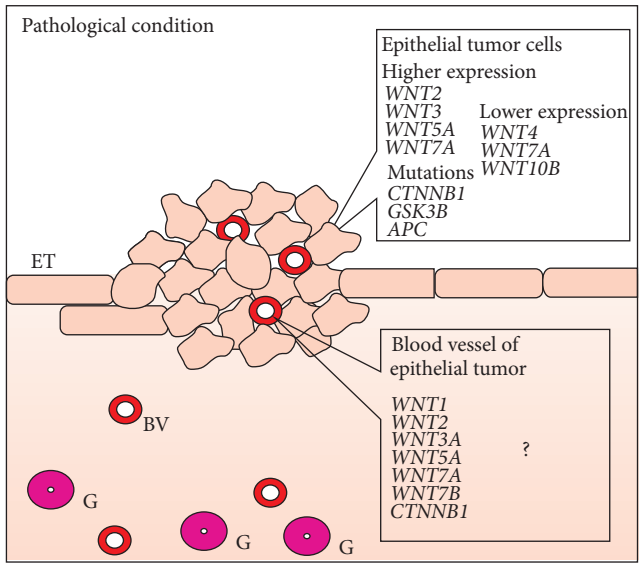

Figure 3: Expression of *WNT* genes in endometrial cancer. ET: epithelial tumor; BV: blood vessel; G: glands.

results of the *in vitro* investigations. *WNT10A* gene expression was decreased in endometrial HEC1B and AN3CA cell lines while WNT10B was increased in Ishikawa cell lines [50].

Mutations of β-catenin gene (*CTNNB1*) were found in many endometrial cancers [51]. According to various reports, 10 to 45% of endometrial cancers present missense mutation of *CTNNB1* [50]. Endometrial tumors with mutation in exon 3 on serine/threonine residue showed predominant nuclear β-catenin accumulation. In this case, blockage of the process of β-catenin degradation results from the lack of its phosphorylation [36, 52, 53]. In rat gliomas [54], human glioblastomas [55], and medulloblastomas [56], nuclear accumulation of β-catenin was observed in endothelial cells of neovessels. Beta-catenin accumulation was observed in the nucleus of malignant changed endometrial cells [36, 52, 53]. However, as far as we know the presence of β-catenin was previously not shown in tumor vascular endothelial cells (Figure 4). However, β-catenin membranous immunoreactivity associated with E-cadherin decreased during transformation of normal endometrium through atypical endometrial hyperplasia to endometrial cancer in parallel with decreased E-cadherin expression in endometrial cancer [57]. *CTNNB1* mutation is observed mainly in endometrioid endometrial cancer [57–59].

Mutations in *KRAS* and/or *CTNNB1*, *GSK-3β*, and *APC* gene are recognized as major alterations in type I endometrial cancer [50]. Upregulation of estrogen receptor signaling causes endometrial hyperplasia and can be a reason of endometrial cancer [29]. WNT signaling activation leads to endometrial and myometrial hyperplasia [32, 60, 61], squamous cell metaplasia without malignant transformation [62], mesenchymal tumors, and endometrial sarcomas [32, 61] in transgenic mouse [62].

DKK1 was highly expressed in benign endometrial tissue and downregulated in endometrial cancer [63]. Treatment of Ishikawa cell line with DKK1 lowered the level of active β-catenin as the result of Wnt signaling pathway inhibition through binding to LRP5/6 [63]. DKK1 is positively correlated with histological differentiation and clinical stage of endometrial cancer [63]. *DKK3* gene expression was found to be decreased in endometrial cancer. It correlated with advanced stage and high risk clinicopathological factors [51]. High expression of *Dkk3* gene reduced motility and proliferation of the cells in *in vitro* experiments [51].

5. Crosstalk of WNT Proteins and Other Factors in Endometrial Angiogenesis

5.1. Angiogenesis in Endometrium. Vascular system is a network of arteries, capillaries, and veins for transport of gases and macromolecules. Vasculogenesis is a process of *de novo* formation of capillary bed through differentiation, proliferation, and migration of precursor cells (angioblasts) [64]. Formation of new blood vessels from already existing capillaries is called angiogenesis [65]. Angiogenesis is a two-step, physiological process essential for proper endometrial functioning [66]. Blood vessels have to be repaired after menstrual phase of the menstrual cycle [66]. Capillaries grow, mature, and coil during the proliferative and secretory phase [66], when endometrial blood flow and permeability of endometrial microvessels become rapidly increased by high levels of estrogens at the late phase of cycle [67]. It is highly probable that vessel growth in human endometrium occurs by nonsprouting mechanism, elongation in response to metabolic demands of surrounding cells [68] and intense hypoxia in the luminal portion of the endometrium on day 2 of the cycle, with negligible detection by d5 [69]. Endothelial cells which form capillary bed are under influence of (i) factors produced by surrounding tissue [65] and/or (ii) angiogenic factors that circulate in blood and their levels fluctuate during menstrual cycle [70]. Growth factors (VEGF, EGF, FGF, NP-1, and angiopoietin) and their receptors (VEGFR, EGFR, FGFR, and IGFR) can both positively or negatively influence this process. The common denominator for the process of angiogenesis occurring in the endometrium is hypoxic environment and hypoxia inducible factor (HIF) stimulation of VEGF expression [71].

5.2. Participation of WNT Proteins in Blood Vessel Formation. Participation of WNT proteins in the process of differentiation of cells of hematopoietic and endothelial cell lineage as well as vasculogenesis and angiogenesis is apparent [72]. It has been demonstrated that sustained WNT pathway activation can be utilized to generate endothelial progenitors from mesodermal lineage of embryonic stem cells in *in vitro* conditions [73]. Moreover, coculture of human embryonic stem cells with *Wnt1*-overexpressing cells accelerated differentiation of mesoderm germ layer into hematoendothelial cells *via* activation of canonical WNT signaling [74]. Presence of WNT1 upregulated WNT/β-catenin signaling, bovine aortic endothelial cells proliferation, and capillary stability under *in vitro* conditions [75]. On the other hand, WNT1 was found to inhibit proliferation of endothelial cells [76].

The role of the WNT2 protein in angiogenesis is less clear. Increased expression of WNT2 protein and FZD-5 receptor

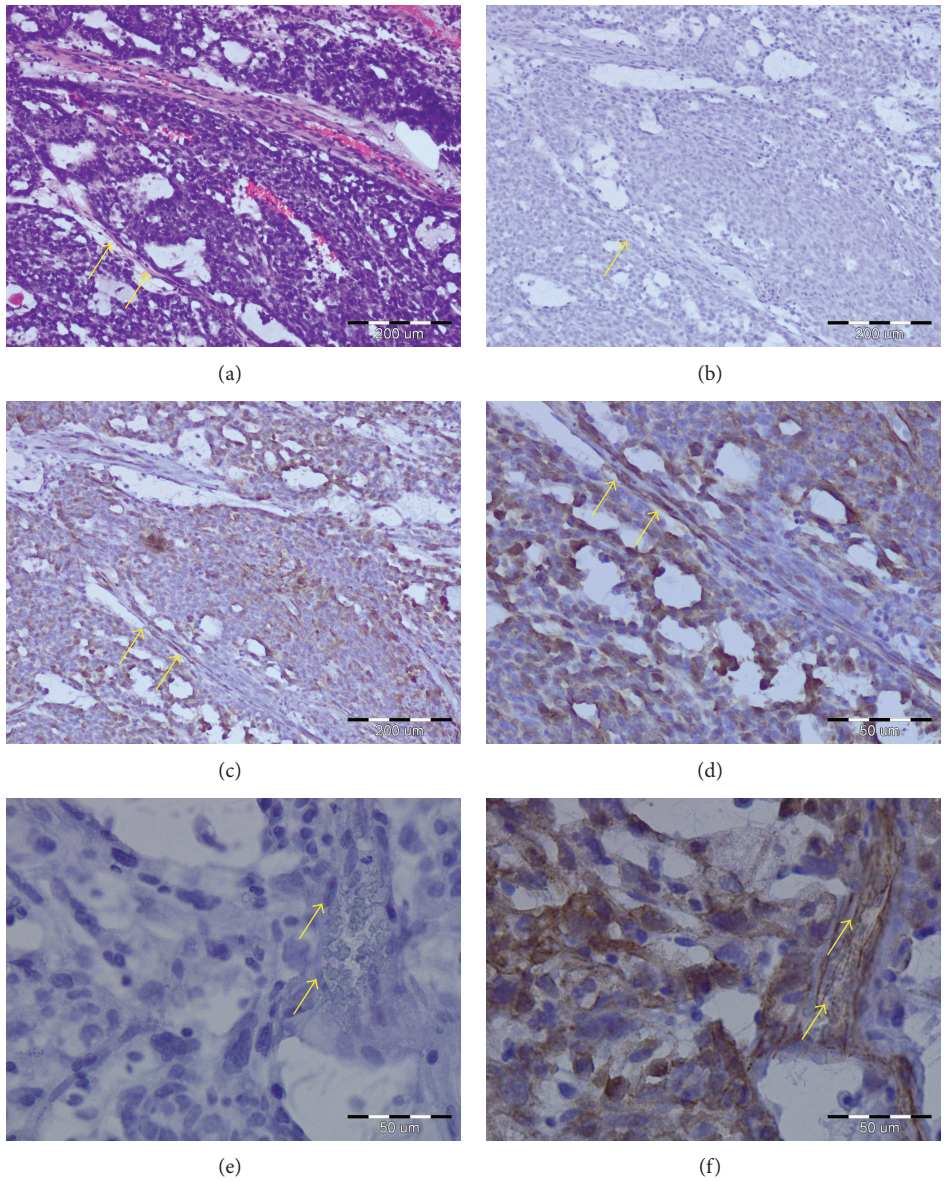

Figure 4: Examples of immunohistochemical staining confirming presence of β-catenin in adenocarcinoma endometrioid (G3) cells and tumor vascular endothelial cells (arrow). Hematoxylin-eosin staining (a); negative controls in magnification ×20 (b) and ×40 (e); positive staining (BD Transduction Laboratories; Cat. number 610153) in magnification ×20 (c) and ×40 (d, f).

caused defects in the vasculature of murine placenta and changed blood flow in the mouse yolk sac [77, 78] as a reduced number of the fetal capillaries were observed [79]. However, expression of *WNT2* gene had no impact on WNT/β-catenin signaling activation, endothelial cells proliferation [72, 75], or capillary length [75]. Differentiation of endothelial cells from mouse embryonic stem cells is suspected to be controlled by Wnt2 and Wnt11 [39].

WNT3A was shown to be direct, VEGF-independent, inducer of endothelial cell proliferation [72, 80] and migration [80].

WNT5A is required for endothelial differentiation of embryonic stem cells and transformation of mouse embryonic stem cells into immature endothelial progenitor cells, taking part in healing process of damaged endothelium [39]. Acting on endothelial cells through autocrine regulation [81], WNT5A can decrease cell number and capillary length. However, these effects were not observed after activation of WNT/β-catenin signaling [75]. Moreover, WNT5A protein did not stimulate human umbilical vein endothelial cells (HUVEC) migration and proliferation [75]. Wnt5a and Wnt10b induced FZD-5-mediated angiogenesis in a mice yolk sac [79].

WNT7 proteins were shown to promote normal angiogenesis in ventral regions of the CNS in mouse [82]. Specifically *Wnt7a* but not VEGF promotes migration and stimulates expression of blood-brain barrier specific transporters of glucose (GLUT-1) in mouse brain endothelial cell line [82].

Moreover, in *Wnt7b* gene deficient mice, loss of *Wnt7b* gene resulted in defective smooth muscle component of the major pulmonary vessel differentiation, degradation of vessel's wall, and perinatal hemorrhage [83].

Inhibition of the expression of *CTNNB1* gene in endothelial cells affected the formation of vasculature of head of mouse embryos, large vitelline and umbilical vessels, and the vasculature of the placenta [84]. As a result of β-catenin absence, significant reduction in cell junctions organization and hemorrhage was observed [84].

5.3. Hypothetical Involvement of the Wnt/β-Catenin Pathway in Endometrial Angiogenesis. Data provided in the proceeding chapter clearly indicate the participation of WNT proteins in the recruitment, proliferation, and migration of endothelial cells in healthy subjects and cancer patients. In the endometrium, development of the vascular network occurs simultaneously with epithelial and stromal cells expansion, expression, and influence of angiogenic factors that circulate in blood and fluctuate in menstrual cycle phase-dependent manner [70]. Regulation of endothelial cell growth and fate was shown to be regulated by reciprocal interactions between mesenchymal and endothelial cells [81]. Even if estradiol was shown to inhibit angiogenesis under *in vivo* conditions [85], this effect was not caused by direct action on endothelial cells because they do not have estrogen receptors [86]. Therefore, they cannot respond to this potential inhibitor of angiogenesis. Thus, it is highly probable that in endometrium capillaries are under influence of other external factors which compensate or antagonize the influence of estradiol. We suggest that WNT proteins are perfect candidate to be such a mediator.

We can hypothesize (Figure 5) that it might be probable that in physiological condition WNT5A can participate in endothelium recovery, rather than angiogenesis process, as it takes part in healing of the damaged endothelium [39], but not in proliferation and migration of the endothelial cells [81] or increasing capillary length [75]. WNT7A might be chemoattractant for endothelial cells in the process of physiological endometrial angiogenesis, as it is a factor of epithelial origin. *WNT7A* gene expression was upregulated during proliferative phase of the estrous cycle and downregulated in the secretory phase [26]. β-catenin can function in the endometrium either directly on endothelial cells or indirectly through its action on endometrial cells where it promotes the expression of VEGF [87] or endothelin 1 [39]. However, this hypothesis requires experimental confirmation.

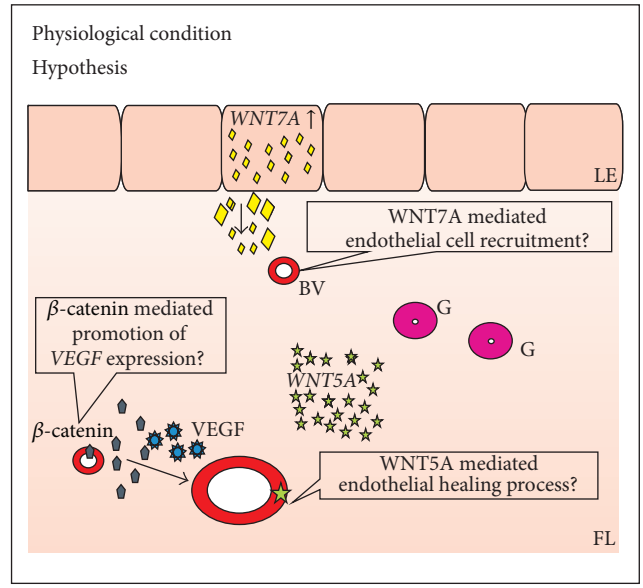

Figure 5: Hypothetical involvement of WNT and β-catenin proteins in endometrial angiogenesis. LE: luminal epithelium; FL: functional layer; ET: epithelial tumor; BV: blood vessel; G: glands.

6. Conclusions and Future Perspectives

The participation of WNT proteins, β-catenin, and inhibitors and inducers of WNT signaling in the process of endometrial angiogenesis is largely unknown. The main task which should be now undertaken should concentrate on defining which WNT, receptors, inhibitors, and signaling pathway are activated in the endothelial cells of the blood vessel of the endometrium. The spatiotemporal pattern of expression of elements of WNT signaling system should also be established. Differences in normal and pathological state should be also considered. Conducting this research will help to determine the angiogenic potential of the WNT family of proteins, will allow for a better understanding of the mechanism of formation of vessels in the endometrium, and will help to determine whether WNT proteins can be potential target for antiangiogenic therapy directed mainly against transcription factors as, for example, β-catenin. We are aware that our hypothesis does not present the experimentally verified information. However, we believe that our suggestion will contribute to the discussion and to some extent the development of research on the role of WNT proteins in the formation of blood vessels in the dynamically changing uterus.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jolanta Kiewisz and Tomasz Wasniewski equally contributed to this paper.

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Research Article

Profiling of Selected MicroRNAs in Proliferative Eutopic Endometrium of Women with Ovarian Endometriosis

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It has been well documented that aberrant expression of selected microRNAs (miRNAs) might contribute to the pathogenesis of disease. The aim of the present study is to compare miRNA expression by the most comprehensive locked-nucleic acid (LNA) miRNA microarray in eutopic endometrium of patients with endometriosis and control. In the study we recruited 21 patients with endometriosis and 25 were disease-free women. The miRNA expression profiles were determined using the LNA miRNA microarray and validated for selected molecules by real-time PCR. We identified 1198 human miRNAs significantly differentially altered in endometriosis versus control samples using false discovery rate of <5%. However only 136 miRNAs showed differential regulation by fold change of at least 1.3. By the use of selected statistical analysis we obtained 45 potential pathways that might play a role in the pathogenesis of endometriosis. We also found that natural killer cell mediated cytotoxicity pathway was found to be inhibited which is consistent with previous studies. There are several pathways that may be potentially dysregulated, due to abnormal miRNA expression, in eutopic endometrium of patients with endometriosis and in this way contribute to its pathogenesis.

1. Introduction

Endometriosis is relatively common, benign gynecological disease in which endometrial tissue ectopically implants in a location outside the uterine cavity. The most common clinical symptoms include pelvic pain and infertility which can seriously influence the quality of life of affected patients [1].

Despite the current controversy regarding the pathophysiology of this disease, Sampson's theory explains the existence of endometrial cells in the peritoneal cavity by retrograde menstruation. Several factors such as increased chemokines and matrix metalloproteinases in the peritoneal fluid (PF), angiogenesis, tumor suppressor, and oncogenes as well as upregulating of proinflammatory cytokines may facilitate the pathogenesis of endometriosis [2–6]. It is therefore assumed to be a complex process, in many aspects characteristic of systemic disease. It has now also become widely accepted that molecular aberration within the eutopic endometrium may predispose a subgroup of women to the development of endometriosis [7].

The molecular changes that could potentially lead to abnormal growth of endometrium outside uterus include microRNAs (miRNAs) expression fluctuations [8]. MicroRNAs are a large class of endogenous, single-stranded, and short ncRNA (noncoding) of approximately 22 nucleotides in length that play a key role in regulating gene expression through interaction with mRNA of protein-coding genes [9]. miRNA expression is tissue- and cell-specific and it was demonstrated that miRNAs are important in endometriosis and associated reproductive conditions [10].

In our recent study we also showed that, out of 667 miRNAs, two molecules, namely, hsa-miR-483-5p and hsa-miR-629*, are significantly downregulated in eutopic endometrium of patients with ovarian endometriosis, which could be a consequence of an early defect in the physiological activity of the proliferative endometrium [11]. Since there have been only few studies, in the available literature, which concerned miRNA profiling of eutopic endometrium (actually mostly luteal) [12, 13], and the technology since our last publication has greatly developed, we decided to expand our previous work and use more robust microarray technique which facilitates expression profiling of more than 2000 miRNAs. The aim of the present study is to examine possible differential regulation as well as putative pathways that might be regulated by abnormal miRNA expression in the proliferative eutopic endometrium of patients with advanced ovarian endometriosis.

2. Material and Methods

Patients ($n = 46$) scheduled for laparoscopy for adnexal mass or infertility at the Medical University of Bialystok were recruited to participate in this study. Endometrial biopsies were collected using Pipelle suction curettes. Endometrial tissue samples were classified by histological dating according to the method of Noyes et al. [14] and only patients in the proliferative phase (days from 6th to 13th) of the cycle were included in the study.

Patients with endometriosis (Group I, $n = 21$) stages from III to IV were diagnosed by laparoscopic findings according to the revised American Fertility Society classification of endometriosis [15] and each case was confirmed by histopathology. As a control (Group II, $n = 25$) we used endometrial tissue from patients without any endometriosis visible during laparoscopy.

All women had regular menstrual cycles (28–30 days) and were not taking any medication for at least 3 months prior to operation. We excluded patients with autoimmune disease, pelvic inflammatory disease, adenomyosis, fibroids, and dysfunctional uterine bleeding. The study was approved by the Institutional Review Board of Medical University of Bialystok and informed consent was obtained from each patient.

The collected tissue was placed separately in buffered formalin for histopathological studies and in RNA later (Sigma-Aldrich, Poland) for molecular analysis. The latter was stored for 24 hours in +4°C and then tissues were transferred and stored in −80°C. Total RNA was extracted using the *mir*Vana miRNA Isolation Kit (Ambion, Life Technologies, Poland). RNA quality was assessed with Agilent Bioanalyzer 2100 and Agilent RNA 6000 Nano kit (Agilent Technologies, Perlan, Poland) and samples chosen for further analysis showed minimum sign of degradation as judged by the RNA integrity number (RIN), which was above 9 for all samples. RNA concentrations were measured on a NanoDrop 2000c (Thermo Scientific, Biotech, Poland).

2.1. miRNA Expression Profiling Using Exiqon Microarrays. For the purpose of miRNA microarray screening we chose 10 samples of patients with advanced endometriosis and 11 controls. Microarray analysis was conducted as single-channel Hy3 experiments on Exiqon's miRCURY LNA (locked-nucleic acid) microRNA Array 7th generation—hsa, mmu and rno. Exiqon arrays contain 3100 capture probes, complementary to most human, mouse, rat, and their related viral sequences from the v.19.0 release of miRBase. The arrays also contain 25 proprietary human miRPlus sequences not yet in miRBase. 500 ng RNA sample was labelled with a Hy3 fluorophore (Exiqon, Denmark). Labelling reactions were performed using Exiqon's miRCURY LNA microRNA Hi-Power Labeling Kit with the use of synthetic spike controls, Spike-in microRNA Kit v2 (Exiqon, Denmark), according to the manufacturer's protocol. Hybridization of labeled RNA to the array was performed in SureHyb chambers (Agilent Technologies, USA) for 16 hours at 56°C. Slides were washed according to manufacturer's instructions and scanned at 10 μm resolution using an Agilent G2505C DNA Microarray Scanner. Raw data were generated using Imagene 9.0 software (BioDiscovery, Inc., USA), using an FE protocol available on demand from Exiqon.

2.2. Quantitative PCR, Real-Time RT-PCR (qPCR). The expression levels of the selected microRNAs and the assay miRBase IDs (miRBase Accession Number) are hsa-miR-4714-5p (MIMAT0019822), hsa-miR-4284 (MIMAT0016915), hsa-miR-5193 (MIMAT0021124), hsa-miR-4454 (MIMAT0018976), hsa-miR-3680-5p (MIMAT0018106), hsa-miR-3667-5p (MIMAT0018089), hsa-miR-23a-3p (MIMAT0000078), hsa-miR-23b-3p (MIMAT0000418), hsa-miR-5187-3p (MIMAT0021118), hsa-miR-3152-5p (MIMAT0019207), and hsa-miR-30d-5p (MIMAT0000245) which were evaluated using the miRCURY LNA Universal RT microRNA PCR system (Exiqon, Denmark). In the first step, we have conducted the one first-strand cDNA synthesis reaction, which provided template for all microRNA real-time PCR assays. The cDNA for each RNA sample was obtained using the Universal cDNA Synthesis Kit II (Exiqon, Denmark) according to the manufacturer's instructions. Levels of miRNAs were quantitated using individual miR CURY LNA Universal RT microRNA PCR Assays (Exiqon, Denmark).

The conditions for qPCR were as follows: 95°C for 10 min and 45 cycles of 95°C for 10 sec followed by 60°C for 1 min. Finally a melting curve analysis was performed with denaturation at 95°C for 15 s and 60°C for 15 s followed by a temperature gradient from 60 to 95°C for 20 min and a final denaturation at 95°C for 15 s. U6 snRNA was used as the endogenous control. LNA PCR amplification reactions for each miRNA molecule were repeated independently three times. Quantitative real-time PCR analysis was performed using the Applied Biosystems 7900HT System (Life Technologies, Foster City, CA).

Gene expression values were calculated based on the 2(−Delta Delta C(T)) method, where one sample was designated the calibrator, through which all other samples were analyzed [16]. Briefly, ΔCT represents the threshold cycle of the target minus that of U6 snRNA and ΔΔCT represents the ΔCT of each target minus that of the calibrator. Relative

quantities were determined using the equation: RQ = $2^{-\Delta\Delta CT}$. For the calibrator sample, that is, control RNA from eutopic endometrium, the equation is relative quantity = 2^{-0}, which is 1; therefore, every other sample is expressed relative to this.

2.3. Statistical Analysis. All data analyses were performed in R statistical environment (http://www.r-project.org/) and relevant Bioconductor software [17].

The raw microarray data were preprocessed with *vsn*2() function implemented in *vsn* package [18]. The *vsn* function was used with default settings. Differentially expressed miRNAs were identified with *limma* [19]. In order to associate miRNA with mRNA we used annotations from 6 databases: miRBase, targetScan, miRanda, tarBase, mirTarget2, and picTar. Probes not associated with human mRNA were not analyzed further. The potential influence of miRNA on mRNA expression was visualized using signaling pathway definitions.

In our study we used signaling pathway definitions from KEGG (*Kyoto Encyclopedia of Genes and Genomes* [20]). The potential miRNA influence on mRNA expression was also used to analyze GO (gene ontology) terms potentially overrepresented in disturbed genes.

To assess perturbation of signaling pathways we applied SPIA (*Signaling Pathway Impact Analysis* [21]). We used the potential influence of miRNA on mRNA expression instead of gene expression data.

While comparing two groups for quantitative data, Mann-Whitney-Wilcoxon test was used due to the nonnormal distribution of the tested variables. The significance level was equal to 0.05. The calculations have been carried out by means of Microsoft Excel spreadsheet and STATISTICA, StatSoft, Inc. Version 7.1. statistical package (data analysis software system).

3. Results

Patients clinical characteristics are presented in Table 1.

We identified 1198 human miRNAs significantly differentially altered in endometriosis versus control samples using false discovery rate of <5% (Table I supplement in the supplementary Material available online at http://dx.doi.org/10.1155/2015/760698).

Volcano plot (Figure 1) presents results of expression analysis for all analyzed miRNAs, while the heatmap (Figure 2) shows expression profiles only of significantly expressed miRNAs.

The associations between miRNA and mRNA are presented in the zipped supplement directory (called Table II supplement).

We obtained 45 potential pathways from the KEGG (Kyoto Encyclopedia of Genes and Genomes) database and particularly mTOR and VEGF signaling pathway caught our attention due to its close potential relation to pathogenesis of endometriosis (Figures 3 and 4, resp.).

The significantly overexpressed GO (gene ontology) terms are presented in Table 2. We found six potential cellular processes that involve protein synthesis to be potentially regulated by influence of miRNA on mRNA, including translational elongation and termination, protein targeting and localization to endoplasmic reticulum, cotranslational protein targeting to membrane, SRP- (signal-recognition particle-) dependent cotranslational protein targeting to membrane, and establishment of protein localization to endoplasmic reticulum.

The SPIA showed perturbation of potentially nine pathways in two analyzed groups. Most interestingly natural killer cell mediated cytotoxicity pathway was found to be inhibited in eutopic endometrium of patients with endometriosis as compared with control (Table 3).

3.1. Validation by RT-PCR. Following the selection of miRNAs by fold change filtering (fold change > 1.3), we found that there were 136 upregulated miRNAs and no downregulated miRNAs in the eutopic endometrium of patients with advanced ovarian endometriosis compared with the eutopic endometrium.

We then validated 11 selected miRNAs but we were not able to observe clear statistical differences as to the expression

Table 1: Clinical characteristics of patients.

	Endometriosis ($n = 21$)	Control ($n = 25$)
Age (years)	31.35 ± 0.90	30.73 ± 0.91
Infertility, *n* (%)	9 (50)	16 (64)
Primary	7	8
Secondary	2	8
Duration of infertility (months)	48.2 ± 19.4 (23–71)	50.8 ± 15.8 (13–71)
Ovarian cysts, *n* (%)		
Endometrial	21 (100)	—
Simple	—	9 (36)

Data are mean ± SEM. Ranges are provided for "duration of infertility."

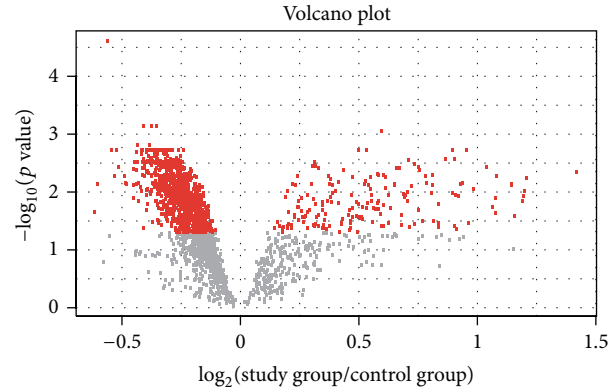

Figure 1: Volcano plot for all analyzed human miRNAs. The *x*-axis (horizontal) is the fold change between endometriosis and control samples (on a log scale, so that up- and downregulation appear symmetric), and *y*-axis represents the *p* value for a test of differences between samples (most conveniently on a negative log scale, so smaller *p* values appear higher up). Red dots represent miRNAs significantly differentially expressed.

Table 2: Gene ontology (GO) terms overrepresented in eutopic endometrium of patients with endometriosis and control.

GO term	Adjusted p values for overrepresentation analysis for miRNAs with higher expression in endometriosis	Adjusted p values for overrepresentation analysis for miRNAs with higher expression in control
Translational elongation	1	0.077
Translational termination	1	0.077
Cotranslational protein targeting to membrane	1	0.077
SRP- (signal-recognition particle-) dependent cotranslational protein targeting to membrane	1	0.077
Protein targeting to endoplasmic reticulum	1	0.077
Protein localization to endoplasmic reticulum	1	0.077
Establishment of protein localization to endoplasmic reticulum	1	0.077

The first column contains names of significantly overrepresented GO terms. Both the second and the third columns contain adjusted p values for overrepresentation analysis (Fisher's exact test). The second column presents results for genes associated with miRNA that have higher expression in endometriosis (of at least 20%) and the third column presents results for genes associated with miRNA that present higher expression in control samples (of at least 20%). GO terms with adjusted p values <0.25 were recognized as significantly overrepresented.

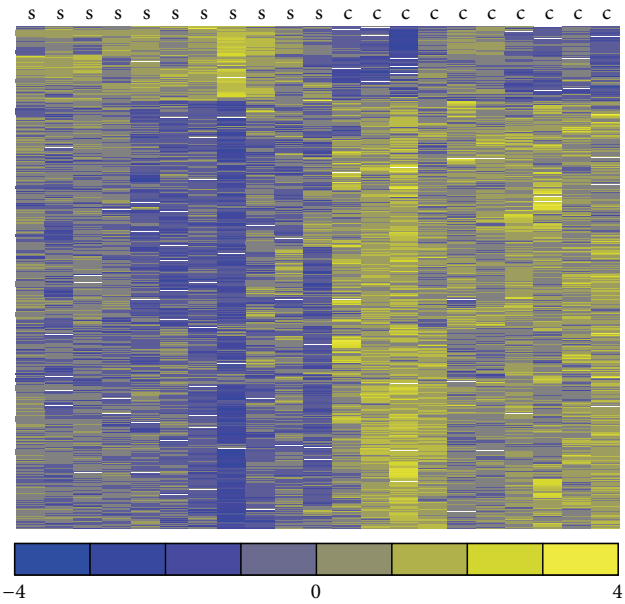

Figure 2: Heatmap display of significantly expressed miRNAs. Heatmap representation of expression data for miRNAs significantly altered in endometriosis samples compared with control samples (adjusted p value < 0.05). Columns correspond to samples and rows correspond to individual miRNAs. For a given miRNA an average value was computed and subtracted from each observation. The yellow color marks a higher expression (over average expression in two groups), while the blue color represents a lower expression (again with regard to average expression). The color scale represents the magnitude of changes. "s" stands for endometriosis samples and "c" stands for control samples.

of any of the chosen molecule (Table 4) between studied groups. We found that, specifically for three miRNAs, that

Table 3: Signaling Pathway Impact Analysis (SPIA) showing perturbation of signaling pathways in eutopic endometrium of patients with endometriosis versus control.

The name of the pathway	Adjusted p values	Status
Alcoholism	0	A
Olfactory_transduction	0	A
Viral_carcinogenesis	0	I
Systemic_lupus_erythematosus	0.01	A
Chronic_myeloid_leukemia	0.01	A
Cytosolic_DNA-sensing_pathway	0.01	A
Natural_killer_cell_mediated_cytotoxicity	0.01	I
RNA_transport	0.03	I
Neurotrophin_signaling_pathway	0.04	A

The first column shows names of significantly impacted pathways, the second column contains adjusted p values, and the third shows type of impact [activation (A)/inhibition (I)] in the analyzed group of samples with reference to the control group.

is, miR-5187-3p, miR-3152-5p, and miR-30d-5p, there exist differences with border significance within 0.05 (Table 4).

4. Discussion

Although in our study we found more than 1000 miRNAs that are significantly differentially regulated between eutopic endometrium of patients with and without endometriosis, the differences, either up- or downregulated, were not higher than 2.6-fold in any case. The problem with obtaining clearly significant qPCR results may point to reservations that we may have as to the expression array results which can indirectly imply that some of the results might be false positives and that there are a number of variables that must also

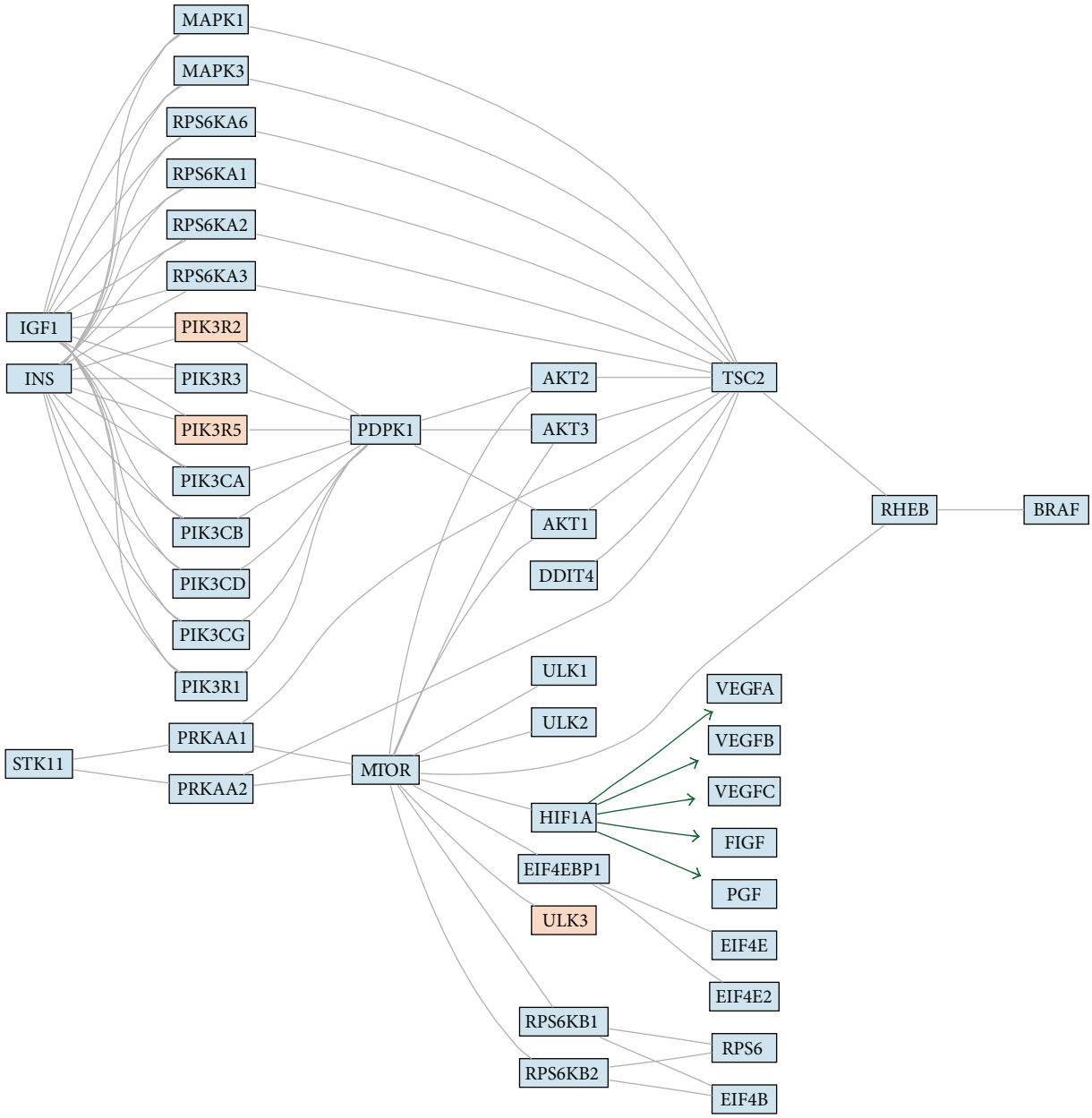

Figure 3: The figure shows signaling pathways (3: mTOR and 4: VEGF) with marked hypothetical influence of miRNA. The colors present expression of miRNAs assigned to a particular gene. The blue color stands for higher expression in control samples, and the red color stands for higher expression endometriosis. The color scale represents the magnitude of changes. The green arrows represent connections start from transcription factor and which hypothetical expression changes are consistent with hypothetical expression changes of genes regulated by this transcription factor.

be considered. However, we must emphasize that although qPCR is the method of choice of confirming gene expression changes, the current technology contains a vital limitation related to experiments of this nature [22]. Specifically, it is not sensitive enough to accurately detect low, but statistically significant, fold changes of around 2-fold [23, 24] that are typical for miRNAs [25, 26]. It is more and more accepted that with the advent of new quantitative digital PCR technologies it is much more likely to detect small miRNA differences [27, 28]. Interestingly we found that for 3 validated miRNAs it followed the same trend, up or down, similar to the array results.

Our study showed little concordance with the endometriosis-associated miRNAs identified by two previous published studies, which also had minimal convergence with one another.

In the first miRNA eutopic versus normal endometriosis study, Pan et al. profiled the expression of 287 miRNAs in paired early-mid secretory eutopic and ectopic endometrium and isolated endometrial cells from women with stage III

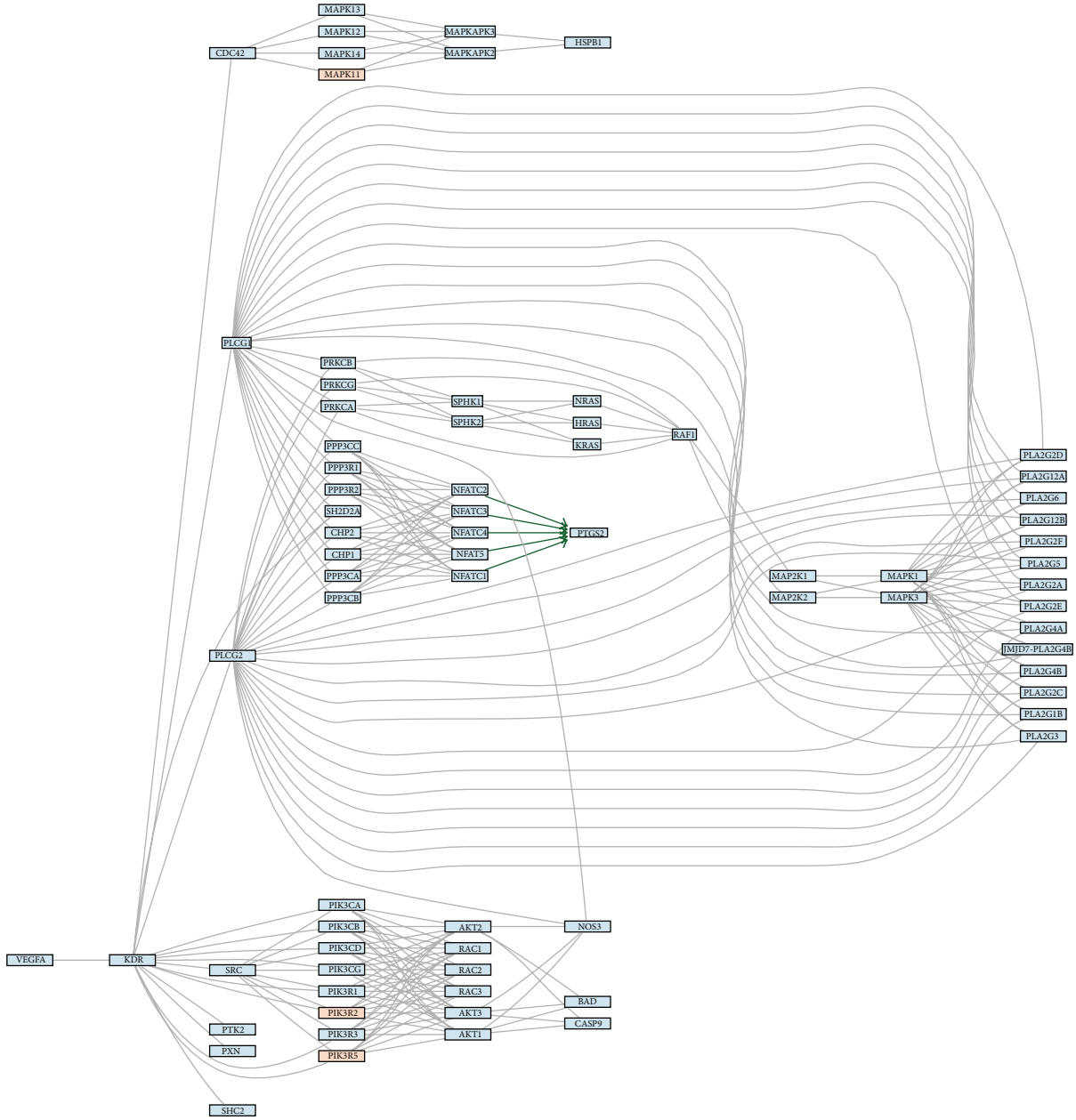

Figure 4: The figure shows signaling pathways (3: mTOR and 4: VEGF) with marked hypothetical influence of miRNA. The colors present expression of miRNAs assigned to a particular gene. The blue color stands for higher expression in control samples, and the red color stands for higher expression endometriosis. The color scale represents the magnitude of changes. The green arrows represent connections start from transcription factor and which hypothetical expression changes are consistent with hypothetical expression changes of genes regulated by this transcription factor.

endometriosis and compared to control by the use of mirVana miRNA Bioarray. They also aimed to compare the expression of selected miRNA between isolated endometrial stromal cell (ESC) and glandular epithelial cell (GEC).

It was found that 65 of these miRNAs were identified to be expressed above the threshold levels set during the analysis in the endometrium of women without endometriosis. By the use of ANOVA it was also identified that there are 48 miRNAs which are differentially expressed between different combinations of eutopic and ectopic endometria. Specifically it was observed that there exists a progressive decline in miRNAs numbers from endometrium of women without endometriosis to eutopic endometrium, ectopic endometrium, and ectopic endometrium without paired eutopic tissue (miR23a and miR23b). It is somewhat confusing since it shows that there might be potential differences between ectopic endometrium of patients in whom eutopic endometrium was collected as compared with patients where this procedure was not performed. It was also shown that 32 miRNAs are differentially expressed in ESC and GEC, a significantly

Table 4: Relative quantity (RQ) values of selected miRNA as validated by real-time PCR in patients with endometriosis ($n = 42$) and control ($n = 25$).

Number	miRNA	Endometriosis median (minimum–maximum values)	Control median (minimum–maximum values)	p value	Fold change difference between endometriosis and control by miRNA microarrays
1	miR-4454	0.34 (0.07–2.07)	0.41 (0.14–3.5)	0.34	1.51
2	miR-4714-5p	0.63 (0.05–9.9)	0.9 (0.01–4)	0.9	1.86
3	miR-5193	0.29 (0.04–11.3)	0.51 (0.04–22.9)	0.13	1.93
4	miR-4284	1.88 (0.1–10.3)	1.6 (0.2–15.7)	0.83	2.28
5	miR-5187-3p	0.28 (0.05–3.2)	0.20 (0.07–0.69)	0.051	1.41
6	miR-3680-5p	0.8 (0.07–31.6)	1.2 (0.06–11.2)	0.96	2.21
7	miR-3667-5p	0.68 (0.06–18.19)	1.8 (0.07–8.5)	0.63	2.3
8	miR-3152-5p	0.17 (0.03–1.4)	0.27 (0.002–2.8)	0.048	1.44
9	miR-23a-3p	1.22 (0.02–11.3)	1.17 (0.03–12.3)	0.48	1.55
10	miR-23b-3p	0.4 (0.06–1.7)	0.5 (0.1–2.2)	0.44	2.08
11	miR-30d-5p	0.22 (0.05–21)	0.42 (0.1–40)	0.05	1.37

lower numbers when compared with endometrium of women without endometriosis. The obvious limitation of the above study was very limited sample size, a total of 16 samples including those with and without endometriosis as well as those without paired eutopic tissue, and restriction to only second phase of the cycle [13].

On the other hand, it was also more recently shown in advanced (III-IV) disease that miR23a and miR23b were significantly decreased in proliferative ectopic and eutopic endometrium compared with normal endometrium [29]. It was one of the reasons to choose above two molecules in our validation phase of the study; nonetheless were not able to observe any significant differences between eutopic endometria of patients with and without endometriosis.

It was also shown that in the early secretory endometrium (ESE), by the use of LNA microarray (which consisted of Tm-normalized capture probes for 1488 distinct miRNAs), there are only 6 miRNAs which were differentially expressed with a fold change of >1.5 in the ESE from women with versus without endometriosis [12]. Downregulated miRNAs included miR-9, miR-9*, miR-34b*, miR-34c-5p, miR-34c-3p, and the unannotated miRPlus_42 780. miR-9 represented the most significantly dysregulated miRNA. The majority of miRNAs were unchanged or not expressed in endometrium, in agreement with the previous data demonstrating spatiotemporal-specific expression of a high percentage of miRNAs. The validation by qRT-PCR showed that the trends for down-regulation of miRNA expression were consistent in all four qRT-PCR measurements (that were chosen for validation) and significant for three of the four miRNAs. Interestingly miR-9* (5′-end form), as opposed to miR-9 (3′-end form), did not demonstrate statistically significant difference in expression between ESE from women with versus without endometriosis.

The above study is strengthened by the stringency of the surgical confirmation of presence or absence of disease and by the inclusion of only biopsy confirmed, moderate-severe (rAFS III-IV) stage endometriosis among the affected cohort. On the other hand, our study is strengthened by the use of microarray technology employing locked-nucleic acid (LNA) probes. Mature miRNAs are approximately 22 nucleotides in length, and this short length presents problems of specificity in their detection and localization. Microarray analysis using LNA probes allows very sensitive detection of these short-coding sequences [30].

Only very recently there have been published two studies which yielded quite different results. Shi et al. studied endometriotic, eutopic, and normal endometrial tissues in the proliferative phase of the cycle [31]. They used LNA microarrays which contained >1,700 capture probes covering miRNAs listed in miRBase v. 14.0. The selection of miRNAs was made by fold change >2 and it was found that, compared with the normal endometrium, 36 miRNAs were downregulated with no upregulated miRNAs in the eutopic endometrium of patients with endometriosis. Since, among these differentially expressed miRNAs, miR-183, miR-215, and miR-363 were found downregulated in both the ectopic and eutopic tissues, the authors selected miR-183 for validation of further functional studies. In our study we could not confirm differential expression of miR-183.

