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Review Article

The role of estrogen in the pathophysiology of tubal ectopic pregnancy

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Abstract: 17β-estradiol, acting through estrogen receptors α and β, plays a fundamental role in the regulation of Fallopian tube cell homeostasis and in the modulation of normal tubal physiological processes. Fluctuations in E2 levels also play crucial roles in the initiation or progression of numerous human diseases. Fallopian tube malfunction often results in tubal ectopic pregnancy, which is one cause of maternal morbidity and mortality in women. Several factors have been proposed to be associated with increased risk of tubal ectopic pregnancy, but whether these factors are the cause of, or are merely symptoms of, such pregnancies remains unresolved due to the lack of knowledge in regards to the mechanisms by which embryos inadvertently implant in the Fallopian tube. This review summarizes recent findings, including data from our own laboratory, on E2 metabolism and estrogen receptor (ER) subtype expression within the Fallopian tube in humans and rodents. This review also outlines several important, unresolved questions in the field that, once addressed, could offer important clues into how E2/ER signaling contributes to the pathology of tubal function. A better understanding of the specific functions of estrogen receptor subtypes *in vivo*, as well as of the mechanism and consequences of receptor subtype interactions is critical to understanding their respective roles in Fallopian tube physiology and in the pathophysiology and etiology of tubal ectopic pregnancy.

Keywords: 17β-estradiol, estrogen receptor subtypes, human, infertility, tubal ectopic pregnancy

Introduction

The main functions of the mammalian Fallopian tube are to facilitate fertilization of the gamete, transport the early embryo to the uterus, and establish a normal intrauterine pregnancy [1, 2]. Compelling evidence suggests that the morphology and the functional integrity of the Fallopian tube is estrogen dependent [2]. The biological effects of 17β-estradiol (E2) are mediated through activation of the estrogen receptor (ER) [3], a ligand-dependent transcription factor belonging to the nuclear hormone receptor super-family [4]. Two structurally related ER subtypes, ERα (ESR1) and ERβ (ESR2), are encoded on chromosome 6 and chromosome 14, respectively, and appear to have overlapping [4] tissue/cell expression profiles and ligand specificities [5-8]. Moreover, when ER subtypes are co-expressed in tissues and cells *in vivo*, homodimers and/or heterodimers of ERα and ERβ contribute to the complexity of ER action [4]. Recent studies have led to the identification of a third subtype, ERγ, that is specifically expressed in fish ovaries and testes [9, 10], but the existence and possible functions of this subtype in mammals have not been established. Furthermore, some evidence exists for estrogen-responsive G protein-coupled membrane receptors such as GPR30, but such interactions have not been well characterized [11].

While there is a little doubt that physiological levels of E2 are important for normal tubal function, the role of aberrant E2-mediated ER signaling in the Fallopian tube in disease progression

has been a matter of debate [12]. Furthermore, it is interesting to note that the biological activity of E2 is linked to both inhibition and stimulation of the interactions between progesterone (P4) and progesterone receptor (PR) in the female reproductive tract [13, 14]. The regulation of some P4-responsive genes is linked to specific tubal cell functions [15], and because successful uterine implantation requires a balance between E2 and P4 action [16] alterations of both ERs and PRs are currently being investigated in terms of their roles in tubal implantation.

Ectopic pregnancy, defined as a pregnancy in which the embryo becomes implanted outside the uterus, is a major cause of human maternal morbidity and mortality [17, 18]. Ectopic pregnancy accounts for approximately 1.5 to 2% of all pregnancies [18] and for 4 to 10% of pregnancy-related deaths [19] in the Western world, and it is a growing problem in developing countries [20] although the proportion of ectopic pregnancy-related mortality is much less due to significant maternal morbidity/mortality in conjunction with child birth. More than 98% of ectopic pregnancies occur in the Fallopian tube and these are difficult to diagnose until symptoms occur, mostly due to intra-abdominal bleeding [12, 18]. Current therapies focus primarily on methotrexate treatments and surgical interventions, but a better understanding of the pathogenesis of the disease could avoid these by providing better prediction and prevention in at-risk women [18, 21]. Animal studies have contributed to increase our knowledge in the Fallopian tube biology, but none of the animal models that are currently available completely reproduce all aspects of tubal ectopic pregnancy in humans [12]. Thus the most pressing question facing clinicians and investigators remains that of why some women develop tubal ectopic pregnancies and others do not. Understanding the cellular and molecular processes behind the implantation of embryos in the Fallopian tube will lead to the development of new and better diagnostic and therapeutic methods for the prevention and treatment of ectopic pregnancies [22].

