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*Review Article*

# Linking DNA methylation to the onset of human tubal ectopic pregnancy

Lei Wang[1,2], Yi Feng[3,4], Shien Zou[5], Mats Brännström[6], Lin He[1,2], Håkan Billig[3], Ruijin Shao[3]

*[1]Institutes of Biomedical Sciences at Fudan University, Shanghai 200032, China; [2]Bio-X Center, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders, Ministry of Education, Shanghai Jiao Tong University, Shanghai 200032, China; [3]Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg 40530, Sweden; [4]Department of Integrative Medicine and Neurobiology, State Key Lab of Medical Neurobiology, Shanghai Medical College and Institute of Acupuncture Research (WHO Collaborating Center for Traditional Medicine), Institute of Brain Science, Fudan University, Shanghai 200032, China; [5]Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200011, China; [6]Department of Obstetrics and Gynecology, Institute of Clinical Sciences, The Sahlgrenska Academy, University of Gothenburg, Gothenburg 41345, Sweden*

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**Abstract:** Ectopic pregnancy is a common reproductive disorder of unknown etiology and is a leading cause of maternal and fetal mortality. Because of the asymptomatic nature of early tubal ectopic pregnancy and the lack of specific biomarkers for early diagnosis, a better understanding of the complex cellular and molecular interactions that contribute to tubal ectopic pregnancy is required. DNA methylation is the most studied epigenetic process in various tissues and cells, and the goal of this article is to provide a brief review of recent work describing the potential mechanisms of DNA methylation and the biological function of such methylation in normal intrauterine pregnancy. Further, novel findings from our laboratory highlight the possible role of DNA methylation in human Fallopian tube dysfunction and suggest a possible correlation between methylation of estrogen receptor α in women and the occurrence of tubal ectopic pregnancies.

**Keywords:** DNA methylation, DNMT, implantation, ectopic pregnancy, fallopian tube

## Challenges in the understanding of the mechanisms behind human ectopic pregnancy

Human ectopic pregnancy (EP) is the second most common cause of pregnancy-related first trimester deaths [1, 2] and complicates up to 2% of all pregnancies in Europe and the USA [3]. Approximately 98% of EPs occur in the Fallopian tube [3]. In mammals, transport of the female and male gametes, fertilization, initial embryonic development, and transport of the embryo to the uterus take place in the Fallopian tube [4]. After fertilization, a precise timing of the transport of the embryo is required for proper intrauterine implantation. Because the Fallopian tubes are not accommodated to hold a growing embryo, the implantation and growth of the embryo will cause the Fallopian tube to rupture if the EP is not surgically or medically treated [1]. Women with a tubal EP have an increased rate of infertility and an increased risk for future tubal EPs [2]. A major limitation to understanding the pathophysiology of EPs is the time from the initiation of the EP to the onset of EP-related symptoms. Although multiple factors have been proposed to be associated with an increased risk of tubal EP [3, 5, 6], no experimental studies have firmly established causative roles for any of the factors implicated in the pathogenesis of EP. These putative predisposing risk factors should, therefore, be interpreted with caution [3] because analysis is limited by the paucity of available studies, small study populations, and conflicting results. The results obtained to date suggest that other, as yet unexamined, factors may be involved in the pathogenesis of EP [5, 6].

Although our understanding of tubal physiology is extensive [7], the data available from human

Fallopian tube studies are generally limited to being descriptive and speculative and result in a fragmented picture of tubal function in humans. This is in part due to the unfeasibility of obtaining Fallopian tube tissues from women at the same gestational stage of intrauterine pregnancy [6]. Thus the physiological role of the Fallopian tubes in maintaining normal intrauterine implantation and pregnancy is not fully understood. Animal studies have contributed to our knowledge of Fallopian tube biology, but none of the animal models that are currently available reproduce tubal EP in humans [8].

Determining the cellular and molecular mechanisms responsible for the development and progression of abnormal pregnancy are among the most challenging topics in the field of female reproductive biology. The influx of new findings from studies of human and rodent Fallopian tubes under physiological conditions [5, 9-12], however, is providing significant opportunities to increase our understanding of how tubal implantation occurs at the cellular and molecular levels. This knowledge will likely lead to novel clinical methods for preventing and controlling the initial processes of tubal EP in humans [3].