Second recent study concentrated on miRNA expression profile in relation to selected angiogenesis factors [32]. In their study GeneChip miRNA 2.0 Affymetrix array platform (that contains 1105 probes for mature human miRNAs) was used for screening and validation was performed by using LNA RT-PCR. The major limitation of this study is very low number of studied samples; that is, seven eutopic endometrial tissues and three ovarian endometrioma tissues were compared to five endometrial tissues from healthy controls. When the three sample categories were compared, they found 156 mature miRNAs that were differentially expressed at least 1.3-fold in ovarian endometrioma or in eutopic endometrium or in both tissues compared with healthy tissue. The results are quite surprising since it looks like there are 156 miRNAs

which are common for ovarian endometrioma and eutopic endometrium from the same patients and this is significantly different from eutopic control endometrium. It is generally accepted that histological structure and biology of eutopic and ectopic endometria are generally different and it is highly unlikely that there are exactly 156 miRNAs which would have the same coexpression pattern. On the other hand, it is stated in the same results section that supervised hierarchical clustering of differentially expressed miRNAs showed similar patterns in control and eutopic endometrium, with ovarian endometrioma clustering separately from control and eutopic endometrium. And in this case (as opposed to 156 profiles) it would be logical since eutopic and ectopic endometria should substantially differ in gene expression profile.

In our own previous study we used different technique, based on TaqMan miRNA microfluidic cards and validated by TaqMan real-time PCR, and we found in similar samples that out of 667 miRNAs there are eventually two, that is, hsa-miR-483-5p and hsa-miR-629*, which are significantly downregulated in patients with endometriosis [11]. In the present study we used different methodology and could not observe significant difference as to any of the above cited molecules between studied groups. The differences are not actually surprising in view of the most recent and comprehensive study by Mestdagh et al. where they evaluated quantitative miRNA expression platforms in the microRNA quality control and systematically compared 12 commercially platforms, including all microarrays (also TaqMan and LNA Exiqon). They observed substantial interplatform differences when evaluating differential miRNA expression with an average validation rate of only 54.6% for differentially expressed miRNAs. One of the most unexpected findings, of this enormous comparative study, was low concordance of differential expression [33].

Our *in silico* analysis, based on KEGG database (resource that integrates genomic, chemical, and systemic functional information) showed that there are potentially several pathways dysregulated by differentially regulated miRNAs and some of them like mTOR (mammalian target of rapamycin) or VEGF (vascular endothelial growth factor) have been previously studied in endometriosis [3, 6, 34–36].

In one of our studies we found that out of 15 different mTOR related genes including *NF1, RHEB, mTOR, PTEN, TSC1, TSC2, KRAS, S6K1, TP53, EIF4E, LKB1, PIK3CA, BECN1, 4EBP1,* and *AKT1* there are at least 2, that is, *AKT1* and *4EBP1,* which were found to be upregulated in eutopic endometrium of patients as compared with control [3]. More recently we also studied 84 angiogenesis-related genes, with different VEGF types, and found at least 5 to be differentially regulated in the eutopic endometrium [6].

On the other hand, the most interesting finding of our study comes from Signaling Pathway Impact Analysis which showed that out of nine potentially perturbed pathways in two analyzed groups it is natural killer cell mediated cytotoxicity which is inhibited in eutopic endometrium of patients with endometriosis. The inhibition of natural killer cells activity has been widely accepted [37, 38] in endometriosis; however, very few studies concentrated on the potential disturbances existing in the eutopic endometria of patients with endometriosis. As mentioned earlier our results can be partly complicated by additional limitations which have been previously discussed [3–6].

Briefly the problem may be the structure of control group, which is limited to infertile patients and simple ovarian cysts. It is therefore difficult to evaluate whether control group is a healthy one alone since infertility and cysts themselves might to some degree influence expression of genes in endometrium. We also think that the result could also represent the heterogenous nature of whole endometrium tissue which is composed of different cellular compartments which create the potential for a large amount of biological variation. On the other hand, we intentionally decided not to perform laser capture microdissection (LCM) since there are several reasons, described in the discussion section of the article by Borghese et al. [39], why this relatively modern histopathological technique is not necessarily feasible in the studies on endometriosis.

As we already discussed it previously in our publications [3–6] it is important to mention that in Poland it is impossible to collect the most suitable healthy control samples in endometriosis research, like those taken during sterilization, since it is an illegal form of contraception. On the other hand, it is also very difficult to evaluate whether even the most proper control group is a healthy one alone. It was shown that 6% of cases, where macroscopically normal pelvic anatomy was found, presented microscopic endometriotic lesions [40].

In summary, we found that there are at least 136 miRNAs with differential expression fold change of at least 1.3 that could be involved in the pathogenesis of endometriosis. It seems however that current quantitative PCR methods are not always sensitive enough to detect differences in the expression of miRNA of less than 2.5. It is hoped that with advent of digital PCR it will be possible to more effectively detect small miRNA expression differences which may have significant impact on the discovery of potential biomarkers. We also found that potential several pathways, including mTOR, VEGF, natural killer cell cytotoxicity, and at least 6 potential cellular processes, involved mainly in protein synthesis, may be regulated by abnormal miRNAs expression. The confirmation of their potential role requires further functional studies.

5. Conclusions

We identified several miRNAs and potentially new pathways that may be abnormally regulated in eutopic endometrium of patients with endometriosis which may contribute to the pathogenesis of this debilitating disease.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

P. Laudanski had an idea that designated the study, participated in the laboratory work, analysed the data, and wrote the

paper. R. Charkiewicz performed molecular biology experiments, especially RNA and protein isolation as well as PCR array experiments. A. Tolwinska helped in the interpretation and analysis of data as well as performing PCR array experiments and writing the paper. J. Szamatowicz helped in the collection of samples and molecular biology interpretation and analysis of data. A. Charkiewicz performed molecular biology experiments, especially RNA and protein isolation as well as PCR array experiments. J. Niklinski coordinated molecular biology experiments and analysed the data.

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Research Article

Lysophosphatidic Acid Inhibits Apoptosis Induced by Cisplatin in Cervical Cancer Cells

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Cervical cancer is the second most common cause of cancer death in women worldwide. Lysophosphatidic acid (LPA) level has been found significantly increased in the serum of patients with ovarian, cervical, and colon cancers. LPA level in cervical cancer patients is significantly higher than in healthy controls. LPA receptors were found highly expressed in cervical cancer cells, suggesting LPA may play a role in the development of cervical cancer. The aim of this study is to investigate the effect of LPA on the apoptosis induced by cisplatin (DDP) in cervical cancer cell line and the underlying changes in signaling pathways. Our study found that cisplatin induced apoptosis of Hela cell through inhibiting expression of Bcl-2, upregulating the expression of Bax, Fas-L, and the enzyme activity of caspase-3 ($p < 0.05$); LPA significantly provided protection against the apoptosis induced by cisplatin by inhibiting the above alterations in apoptotic factor caused by cisplatin ($p < 0.05$). Moreover, PI3K/AKT pathway was found to be important for the LPA antiapoptosis effect, and administration of PI3K/AKT partially reversed the LPA-mediated protection against cisplatin-induced apoptosis ($p < 0.05$). These findings have shed new lights on the LPA bioactivity in cervical cancer cells and pointed to a possible sensitization scheme through combined administration of PI3K inhibitor and cisplatin for better treatment of cervical cancer patients, especially those with elevated LPA levels.

1. Introduction

Cervical cancer is the second most common type, and the second most cause of deaths, of malignancy, in females worldwide. An estimated 500,000 new cases of cervical cancer are diagnosed, leading to 280,000 deaths, each year worldwide. The highest incidences of cervical cancers occur in developing countries. While surgery and chemoradiotherapy can cure 80%–95% of women with early stage cervical cancer, the recurrence and metastasis events are often associated with poor prognosis. In addition to the efforts for more effective prevention, new diagnosis and treatment modalities are urgently needed for better management of this life-threatening disease.

High levels of lysophosphatidic acid (LPA) were firstly found in the ascites of ovarian cancers patients [1, 2]. LPA is known as an "ovarian cancer activating factor" to exert a growth factor-like effect through binding to 4 specific G protein-coupled receptors (LPA1-4). The biological activities of LPA in ovarian cancer have been studied for many years.

Increased level of lysophosphatidic acid is also found in patients with acute myocardial infarction. LPA has been implicated in the development of the cardiovascular system, assisting in its progression to a fully functional state [3, 4]. LPA is a bioactive glycerophospholipid generated and released by platelets, macrophages, epithelial cells, and tumor cells. LPA modulates a broad range of cellular responses, including alterations of cell proliferation, protection against apoptosis, modulation of chemotaxis, and transcellular migration [5, 6], thereby affecting the survival of ovarian cancer cells, macrophages, fibroblasts, and neonatal cardiac myocytes. The significant role of LPA in triggering these cellular responses has implicated LPA in tumor progression.

It has also been reported that LPA is increased in the plasma of cervical cancer patients [2]. Xu et al. found that stage I and stage IV cervical cancer patients had significantly higher plasma LPA levels than normal controls. Elevated LPA levels were detected in all the 6 cervical cancer patients examined [2]. In addition, there was an increased ratio of total LPA/lysophosphatidylinositol (LPI) [7]. Similarly, Shen et al. reported that the ratio of unsaturated LPA/LPI subspecies was significantly higher in patients with cervical cancer than in healthy controls [7]. The significantly increased LPA in the plasma of patients of cervical cancer points to its possible role(s) for the development of this malignancy. Indeed, LPA receptors were also found to be extensively expressed in cervical cell lines including Hela, CaSki, and Siha [8–11]. Previous studies from this group confirmed a high expression level of LPA receptors, especially the LPA receptor 2, in Hela cells [12], providing a basis for using this cell line as a study model to investigate the LPA bioactivity and the underlying pathways.

Cisplatin (DDP) has been used as the first line chemotherapy drug for adjuvant treatment of cervical cancer patients. Cisplatin-induced DNA damage activates multiple signaling pathways leading to cell apoptosis [13–15]. DNA damage caused by cisplatin induces the phosphorylation and stabilization of p53 [16]. p53 promotes cisplatin-induced apoptosis by antagonizing the antiapoptotic effect of Bcl-xL [17]. Phosphatidylinositol 3-kinase/AKT pathway is also involved in apoptosis regulation. Yan et al. found that suppression of PI3K/AKT pathway caused apoptosis in the HepG2 human hepatoma cell line [18]. On the other hand, Wang et al. found that LPA protects bone marrow-derived mesenchymal stem cells (BMSCs) against hypoxia- or serum deprivation-induced apoptosis [19]. LPA rescues H_2O_2-induced apoptosis by activating ERK1/2 and PI3K/AKT pathways in mesenchymal stem cells [19]. However, LPA effect on the apoptosis in cervical cancer cells and the potential mechanism remains unclear.

In this study we investigate how LPA-triggered cell responses may affect the cell apoptosis induced by cisplatin in a cervical cancer cell line. We characterized the effects of LPA on cell apoptotic factors including Bcl-2, Bax, and caspase-3 in Hela cells treated with cisplatin. The influence of LPA on the upstream pathway PI3K/AKT was also determined. The findings provide insights on the general bioactivity as well as the chemotherapeutic concerns of LPA produced by cervical cancer cells.

2. Materials and Methods

2.1. Materials. 1-Oleoyl LPA (18 : 1 LPA) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Inhibitor of phosphoinositide 3-kinase (PI3K), LY290042, and inhibitor of mitogen-activated protein kinase (MAPK), PD98059, were from Cell Signaling (Beverly, MA, USA). Rho kinase inhibitor, Y-27632, was from Biomol (Beverly, MA, USA). Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mL/L fetal bovine serum (FBS), streptomycin (100 mg/L), and penicillin (100 KU/L) at 37°C in 50 mL/L CO_2 incubator. Cells were serum starved for 12 hours before LPA treatment.

2.2. Cell Proliferation Assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetry assay was employed to measure cell proliferation. Hela cells (2×10^3/well) were seeded at 96-well plate. After being starved for 12 hours, cells were fed with DMEM containing LPA supplemented with 1 g/L bovine serum albumin. Following 24, 48, 72, and 96 hours of culture, 20 μL of MTT solution (5 g/L) was added to each well. Four hours later, the medium was removed and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. Absorbance was measured at 490 nm on a Microplate Reader (EXL800). Each assay was performed in quintuplicate.

2.3. Annexin V Staining and Flow Cytometry Assay. Cells were starved in serum-free DMEM for 12 hours and treated with different concentrations of LPA. For kinase inhibitor experiments, LY294002 (50 μmol/L), PD98059 (10 μmol/L), and Y-27632 (10 μmol/L) were applied to cells 30 min before addition of LPA. Cells were resuspended in binding buffer (10 mmol/L HEPES/NaOH, pH7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) and stained with 5 μL of annexin-FITC and 5 mg/L propidium iodide (PI). Following washing, cells were analyzed with flow cytometry (FACS Calibur cytometer, BD Biosciences, USA) and CellQuest (BD Biosciences) for quantification of cell apoptosis. The experiment was performed in triplicate and repeated twice.

2.4. Caspase-3 Activity Assay. Activity of caspase-3 was detected using the CaspACE Assay System (Promega, USA). Briefly, cell lysates from treated and untreated control cells were prepared. Cell lysates were centrifuged at 15,000 ×g for 20 min at 4°C, and the supernatant was collected. The assay was performed in a total volume of 100 μL in 96-well plates. Cell extracts with an equal amount of protein (50–100 μg of total protein) were mixed with specific colorimetric substrate (DEVD-pNA) for caspase-3. The mixture was incubated at 37°C for 4 h according to the manufacturer's protocol. The absorbance was measured at a wave length of 405 nm.

2.5. Western Blotting. Antibodies against AKT (1 : 200 dilution times) and phosphorylated-AKT (1 : 200 dilution times) were purchased from Cell Signaling Transduction (USA). Antibodies against Fas (1 : 200 dilution times), Bcl-2 (1 : 200 dilution times), and Bax (1 : 200 dilution times) were purchased from Santa Cruz. After treatment, cells were rinsed

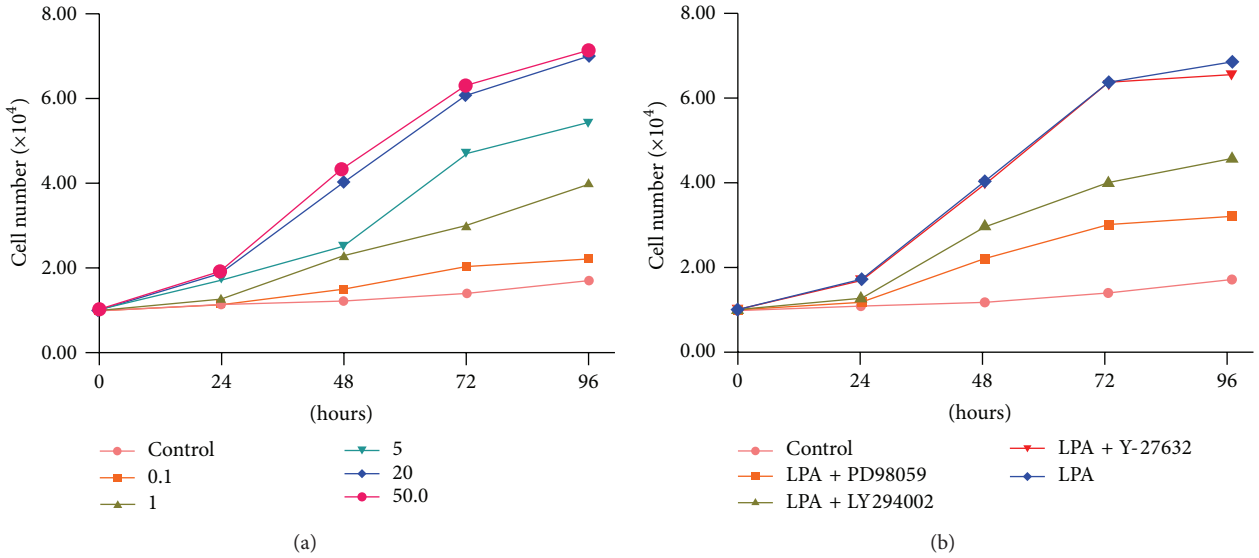


Figure 1: LPA effects on cell proliferation of Hela cells. (a) Hela cells were treated with different doses and time of LPA; MTT essay was performed. LPA significantly stimulated the proliferation of Hela cells in a dose-dependent and time-dependent manner. (b) Effect of inhibitors on LPA stimulation of cell proliferation. Hela cells were treated with LPA at 20 μM plus different inhibitors for different time periods before MTT essay. PD98059 (MAPK inhibitor, at 10 μM), PD98059 (MAPK inhibitor, at 10 μM), and Y-27632 (Rho inhibitor, at 10 μM) were applied, respectively. MAPK inhibitor, PD98059, significantly blocked the stimulation effect of LPA on cell proliferation in Hela cells. PI3K inhibitor, LY294002, partially blocked the LPA effect on cell proliferation.

with ice-cold PBS and then lysed in SDS sample buffer. Samples were resolved in electrophoresis using 10–12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Immunoblot analyses were carried out using the appropriate antibodies. Specific proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ).

2.6. Statistical Analysis. Statistical significance was assessed by one-way ANOVA using SPSS13.0 software. The Band in the Western blotting was quantified with software Quantity One. Data are presented as the means ± standard error. $p \leq 0.05$ was used as standard for statistical significance.

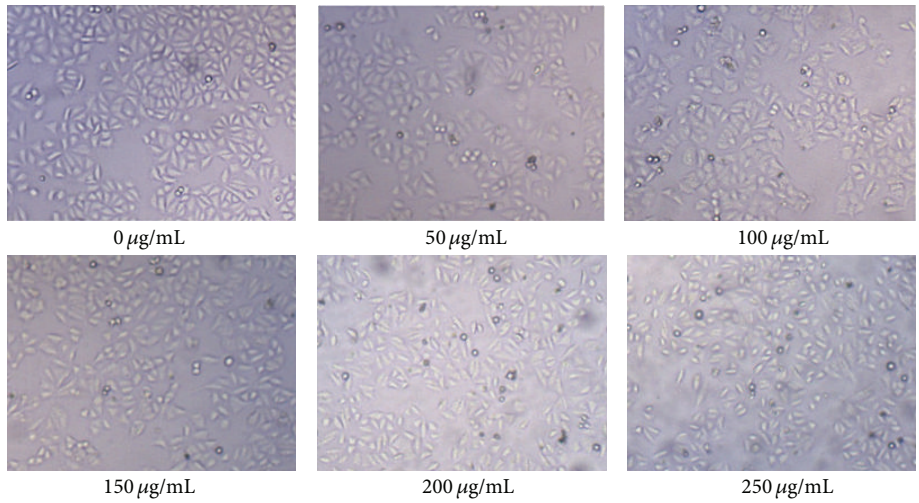
3. Results

3.1. Effects of LPA on Proliferation of Hela Cell. Cells were treated with LPA at different concentrations including 0 μM, 0.1 μM, 1 μM, 5 μM, 20 μM, and 50 μM for different time periods including 24 hours, 48 hours, 72 hours, and 96 hours. It was found that LPA significantly stimulated the proliferation of Hela cells ($p < 0.05$). Between 0.1 μM and 20 μM, the higher the LPA concentration is, the greater the stimulation effect on cell proliferation was observed. There was no significant difference between the stimulation effect of 20 μM LPA and 50 μM LPA on cell proliferation. The stimulation effect of LPA on proliferation of Hela cells was also increased along with the time. The longer the exposure time, the greater the stimulation effect of LPA (Figure 1(a)).

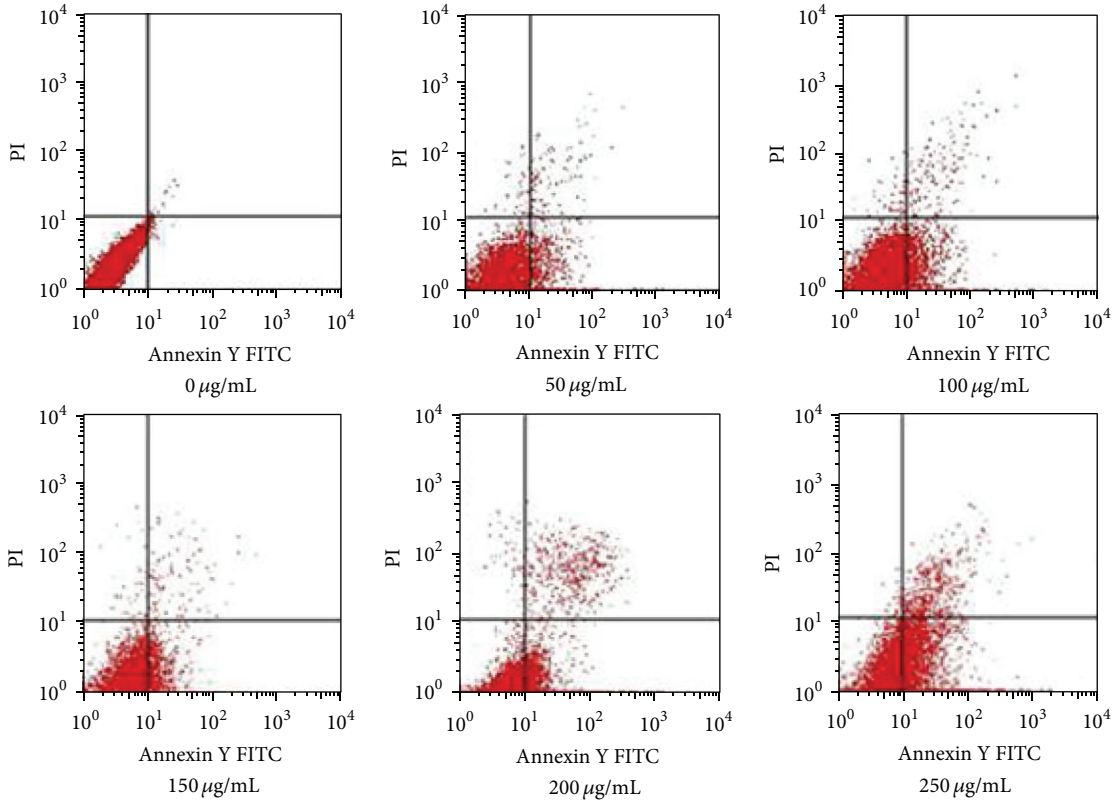
Several inhibitors were employed to determine which signaling transduction pathway was involved in the LPA effect on Hela cell proliferation. The inhibitors include LY294002 (PI3K inhibitor), PD98059 (MAPKinase inhibitor), and Y-27632 (Rho kinase inhibitor). Cells were treated with 20 μM of LPA plus different inhibitors. It was found that PD98059 (MEK1 inhibitor) significantly blocked the stimulation effect of LPA on the proliferation of Hela cell ($p < 0.05$). LY294002 (PI3K inhibitor) also partially blocked the stimulation effect of LPA on the proliferation of Hela cell (Figure 1(b)). The results suggested that Ras/Raf-MAPK signal pathway may be involved in the LPA stimulation of Hela cells proliferation.

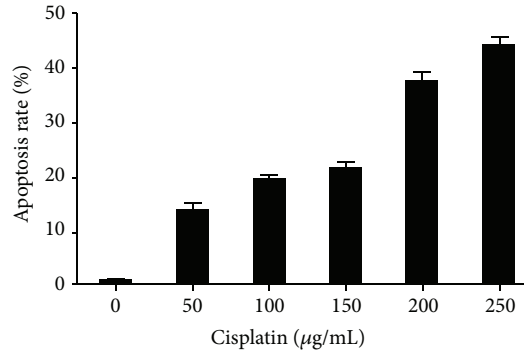
3.2. Effect of LPA on Apoptosis Induced by Cisplatin. Following treatment with increasing concentrations of cisplatin (50–250 μg/mL) for 8 hours, many floating cells were observed (Figure 2(a)), indicating the occurrence of extensive cell deaths. To confirm the cells died of apoptosis, Hela cells treated with cisplatin were subject to flow cytometry analysis and the assay of caspase-3 activity. Similar levels of apoptosis were detected by flow cytometry and measurement of caspase-3 activity. The apoptosis rates elevated with the increase of cisplatin concentrations. The apoptosis rate was 0.96 ± 0.07%, 13.65 ± 1.36%, 19.49 ± 0.75%, 21.44 ± 1.16%, 37.48 ± 1.65%, and 44.15 ± 1.33% when cisplatin was applied at 50, 100, 150, 200, and 250 μg/mL, respectively (Figure 2(b)).

To examine the LPA effects on cisplatin-induced apoptosis, Hela cells were treated by 200 μg/mL cisplatin alone or plus LPA for 4 hours. It was found that apoptosis induced by cisplatin was significantly inhibited by LPA. Moreover, this inhibition displayed a dose-dependency. The inhibition for cell apoptosis became significant ($p < 0.05$) when the concentration of LPA was ≥20 μM (Figure 2(c)).



(a)





(b)

Figure 2: Continued.

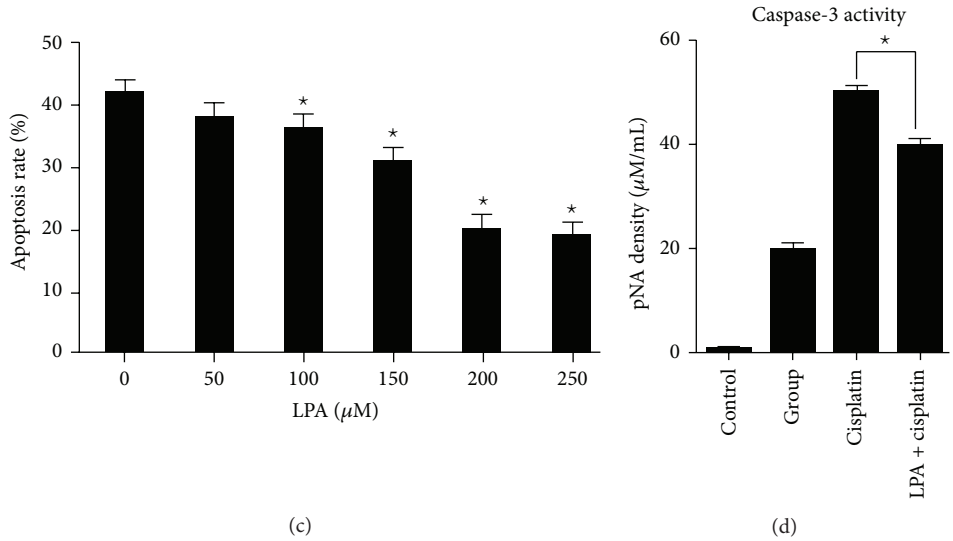


Figure 2: LPA effects on apoptosis induced by cisplatin in Hela cell. (a) Cell pictures (×20). Hela cells were treated with cisplatin at different doses. After 8 hours of treatment with cisplatin, many floating cells were observed. The bigger the dose of cisplatin, the more the floating cells. (b) Flow cytometry detection of apoptosis with Annexin V/PI staining. Hela cells were treated with cisplatin at different doses for 4 hours. Apoptotic cells were detected and quantified with flow cytometry. Increased apoptosis following cisplatin treatment was observed. (c) Quantification of LPA effects on apoptosis induced by cisplatin. Hela cells were treated with cisplatin (at 200 μg/mL) and LPA (at different concentrations) for 4 hours. The apoptotic cells were detected by flow cytometry and the apoptosis rate of Hela cells was quantified and shown. Cell apoptosis induced by cisplatin was significantly inhibited by LPA. (d) Caspase-3 activity. Hela cells were treated with 200 μg/mL of cisplatin plus LPA at 20 μM for 4 hours. The caspase-3 activity was measured and shown. Cisplatin dramatically increased the caspase-3 activity, but the upregulated activity of caspase-3 was partially reversed by LPA treatment.

In caspase-3 activity assay, Hela cells were treated with 200 μg/mL of cisplatin alone or plus 20 μM of LPA for 4 hours. It was found that cisplatin treatment significantly increased the caspase-3 activity, but the upregulation of caspase-3 activity was partially reversed by LPA (Figure 2(d)).

3.3. LPA Effects on the Expression of Apoptosis Proteins. Since cisplatin induced apoptosis in cervical cancer Hela cell, we further characterized alterations of key proteins involved in the apoptotic pathway. Western blotting showed that the expression of Fas-1 was significantly increased by cisplatin treatment in a dose-dependent manner (Figure 3(a)). Expression of the antiapoptotic protein, Bcl-2, was significantly inhibited by cisplatin. Moreover, the inhibition of Bcl-2 expression was dependent on the dose of cisplatin (Figure 3(b)). Expression of another important apoptotic protein, Bax, was also significantly increased by cisplatin treatment (Figure 3(c)). Since LPA appeared to rescue Hela cells from apoptosis induced by cisplatin, we examined the LPA effect on the change of apoptosis-related proteins induced by cisplatin. Western blotting showed that the increase of Fas-1 expression induced by cisplatin was significantly inhibited by LPA (Figure 4(a)). Moreover, LPA treatment restored the inhibition of Bcl-2 expression by cisplatin (Figure 4(b)) in comparison to cells treated with cisplatin alone (Figure 2(b)). In addition, LPA significantly inhibited the increased Bax expression induced by cisplatin. The increase of Bax expression induced by cisplatin was significantly inhibited by LPA (Figure 4(c)). These results indicated that LPA was able to block the apoptotic effects of cisplatin by reversing the changes in apoptotic proteins caused by cisplatin treatment.

3.4. Signaling Transduction Pathways Involved in the LPA Inhibition of Apoptosis Induced by Cisplatin. Since several studies have shown cell apoptosis is associated with the activation of PI3K/AKT, Ras/MAPK, and RHO/GEFs/RHOA pathways, we applied specific inhibitors specifically targeting these pathways to elucidate the mechanism underlying the LPA antiapoptotic activity. Cells were treated by 200 μg/mL cisplatin alone or plus 20 μM LPA and, additionally, different inhibitors including PD98059 (MAPK inhibitor, at 10 μM), LY294002 (PI3K inhibitor, at 10 μM), or Y-27632 (Rho inhibitor, at 10 μM). It was found that the PI3K inhibitor LY294002 significantly reversed the protection effect of LPA on apoptosis induced by cisplatin ($p = 0.003$), while MAPK inhibitor PD98059 and Rho inhibitor Y-27632 had no significant effect (Figures 5(a) and 5(b)). These results suggested that the LPA effect on apoptosis induced by cisplatin were likely mediated by the PI3K/AKT signaling pathway, but not Ras/MAPK and RHO/GEFs/RHOA pathways.

3.5. Effects of LPA on the Phosphorylation of AKT. Since AKT inhibitor significantly reversed the LPA protection of cell apoptosis caused by cisplatin, we subsequently investigated if LPA could activate the PI3K/AKT pathway by increasing the phosphorylation of AKT. Cells were treated by LPA at different concentrations including 0 μM, 0.1 μM, 1 μM, 5 μM,

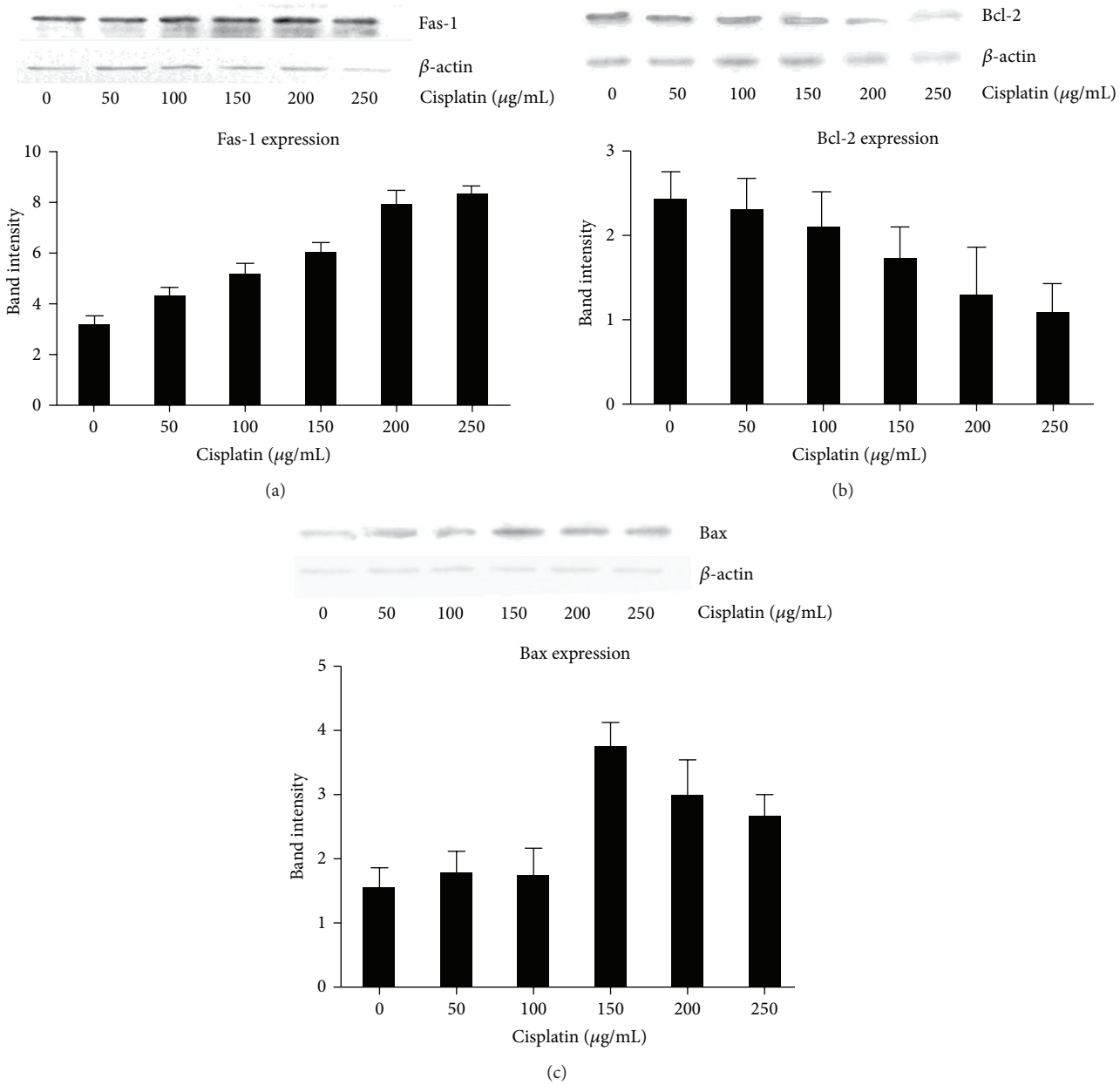


Figure 3: Western blotting analysis of apoptotic proteins in cells treated with cisplatin. Hela cells were treated with cisplatin at different doses including 0.50 μg/mL, 100 μg/mL, 150 μg/mL, 200 μg/mL, and 250 μg/mL, for 4 hours. β-actin was used as a protein loading control. The resultant bands were subject to densitometry analysis for quantification. (a) Western blotting of Fas-L. The expression of Fas-L was significantly induced by cisplatin in a dose-dependent manner. (b) Western blotting of Bcl-2. The expression of Bcl-2 was significantly inhibited by cisplatin in a dose-dependent manner. (c) Western blotting of Bax. The expression of Bax was significantly induced by cisplatin in a dose-dependent manner.

20 μM, and 50 μM of LPA for 1 hour (Figure 6(a)). The results showed that LPA treatment led to an increased phosphorylation of AKT in a dose-dependent manner. In addition, LPA-mediated activation of AKT was also characterized by the time-dependent manner. When cells were treated with 20 μM of LPA for different time, AKT phosphorylation gradually increased, reaching the peak at the 2-hour time point. There was a significant difference between control and the LPA-treated groups at 5 min, 10 min, 30 min, 1 hour, and 2 hours (Figure 6(b)). These results indicated that LPA protection effect on the apoptosis induced by cisplatin may be related to the phosphorylation of AKT.

3.6. Effects of LPA on the Phosphorylation of ERK. Since MAPK inhibitor, PD98059, significantly inhibited the proliferation stimulated by LPA, which suggested ERK/MEK/MAPK pathway was involved in the LPA functions in the proliferation in Hela cells, we investigated whether LPA can

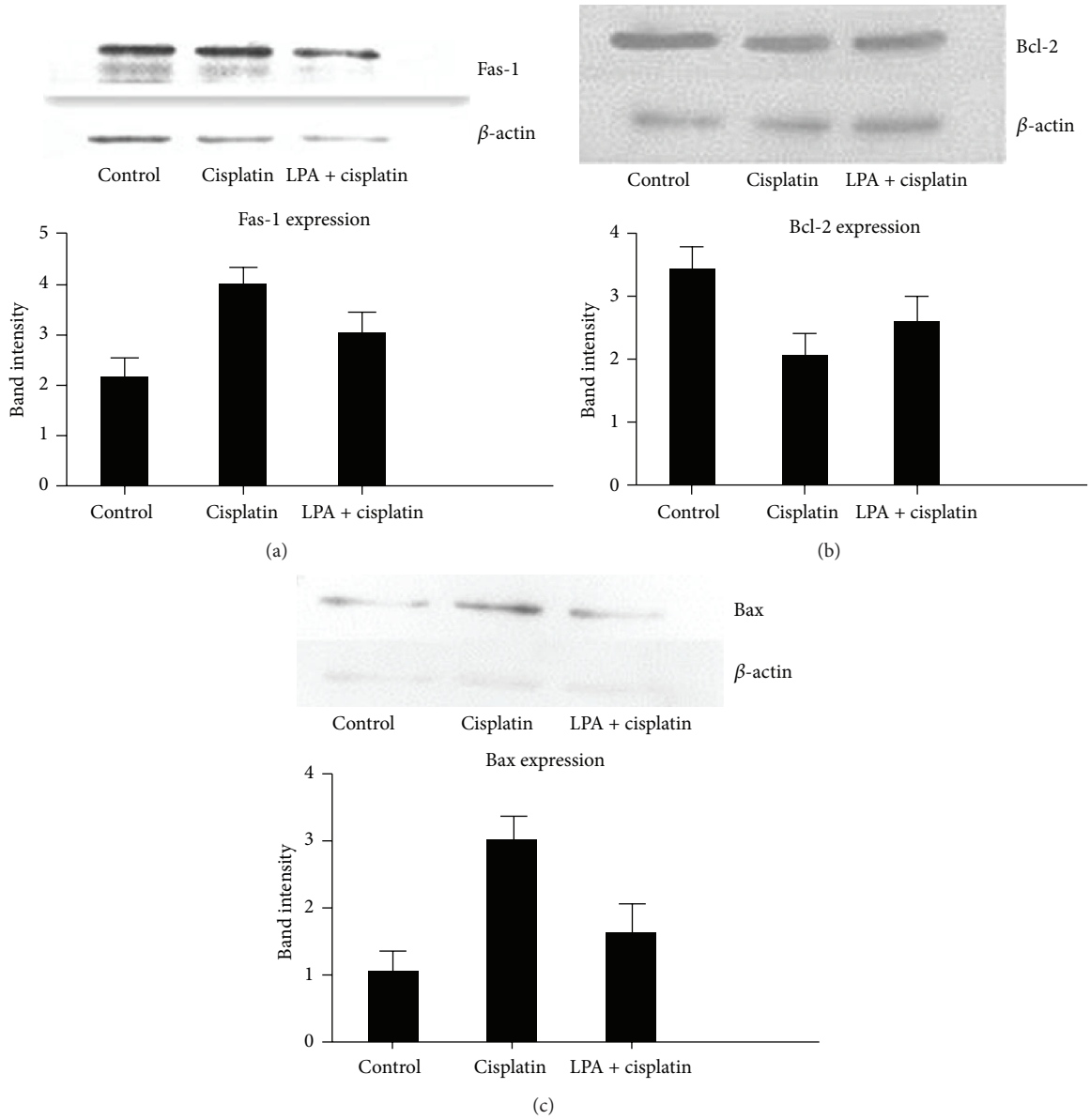


Figure 4: LPA effects on the expression of apoptotic proteins induced by cisplatin. Hela cells were treated with cisplatin (at 200 μg/mL) alone or plus LPA (at 20 μM) for 4 hours. Western blotting was used to determine the expression of apoptosis related proteins. β-actin was used as a protein loading control. (a) Western blotting of Fas-L. The increased Fas-L expression induced by cisplatin was significantly reversed by LPA treatment. (b) Western blotting of Bcl-2. LPA restored the Bcl-2 expression which was inhibited by cisplatin. (c) Western blotting of Bax. The upregulation of Bax expression induced by cisplatin was significantly inhibited by LPA.

activate the phosphorylation of ERK. The results showed that LPA significantly activated the phosphorylation of ERK in a dose-dependent manner. Cells were treated with LPA at different concentrations including 0 μM, 0.1 μM, 1 μM, 5 μM, 20 μM, and 50 μM for 1 hour. The effect on ERK phosphorylation of LPA was increased along with the increase of LPA concentration (Figure 7(a)).

LPA also activated ERK in a time-dependent manner. Cells were treated by 20 μM LPA at different time points including 0 min, 5 min, 10 min, 30 min, 1 hour, 2 hours, 3 hours, and 4 hours. ERK phosphorylation reached the peak at 3 hours following LPA treatment (Figure 7(b)).

4. Discussion

In the intrinsic or mitochondrial pathway of apoptosis, Bcl-2 is able to prevent cell apoptosis, while a related protein, Bax, can migrate to the surface of the mitochondrion where it inhibits the protective effect of Bcl-2 by interrupting the membrane structure, causing the release of cytochrome C and caspase activation. In the extrinsic or death receptor pathway of apoptosis, Fas and the TNF receptor are integral membrane proteins receiving apoptotic signals. Upon binding by ligand, they transmit signals to the cytoplasm, which leads to activation of caspase and phagocytosis.

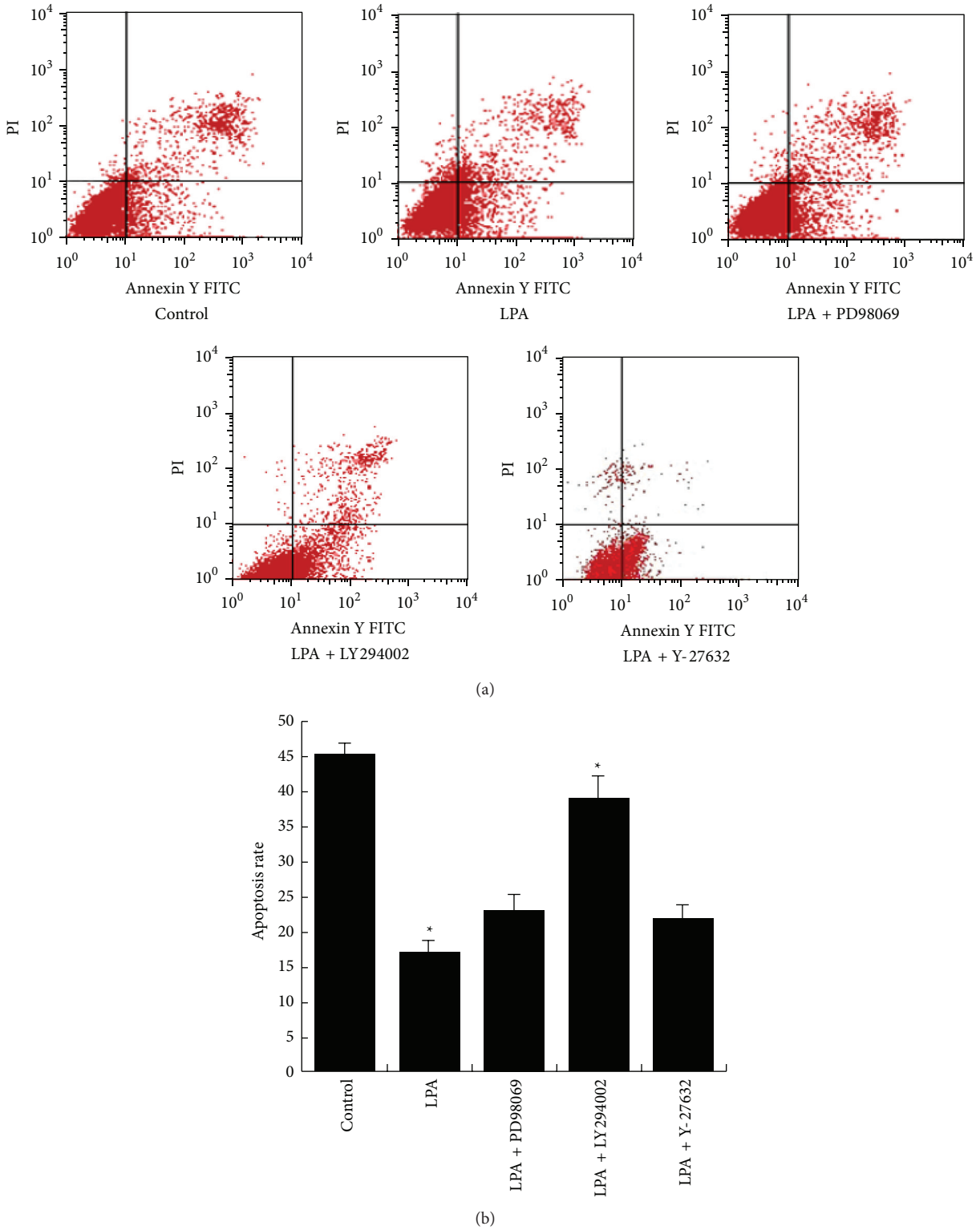


Figure 5: Flow cytometry detection of cell apoptosis by Annexin V/PI staining. (a) Hela cells were treated with cisplatin (200 μg/mL) and LPA (20 μM), alone or in combination with different inhibitors for 4 hours. Cisplatin and LPA were used in each inhibitors-treated group. Inhibitors including PD98059 (MAPK inhibitor, at 10 μM), PD98059 (MAPK inhibitor, at 10 μM), and Y-27632 (Rho inhibitor, at 10 μM) were applied, respectively. (b) The apoptosis rate of Hela cells was quantified. Cisplatin-treated group served as control. PI3K inhibitor LY294002 significantly reversed the LPA effect on apoptosis induced by cisplatin, while MAPK inhibitor PD98059 and Rho inhibitor Y-27632 had no significant effect.

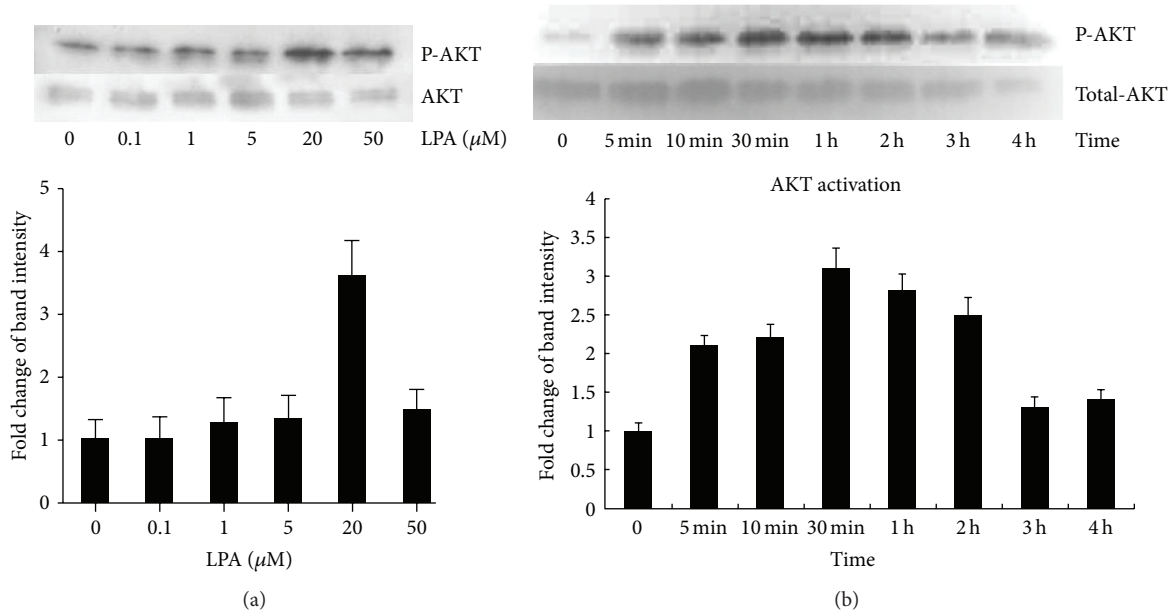


Figure 6: Western blotting analysis of phosphorylated AKT in cells treated with LPA. (a) Hela cells were treated with LPA at different concentrations including 0, 0.1 μM, 1 μM, 5 μM, 20 μM, and 50 μM for 1 hour. Western blotting was conducted to detect the expression of phosphorylated AKT. Total AKT was detected and the results were used as protein input control. Densitometry analysis was carried out for quantification purpose. LPA treatment led to increased phosphorylation of AKT in a dose-dependent manner. (b) Time-response effect of LPA on the phosphorylation of AKT. Hela cells were treated with LPA at 20 μM for different times including 5 min, 10 min, 30 min, 1 hour, 2 hours, 3 hours, and 4 hours. Western blotting was used to detect the expression of phosphorylated AKT. Compared to the total AKT, LPA treatment activated the phosphorylation of AKT in a time-dependent manner.