In this review, we give special attention to what is known about E2 metabolism and ER subtype expression and the systems they regulate within the Fallopian tube. We also indicate the questions surrounding Fallopian tube dysfunction that remain and need to be addressed.

E2 metabolism

E2 is synthesized primarily by the ovary and is the principle estrogen in the circulation [23]. In humans, growing follicles in the ovary produce increasing amounts of E2 that peak at ovulation at the end of the proliferative phase [23]. The Fallopian tube is a dynamic, steroid-responsive tissue [1, 2] and it has been reported that high E2 levels are able to prevent initial epithelial deciliation in human Fallopian tubes *in vitro* [24]. Several studies have shown that E2 regulates the ciliary beat frequency in guinea-pig Fallopian tubes *in vivo* [25, 26], and regulates tubal protein secretion in human and rodent Fallopian tubes *in vivo* [27, 28] and *in vitro* [29]. In rats, circulating E2 levels appear to reflect local tubal E2 levels during embryonic transport [30]. E2 production is also highly upregulated during human pregnancy [31], and it has been suggested that estrogen, in addition to progesterone, regulates the implantation process in most mammalian species [16]. In mammals, E2 is involved in blastocyst hatching, an early implantation event during the establishment of uterine pregnancy [32], and a delayed implantation mouse model provides evidence that E2 is critical for the attachment of the embryo to the uterine luminal epithelium [33] during the window of uterine receptivity for implantation [34]. Although the specific etiology of tubal ectopic pregnancy is unknown, several related risk factors have been proposed such as endometriosis [35], an E2-dependent disease [36, 37], and it has been reported that women treated with diethylstilbestrol (DES, a synthetic estrogen agonist) have an increased rate of ectopic pregnancies in the Fallopian tube [38]. Moreover, changes in the E2/P4 ratio (high concentrations of estrogens and/or low progesterone concentrations) have been suggested to disturb embryonic motility in the Fallopian tube and lead to ectopic pregnancy [39, 40]. Given the diverse functions of E2 in normal female reproduction, abnormal E2 levels may promote an inappropriate tubal implantation through deleterious effects on tubal function.

Intracellular levels of E2 are determined by its relative rates of synthesis and breakdown. Steroid hormone synthesis is controlled by several highly substrate-selective cytochrome P450 enzymes and a number of steroid dehydrogenases and reductases. Synthesis of E2 requires cytochrome P450 aromatase (*CYP19A1*), which is responsible for the aroma-

tization of androgens (testosterone or androstenedione) to make estrogens [41]. Two types of 17β-hydroxysteroid dehydrogenases (*HSD17B1* and *HSD17B2*) are also implicated in estrogen synthesis, metabolism, and activity in a variety of tissues [42] with *HSD17B1* catalyzing the reversible transformation of the less biologically active estrone (E1) to E2. Interestingly, *CYP19A1 and HSD17B1* have been identified in human and rhesus monkey Fallopian tubes [43, 44], and based on these results our lab has investigated the steroidogenic enzymes that lead to local changes in E2 levels in the human Fallopian tube during the menstrual cycle. We have found that there are no significant changes in *CYP19A1, HSD17B1 and HSD17B2* mRNA expression levels during the ovulation period and the midsecretory phase (data not shown). These results suggest that the Fallopian tube might not produce the endogenous E2 in humans during the menstrual cycle. Although local E2 levels in the tubal cells and fluids have not yet been measured, it is known that both E2 and P4 levels are increased after ovulation during normal menstrual cycles whereas E2 levels are low and P4 levels are high during intrauterine pregnancy [23]. Because circulating E2 levels are higher in women with tubal ectopic pregnancy than non-pregnant women [45, 46], abnormally elevated E2 levels or the imbalance of the E2/P4 ratio may interrupt the tubal microenvironment leading to embryo implantation, thus relating increased E2 levels to increased risk of ectopic pregnancy.