## The biological roles of DNA methylation

DNA methylation is a biochemical process that is important for normal development [13]. It involves the addition of a methyl group to the carbon at position 5 of the cytosine pyrimidine ring or the nitrogen at position 6 of the adenine purine ring. In prokaryotes, DNA methylation occurs on both cytosine and adenine bases, but in eukaryotes methylation occurs only on cytosine bases. In humans DNA methylation results in the formation of 5-methylcytosine (5-mC), and it is estimated that between 60 and 90% of all cytosine-phosphate-guanine (CpG) dinucleotide sequences in the human genome are methylated [14]. 5-mC is an epigenetic marker that can regulate genomic activity and can be maintained throughout mitosis and meiosis [15].

The addition of methyl groups changes the biophysical characteristics of the DNA and inhibits the recognition of DNA by some proteins and permits the binding of others [13]. In mammals DNA methylation is tightly controlled by the DNA methyltransferases (DNMTs) DNMT1, DNMT3a, and DNMT3b (**Figure 1A**). DNMT1 is the maintenance methyltransferase and copies pre-existing methylation patterns onto the new DNA strand during DNA replication. DNMT3a and DNMT3b are the *de novo* methyltransferases and are mainly responsible for introducing cytosine methylation at previously unmethylated CpG sites [16].

5-mC has been found in every vertebrate examined, and in adult somatic tissues DNA methylation typically occurs in a CpG dinucleotide context [13, 16]. 5-mC reduces gene expression by interfering with the binding of transcription factors and other proteins of the transcription complex that recognize cytosine bases in the major groove of certain DNA sequences. The majority of known transcription factors have binding sites that recognize GC-rich DNA sequences, and the recognition elements for many transcription factors contain CpG dinucleotides. Under normal conditions, transcriptional factors bind to the CpG elements in the promoter regions of genes and activate gene transcription. Under disease conditions in which DNA methylation is upregulated, excessive methylation of CpG dinucleotides disrupts binding of these factors and transcription is repressed (**Figure 1B**) [13, 17]. Although DNA methylation generally silences the gene expression and loss of DNA methylation is associated with increased gene expression, exceptions to this rule are beginning to emerge [16].

Evidence has shown that DNA methylation is a dynamic epigenetic mechanism that plays a significant role in regulating tissue- and cell-specific gene expression [17]. On the genomic level, microarray-based approaches and restriction landmark genome scanning have identified differentially methylated regions in specific tissues that display an inverse correlation with gene expression [13, 17]. On the single gene level, an ever-increasing number of genes have been found to be regulated by DNA methylation during early development, in adult somatic cells, and during disease progression [16, 17]. Within certain tissues, different cell types have been shown to have different DNA methylation statuses, and this is exemplified in human placenta [18] and breast tissues [19]. Tissue- and/or cell-specific gene regulation may be the result of the recruitment of sequence-specific transcription factors that are essential for tissue-specific gene expression [15, 16], and