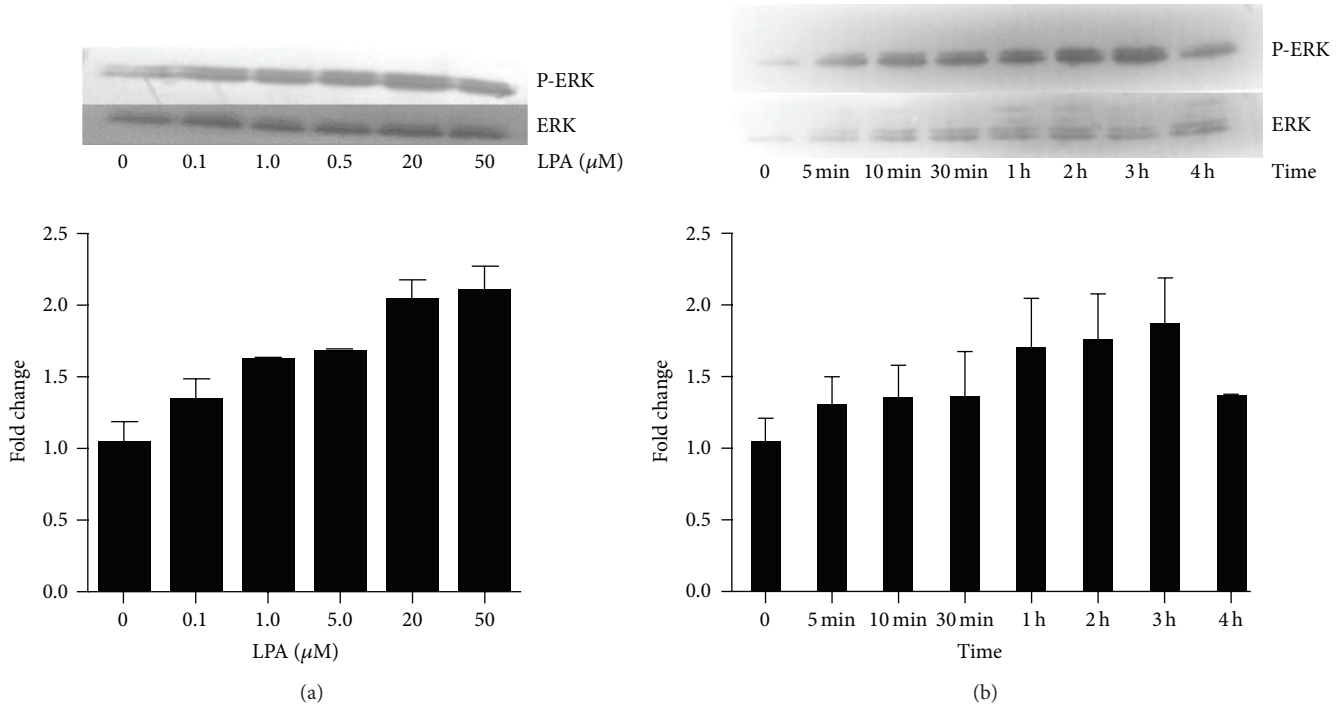


Figure 7: Western blotting analysis of phosphorylated ERK in cells treated with LPA. (a) Hela cells were treated with LPA at different concentrations including 0, 0.1 μM, 1 μM, 5 μM, 20 μM, and 50 μM, for 1 hour. Western blotting was performed to detect the levels of phosphorylated ERK. Total ERK was used as protein input control. The density of bands was quantified and compared. LPA activated the phosphorylation of ERK in a dose-dependent manner. (b) Hela cells were treated with LPA at 20 μM for different times including 5 min, 10 min, 30 min, 1 hour, 2 hours, 3 hours, and 4 hours. A time-dependent increase in the ERK phosphorylation was detected.

Some studies found that cell apoptosis induced by cisplatin was mediated by death receptors signaling pathway [20, 21], while others claimed that it was mediated by mitochondrial pathway [22, 23]. The discrepancy in previous studies may be caused by the divergent cell lines, methodologies, and conditions used by different groups. In this study, we observed that cisplatin significantly induced the apoptosis of Hela cells in a concentration-dependent manner. Cisplatin treatment was accompanied by increased Fas-1 and Bax expression and the increased enzyme activity of caspase-3. Meanwhile cisplatin inhibited Bcl-2 expression. These observations suggested that cisplatin-induced apoptosis of Hela cell may be mediated by both intrinsic and extrinsic pathways. Indeed, extensive cross talks between the two pathways have been identified [24, 25]. Simultaneous activation of the two pathways has been observed in many types of cells following various treatments.

Our previous study found that both ovarian cancer cells and peritoneal mesothelial cells contribute to the elevated LPA levels in ovarian cancer ascites. Under normal culture conditions, ovarian cancer cells and prostate cancer cells, but not breast cancer cell lines, constitutively synthesize and release significant amounts of LPA to the culture medium. A major finding of this study is that LPA was able to rescue the Hela cells apoptosis induced by cisplatin. The protective role of LPA against apoptosis was observed in ovarian cancer cells [26]. We observed that LPA treatment blocked the alterations of Bcl-2, Fas-1, and Bax expression that were induced by cisplatin. LPA also significantly inhibited the upregulation of the caspase-3 enzyme activity induced by cisplatin. Our observations of LPA effect on cervical cancer were consistent with other reports on LPA effects in different kinds of cells. Meng et al. found that LPA may protect epithelial ovarian cancer from immune cell attack and cytoskeleton disrupting reagents induced apoptosis through multiple pathways and LPA inhibited anti-Fas-induced apoptosis enhanced by actin depolymerization [26, 27]. Actin depolymerization accelerated caspase-8 activation, while LPA inhibited the association and activation of caspase-8 at the DISC in epithelial ovarian cancer OVCAR3 cells. Thus, our observations have raised an interesting question on the potential role of LPA for the development of chemoresistance by cervical cancer cells. In addition, could LPA be an effective biomarker for the prediction of chemoresistance in cervical cancer? Further studies are required to answer these questions.

In this study, three specific kinase inhibitors were applied to determine the signaling pathways involved in the protection effect of LPA on apoptosis. It was found that PI3K inhibitor (LY290042) almost completely blocked the LPA protection against apoptosis induced by cisplatin. Subsequent experiments confirmed that LPA treatment of Hela cells led to increased phosphorylation of AKT in a dose- and time-dependent manner. This indicated that LPA protected Hela cells from apoptosis induced by cisplatin. This effect of LPA may be through the activation of PI3K/AKT pathway. This finding appears to be consistent with published data showing that PI3K/AKT pathway plays a key role in the regulation of cell apoptosis [28, 29]. Wan et al. observed that PI3K inhibition augmented staurosporine-induced apoptosis in the endometrial carcinoma cell line [28]. In that experiment, staurosporine reduced the levels of phosphorylation in AKT and Bad; In a study by Kim et al. 2,4-bis(p-hydroxyphenyl)-2-butenal (HPB242) induced apoptosis via the inhibition of PI3K/AKT pathway in human cervical cancer cells. HPB242 treatment decreased phosphatidyl inositol 3-kinase and p-AKT expression levels, demonstrating that this survival pathway may also be inhibited by HPB242 [30]; In another study, Cui et al. found that MiR-125b inhibited tumor growth and promoted apoptosis of cervical cancer cells by targeting phosphoinositide 3-kinase catalytic subunit delta. Overexpression of MiR-125b in Hela cervical cancer cells induced apoptosis and downregulated the expression of PIK3CD and Phospho-AKT [31]; Kang et al. found that thioridazine induces apoptosis by targeting the PI3K/AKT/mTOR pathway in cervical cancer cells [32]; Thanaketpaisarn et al. found that Artesunate enhanced TRAIL-induced apoptosis in human cervical carcinoma cells through inhibition of the PI3K/AKT and NF-κB signaling pathways [33]; Wang et al. found that Stathmin is involved in arsenic trioxide-induced apoptosis in human cervical cancer cell lines via PI3K linked signal pathway. As2O3-induced stathmin downregulation is mediated through the phosphatidylinositol-3-kinase (PI3K) signaling pathway. PI3K inhibitor effectively attenuated the stathmin downregulation and cell apoptosis upon As2O3-treatment [34]; Recurrent and metastatic cervical cancers often acquire chemoresistance to cisplatin. Our finding pointed to a possible sensitization scheme through combined administration of PI3K inhibitor and cisplatin.

It should be pointed out that while our current study focused on the apoptotic pathways and mechanisms, the LPA effects on cell cycle control and cell proliferation should not be excluded. In fact the PI3K/AKT pathway is known to play an active role in the control of cell cycle [35]. We found that MEK1 inhibitor (PD98059) significantly inhibited the effect of LPA on the proliferation of Hela cells. Ras/Raf1/MEK/ERK signal transduction pathway is one of the most important members of the mitogen-activated protein kinase pathways regulating cell proliferation [36]. MEK1, a MAPK, represents a key molecule of the Ras/Raf1/MEK/ERK signal pathway [37]. Several lines of evidence suggest that, in cervical cancer, the Ras/Raf1/MEK/ERK pathway, but not the JNK pathway or the p38 MAPK pathway, is the major regulator of cell proliferation [38–40].

In summary, we demonstrated that cisplatin induced apoptosis of Hela cell via the downregulation of Bcl-2 and upregulation of Bax, Fas-L, and increased enzyme activity of caspase-3. LPA was able to block the alterations in the apoptotic factors caused by cisplatin and, ultimately, reversed the apoptosis induced by cisplatin. Cisplatin-induced cell apoptosis appeared to be partially dependent on PI3K/AKT pathway, and AKT inhibitor was able to reverse the protective effects of LPA against cisplatin-induced apoptosis. LPA stimulated the proliferation of Hela cells through the Ras/Raf1/MEK/ERK pathway. These data enriched our knowledge regarding the LPA activity in cervical cancer cells. In addition, the finding on the positive impact of PI3K/AKT inhibitor on the apoptotic effects of cisplatin has provided useful information for the design of sensitization strategy in

cervical cancer patients, especially those with high levels of LPA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yanxia Sui and Ya Yang contributed equally to this work.

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Review Article

EMT-Inducing Molecular Factors in Gynecological Cancers

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Gynecologic cancers are the unregulated growth of neoplastic cells that arise in the cervix, ovaries, fallopian tubes, uterus, vagina, and vulva. Although gynecologic cancers are characterized by different signs and symptoms, studies have shown that they share common risk factors, such as smoking, obesity, age, exposure to certain chemicals, infection with human immunodeficiency virus (HIV), and infection with human papilloma virus (HPV). Despite recent advancements in the preventative, diagnostic, and therapeutic interventions for gynecologic cancers, many patients still die as a result of metastasis and recurrence. Since mounting evidence indicates that the epithelial-mesenchymal transition (EMT) process plays an essential role in metastatic relapse of cancer, understanding the molecular aberrations responsible for the EMT and its underlying signaling should be given high priority in order to reduce cancer morbidity and mortality.

1. Introduction

Epithelial-mesenchymal transition (EMT) is the conversion of epithelial cells to a mesenchymal phenotype, characterized by a loss of epithelial markers, such as E-cadherin, β-catenin, occludin, claudin, plakophilin, cytokeratin, and desmoplakins [1–3], and a gain of mesenchymal markers, such as N-cadherin, vimentin, and fibronectin [1, 3]. Studies have shown that dysregulation of certain transcription factors, oncogenes, tumor suppressors, miRNAs, and growth factor signaling can trigger the initiation of EMT, which is considered a major contributor to the poor prognosis in gynecological cancer.

Ovarian cancer is the most lethal gynecological cancer and tumor metastasis is responsible for its large disease mortality rate. The American Cancer Society (ACS) estimates 21,980 new cases of and 14,270 deaths from ovarian cancer in the USA in 2014 [4]. Ovarian cancer can start from epithelial cells, germ cells, and stromal cells. Platinum complexes and taxanes are usually the first treatment option given. However, about 20~30% of patients show resistance to the platinum-based chemotherapy [5, 6] and about 70~90% of patients who responded to the treatment experience a recurrence of cancer within a window of months to years [5, 7].

The ACS estimates 12,360 new cases of and 4,020 deaths from cervical cancer in the USA in 2014 [4]. Cervical cancer starts in a woman's cervix, which connects the lower part of the uterus to the vagina. HPV types 16, 18, and 31 cause most cervical cancers and vaccinations against HPV as well as the usage of condoms during sexual contact play a role in preventing HPV transmissions. The ACS recommends the administration of a Pap test every 3 years for women aged 21–29 and every 5 years for women aged 30–65 to detect malignancies and cervical intraepithelial neoplasias; early detection is key for good prognosis. While the incidence of cervical cancer has decreased in the past 40 years, it continues to be one of the most common gynecologic cancers in women [2].

Uterine cancer is commonly called endometrial cancer because it mostly starts in the endometrium, the inner lining of the uterus. The development of endometrial cancer is most prevalent in postmenopausal women. For populations within this category, it is highly recommended to have a pelvic exam every year and to report any vaginal bleeding as soon as possible to prevent the cancer metastasis. The ACS estimates 52,630 new cases of and 8,590 deaths from endometrial cancer in the USA in 2014 [4].

Table 1: Factors involved in EMT of gynecological cancers.

	Cervical cancer	Ovarian cancer	Endometrial cancer	Vulvar cancer	Vaginal cancer
Transcriptional regulators	Snail/Slug/Smuc Zeb1/Zeb2 Twist1/Twist2 E47	Snail Zeb1/2 Twist1 KLF4 ETV5 HMGA2	Snail/Slug Zeb1 Twist1/Twist2 KLF17 ETV5 HMGA2	Snail/Slug Twist2	
Growth factors	TGF-β EGF	TGF-β EGF /HB-EGF VEGF HGF FGF	TGF-β EGF VEGF IGF1		
Oncogenes	HPV16 E6/E7 Sam68 AEG1 FTS	PIK3CA AKT2	BMI-1		
Tumor suppressors	Klotho SFRPs	P53 SFRPs RASAL2	P53		
miRNAs	miR-200 family miR-155 miR-361-5p	miR-200 family miR-181 miR-20a miR-214	miR-200 family miR-155 miR-130		
Other molecular factors	β1-integrin receptors MMPs 7/9 IL6 RhoC Gelsolin TBLR1 TACC3 KCC3 EphB2 Nogo-B TNF-α	β1-integrin receptors MMP 9 IL6 RhoC MUC4/16 ET-1 BMP4 CCR7 TG2 PAK1 MTA1 Sema3E Gb2 FN1	ERα TrkB PR	ERα/β	

According to the ACS, about 4,850 new cases of and 1,030 deaths from vulvar cancer will occur in the USA in 2014 [4]. Cancer of the vulva is a rare disease. It forms in a woman's external genitalia and makes up 3 to 5% of all gynecologic malignancies [8]. Most vulvar cancers occur in older women after the development of precancerous variations called vulvar intraepithelial neoplasias (VIN) that last for several years before developing into cancer. It also can affect younger women infected with HPV types 16, 18, 33, and 35 [9].

Vaginal cancer is unusual and accounts for less than 2% of gynecologic malignancies with an expected 3170 new cases and 880 deaths in the United States in 2014 [4]. About 70% of cases of vaginal cancer are squamous cell carcinomas, which begin in the epithelial lining of the vagina. The main risk factors are HPV subtypes 16 and 18. In order to prevent this type of cancer, vaccination and the routine Pap test are highly recommended.

An increase in cellular survival, migration, invasion, metastasis, recurrence, and drug resistance is observed in gynecological cancers and may be due to EMT [2, 10–14]. Therefore, we strongly believe that investigating the dysregulation of molecular networks responsible for EMT and its consequences may be critical to a better understanding of the etiology of the cancers and development of new therapeutic modalities. In this review, we will discuss the current knowledge regarding factors involved in EMT in each of the gynecological cancers (Table 1).

2. Transcription Factors

Several studies have shown the importance of the Snail family of transcription factors in inducing EMT in cervical cancer, endometrial cancer, ovarian cancer, and vulvar cancer [1, 3, 9, 15]. The Snail family consists of zinc finger-containing transcription factors and includes Snail (SNAI1), Slug (SNAI2), and Smuc (SNAI3) [1]. In cervical cancer cells, Snail inhibits the expression of claudins, occludin, and thrombomodulin [1, 16, 17]. Snail and Smuc have both been

associated with lymph node metastasis [15]. In endometrial cancer cells, Snail is overexpressed in both primary and metastatic tumors, and both Snail and Slug reduce E-cadherin expression [3]. Overexpression of Snail in ovarian cancer cells induced mesenchymal markers, such as tea-shirt zinc finger homeobox (TSHZ1), collagen type Vα, and fibrillin-1 (FBN-1), while suppressing E-cadherin, myosin-5C, keratin-18, keratin-8, annexin-A3, and suppressor of tumorigenicity 14 (ST14) [18]. In vulvar cancer, both Snail and Slug inhibit the expression of E-cadherin and increase the expression of vimentin [9].

Zeb1/2 (collectively known as SIP1) are two-handed zinc factors that have been shown to cause EMT. In cervical cancer cells [1] and ovarian cancer cells [19], both transcription factors reduce the expression of E-cadherin. Zeb1 also induced the upregulation of vimentin in cervical cancer [2]. Its nuclear expression in cervical cancer cells was positively associated with increased invasiveness, pelvic lymph node metastasis, and late FIGO staging [20]. In ovarian cancer cells, overexpression of Zeb1 induced mesenchymal markers TSHZ1 and FBN1, thus promoting EMT [18]. In endometrial cancer, Zeb1 decreased the expression of E-cadherin [3].

Twist1/2 are basic helix-loop-helix transcription factors sharing 66% structural homology and repress E-cadherin expression in cervical cancer [1, 21]. Twist1 is a master regulator of EMT in cervical cancer and its expression is a poor prognostic factor [1, 22]. Its elevated expression has also been associated with chemo- and radiotherapy resistance in cervical cancers [22, 23]. Twist2 expression in cervical squamous cell carcinoma patients is a predictor for metastatic potential [1, 24]. Expression of these transcription factors in cervical cancer cells is responsible for the activation of AKT and β-catenin pathways and for the preservation of stem cell-like characteristics of the cells [1, 25]. Twist overexpression has been demonstrated in invasive endometrial carcinomas and is associated with a lower patient survival rate [3, 26]. It represses the expression of E-cadherin [3]. In ovarian cancer, Twist1 overexpression promotes the expression of N-cadherin and reduces the expression of keratin-8 and E-cadherin [18]. Twist2 plays a role in the induction of EMT in vulvar cancer and has been shown to downregulate E-cadherin [9].

It has been shown that the expression of transcription factor Kruppel-like factor 17 (KLF17) induces the expression of Twist in endometrial cancer cells [27]. This led to the subsequent activation of EMT, increased cell invasion, and drug resistance [27]. Both Twist and KLF17 are upregulated in endometrial cancer cells [27]. KLF4, a transcription factor related to KLF17, acts as a tumor suppressor by inhibiting cell proliferation, migration, and invasion through attenuating transforming growth factor-β (TGF-β) induced EMT in ovarian cancer cells [28].

Ets variant 5 (ETV5) belongs to the polyoma enhancer activator 3 (PEA3) family of transcription factors and has been found to induce EMT in endometrial cancer cells by increasing the transcription of Zeb1 [14]. Endometrial cancer cell lines that had undergone EMT accompanied by modified cell adhesion molecules and cytoskeleton reorganization were found to have upregulated ETV5 [14, 29]. ETV5 has also been shown to increase the transcription of matrix metalloproteinases 2 (MMP2) and HEP27, which allow for cellular invasion by degrading extracellular matrix and prevent apoptosis in tumor cells, respectively [14, 30, 31].

There is new evidence that E47 and high-mobility group AT-hook 2 (HMGA2) could play a role in inducing EMT in gynecologic cancers. E47 is a basic helix-loop-helix transcription factor that represses the expression of E-cadherin in cervical cancer cells [1]. HMGA2 represses the expression of E-cadherin in endometrial carcinoma cells [3, 32] and is inversely correlated with the expression of tumor suppressor lumican (LUM), an inhibitor of EMT, in ovarian cancer [33].

3. Growth Factors

TGF-β has been suggested to be associated with the progression of ovarian [34, 35], endometrial [14], and cervical cancers [1]. Overexpression of TGF-β (TGF-β1, TGF-β2, and TGF-β3) in ovarian cancer activates a TGF-β receptor/Ras-Related C3 botulinum toxin substrate 1 (RAC1)/Smad-dependent signaling pathway [36, 37] and in cervical cancer activates mitogen-activated protein kinases (MAPK), Smad, Wnt, tumor necrosis factor-α (TNF-α), and nuclear factor-κB (NF-κB) pathways [2, 38], promoting EMT.

Epidermal growth factor (EGF) has been shown to be a potent EMT inducer in a variety of solid tumors, including cervical [39], ovarian [10], and endometrial [14] cancer. In ovarian cancer cells, EGF binds to the EGF receptor (EGFR), thus inducing EMT activation through phosphorylation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT3) pathway. Yagi et al. also showed that heparin-binding epidermal growth factor-like growth factor (HB-EGF) is involved in ovarian cancer metastasis through EMT [40]. Lee et al. have reported that the chronic treatment of EGF in cervical cancer cells increased phosphorylation of GSK-3β, a regulator of Snail [41], inducing EMT [39]. The chronic treatment of EGF in cervical cancer cells has been shown to increase phosphorylation of glycogen synthase kinase-3 (GSK-3β), a regulator of Snail [41], thus inducing EMT [39]. The suppression of EGF signaling in endometrial cancer cells with EGF inhibitor AG1478 represses EMT and cellular migration and invasion [24].

High levels of hepatocyte growth factor (HGF) and its tyrosine kinase receptor (cMET) are found in malignant ovarian cysts and ascitic fluid from women with ovarian carcinomas [42]. HGF, through activation of the MAPK and phosphatidylinositide-4,5-bisphosphate 3-kinase (PI3K)/AKT pathways, promotes phosphorylation of p70S6K, which induces EMT in ovarian cancer cells by increasing expression of Snail and MMP9 [43, 44].

Vascular endothelial growth factor (VEGF) is an angiogenesis stimulator and its increased expression is associated with tumor growth in endometrial [14] and ovarian cancers [45]. VEGF allows cancers to grow by enabling the development of new blood vessels for nutrient and oxygen supply and metastatic spread. Ptaszynska et al. showed that, in metastatic ovarian cancer cells, VEGF stimulates the expression of protein autotaxin (ATX) [45], a potent motility factor, which produces the bioactive lysophospholipid (LPA) responsible

for regulating cellular migration, cell-cell interactions, and inhibiting apoptosis [46]. Furthermore both VEGF and TGF-β1 could be responsible for aberrant expression of Ras homolog gene family, member C (RhoC), which is involved in the EMT of ovarian cancer cells [47]. In endometrial cancer, Mirantes et al. showed that VEGF and insulin-like growth factor-1 (IGF1) activate Snail, Slug, Twist, and E47 [3].

Fibroblast growth factor 2 (FGF2), which is a member of the FGF family, induces ovarian cancer cell invasion by the activation of the PI3K/Akt/mammalian target Of rapamycin (mTOR) and MAPK/extracellular signal-regulated kinase (ERK) signaling pathways. This subsequently increases the expression of Slug and ZEB1, which are responsible for E-cadherin downregulation [48].

4. Tumor Suppressors

p53 is a well-known tumor suppressor that acts as an inhibitor of EMT [49]. p53 gain-of-function mutants (R273H, R175H, or C135Y) showed downregulation of miR-130b and subsequently increased the expression of Zeb1 and induced EMT in endometrial cancer [50]. Loss-of-function of p53 was also observed in 96% of high-grade serous ovarian cancers [51]. In fact, loss of p53 through Twist activation [52] or its abrogation is associated with mammalian DNA methyltransferase (DNMT) and promoter methylation that represses E-cadherin [53], promoting EMT.

Klotho, a single-pass transmembrane protein, was originally identified as a suppressor of aging [54] and acts as a modulator for insulin [55] and insulin-like growth factor-1 receptor (IGF-1R) [56] signaling and a coreceptor for FGF23 [57]. Mice lacking Klotho have a short lifespan [54] while high levels of Klotho increase lifespan [58]. Studies have demonstrated its role as a tumor suppressor [56, 59, 60]. Klotho has been shown to be downregulated in cervical cancer due to epigenetic silencing of the promoter region [61] and its downregulation is associated with cervical cancer invasiveness [62]. Overexpression of Klotho in cervical cancer cells upregulates E-cadherin and downregulates N-cadherin, Slug, and Twist [62]. In addition, overexpression of Klotho inhibits cell motility and invasiveness by suppressing expression of MMP-7 and MMP-9 and the Wnt/β-catenin pathway [62].

Secreted frizzled-related proteins (SFRPs) are extracellular signaling molecules that inhibit the Wnt pathway [63] and are methylated in cervical cancer cells [2, 64]. Overexpression of SFRPs reduces the expression of Twist, Slug, and Snail and upregulates E-cadherin, thus reducing the invasive ability of cervical cancer cells [2, 65]. In ovarian cancer, an *in vitro* study conducted by Su et al. shows that silencing SFRP5 through methylation induces EMT through Twist and activates AKT2 [66].

RAS protein activator-like 2 (RASAL2), a GTPase activating protein (GAP) that has been identified in ovarian cancer as a tumor suppressor, plays an important role in EMT and metastasis [67]. Reduced levels of RASAL2 mRNA activate the Ras-ERK pathway *in vivo* and *in vitro* [67]. Mutations in RAS are very common in malignant diseases and increase the pathological grades of the disease by suppressing E-cadherin and elevating vimentin expression [67], suggesting that downregulation of RASAL2 may promote EMT activation [67].

5. Oncogenes

HPV types 16, 18, 33, and 35 have been associated with the development of cervical and vulvar cancers [9]. HPV 16 E6/E7 oncoproteins induce the development of cervical intraepithelial neoplasias [2, 68]. Transfection of normal human foreskin keratinocytes with HPV16 E7 induced the EMT program [1, 69] and cervical cancer cells transfected with HPV16 E6/E7 oncogenes experienced downregulation of E-cadherin and upregulation of vimentin [2, 70]. Lastly, exposure to low-dose estrogen and FGF along with HPV16 E7 transfection induced the production of invasive cervical cancer cells [2, 70].

Src-associated substrate in mitosis of 68 kDa (Sam68) is a member of the signal transducer and activator of RNA (STAR) family of RNA-binding proteins [71] and is involved in various cellular processes, including T cell receptor [72] and insulin receptor signaling [73], transcriptional regulation [74], mRNA processing [75], TNF-induced NF-κB activation, and apoptosis [76]. Li and colleagues have shown that Sam68 is overexpressed in cervical cancer cell lines and tissues and its expression is associated with pelvic lymph node metastasis [77]. Also, its cytoplasmic subcellular localization is associated with poor overall survival of cervical cancer patients [77]. Depletion of Sam68 inhibits migratory and invasive potential of cervical cancer cells as well as the expression of vimentin and fibronectin, possibly through suppressing the Akt/GSK3β/Snail pathways [77].

Astrocyte-elevated gene 1 (AEG1) was identified as an HIV-inducible gene in astrocytes [78] and has been implicated in the development, progression, and pathogenesis of various tumors [79–81]. Overexpression of AEG1 resulted in downregulation of E-cadherin and upregulation of N-cadherin and vimentin, thus increasing invasive capability of cervical cancer cells [2, 82]. Also, it is suggested that AEG1 could potentially play a role in the resistance of cervical cancer cells to paclitaxel and cisplatin chemotherapy [2, 82].

Fused-toe homologue (FTS) was originally identified as a gene deleted in the Fused toes mouse mutation [83]. FTS has been involved in uterine cervical carcinogenesis and correlates positively with staging and grading of cervical cancer [2, 84]. Induction of FTS expression reduces expression of E-cadherin and claudin and upregulates expression of N-cadherin and Slug [2, 85]. Knockdown of FTS inhibits EGF-induced EMT and migratory ability of cervical cancer cells [2, 85].

B lymphoma mouse Moloney leukemia virus insertion region 1 (BMI-1) activates EMT in many human cancers and is overexpressed in endometrial cancer [86]. Endometrial cancer cells expressing endogenous BMI-1 show increased invasive ability in comparison to those not expressing endogenous BMI-1 [86]. Those expressing BMI-1 expressed spindle-like, fibroblast morphology, reduced E-cadherin

expression, and increased vimentin expression [86]. miR-194 targets BMI-1 and silences its effects, reversing the invasive ability of cells, increasing E-cadherin expression, and decreasing vimentin expression [86]. Furthermore, knockdown of BMI-1 inhibited *in vitro* cell proliferation [86].

6. miRNA

miRNAs are small noncoding RNAs that bind target mRNAs and inhibit gene translation [87]. Studies have shown that the miR-200 family, consisting of miR-141, miR-200a, miR-200b, miR-200c, and miR-429, play a crucial role in EMT. miR-200 is a master regulator of cervical cancer and ovarian cancer EMT [2, 51]. Downregulation of miR-200 in mesenchymal cells of uterine carcinosarcomas and ovarian cancer cells increases the expression of Zeb1/2 [3, 51, 88, 89]. A study conducted by Lei et al. has shown that miR-155 functions as a tumor suppressor in cervical cancer cells [90] and other studies have shown its oncogenic role in a variety of human cancer cells and tumors [91–93]. They also reported that overexpression of miR-155 suppresses the migratory/invasive capability of cervical cancer cells and EGF-induced EMT through upregulation of p53 and downregulation of Smad2 [90]. Interestingly, miR-155 was found to be overexpressed in endometrial cancer cells and to induce EMT [14]. It is thought that its mode of action is related to TGF-β. When normal murine mammary gland epithelial cells were treated with TGF-β, they underwent EMT and also upregulated miR-155 [14, 94]. The ectopic expression of miR-155 led to increased cellular invasiveness and inhibited the formation of tight junctions [14, 94]. Future work should be done to pinpoint the differences in pathways that cause miR-155 to be an EMT suppressor in cervical cancer but an EMT activator in endometrial cancer. miR-361-5p is elevated in cervical cancer cells and promotes cell proliferation, lymph node invasion, and metastasis through EMT [2, 95]. miR-214 [96] and miR-20a [97] promote EMT by downregulation of PTEN in ovarian cancer. Also miR-181a acts as an inducer of TGF-β by Smad7 inhibition and promotes EMT in epithelial ovarian cancer [98].

7. Other Molecular Factors

Endothelin-1 (ET-1) is a vasoconstrictor peptide that binds two G-protein-coupled transmembrane receptors (ET_A and ET_B) [99, 100] and has been implicated in EMT in ovarian tumors [100]. In particular, elevated levels of ET-1 activate ET_A receptor, which activate an integrin-linked kinase- (ILK-) mediated signaling pathway. This causes the inhibition of GSK-3β and the E-cadherin promoter and increased levels of β-catenin, N-cadherin, and Snail, which drives the cells to a fibroblastoid and invasive phenotype [100]. Also, Colas et al. showed that, in endometrial cancer, ILK signaling activates Snail, Slug, Twist, and E47, promoting EMT [14].

Bone morphogenetic protein 4 (BMP4) belongs to the TGF-β superfamily proteins and is highly expressed in ovary cell types [101]. BMP4 in ovarian cancer cells induces EMT via the (BMP4)/anaplastic lymphoma kinase (ALK) pathway, increasing the expression of mRNA and protein levels of Snail and Slug and downregulating E-cadherin [19, 101, 102].

Transmembrane protein receptors, such as integrin, are also involved in cervical [103] and ovarian tumor progression [104]. Integrins consist of 18α and 8β subunits and they play a major role in cell migration [105]. In cervical cancer, αv β3 and αv β6 are associated with decreased patient survival [103]. In ovarian cancer, it has been found that α5β1 integrin binds to fibronectin to induce EMT [37].

MMPs are a family of zinc-required matrix-degrading enzymes. Several groups have found that the increased expression of MMPs can predict tumor aggressiveness and poor patient survival [106, 107]. A study has shown that cervical cancer cells treated with MMP7 and MMP9 adopt mesenchymal characteristics with high migratory ability [2, 108]. The inhibition of MMP expression causes the inhibition of Vim1, fibronectin, and Snail expression [2, 108]. This suggests that MMPs play a direct role in inducing EMT processes.

Mucins 4/16 (MUC4/16) are identified as tumor antigens in epithelial ovarian cancer. Overexpression of MUC16 in epithelial ovarian cancer downregulates E-cadherin and upregulates N-cadherin and vimentin, thus promoting tumor metastasis [22]. In ovarian cancer cells, MUC4 overexpression upregulates Snail, focal adhesion kinase (FAK), Twist1/2, and MAPK signaling cascade proteins and downregulates E-cadherin and CK18 expression [109].

Chemokine receptor 7 (CCR7) is constitutively expressed in epithelial ovarian cancer. CCR7 and its ligand CCL19/21 participate in EMT development, thus promoting ovarian cancer metastasis [110].

Interleukin 6 (IL6) is a proinflammatory cytokine that induces EMT in cervical and ovarian cancers [2, 111]. In cervical cancer, IL6 promotes EMT activation via the STAT3 pathway [2, 112]. In ovarian cancer, increased levels of EGF stimulate IL6 secretion, which promote the mobility and resistance to chemotherapy via the JAK/STAT3, SHP-2/Ras, MAPK, and PI3K/Akt signaling pathways [111, 113].

Rho-GTPases play a key role in cervical cancer EMT due to its induction of the downregulation of cell adhesion molecules and cytoskeleton reorganization [1]. RhoC is involved in the reorganization of the actin cytoskeleton and in the regulation of cell shape, motility, and attachment [47, 114]. Stable expression of RhoC enhanced migratory, invasive, and tumor-forming abilities of cervical cancer cells [1, 115]. RhoC has been reported to be a downstream effector of Notch receptor [115]. The knockdown of RhoC and Notch receptor caused decreased expression of fibronectin and actin stress fibers during wound healing [1, 115]. Gou et al. showed that overexpression of RhoC downregulates E-cadherin and upregulates B-cadherin and α-SMA, thus promoting cell migration, invasion, and lamellipodia formation, and suggested that RhoC-mediated EMT could be initiated by TGF-β1 and VEGF [47].

Gelsolin is an actin-binding protein that is upregulated in cervical cancer cells [1, 116]. Cervical cancer patients expressing gelsolin had a decreased 5-year survival rate in comparison to those not expressing gelsolin [1, 116]. Decreased gelsolin expression caused decreased cell migration, MMP2

expression, vimentin, and upregulated E-cadherin expression in cervical cancer cells [1, 116].

Cell surface receptor tyrosine kinase (TrKB) and its ligand, brain-derived neurotrophic factor (BDNF), are upregulated in endometrial carcinoma in comparison to normal epithelial cells [117]. High TrKB levels are associated with EMT phenotype and lymph node metastasis, and its knockdown resulted in the decreased migratory and invasive abilities of the endometrial cancer cells [117].

The expression of transducin β-like-related 1 (TBLR1), a transcriptional cofactor, in cervical cancer cells induces the expression of Vim1 and fibronectin while reducing the expression of β-catenin [2, 118]. It activates the NF-κB and Wnt/β-catenin pathways to upregulate expression of Snail and Twist [2, 118]. Furthermore, TBLR1 expression has been shown to induce the invasiveness of cervical cancer cells [2, 118].

Transforming, acidic coiled-coil protein 3 (TACC3) plays a role in the interaction of actin with microtubules and regulates the centrosome during cell mitosis. We have demonstrated that EGF/EGFR signaling is critical for TACC3-mediated EMT in cervical cancer [119].

K+/Cl− cotransporters (KCC) play an important role in the regulation of cell volume and cytoplasmic chloride, transepithelial transport [120, 121], and cervical tumorigenesis [122, 123]. Hsu et al. have reported that KCC3, one of the KCC isoforms (KCC1–KCC4), is significantly elevated in primary cervical carcinomas and its overexpression causes EMT, accompanied by cell morphological changes, downregulation of E-cadherin and β-catenin, upregulation of vimentin, and enhancement of cell proliferation and invasiveness in cervical cancer cells [124].

Erythropoietin-producing human hepatocellular carcinoma (Eph) receptor B2 is a member of the largest family of receptor tyrosine kinases in the human genome [125] and is phosphorylated when its ligand binds, subsequently triggering downstream signaling cascades [126]. Gao et al. have reported that EphB2 is overexpressed in cervical cancer and its expression is associated with tumor progression [127]. In addition, ectopic expression of EphB2 in cervical cancer cells results in an increase in cell migration/invasion and EMT signature fibroblast-like morphology, loss of cell-to-cell contact, and downregulation of E-cadherin. Its ectopic expression also resulted in upregulation of mesenchymal markers (vimentin, CDH2, and fibronectin) and EMT inducers (Snail1, Snail2, and Twist2), as well as an acquisition of stem cell properties via activation of the R-Ras pathway, whereas its depletion has an opposite effect [127].

Nogo isoforms (Nogo-A, Nogo-B, and Nogo-C) belong to the reticulum superfamily and share a conserved reticulum homology domain (RHD), which contains a 66 aa loop domain, Nogo-66 [128]. Nogo-B was identified as a novel human apoptosis-inducing protein [129] and its overexpression has been shown to induce apoptosis through endoplasmic reticulum stress-specific signaling pathways [130] and to play a role in cell adhesion and migration [131, 132]. Nogo-B expression is elevated in cervical cancers and its expression is correlated with the degree of cervical cancer metastasis [128]. Overexpression of Nogo-B in cervical cancer cells promotes cell migration, invasion, and EMT and inhibits cell adhesion [128]. Nogo-B interacts with Fibulin-5, a member of the Fibulin family [133] which contains a conserved Arg-Gly-Asp (RGD) motif that binds to integrins, mediates endothelial cell adhesion, and suppresses angiogenesis [134]. Downregulation of Fibulin-5 inhibits cell migration/invasion, upregulates E-cadherin, and downregulates mesenchymal markers (N-cadherin and vimentin) as well as EMT inducers (Snail, Twist1, Zeb1, and Zeb2) [128].

Estrogen receptors α (ERα) and β (ERβ) have been recognized in the normal vulvar epithelium [135, 136]. Zannoni et al. showed that changes in both ERα and ERβ expression, likely due to low estrogen, induce EMT in vulvar squamous cell carcinomas, which is associated with a significant reduction in the expression of E-cadherin [136]. In endometrial cancer the reduction of ERα correlated with the activation of Wnt, Sonic hedgehog, and TGF-β signaling and reduced patient survival [137]. In ovarian cancer, 17β-estradiol (E2) promotes EMT via an ERα-dependent pathway [138]. In the presence of E2, Snail and Slug are significantly upregulated while E-cadherin is downregulated [138].

Progesterone receptors (PR) have been indicated as potential EMT suppressors in endometrial cancer [14]. PR expression in endometrial cancer is a good prognostic factor for patients and is associated with successful treatment with medroxyprogesterone acetate (MPA) [14, 139]. This may be due to the fact that a loss of progesterone signaling induces EMT. It was shown that MPA treatment in PR-modulated cells downregulates the expression of mesenchymal cell markers, migration, and several signaling pathways, including EGF, IGF1, IL6, integrin/ILK, platelet-derived growth factor (PDGF), TGF-β, VEGF, and Wnt/β-catenin signaling pathways [14, 140].

Tissue transglutaminase (TG2), a multifunctional protein involved in cellular adhesion, promotes EMT [19] in ovarian carcinoma by activating NF-κB complex, which induces Zeb1 [141]. It also upregulates MMP2 secretion [142] and downregulates E-cadherin [143].

p21-activated kinase (PAK1), a serine/threonine protein kinase, coordinates cell morphology and motility [144] and is targeted by miRNA 222 [145]. PAK1 is highly expressed in primary ovarian cancer and downregulates E-cadherin through Snail [87].

Metastasis-associated gene 1 (MTA1) is considered a critical factor in tumor metastasis [146]. Knockdown of MTA1 decreased migratory, invasive, and adhesive capabilities of cervical cancer cells as well as the expression of E-cadherin and p53 [147]. In ovarian cancer, overexpression of MTA1 promotes oncogenic transformation and downregulates E-cadherin by increasing expression of Snail and Slug [148].

Semaphorin 3E (Sema3E) is a secreted molecule that controls angiogenesis [149] and tumor cell survival and serves as a ligand for Plexin D1 [150]. High levels of Sema3E are found in high-grade ovarian endometrioid carcinoma [151]. Sema3E/Plexin D1 promotes increased migratory and invasive potential and metastatic growth of ovarian endometrioid carcinoma cells [151]. Furthermore, Sema3E/Plexin D1 induces EMT through nuclear localization of Snail and PI3K

and ERK/MAPK signaling pathways, which play an important role in the Sema3E/Plexin D1-mediated EMT process in ovarian endometrioid carcinoma cells [151].

GRB2-associated-binding protein 2 (Gab2), a member of the Gab/DOS family of scaffolding adapter proteins [152], is highly expressed in ovarian cancer [153]. Overexpression of Gab2 reduced expression of E-cadherin and increased Zeb1 expression and cell migratory and invasive abilities through the activation of the PI3K pathway in ovarian cancer cells [153].

8. Conclusion

Metastasis is the major cause of death from all gynecological cancers. Many studies have found that EMT plays a central role in cancer metastasis by deregulating the molecular network. This allows the downregulation of epithelial markers, upregulation of mesenchymal markers, and increased migration, invasion, cell survival, metastasis, and recurrence.

The knowledge of all factors that contribute to the activation of EMT opens a door to new therapeutic strategies that can inhibit metastasis in all gynecological cancers. Several studies have found that EMT could be pharmacologically targeted. Selumetinib, a small molecule MEK inhibitor, is able to suppress EMT in patients with frequent low-grade serous epithelial ovarian cancer [154]. ILK inhibitors, KP392, QLT0267, and T315, can suppress ILK- EMT activity in epithelial ovarian cancer [155]. *In vitro* studies have also shown that the EGF inhibitor, AG1478, causes EGF-induced EMT in cervical, ovarian, and endometrial cancer [155]. Additionally, it has been found that inhibitors of FAK (focal adhesion kinase, induced by MUC4-overexpressing cells) [109], ETaR (zibotentan), and TG2 (KCC-009) suppress EMT in ovarian cancer [155]. Furthermore, vaccines and antibodies against mucins (MUC16) can limit tumor metastasis in ovarian carcinoma [113]. New improvements have been made in targeting EMT via epigenetic or miRNA control in epithelial ovarian cancer. For example, restoration of the miR-200c family negatively regulates the EMT process [154]. HPV vaccine targeting HPV16 E6/E7 antigens in patients with high-grade cervical dysplasias actually caused regression of the dysplasias [156]. Lapatinib, a drug used in endometrial cancers, blocks EGFR tyrosine kinase, thereby preventing EGF signaling [157]. The rapid understanding of molecular dysregulation involved in EMT activation creates the hope for future approaches to more personalized treatments that will have a positive impact on survival rate.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Loredana Campo and Catherine Zhang contributed equally.

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Clinical Study

Analysis of the Influence of Hormone Replacement Therapy on Osteocalcin Gene Expression in Postmenopausal Women

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Background. Osteocalcin (OC) contributes to the process of bone mineralization. Present study was designed to investigate the changes in OC gene expression of postmenopausal women treated with hormone replacement therapy (HRT). Study was also designed to evaluate OC gene expression in cells which are not part of connective tissue. *Material and Methods.* Research was carried out on 30 postmenopausal women not treated and 30 treated with HRT. Examination of OC gene expression was conducted on peripheral blood lymphocytes (PBL) and buccal epithelial lining (BEL). Densitometry was conducted on femur and mandible. *Results.* Tests revealed OC gene expression in BEL and PBL. BMD was higher in groups treated with HRT. Assessment of correlation between the OC gene expression in BEL and BMD of mandible revealed significant positive relation. *Conclusions.* OC gene expression can be stated BEL and PBL. Analysis of correlation between OC gene expression in oral cavity and mandible BMD showed significant correlation between local OC expression and local bone metabolism. The relation between OC gene expression and bone metabolism is complex and further research is needed to clear all of the uncertainties.

1. Introduction

Bone metabolism is a dynamic process which involves bone formation and resorption. Osteoblasts are responsible for bone apposition, while osteoclasts participate in the process of bone resorption. Constant reconstruction of bone tissue is known as remodeling. There are biochemical markers which may be used during the process of monitoring of bone metabolism. Enzymes and proteins released into the circulation during osteoblastic and osteoclastic activity may reflect changes which take place in the skeleton. Changes in concentrations of bone metabolism markers may be used in order to assess the rate of bone turnover [1]. Information about the status of connective tissue is essential for the assessment of the rate of decrease of bone mineral density (BMD), estimation of the risk of bone fracture, and monitoring of connective tissue diseases therapy.

Osteocalcin (OC, bone gamma-carboxyglutamic acid-containing protein (BGLAP)) is a noncollagenous bone matrix polypeptide encoded by the *BGLAP* gene, which is located on chromosome 1 (1q22) [2, 3]. It is produced mainly by osteoblasts. OC is a vitamin K-dependent and vitamin D-dependent protein. Osteocalcin gene expression is controlled by modularly distributed basal and hormone-responsive elements, which are located in DNase I-hypersensitive sites which are present in bone-derived cells [4]. Osteogenesis is mainly controlled by core binding factor alpha 1 (*Cbfa1*), also known as runt-related gene 2 (*Runx2*), which is the transcript factor. *Cbfa1* activates OC gene expression by osteogenic cells [5]. Other important elements which regulate OC gene expression are recognized by the 1α,25-dihydroxyvitamin D_3 receptor (VDR) complex upon ligand activation. This VDR element is located in the distal region of the Osteocalcin promoter and leads to increase in OC gene transcription.

In a carboxylated form, OC binds with calcium and contributes to the process of bone mineralization [6]. Other systemic functions of OC are decrease of visceral fat, increase of energy expenditure, and increase of the number of pancreatic β-cells leading to growth of insulin secretion [7].

Increased levels of circulating Osteocalcin can be stated in metabolic diseases connected with accelerated bone turnover, such as the osteoporotic changes in postmenopausal women, particularly in the case of rapid bone loss. Other pathologies which affect the OC concentration include osteomalacia, Paget's disease, thyrotoxicosis, primary hyperparathyroidism, and renal osteodystrophy [8]. Half-life of BGLAP is short and reaches several minutes and its concentration fluctuates considerably during the day (concentration is maximal in the middle of the night and minimal in the morning).