Estrogen receptor localization and regulation

The main cell types of the Fallopian tube are ciliated and secretory epithelial cells, smooth muscle cells, immunocompetent cells such as leukocytes, and blood vessel cells [1, 12]. The ratio of ciliated epithelial cells to secretory epithelial cells in the Fallopian tube is different in different regions of the tube [47, 48]. Moreover, the activities and functions of different tubal cells change throughout the menstrual or estrous cycle and communication among tubal cells allows for normal tubal functions [2, 12]. In rodents, ERα is the predominant ER subtype in the Fallopian tube [5, 28, 48] and it has been shown that the expression of ERα is regulated during both development and the estrous cycle [49-53]. Studies of tubal ER subtypes and cell marker protein expression in rodents using dual immunofluorescence analysis indicate that ERα is localized in ciliated and secretory epithelial cells as well as smooth muscle cells, and ERβ is expressed in ciliated but not secretory epithelial cells [28, 48]. In contrast to the predominant expression of ERα that is observed in rodent Fallopian tubes, both ERα and ERβ are co-expressed at similar levels in normal human Fallopian tubes [54-56]. There is, however, some evidence suggesting that the two ER subtypes are regulated by different mechanisms. During the menstrual cycle, the levels of ERβ expression fluctuate in response to high circulating E2 levels whereas the levels of ERα expression are not altered [54, 55]. These results illustrate that the cell type-specific localization and regulation of ER subtypes in the Fallopian tube varies among species. Mice lacking ERα, ERβ, or both subtypes [57] have different reproductive phenotypes, demonstrating a complex tissue- and cell-specific interplay between the two receptors [58]. Interestingly, ERα is frequently lost in the implantation and non-implantation site (our unpublished data) of the Fallopian tube in women who have suffered from ectopic pregnancy [54, 59]. These results suggest that changes in the expression level of one ER subtype may disrupt the equilibrium among ER dimers in the tubal disease state and thereby lead to Fallopian tube dysfunction. Generally, ligand binding to ERs results in the dissociation of heat shock proteins thereby allowing the receptors to form dimers (ERα/ERα, ERβ/ERβ or ERα/ERβ) [4], and there is evidence that two ER subtypes can modulate each other's activities under some circumstances [60]. In contrast to ERβ, common polymorphisms in the human ERα gene have been associated with female infertility [61], and ERα serves as a dominant regulator in Fallopian tube development [49]. The relative levels of ERα and ERβ within target cells may contribute to the nature and magnitude of functional responses to E2, thus the biological impacts of such ER subtype ratios in terms of Fallopian tube function needs to be determined. However, ERα, which appears to be important for tubal transport, cannot be studied in depth because deletion of ERα in mice results in blockage of ovulation and infertility [57]. The effects of a loss of ERα signaling on tubal function will, therefore, need to be determined by development of tubal-specific ERα knockouts. Future research should be directed towards deciphering the functional, rather than the cellular, characteristics of tubal ER subtypes *in vivo under conditions of* tubal ectopic

pregnancy.

In both humans and mice, uterine natural killer (uNK) cells are the most abundant lymphocytes and are functionally distinct from mature circulating NK cells [62]. Several studies have shown that, in humans, uNK cells within the endometrium reach peak numbers during embryo implantation and the initiation of pregnancy [63, 64]. While uNK cells express ERβ but not ERα [65], it has been shown that uNK cells are absent from the implantation site and the non-decidualized tubal wall in women with EP [66]. Moreover, in women suffering from diseases associated with excess estrogen, such as endometrial hyperplasia, the endometrium lacks uNK cells [62] suggesting that estrogen regulates the production of uNK cells. At present, however, it is unclear whether loss of ERβ-expressing NK cells is a direct consequence of tubal ectopic pregnancy.

Neuroeffector cells of smooth muscle activity, called oviduct interstitial cells of Cajal (ICC-OVI), have been identified in the Fallopian tube of humans [67] and mice [68, 69]. Human [70] and rat [71] ICC-OVI are located in the smooth muscle layer and express ERs. Moreover, animal studies show that treatment with methotrexate, a chemotherapeutic agent used in the treatment of unruptured tubal ectopic pregnancy, decreases tubal ER expression in rats [71], and decreases the number of ICC-OVI and diminishes tubal smooth muscle contractility in rabbits [72]. However, the potential role of ER-expressing ICC-OVI in human Fallopian tube dysfunction has not yet been determined.

Interestingly, several splice variants and other truncated forms of both ERα and ERβ have been identified in human and rodent Fallopian tubes [28, 48, 54-56, 73]. This suggests that the tissue- and cell-specific responses to estrogen may at least in part be due to differences in the expression and regulation of ERα and β isoforms. Because isoforms of both ERα and β might differ in their bioactivity from the wild-type receptor [14], the roles of these isoforms in E2-mediated responses under normal circumstances in the Fallopian tube should be the focus of future research.