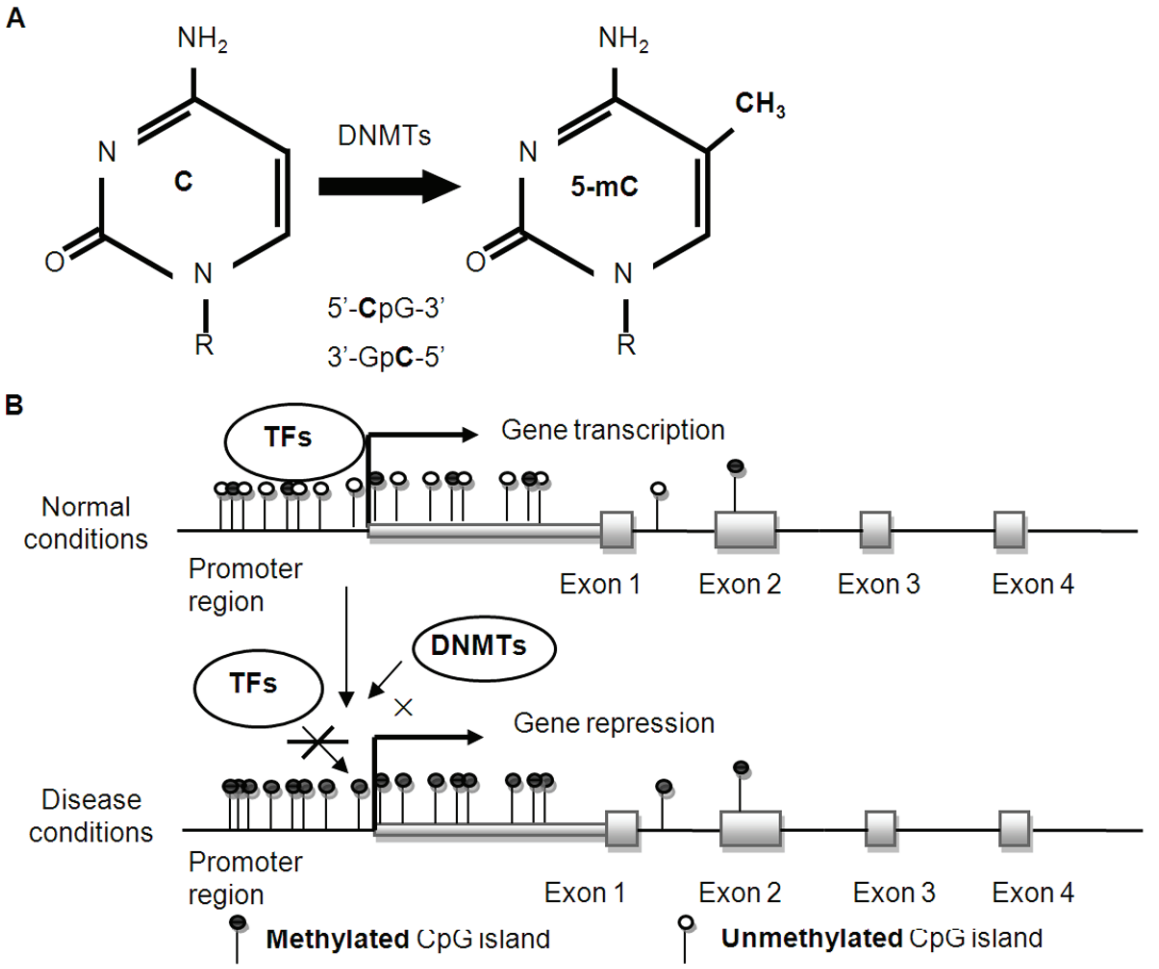


**Figure 1.** A. DNMTs convert cytosine into 5-methylcytosine. B. Under normal conditions, most CpGs within a CpG island are unmethylated and binding of TFs initiates gene transcription. However, when DNMTs convert unmethylated CpG islands into methylated CpG islands, TFs can no longer bind leading to gene repression. C, cytosine; 5-mC, 5-methylcytosine; DNMTs, DNA methyltransferases; CpG, cytosine-phosphate-guanine; TFs, transcription factors.

aberrant DNA methylation may disrupt this specificity and result in the development of complex diseases such as cancer [20].

### The impact of DNA methylation on normal intrauterine pregnancy

The implantation process requires that the embryo attaches to the receptive endometrial epithelium, traverses the cells of the epithelial lining, and invades into the endometrial stroma of the uterus [21]. There is increasing evidence that epigenetic mechanisms, including DNA methylation, are involved in the regulation of endometrial changes during the menstrual cycle [22-24], the implantation process [25-28], and early embryo development [29]. These mechanisms, therefore, make important contributions to normal pregnancy outcomes. For instance, the expression levels of DNMT1, 3a, and 3b are higher in the proliferative phase than the secretory phase [22-24]. Moreover, *in vitro* exposure to 17β-estradiol (E2) and/or progesterone (P4) has been shown to alter the levels of DNMT1, 3a, and 3b mRNA and protein in human endometrium in a time-dependent manner [24]. Preliminary results from our lab suggest that decreases in endogenous E2 and P4 levels are associated with decreases in endometrial DNMT1, 3a, and 3b protein levels in post-menopausal women (preliminary with unpublished). Thus, it is tempting to postulate that expression of DNMTs is likely regulated by E2 and P4 in women during intrauterine pregnancy, a time when circulating E2 and P4 levels are markedly elevated.