Osteocalcin is mainly produced by mature osteoblasts, but OC expression was also detected in other types of cells. OC immunoexpression was stated in the subcutaneous adipose tissue, used during the treatment of osteolytic bone defects [9]. What is more, the phenomenon of epithelial-to-mesenchymal transition can cause the expression of OC in epithelial tissue. The epithelial-to-mesenchymal transition is crucial, inter alia, for the process of embryonic craniofacial development or wound healing. Transformation of epithelial cells into mesenchymal can lead to recruitment of new osteoblasts or chondrocytes. The process of epithelial-to-mesenchymal transition can be observed during the healing of bone fractures. During this process mesenchymal osteoblasts and chondrocytes which take part in the healing of bony defect stain positive with antibodies which are specific for endothelial markers [10]. Also immune cells are involved in the process of bone healing. Activated immune cells release signaling factors which modulate the function of osteogenic cells. Factors which directly activate lymphocytes are unknown. Expression of major histocompatibility complex (HLA) class II determinants on osteoblasts seems to play an important role in local immune cell activation. HLA positive bone surface cells activate T lymphocytes which affect bone metabolism via multifactorial signaling pathway [11].

The development of postmenopausal osteoporosis is determined by the size of the peak bone mass, which is defined as the highest bone mass achieved during life. Currently, it is believed that 70% of the peak bone mass variation can be attributed to genetic factors. It is suggested that number of specific genes can determine bone mass, its fluctuation, and severity of the resorption process. In addition to environmental and dietary habits affecting skeleton, the bone metabolism is inherited from parents [12]. Polymorphism of several genes encoding the receptors for Osteocalcin is associated with bone density and the risk of fracture [13].

Menopause is defined as termination of menstrual cycles due to the failure of ovarian function (natural menopause—M). Menopausal symptoms also occur after surgical removal of ovaries (ovariectomy—OV)—surgically induced menopause [14]. Cessation of hormonal function of ovaries results in estrogen deficiency, which leads to the development of systemic metabolic disease of bone tissue—postmenopausal osteoporosis. Etiology and pathogenesis of postmenopausal osteoporosis have not been sufficiently explored and still require further investigation.

Hormone replacement therapy (HRT) for a long time has been recognized as a method of treatment in the event to adverse symptoms resulting from the cessation of ovarian endocrine function. HRT is administered in the form of estrogen alone or in the form of estrogen-progestogen therapy. In studies conducted over the past several years, the beneficial prevention of distant effects of menopause, such as osteoporosis or atrophy of skin, caused by HRT has been documented. Estrogen replacement therapy substantially or completely prevents postmenopausal bone loss. If it is used in the early phase of menopause or immediately after ovariectomy, it maintains bone mass. While in women who have started treatment after few years since the last menstrual period, HRT prevents further bone resorption. Estrogen inhibits the release of proinflammatory cytokines and reduces metabolic activity of osteoclasts. It is worth noting that women taking estrogen have fewer fractures than postmenopausal patient who are not treated with HRT [15, 16]. The research work on mechanisms of HRT osteoprotective effect is still carried out in order to potentiate their beneficial effects and minimize complications of estrogen therapy.

The aim of this study was to investigate the expression of Osteocalcin gene in peripheral blood lymphocytes (PBL) and buccal epithelial lining (BEL) of postmenopausal women. Potential differences in the OC gene expression between postmenopausal women treated and not treated with HRT would allow determining if osteoprotective effect of estrogen is exerted, inter alia, by changes in metabolism of OC.

2. Materials and Methods

The research was conducted in a group of 30 postmenopausal women undergoing HRT for 6 months (age range 49–59 years; mean age 53.0 years) (study group). Women from study group were supplemented with combination HRT (estrogen and progesterone in combination). Patients received Femoston in tablets (2 mg of estradiol hemihydrate and 10 mg of dydrogesterone). Estrogen was taken on a continuous basis. Patients received progesterone for the last 14 days of each course (course lasted for 4 weeks). The control group consisted of 30 postmenopausal women, at least 36 months after the last menstruation (age range 53–59 years; mean age 55.4 years). Patients in the control group have never received HRT. Patients were treated in the out-patients gynecological clinic of Public Hospital Number 4 in Lublin (Poland). Control and research groups were divided into four subgroups: M: group of postmenopausal women, OV: a group of women after surgical removal of ovaries, OV + HRT: a group of women after surgical removal of ovaries using HR and M + HRT: a group of postmenopausal women treated with HRT. Patients after ovariectomy underwent surgery at least 36 months before the study was conducted. Surgeries were performed as a way of treatment of diseases of reproductive system. In case of patients with malignant tumors, no metastasis had been discovered. None of patients have received neither chemo- nor radiotherapy at least for 2 years before study. Testing procedures received approval of local ethics committee in Lublin (registration symbol KE-0254/246/2005). The study was carried out in accordance with the ethical principles concluded in the Declaration of Helsinki. All patients gave their consent for the examination and research protocol.

Questions included in the anamnesis chart were as follows: age, occupation, socioeconomic status, date of the last menstrual period, duration of hormone replacement therapy (HRT), addictions, physical activity, medications, and surgeries. Women enrolled for the study had no medical history of fractures. None of patients from control group had been treated for osteoporotic changes. None of patients enrolled for tests had been constantly treated with steroids [17]. Patients that qualified for the study did not suffer from any severe systemic diseases, had no addictions, and had not been treated with any medications on constant basis [18]. Patients enrolled for further test were between 36 and 48 months after last menstrual period. Body mass index was calculated for each patient. Patients qualified for the study had BMI values between 27 and 29 (values classified as overweight, but not obese). Examination of the oral cavity was performed with use of dental mirror and periodontal probe. Clinical examination focused on the presence of any pathological mucosal lesions, dental status, presence of dentures, and oral hygiene. Patients that qualified for further tests did not have any acute inflammatory conditions of oral mucosa and the depth of periodontal sockets was less than 5 mm. Patients that qualified for the study did not require any tooth extractions and presented with average or good oral hygiene. Women that qualified for the study did not use any removable dentures. Samples were collected at fixed hours (7–9 a.m.). Epithelial lining of buccal mucosa was collected and suspended in 5 mL of 0.9% saline and centrifuged for 15 minutes at speed of 3000 rpm in order to separate the epithelium. Obtained buccal epithelium and newly drawn, uncentrifuged blood collected in an EDTA tube were used for analysis of gene expression: a control GAPDH gene and Osteocalcin gene [19]. Total cellular RNA isolation was performed using TriReagent Sigma (method modified by Chomczynski and Sacchi) according to the procedure provided by the manufacturer [20]. Following complete dissociation of the nucleoprotein complex (5 minutes at room temperature), 0.2 mL of chloroform was added, shaken vigorously, incubated for 15 minutes, also at room temperature, and centrifuged at a 12,000 rpm for 20 minutes at 4°C. Lysate was divided into three phases: the organic phase containing protein, interphase containing DNA, and colorless upper aqueous phase containing RNA. After transferring the aqueous phase to new Eppendorf test tubes, 0.5 mL of isopropanol was added. The samples were left for 10 minutes at room temperature and then centrifuged at 12,000 rpm for 10 minutes at 4°C. After removing of the supernatant, RNA residue was washed with 75% ethanol, centrifuged (7500 rpm) for 5 minutes, dried, and then dissolved in H_2O. Obtained RNA was used to prepare cDNA. RNA isolation from blood (1 mL) was preceded by the lysis of erythrocytes in a buffer composed of NH_4Cl 0.8 M, $KHCO_3$ 0.05 M, and EDTA 0.01 M. After 30 minutes of incubation at 4°C, blood was centrifuged (12,000 rpm) for 20 minutes at 4°C. The supernatant was discarded and RNA was isolated from the sediment according to the method described above. cDNA synthesis was performed using a reagent kit: High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), in accordance with the procedure recommended by the manufacturer. 2 μg of total RNA was used per 20 μL reaction. The 2x RT master mix was prepared using the kit components before preparing the reaction plate. During the procedure Kit with RNase Inhibitor and MultiScribe Reverse Transcriptase was used. 10 μL of 2x RT master mix and 10 μL of RNA sample were pipetted into each well of a 96-well reaction plate or individual tube. The plate was centrifuged to spin down the contents and to eliminate any air bubbles. Reverse transcription was performed in thermal cycler. Step 1 of reaction lasted for 10 min in temperature of 25°C. Second face of process lasted for 120 min in temperature of 37°C. Step 3 of reaction lasted for 5 min in temperature of 85°C. cDNA Reverse Transcription Reactions were stored at temperature of −15 to −25°C. cDNA was used for "real-time" PCR in order to determine the level of GAPDH (control gene) and BGLAP (Osteocalcin gene) expression. TaqMan glyceraldehyde-3-phosphate dehydrogenase Control Reagents [Human] and Bone gamma-carboxyglutamate (gla) protein TaqMan Gene Expression Assay (hCG1999357). The oligonucleotide primer sets which were used for PCR analysis of cDNA were human Osteocalcin 5′-ACACTCCTCGCCCTATTG-3′ (forward) and 5′-GATGTGGTCAGCCAACTC-3′ (reverse) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′-ACCACAGTCCATGCCATCA-3′ (forward) and 5′-TCCACCACCCTGTTGCTGT-3′ (reverse).

Evaluation of bone mineral density (BMD) of the femur was carried out at the Laboratory of Densitometry at The Institute of Agricultural Medicine in Lublin, by means of the DPX-A (General Electric Healthcare Technologies Lunar) Prodigy Advance with use of Femur option and absorptiometry of X-rays beams of two energies. Scan time lasted for 30 seconds. The BMD was measured at the level of upper femoral neck. The area of interest was determined by analysis software. Images of the mandible were scanned at level of mental foramen and the average bone density of the area was calculated. Bone density was specified in g/cm^2. In order to evaluate the results of densitometry examination, the T-score index was calculated. T-score index is a ratio of the patient's bone mineral density and the average bone density of a young person. T-score allows stating objectively the loss of bone mass. T-score values characterizing the quality of the bone are defined as follows: healthy bone: T-score greater than (−1), osteopenia: T-score between (−1) and (−2.5), and osteoporosis: T-score of less than (−2.5).

Results were statistically analyzed using STATISTICA software. The arithmetic mean (M) and standard deviation (SD) were calculated. The significance of difference between groups was based on confidence intervals determined by the analysis of variance (ANOVA). The interdependence between selected traits was expressed by Pearson's correlation, which is a measure of the dependence between the variables. The Pearson's correlation indicates level of linear dependence between two variables. The Pearson's correlation coefficient between two variables can be defined as covariance of two variables divided by the product of their standard deviations [21]. Value of (+1) stands for a perfect positive (increasing) linear relationship. Value of (−1) stands for a perfect negative (decreasing) correlation. Values approaching (0) show lack of correlation between variables. A paired t-test was used to determine whether results differed significantly between

Table 1: Information about patients enrolled for the study.

Research group	Sample number	Age [years] (M ± SD)	Control group	Sample number	Age [years] (M ± SD)	Statistically significant differences between results in control and research group ($P < 0.05$)
M + HRT	15	53,27 ± 3,13	M	15	55,20 ± 2,21	No
OV + HRT	15	52,73 ± 2,81	OV	15	55,60 ± 2,10	No
		Time from last menstrual period [months] (M ± SD)			Time from last menstrual period [months] (M ± SD)	
M + HRT	15	43,3 ± 3,8	M	15	41,5 ± 3,9	No
OV + HRT	15	41,5 ± 5,1	OV	15	42,7 ± 4,1	No
		Duration of HRT [months] (M ± SD)			Duration of HRT [months] (M ± SD)	
M + HRT	15	17,0 ± 3,8	M	15	17,6 ± 3,6	No
OV + HRT	15	17,6 ± 4,1	OV	15	16,8 ± 3,5	No
		Body mass index (M ± SD)			Body mass index (M ± SD)	
M + HRT	15	27,73 ± 0,70	M	15	27,87 ± 0,83	No
OV + HRT	15	27,87 ± 0,83	OV	15	27,80 ± 0,77	No

Table 2: Ratio of Osteocalcin to control GAPDH gene relative expression in epithelial lining of the cheek and peripheral blood lymphocytes.

Research group	Sample number	Ratios of Osteocalcin to control GAPDH gene expression level in the epithelial lining of the cheek (M ± SD)	Control group	Sample number	Ratios of Osteocalcin to control GAPDH gene expression level in the epithelial lining of the cheek (M ± SD)	Statistically significant differences between results in control and research group ($P < 0.05$)
M + HRT	15	0,03 ± 0,02	M	15	0,11 ± 0,17	No
OV + HRT	15	0,05 ± 0,05	OV	15	0,35 ± 0,21	Yes
		Ratios of Osteocalcin to control GAPDH gene expression level in peripheral blood lymphocytes (M ± SD)			Ratios of Osteocalcin to control GAPDH gene expression level in peripheral blood lymphocytes (M ± SD)	
M + HRT	15	1,57 ± 1,22	M	15	2,02 ± 2,08	No
OV + HRT	15	2,3 ± 1,72	OV	15	0,67 ± 0,51	Yes

control and research groups. The risk of inference error in the study was 5%, which means that the results were significant if P value was equal to or less than 0.05.

3. Results

Relevant medical information about patients is presented in Table 1. No significant differences between research and control groups were stated in terms of age, time from last menstrual period, duration of hormone replacement therapy, or body mass index (Table 1).

Results of Osteocalcin gene expression in epithelial lining of the cheek revealed slightly higher gene expression in control group, when compared to group treated with HRT. The difference was statistically significant between groups OV + HRT and OV. The highest average OC gene expression was stated in group OV (BGLAP to GAPDH gene ratio = 0.35) and the lowest in group M + HRT (BGLAP to GAPDH gene ratio = 0.03) (Table 2).

Analysis of data concerning the expression of Osteocalcin gene in PBL showed the lowest average level of gene expression in group (OV) (BGLAP to GAPDH gene ratio = 0.67) and the highest in group (OV + HRT) (BGLAP to GAPDH gene ratio = 2.3) and the difference between groups after ovariectomy was statistically significant. BGLAP gene expression was slightly lower in group M + HRT than in group M, but the difference was not statistically significant (Table 2). Comparison of OC gene expression between epithelium of cheek and peripheral blood lymphocytes revealed higher expression in lymphocytes than in epithelial cells (Table 2).

Table 3: T-score of mandible and femoral bone.

Research group	Sample number	T-score of femoral bone (M ± SD)	Control group	Sample number	T-score of femoral bone (M ± SD)	Statistically significant differences between results in control and research group ($P < 0.05$)
M + HRT	15	−0,82 ± 0,35	M	15	−1,89 ± 0,87	Yes
OV + HRT	15	−0,90 ± 0,38	OV	15	−1,66 ± 0,49	Yes
		T-score of the mandible symphyseal area (M ± SD)			T-score of the mandible symphyseal area (M ± SD)	
M + HRT	15	−0,24 ± 0,38	M	15	−0,80 ± 1,41	Yes
OV + HRT	15	−0,63 ± 0,93	OV	15	−1,07 ± 0,74	No

Table 4: Correlation between Osteocalcin to control GAPDH gene expression level and BMD.

Group	Sample quantity	Pearson's correlation between ratio of Osteocalcin to control GAPDH gene expression level in the epithelial lining of the cheek and BMD of mandible—area of teeth 35–45	Pearson's correlation between ratio of Osteocalcin to control GAPDH gene expression level in peripheral blood lymphocytes and BMD of femoral bone	Pearson's correlation between ratio of Osteocalcin to control GAPDH gene expression level in peripheral blood lymphocytes and epithelial lining of the cheek
M	15	−0,3367	0,5797	0,1125
OV	15	0,9846	−0,2088	0,6182
M + HRT	15	0,6877	0,2844	0,8892
OV + HRT	15	0,7774	−0,3063	−0,0688

Results of BMD and T-score evaluation presented in Table 3 reveal that BMD of femur is significantly higher in groups treated with HRT. Analysis of results for symphyseal area of mandible shows significantly higher level of BMD in group M + HRT when compared to group M. In case of patients after surgical ovariectomy, results show that T-score in group treated with HRT is higher than in control group, but the difference is not high enough to be statistically significant.

Assessment of Pearson's correlation between the concentration of OC gene expression in epithelium of the cheek and BMD of mandible revealed significant positive correlation in all groups except in group M. Based on the determined regression line, it can be considered that increase in OC expression resulted in increase of mandible BMD (Table 4). Analysis of the Pearson's correlation for OC gene expression in PBL and BMD of femur showed significant positive relationship only in group M. In group M + HRT linear relationship between Osteocalcin gene expression in the epithelium of the buccal mucosa and in peripheral blood lymphocytes was statistically significant ($r = 0.8892$). This means that, in population of women from group M + HRT, it can be considered that the increase in Osteocalcin gene expression in the epithelium of the buccal mucosa by 0.1 (units) caused an increase in OC gene expression in peripheral blood lymphocytes of 7.07675 (units). Also in the group (OV), correlation between Osteocalcin gene expression in the epithelium and in peripheral blood lymphocytes was high enough to be statistically significant ($r = 0.6182$). This means that, in group (OV), the increase in expression of the Osteocalcin gene in the epithelium by 0.1 (units) caused an increase in Osteocalcin gene expression in peripheral blood lymphocytes of 0.15288 (units). In other groups no significant linear correlation between gene expression in epithelium and blood lymphocytes was stated (Table 4).

4. Discussion

Many clinical factors can significantly influence the bone metabolism. Nutrition, general diseases, body mass index, age, or time from last menstrual period substantially affect the processes of remodeling which take place in connective tissue [22, 23]. In order to avoid inaccuracies in tests results, which could be the result of the impact of factors other than the level of sex hormones on bone status, patients that qualified for the study did not differ significantly in terms of age, physical activity, oral hygiene, general health, oral health status, or duration of hormone replacement therapy.

Cessation of ovarian function exerts crucial influence on the organism. One of the adverse effects of estrogen deficiency is the domination of resorption process over apposition of bone tissue [24]. HRT is a therapeutic method used to compensate for bone loss caused by estrogen deficiency in postmenopausal women, but the pathways by which the HTR influences the bone tissue are still not completely known [25]. Influence of Osteocalcin on BMD has been discussed by many authors. The aim of the present study was to investigate the influence of sex hormones supplementation on the expression of BGLAP gene.

Osteoporosis is a disease of bone tissue, which leads to an increased risk of fracture. It leads to BMD reduction and bone microarchitecture alteration. Osteoporosis starts with decalcification—a condition called osteopenia. Many

previous studies support the theory of an inverse correlation between serum biomarker concentration and the level of estrogen expressed by increased level of serum Osteocalcin. Study conducted by Laloš-Miljuš et al. revealed a difference between concentrations of OC in patients suffering from osteoporosis or osteopenia [26]. Levels of OC in patients with osteopenia were significantly lower (29.26 ± 3.65 ng/mL) than in patients diagnosed with osteoporosis (32.07 ± 6.24 ng/mL). Fluctuations in OC levels in the course of postmenopausal osteoporosis were presented in the study carried out by Gurban and associates [18]. Levels of OC among women who had not been menstruating for at least 15 years reached values of 20.12 ± 0.87 ng/mL, whereas in group where this period was less than 15 years concentrations of OC were significantly lower (15.12 ± 1.55 ng/mL). These results allow for putting forward a thesis that sustained decrease in function of osteoblasts after the last menstrual period is reflected by increased levels of ucOC in blood serum. This assumption may be relevant in the understanding of the OC metabolism. Lower female sex hormone levels cause changes in the OC system, leading to the decrease of OC and in consequence increase in ucOC levels in blood serum. However, not all authors completely agree with this theory. A study carried out by Lewandowski et al. showed that the level of secretion and concentration of Osteocalcin in body fluids was influenced by various factors, such as medicines, general diseases, or diet [27].

Correlation between Osteocalcin gene expression and bone metabolism was previously investigated by Rodrigues et al. [28]. In their study conducted on 64 patients (25 subjected to hip replacement surgery due to pathological fractures and 39 due to osteoarthritis), authors compared the ratios of bone OC/collagen gene expression (OC/COL1A1). The ratio was significantly lower among patients with hip fracture compared to those with osteoarthritis. Low bone OC/COL1A1 expression ratio was a predictor of worse trabecular mechanics and of a hip fracture episode. However, analysis of total OC and ucOC in the serum did not show significant differences between groups. Tests performed in present study proved that expression of OC gene is present both in buccal mucosa and in peripheral blood lymphocytes. The expression of Osteocalcin gene in cells which are not directly connected with bone metabolism is probably associated with constant bone remodeling processes which take place in living organism. Immune cells, which are important part of processes of skeleton destruction and healing, and cells from BEL, which overcome epithelial-to-mesenchymal transition, play significant role in metabolism of connective tissue [9–11]. This finding seems to be important due to the fact that bony material for diagnostic tests is far more difficult to obtain than blood or mucosal lining. What is more, the surgical procedure which is needed in order to obtain bone tissue is more complicated and associated with far more severe complications than collecting blood or epithelium samples. Analysis of BGLAP gene expression in present study revealed a statistically significant difference between the OV + HRT and OV groups. Expression of Osteocalcin in BEL was significantly lower in the OV + HRT group when compared to the OV group. An opposite correlation was observed in case of OC gene expression in PBL. Analysis of Pearson's correlation of BGLAP gene expression in the epithelial lining and PBL revealed a statistically significant positive interdependence in OV and M + HRT groups ($r > 0.5$). What is interesting is that the analysis of Pearson's correlation showed that in terms of OC gene expression in cheek epithelium lining in most of groups the gene expression caused increase in the BMD of mandible. Results for PBL did not reveal similar correlations.

It needs to be emphasized that the local levels of biomarkers, such as OC concentration in saliva, can be significantly influenced by factors such as periodontal status or local inflammations and they must be taken into consideration while qualifying patients for tests. In order to avoid significant impact of local inflammatory conditions on BEL OC gene expression, the present study included patients without any foci of acute inflammation in oral mucosa and the depths of their periodontal sockets were less than 5 mm. Patients enrolled for this study did not require any tooth extractions and presented with average or good level of oral hygiene. Another factor which has significant impact on bone condition is body fat content. Increased aromatization of androgens into estrogens takes place in adipose tissue. Adipose tissue functions as an active endocrine organ. Fat secretes hormones and cytokines such as resistin, leptin, adiponectin, TNF, and IL-6, which exert significant influence on the entire human organism, including bone tissue [29]. Increased aromatization of androgens into estrogens in fat tissue leads to elevation of concentrations of endogenous estrogens. Adipose tissue has no direct effect on the production of Osteocalcin, but it affects its concentration in body fluids through substances such as leptin or secreted cytokines [30]. In the present study, patients from both the control and the study group were matched in terms of BMI. Although all patients in our study had a body mass index higher than 25 (above normal), but none of the patients were categorized as obese (BMI lower than 30). Patients were matched in terms of BMI in order to prevent significant influence of adipose tissue on OC gene expression.

Osteocalcin gene expression can be influenced by many factors such as diet, steroid hormones, or inflammatory cytokines. Moreover, levels of biomarkers significantly changed throughout the day [31]. In their study, Gouveia et al. demonstrated the influence of thyroid hormones on the expression of OC [32]. OC secretion is influenced not only by estrogen but also by other hormones, such as thyroid hormones, growth hormone, or glucocorticoids [32, 33]. Patients included in the study did not receive any medications or dietary supplementation. Samples were collected at the same hours in order to avoid fluctuations in gene expression. It is impossible to eliminate all factors influencing OC level fluctuations. Moreover, levels of bone turnover biomarkers such as Osteocalcin are not specific for diseases of bone metabolism. Fluctuations in the expression of BGLAP indicate changes in bone metabolism regardless of their cause. Increased levels of OC may appear not only in osteoporosis but also in other diseases of bone tissue, such as hyperparathyroidism, Paget's disease, bone neoplasm, or avitaminosis. A study by Lee et al. showed that the level of

OC may be significantly influenced by metabolic disorders and could be a predictor of abdominal obesity or insulin resistance [34]. Ducy reported that Osteocalcin-null mice exhibit increased apposition of bone tissue without impaired osteolysis. This finding may suggest that interaction between recruited osteoblasts and osteoclast in the process of bone remodeling is complex and not completely dependent on the OC expression [5]. These factors greatly limit use of OC as a reliable indicator of osteoporosis in menopausal women. The analysis of correlation of OC gene expression and BMD in present study clearly indicates that it is difficult to state a significant correlation between status of skeleton mineralization and OC gene expression in PBL. Results from limited, local area, which is oral cavity, showed more significant relationship between OC gene expression and BMD of mandible. It needs to be stressed out that the OC gene expression was slightly lower in groups treated with HRT than in control groups. Further investigations on a broader group of patients are needed to verify process influencing the metabolism of OC in menopausal women.

5. Conclusions

(1) OC gene expression can be stated in both peripheral blood lymphocytes and epithelial lining of cheek.

(2) Hormone replacement therapy does not significantly influence OC gene expression in PBL/epithelial lining of women during natural menopause.

(3) HRT has significant influence on OC gene expression in PBL/epithelial lining of women during surgically induced menopause.

(4) The level of OC gene expression in blood stream is highly variable and seems to be influenced by many systemic factors, which makes it difficult to use for diagnostic purposes. Analysis of correlation between OC gene expression in inflammation-free area of oral cavity and BMD of mandible allowed stating of significant relationship between OC and bone metabolism.

(5) The relation between OC expression and bone metabolism is complex and further research is needed to clear all of the uncertainties.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Decorin-Mediated Inhibition of Human Trophoblast Cells Proliferation, Migration, and Invasion and Promotion of Apoptosis *In Vitro*

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Preeclampsia (PE) is a unique complication of pregnancy, the pathogenesis of which has been generally accepted to be associated with the dysfunctions of extravillous trophoblast (EVT) including proliferation, apoptosis, and migration and invasion. Decorin (DCN) has been proved to be a decidua-derived TGF-binding proteoglycan, which negatively regulates proliferation, migration, and invasiveness of human extravillous trophoblast cells. In this study, we identified a higher expression level of decorin in severe PE placentas by both real-time reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC). And an inhibitory effect of decorin on proliferation, migration, and invasion and an enhanced effect on apoptosis in trophoblast cells HTR-8/SVneo and JEG-3 were validated *in vitro*. Also the modulations of decorin on trophoblast cells' metastasis and invasion functions were detected through regulating the matrix metalloproteinases (MMP2 and MMP9). Thus, we suggested that the contribution of decorin to the modulation of trophoblast cells might have implications for the pathogenesis of preeclampsia.

1. Introduction

Preeclampsia (PE) has been proved to be a crucial cause for the increased maternal and perinatal mortality and morbidity, with a worldwide considerable incidence of 2–8% [1]. It is known as a pregnancy-specific disease with a new occurrence of hypertension and proteinuria during the second stage of pregnancy as its main clinical characteristic. The release of the symptoms happens only along with the delivery of the baby and placentas [2]. Since the enigmatic prognosis of this disease, more and more research has been continuously done to overcome its adverse outcomes.

As for the pathogenesis of PE, the hypotheses including inflammatory cytokines theory [3–5], insufficient remodeling of the maternal spiral artery [6–8], dysfunctional oxidative stress [9, 10], and genetic and dietary reasons [11] are involved. The dysfunctional state of oxidative stress is reported to activate a series of apoptotic signaling pathways which used to keep balance under normal circumstances [12]. And the aberrant villous trophoblast apoptosis has been discovered to be associated with the pathogenesis of PE [13]. Also the impaired remodeling of the maternal spiral artery contributes importantly to PE development, which strongly relies on the decreased migration and invasion potential of placental extravillous trophoblast (EVT) cells [14]. In normal conditions, the EVT cells migrate to the endovascular of the artery in order to invade and replace it, which is known as the endovascular transformation process. Then large diameter, low resistance vessels which could provide steady perfusion to the placenta and baby appear as the consequence of this process [15, 16]. Thus we postulate that the increase of trophoblast cells apoptosis and decrease of migration and invasion ability were closely related to the poor placental implantation and abnormal spiral artery remodeling in pregnancy.

Decorin, primarily synthesized by fibroblasts and myofibroblasts, is a member of the small leucine-rich proteoglycan (SLRP) family [17, 18]. Borbely et al. indicated that decorin was implicated in the invasive activity of EVT cells in pathology of both healthy and disordered placentas [19]. It has been reported that decorin might contribute to the regulation of trophoblast cells' migration and invasion potentials in the mammal placentas [20, 21]. Also, decorin is known to be a functional component of the extracellular matrix with biological functions such as regulating collagen fibrillogenesis and controlling cell proliferation by upregulating p21 [22, 23]. It binds to collagens types I, II, and IV *in vivo* and promotes the formation of fibers with increased stability and changes in solubility [24, 25]. Therefore decorin may contribute to the production of fibers during the remodeling of spiral arteries. However, the detailed influence of decorin on trophoblast cells functions and its involvement in the pathogenesis of PE remain deeply explored.

So in this study, to pursue the effect of decorin gene on trophoblast cells biological functions during PE, we overexpressed decorin gene in trophoblast cells HTR-8/SVneo and JEG-3 cells to identify the role of decorin-mediated cell growth, migration and invasion, and apoptosis *in vitro*.

2. Materials and Methods

2.1. Patients and Clinical Samples Collection. A group of primipara women aged 20–36 years who were hospitalized for cesarean delivery during December 2011 to March 2012 in our hospital (The First Affiliated Hospital of Nanjing Medical University) was selected for the placenta samples collection. The tissues were washed with sterile phosphate-buffered saline immediately after delivery from the maternal uterus and then kept in the liquid nitrogen until RNA extraction. We obtained the signed version of informed consents from all the women. All trials were approved by the ethics board of our hospital complied with the principles of Declaration of Helsinki guidelines.

2.2. Immunohistochemistry Staining. We used immunohistochemistry (IHC) to semiquantitatively and qualitatively detect the decorin protein expression according to the standard method. Briefly, sections of placenta tissues were incubated in 0.3% H_2O_2-methanol for 20 min to block endogenous peroxidase activity after being deparaffinized and dehydrated by xylene and rehydrated by 100% alcohol. Then we irritated the sections in 0.1 M citrate buffer in a microwave oven for antigen retrieval and incubated them with 10% bovine serum albumin to block nonspecific antibody binding. The primary antibody (rabbit anti-DCN; 1 : 500, Santa Cruz Biotechnology) and peroxidase-conjugated secondary antibody (1 : 1,000; Beijing ZhongShan Biotechnology CO., Beijing) were utilized to incubate the sections in turn. Finally, a digital photomicrograph was applied to capture photos of stained sections.

2.3. Cell Culture and Treatment. One of the cell lines used in this study is HTR-8/SVneo [26], which is derived from a short-lived primary EVT and was kindly provided by Dr. Charles Graham, Queen's University, Canada. With the similarity, it has been adopted to simulate trophoblast cells in a number of researches [27, 28]. It was cultured in an incubator with temperature of 37°C and 5% humidified CO_2 perfusion. The medium is RPMI1640 which is supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

The other cell line choriocarcinoma cell JEG-3 (bought from Cell Bank of Chinese Academy of Sciences in Shanghai) was maintained in DMEM medium, with the same condition mentioned above. Both cells were transiently transfected with overexpression plasmids of decorin (pEGFP-DCN) and an empty vector (pEGFP-N1) as control. The overexpression plasmid of decorin was conducted by Invitrogen Inc. After transfection for 48 hours, we collected the cells to test the transfection efficiency by observing the fluorescence efficiency under a fluorescence microscopy and overexpression efficiency via quantitative real-time PCR (qRT-PCR), respectively.

2.4. Cell Proliferation Assays. We used a MTT kit (Sigma) and colony formation assay to analyze the viability of both cells. According to the MTT instructions, an enzyme-linked immunosorbent assay plate reader was used to measure the absorbance of treated cells at 490 nm. As to the colony formation assay, we sowed 500 cells into each 6-well plate with 10% FBS medium for 2 weeks. After being fixed with methanol, the cell colonies were stained with 0.1% crystal violet (Sigma) for 15 minutes. The number of stained colonies was counted under an inverter microscope. Random experiments were done more than 3 times.

2.5. In Vitro Cell Migration and Invasion Assays. After being transfected as mentioned previously for 24 hours, 5×10^5 cells were resuspended in 1% FBS medium and placed into the upper well of a transwell chamber (Millipore, Billerica, MA), while 10% FBS medium was added into the lower well as a chemoattractant. The diameter of the membrane pore of the transwell chamber is 8 μm. There exists little difference between migration and invasion assays in the following steps. For invasion assay, the upper chamber was coated with 100 μL growth factor reduced matrigel (BD Biosciences, Oxford, UK) and allowed to set at 37°C for at least 30 min in advance, while the migration asssay was not coated. After another 24-hour culture, the number of cells that migrated to the lower surface was fixed by crystal violet and examined.

2.6. Western Blotting (WB) Analysis. After treatment, cells were lysed by using RIPA protein extraction reagent (Beyotime) which is supplemented with a protease inhibitor cocktail (Roche) and phenylmethylsulfonyl-fluoride (Roche). The concentration of proteins in each sample was tested by a Bio-Rad protein assay kit. The protein extractions (50–100 μg) were separated by polyacrylamide gel electrophoresis containing 10% sodium dodecyl sulfate and then transferred to polyvinylidene difluoride membranes or 0.22 mm nitrocellulose (Sigma). Then we used specific antibodies in

Table 1: Clinical characteristics of normal and preeclamptic pregnancies.

Variable	PE ($n = 30$)	N ($n = 30$)	P^a value Control vs PE
Maternal age	30.2 ± 5.7	30.6 ± 3.5	$P > 0.05$ (0.1388)
Proteinuria (g/day)	6.32 ± 0.85	<0.3	$P < 0.01$ (0.0065)
Gestational age (week)	36.5 ± 3.7	39.1 ± 1.2	$P > 0.05$ (0.0976)
Systolic blood pressure, mm Hg	169 ± 20.1	112 ± 6.8	$P < 0.01$ (0.0037)
Diastolic blood pressure, mm Hg	115 ± 12.8	77 ± 7.1	$P < 0.01$ (0.0094)
Body weight of infant (g)	2582 ± 740	3322 ± 413	$P < 0.05$ (0.0373)
CRP (C-reaction proteins)	8.1 ± 3.1	5.9 ± 2.9	$P > 0.05$ (0.0966)

All results are presented as mean ± SD. SD: standard deviation.
[a]Obtained by 1-way analysis of variance using SPSS 13.0 software (SPSS Inc., Chicago, IL).

a concentration of 1 : 1000 (DCN, Santa Cruz; Caspase-3, Bcl-2, MMP2, and MMP9, Cell Signaling Technology) to incubate them.

The horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1 : 1,000; Beijing Zhong-Shan Biotechnology CO., Beijing) was adopted as the secondary antibody. In order to visualize the bands, the ECL chromogenic substrate was used. The intensity of the bands was quantified by Quantity One software (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1 : 1000, Santa Cruz) was applied as control.

2.7. RNA Extraction and Real-Time RT-PCR. Trizol reagent (Invitrogen Life Technologies) was utilized for total RNA extraction from cells and placenta tissues. The RNA (1 mg) was reversed to cDNA by using a Reverse Transcription Kit (Takara). The qRT-PCR with the use of An ABI 7500 and the reagent of SYBR Premix Ex Taq (TaKaRa) was to determine the expression levels of amplification product. We applied GAPDH as internal control. The sequences of the primers are as follows: DCN (Forward: 5′-CGAGTGGTCCAGTGTTCTGA-3′, Reverse: 5′-AAAGCCCCATTTTCAATTCC-3′); GAPDH (Forward: 5′-GACTCATGACCACAGTCCATGC-3′, Reverse: 5′-AGAGGCAGGGATGATGTTCTG-3′).

2.8. Flow Cytometry (FCM). After transfection the HTR-8/SVneo and JEG-3 cells were harvested to analyze the apoptosis by flow cytometry (FACScan; BD Biosciences) equipped with CellQuest software (BD Biosciences). The annexin V-APC and 7-amino-actinomycin (7-AAD) (BD Biosciences) were used to label the cells. The number of cells including living, necrotic, early apoptotic, and lately apoptotic cells was counted. And the early and lately apoptotic cells were chosen for further comparison.

As to cell-cycle analysis, cells after treatment were stained with propidium oxide by the Cycle Test Plus DNA Reagent Kit (BD Biosciences) according to the protocol and then analyzed by FACScan (BD Biosciences). The cells were sorted into G0-G1, S, and G2-M phase and the percentages of each phase were counted and compared. These assays were repeated more than three times.

2.9. Statistical Analysis. A SPSS 17.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The patients' clinical data analysis was processed by One-Way ANOVA, and as to the cells experiments, paired samples t-test was used. The data appeared as mean ± SD (standard deviation, SD). P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Clinical Characteristics and Expression Level of Decorin in Human Placenta and Normal Tissues. The expression level of decorin was detected in 9 PE and 12 normal placenta tissues by using immunohistochemical staining. The results showed that decorin protein was greatly upregulated in PE but was expressed at lower level in normal placenta tissues (Figures 1(a) and 1(b)). Also, the qRT-PCR analysis was conducted by comparing 30 PE placentas to 30 normal pregnant ones. The expression level of decorin mRNA was also significantly higher in PE placentas than that of the normal ones (Figure 1(c)). Table 1 shows the patients' clinical characteristics in detail.

3.2. Exogenous Overexpression of Decorin in HTR-8/SVneo and JEG-3 Cells. The HTR-8/SVneo and JEG-3 cells that were sowed into 6-well plates previously were transfected with overexpression plasmids targeting decorin. The overexpression efficiency was detected by both qPCR (Figure 1(d), $P < 0.01$) and Western blotting assay (Figures 1(e) and 1(f), $P < 0.01$) in both cells after being transfected with pEGFP-DCN and empty vector for 48 h. The qPCR presented a 2091-fold and 1708-fold overexpression of decorin by pEGFP-DCN and WB analysis showed a 15% and 43% upregulation of decorin as compared to control in HTR-8/SVneo and JEG-3 cells, respectively. Of course, the transfection efficiency was detected by observing the fluorescence efficiency (more than 75%) under a fluorescence microscopy in both cells (Figures 1(g) and 1(h), $P < 0.05$). These results indicated that the overexpression of decorin was effectively in our study.

3.3. Modulation of Decorin Expression in Cell Migration and Invasion In Vitro. It was determined by transwell assays that the migratory and invasive capacity of cells transfected with pEGFP-DCN reduced by approximately 37.6% and 51.88% of HTR-8/SVneo cells (Figures 2(a), 2(b), and 2(c), $P < 0.01$), respectively, and 57.3% and 34.8% of JEG-3 cells (Figures 2(d), 2(e), and 2(f), $P < 0.01$), respectively, as compared to

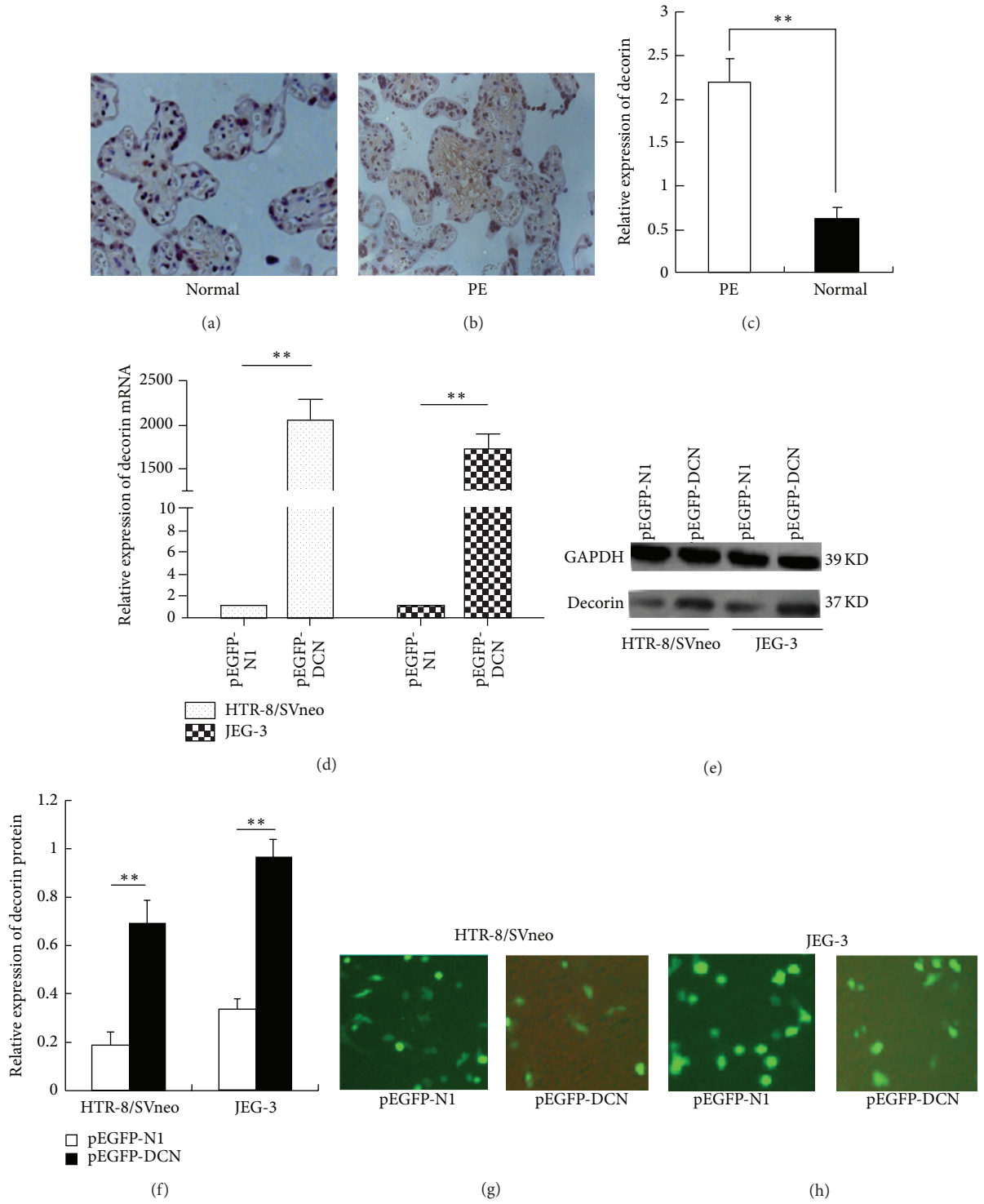


Figure 1: Expression of decorin in preeclampsia placentas compared with normal and decorin-overexpression efficiency in trophoblast cells. (a) Relative expression of decorin was 71.8% higher in preeclampsia placenta tissues compared to the normal pregnancies, as determined by qRT-PCR. (b) The mRNA expression of decorin in HTR-8/SVneo and JEG-3 cells transfected with pEGFP-DCN, detected by qRT-PCR. ((c) and (d)) The protein expression of decorin in HTR-8/SVneo and JEG-3 cells transfected with pEGFP-DCN, detected by Western blotting. Values are represented as mean ± SEM ($^{**}P < 0.01$).

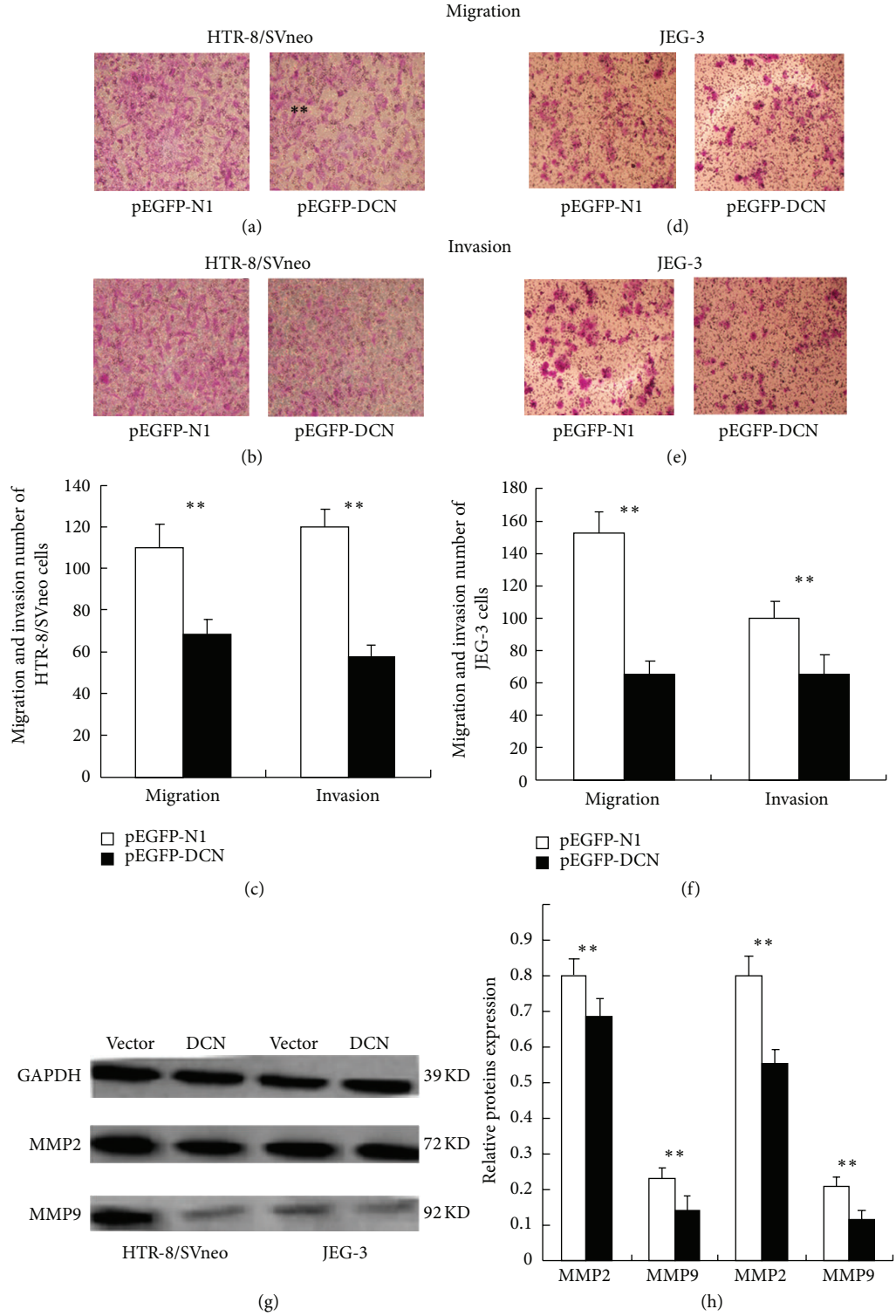


Figure 2: The migration and invasion capacity of trophoblast cells transfected with pEGFP-DCN and control. ((a) and (b)) HTR-8/SVneo cells treated with decorin overexpression presented significantly inhibited migration (a) and invasion (b) potentials compared to control. (c) The histogram showed the statistical data of (a) and (b). (d) The migration ability of JEG-3 cells treated with decorin overexpression was significantly lower than that of the control, as determined by transwell assays. (Values are mean ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$.) (e) The invasion ability of JEG-3 cells treated with decorin overexpression was significantly lower than control. (f) The histogram showed the statistical data of (e) and (f). ((g) and (h)) Western blotting analysis of MMP2 and MMP9 protein in pEGFP-DCN or empty vector transfected HTR-8/SVneo and JEG-3 cells. (Values are mean ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$.)