Estrogen-related tubal functions and molecular changes

Adrenomedullin (ADM) is a polypeptide that is structurally similar to calcitonin, calcitonin-gene-related peptide, and amylin [74]. ADM is expressed in epithelial cells of the human and rat Fallopian tube [75-77] and the levels of ADM in the tube are approximately 3- to 4-fold higher than those of the ovary and uterus [77]. There is evidence suggesting that circulating ADM levels increase and decrease along with circulating E2 levels in humans during the menstrual cycle [78], and *in vivo* and *in vitro* studies have shown that treatment with E2 leads to up-regulation of ADM expression in human and rat Fallopian tubes [75, 77]. Recently, a series of studies performed on human and rat Fallopian tubes indicated that ADM increases ciliary beat frequency and decreases smooth muscle contractility [75, 79, 80]. These results suggest that E2-induced ADM could be important in normal tubal cells and that the decrease in ERα expression by Fallopian tube cells in women suffering from ectopic pregnancy [54, 59] may result in a loss of ADM expression and subsequent tubal dysfunction.

Exogenous treatment with E2 enhances oocyte transport in the rodent Fallopian tube [73, 81, 82]. On the other hand, excessive exposure to other nonsteroidal, weak estrogenic compounds such as bisphenol-A (BPA) causes embryos to be retained in the mouse Fallopian tube [83]. This discrepancy may possibly be explained by different ER subtypes being involved in the responses to E2 and BPA. For example, while E2 is nonselective and the most potent natural ligand for both ERα and ERβ [4], BPA either acts as an ERβ agonist or plays dual roles as an ERα agonist or antagonist in a cell type-dependent manner [84]. Indeed, our lab has shown that *in vivo* treatment with 4,4',4-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) tris-phenol (PPT, an ERα-selective agonist) or 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN, an ERβ-selective agonist) result in opposite effects on oocyte transport in the rat Fallopian tube [73]. Thus, it is perhaps useful to think of the two ER subtypes as "competence factors" for Fallopian tube transport.

An important question that needs to be addressed is whether or not women using ER antagonist medications show elevated gamete/embryo transport and/or incidences of tubal ectopic pregnancy. One example of such a drug is clomiphene citrate (CC), a nonsteroidal, tissue-selective ER modulator that acts as an ER antagonist in female reproductive tissues [38].

CC is used to treat anovulatory infertility, particularly polycystic ovary syndrome [85], and is the drug of choice for ovulatory women undergoing *in vitro* fertilization previously [24]. Although CC has limited dose-dependent side effects, intermittent use over an extended period of time is associated with an increased frequency of tubal ectopic pregnancy in humans [83, 86-88] and raises the possibility that such treatment causes Fallopian tube abnormalities. Supporting this hypothesis, our lab has shown that isthmus-specific apoptosis of epithelial cells and activation of ERβ act in parallel to block oocyte transport through rat Fallopian tubes in response to chronic CC therapy [73]. These results suggest that CC-induced epithelial cell death might be implicated in the tubal occlusions that lead to tubal ectopic pregnancy.

As master regulators of tubal functions, ERs govern the expression of downstream target genes, which is an ordered, sequential process. The binding of E2 to their receptors leads to activation of downstream signaling pathways [3, 4]. After binding to the specific estrogen response elements (ERE) on DNA, ERs can modulate target gene transcription directly by interacting with components of the transcriptional machinery or indirectly by recruiting and interacting with of a number of primary and secondary transcription factors such as activating protein-1 and c-jun/c-fos [89] or coregulators such as chromatin remodelers [3]. Genetic ablation of the ERα and/or ERβ genes in mice [58] shows that ERα and ERβ can regulate different genes and have different functions [3]. Several studies have shown that changes in gene expression in Fallopian tube cells are associated with estrogen-induced tubal transport and development [81, 90]. Therefore, changes in the tubal ERα expression in women with ectopic pregnancy [54] should result in a dysregulated gene expression pattern in the Fallopian tube and subsequent ectopic implantation. ERα has been shown to serve as a dominant regulator in Fallopian tube development in rats [50] and the next phase of research will focus on identifying, at a molecular level, the downstream ER targets that are involved in the regulation of normal tubal function.