Uterine implantation has been shown to alter the expression of various genes in the human endometrium and rodent uterus [21]. *In vivo* treatment of mice with 5-Aza-2'-deoxycytidine, a DNA methylation inhibitor, result in the reduction of intrauterine implantation [25] highlighting the role of DNA methylation in normal implantation. Moreover, considerable evidence from both *in vivo* and *in vitro* studies suggests that DNA methylation has a biphasic effect on the regulation of the expression of several essential endometrial genes, such as oestrogen and progesterone receptors, in human endometrial stromal cells and the mouse uterus [25, 28]. In addition, successful implantation depends on a complex and sophisticated interaction between the competent embryo and the receptive endometrium in humans [21]. DNMTs are essential for normal embryonic development because individual *Dnmt1*-, *3a*-, or *3b*-null mice show embryonic lethality or postnatal death [29]. Several clinical studies have shown that epigenetic changes very often are associated with adverse pregnancy outcomes [30].

### New findings in the human Fallopian tube

The human Fallopian tube consists of an inner mucosal layer (the endosalpinx) that is supported by the lamina propria (a loose connective tissue), a muscular layer (the myosalpinx), and a serosal coat (the mesosalpinx) [31]. These different tissue/cell layers are mainly composed of epithelial cells, smooth muscle cells [32]. Although it is still debatable whether the tubal stromal cells undergo decidualization in the Fallopian tube, stromal cells within the lamina propria have been shown to transform into decidual cells during the development of EP [33]. The global DNA methylation status in normal human Fallopian tubes has recently been reported [34], but it is unknown whether aberrant DNA methylation will be present when the Fallopian tube becomes dysfunctional. To our knowledge, no studies have examined the expression pattern of DNMTs in the Fallopian tube under both physiological and disease conditions.

Direct comparison of DNMT expression and global DNA methylation status in the Fallopian tube between tubal and gestational age-matched intrauterine pregnancies is difficult. However, in our laboratory we have recently begun to investigate the expression levels of DNMT1, 3a, 3b and the levels of 5-mC in Fallopian tube biopsies from non-pregnant women in mid-secretory phase (the implantation window) and from both the implantation and non-implantation sites in women with EP. Real-time RT-PCR analysis showed that mRNA levels of DNMT1 and tet methylcytosine dioxygenase 1 (TET1) did not change significantly between the implantation and non-implantation sites in women with EP; however, DNMT3a and 3b mRNA expression was significantly higher in the EP implantation site (**Figure 2**). Immunohistochemical assessments revealed that both DNMT1 and 5-mC were present in the nuclei of tubal epithelial and stromal cells taken from non-pregnant women during their mid-secretory phase (**Figure 3B1** and **D1**). We observed no expression of DNMT3a in these tissues (**Figure 3C1**). In Fallopian tube tissue samples from the non-implantation site in women with EP, DNMT1 immunoreactivity was observed in the cytoplasm of epithelial cells (**Figure 3B2**) along with 5-mC (**Figure 3D2**), but the level of 5-mC was slightly reduced from that seen in the samples from non-pregnant women in mid-secretory phase (**Figure 3D1**). No immunoreactivity was seen for DNMT1 or 5-mC in the epithelial cells from the EP implantation site (**Figure 3B3** and **3D3**). One interesting observation was that immunoreactivity of DNMT3a was found to be absent in the epithelial and stromal cells in the mid-secretory phase of non-pregnant women and from the non-implantation site in women with EP (**Figure 3C1** and **C2**), but high levels of immunoreactivity were observed in the nuclei of epithelial cells in the EP implantation site (**Figure 2C3**). DNMT3b was difficult to detect in any of the tubal cells (data not shown). There were no changes in expression of DNMT1- an3d 5-mC-positive or DNMT3a- and 3b-negative smooth muscle cells in response to either non-pregnant or pregnant conditions (data not shown).