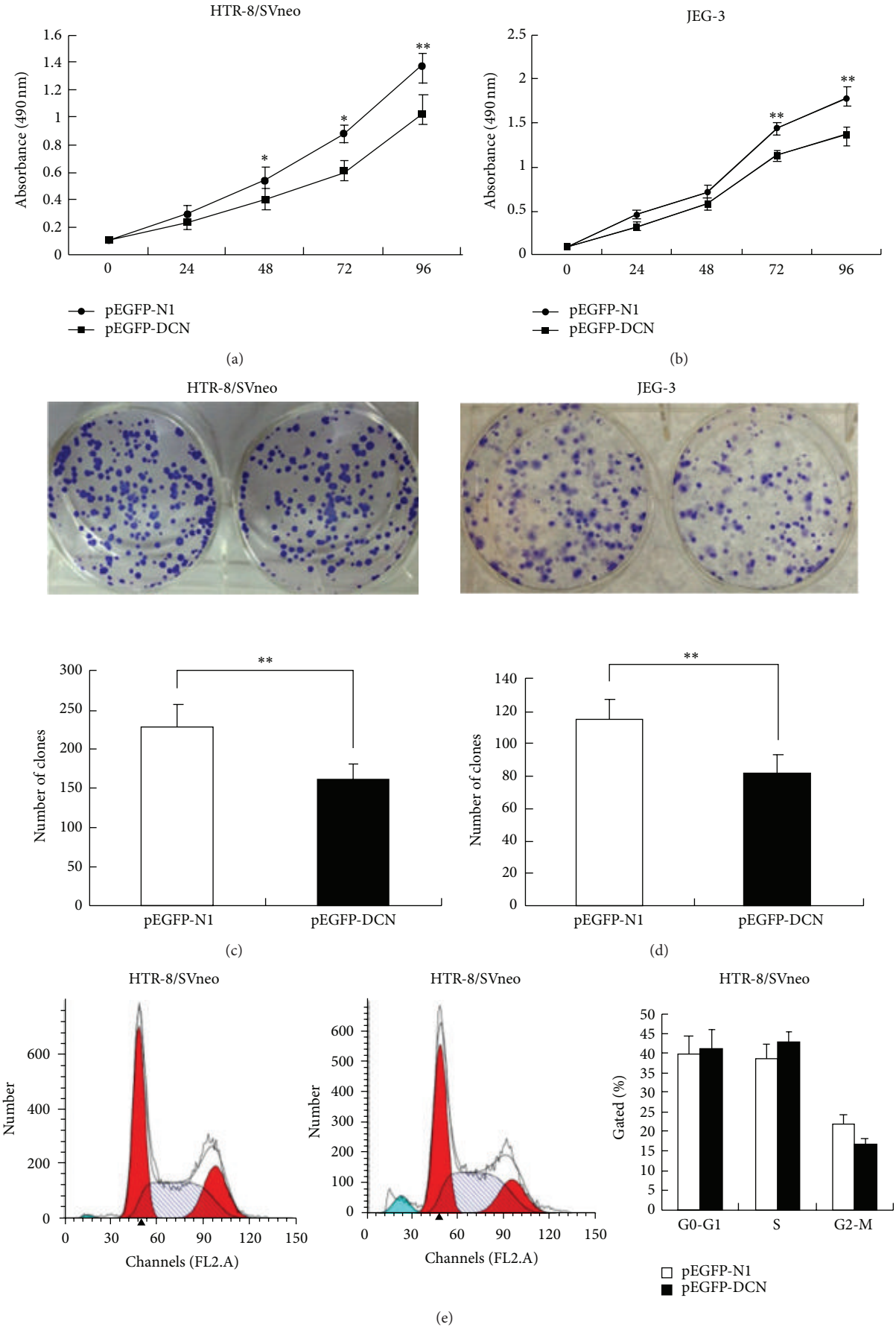
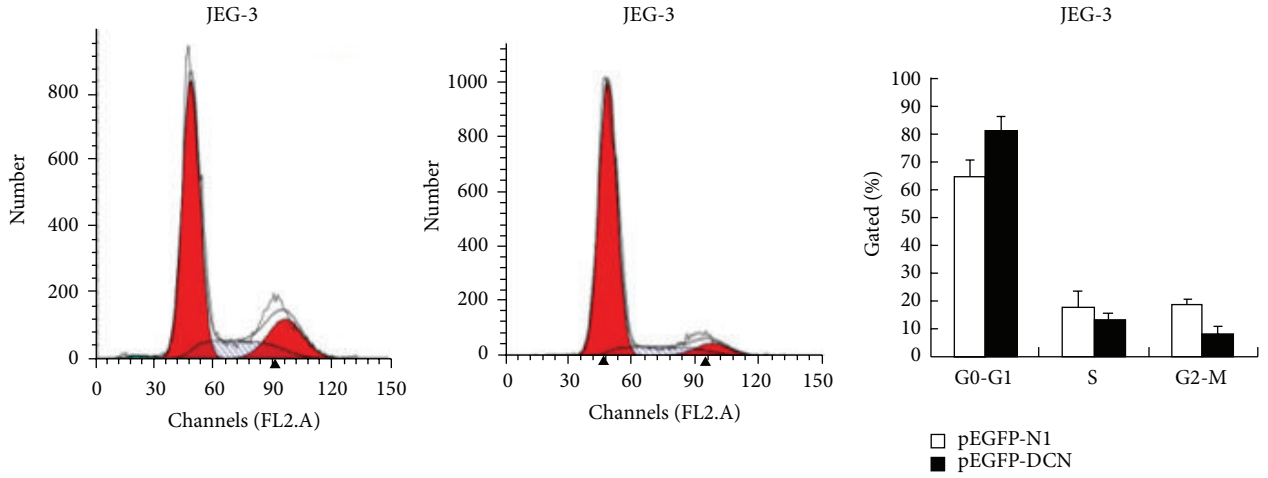


Figure 3: Continued.



(f)

Figure 3: Effects of decorin on growth and proliferation of trophoblast cells. ((a) and (b)) The proliferation ability of HTR-8/SVneo and JEG-3 cells was inhibited in pEGFP-DCN group compared to control, as identified by MTT assays. ((c) and (d)) Colony-forming assay showed a decrease of cells proliferation in pEGFP-DCN group compared to empty vector both in HTR-8/SVneo and in JEG-3 cells. ((e) and (f)) Cell-cycle analysis was performed 48 h following the treatment of HTR-8/SVneo and JEG-3 cells with pEGFP-DCN or empty vector. The DNA content was quantified by flow cytometric analysis. Values are represented as mean ± SEM ($^{**}P < 0.01$).

that of control. Moreover, matrix metalloproteinases, MMP2 and MMP9, also presented a decrease under the influence of decorin overexpression in both cells (Figures 2(g) and 2(h), $P < 0.01$). Thus, these data proved that decorin might be closely associated with the inhibition of migration and invasion behaviors in trophoblast cells.

3.4. Overexpression of Decorin Inhibited Cell Growth and Proliferation and Promoted Cell Apoptosis In Vitro. The significant increase of decorin expression in PE placenta samples prompted us to explore the possible biologic significance of decorin in the pathogenesis of PE. To determine whether decorin affects trophoblast cells growth, we conducted MTT assay to detect cell growth viability in pEGFP-DCN transfected HTR-8/SVneo and JEG-3 cells compared to that of control (Figures 3(a) and 3(b), $P < 0.01$). Also, the impact of decorin on cell proliferation was assessed by colony formation assay. According to the colony formation assay, we found that cells transiently transfected with pEGFP-DCN had significantly reduced proliferation of cells compared with that of cells transfected with pEGFP-N1 (Figures 3(c) and 3(d), $P < 0.01$). Additionally, flow cytometric analysis was used to further examine whether the inhibition of decorin on cell proliferation reflected cell-cycle arrest. The cell-cycle analysis showed that cells transfected with pEGFP-DCN had an obvious cell-cycle arrest at the G1-G0 phase with a decreased G2–S-phase compared to that of negative control (Figures 3(e) and 3(f), $P < 0.01$).

Furthermore, in order to evaluate whether the trophoblast cells growth and proliferation potential was affected by cell apoptosis, we performed flow cytometry to detect the apoptotic cells and Western blotting assays to identify apoptotic proteins in both cells treated with pEGFP-DCN. When HTR-8/SVneo and JEG-3 cells were transfected with pEGFP-DCN, a significant increase of apoptosis was observed as compared to control (Figures 4(a), 4(b), 4(c), and 4(d), $P < 0.01$). And the apoptotic protein cleaved Caspase-3 was signifiantly increased in cells transfected with pEGFP-DCN while the antiapoptotic protein Bcl-2 decreased (Figures 4(e) and 4(f), $P < 0.01$). These results indicated that enhanced decorin expression could repress trophoblast cells growth and proliferation and promote cells apoptosis.

4. Discussion

There have been more and more reports that evidenced decorin's inhibitory effects on tumorigenesis and overexpressed decorin could inhibit cancer cells growth and metastasis and promote apoptosis [29–32], while fewer reports [21] existed referring to its role in trophoblast cells' functions, even pathogenesis of PE. Herein, we verified by qRT-PCR that decorin mRNA was markedly increased in PE placentas than in normal pregnancy. And a positive relationship between the expression of decorin proteins detected by IHC and PE suggests that decorin may be involved in pathogenesis of PE. Thus a series of studies about the role of decorin in the biological functions of trophoblast cells involved in PE pathogenesis were conducted by us. These results may not be consistent with the results by Chui et al. who reported a reduced expression of decorin in 21 cases of PE compared to normal [33]. We thought this may be attributed to the fewer samples cases and ethnic differences. However, the effect of decorin on the biological functions of EVT cells is consistent with other researchers [20].

The *in vitro* data in our study proved that the growth and proliferation of the HTR-8/SVneo cells were decreased and their apoptosis rate was increased when decorin was upregulated in trophoblast cells HTR-8/SVneo and JEG-3. These assays evidenced that decorin could modulate the biological

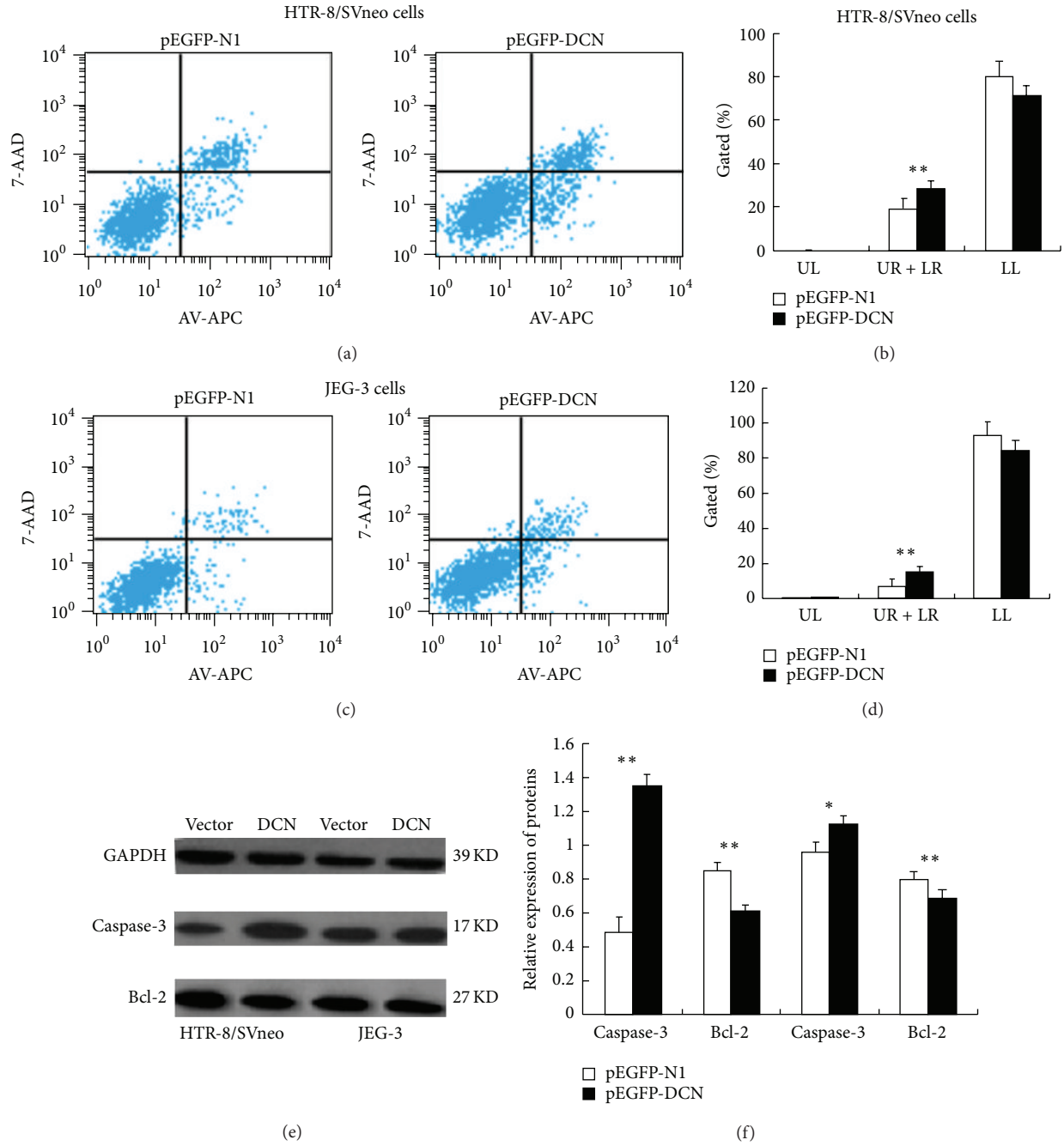


Figure 4: Cell apoptosis detection by flow cytometry and Western blotting assays. ((a) and (b)) HTR-8/SVneo cells transfected with pEGFP-DCN showed a significantly higher rate of apoptosis by flow cytometry. ((c) and (d)) JEG-3 cells transfected with plasmid overexpressing decorin showed a significant increase in apoptotic rate as compared to that of the empty vector as demonstrated by flow cytometry. ((e) and (f)) Western blotting analysis of apoptotic protein in cells transfected with pEGFP-DCN displayed an increase of cleaved Caspase-3 (17KD), while it displayed a reduction of Bcl-2 (27KD). (Values are mean ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$.)

activity of trophoblast cells by inhibiting cells growth. The mechanism of cell growth modulation by decorin may be through interaction with growth factor receptors at the cell surface. It was reported that decorin could modulate and induce signal transduction along pathways involving the EGFR [34] and the IGFR [35] among others. On the other hand, Guidetti et al. proved that exogenous recombinant decorin or de novo expression of decorin could downregulate the endogenous expression of proangiogenic factor, VEGF, as well as that of fibroblast growth factor-2 (FGF-2) [36] and suppressed the tumorigenicity of human colon carcinoma cells both *in vitro* and *in vivo*. However, the detailed pathways through which decorin regulates trophoblast cells' biological behaviors in PE need to be multidirectionally explored.

Generally, the disorder of maternal spiral arteries remodeling might contribute to the pathogenesis of PE. And

our findings in this work indeed indicated that decorin overexpression could inhibit trophoblast cells migration and invasion and promote apoptosis. Decorin, localized to the placental fetal blood vessel walls, is involved in trophoblast cells migration and invasion [20] and endothelial cell development as well. PE is associated with reduced perivascular and endovascular trophoblast cell invasion of the maternal spiral arteries [37] and increased cells apoptosis. These findings indicated that decorin might be one of the crucial factors for the conversion from endometrial epithelial cells to trophoblast cells in remodeling of maternal spiral arteries.

MMPs, a family of zinc-dependent proteolytic enzymes, are expressed in extravillous trophoblasts and involved in the process of trophoblast cells degrading the extracellular matrix and remodeling normal structure [38]. Reduced MMPs levels in the deciduas and placentas were observed in PE women and contribute importantly to the invasion of extravillous trophoblasts into the spiral arteries wall [39]. Thus, the modulation of decorin on trophoblast cells' metastasis and invasion functions may be through interacting with MMP. Then in our study, we found a block of MMP2 and MMP9 proteins after trophoblast cells transfected with decorin-overexpression plasmid. Therefore, the disrupted expression of decorin might cause implications for inadequate conversion of maternal spiral arteries through acting with MMPs, leading to placental abnormalities or PE. However, the detailed modulation mechanism remains unclear.

In conclusion, our study showed that decorin was significantly upregulated in PE placentas and was involved in regulating trophoblast cells biological functions. Decorin might be associated with the pathogenesis of PE and further insights into the deep basis of its function and clinical implications may contribute to the early diagnosis and treatment of PE.

Disclaimer

The authors alone are responsible for the content and writing of the paper.

Conflict of Interests

The authors report no conflict of interests.

Authors' Contribution

Yanfen Zou and Xiang Yu contributed equally to this work and should be regarded as joint first authors.

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Review Article

Preimplantation Exposure to Bisphenol A and Triclosan May Lead to Implantation Failure in Humans

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Endocrine disrupting chemicals (EDCs) are chemicals that have the capacity to interfere with normal endocrine systems. Two EDCs, bisphenol A (BPA) and triclosan (TCS), are mass-produced and widespread. They both have estrogenic properties and similar chemical structures and pharmacokinetic features and have been detected in human fluids and tissues. Clinical evidence has suggested a positive association between BPA exposure and implantation failure in IVF patients. Studies in mouse models have suggested that preimplantation exposure to BPA and TCS can lead to implantation failure. This paper reviews the relationship between preimplantation exposure to BPA and TCS and implantation failure and discusses the remaining problems and possible solutions.

1. Introduction

During the last few decades, the incidence of human infertility has significantly increased in many countries, such as the United States and China [1–5]. The rate of this increase is too rapid to be explained by genetic mutations. More than 10% of infertile couples suffer from infertility of an unexplained nature [6]. The women in these couples have normal ovulatory cycles and hormonal profiles and no organ pathologies. Their partners show no evidence of semen quality problems. In the meantime, the production of many artificial chemicals such as plastics has been increased [7].

Some chemicals which have been widely used for decades have recently been found to have the ability to disrupt endocrine function in humans. They are called endocrine disrupting chemicals (EDCs). Bisphenol A (BPA) and triclosan (TCS) are EDCs with similar chemical structures to 17β-estradiol [8] (Figure 1). They have recently been noticed due to their ubiquitous presence in the environment and in human fluids and tissues [9–23]. BPA is the monomer used in the production of polycarbonate plastics and some epoxy resins. It is one of the most-produced chemicals worldwide, with over six billion pounds produced each year [21]. TCS is an antimicrobial additive used in many personal care and household products.

Many studies have suggested that BPA exposure is associated with female infertility [15–17]. However, the association between TCS exposure and female infertility remains unknown. Mice have been used as an animal model to study the association between the exposure to these two chemicals and infertility [24–28].

Subtle changes in estrogen levels can lead to implantation failure in humans and mice [29, 30]. BPA and TCS have estrogenic activity in vitro and in vivo [21, 31]. BPA binds to both ERα and ERβ [32–35]. Both BPA and TCS have many biological effects mediated via estrogen receptors [36–39]. Thus, BPA and TCS may cause implantation failure due to their ability to mimic estrogen in humans [40–42]. In human beings, from oocyte maturation to implantation, the biological features of the oocyte and the embryo change

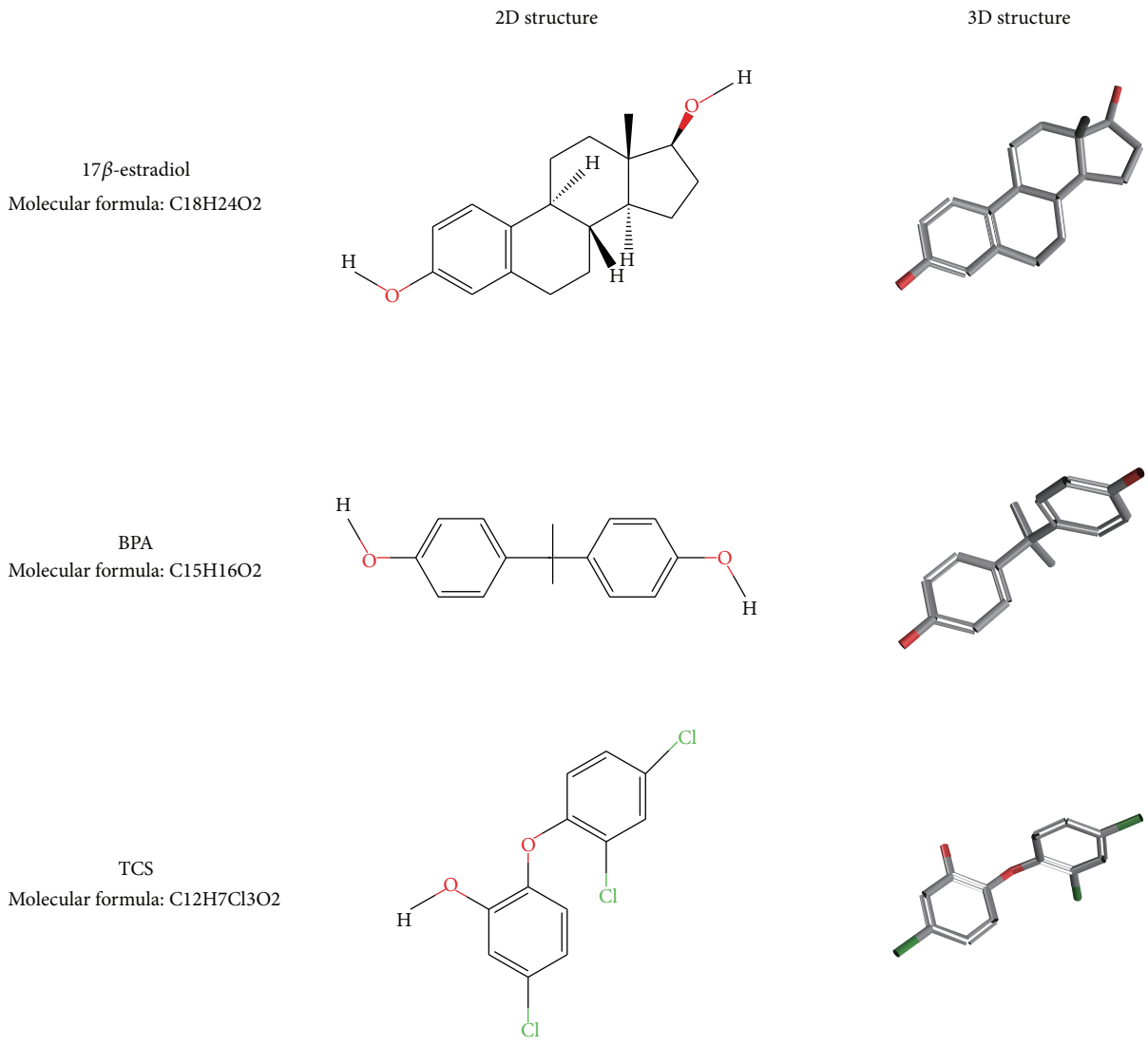

Figure 1: Chemical structures of BPA, TCS, and 17β-estradiol.

dramatically. The levels of sex hormones, such as estrogen, progesterone, and androgen, and their receptors also change dramatically. Thus, the sensitivity of the female reproductive system to BPA and TCS may vary depending on the time of exposure. It has been reported that, in mice, preimplantation exposure to the same amount of BPA or TCS on gestational day 2/3 is more potent to induce embryo implantation failure than exposure on gestational day 0/1 [27, 28]. Thus, in mice, gestational day 2-3 may be a sensitive window for BPA and TCS. Exposure to these two endocrine disruptors during a sensitive window might lead to implantation failure. However, in human beings, the sensitive window for these EDCs still needs further investigation.

2. Exposure and Detection of BPA and TCS in Humans

2.1. The Route and Amount of Exposure to BPA and TCS in Humans. There is a trend of increased exposure to endocrine disrupting chemicals, including BPA and TCS. In 2008, it was reported that daily exposure of BPA to humans is below 0.1 μg/kg/day for the majority of the population [43]. In 2011, Taylor et al. suggested that total daily human exposure of BPA is via multiple routes and is much higher than previously assumed based on animal studies and pharmacokinetic features of BPA in human and animal [44]. Recently, Lassen et al. [45] reported that the median daily intake of BPA among 33 Danish men is approximately 27 ng/kg/day.

Although BPA and TCS can be absorbed orally, dermally, and by inhalation [46], the majority of absorption occurs via ingestion. It is estimated that 90–99% of BPA exposure in adults and children is from food [47–49]. Allmyr et al. [50] suggested that oral care products are probably the most important means of exposure to TCS in adults because brushing teeth with TCS-containing toothpaste has been shown to result in a large and rapid uptake of TCS. However, the percentage of TCS ingested orally in relation to total TCS absorption is not known.

Table 1: The distribution and amount of BPA and TCS in human tissue.

Chemical	Tissue type	Concentration	References
BPA	Serum (adult)	~1-2 ng/mL	[22, 81]
	Serum (fetal)	~1-2 ng/mL	[22]
	Breast milk	0.61 ± 0.20 ng/mL	[82]
	Colostrum	3.41 ± 0.13 ng/mL	[83]
	Follicular fluid	~1-2 ng/mL	[22]
	Amniotic fluid (full term)	~1-2 ng/mL	[22]
	Amniotic fluid (15–18-week gestation)	8.3 ± 8.7 ng/mL	[22]
	Urine	2.75–3.3 μg/g creatinine (~3.00 ng/mL)	[84, 85]
	Brain	0.91 ng/g	[10]
	Adipose tissue	3.78–5.83 ng/g	[10, 86]
	Liver	1.48 ng/g	[10]
TCS	Serum	4.1–19 ng/g	[50]
	Breast milk	1.3 ± 2.7 ng/g fresh weight	[87]
	Urine	3.55 μg/g creatinine (3.77 ng/mL)	[84]
	Adipose tissue	0.61 ng/g	[10]
	Liver	3.14 ng/g	[10]

Rodricks et al. [51] estimated the amount of human daily exposure to TCS from two approaches. One approach is based on the estimation of the combination of daily intake products, and the other is based on biomonitoring data from human volunteers. The total intake of TCS per day from the consumer products evaluated were 0.047, 0.065, and 0.073 mg/kg/day for men, women, and children, respectively [51]. The daily TCS intake estimates based on the 50th percentile urinary concentrations of TCS reported in the NHANES (National Health and Nutrition Examination Survey) 2003-2004 were approximately 0.0002, 0.0002, and 0.0001 mg/kg/day for men, women, and children, respectively [51]. The estimates based on the 95th percentile urinary concentrations of TCS were approximately 0.009, 0.007, and 0.004 mg/kg/day [51]. The estimated exposure level of TCS based on biomonitoring data is much higher than the product-based TCS intake estimates and suggests that actual TCS intakes are lower than the product-based estimates.

People living in different regions or having different living habits are probably exposed to different amounts of these two EDCs. And the human exposure level of BPA and TCS remains unclear and needs further investigation.

2.2. Distribution and Amount of TCS and BPA in Human Tissue. BPA and TCS have been detected in the blood, breast milk, urine, adipose tissue, liver, and brain of most human volunteers [9–21]. BPA has also been detected in human amniotic fluid and follicular fluid [22, 23]. The distribution and amount of BPA and TCS in the human body are listed in Table 1. It is interesting that BPA has an ~5-fold higher concentration at 15–18-week gestation, which must be considered in evaluating the potential for human exposure to BPA [22]. The concentrations of BPA in serum, breast milk, follicular fluid, amniotic fluid (full term), and urine are very close, suggesting that BPA is distributed evenly in human fluids. The distribution and amount of BPA and samples suggest a profile of ubiquitous presentation in human body.

3. Pharmacokinetics of BPA and TCS

3.1. BPA. Völkel et al. [52] administered 5 mg radioactive BPA/person (54–90 ug/kg body weight) and reported that this BPA was completely eliminated from the body within 24 h. Maximum plasma concentrations were reached 80 min after dosing and rapidly declined for the next 6 h. BPA is only detected in its glucuronidated form and not as free BPA. This study indicates that BPA was absorbed from the gastrointestinal tract quickly, conjugated with glucuronic acid in the liver, and BPA-glucuronide was rapidly filtered from the blood by the kidneys and excreted in the urine [52]. Acute studies in both mice and humans indicate rapid metabolism and clearance of BPA [44, 52, 53].

3.2. TCS. The absorption of TCS following oral administration in both humans and mice is rapid and efficient [51]. Maximum plasma concentrations were achieved 1 to 3 h following administration in humans and 1 to 4 h in mice, respectively [51, 54]. In humans, TCS does not accumulate in the blood [55]. Nearly all of the TCS absorbed is metabolized to sulfate and glucuronide conjugates in both humans and mice [51, 56, 57]. In humans, about 80–85% of the administered dose in volunteers is excreted in the urine (71–80%) or feces (5–7%) in the form of conjugated metabolite, and the elimination half-life of TCS is estimated to be approximately 10 to 20 h [51, 54]. Most of the absorbed TCS can be excreted from the human body less than 24 h after exposure [54].

In short, the pharmacokinetic features of TCS are very similar to BPA in both humans and mice. In both humans and mice, TCS can be rapidly and nearly completely absorbed, metabolized to glucuronide and sulfate conjugates, and excreted.

4. Preimplantation Exposure to BPA and TCS Can Cause Implantation Failure

Accumulating evidence suggests that there is an association between women's exposure to BPA and female infertility. Moreover, animal model studies have suggested that exposure to BPA, TCS, or both BPA and TCS during the preimplantation period could lead to implantation failure in mice.

4.1. Clinical Studies. In 2005, Sugiura-Ogasawara et al. [17] reported that BPA is associated with recurrent miscarriage in humans. Recently, Ehrlich et al. [15] reported a significant linear dose-response association between increased urinary BPA concentrations and a decreased number of oocytes (overall and mature), a decreased number of normally fertilized oocytes, and decreased peak serum estradiol levels. The mean number of oocytes and normally fertilized oocytes decreased by 24 and 27%, respectively, for the highest versus the lowest quartile of urinary BPA (trend test $P < 0.001$ and 0.002, resp.). Women with urinary BPA above the lowest quartile had decreased blastocyst formation (trend test $P = 0.08$). Ehrlich et al. [16] also claimed a positive linear dose-response association between BPA urinary concentrations and implantation failure.

The correlation between the concentration of TCS in pregnant women and female infertility remains unclear due to a lack of investigation. However, the similarity of the distribution, chemical structures, and estrogenic activity of BPA and TCS suggests the possible involvement of TCS in implantation failure.

4.2. Animal Studies. Implantation failure could be caused by the embryo itself, inadequate uterine receptivity, or defects in communication between the embryo and the endometrium. It is generally assumed that the embryo itself is probably only responsible for one-third of IVF failures, and the other two-thirds of implantation failures occur due to impaired uterine receptivity or defects in embryo-endometrium communication [58–60]. Animal models have been used to investigate the effect of preimplantation exposure of BPA and TCS on implantation.

Crawford and deCatanzaro [28] studied the impact of preimplantation exposure to BPA, TCS, and BPA and TCS on implantation rates in mice. They found that exposure to TCS on the level of 523/785 mg/kg/day on gestational days (GD) 1–3 could significantly reduce implantation rates by 30%/40% [28]. They also found that although doses of 4 mg BPA (122 mg/kg) and 9 mg triclosan (262 mg/kg) on GD 1–3 were individually ineffective, in combination they reduced the number of implantation sites and also increased gestation length [28]. Xiao et al. [24] showed that preimplantation exposure to 100 mg/kg/day BPA on gestational days 0.5–3.5 can reduce implantation rates to zero by affecting uterine receptivity, embryo transport, and preimplantation embryo development in mice. Berger et al. [25] reported that preimplantation exposure to 200/300 mg/kg/day BPA on gestational days 1–4 can reduce implantation sites by 70%/100%.

Takai et al. [61] showed that, at 100 uM, BPA could inhibit the development of preimplantation embryos in vitro. At lower, more environmentally relevant concentrations (1 nM and 3 nM), BPA has stimulatory effects on embryo development in mice.

4.3. Possible Mechanisms. Uterine receptivity and embryo development are both critical for successful implantation. Coordinated actions of progesterone and estrogen play a critical role in creating a receptive uterine environment, embryo development, and embryo migration through the oviduct [62, 63]. Estrogen and progesterone actions are critical in the regulation of uterine cell proliferation, establishing a window of receptivity for blastocyst implantation [64, 65]. In mice, this window is very narrow and sensitive to changes in steroid levels [29, 62]. Small increases in estradiol levels can alter uterine PR (progesterone receptor) and gene expression, causing the uterus to enter a refractory state and thereby decreasing the probability of successful implantation [29]. Kim et al. [66] claimed that, through nuclear ER-dependent ERK1/2 phosphorylation, both E2 and BPA can rapidly and transiently induce Egr1 which may be important for embryo implantation and decidualization in mouse uterus. Recently, Mannelli et al. [67] reported that BPA perturbed the expression of ERα, ERβ, PRA, PRB, and hCG/LH-R, reduced the mRNA transcription of dPRL, and stimulated secretion of MIF in human endometrial stromal cells in vitro.

Several studies have shown that BPA can impair the development of mouse embryos and that this effect can be counteracted by Tamoxifen (an estrogen receptor modulator) [24, 61, 68]. Thus, the impairment of BPA on the embryo is probably mediated by the estrogen receptor.

BPA, TCS, and 17β-estradiol have similar chemical structures and they are all fat-soluble chemicals. BPA and TCS both have estrogenic activity, share similar pharmacokinetic features, and can be detected in human fluids and tissues. Evidence from clinical studies and animal models supports the assumption that preimplantation exposure to BPA and TCS could lead to implantation failure in humans.

In addition, preimplantation exposure to BPA can non-monotonically change the expression of the ERα (estrogen receptor α) and PR (progesterone receptor) in the uterus of mice [25]. Thus it seems that BPA might interfere with the coordinated actions of progesterone and estrogen and impair the receptivity of the uterus and embryo migration. Xiao et al. [24] reported that preimplantation exposure to BPA affects embryo transport, preimplantation embryo development, and uterine receptivity in mice.

BPA also can increase the luminal area and luminal cell height of the mouse uterus on gestational day 6 following subcutaneous injections of BPA on days 1–4 of gestation [25]. These morphological changes in the uterus could have implications for the success of blastocyst implantation.

Varayoud et al. [69] reported that neonatal exposure to BPA alters rat uterine HOXa10 and its downstream gene expression and reduces the number of implantation sites compared to the control group. Bromer et al. [70] claimed that BPA exposure in utero on gestational days 9–16 (after implantation and in the middle of the pregnancy) can upregulate

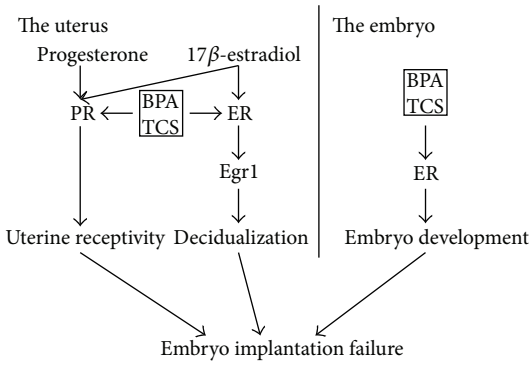

Figure 2: Possible mechanism by which BPA and TCS may affect embryo implantation.

the expression of HOXa10 in the uterus of female offspring in mice. However, whether preimplantation exposure to BPA affects the expression of HOXa10 and its downstream genes has not been investigated.

Some studies have suggested that exposure to BPA could affect the meiotic maturation of oocytes in humans and mice [71–76].

Crawford and deCatanzaro [28] suggested that preimplantation exposure to TCS can also cause implantation failure in mice and that TCS can act in conjunction with BPA. However, the mechanism of this implantation failure in humans and mice is still unknown. The possible mechanism by which the two chemicals may affect embryo implantation is illustrated in Figure 2.

5. Summary and Suggestions for Further Studies

In the clinic, fertilization can only be confirmed afterwards. There is no way to detect embryo implantation failure in the clinic except for patients undergoing in vitro fertilization. The most common way to diagnose pregnancy is by testing human chorionic gonadotrophin (HCG) in urine samples. However, HCG is secreted by the syncytiotrophoblast and is detectable in maternal blood two days after the implantation of the embryo [77]. Thus, it is possible that many women did not know that they had a fertilized embryo which failed to implant into their endometrium because no HCG was secreted. Even if there was vaginal bleeding and they went to see a gynecologist, it will only be seen as ovulation bleeding which is very common in the clinic. Most of the time, when a woman wants to know if she is pregnant she will do a urine pregnancy test. However, a detectable level of HCG in the urine requires the embryo to survive for at least a week after implantation. Since BPA and TCS can be absorbed and excreted quickly and do not accumulate in the human body [44, 51–53], a change in habits like starting or stopping using TCS-containing toothpaste or using plastic food containers can cause a fluctuation in the levels of these two chemicals in the human body. This means that if the exposure ceased during the sensitive time frame—for example, the woman has used up her TCS-containing toothpaste and bought some new TCS-free toothpaste or lost her plastic bottles which leak BPA—she could have a relatively low level of TCS and BPA in her body during this time and become pregnant. In the clinic, a woman cannot be defined as infertile unless she has attempted unprotected coitus for at least one year without becoming pregnant. This means that perhaps BPA and TCS have caused more miscarriages than we have realized.

However, the most sensitive time for BPA and TCS to influence implantation remains unknown. Although the preimplantation period might be a sensitive time frame for BPA and TCS exposure, it might not be the most sensitive and important one.

The procedure of in vitro fertilization and embryo transfer (IVF/ET) has provided some possible ways to identify the most dangerous time frame for TCS and BPA exposure in humans. Only in those patients would we know the exact time of fertilization, the condition of the oocyte prefertilization, and the embryo's preimplantation.

Recently, Ehrlich et al. [15, 16] studied the association between BPA exposure levels and the clinical outcomes of IVF patients as mentioned above. However, their study did not measure the exposure level of TCS in those patients or the chromosomal condition of the oocytes before fertilization using techniques such as biopsy of their first polar body or the discarded GV (germinal vesicle) oocytes (i.e., immature oocytes). Oocytes with meiotic abnormalities can look normal and even become fertilized, but they lead to a low fertilization rate or a low implantation rate if they are fertilized [78, 79].

Many studies have investigated the association between BPA exposure and clinical outcomes in IVF patients [14–16, 18]. However, the menstrual cycles of IVF patients are not natural but are altered using hormones to obtain more ova and the optimal endometrium state during embryo transfer. Since it is likely that BPA and TCS act through estrogen receptors, it is best to study their effect under natural conditions where the status of estrogen receptors and the amount of estrogen in vivo are less affected by exogenous hormones. For example, one could conduct the study in IVF patients undergoing natural cycles. Since only patients with stable menstrual cycles would be included in this procedure, the samples may be more monotonous. Also, most of the animal studies have used the subcutaneous route for exposure [25, 27, 28, 69]. However, human exposure to BPA and TCS is mainly orally. So either gavage or food and drink might be a better exposure procedure for animal models.

It is not easy to maintain low TCS and BPA exposure levels due to their widespread existence. And recently it has been reported that BPA-free plastic products also release other chemicals with estrogenic activity [80]. Fortunately, the pharmacokinetic features of these two chemicals suggest that the fertility rate of women could probably be raised just by minimizing contact with BPA- and TCS-containing products during sensitive time frames such as the preimplantation period. However, the other part of reproduction, including the period of the second meiosis of the oocyte, cannot be

excluded. If we could determine the most sensitive time frame for these two chemicals, we might not need to find a counteractive drug.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

MiR-183 Regulates ITGB1P Expression and Promotes Invasion of Endometrial Stromal Cells

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We applied in the previous study miRNA microarray screening analysis to identify several differentially expressed miRNAs, including miR-183 in normal, eutopic, and ectopic endometrium. Knockdown of miR-183 expression induced the invasiveness and inhibition of apoptosis in endometrial stromal cells. The current study aims to identify the miR-183 targets with relevance to cell functions in endometrial stromal cells, to verify the interaction of miR-183 with its target genes, and to confirm the role of miR-183 in the process of endometriosis. Using microarray analysis, we identified 27 differentially expressed genes (19 were upregulated and 8 downregulated), from which we selected 4 downregulated genes (ITGB1, AMIGO2, VAV3, and PSEN2) based on GO databases for functional analysis and significant pathway analysis. Western blotting analyses showed that integrin β1 (ITGB1), but not AMIGO2, was affected by miR-183 overexpression, whereas no protein expression of VAV3 and PSEN2 was detected. Luciferase reporter assay verified that ITGB1 is a target gene of miR-183. Moreover, we found that ITGB1 is overexpressed in the endometrium of endometriosis patients. Furthermore, overexpression of ITGB1 rescued the repressive effects of miR-183 on the invasiveness of endometrial stromal cells. These findings, together with the fact that ITGB1 is a critical factor for cell adhesion and invasiveness, suggest that miR-183 may be involved in the development of endometriosis by regulating ITGB1 in endometrial stromal cells.

1. Introduction

Affecting 6–10% of women at reproductive age, endometriosis is a common chronic gynecological disorder associated with infertility, severe pelvic pain, and menstrual disorders [1]. Despite intensive research efforts, the pathogenic mechanism of endometriosis remains poorly understood. So far, it is still a highly underdiagnosed disease, and the current surgical and hormonal treatment of endometriosis is not effective and recurrence is often observed.

MicroRNAs (miRNAs) are small, noncoding regulatory RNAs that regulate the stability and translation of mRNAs by inhibiting ribosome functions, deadenylating the poly(A) tail, and degrading target mRNA [2]. Aberrant miRNA expression has been linked to a variety of human diseases such as gynecological diseases, cancers, inflammatory diseases, and cardiovascular disorders [3].

A single microRNA molecule is able to modulate multiple target genes; thus it is involved in multiple cell functions including proliferation, invasiveness, and differentiation [4]. In 2007, Pan et al. detected deregulated expression of miRNAs in paired ectopic and eutopic endometrial tissues using microarray analysis. From then on, many studies have confirmed the aberrant expression patterns of miRNAs in ectopic as well as eutopic endometrial tissues from endometriotic patients. MiR-520, miR-199a, miR-23a, miR-23b, miR-145, miR-196, miR-10, miR-20a, let-7, miR-126, and miR-135 are among the most studied miRNAs in endometriosis. Adammek et al. reported that miR-145 inhibited endometriotic cell proliferation, invasiveness, and stemness by targeting pluripotency factors, cytoskeletal elements, and protease inhibitors [5]. MiR-196b was implicated in HOXA10 expression and in the proliferation of endometriotic stromal cells [6]. Loss of eutopic endometrial miR-451 expression is

associated with decreased establishment of endometriosis in a mouse model [7]. These data landed strong support for an important role of miRNAs in the development of endometriosis through regulating cell proliferation, cell apoptosis, cell migration, cell invasiveness, and estrogenic signaling.

In a previous study, we identified several differentially expressed miRNAs among normal, eutopic, and ectopic endometrium. MiR-183 was found to be the most downregulated miRNA in both the ectopic and eutopic tissues compared to normal endometrium. Functional studies indicated that miR-183 may contribute to endometrial stromal cell apoptosis and impose a negative regulatory impact on cell invasiveness, but it has no effect on endometrial stromal cell proliferation. The study suggested that aberrant miR-183 expression may be involved in the development and progression of endometriosis [8].

In the present study, we aim to identify the functional targets of miR-183 using an overexpression model. We examine if miR-183 exerts its effects by directly binding to and inhibiting the ITGB1 promoter activity. In addition, we investigate if miR-183 could regulate stromal cell invasiveness. The findings from these experiments will help us to better understand the miR-183-mediated molecular mechanism in endometric stromal cells.

2. Materials and Methods

2.1. Tissue Acquisition and Cell Culture. All the eutopic and normal endometrial tissues were obtained at the Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University by uterine curettage from patients with or without endometriosis. None of the patients had received preoperative hormonal therapy, and all samples were histologically confirmed by pathologists. All samples were collected from the proliferative phase of the menstrual cycle. The phase of the menstrual cycle was determined based on histologic evaluation of the endometrium with the assistance of pathologists. The average age of the patients was 30.5 ± 5.2. Patients consented to tissue donation prior to surgery. The research project and consent form were approved by the hospital ethics committee. Each sample was divided into two parts: one part was snap-frozen and stored at −20°C and later used for mRNA and protein extraction; the other part was rinsed thoroughly with cold PBS and directly subjected to digestion and stromal cell isolation.

The endometrial stromal cells (ESC) from normal women without endometriosis were used as control. Tissue digestion and stromal cell isolation and culture were performed according to the method described in previous publication [9].

2.2. MiR-183-Lentivirus Construction and Transduction. The lentivirus gene transfer vector carrying precursor of hsa-miR-183 (Genbank accession number: MIMAT0000261) and encoding green fluorescence protein (GFP) were constructed by Genechem Co., Ltd., Shanghai, China, and confirmed by DNA sequencing. The primers of RNA were 5′-GAGGATCCCCGGGTACCAAGGGAGTGGGCAGGCTA and 5′-ATAAGCTTGATATCGTCCCTGCACCCTTGGAAGCA. The recombinant lentivirus for miR-183 overexpression (miR-183-lentivirus) and the control lentivirus (GFP-lentivirus) were prepared and titered to $5.0E + 8$ TU/mL (transfection unit per mL).

The sequence of inhibitor of hsa-miR-183-5p was TATGGCACTGGTAGAATTCACT. The recombinant lentivirus of miR-183-5p inhibitor (In-miR-183 lentivirus) and the control lentivirus (GFP-lentivirus) were prepared and titered to $4.0E + 8$ TU/mL (transfection unit per mL).

ESC from women without endometriosis were plated in 6-well plates (5×10^4 cells/well) overnight. The lentiviruses were diluted in 0.2 mL complete medium containing polybrene (8 mg/mL) and added to the cells for 12 h of incubation at 37°C, followed by incubation in 0.3 mL of freshly prepared polybrene-DMEM for 24 h. The media were replaced with fresh DMEM and the cells were cultured for 3 days. The lentivirus transduction efficiency of ESC was determined by the detection of GFP signals under a fluorescence microscope at 72 h after transduction. The miR-183 expression in stably transduced ESC was measured by real-time PCR. The ESC transfected with miR-183-lentivirus, In-miR-183-lentivirus, and GFP-lentivirus were kept for further analysis.

2.3. RNA Extraction and Microarray. For the microarray analyses, groups were divided into the ESC with miR-183 overexpression and the control ones. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Gene expression profiling was conducted using PrimeView Human Gene Expression Array (Affymetrix). The array contains 530,000 probes covering more than 36,000 transcripts and variants, which represent more than 20,000 genes mapped through RefSeq or via UniGene annotation. All subsequent technical procedures and quality controls were performed by Genechem Co., Ltd., Shanghai, China. The arrays were scanned using a GeneChip Scanner 3000 (Affymetrix, Inc., CA, USA). Raw data were extracted from the scanned images and analyzed using GeneSpring GX software version 11.5 (Agilent Technologies, CA, USA).

The data were normalized using the PLIER default protocol. Significant differentially expressed genes were analyzed using an unpaired t-test. Hierarchical cluster analysis was used to assess correlations among samples for each identified gene set with Euclidean distance and average linkage statistical methods.

2.4. Microarray Data Analyses. Differentially expressed genes were uploaded to the Ingenuity database (Ingenuity Systems, Redwood City, CA, USA) for pathway and functional analyses. Genes were further annotated and classified based on the Gene Ontology (GO) consortium annotations from the GO Bostaurus database using GOEAST (Gene Ontology Enrichment Analysis Software Toolkit).

2.5. Western Blotting. ESC proteins were extracted with RIPA buffer [(50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/o) sodium deoxycholate, and 0.1% (w/o)

sodium dodecyl sulphate (SDS)] supplemented with a protease inhibitor cocktail (Sigma). After incubating on ice for 30 min, cell debris was removed by centrifugation at 12,000 g for 10 min. Cell lysates were resolved in 12% SDS-PAGE electrophoresis and transferred to the PVDF membrane (Bio-Rad, Hercules, CA). Nonspecific binding was blocked by incubating the membranes in Tris-buffered saline containing 5% nonfat milk (TBST, 50 mmol/L Tris–HCl, 150 mmol/L NaCl, and 0.1% Tween-20) for 1 h at room temperature. Membranes were incubated overnight at 4°C with AMIGO2 antibody (1 : 400, RD), integrin β1 antibody (1 : 1000, Cell Signaling), Presenilin 2 antibody (1 : 500, ABCAM), and VAV3 antibody (1 : 500, ABCAM). After three washes with TBST, the membranes were incubated for 1 h at room temperature with the horseradish peroxidase-labeled secondary antibody. The signals were visualized with chemiluminescence and quantified with Quantity-One (Bio-Rad, America).