More importantly, the downstream signaling pathways of the ER subtypes may also crosstalk with each other, complicating the roles of E2/ER complexes as transcription factors in the occurrence of tubal ectopic pregnancy. Selective ER subtype agonists and antagonists may prove to be very valuable tools for deciphering the specific roles of ERα and ERβ in the Fallopian tube under physiological conditions [28, 48, 73, 82] and disease states. Specifically, delineating the downstream signaling pathways activated by E2/ERα and/or E2/ERβ complexes in epithelial cells versus smooth muscle cells, and in ciliary beating versus muscular contraction, will help in the development of novel strategies that selectively affect subsets of E2 responses associated with tubal dysfunction and the initiation and development of tubal ectopic pregnancy. While much knowledge has been gained in understanding the mechanisms of the implantation process in mouse uteri by using loss-of-function mutations of specific genes [31], we still know comparatively little about the molecular players that orchestrate uterine implantation and pregnancy events in humans due to ethical constraints and a lack of sufficient amounts of tissue samples. It is known that the Fallopian tube and uterine endometrial-subendometrial layers are of the same embryological origin and that women with tubal ectopic pregnancy do not have any morphological abnormalities in the tubal implantation site [35].

Tubal epithelial cells produce and secrete numerous protein molecules that participate in the regulation of events that precede fertilization as well as support early embryonic development [91]. A number of secretory proteins such as insulin-like growth factor-1 (IGF-1) appear responsive to the cyclic influence of E2 [92]. IGF-1 facilitates normal embryo implantation, and disturbances of IGF-1 levels reduce pregnancy rates [86]. A lack of IGF-1 stimulation in ERα knockout mice demonstrated that ERα is required for mediating IGF-1 activities such as uterine epithelial cell growth and proliferation [93]. Human and rodent tubal epithelial cells express IGF-1 [28, 87, 88] and our previous studies have shown that E2 enhances tubal epithelial cell IGF-1 synthesis and secretion in rodents in an ERα-dependent manner [28, 88]. Estrogen-responsive elements exist in the regulatory regions of the IGF-I gene [94] suggesting that E2 regulates IGF-1 via a direct transcriptional mechanism. As a downstream target of ERα, the role that IGF-1 may play in Fallopian tube transport and implantation is a prime target for further research efforts.

In addition to the E2-mediated/ER-dependent effects on tubal transport discussed above, E2 also regulates transcription via non-genomic pathways such as the activation and production of intracellular cyclic adenosine monophosphate (cAMP) during oocyte transport in rats [95]. How this observation relates to impairment of tubal function leading to tubal ectopic pregnancy is not known and warrants further study.

Conclusion

E2-mediated ER activity is implicated not only in the regulation of normal reproductive functions [23], but also in the development and progression of numerous human diseases [3]. Fallopian tube malfunction may result in tubal ectopic pregnancy [18], one of the most common gynecologic disorders, but little is known about the biological events leading to its occurrence. No biomarkers with high predictive value of tubal ectopic pregnancy have been identified [18], and an understanding of the development of human tubal ectopic pregnancy at the cellular and molecular levels is currently hampered by a lack of appropriate animal models [12]. Given the complexity of E2's actions in the Fallopian tube, it is likely that both genomic and non-genomic pathways influence different aspects of tubal function. Specific E2 responses are dependent on (1) the amount of available E2, (2) the expression pattern and dimerization of ER subtypes, and (3) the different ER subtype downstream targets. Tubal implantation may result from molecular and functional aberrations in the Fallopian tube, abnormal sex steroid responsiveness, or an improper tubal fluid environment [22]. There is currently no accurate method to predict ectopic pregnancy or to identify what treatments for tubal dysfunction should be undertaken to prevent ectopic pregnancy. ERs are important therapeutic targets and while estrogenic agents are widely used for emergency contraception [96] and menopausal hormone replacement therapy [97].

Despite the huge strides made in understanding the molecular mechanisms underlying the potential risk factors involved in ectopic pregnancies [18], several questions still remain to be addressed. For example, how are Fallopian tube functions such as early embryo transport controlled in humans? Do they occur high or low E2 levels and are there different types of tubal cells involved in these processes? How do the initial steps in ER signaling and its downstream targets in tubal cells lead to onset of tubal ectopic pregnancy? Because studies from genetic knockout experiments show that uterine expression of ERα, but not ERβ, is required for implantation [58], defining the mechanisms by which E2-mediated, ER subtype-specific interactions lead to tubal implantation will be a key question for future studies.

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