Although a primary role of DNA methylation in the Fallopian tube itself cannot be dismissed, the presence of distinct DNMT1, 3a and 5-mC expression patterns at the implantation site suggests that an implanted blastocyst exerts a paracrine influence on the DNA methylation status of the Fallopian tubes in a cell type-specific manner. In light of our understanding of the importance of embryo-endometrium inter-

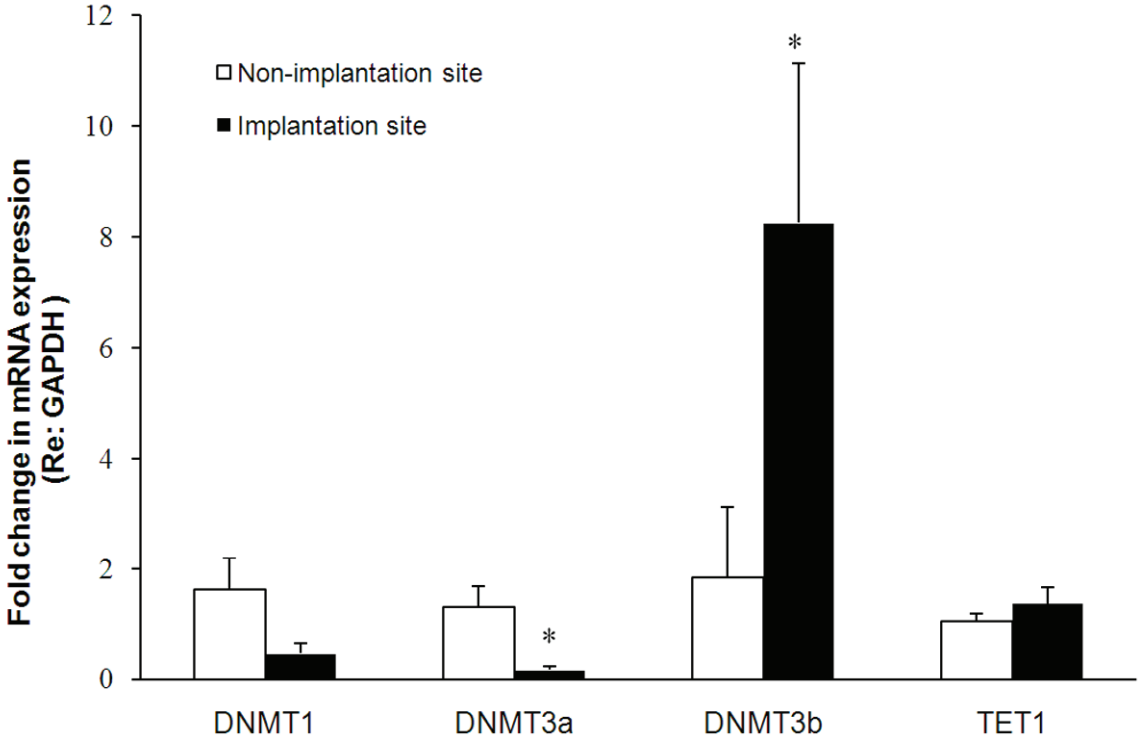


**Figure 2.** Change in mRNA levels of DNMTs in the implantation and non-implantation sites of women with ectopic pregnancy. Fallopian tubes (n = 10) were obtained from the Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University and were analyzed for DNMT1, DNMT3a, DNMT3b, and tet methylcytosine dioxygenase 1 (TET1) mRNA levels by qRT-PCR. mRNA levels of each gene are relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in the same samples. Values are mean ± SEM. Significance was tested by one-way ANOVA with Bonferroni correction when appropriate. *$P < 0.05$.

action for normal implantation and pregnancy [21], it can be indirectly assumed that molecules that derive from the developing blastocyst and affect embryo-tubal communication may favour tubal implantation. However, we do not know how the changes in DNA methylation patterns are relevant to the initiation and progression of tubal EP. The epithelial layer of the Fallopian tube usually avoids implantation by preventing the early embryo from physically interacting with the epithelial cells [7]. Our hypothesis is that the delay of the mature embryo in entering the uterus due to tubal cell damage and/or tubal microenvironmental changes increases the risk of EP [6].