2.6. Luciferase Assay. The mutant construct of ITGB1 3′UTR was obtained by introducing the mutation into the 7 nucleotides (GUGCCAUU) of the seed region for miR-183. The mutant construct of AMIGO2 3′UTR was obtained by introducing two mutations into the 7 nucleotides (GUGCCAUA) (UGCCAUA) of the seed region for miR-183. The miR-183 target sequences in the coding region of ITGB1/AMIGO2 were amplified by PCR and cloned into GV143 that contained a firefly luciferase reporter gene. Wild-type ITBG1/AMIGO2 3′UTR or mutant ITBG1/AMIGO2 3′UTR and the empty 3′UTR vector were cotransfected into ESC. Cell transfection using Lipofectamine 2000 and normalization for transfection efficiency was performed according to the recommendation of manufacturer (Invitrogen). Luciferase activity was measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI).

2.7. Quantitative RT-PCR. Endometrial tissue was minced with a scalpel blade. Total RNA was isolated using TRIzol (Takara, Otsu, Shiga, Japan) and cDNA was synthesized using the SYBR Prime Script RT-PCR kit (Takara) on the ABI Prism 7500 Sequence Detection System according to manufacturer's instructions. The housekeeping gene β-actin was used for normalization. The primers used were 5′-TCGTCACGTTCCGGTTATTC (sense) and 5′-CTTTTACTTACGGTTTACCC (antisense) for ITGB1; 5′-CCTCGCCTTTGCCGATCCG (sense) and 5′-GCCGGAGCCGTTGTCGACG (antisense) for β-actin. The cycling conditions were 94°C for 1 min, 35 cycles of 94°C for 30 sec, 66°C for 30 sec, and 68°C for 3 min, followed by extension at 68°C for 3 min. Quantitative PCR was carried out using SYBR-Green JumpStart Taq ReadyMix (Sigma) and the 7300 Real-Time PCR Detection System (ABI). The results were analyzed using the comparative threshold cycle (CT) method.

2.8. Rescue Experiment. To further validate direct targeting of ITGB1 by miR-183, functional rescue experiment was performed by cotransfection with miR-183 mimic and plasmid constructs expressing ITGB1 in ESC using Lipofectamine 2000 (Invitrogen) as described above. The expression plasmid pcDNA-ITGB1, encoding human ITGB1, was purchased from Genechem. The presence of complete ITGB1 coding regions was confirmed by DNA sequencing.

2.9. Invasion (Matrigel) Chamber Assay. ESC (2.5×10^4) were seeded on a transwell insert coated with extracellular matrix (ECM) (8 mm pore size, 24-well format; Corning Costar) in 2% FBS medium. Complete medium (10% FBS) was added to the lower chamber. To determine the amount of invasion, cells were incubated for 24 h and then removed from the upper chamber using a cotton swab. The invaded cells on the underside of the insert were fixed with methanol, stained with crystal violet for 2 min, and rinsed with phosphate buffered saline (PBS). The undersides of the membrane were photographed to compare the number of invaded cells per insert. The transmigrated cells were counted using a light microscope. Invasive cells were scored by counting 10 random high-power fields per filter.

2.10. Statistical Analyses. Data represents mean ± SEM from at least 3 independent experiments. Difference between experimental and control groups was determined by Student's t-test while one-way ANOVA analysis was employed to compare three or more groups. $P < 0.05$ was considered as statistical significance.

3. Results

3.1. Gene Expression Profiling following miR-183 Overexpression. In order to screen target genes in response to miR-183, we used microarrays representing more than 20,000 genes mapped through RefSeq or via UniGene annotation. We studied gene expression alterations (up- or downregulation) at 24 h after transfection. The changes of gene expression in miR-183-overexpressing endometrial stromal cells were analyzed. Differential expression was found in 27 genes at P value < 0.05 with folds of change ≥ 1.5. Of these, 19 were upregulated and 8 downregulated (ITGB1, AMIGO2, VAV3, PSEN2, LHFPL2, HS2ST1, AHSA2, and UQCRB). Results of hierarchical cluster analyses of these genes are shown in Figure 1 and supplementary 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/340218.

3.2. Functional Analysis with GO Databases. By using the Gene Ontology (GO) database, we systematically extracted and analyzed the information of three GO categories, "biological process," "molecular function," and "cellular component." It was revealed that the identified genes were involved in hemophilic cell adhesion (ITGB1, AMIGO2), cell-cell adhesion (ITGB1, AMIGO2), cell migration (ITGB1, MYH9), positive regulation of catalytic activity (PSEN2, SHC1), and proteolysis (MYH9, PSEN2) (Table 1).

3.3. Significant Pathway Analysis. Significant pathway analysis revealed that the gene expression alterations in endometrial stromal cells were involved in pathways of PTEN (ITGB1, SHC1), TFF (ITGB1, SHC1), ECM (ITGB1, SHC1),

Table 1: List of genes with fold of change ≥1.5 ($P < 0.05$) and their biological functions.

Gene	Gene set name	P
ITGB1, AMIGO2	Hemophilic cell adhesion	$1.97E-03$
ITGB1, AMIGO2	Cell to cell adhesion	$5.02E-02$
ITGB1, MYH9	Cell migration	$6.10E-02$
PSEN2, SHC1	Positive regulation of catalytic activity	$1.52E-01$
MYH9, PSEN2	Proteolysis	$1.91E-01$

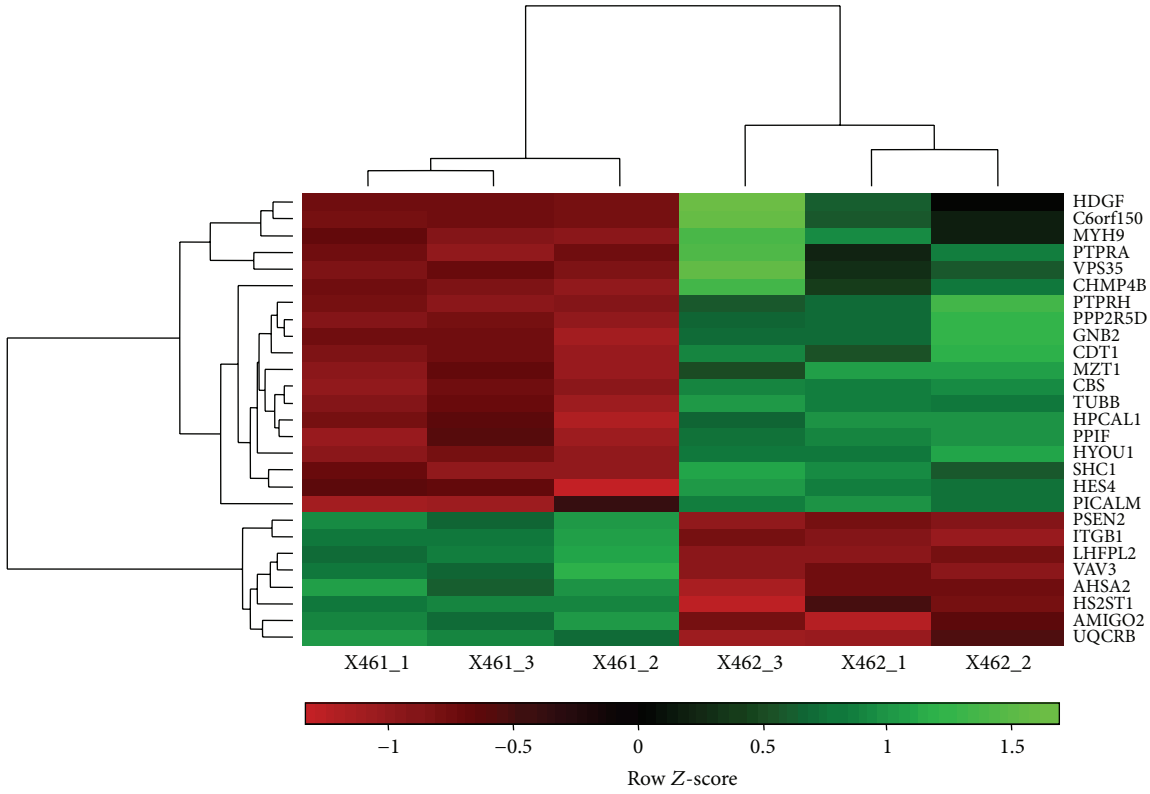

Figure 1: Hierarchical clustering of differentially expressed genes in miR-183-overexpressing endometrial stromal cells versus control cells. Gene expression profiling was conducted using PrimeView Human Gene Expression Array. Raw data were extracted from the scanned images and analyzed with GeneSpring GX software version 11.5. The data was normalized using the iterative PLIER default protocol. Differentially expressed genes were analyzed with the unpaired Student t-test. Statistically different genes ($P < 0.05$) with a greater than 1.5-fold increase or decrease were recorded. Red color indicates low expression; green color indicates relatively high expression.

ERK (ITGB1, SHC1), integrin (ITGB1, SHC1), pathogenic *Escherichia coli* infection (ITGB1, TUBB), chemokine signaling pathway (SHC1, VAV3, and GNB2), focal adhesion (ITGB1, SHC1, and VAV3), regulation of cytoskeleton (ITGB1, VAV3, and MYH9), leukocyte transendothelial migration (ITGB1, VAV3), natural killer cell-mediated cytotoxicity (SHC1, VAV3), and Alzheimer's disease (UQCRB, PSEN2) (Table 2).

3.4. Confirmation of Microarray Data by Western Blotting. Because of the inhibitory property of miRNA on target genes, we chose from the list of 8 downregulated genes (ITGB1, AMIGO2, VAV3, PSEN2, LHFPL2, HS2ST1, AHSA2, and UQCRB) in miR-183-overexpressing cells. Biological function analysis using GO databases revealed that ITGB1 and AMIGO2 were involved in cell adhesion and/or cell migration. These two genes were selected for further study. PubMed reports showed VAV3 and PSEN2 were both involved in cell invasion [10, 11], and these two genes were included as study targets as well.

Western blotting (Figure 2) was used to confirm their alterations on protein levels. Using the initial sample sets and the criteria of ≥1.5-fold of change, integrin $\beta 1$ yielded consistent results by the two technologies. Compared to the control group, the levels of integrin $\beta 1$ showed a significant decrease in the miR-183-overexpression group, whereas the miR-183-downexpressing group ($P < 0.05$) showed a sharp increase. Although endometrial stromal cells express AMIGO2 protein, the amount was largely unchanged in the miR-183 up- or downexpression groups, suggesting it was not regulated by miR-183. On the other hand, no protein expression of

Table 2: List of genes with fold of change ≥1.5 ($P < 0.05$) and the pathways involved.

Gene	Gene set name	Pathways	P
ITGB1, SHC1	BIOCARTA_PTEN_PATHWAY	PTEN dependent cell cycle arrest and apoptosis	$3.08E-03$
ITGB1, SHC1	BIOCARTA_TFF_PATHWAY	Trefoil factors initiate mucosal healing	$4.19E-03$
ITGB1, SHC1	BIOCARTA_ECM_PATHWAY	Erk and PI-3 kinase are necessary for collagen binding in corneal epithelia	$5.46E-03$
ITGB1, SHC1	BIOCARTA_ERK_PATHWAY	Erk1/Erk2 Mapk signaling pathway	$7.39E-03$
ITGB1, SHC1	BIOCARTA_INTEGRIN_PATHWAY	Integrin signaling pathway	$1.34E-02$
ITGB1, TUBB	KEGG_PATHOGENIC_ESCHERICHIA_COLI_INFECFECTION	Pathogenic *Escherichiacoli* infection	$3.06E-02$
SHC1, VAV3, GNB2	KEGG_CHEMOKINE_SIGNALING_PATHWAY	Chemokine signaling pathway	$5.75E-02$
ITGB1, SHC1, VAV3	KEGG_FOCAL_ADHESION	Focal adhesion	$6.59E-02$
ITGB1, VAV3, MYH9	KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	Regulation of actin cytoskeleton	$7.82E-02$
ITGB1, VAV3	KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATRATION	Leukocyte transendothelial migration	$1.05E-01$

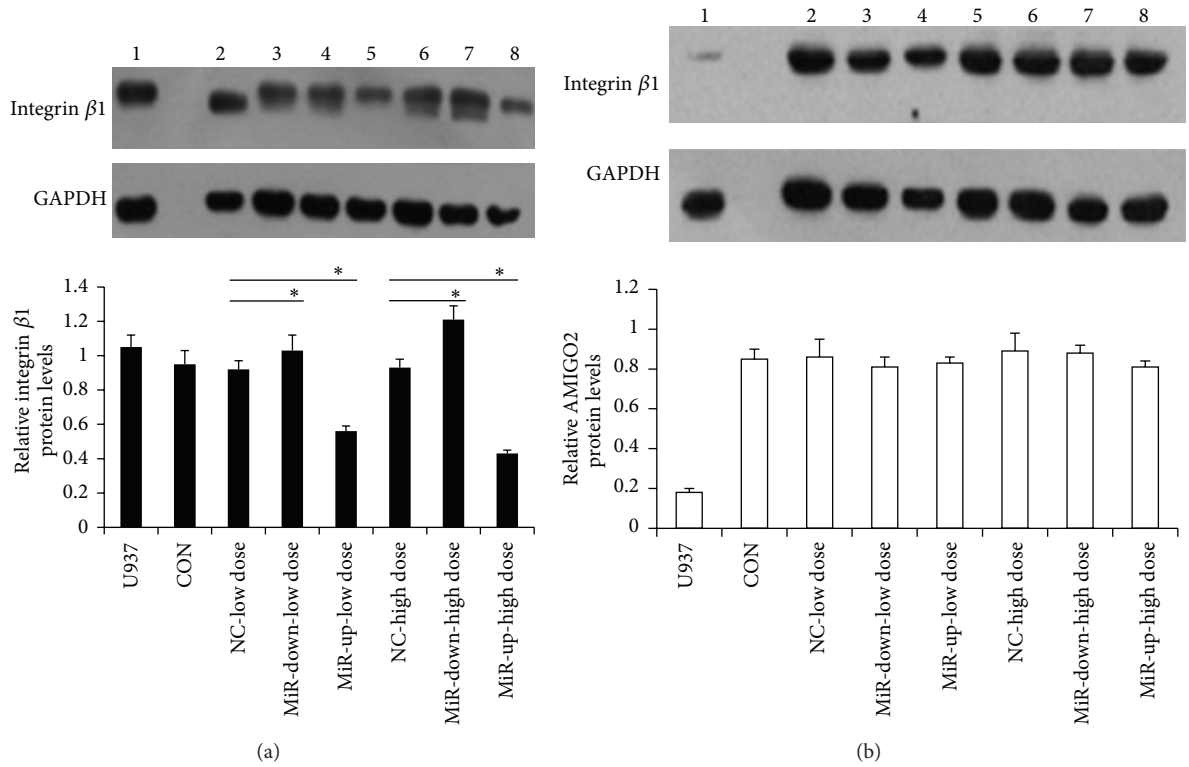

Figure 2: Confirmation of microarray data with Western blotting analyses. The endometrial stromal cells transfected with miR-183-lentivirus (low dose or high dose), In-miR-183-lentivirus (low dose or high dose), and GFP-lentivirus (NC, normal control) were used to examine the expression alterations of integrin β1 (a) and AMIGO2 (b). β-actin served as protein loading control. Compared with the control group, integrin β1 expression in the miR-183-overexpression group was significantly decreased, whereas it increased sharply in cells with low miR-183 expression. AMIGO2 was largely not regulated by miR-183. $^{*}P < 0.05$ when compared to the negative control. Error bars represent ± SEM.

Presenilin 2 and VAV3 was detected in endometrial stromal cells (data not shown) and these two genes were not further studied.

3.5. MiR-183 Directly Targets ITBG1, but Not AMIGO2. To investigate if ITBG1 and AMIGO2 are the direct targets of miR-183, we performed luciferase assay to determine whether miR-183 binds to the 3′UTR of ITBG1/AMIGO2 mRNA (Figure 3(a)). Our results showed that reporter plasmid containing the miR-183 targeting sequence of ITGB1 mRNA displayed a significantly decreased luciferase activity in cells transfected with miR-183 ($P < 0.05$), whereas luciferase

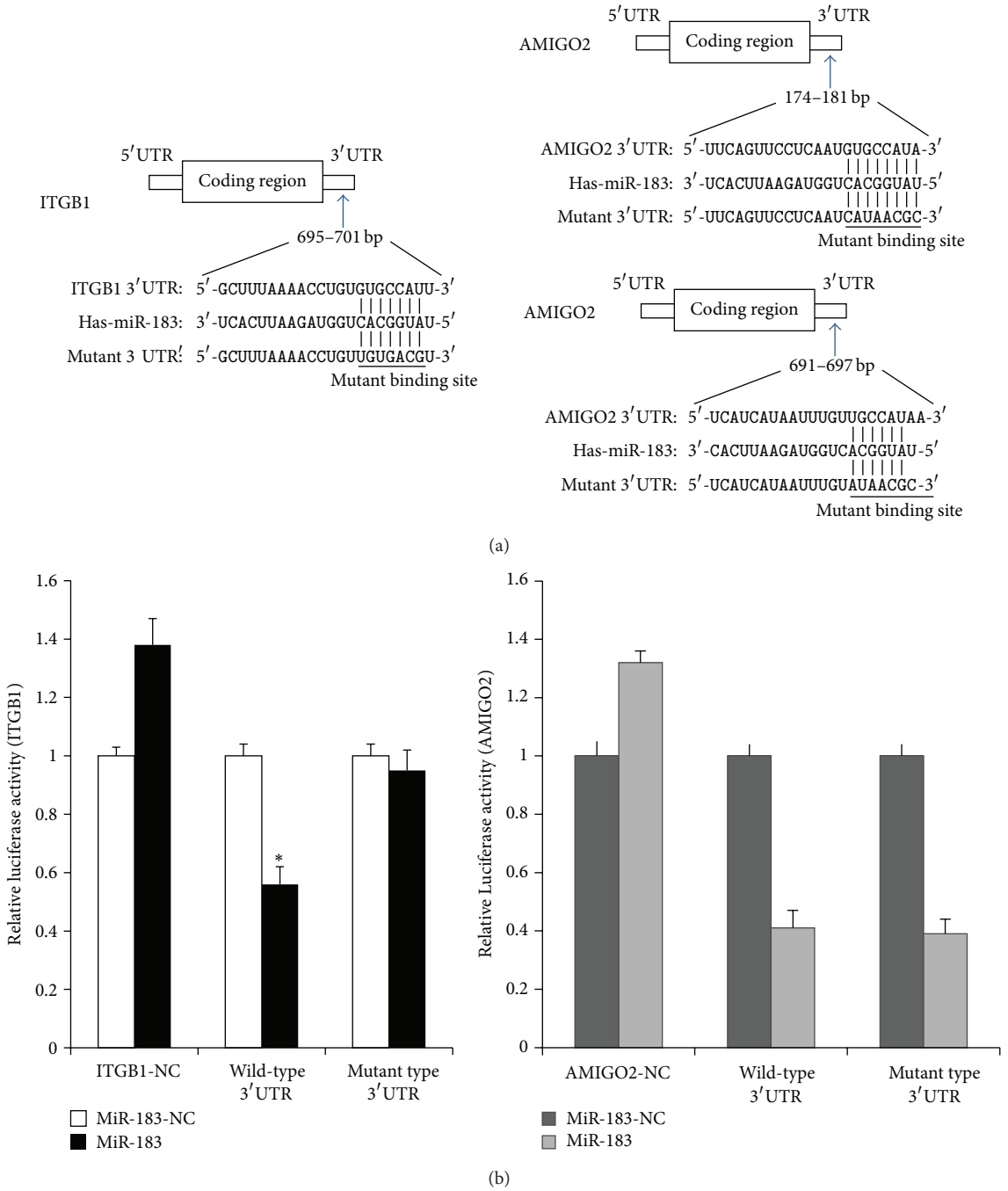

Figure 3: ITGB1, but not AMIGO2, is the direct target of miR-183. (a) The binding site of miR-183 in the 3′UTR of ITGB1 (top) and AMIGO2 (bottom) 3′UTR, along with the mutant construct in the predicted binding region. (b) MiR-183, but not AMIGO2, directly targets ITBG1. Endometrial stromal cells were cotransfected with wild-type reporter containing the ITBG1/AMIGO2 3′UTR or mutant ITBG1/AMIGO2 3′UTR plus miR-183 or negative control using Invitrogen Lipofectamine 2000 reagents. Luminescence was measured after 24 hours of transfection. Reporter plasmid expressing ITGB1 mRNA that contains sequences potentially targeted by miR-183 displayed a significantly decreased luciferase activity in cells transfected with miR-183 ($P < 0.05$), whereas luciferase activity of reporter plasmid containing mutant sequence of ITGB1 was not changed following forced expression of miR-183. $^{*}P < 0.05$ when compared to the negative control. Error bars represent ± SEM.

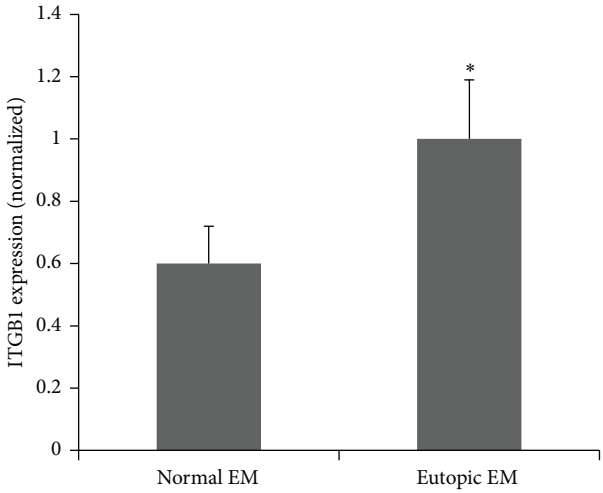

Figure 4: Validation of ITGB1 expression in eutopic endometrium with endometriosis ($n = 19$) versus normal group ($n = 18$) by qPCR. The results were analyzed using the comparative threshold cycle (CT) method. Gene expression data is presented as the fold of change relative to the levels of reference gene. $^{*}P < 0.05$ when compared to the normal group. Error bars represent ± SEM.

activity of reporter plasmid containing mutant sequence of ITGB1 was not changed following forced expression of miR-183 (Figure 3(b)). We found no significant change when compared to the luciferase activity between cells transfected with wild-type or mutated AMIGO2 3′UTR ($P > 0.05$, Figure 3(b). Thus, these data suggested that miR-183 negatively regulates the expression of ITBG1 by directly targeting their 3′UTR, whereas miR-183 does not directly target AMIGO2 3′UTR.

3.6. ITGB1 Is Overexpressed in Endometrium from Endometriosis Patients. Real-time RT-PCR results indicated that ITGB was significantly increased in the eutopic endometrial tissues from patients with endometriosis ($n = 19$) compared with endometrium from normal women ($n = 18$) ($P < 0.05$) (Figure 4).

3.7. Overexpression of ITGB1 Rescued the Repressive Effects of miR-183 on Endometrial Stromal Cells. To ascertain that miR-183 regulates the function of endometrial stromal cells through its interaction with ITGB1, a rescue experiment was performed. Overexpression of ITGB1 partially rescued the repressive effects of miR-183, leading to elevated invasion abilities in the cells (Figure 5). This data indicated that miR-183 targets ITGB1, which in turn led to negative regulation on the invasive activity of endometrial stromal cells.

4. Discussion

In the previous study, we performed miRNA microarray screening and identified several differentially expressed miRNAs in the normal, eutopic, and ectopic endometrium. Among these miRNA species, miR-183 was found to be downregulated in the ectopic and eutopic tissues. Functional analysis indicated that miR-183 promoted endometrial stromal cell apoptosis and had a negative regulatory impact on the invasive ability of cells, although it had no effect on cell proliferation [8]. These findings suggested that the downregulation of miR-183 expression might be involved in the pathogenesis of endometriosis. The molecular mechanisms, however, remain to be characterized.

MiR-183 is a member of a miRNA family (miR-183, miR-182, and miR-96) that clusters within a 2–4 kb region at chromosome 7q32. These miRNAs are known to regulate cell differentiation, apoptosis, motility, adhesion, and invasion [12]. Moreover, it has been reported that miR-183 is upregulated in colorectal cancer, prostate cancer, and hepatocellular carcinomas and downregulated in ovarian cancer, breast cancer stem cells, and osteosarcomas, pointing to varied and cell type-dependent function of miR-183. Several target genes of miR-183 including ITGB1, Taok1, Ezrin, EGR1, PDCD4, and LRP6 have been identified and validated [13–19].

To systematically investigate the potential mechanism by which miR-183 may contribute to the development of endometriosis, we performed microarray analysis to identify the target genes with relevance to cell functions in miR-183-overexpressing endometrial stromal cells. Eight genes were found to be downregulated in endometrial stromal cells with miR-183 overexpression. Among them, the GO databases, pathway analyses, and PubMed reports pointed to important association of 4 genes (ITGB1, AMIGO2, VAV3, and PSEN2) with cell adhesion, cell migration, and cell invasiveness. Western blotting results showed that endometrial stromal cell secreted integrin β1 and AMIGO2, but not VAV3 and Presenilin 2. The protein expression of integrin β1 appeared to be a direct regulation target of miR-183, which has been proved in human trabecular meshwork cells, human diploid fibroblasts, and hela cells [19]. AMIGO2 was not directly regulated by miR-183.

According to Sampson's theory, endometriosis is thought to be initiated via retrograde endometrium into peritoneal cavity during menstruation. However, while this phenomenon occurs in approximately 75–90% of women, far fewer suffer the disease, suggesting that additional factors must contribute to the process. Invasion to ectopic locations is the key step for the initiation of endometriosis, during which adhesion molecules play an important role. The integrin family includes glycoproteins that form dimeric structure and mediate cell attachment to the ECM components [20]. It has been reported that β1 integrin-depleted cells had reduced invasive capabilities, and β1 integrin signaling was involved in cancer cell invasion [21]. Integrin β1 is constitutively expressed in endometrial stromal cells and endometriotic lesions [22]. Increased integrin β1 protein expression in endometrial stromal cells was observed in endometriotic compared to normal endometrial tissues [23]. It was reported that downregulation of prostaglandin E2 receptors EP2 and EP4 led to inhibited adhesion of human endometriotic epithelial and stromal cells. Importantly, this inhibition could be mediated through suppression of integrin-mediated mechanisms [24]. This finding provided a strong evidence

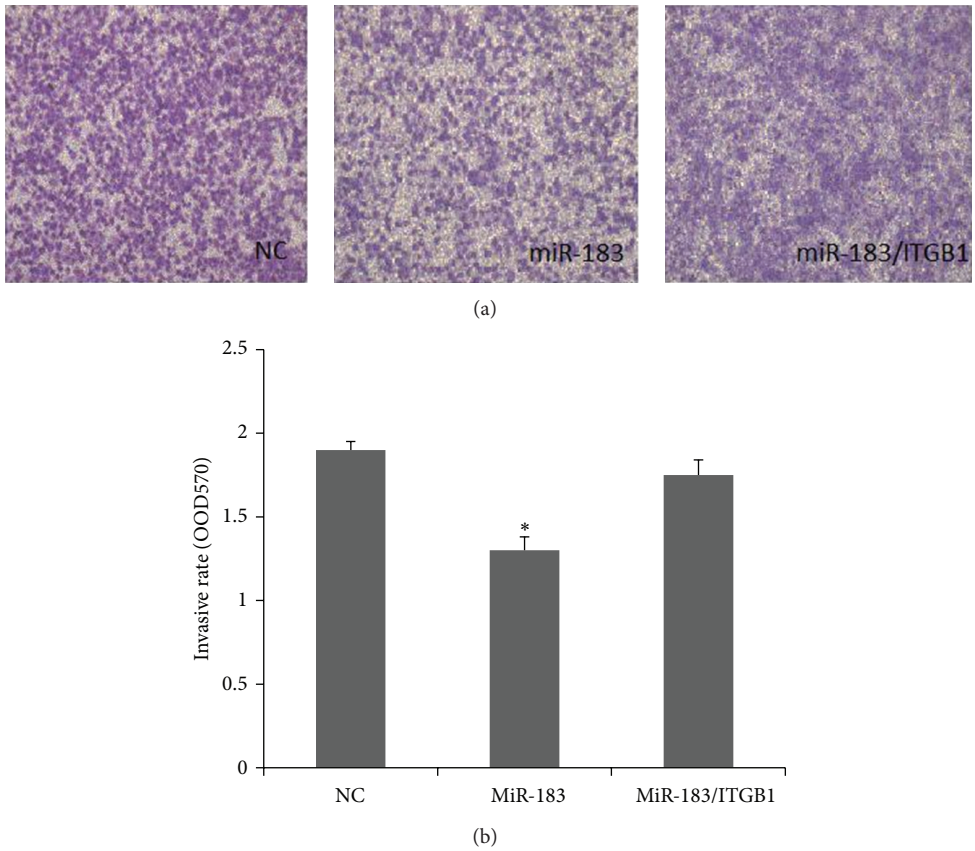

Figure 5: Overexpression of ITGB1 rescued the repressive effects of miR-183 on endometrial stromal cells, leading to elevated invasive abilities in transwell assays. Endometrial stromal cells were infected with miR-183-lentivirus, miR-183/ITGB1-lentivirus, and GFP-lentivirus in upper wells. After 24 hours, the number of cells that invaded through Matrigel was counted in at least 10 fields per well. (a) Representative photographs show that miR-183 inhibited the invasiveness of endometrial stromal cells, whereas ITGB1 partially rescued the repressive effects of miR-183. (b) Cell counting results indicate that ITGB1 overexpression rescued the repressive effects of miR-183. $^{*}P < 0.05$ when compared to the negative control. Error bars represent ± SEM.

for the significance of integrins in the adhesion function of endometrial stromal cells.

AMIGO2 (ALI1, DEGA) was first reported to be preferentially expressed in the central nervous system. Sequence analyses revealed that the protein contains seven leucine-rich repeats, one IgC2-like loop, and a transmembrane domain and displays homology to Kek and Trk families [25–27]. In the gastric adenocarcinoma cell line, AMIGO2 was reported to be involved in cell adhesion, extracellular matrix, and basement membrane formation [28]. AMIGO2 was also implicated in oral carcinogenesis, by preventing the arrest of cell cycle progression at the G1 phase through inhibiting the expression of cyclin-dependent kinase inhibitors. However, little is known about the role of AMIGO2 in the development of endometriosis.

To investigate whether miR-183 directly targets ITGB1 and AMIGO2, dual luciferase reporter assays were carried out. We found that miR-183 negatively regulated the expression of ITBG1 by directly targeting its 3′UTR but might not directly regulate the expression of AMIGO2. We further detected differential expression of ITGB1 in endometrium with or without endometriosis by quantitative RT-PCR. The higher expression of integrin β1 in endometrium tissues in endometriosis was confirmed, which is consistent with the result for higher integrin β1 expression in ectopic stromal cells than normal endometrial stromal cells. Previous studies have shown that integrin β1 mediates cell adherence to extracellular matrix [29]. Indeed, the rescue experiment indicated that overexpression of ITGB1 blocked the repressive effects of miR-183 on endometrial stromal cell invasiveness, which provided a critical evidence supporting that the lower expression of miR-183 may promote ITGB1 expression and contribute to the development of endometriosis.

In conclusion, altered miR-183 expression may cause deregulation of its target gene ITBG1, affecting the cell adhesiveness and invasiveness of endometrial stromal cells, which in turn lead to pathogenesis of endometriosis. These findings revealed a novel pathologic role of miR-183 for endometriosis. It would be of great interest to investigate if these alterations may serve as biomarkers for the prediction

and/or treatment of endometriosis. The regulation of miR-183 and the exact mechanism by which miR-183 regulates ITBG1 expression remain to be characterized.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Paracrine Regulation of Steroidogenesis in Theca Cells by Granulosa Cells Derived from Mouse Preantral Follicles

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Interaction partners of follicular cells play a significant role in steroidogenesis, follicular formation, and development. Androgen secreted by theca cells (TCs) can initiate follicle development and ovulation and provide precursor materials for estrogen synthesis. Therefore, studies on ovarian microenvironment will not only lead to better understanding of the steroidogenesis but also have clinical significance for ovarian endocrine abnormalities such as hyperandrogenism in polycystic ovary syndrome (PCOS). This study applied the Transwell coculture model to investigate if the interaction between granulosa and theca cells may affect androgen production in theca cells. Concentrations of testosterone and androstenedione in the spent medium were measured by radioimmunoassay and enzyme linked immunosorbent assay, respectively. The results show that the coculture with granulosa cells (GCs) increases steroidogenesis in TCs. In addition, testosterone and androstenedione productions in response to LH stimulation were also increased in the coculture model. Significantly increased mRNA expressions of steroidogenic enzymes (*Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b2*) were observed in the cocultured TCs. Thus, GCs were capable of promoting steroidogenesis and LH responsiveness in TCs. This study provided a basis for further exploration of ovarian endocrine mechanism and pathologies.

1. Introduction

Oocyte, granulosa cells (GCs), and theca cells (TCs) are the main components of follicles, and the functions and interactions among them play crucial role(s) in steroidogenesis, follicular development, and atresia [1]. Oocyte-granulosa cell communications, largely carried out by gap junctions, are essential for the female gametogenesis and formation and maturation of follicles as well as embryonic development after fertilization. Some paracrine factors such as growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) are specifically secreted by oocyte. These factors regulate follicle formation and GCs/TCs proliferation and differentiation [2, 3]. On the other hand, insulin-like growth factor-I (IGF-I), activin, and inhibin secreted by GCs are autocrine/paracrine regulators contributing to the modulation of ovarian function [4]. TCs do not only support the structure and functionality of the developing follicle but also provide androgen for estrogen biosynthesis in GCs, which stimulates the early follicle growth [5, 6].

Androgen is mainly secreted from ovary and adrenal gland. In ovaries, the steroidogenesis is accomplished by both the granulosa cells and theca cells. The synthesis of various steroids is determined by cell-specific expression of a series of steroidogenic enzymes. The transportation of cholesterol into the mitochondria is facilitated by STAR which regulates the production of androgens. It is established that the androgen-secreting theca cells express P450Scc (CYP11A1), P450C17 (CYP17), and HSD3B2, all of which are key enzymes essential for androgen biosynthesis. In hyperandrogenemia patients, the circulatory testosterone and androstenedione are primarily produced by follicular TCs of ovary [7, 8]. Although some investigator observed that oocyte-derived factors such as GDF-9 directly upregulated *Cyp17* mRNA and protein expression in rats [9, 10] and paracrine regulations of GCs may also enhance steroid production in whole follicles, many

aspects of the signaling network among different groups of follicular cells, especially their effects on steroid synthesis, remain to be investigated.

In vitro studies have shown that, as a key feature of TCs, the increment of androgen production was driven by LH in a dose-dependent manner [5, 11, 12]. Although these findings suggested that increased LH secretion may be pivotal for the upregulation of androgen production, some other studies in women with polycystic ovary syndrome (PCOS) found that its main effect was to promote the conversion of progesterone to androgen [13]. There appears a complex network of signals between GCs and TCs on the regulation of androgen production under different pathophysiological conditions. Unfortunately, studies on GCs regulation of the steroidogenesis in TCs in early follicle development are scattered and the regulatory mechanism remains poorly understood [14]. Specifically, literature search failed to find any systematic investigation focusing on the paracrine regulation of TCs' steroidogenesis by GCs using cells derived from mouse preantral follicles.

The objective of this study is to explore the functional interactions between GCs and TCs and their involvement in steroid metabolism. We applied the Transwell coculture model to determine the effects of GCs on the steroidogenic function of TCs in the mouse preantral follicles by measuring the change of androgen concentration in response to LH stimulation. Results of the study will provide basic information on the significance of cell interactions for steroidogenesis in ovary.

2. Materials and Methods

2.1. Animals. Immature Female ICR mice for research (21 days old) were obtained from Lab Animal Center of Weifang Medical University. Mice were housed under controlled conditions with a daily lighting (12 h light and 12 h darkness). Food and water were provided *ad libitum*. All animal management procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

2.2. Cell Isolation and Culture. TCs were isolated from mice aged 21 days and cultured following published protocols with minor modifications [15, 16]. Briefly, isolated ovaries were immediately transferred to the prewarmed Medium 199 (GIBCO, Grand Island, NY) containing 0.1% BSA, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Ovaries were cleaned of any surrounding tissue and repeatedly punctured with 29 G needles to release GCs. The remaining ovarian tissues were incubated in Medium 199 supplemented with 4 mg/mL collagenase I (Worthington Biochemical Corp, Lakewood, New Jersey), 10 μg/mL DNase I (Worthington Biochemical Corp, Lakewood, New Jersey), and 0.1% BSA for 60 min at 37°C following three times of washing with Medium 199. Then the TCs were placed in Medium 199 containing 0.1% BSA, 1% FBS, 1% insulin-transferrin-selenium (ITS) (GIBCO, Grand Island, NY, USA), 100 IU/mL of penicillin, and 100 μg/mL streptomycin. After overnight incubation, the wells were washed with Medium 199 to remove unattached cells.

For GCs collection, the released cells from the above steps were placed in prewarmed medium of DMEM/F12. Following three times of washing, the GCs were transferred to the conditioned DMEM/F12 medium containing 0.1% BSA, 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. After overnight incubation, the wells were washed with DMEM/F12 medium to remove unattached cells. Cellular morphology was observed under the microscope.

2.3. Immunofluorescence Detection of CYP17A1 and FSHR. TCs were seeded on coverslip following washing with Medium 199 to remove unattached cells after overnight incubation. For the identification of TCs and GCs, a CYP17A1 antibody, a marker enzyme specifically expressed in TC, was used to verify the purity and identify the cells. Icy acetone was added and a coverslip was placed at −20°C for 10 minutes. After washing and permeabilization with PBS containing 0.5% Triton X-100, the cells were blocked with goat serum (Zhongshan Jinqiao Biotechnology Co. Ltd., Beijing, China) for 1 h in a humidified chamber at 37°C. Subsequently, slides were incubated with CYP17A1 antibody (1 : 200, Santa Cruz, CA) or FSHR antibody (1 : 200, Bioworld Technology, Inc., Minnesota) overnight at 4°C. Slides were incubated with secondary fluorescent FITC (1 : 100, Boster Biotechnology Co. Ltd., Wuhan, China) for 1 h in a humidified chamber at 37°C. After washing and nuclear staining with Hoechst for 10 seconds, the staining was stopped. The slides were seal-capped with Debico and observed under a fluorescence microscope.

2.4. Transwell Coculture of GCs and TCs. GCs were seeded in Transwell (3 μm pore size, 24 mm diameter; Corning, NY) inner membrane at a density of 3×10^5 and cultured with 1 mL DMEM/F12 supplemented with 0.1% BSA and antibiotics. After transfer into 6-well plate, TCs were seeded at a density of 1×10^5 with Medium 199 supplemented with 0.1% BSA, 1% ITS, and antibiotics. For androgen assay and quantitative real-time PCR analyses, cells were cultured in serum-free medium for 72 h.

To assess the effect of GCs on LH responsiveness after being cocultured for 24 h, LH was added at increasing concentrations (0, 0.01, 0.1, or 1 IU/mL) and culture continued for 48 h. Then the medium was collected for subsequent measurement.

2.5. Androgen Assays. The spent medium was collected at 24 h, 48 h, and 72 h. After centrifugation at 2500 ×g at 4°C for 15 minutes, the supernatant was stored at −80°C for androgen measurement. The concentrations of testosterone and androstenedione were determined using radioimmunoassay (RIA) kit (Beifang Biotechnique Institute, Beijing, China) and enzyme linked immunosorbent assay (ELISA) kit (J&L Biological, Shanghai, China), respectively, according to manufacturer's protocols.

Table 1: Primer sequences used for real-time PCR.

Accession number	Gene	Sequence 5′-3′	Size (bp)
NM_011485.4	*Star*	F: CCACCTGCATGGTGCTTCA R: TTGGCGAACTCTATCTGGGTCTG	142
NM_019779.3	*Cyp11a1*	F: GGCACTTTGGAGTCAGTTTACAT R: GTTTAGGACGATTCGGTCTTTCTT	186
NM_007809.3	*Cyp17a1*	F: TCTGGGCACTGCATCACG R: GCTCCGAAGGGCAAATAACT	124
NM_153193.3	*Hsd3b2*	F: GCCCCTACTGTACTGGCTTG R: TCCCGATCCACTCTGAGGTT	212
NM_001289726.1	*GAPDH*	F: AGGTTGTCTCCTGCGACTTCA R: GGGTGGTCCAGGGTTTCTTACT	187
NM_013582.2	*LHR*	F: GAGACGCTTTATTCTGCCATCT R: CAGGGATTGAAAGCATCTGG	119

2.6. Quantitative Real-Time PCR. TCs were harvested and RNA was extracted using Trizol reagents (Invitrogen, Carlsbad, CA). RNA was measured with spectrometry for OD260/280 and cDNAs were prepared with PrimeScript Reverse Transcription Kit (Takara, Dalian, China) according to manufacturer's recommendations. Real-time PCR was performed with StepOne Plus Real-Time PCR System (Applied Biosystems, USA) using SYBR Premix Ex Taq II (Takara, Dalian, China). Primers were designed for each gene using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). The designation and sequences of specific PCR primers of *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b2*, and *LHR* expression were listed in Table 1. Primers were synthesized by Invitrogen (Invitrogen Trading Co. Ltd., Shanghai, China).

Each sample was assayed in duplicate using 1 μL cDNA, 10 pmol primer, and 1 μL SYBR Green Master Mix (Applied Biosystems, USA) in a total volume of 20 μL. *GAPDH* mRNA was measured and the results served as internal control. PCR conditions were initial denaturation at 95°C for 1 min followed by 40 cycles of amplification: denaturation at 95°C for 15 sec, annealing at 60°C for 1 min, and extension at 72°C for 3 min. Melting curve analysis was performed to confirm the specificity of amplification. The difference of CT between steroidogenic enzymes and *GAPDH* was defined as ΔCT. The difference of ΔCT between experimental and control groups was defined as ΔΔCT. The relative quantity was determined by $2^{-\Delta\Delta CT}$ [17].

2.7. Statistical Analysis. All data were expressed as mean ± SEM from at least three independent experiments. For comparison of quantitative data of two groups, Student's *t*-test was used, and for multiple comparisons, one-way ANOVA (SPSS 16.0) was used. $P < 0.05$ was considered as reaching a statistically significant level.

3. Results

3.1. Verification on the Purity of Theca Cells. After overnight incubation, the wells coated with TCs were washed with medium to remove unattached cells. The cellular morphology and confluence of TCs were observed under the microscope. Typically, the attached cells looked thin and dendritic, indicating their TC origin. By a series of titration experiments, the density of TCs was adjusted to approximately 70% confluence. The purity of TCs was further verified with immunofluorescence staining using a TC-specific marker Cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1, also known as 17a-hydroxylase/17, 20-lyase), and the GC-specific marker Follicle Stimulating Hormone Receptor (FSHR) for reverse identification. The results in Figure 1 demonstrated that more than 90% of the cells were stained positive for CYP17A1, confirming these cells' TC origin. As FSHR is a GC-specific marker not TCs, we used it to confirm that the extracted cells were TCs and contain almost no GCs by reverse identification. Results from control experiments using FSHR antibody did not show significant fluorescence signal, indicating that these TCs cultures were largely free of GC contamination.

3.2. Effect of Granulosa Cells on the Steroidogenesis in Theca Cells. Concentrations of testosterone and androstenedione in the spent medium were measured at 24 h, 48 h, and 72 h using RIA and ELISA, respectively. When cocultured with GCs, TCs produced an increased amount of testosterone and androstenedione than TCs cultured alone ($P < 0.05$, Figure 2(a) for testosterone and Figure 2(c) for androstenedione). Interestingly, when TCs were cultured alone, the production of testosterone and androstenedione showed a declining trend along time, which may reflect the adaptation change to *in vitro* conditions lacking the supports from other cell types. In the spent medium of GCs cultured alone, the levels of testosterone (Figure 2(b)) and androstenedione (Figure 2(d)) were both diminished in comparison with TCs. And no alterations were observed following different time of *in vitro* culture ($P > 0.05$). Taken together, these results indicated that GCs could promote TCs production of testosterone and androstenedione.

3.3. Expression of Steroidogenic Enzymes in the Cocultured Model. To investigate the regulatory pathway leading to the

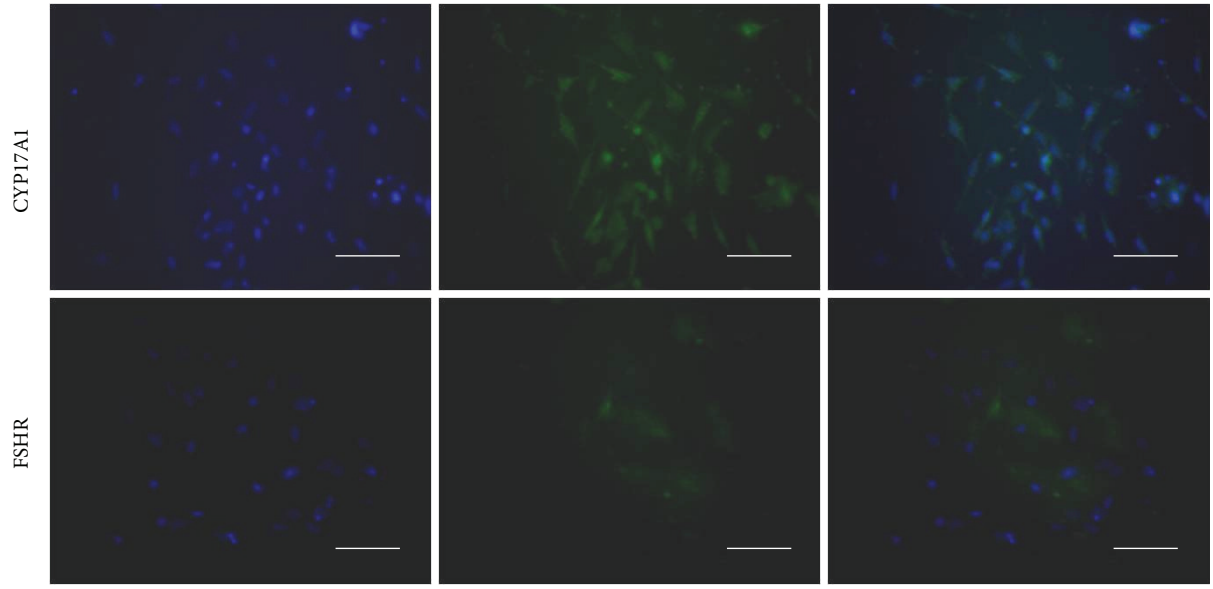

Figure 1: Immunofluorescence staining of CYP17A1 and FSHR in theca cells. Scale bar = 100 μm. The purified cells were examined with immunofluorescence method after 24 h. By a series of titration experiments, the density of TCs was adjusted to approximately 70% confluence. FITC (green) staining CYP17A1 indicated the TCs origin. Hoechst staining (blue) indicated the location of cell nuclei. More than 90% of the cells were stained positive for CYP17A1, confirming the purity of TC cells population. Weak staining with FSHR antibody indicated that these TCs cultures were largely free of GCs contamination.

increased androgen production, quantitative real-time PCR was performed to determine the expression of genes involved in androgen synthesis *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b2* at 24 h, 48 h, and 72 h. As shown in Figure 3, the results revealed that the expressions of all these genes were increased in the cocultured model compared with TCs cultured alone ($P < 0.05$). Thus, increasing expression of the genes involved with androgen synthesis appeared to account for the increased androgen production. These results indicated that paracrine factors secreted from GCs can pass the Transwell insert membrane and regulate the transcription of steroidogenic enzymes.

3.4. Effects of Granulosa Cells on the Expression of LHR mRNA in Theca Cells. While the *LHR* mRNA expression of TCs in the cocultured model manifested no difference compared with TCs cultured alone at 24 h ($P > 0.05$, Figure 4(a)), at 48 h the *LHR* mRNA expression of TCs increased significantly ($P < 0.05$, Figure 4(a)), indicating that paracrine factors secreted from GC cells could stimulate the *LHR* mRNA expression in TCs. The subsequent disappearance of difference between TCs cultured alone and cocultured model may reflect the suboptimal conditions related to *in vitro* environment. The cocultured GCs could enhance the capacity of LH responsiveness through increasing *LHR* mRNA expression in TCs.

It was reported that, in TCs, LH upon binding to LH receptor could regulate the expressions of a series of synthesis enzymes through activation of cAMP/PKA signaling pathway, facilitating the synthesis and secretion of steroids [18, 19]. To determine the effect of GCs on androgen production by TCs in response to LH, the levels of testosterone and androstenedione in the spent medium were measured following stimulation with LH. The results revealed that testosterone and androstenedione production significantly increased in response to different concentrations of LH in the cocultured model ($P < 0.05$, Figures 4(b) and 4(c)). However, there was no difference in the amount of testosterone ($P > 0.05$, Figure 4(b)) or androstenedione ($P > 0.05$, Figure 4(c)) even in the presence of LH when TCs were cultured alone.

4. Discussion

On the basis of two-cell/two-gonadotropin theory of ovarian steroid synthesis, androgen production is initiated by steroidogenic enzymes (STAR, P450Scc, P450C17, and HSD3B2) in TCs and then subsequently aromatized in GCs [20]. Based on the findings that TCs androgenic pathways can be regulated by GCs via a paracrine pathway [21], we attempted to confirm that this phenomenon exists in mouse preantral follicles using the cocultured model. In this Transwell coculture model, hormones and paracrine factors secreted from follicular cells such as steroids, growth factors, and cytokines can pass the Transwell insert membrane. The cocultured model can mimic to a certain extent the local ovarian steroidogenesis by supporting similar structural integrity of follicles. Through measuring the steroidogenesis in this model, we can observe the effects of extensive cross talk and intercellular signaling between GCs and TCs, validating the efficacy of the model for studying the cell-cell interactions. In future, the coculture can be applied to investigate the significance of interactions between GCs and TCs under various pathological conditions such as hyperandrogenemia

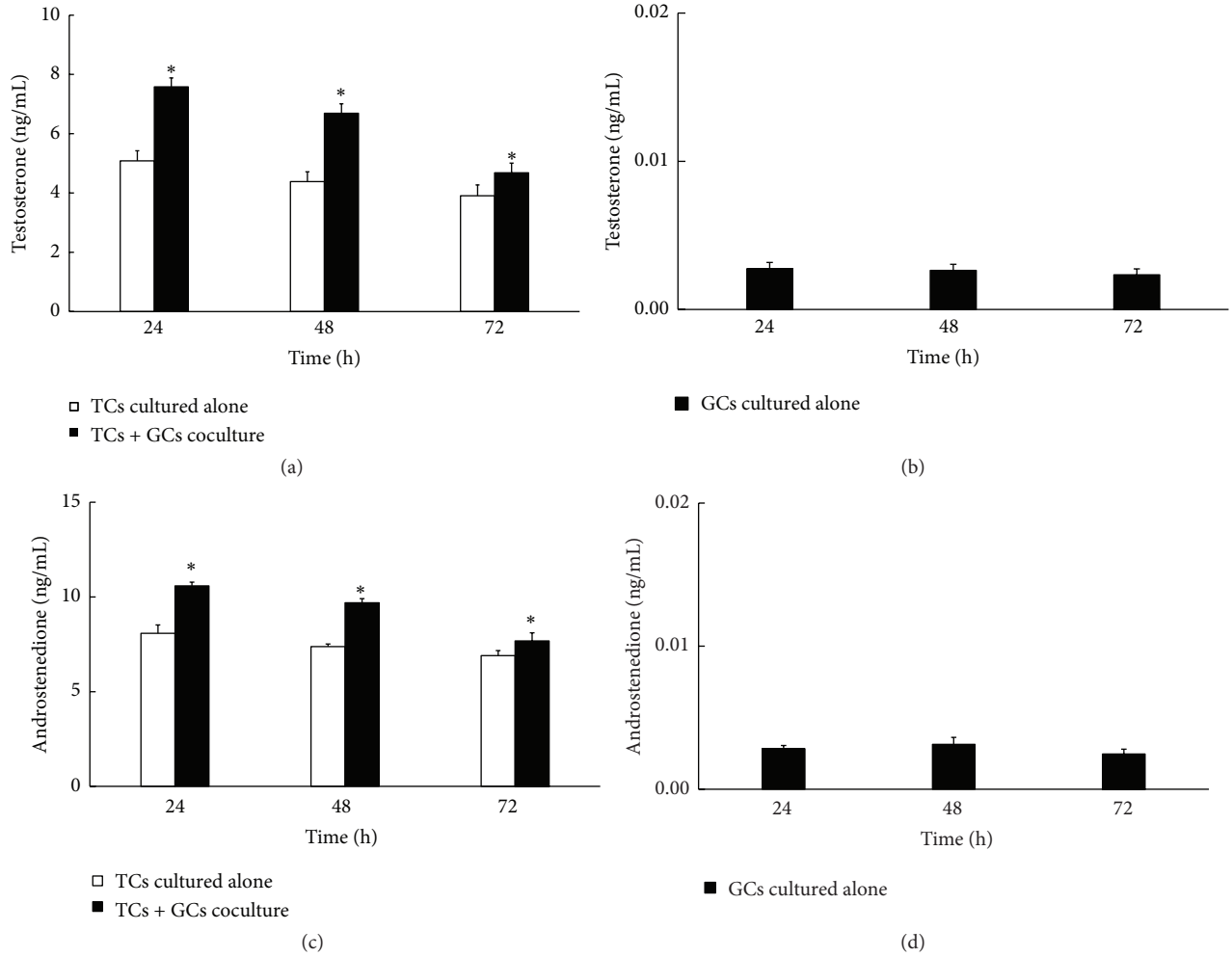

Figure 2: Effect of GCs on steroidogenesis in TCs. Concentrations of testosterone and androstenedione in the spent medium were measured by radioimmunoassay and enzyme linked immunosorbent assay, respectively. (a) and (c) An increased amount of testosterone and androstenedione was observed in cocultured model. (b) and (d) GCs produced diminished levels of testosterone and androstenedione, respectively. Note the scale difference between (a) and (c) as well as between (b) and (d). Data were expressed as mean ± SEM of three independent experiments. The asterisk marked the statistically significant difference.

in PCOS [22] where the expression of steroidogenic enzymes was much increased [23].

Parrott and Skinner reported that GCs could promote ovarian interstitial cells recruitment to form theca cell layers around follicles and that inhibin secreted from GCs could induce maturation of the dominant follicle and the production of androstenedione [24]. These findings indicated that GCs probably provide some essential regulating factors for TCs on steroidogenesis, such as steroids, growth factors, cytokines, and extracellular matrix and maintain the ability of TCs for androgen synthesis by paracrine regulation. In this study, we evaluated the functions of ovarian cells by measuring the change in androgen concentrations. When cocultured with GCs, TCs produced an increased amount of testosterone and androstenedione than TCs cultured alone. This increase was driven by enhancing the expressions of steroidal synthetase (STAR, P450Scc, P450C17, and HSD3B2) in TCs. As more P450Scc was expressed, cholesterol is converted to pregnenolone and progesterone. When precursor of steroid runs out, both testosterone and androstenedione productions showed a declining trend over time. This illustrates that not only oocyte-derived factors but also GCs-derived factors participate in steroid production in ovaries. How and by what pathway these autocrine/paracrine regulators derived from these follicular cell types exert effects on steroidogenesis still needs further investigation.

To determine the effect of GCs on androgen productions of TCs in response to LH, acquisition of LH responsiveness in preantral follicular TCs from the mouse ovary was evaluated. In this study, we found that the cocultured GCs could enhance the capacity of LH responsiveness on increasing *LHR* mRNA expression in TCs. The presence of GCs led to a significant increase on *LHR* mRNA expression of TCs at 48 h but manifested no difference compared with TCs cultured

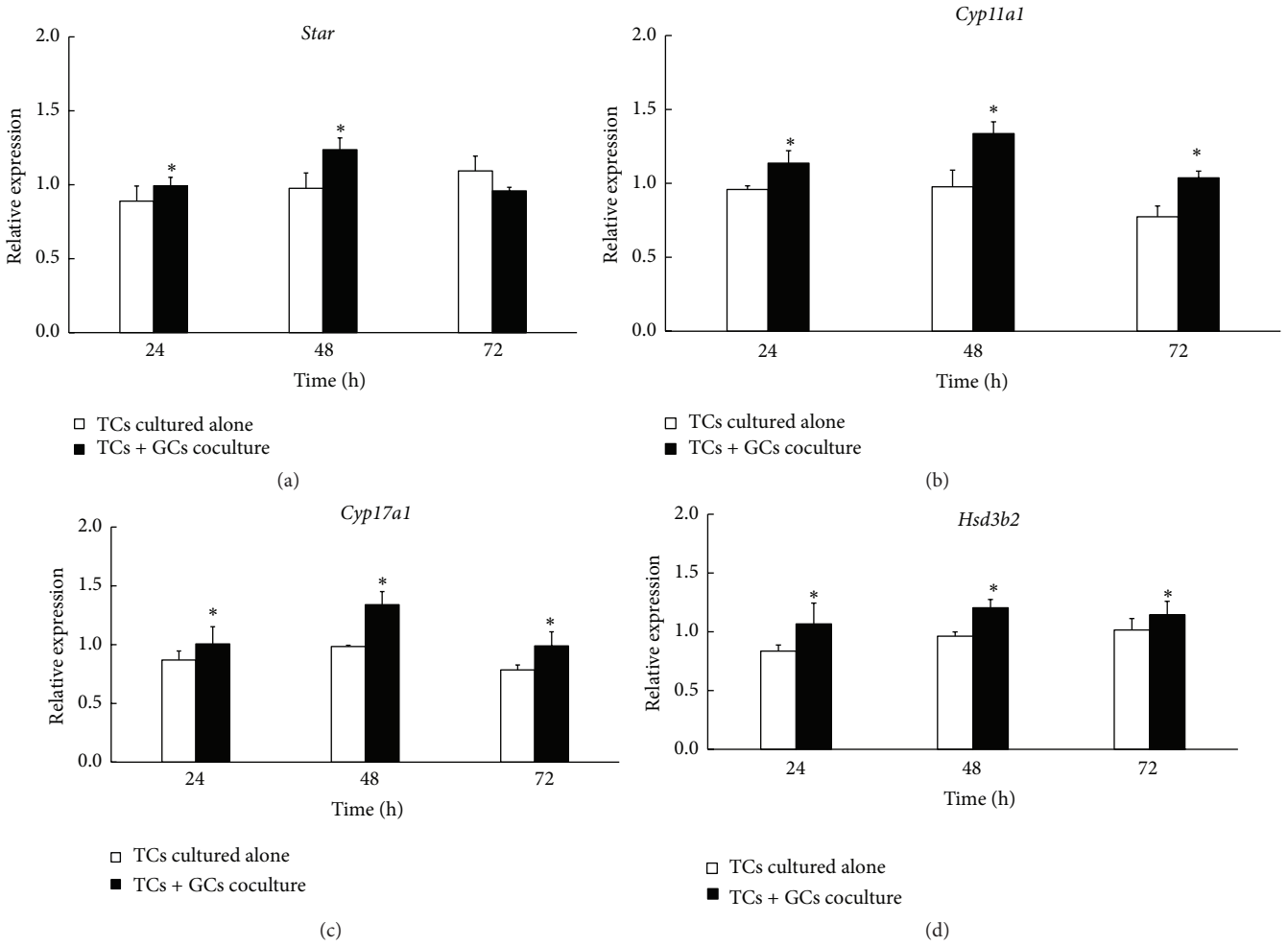

Figure 3: Effect of granulosa cells on mRNA expression of genes encoding steroidogenic enzymes (*Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b2*) in TCs. After TCs were cultured up to 72 h, the expressions of *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b2* were measured by quantitative real-time PCR (*GAPDH* as internal control). Expressions of all these genes were increased in the cocultured model compared with TCs cultured alone. Data were the mean ± SEM of three independent experiments where all the samples were repeated in thrice. The asterisk showed the statistically significant difference. $^{*}P < 0.05$.

alone at 24 h and 72 h most likely due to the suboptimal *in vitro* culture conditions, for example, the lack of support by LH and other hormones.

It is generally accepted that LH responsiveness and responses to LH on androgen production are two well-established functions of TCs in ovary [25–27]. By cAMP/PKA signal pathway, LH could regulate the expression of a range of androgen synthetases and facilitates the transcription of *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b2* [28]. Previous studies have shown that the capacity of TCs on androgen synthesis and secretion in response to LH appears to be dose-dependent [5, 11, 12]. These findings are consistent with the clinical observation in women with PCOS that elevated serum LH levels positively correlate with serum testosterone concentrations [29, 30]. Rosenfield and Bordini [31] found that moderate levels of testosterone appear to stimulate LH production at both the hypothalamic and pituitary levels, while high levels of testosterone suppress the effect of LH. Another study indicated that the level of testosterone was low, while androstenedione was high in PCOS patients [32]. In our study, when treated cocultured model with different concentrations of LH (0-1 IU/mL), concentrations of testosterone and androstenedione increased in low concentrations of LH but no further increase was observed at high concentrations. These results were in accordance with previous studies, which suggested that excessive testosterone may suppress the response to LH [5, 31, 32]. Taken together, these findings indicated that GCs could strengthen the capacity of LH by facilitating the transcription of *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b2*, therefore inducing androgen synthesis in TCs.

As an important component of a follicle, TCs exert certain effects on steroidogenesis, follicular development, and atresia. In the present study, the *in vitro* cocultured model including GCs and TCs from mouse preantral follicles recapitulates some native ovary function. However, it is unable to completely represent *in vivo* microenvironment of whole follicles. Furthermore, it is not yet known what effects TCs-derived factors may exert on GCs and oocytes. Therefore, the

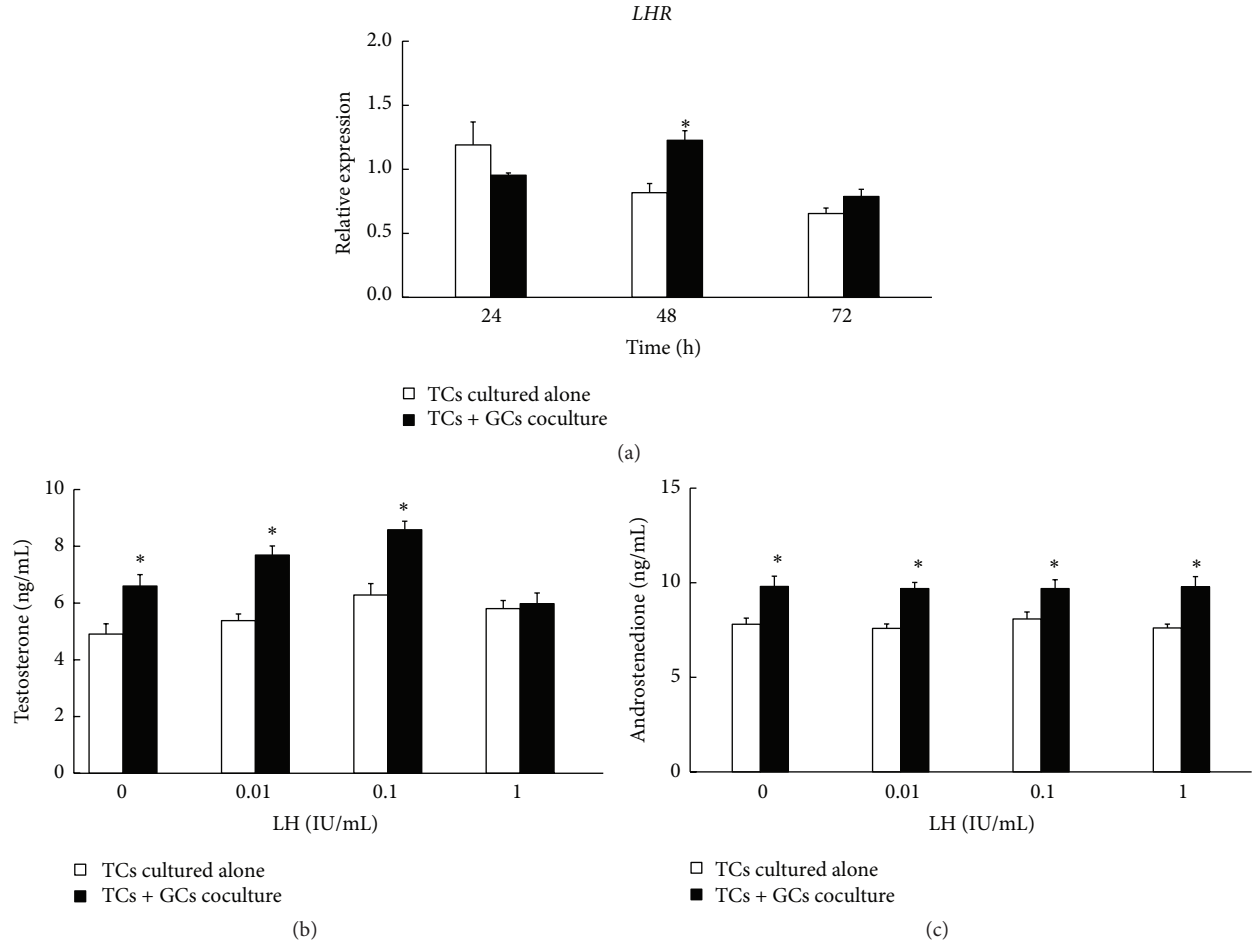

Figure 4: (a) Effect of granulosa cells on mRNA expression of *LHR* in TCs cells and LH stimulation of androgen production in TCs in the cocultured model. The *LHR* mRNA expression of TCs in cocultured model increased significantly at 48 h but manifested no difference compared with TCs cultured alone at 24 h and 72 h (*GAPDH* as internal control). (b) Effect of GCs on testosterone production responded to LH in TCs cells after being cocultured for 48 h. Testosterone production significantly increased in response to low concentrations of LH (0–0.1 IU/mL) in the cocultured model but no increase was observed at high concentration (1 IU/mL). (c) Effect of GCs on androstenedione production responded to LH in TCs cells after being cocultured for 48 h. Androstenedione production significantly increased in response to different concentrations of LH (0-1 IU/mL) in the cocultured model. Data were expressed as mean ± SEM of three independent experiments. The asterisk showed the statistically significant difference. $^{*}P < 0.05$.

function and mechanism of TCs exerted in the ovary as well as the interaction between TCs with GCs and oocytes deserve further exploration *in vivo*.

In conclusion, the present results demonstrated the key role of granulosa cells for the steroid production in theca cells. The Transwell coculture model has provided a technical basis for studying some features of interactions between follicular cell groups under different pathophysiological conditions such as that of hyperandrogenemia.

Disclosure

Xiaoqiang Liu and Pengyun Qiao are co-first authors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effects of Mitochondrial Uncoupling Protein 2 Inhibition by Genipin in Human Cumulus Cells

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UCP2 plays a physiological role by regulating mitochondrial biogenesis, maintaining energy balance, ROS elimination, and regulating cellular autophagy in numerous tissues. But the exact roles of UCP2 in cumulus cells are still not clear. Genipin, a special UCP2 inhibitor, was added into the cultural medium to explore the roles of UCP2 in human cumulus cells. There were no significant differences in ATP and mitochondrial membrane potential levels in cumulus cells from UCP2 inhibiting groups as compared with the control. The levels of ROS and Mn-SOD were markedly elevated after UCP2 inhibited Genipin. However, the ratio of reduced GSH to GSSG significantly declined after treatment with Genipin. UCP2 inhibition by Genipin also resulted in obvious increase in the active caspase-3, which accompanied the decline of caspase-3 mRNA. The level of progesterone in culture medium declined obviously after Genipin treatment. But there was no significant difference in estradiol concentrations. This study indicated that UCP2 is expressed in human cumulus cells and plays important roles on mediate ROS production, apoptotic process, and steroidogenesis, suggesting UCP2 may be involved in regulation of follicle development and oocyte maturation and quality.

1. Introduction

Mammalian ovarian follicles are highly specialized structures that support the growth and development of oocytes. Bidirectional communication between the oocyte and its surround granulosa cells is essential for the growth and development of both follicle and oocyte [1]. There are two types of granulosa cells: the cumulus cells (CCs) and the mural granulosa cells (MGCs). The mural granulosa cells, located in the basal membrane of the follicles, keep in lesser close contact with oocyte due to the distance. Yet, CCs are closer to the oocyte and maintain a proximity relationship via transzonal processes and gap junctions with the oocyte, providing nutrients, maturation-enabling factors, and an optimal microenvironment to ensure successful maturation and further developmental competence [2, 3]. This close relationship between the oocyte and CCs implies that the CCs may serve as a biomarker for oocyte maturation and quality [4].

Providing a steady source of ATP plays a vital role in most cellular functions. Likewise, both in CCs and oocytes, energy in the form of ATP is thought to be critical for the processes of follicle growth, oocyte maturation, and fertilization and ensuing embryo development [5]. Mitochondria are the primary energy-generating system in most eukaryotic cells, including CCs and oocyte. Yet, unlike in most somatic cells where energy is produced via glucose, the oocyte is specialized with pyruvate as the main energy substrate [6, 7]. CCs have a special role to metabolize glucose into pyruvate, which then is transferred into oocyte [6]. Besides, the reactive oxygen species [8] is the inevitable byproduct of mitochondrial oxidative metabolism. But excessive amount of ROS, caused by mitochondria dysfunction or depletion of enzymatic antioxidant system, induces cellular oxidative stress (OS), promote apoptosis, and damage the quality of CCs and oocyte [9]. Together, these previous studies suggested that keeping a mitochondrial homeostasis in CCs is critical for oocyte metabolism and quality.

The uncoupling protein 2 (UCP2) belongs to the mitochondrial anion transporter superfamily that uncouple oxidative phosphorylation and regulate ATP synthesis [10]. The precise biochemical function of mitochondrial UCP2 is still a matter of debate. Accumulating literatures have showed that UCP2 plays a positive physiological role by regulating mitochondrial biogenesis, maintaining energy balance, keeping calcium homeostasis [11], ROS elimination [12], and regulating cellular autophagy [13] and, thereby, provides cellular protection and possibly anti-aging [14]. But some other studies that used inhibitors, knockdown, or mutagenesis methods indicated UCP2 might have many deleterious effects and were involved in pathogenesis of numerous diseases, such as cardiovascular diseases [15], type 2 diabetes mellitus [8], obesity [16], polycystic ovary syndrome (PCOS) [17], and various cancers [18].

Rousset et al. first reported that UCP2 is expressed in the female mouse reproductive tract, which was detected in ovary, oviduct, and uterus [19]. Roles of UCP2 in female reproductive tract were concerned by a few studies. The correlation of ovarian UCP2 with PCOS has been found by Liu et al. [17], and they observed that UCP2 in MGCs was strongly associated with the expression of P450scc protein, a key steroidogenic enzyme, suggesting that UCP2 may be involved in the pathogenesis of PCOS by altering androgen synthesis [17]. A previous study suggested that UCP2 was also closely related with the ROS generation and oocyte developmental potential [20]. The patients whose UCP2 was under the mean-level have higher ROS level in granulosa cells and impaired oocyte quality. In addition, authors also demonstrated that the expression of UCP2 in ovaries is correlated with female age. Levels of UCP2 from younger women were higher than that of advanced-age women [20]. The association between UCP2 expressing with fetal development in uterus and endometrium has also been confirmed [21, 22]. In the late stage of pregnancy, the expression of UCP2 in uterine would upregulate by several folds, suggesting UCP2 also plays some roles on fetal growth [22].

Yet, so far, the exact roles of UCP2 in CCs are still not clear. In this present study, to analyze the roles of UCP2 played in CCs, we introduced Genipin, a special UCP2 inhibitor to inhibit UCP2 functions. Genipin is extracted from the fruit of Gardenia and has been used to relieve symptoms of type II diabetes in traditional Chinese medicine [16]. Previous studies have shown that Genipin can decrease mRNA and protein level of UCP2, inhibit mitochondrial proton leak, promote mitochondrial membrane potential, and increase ATP synthesis [16]. The contents of ATP and mitochondrial membrane potential, levels of ROS and antioxidants, and apoptosis related protein caspase-3 are quantified in cultured CCs *in vitro*. In addition, the concentrations of estradiol and progesterone in culture medium are measured.

2. Materials and Methods

2.1. Patient Recruitment and Informed Consent. CCs were isolated from the infertile women who were treated with ICSI-ET at the Reproductive Medicine Center of the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, China. This study was approved by the Medical Ethical Committee of the hospital. Written informed consent was obtained from all couples participating in this study.

The long downregulation protocol was adopted for all the patients. All women were downregulated with a GnRH agonist on mid-luteal phase. When optimally downregulated, recombinant FSH (Merck Serono, Switzerland) was started on day 3 of the menstrual cycle. When one follicle size reached 18 mm or two follicles size reached 17 mm or three follicles size reached 16 mm, human chorionic gonadotropin (hCG, Merck Serono, Switzerland) was administered. At 36 hrs after administration of hCG, the follicles were aspirated through transvaginal ultrasound retrieval.

2.2. Isolation of Cumulus Cells and Cells Culture. CCs were separated mechanically from oocytes by mechanically pipetting for less than 1 min in 0.1% hyaluronidase solution and then transferred into 15 mL centrifuge tubes. The tubes were centrifuged at 1500 rpm for 5 min and the supernatant was decanted. The cells were washed twice with culture medium (DMEM/F12, Sigma-Aldrich, USA) containing 10% of fetal bovine serum (Sijiqing Bioen-gineering Material Co., Ltd., China) then cultured into 24-well culture plates. Cells were incubated at 37°C with 5.0% CO_2 for 48 h with Genipin.

2.3. Cell Viability Measure. The cell count kit-8 (CCK, Beyotime, China) was used to quantitatively identify the cellular viability. The cultured CCs were rinsed twice with PBS, and then DMEM/F12 with 10% CCK-8 was added to these samples in a separate volume of 0.2 mL. After incubation for one hour, the absorbance was measured at 450 nm under a microplate reader (Bio-TEK, USA). Four parallel replicates of each sample at each time point were prepared during this cell viability assay.

2.4. Measurement of Mitochondrial Membrane Potential. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazoyl carbocyanine iodide, Anaspec Inc., USA) was a lipophilic cation and was used to assess the mitochondrial status in cells. CCs were incubated in culture medium with JC-1 at a concentration of 2.5 μg/mL in a humidified incubator at 37°C, 5% CO_2 for 20 min. CCs were washed twice at room temperature and centrifuged at 2000 rpm for 5 min. Then the cells were resuspended in 0.5 mL of PBS and analyzed them by BD FACSCalibur flow cytometry (BD, USA).

2.5. Measurement of ATP. The amount of ATP was measured by the ATP detection kit (Beyotime, China). CCs were lysed on ice with 100 μL lysis buffer from ATP detection kit. After being centrifuged at 12,000 g for 5 min at 4°C, the supernatant was transferred to a new 1.5 mL tube for ATP test. Protein concentrations were determined by using a BCA Protein Assay Reagent Kit (Beyotime, China). The luminescence from a 100 μL sample was assayed in a luminometer together with 100 μL of ATP detection buffer from the ATP detection kit. The standard curve of ATP concentration was prepared from a known amount (1 nM–10 μM).

2.6. Measurement of Intracellular ROS. The intracellular ROS of CCs was detected using DCHFDA. The CCs were incubated with 10 mM DCHF-DA in DMEM medium for 30 min at 37°C to allow cellular incorporation and then washed and resuspended in PBS. The ROS levels were detected by a microplate reader at 488 nm (Bio-TEK, USA).

2.7. Measurement of Lipid Peroxidation (MDA). The lipid peroxidation in CCs was measured by Lipid Peroxidation MDA Assay Kit (Beyotime, China). CCs were lysed and homogenized and then centrifuged at 20000 ×g at 4°C for 10 min, and then the supernatants were collected. Protein concentrations were determined by using a BCA Protein Assay Reagent Kit (Beyotime, China). A 200 μL of thiobarbituric acid (TBA) reagent was added into 100 μL of the suspension in 96-well plates. The mixture was treated in a boiling water bath for 15 min. After cooling, the suspension was centrifuged at 1,000 ×g at room temperature for 10 min and the supernatant was separated, then the absorbance was measured at 530 nm under a microplate reader (Bio-TEK, USA).

2.8. Measurement of Mn-SOD Activity. Superoxide dismutase (SOD) activity was measured using a Cu-Zn/Mn-SOD assay kit (WST) (Beyotime, China). Briefly, the Mn-SOD activity was measured by reduction rate inhibitions of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium and monosodium salt (WST-8) adding Cu-Zn-SOD inhibitor A and inhibitor B to inactivate Cu-Zn-SOD activity. Mn-SOD activity was expressed as units per mg of protein (one unit was defined as the amount of enzyme hat inhibited WST-1 reduction by 50%). Protein concentrations were determined by using a BCA Protein Assay Reagent Kit (Beyotime, China). The sample was diluted with PBS by 1 : 1, and then mix with the working solution in a 96-well plate. After incubated at 37°C for 20 mins, the absorbance was measured at 450 nm under a microplate Reader (Bio-TEK, USA).

2.9. Colorimetric Determination of Reduced and Oxidized Glutathione. The reduced GSH and oxidative GSH (GSSG) concentrations in CCs were measured by using a GSH/GSSG kit (Beyotime, China). The GSSG and GSH standards were prepared in 5% metaphosphoric acid. Cell samples were prepared in 5% metaphosphoric acid with or without 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate, a GSH-specific scavenger. Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid) reacts with GSH to form a product with an absorption maximum at 412 nm. GSSG was determined using glutathione reductase to reduce GSSG to GSH followed by reaction with Ellman's reagent.

2.10. Measurement of Estradiol and Progesterone Concentrations. The CCs culture medium was collected and stored at −20°C until the progesterone or estradiol was assay. Progesterone concentrations in the medium were measured by a specific radioimmunoassay (RIA). The sensitivity of the assay was 3.5 pg/tube, and the intra-assay and interassay coefficients of variation were 8.7 and 6.3%, respectively. Estradiol concentrations in the medium were measured by enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY), and the intra-assay and interassay coefficients of variation were less than 4%.

2.11. Real-Time PCR. At the beginning of the experiment, cells were harvested at the indicated time points for the extraction of RNA, and 800 μg of total RNA was reverse-transcribed into cDNA (TRizol, Invitrogen). To measure mRNA expression of CX 43 and caspase-3, RT-PCR using the SYBR green (Takara) was used according to the manufacturer's instructions. The following primers were used: caspase-3: GCCGTGGTACAGAACTGGACT, GCACAAAGCGACTGGATGAA. β-actin was used as an internal reference and its primers: TGACGTGGACATCCGCAAAG and CTGGAAGGTGGACAGCGAGG.

2.12. Western Blotting. Proteins from CCs were isolated with RIPA lysis buffer (20 μL) (Beyotime, China). The mixture was centrifuged at 12000 g for 20 min at 4°C and the supernatant transferred into 1.5 mL Eppendorf tube. Protein concentrations were determined using a BCA Protein Assay Reagent Kit (Beyotime, China). Caspase-3 protein was quantified by western blotting. The primary antibody to caspase-3 was used at a concentration of 1 : 1000 (Cell Signaling Technology, USA). The mouse monoclonal antitubulin antibody (Beyotime, China) was used for normalization. Secondary antibodies were conjugated with horseradish peroxidase, and the signals were detected using SuperSignal West Pico (Pierce, USA).

2.13. Statistical Analysis. All experiments were performed in triplicate. Data are presented as the mean ± SE. The results were analyzed with Student's t-test or by one-way *ANOVA*. P values < 0.05 were considered as significant using SPSS 13.0 software (Chicago, IL, USA).

3. Results

3.1. Effects of UCP2 Inhibition by Genipin on Cellular Viability. A CCK8 assay was used to determine the appropriate concentration of Genipin choice in the study, which would not result in significant cytotoxicity on CCs. As shown in Figure 1(a), cultivating CCs in Genipin concentrations 0, 10, 20, and 50 μM, there were not significantly impacts on cellular morphology, size, and number. But the cell viability and cellular morphology were changed significantly when the Genipin concentration reached 100 μM ($P < 0.05$, Figure 1(a)). So, the 50 μM Genipin was choice in this study.

3.2. Effects of UCP2 Inhibition by Genipin on Activity of OXPHOS. To determine the effects of UCP2 on the activity of OXPHOS, the ATP content and levels of mitochondrial membrane potential in CCs were measured. In Figures 1(b) and 1(c), there were no significant differences in both ATP contents and mitochondrial membrane potential levels were

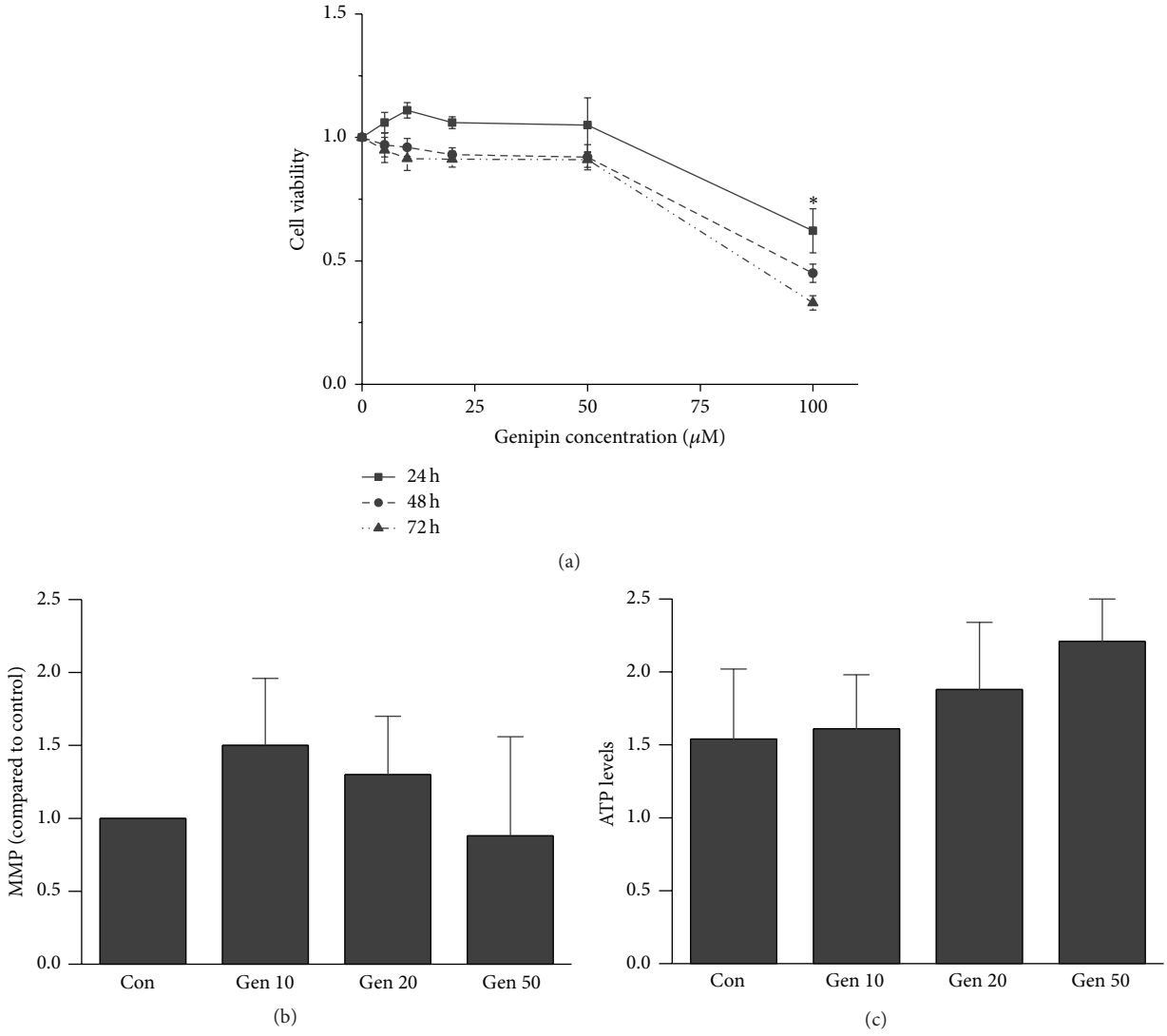

Figure 1: Effects of UCP2 inhibited by Genipin on cell viability, contents of ATP, and mitochondrial membrane potential (MMP). No significant differences were found in cell viability, contents of ATP, and mitochondrial membrane potential of CCs from UCP2 inhibited by Genipin as compared with their controls. Con means control and MMP means mitochondrial membrane potential.

found in all Genipin treated groups as compared with the control ($P > 0.05$).

3.3. Effects of UCP2 Inhibition by Genipin on Oxidative Stress. To determine effects of UCP2 on cellular redox status in CCs, Mn-SOD activity and the levels of the ROS, ratio of GSH/GSSG, and MDA concentration were evaluated, respectively. The intracellular ROS generation in CCs was markedly elevated after CCs were treated with 20 and 50 μM Genipin ($P < 0.05$, Figure 2(a)), which might feedback induce an increase of Mn-SOD in 20 and 50 μM Genipin treated groups ($P < 0.05$, Figure 3(b)). And obviously decreases were detected in the ratio of reduced GSH to GSSG in 20 and 50 μM Genipin treated groups too ($P < 0.05$, Figure 2(d)). In contrast, the MDA concentration increased significantly after being treated with 50 μM Genipin as compared with control ($P < 0.05$, Figure 2(c)).

3.4. Effects of UCP2 Inhibition by Genipin on CCs Apoptosis. To evaluate the effects of UCP2 on CCs apoptosis, the protein and gene of caspase-3, an executioner caspase, were, respectively, assessed. Two types caspase-3 protein were observed by WB, a proactive caspase-3 (32 kD) and an active caspase-3 (17 kD). UCP2 inhibition resulted obviously in an increase in expression of the active caspase-3, which was associated with the decline of proactive caspase-3 in 20 and 50 μM Genipin treated groups as compared with control ($P < 0.05$, Figure 3(a)). The expression of mRNA of caspase-3 has the same tendency as showed in caspase-3 protein ($P < 0.05$, Figure 3(b)).

3.5. Effects of UCP2 Inhibition by Genipin on Steroidogenesis. To evaluate the effects of UCP2 on steroid hormone secretion of CCs *in vitro*, the concentrations of estradiol and progesterone in culture medium were measured. A decline

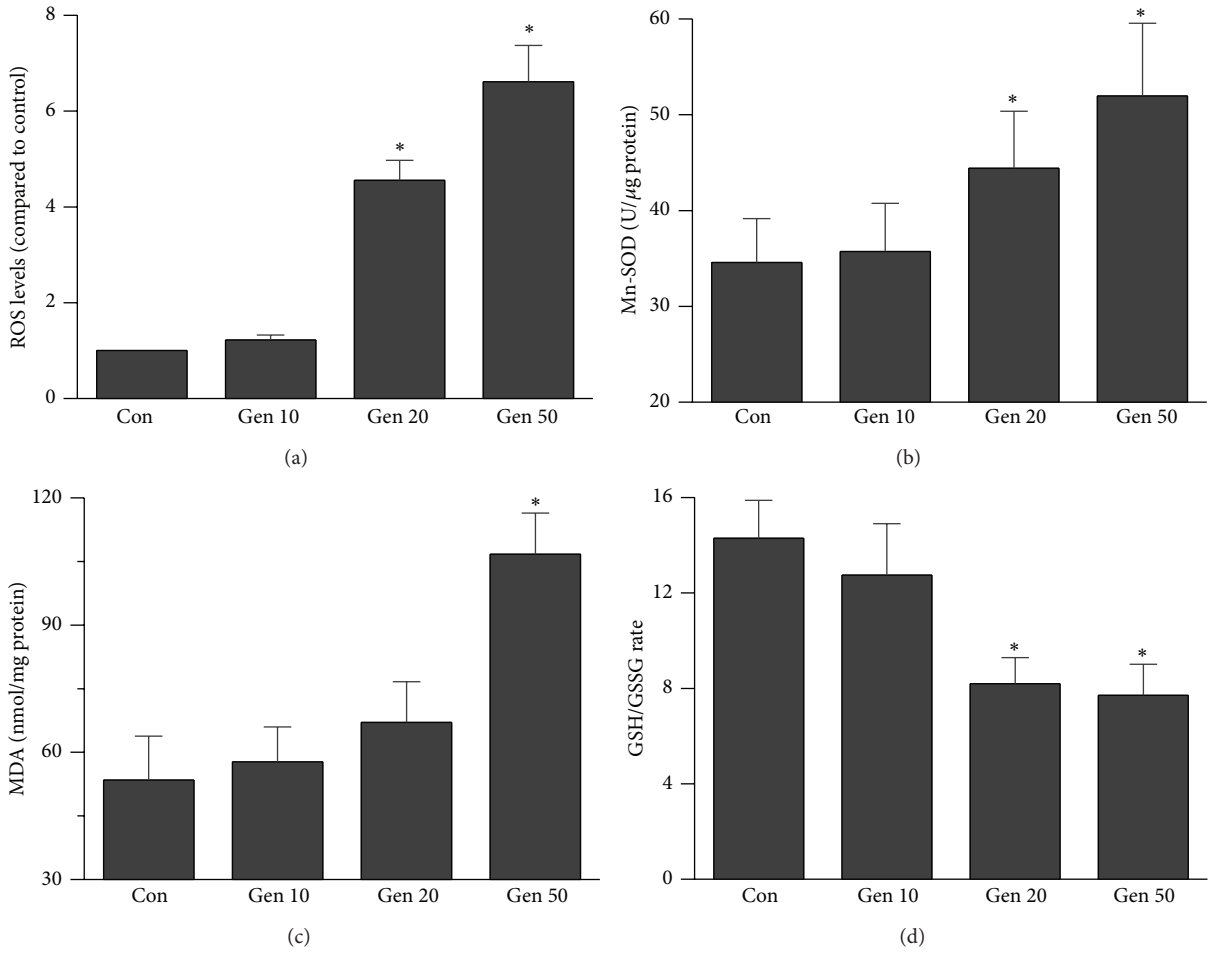

Figure 2: Effects of UCP2 inhibited by Genipin on oxidative stress. (a) The level of ROS in CCs has an increasing tendency after UCP2 inhibiting. (b) The Mn-SOD activity has an increasing tendency after UCP2 inhibiting. (c) The MDA level has an increasing tendency after UCP2 inhibiting. (d) The ratio of reduced GSH to GSSG has a decline tendency after UCP2 inhibiting. Con means control, asterisks indicate significant difference between control and treatment group at $P < 0.05$.

of concentrations of estradiol was found, but no significant difference was detected in all Genipin treated groups as compared with control ($P > 0.05$, Figure 4(a)). On the contrary, the level of progesterone in culture medium has a decline tendency after Genipin treatment, and significant differences were detected in 20 and 50 μM Genipin treated groups ($P < 0.05$, Figure 4(b)).

4. Discussion

Numerous researches observed that UCP2 plays key roles in regulation of ATP production and maintaining cellular energy balance. Regulating UCP2 expression by UCP2 inhibitors or analogues, such as FCCP or DNP, can alter cellular ATP contents in many cell types [16, 23]. However, in this study, UCP2 inhibition by Genipin does not lead to a visible change in ATP levels in CCs. This is consistent with several previous studies, in which overexpression of UCP2 did not alter the ATP contents of mouse brain cell [24]. No significant alteration in activity of OXPHOS was detected after UCP2 suppression, implying that regulatory functions of ATP generation or maintaining the energy balance might not be the primary role of UCP2 in human CCs, where it may play roles priority in reducing ROS generation, promoting apoptosis, or some others. Besides, some previous studies demonstrated that, apart from OXPHOS, other pathways, including the glycolysis and the pentose phosphate pathway (PPP), are confirmed to be involved in ATP production in CCs [25], which may help explain our results.

The oxidative damage of CCs or in follicular fluid is observed with a negative association with oocyte maturation, fertilization, and ensuing embryo developmental potential, as well as IVF/ICSI results [9, 26, 27]. A previous study suggested that overgeneration of ROS, caused by mitochondria dysfunction or depletion of enzymatic antioxidant system, could inhibit steroidogenic enzymes activity in MGCs and CCs [28]. Mild mitochondrial uncoupling has been proposed as a mechanism to decrease ROS generation, in which UCP2 inhibiting or overexpression can markedly alter ROS production in numerous tissues [24, 29]. In CCs, we found the same

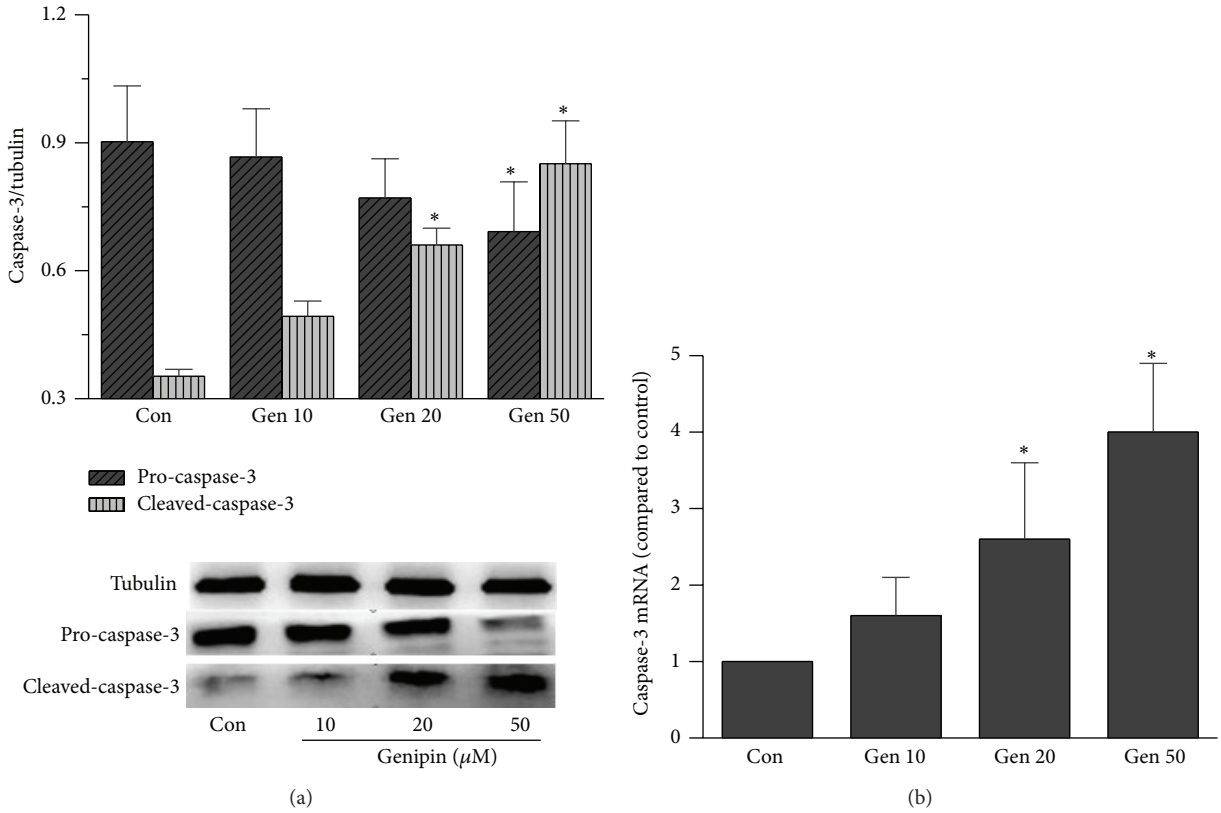

Figure 3: Effects of UCP2 inhibited by Genipin on CCs apoptosis. (a) The caspase-3 protein was quantified by Western blotting. The proactive caspase-3 declined obviously, and the active caspase-3 markedly increased after UCP2 inhibition. (b) Caspase-3 gene was quantified by RT-PCR, which markedly increase after UCP2 inhibition. Con means control, asterisks indicate significant difference between control and treatment group at $P < 0.05$.