It has been reported that ovulation increases double-strand DNA breaks in tubal epithelial cells in mice *in vivo*, and the same study also shows that alteration of the DNA repair activity rather than an increase in the rate of apoptosis is occurs in these damaged epithelial cells [35]. DNA methylation plays an important role in DNA repair, and DNMT1, an ancestral DNA repair protein, is recruited to sites of DNA damage when DNA repair processes are activated [36]. It is possible, therefore, that loss of DNMT1 expression may result in the interruption of DNA damage repair in epithelial cells after ovulation and may allow the embryo to implant in the Fallopian tube through DNA methylation switching. The potential function of DNMT1 in tubal epithelial cells during the transport of the early embryo remains to be explored.

Although there are anatomical differences between the Fallopian tubes and the uterus in humans, the Fallopian tube and uterine endometrial-subendometrial layers are of the same embryological origin [37]. Tubal EP presents a morphologically normal blastocyst at the tubal implantation site as would be seen in an intrauterine pregnancy [38], and it is possible that dynamic regulation of DNMT1/3a expression and 5-mC formation in the Fallopian tubes of women with EP may mirror the biological changes that occur in the uterus during implantation. Indeed, in agreement with our data (**Figure**

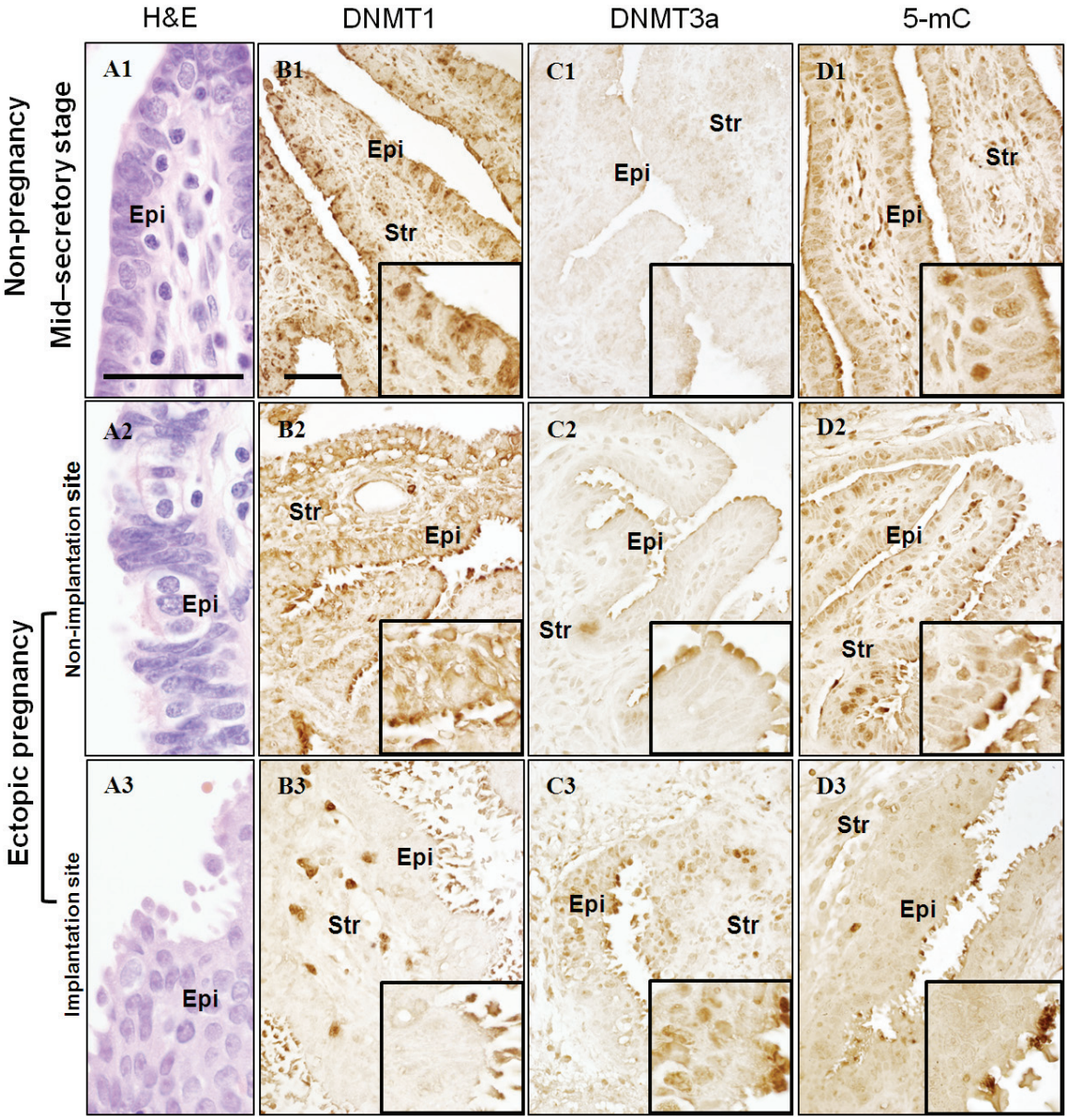


**Figure 3.** Comparison of endogenous DNMT1, DNMT3a, and 5-methylcytosine localization in human Fallopian tubes. Human Fallopian tube biopsies from non-pregnant women at mid-secretory stage (n = 7) and from the implantation and non-implantation sites of women with ectopic pregnancy (n = 8) were obtained from the Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University and fixed in formalin and embedded in paraffin. The histology of haematoxylin and eosin-stained human tubal biopsy samples is indicated in panels A1–3. Mouse anti-DNMT1 (ab92453, 1: 100), mouse anti-DNMT3a (ab13888, 1: 100), and mouse anti-5-methylcytosine (5-mC, ab73938, 1: 100) were obtained from Abcam (Cambridge, UK). The localization of DNMT1 (B1–3), DNMT3a (C1–3), and 5-mC (D1–3) was detected by a peroxidase-antiperoxidase detection method using a single 3,3'-diaminobenzidine (DAB) as the chromogen. Representative micrographs show that DNMT1 immunoreactivity is heterogeneously distributed in the nuclei and cytoplasm of epithelial cells and in the nuclei of stromal cells in the mid-secretory stage (B1). Increased cytoplasmic expression of DNMT1 is observed in the cytoplasm of epithelial cells in the non-implantation site (B2), and mainly nuclear immunostaining of DNMT1is seen in stromal cells in the implantation site (B3). DNMT1 immunostaining in the apical portion of epithelial cells (i.e., cilia) is consistently observed in mid-secretory stage (B1) and tubal EP (B2 and B3). Representative micrographs show that very low DNMT3a immunoreactivity is limited to a few apical epithelial cells in the mid-secretory stage (C1), and DNMT1 immunoreactivity is increased in the apical epithelial cells in the non-implantation and implantation sites (C2 and C3). However, DNMT3a immunoreactivity is increased selectively in the nuclei of epithelial cells in the implantation site (C3). Representative micrographs show that although 5-mC immunostaining densities are slightly different, 5-mC

immunoreactivity is homogeneously distributed in the nuclei of epithelial cells and stromal cells in the mid-secretory stage (D1) and the non-implantation site (D2). 5-mC immunoreactivity is highly enriched in cilia in mid-secretory stage (D1) and tubal EP (D2 and D3), but 5-mC immunoreactivity is absent in epithelial cells and rarely detected in stromal cells at the implantation site (D3). Enhanced magnifications of the images are shown in the lower right corner. Sections that were exposed to mouse IgG were used as negative controls (data not shown). Epi, epithelial cells; Str, stromal cells. Scale bar, 50 μm.

3B3) a recent *in vitro* study has shown that the level of DNMT1 is increased in human endometrial stromal cells during decidualization, which is the process by which the trophoblast cells invade the endometrium and establish the formation of the placenta [39].

## Clues for DNA methylation of ERα in the Fallopian tube

Many of the functions attributed to the Fallopian tube are regulated by a variety of endogenous molecular mediators, including steroid hormones [4]. 17β-estradiol plays a crucial role in the intricate process of implantation [40]. For example, E2 promotes blastocyte hatching, which occurs early during uterine implantation [41]. Several studies using a delayed-implantation mouse model have illustrated the E2-dependent attachment of the embryo to the receptive uterus for implantation [43, 44]. In addition, changes in gene expression in Fallopian tube cells are associated with oestrogen-induced tubal transport and development [42, 43]. The effects of oestrogens are mediated through the nuclear estrogen receptors (ERs), which regulate transcription of target genes through binding to specific DNA target sequences [40], and blastocysts fail to implant in *Esr1 (ERα)*-null female mice following donor embryo transfer [44]. This suggests that functional ERα is required for normal intrauterine implantation in mice.