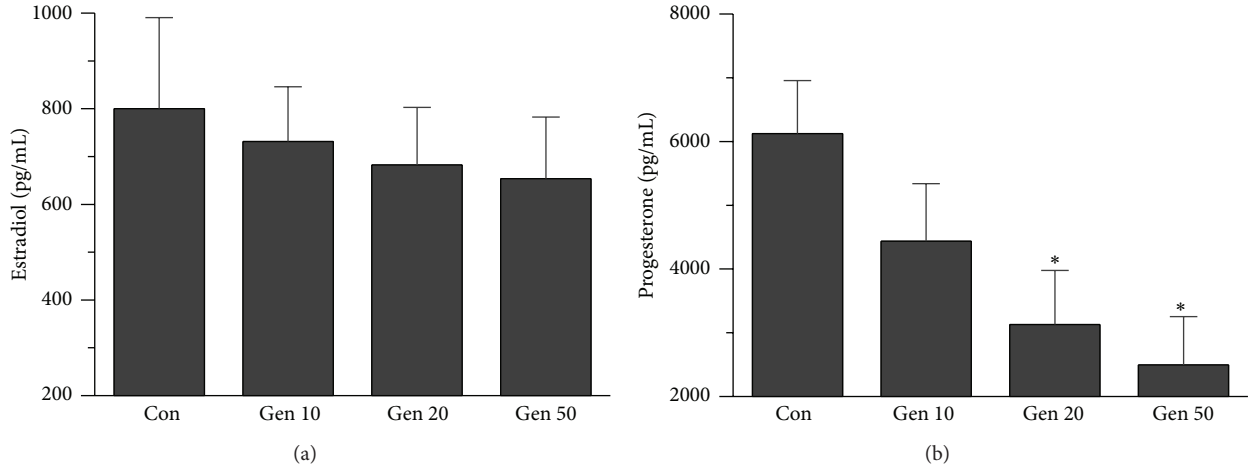

Figure 4: Effects of UCP2 inhibited by Genipin on steroidogenesis. (a) A slight decline of concentrations of estradiol in culture medium was found, but no significant difference was detected between UCP2 inhibited by Genipin and the control. (b) The level of progesterone in culture medium decline obviously after Genipin treated. Con means control, asterisks indicate significant difference between control and treatment group at $P < 0.05$.

effects. UCP2 inhibited by 20 and 50 μM Genipin markedly increase mitochondrial ROS production. High ROS production often leads to compensatory upregulation of antioxidant responses, which can also be observed in this study. UCP2 suppression increases ROS generation leading to increasing Mn-SOD activity feedback. However, the ratio of GSH/GSSG reduces obviously and is concomitant with an increase of the MDA level, suggesting that UCP2 suppression can aggravate the oxidative injury in CCs. Our result is consistent with a former research in MGCs [20], which suggested there is a UCP2-ROS mutual-regulation system in granulosa cells.

Correlation of UCP2 with apoptosis has also been observed in many type cells, where it endows an antiapoptotic property [30]. Inducing UCP2 overexpression can inhibit apoptosis process [31]. In contrast, inhibition or knockdown UCP2 can promote apoptosis [30]. In our studies, we observed that treatment with 20 and 50 μM Genipin, the active form of caspase-3, increased significantly, suggesting UCP2 suppression by Genipin promotes apoptosis in CCs. Our results also support a previous observation in cultured MGCs, which UCP2 inhibited by exogenous ATP, resulting in an increase in the apoptotic rate and a decrease of the cellular antioxidant capability [32].

For steroidogenesis, the initial step occurs within the mitochondrion. The intact mitochondrial structure and functions are the requirement of steroidogenesis [33]. So, in this study, it is not unexpected that UCP2 inhibition by Genipin can affect steroid hormone levels in CCs culture medium, especially the progesterone level, a hormone essential for implantation and maintenance of pregnancy in mammals. Roles of UCP2 regulating steroidogenesis have been observed in a former study. UCP2 can inhibit androgen synthesis in MGCs of PCOS patience [17], and inducing UCP2 overexpression *in vitro* would increase testosterone synthesis in both normal and PCOS granulosa cells.

In conclusion, our results demonstrated that *in vitro* UCP2 plays important roles on mediate ROS production, apoptotic process, and steroidogenesis. UCP2 inhibition results in increase of cellular ROS generation, disturbs cellular redox status, and induces apoptosis, as well as impairing progesterone production, but does not significantly affect the activity of OXHPOS. But we have to recognize that, apart from UCP2 inhibiting, Genipin might have some other nonspecific effects, which might affect our conclusions. So, further study is required to use other methods such as UCP2-siRNA to explore the impacts of UCP2 on CCs and its impact on oocyte maturation and fertilization and ensuing embryonic development potential *in vitro* and *in vivo*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

BRCA1 185delAG Mutation Enhances Interleukin-1β Expression in Ovarian Surface Epithelial Cells

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Familial history remains the strongest risk factor for developing ovarian cancer (OC) and is associated with germline BRCA1 mutations, such as the 185delAG founder mutation. We sought to determine whether normal human ovarian surface epithelial (OSE) cells expressing the BRCA1 185delAG mutant, BRAT, could promote an inflammatory phenotype by investigating its impact on expression of the proinflammatory cytokine, Interleukin-1β (IL-1β). Cultured OSE cells with and without BRAT were analyzed for differential target gene expression by real-time PCR, western blot, ELISA, luciferase reporter, and siRNA assays. We found that BRAT cells expressed increased cellular and secreted levels of active IL-1β. BRAT-expressing OSE cells exhibited 3-fold enhanced IL-1β mRNA expression, transcriptionally regulated, in part, through CREB sites within the (−1800) to (−900) region of its promoter. In addition to transcriptional regulation, BRAT-mediated IL-1β expression appears dualistic through enhanced inflammasome-mediated caspase-1 cleavage and activation of IL-1β. Further investigation is warranted to elucidate the molecular mechanism(s) of BRAT-mediated IL-1β expression since increased IL-1β expression may represent an early step contributing to OC.

1. Introduction

Ovarian cancer (OC), an inflammation associated cancer, is the deadliest gynecologic malignancy and is the 9th most common cancer among women [1]. Approximately 22,240 new cases are diagnosed and 14,030 women die of the disease annually [1]. Since most OC are diagnosed in late stage, the 5-year survival is approximately 27% [1].

Most OCs are epithelial ovarian carcinomas traditionally thought to arise from the ovarian surface epithelium (OSE) [2]; though more recently, studies have suggested that OC may arise from the fallopian tube epithelium [3, 4]. The etiology of the disease is not completely understood, but family history (FH) is the strongest risk factor for the development of epithelial OC [5]. Hereditary OCs are often associated with mutation of the tumor suppressor breast cancer susceptibility gene 1 (BRCA1) [6]. Carriers of the BRCA1 mutation have a 30% risk of developing OC during their lifetime [7]. BRCA1 plays a role in DNA damage response, cell cycle signaling, recruitment of chromatin modifying proteins, interaction with transcription factors, and ubiquitin ligase activity [8]. Loss of these functions may contribute to the development of cancer by promoting genomic instability and accumulation of cancer-causing mutations. Mutation of the BRCA1 gene can also result in either "loss of function" or "gain of function" with appearance of novel truncated protein products, respectively (reviewed in [9]). Among possible gain-of-function BRCA1 mutations, the 185delAG mutation is one of the most common founder mutations and is associated with a 66% lifetime risk of developing OC [10].

The 185delAG BRCA1 truncated mutant, BRAT, is the result of a deletion of two nucleotides in the second exon of the BRCA1 gene leading to a reading frame shift and a premature stop codon at position 39. Previously, we

demonstrated that human OSE cells with the BRAT mutation exhibited enhanced apoptosis and caspase-3 activation [11] as well as diminished levels of phosphorylated Akt, cellular inhibitor of apoptosis 1 (cIAP1), and X-linked inhibitor of apoptosis protein (XIAP) [12]. We also found that BRAT-mediated maspin expression was correlated with enhanced chemosensitivity [13] which is in agreement with clinical reports of increased survival in patients with elevated maspin levels [14]. Microscopic examination of ovarian specimens obtained following prophylactic oophorectomy from women with FH of OC indicated that greater than 85% presented with two or more abnormal OSE histologic features such as surface epithelial pseudostratification, surface papillomatosis, cortical invaginations of OSE, epithelial inclusion cysts, and epithelial hyperplasia [15]. Likewise, overtly normal OSE from women with a FH of breast and/or OC (FHOSE) in culture show an increased autonomy of the epithelial phenotype in terms of expression of the epithelial differentiation marker CA125 [16], persistence of an epithelial morphology [16], and reduction in epithelial-mesenchymal conversion as noted by the maintenance of high keratin expression, but a reduction of collagen type II expression compared with no family history (NFH) OSE [17]. Since OSE become more committed to an epithelial phenotype in the course of carcinogenesis, these reports coupled with our previous findings suggest that preneoplastic characteristics may already exist in overtly normal OSE in some women with a strong FH of breast and ovarian cancer.

Inflammation of the ovarian epithelium has also long been associated with increased risk for OC [18–21]. By promoting a local pelvic inflammatory reaction, endometriosis has been associated with increased risk for endometrioid adenocarcinoma and clear cell carcinoma of the ovary [22–25]. Likewise, chronic pelvic inflammatory disease, often resulting from infection, also supports a role for inflammation and increased risk for OC [26, 27]. Lastly, epidemiological studies suggest that incessant ovulation causes rapid cycles of OSE division associated with repeated ovulatory traumatization and repair of the ovulatory defect [28]. Ultrastructural and histochemical studies of OSE *in situ* have shown that OSE migrates and proliferates extensively during repair of the OSE after ovulation [29, 30]. During ovulatory repair, OSE is exposed to proinflammatory mediators such as cytokines, chemokines, matrix-remodeling enzymes, and various growth factors that can result in an increased risk for malignant transformation [31, 32]. Reports of OC associated with ovarian hyperstimulation due to fertility drugs [33, 34] further support a role for inflammatory traumatization in OC.

Interleukin-1 beta (IL-1β) is a proinflammatory cytokine mainly produced by monocytes and macrophages [35], but which can also be produced by endothelial cells, fibroblasts, and epidermal cells in response to bacterial or innate immunity stimulation [36]. Interestingly, normal and malignant epithelial ovarian cells also produce IL-1β [2]. IL-1β is translated into a 31 kDa inactive precursor form that is cleaved intracellularly by caspase-1 into an active 17 kDa secreted form [35]. The aim of this study was to investigate the *in vitro* production of IL-1β in human OSE cell lines carrying the 185delAG BRCA1 mutation in order to determine whether enhanced IL-1β in these cells could contribute to an inflammatory phenotype.

2. Materials and Methods

2.1. Cell Culture and Transfection. The following SV 40-Large T-Antigen transfected human OSE (IOSE) cell lines were used: IOSE118 derived from a normal patient with a FH of breast and/or OC with wtBRCA1 status confirmed [13]; IOSE 121 derived from a normal patient with NFH of breast and/or OC though BRCA1 mutation status was not determined; and IOSE 3261-77 and IOSE 1816-686 derived from normal patients with a FH of breast and/or OC as well as confirmed carriers of the BRCA1 185delAG mutation. IOSE cells were cultured in Medium 199/MCDB 105 (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS) and gentamicin. Stable PCDNA (pcDNA3.1) and BRAT clones (BRATc1, BRATc2) were generated by transfection of 2.5×10^5 IOSE 118 cells with 2.5 μg of pcDNA3.1 or Flag-BRAT with G418 resistance gene as previously described [13] using Program X-005, Kit V, and the Nucleofector device (Amaxa/Lonza, Walkersville, MD). Stable cells were maintained in 1 mg/mL G418 selection media and BRAT clones were confirmed to express BRAT by RT-PCR [13]. All cells were incubated at 37°C with 5% CO_2. For knockdown studies, cells were cotransfected with 1.5 μg ON-TARGETplus siRNA (siCREB, siCON) from Dharmacon (Chicago, IL). For treatment with IL-1 receptor antagonist (IL-1Ra), stable BRAT clones were washed with phosphate buffered saline (PBS) and media containing 0.1% FBS were added to the cells along with varying concentrations of IL-1Ra (R&D Systems, Inc., Minneapolis, MN). After 6 hours, the media were removed and RNA was extracted from the cells for PCR analyses as described below.

2.2. Western Immunoblot. Cells were washed in PBS, trypsinized, pelleted, and washed 1-2 times in cold PBS. Cells were lysed for 30 minutes on ice in modified CHAPS buffer, and the lysate was centrifuged at 115,000 ×g, at 4°C for 1 hour. Then 30 μg of protein was separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes, dried, and blocked in 5% milk in Tween 20-Tris buffered saline. Blots were incubated in their respective primary antibodies overnight, followed by incubation with a horseradish peroxidase- (HRP-) conjugated secondary antibody (Fisher, Pittsburgh, PA), and developed via enhanced chemiluminescence substrate (ECL) (Pierce/Fisher, Pittsburgh, PA). Antibodies used were IL-1β (1 : 1000) Cat. # 2022 (Cell Signaling Technology, Beverly, MA), Actin clone AC-40 (1 : 10,000) Cat. # 4700 (Sigma, St. Louis, MO), Apoptosis-associated Speck-like protein containing a C-terminal caspase recruitment domain (ASC) (1 : 1000) Cat. # sc-271054 (Santa Cruz Biotechnology, Inc., Dallas, TX), Caspase-1 (1 : 1000) Cat. # 2225 (Cell Signaling Technology, Beverly, MA), CREB (1 : 1000) Cat. # 9104 (Cell Signaling Technology, Beverly, MA), phospho-CREB (1 : 1000) Cat. # 9196 (Cell Signaling Technology, Beverly, MA), NACHT, LRR, and PYD domains-containing

protein 3 (NALP3) (1 : 1000) Cat. # sc-134306 (Santa Cruz Biotechnology, Inc., Dallas, TX). Western blot quantification was done by ImageJ software normalizing band strength to the respective actin band.

2.3. Enzyme-Linked Immunosorbant Assay. For conditioned media (CM) analyses, medium containing 0.1% FBS was added to cells 24 hours after transfection/plating. After 24 hours, cells were counted and CM were collected and centrifuged to remove debris, aliquoted, and stored at (−80°C). For the IL-1β Enzyme-Linked Immunosorbant Assay (ELISA), CM were also concentrated 17x using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, Billerica, MA) before storage at (−80°C). To assess the presence of mature IL-1β, an ELISA (R&D Systems, Inc., Minneapolis, MN) was performed on concentrated CM samples in triplicate according to the manufacturer's protocol. Fluorescence was read on an ELx800 Absorbance Microplate Reader (Biotek, Winooski, Vermont) using Gen5 Data Analysis Software (Biotek, Winooski, Vermont). Resultant values were derived from a standard curve and expressed as the mean IL-1β concentration of triplicate samples ± standard error. When cell viability varied significantly, IL-1β concentration was normalized to average cell number at time of CM collection.

2.4. Polymerase Chain Reaction. RNA samples were isolated using TRIzol reagent from Invitrogen (Carlsbad, CA) as per the manufacturer's protocol and DNAse-treated.

For BRAT semiquantitative PCR to confirm transfection, 1 μg total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA as previously described [13]. The cDNA samples were amplified using the Applied Biosystems GeneAmp RNA PCR Core Kit (Foster City, CA). Primers used were Flag-BRAT sense (CGATGACAAAATGGATTTATCTGC), Flag-BRAT antisense (GAGACAGGTTCCTTCATCAACTCC), actin sense (GGGAATTCAAAACTGGAACGGTGAAGG), and actin antisense (GGAAGCTTATCAAAGTCCTCGGCCACA). The amplified products were separated by electrophoresis on a 10% polyacrylamide gel, stained with SYBR Green (Lonza, Rockland, ME), and photographed with the Kodak EDS 120 Digital Analysis System. The net intensity of each band was normalized to the respective endogenous control band.

For quantitative PCR, 100 ng total RNA was reverse-transcribed to generate single-strand cDNA as previously described [13]. The cDNA samples were amplified in triplicate using Fast SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems Step One Plus instrument. Primers used were IL-1β sense (TCCAGGGACAGGATATGGAG), IL-1β antisense (TCTTTCAACACGCAGGACAG), and actin (same as above). RQ (relative mean mRNA expression level) was calculated by the Step One software version 2.0. Using standard curves constructed for target and endogenous control genes, an arbitrary quantitative gene expression value was determined from the threshold cycle (Ct) for each gene for each sample. Target gene values were normalized to control gene values, and fold difference was determined by dividing by the designated reference/calibrator sample.

2.5. Dual-Luciferase Assay. Stable 118 pcDNA3.1 or BRAT clones were transfected with 0.15 μg Renilla luciferase reporter and 1.5 μg IL-1β luciferase promoter deletion constructs pIL1(4.0 kb)LUC (−4000), pIL1(3.1 kb)LUC (−3100), pIL1(1.8 kb)LUC (−1800), pIL1(0.9 kb)LUC (−900), and pIL1(0.5 kb)LUC (−500). Twenty-four hours later, cells were collected in Promega Passive Lysis Buffer and subjected to two freeze-thaw cycles. Lysates were centrifuged at 10,600 ×g for 1 minute at 4°C, and the supernatant was collected. Luciferase activity was assessed in triplicate using a manual luminometer and the Promega Dual Luciferase Assay System according to the manufacturer's protocol. For knockdown reporter studies, siRNA was cotransfected and cells were collected 48 hours after transfection.

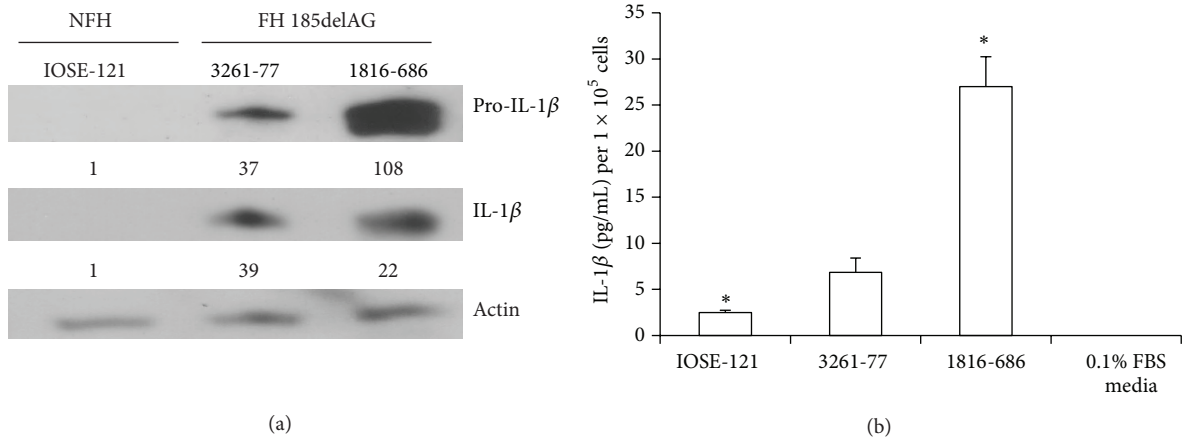
2.6. Immunoprecipitation. Cells from stable 118 pcDNA3.1 and BRAT clones were washed with PBS and incubated on ice with RIPA lysis buffer containing protease inhibitors. After 15 minutes, the cells were removed by scraping, the lysates were incubated for 60 minutes on ice, and then the lysates were centrifuged at 10,000 ×g for 10 minutes at 4°C. Two micrograms of primary antibody was incubated with 1 mg of whole cell lysate for 2 hours at 4°C. Protein A/G agarose suspension was added to lysate and antibody mixture followed by incubation at 4°C on a rocker overnight. The suspension was collected by centrifugation at 500 ×g for 2 minutes at 4°C. The pellet was washed with RIPA buffer containing protease inhibitors followed by analysis by western immunoblotting.

2.7. Statistics. For real-time PCR, relative gene expression was expressed as the average fold change (RQ) of RQ_{min} and RQ_{max} calculated as $2^{-(\Delta\Delta Ct+T\times SD(\Delta Ct))}$ and $2^{-(\Delta\Delta Ct+T\times SD(\Delta Ct))}$, respectively, at the 95% confidence interval, for 5 degrees of freedom and where SD = standard deviation, T = confidence level and Ct = cycle threshold. For ELISA and reporter assays, Student's t-tests were performed to assess statistical difference between means of triplicates ± standard error from three separate experiments.

3. Results

3.1. IL-1β Levels Are Increased in IOSE Cells Carrying the BRCA1 185delAG Mutation. To determine the relationship between the BRCA1 185delAG mutation and protein levels of IL-1β, we compared IL-1β protein levels in human OSE cell lines that endogenously carry this mutation to those with wild-type BRCA1. Both FH IOSE 3261-77 and 1816-686 cell lines with confirmed 185delAG mutation had 7-fold and ≥13-fold higher active IL-1β protein levels, respectively, as measured by western blot than the NFH cell line (IOSE-121) (Figure 1(a)). Likewise, 3261-77 and 1816-686 cell lines had 2- and 10-fold, respectively, higher levels of secreted IL-1β protein in their CM as measured by ELISA than the IOSE-121 cell line (Figure 1(b)).

3.2. BRAT Transfection Increases Levels of IL-1β. To confirm that increased intracellular and secreted IL-1β levels are related to the presence of BRAT, we analyzed IOSE-118 cells



(a) (b)

Figure 1: IL-1β protein levels are increased in 185delAG BRCA1 mutation carriers. (a) Normal NFH and FH BRCA1 185delAG IOSE cells were analyzed for precursor (pro-) and cleaved IL-1β protein expression via western blot. Blots were then stripped and probed for β-actin as a loading control. Values represent relative densitometry. (b) Cells were plated in triplicate at similar densities and conditioned media were collected as described. IL-1β ELISA activity assay was performed in triplicate and the results are expressed as the mean ± standard error. Symbol (∗) denotes statistical significance at the 0.04 confidence level.

stably transfected with PCDNA or Flag-BRAT for IL-1β message and protein levels. BRATc1 had approximately 3-fold higher levels of IL-1β RNA as measured by real-time PCR than the pcDNA3.1 cell line (Figure 2(a)). Furthermore, densitometric analyses indicated that 22% of cellular pro-IL-1β in BRAT cells was cleaved into its active form while only 5.5% of cellular pro-IL-1β in pcDNA3.1 cells was cleaved into its active form (Figure 2(b)). Lastly, BRATc1 and BRATc2 both had 2-fold and 1.5-fold, respectively, higher levels of secreted IL-1β protein levels as measured by ELISA than the pcDNA3.1 cells (Figure 2(c)).

3.3. BRAT-Mediated IL-1β Promoter Activity Is Partially Mediated by CREB. To determine which region of the *IL-1β* promoter was required for the BRAT-mediated *IL-1β* promoter activation, we utilized dual-reporter assays in BRAT cells using five truncated *IL-1β* promoter luciferase reporter plasmids. There was no difference in reporter activity between deletion constructs (−4000), (−3100), and (−1800) (Figure 3(a)). However, reporter activity was diminished by 80% between deletion constructs (−1800) and (−900) (Figure 3(a)) suggesting that the 900 bp region between the (−1800) construct and the (−900) construct contributes to BRAT-mediated *IL-1β* promoter activity.

To further evaluate BRAT-mediated *IL-1β* promoter activation, we performed an online transcription factor prediction analysis of the 900 bp promoter region identified earlier to determine potential transcription factor binding sites using the prediction software, PROMO [37, 38]. Our analyses identified two potential CREB binding sites within the 900 bp promoter region. Further, densitometric analyses of western immunoblots performed for phospho-CREB and CREB levels revealed 30% increased levels of phospho-CREB in BRAT cells (Figure 3(b)). Based on this observation, we used pooled siRNA to knockdown CREB and western blot to confirm CREB silencing. We found that CREB knockdown resulted in partial loss of *IL-1β* promoter activity using the (−1800) reporter plasmid (Figure 3(c)).

3.4. Caspase-1, ASC, and NALP3 Protein Levels Are Increased in BRAT Cells. To confirm intracellular cleavage of the inactive IL-1β into its active form, we measured caspase-1 levels, the known intracellular activator of IL-1β. Stably transfected pcDNA3.1 cells had about 3-fold more inactive precursor caspase-1 protein levels than BRAT cells as seen by western blot (Figure 4(a)). However, stably transfected BRAT cells showed up to a 50-fold increase in active caspase-1 by western blot (Figure 4(a)). Given that BRAT might increase IL-1β activation by caspase-1-mediated cleavage, we sought to determine if the NALP3-ASC inflammasome, which cleaves pro-caspase-1 into its active form, was enhanced in BRAT cells. Western immunoblots confirmed a 3-fold increase of ASC protein levels in BRAT cells compared to the pcDNA3.1 cells (Figure 4(b)). Likewise, immunoprecipitation followed by densitometric analyses showed that, when normalized to cellular protein, both ASC and NALP3-bound ASC protein levels were 44% and 250% higher, respectively, in BRAT cells (Figure 4(c)).

3.5. IL-1Ra Suppresses Proinflammatory Mediators in BRAT Cells. To evaluate the potential autofeedback loop of IL-1β on OSE cells, we treated stably transfected BRAT-containing IOSE cells with the IL-1 receptor antagonist, IL-1Ra. Then, we measured mRNA expression levels of known IL-1β downstream transcription targets: IL-1β, IL-6, and IL-8 by real-time PCR. Treatment of BRAT cells with IL-1Ra resulted in up to a 50% loss of mRNA expression levels of IL-1β, IL-6, and IL-8 (Figure 5).

4. Discussion

The results of the present study show that the 185delAG BRCA1 mutant protein, BRAt, increases IL-1β mRNA and protein levels in immortalized human OSE cell lines. Interestingly, compared to controls, increased levels of IL-1β varied among IOSE cell lines and may reflect differences in BRCA1

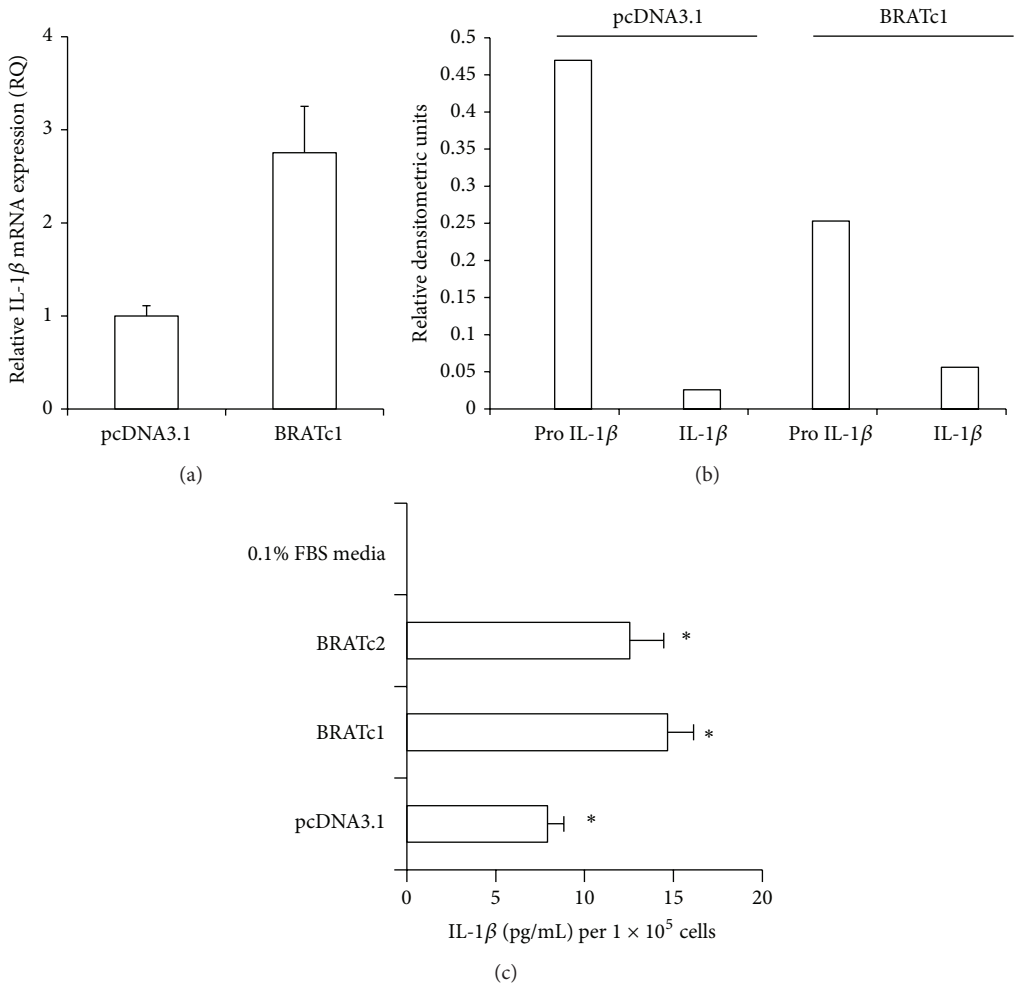


Figure 2: IL-1β protein and message levels are increased in stably transfected BRAT cells. (a) Cells stably expressing BRAT or the pcDNA3.1 cells were plated at equal densities and RNA was isolated, DNAse-treated, and reverse-transcribed. Real-time PCR was performed in triplicate for IL-1β and actin using SYBR Green detection and the data are expressed as the mean fold difference ± standard error. (b) pcDNA3.1 and BRATc1 cells were analyzed for precursor (pro-) and cleaved IL-1β protein expression via western blot. Blots were then stripped and probed for β-actin as a loading control. Values represent relative densitometry. (c) pcDNA3.1, BRATc1, and BRATc2 cells were plated in triplicate at similar densities and conditioned media were collected. IL-1β ELISA activity assay was performed in triplicate and the data are expressed as the mean ± standard error. Symbol (∗) denotes statistical significance at the 0.01 confidence level between BRAT and PCDNA3.1 cells.

185delAG penetrance, BRAt expression, and/or wtBRCA1 expression [11, 13]. Therefore, it is important to note that inherent differences of wtBrca1 expression among cell lines derived from patients endogenously carrying the 185delAG mutation can limit understanding the independent functions of BRAt in these cells. For these reasons, we transitioned our studies into transiently or stably transfected 185delAG cell lines in a confirmed wtBRCA1 background.

We also demonstrated that BRAT-dependent expression is transcriptionally mediated, in part, via CREB binding sites. In addition, we found that BRAT-dependent activation of IL-1β protein by caspase-1 may be enhanced by increased expression of the NALP3-ASC inflammasome. While further investigation is required to discover the exact mechanism of enhanced NALP3-ASC protein expression in BRAT cells, we suspect that ASC expression may be CREB-dependent or AP1-dependent since the current and prior studies indicate BRAT-mediated gene expression through CREB and AP1 sites [39]. Further, CREB binding sites are likely present in the ASC promoter according to commercially available promoter ChIP assay (Qiagen, Valencia, CA) program analysis. Lastly, we confirmed that IL-1β plays a role in expression of proinflammatory mediators, IL-6 and IL-8, via the IL-1 receptor suggesting that BRAT might stimulate a proinflammatory environment that could promote OC oncogenesis.

Establishing animal and cell models of OC oncogenesis has proven to be difficult. Spontaneous development of OC in animal models occurs with a low frequency and highly variable phenotypes [40]. Furthermore, OC lacks a clear molecular profile making it difficult to elucidate a universal cause in OC initiation or progression [41–43]. Loss of some or all wild type functions of the BRCA1 gene product due to gene mutation is commonly associated with enhanced breast cancer and OC risk (reviewed in [9]). *BRCA1* gene

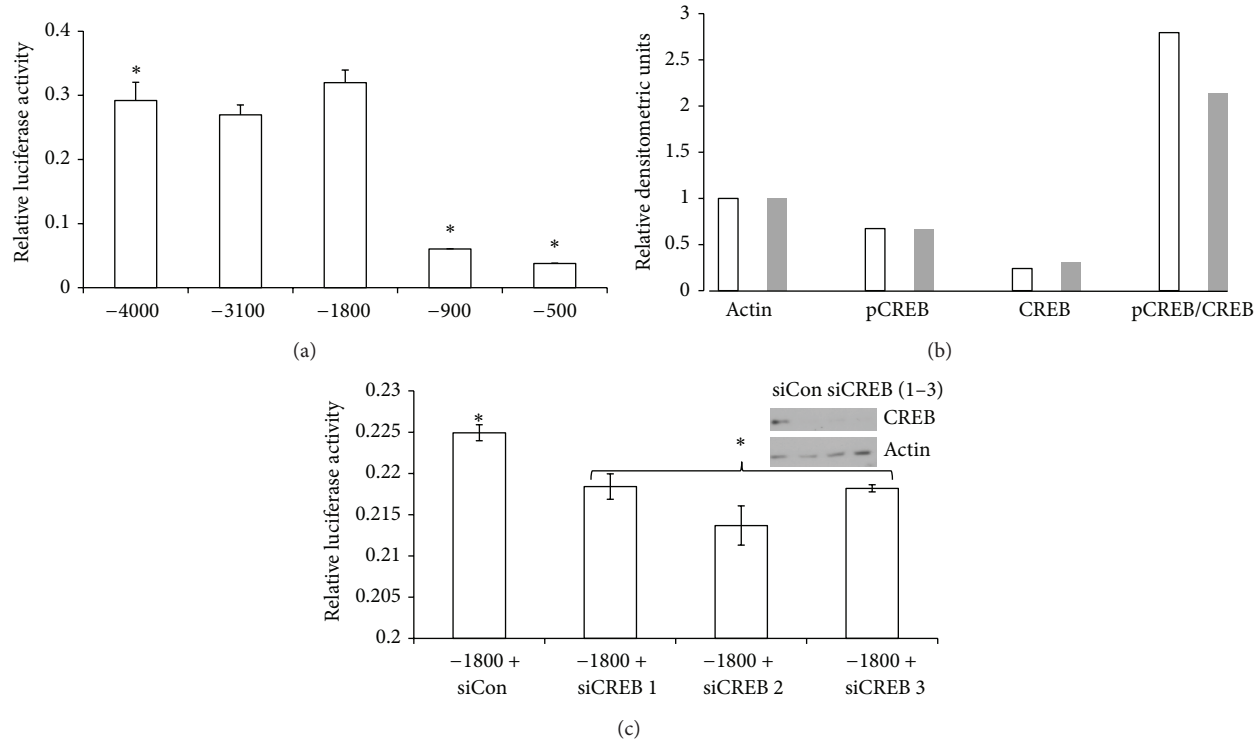


Figure 3: CREB sites within the IL-1β promoter partially mediate enhanced IL-1β mRNA expression in BRAT cells. (a) Cells stably expressing BRAT were transiently transfected with the indicated IL-1β deletion reporter constructs and a Renilla constitutive luciferase reporter plasmid for normalization. Lysates were collected, subjected to two freeze-thaw cycles, and assayed in triplicate on a manual luminometer using Promega's Dual Luciferase Assay Kit. Luciferase activity was normalized to Renilla luciferase activity for each triplicate, averaged, and results are expressed as mean reporter activity ± standard error. (b) Stable BRAT (white bars) and pcDNA3.1 (grey bars) cells were analyzed by western immunoblot for comparison of cellular levels of phospho-CREB, CREB, and actin. The results are expressed in relative densitometric units. (c) Cells stably expressing BRAT cells were cotransfected with nontargeting control siRNA (siCon) or siRNA targeting CREB, collected, and assayed similarly to (a). Protein lysates were collected in parallel for knockdown analyses and CREB protein silencing demonstrated by western blot (inset). Symbol (∗) denotes statistical significance ≤ 0.03 confidence level.

mutations resulting in a premature stop codon are generally susceptible to nonsense-mediated messenger RNA (mRNA) decay. However, two common risk-associated BRCA1 mutations, 185delAG and 5382InsC, were found to be unaffected by mRNA decay [44, 45]. This study utilizes a stable OSE cell line model with intact endogenous biallele *BRCA1* along with the 185delAG BRCA1 mutant transcript. By retaining a BRCA1 wild type background, we are able to conclude that changes observed are due to the presence of the 185delAG BRCA1 mutation and not due to the partial or complete loss of BRCA1. This is in keeping with previous studies that have shown other BRCA1 mutations with independent and novel gain-of-function roles in OC proliferation, chemosensitivity, tumorigenesis, and apoptosis [46, 47].

Inflammation has long been suggested to contribute to tumor initiation, promotion, and progression [48]. Many components of the inflammatory pathway, including free radicals, cytokines, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and vascular endothelial growth factor (VEGF) have been implicated in the development of various malignancies, including OC [49]. Immunohistochemical analyses revealed increased COX-2 expression in nonmucinous ovarian tumors and increased COX-2 expression correlating with poor prognosis [50]. Likewise, the present results are in keeping with others who reported a relation between proinflammatory mediators and increased OC risk. A case-control study by Clendenen et al. confirmed a positive association between circulatory inflammatory cytokines, IL-2, IL-4, IL-6, IL-12, and IL-13, and OC risk [51]. Also, increased serum levels of the inflammatory marker CRP have been associated with an increased risk of OC [20, 52]. Yellapa et al. demonstrated increased expression of the proinflammatory cytokine, IL-16, in serum and tissue of early and late stage OC patients as well as increased IL-16 serum levels during the progression from normal to early and late stage OC progression in hens [53]. Further, since increasing IL-16 serum levels preceded detection of OC by transvaginal ultrasound, it is tempting to speculate that monitoring proinflammatory mediators, such as IL-1β, may signal disease in women at high risk for OC before detection by conventional means.

Epidemiological studies suggest that long-term usage of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with decreased OC risk [54–56]. Further, *in vitro* and *in vivo* use of phytochemicals [57] exert anti-inflammatory activity related to reduced OC progression by stabilizing

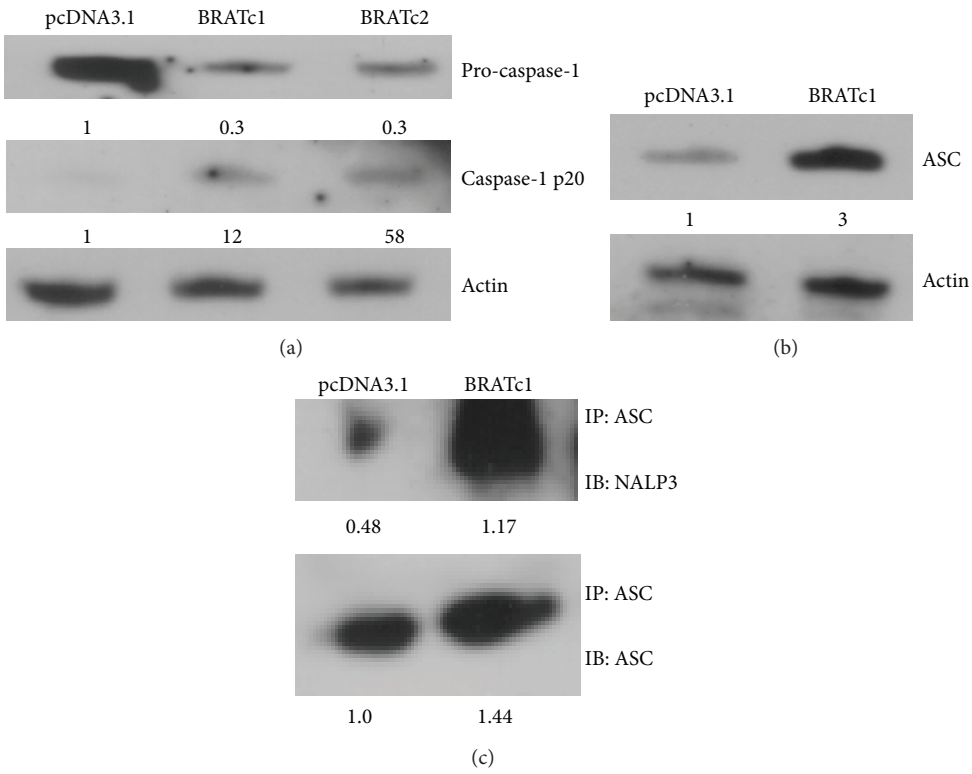


Figure 4: BRAT alters levels of inflammasome constituents. (a) Cells stably expressing pcDNA3.1 or BRAT (BRATc1 and BRATc2) were analyzed for precursor (pro-caspase-1) and cleaved caspase-1 (caspase-1 p20) protein levels via western blot. Blots were then stripped and probed for β-actin as a loading control. Values represent relative densitometric units. (b) Stable pcDNA3.1 or BRATc1 cells were analyzed for ASC protein expression via western blot. Blots were then stripped and probed for β-actin as a loading control. Values represent relative densitometric units. (c) Lysates obtained from stable pcDNA3.1 and BRATc1 cells were immunoprecipitated with ASC antibody and then analyzed via western blot for ASC and NALP3. Values represent densitometric units.

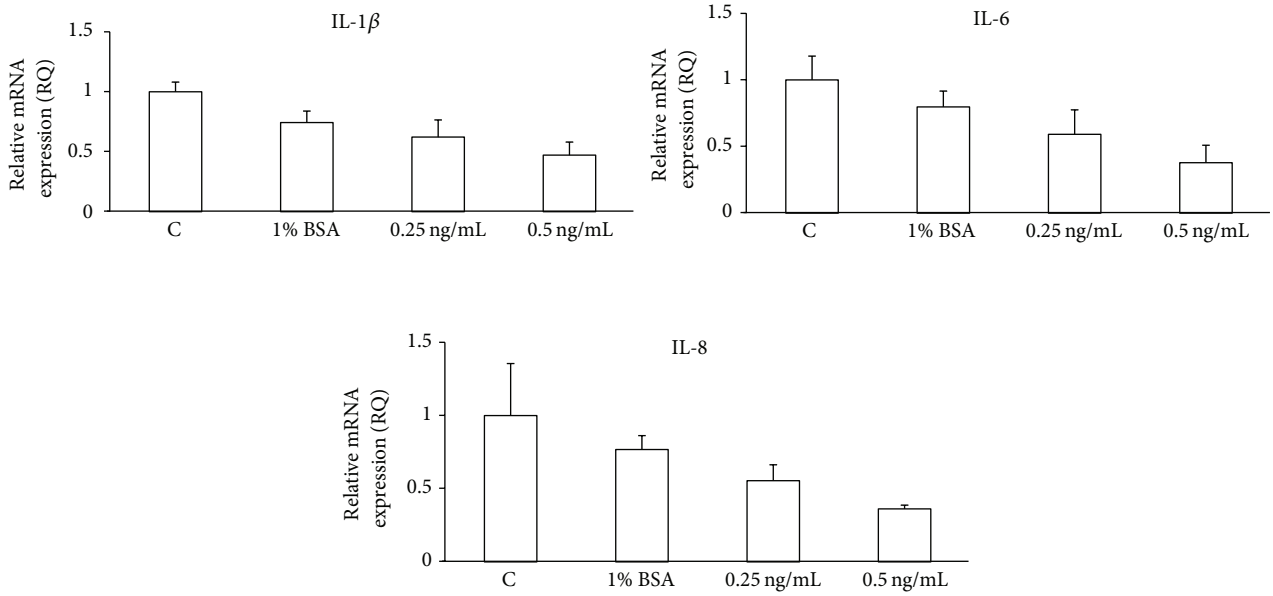


Figure 5: IL-1Ra inhibits IL-1β, IL-6, and IL-8 mRNA expression in BRAT cells. Cells stably expressing BRAT were plated at equal densities, treated with recombinant IL-1Ra, and collected. RNA was isolated, DNAse-treated, and reverse-transcribed. Real-time PCR was performed in triplicate from two separate experiments for IL-1β, IL-6, IL-8, and actin using SYBR Green detection. Data are expressed as the mean fold change in gene expression ± standard error.

p53 [58], enhancing cisplatin sensitivity [59], inhibiting cell growth and VEGF expression [60], and suppressing IL-6 function [61]. There are currently three IL-1 inhibitors used in the treatment of nonmalignant inflammatory conditions: recombinant IL-1Ra, anakinra; IL-1 trap, rilonacept; and monoclonal anti-IL-1β antibody, canakinumab [62]. Consequently, premalignant targeting of the proinflammatory mediator IL-1β and its upstream processors, caspase-1 and/or the NALP3 inflammasome, specifically in 185delAG BRCA1 mutation carriers, may reduce the risk of malignant transformation initiated by chronic inflammation.

Lastly, tumors can develop within and in response to their inflammatory microenvironment. For example, Kim et al. observed localized IL-1 expression among normal, benign, and malignant canine mammary tumors such that IL-1 could not be detected in normal tissue while concentrated IL-1 expression was noted in the stroma of benign tumors and diffuse expression in malignant and metastatic tumors [63]. Similarly, IL-1β secreted by OC cells suppresses p53 expression in cancer-associated fibroblasts [64]. In OC, IL-1β also promotes invasiveness and tumor angiogenesis and induces immune suppression [65]. Constitutive production of IL-1β by human ovarian carcinoma cell lines [66] enhances their invasion capacities by increasing expression of matrix metalloproteinase-1 and stimulating production of proangiogenic factors [52, 67]. Watanabe et al. showed that IL-1β produced by OC cells induced mesothelial cell beta1-integrin-dependent peritoneal metastasis [68]. Increased levels of IL-1β found in the serum and/or the ascites of OC patients have been associated with decreased survival [69]. Likewise, IL-1β can effect OC progression by altering the expression of other proinflammatory cytokines, such as IL-6 which is a downstream target of IL-1β. IL-6 is known to play a major role in OC progression and prognosis and may also be a potential marker of immunological and metabolic changes in OC [70]. Elevated levels of IL-6 have been found in the serum and ascites of OC patients [71] and are also associated with poor prognosis [2]. In support of the data reported herein, we recently found that urinary levels of IL-1β were generally 5 times greater in women with OC compared to healthy controls [72]. Interestingly, while exact BRCA1 mutational status could not be determined, we also found that urinary levels of IL-1β were 3 times higher among women with benign ovarian disease and a FH of breast/ovarian cancer compared with women with benign ovarian disease and lacking a FH of breast/ovarian cancer. Likewise, OC patients with a confirmed FH of cancer had urinary IL-1β levels several fold higher than OC patients without a known FH of cancer. Therefore, chronic presence of IL-1β within the ovarian microenvironment may enhance malignant transformation and underscores the complexity among cancer cells within their microenvironment for tumor progression.

5. Conclusions

Our data identify the proinflammatory mediator, IL-1β, as a novel target in OSE cells expressing the 185delAG BRCA1 mutation. Further, the mechanism by which BRAT regulates IL-1β expression is twofold: (1) CREB-dependent transcriptional control and (2) caspase-1 cleavage of pro-IL-1β. It is interesting to speculate that increases in IL-1B might be limited to 185delAG rather than common to multiple BRCA1 mutations. While we have not been able to study other BRCA1 mutations in similar detail, a recent study by Rebbeck et al. [73] suggests that differences in risk for breast and/or ovarian cancer may be linked to the locations of mutations in BRCA1 and BRCA2. Further, in prior publications, we have shown that targets downstream of 185delAG, including maspin, are unaffected when 185delAG is itself mutated to generate a further BRCA1 mutation [39] suggesting that the 185delAG BRCA1 mutation may promote tumorigenesis by a unique mechanism. Clearly, additional studies on the role of BRCA1 mutations to promote a chronic inflammatory phenotype are warranted since the potential also exists for IL-1β to serve as a predictor of OC and/or as a therapeutic target.

Conflict of Interests

The authors state that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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