ERα serves as a dominant regulator of Fallopian tube development [45]. In humans and rodents, ERα is expressed in the Fallopian tubal cells, with mRNA or protein levels that do not fluctuate during the menstrual cycle in contrast to the oestrous cycle [40]. In women with EP, epithelial ERα expression is frequently lost in the implantation site but not in the non-implantation site in the Fallopian tubes [40]. It is not yet clear whether loss of ERα expression in the tubal implantation site is an cause or a consequence of tubal EP, but it is clear that decreased ERα expression occurs in parallel to decreased DNA methylation in the intrauterine implantation site in folate-deficient mice [27]. Previous studies have demonstrated that elevated DNA methylation of the ERα gene promoter is associated with reduced ERα expression in breast cancer cells *in vitro* [46, 47]. Indeed, many actively transcribed genes have been found with high levels of DNA methylation suggesting that the differential distribution of DNA methylation is crucial to transcriptional regulation [48].

It has not yet been established that epigenetic alterations of the ERα gene participate in the initiation and development of tubal EP in humans. A previous study has shown that recruitment of DNMT3a and 3b parallels to the loss of methylation at an oestrogen-response element [49]. We note that increased DNMT3a expression is associated with reduced ERα expression in the implantation site of the Fallopian tube during EP. This raises the possibility that *de novo* DNA methylation contributes to the inhibition of ERα expression in women with tubal EP. Certainly, establishment of the relationship between promoter DNA methylation patterns and expression of ERα in the Fallopian tube in future studies will aid in understanding how the epigenetic modification of endogenous ERα participates in the pathogenesis of tubal EP.

## Conclusions and perspectives

The triggers for tubal EP are still unknown [3]. A significant challenge in identifying the potential cellular and molecular abnormalities in the Fallopian tube that lead to the onset of tubal implantation is essential. Recent studies reveal roles of DNA methylation in normal intrauterine implantation and early embryo development in humans *in vitro* and in mice *in vivo*. Thus the link between DNA methylation and tubal implantation in humans *in vivo* is an area of keen interest. The data presented here provide new insights into the hypothesis that DNA methylation and DNMTs might play a direct role in the occurrence of tubal EP in women. While unique characteristics of DNA methylation are

clear [15-17], a cause and effect relationship between DNA methylation and tubal EP remains to be determined.

Epigenetic processes begin with DNA methylation, which constitutes an essential mechanism for repression of tissue- and/or cell-specific gene expression [17], and determining the distribution of DNMTs in the human Fallopian tube is likely to be an important key to understanding the role of DNA methylation. However, simply reporting DNMT protein levels and global DNA methylation status is no longer sufficient. Ideally, studies using bioinformatics analyses to decipher the connection of DNA methylation and modified gene-specific patterns for the elucidation of epigenetically regulated pathways associated with the development of tubal EP should be performed in the future. Because the activity of DNMTs and the genes required for intrauterine implantation are hormonally regulated, it is possible that dynamic regulation of epigenetic modification is one of the key mechanisms involved in the biology of female reproduction. Although the biology of ERα in general remains an area that warrants further study [40], its role in intrauterine and tubal pregnancies in humans is of particular interest. Further investigation is required to determine whether the changes seen in DNA methylation status, DNMT expression patterns, and DNMT-mediated ERα regulation in the Fallopian tube contribute to, or are a consequence of, tubal EP.

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Authors' contributions: LW, FY, SZ, and RS participated in the recruitment of patients, conducted the experiments, and made the figures. LW and RS drafted the manuscript. SZ, MB, LH, and HB critically reviewed the manuscript. All authors read and approved the final manuscript.

## Conflict of interest statement

The authors report no conflict of interest.

**Address correspondence to:** Dr. Ruijin Shao, Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg 40530, Sweden. Phone: 46 31 786 3408; Fax: 46 31 786 3512; E-mail: ruijin.shao@fysiologi.gu.se

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