

## —Mini Review—

# How does progesterone support embryo implantation?

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**Abstract:** Pregnancy comprises multiple stages with complex interactions of molecules and cells, and previous studies have clarified that progesterone ( $P_4$ ) is a key player in pregnancy. Several animal experimental models have been established to address the detailed mechanisms of  $P_4$ , and genetically engineered mouse models have especially helped us understand its function.  $P_4$  receptor (PR)-null female mice show no ovulation, while PR co-chaperone FKBP52-null mice exhibit implantation failure with normal ovulation. Moderate supplementation of  $P_4$  rescues implantation failure in FKBP52-deficient mice but does not restore the capability for pregnancy up to full term, resulting in embryo resorption. Supplementation of a large amount of  $P_4$ , however, can rescue pregnancy and provide normal reproductive outcomes until parturition. Mouse studies by our groups, and others, have also shown that epigenetic regulation of uterine  $P_4$ -PR signaling,  $P_4$ -induced molecular cross-talk between the epithelium and stroma and uterine proliferation-differentiation switching are indispensable for successful implantation. Collectively,  $P_4$  orchestrates the whole process of pregnancy in spatiotemporal manners, eventually integrating them toward successful parturition. In this review article, we review the literature on the uterine functions of  $P_4$  in pregnancy, with a special focus on the knowledge gained about embryo implantation by studies utilizing mouse models.

**Key words:** Progesterone, Embryo implantation, Progesterone receptor, Proliferation-differentiation switching

## Introduction

In the developed nations, including Japan, infertility, miscarriage, preterm birth, and other pregnancy-related

disorders have become social and economic concerns due to changes in lifestyle and maternal aging. Despite sincere clinical and research efforts, the underlying mechanisms of pregnancy remain obscure.

Progesterone ( $P_4$ ) is called a “hormone of pregnancy”. It is secreted from the ovarian corpus luteum after ovulation. Successful implantation induces the inhibition of luteolysis and the maintenance of ovarian  $P_4$  secretion. In humans, the main source of  $P_4$  production switches from the ovary to the placenta by the second trimester of pregnancy. Previous studies have shown that  $P_4$  is a key player in each step of pregnancy, including ovulation, fertilization, implantation, decidualization, and pregnancy maintenance [1–8].  $P_4$  is clinically used for luteal support to improve the implantation rate, providing empirical evidence of its significant role in human implantation. It has been reported that 75% of failed conceptions are due to implantation failure [9]. Therefore, it is important to study implantation mechanisms both from the clinical and research aspects. Progestin is a drug clinically known to improve implantation rates [10], and therefore, elucidation the role of  $P_4$  in implantation may ultimately help to discover novel approaches for infertility treatment.

Molecular and cellular functions of  $P_4$  in embryo implantation have been greatly clarified using many animal models [2–8]. In order to understand the physiological roles of  $P_4$  in pregnancy, mouse models are powerful tools. Recently, genetically-engineered mouse models have helped us gain a better understanding of the fundamental functions of  $P_4$  in embryo implantation. In this review article, we focus on the  $P_4$ -associated molecular mechanisms during pregnancy, especially those in implantation, elucidated by studies using several mouse models.

## $P_4$ Signaling in Ovulation and Implantation

$P_4$  acts through progesterone receptor (PR), a nuclear receptor, transcriptionally controlling  $P_4$ -responsive

genes and triggering critical pathways for each pregnancy event including ovulation and implantation [1–4]. The genetic modification of PR in female mice has provided many insights into the role of P<sub>4</sub> during pregnancy. PR-deficient female mice are infertile due to anovulation [11], suggesting that P<sub>4</sub>-PR signaling is essential for ovulation. This model is very useful for analyzing the physiological and pathological molecular pathways in ovulation, but not for the detailed examination of the role of P<sub>4</sub> in implantation and subsequent pregnancy events. Nonetheless, we can understand the hormonal responsiveness of the uterus in PR-null mice by employing ovariectomy with ovarian hormone treatments. Ovariectomized PR-null and wild-type (WT) mice with treated with both estradiol-17β (E<sub>2</sub>) and P<sub>4</sub> show different endometrial statuses of cell proliferation and differentiation [11]. It is widely accepted that proliferation is poorly compatible with differentiation, and distinct switching between proliferation and differentiation have been demonstrated for many different cell types [12–15]. In WT uteri, both attenuated proliferation of endometrial epithelial cells and activated proliferation of stromal cells start simultaneously [12]. Here, we describe this phenomenon as endometrial proliferation-differentiation switching (PDS). In contrast, PR null uteri do not demonstrate PDS, but show epithelial proliferation and poor stromal cellularity [11]. These findings indicate that P<sub>4</sub>-PR signaling leads to endometrial PDS. In the physiological condition when newly-formed corpus lutea produces P<sub>4</sub> after ovulation in WT mice, P<sub>4</sub>-PR signaling governs the uterus and induces endometrial PDS in the preimplantation period. Our recent study clearly demonstrated that an injection of PR antagonist RU486 in the periimplantation period impairs endometrial PDS and blastocyst implantation in WT mice [16]. In addition, PDS in the receptive uterus occurs not only in mice but also in humans [16]. Without exception, the literature reports that all types of genetically-modified mice lacking PDS in the periimplantation period have implantation failure [2–4, 8], strongly suggesting that PDS is a marker of uterine receptivity. Furthermore, PR has two isoforms, PR-A and PR-B, and previous studies have demonstrated that PR-A is primarily responsible for uterine function during pregnancy, contributing to the endometrial PDS [17, 18]. However, it is assumed that PR-B does not have a critical function in pregnancy, because systemic ablation of PR-B does not induce any problems in pregnancy outcome [17, 18]. Thus, the signaling of P<sub>4</sub>-PR, especially of P<sub>4</sub>-PR-A, controls endometrial PDS as well as receptivity to embryo implantation.

### Gene Modified Mouse Models for Analyzing Implantation

Appropriate PR function depends on the stability of the PR complex. The functionally mature PR complex consists of a receptor monomer, a 90-kDa heat shock protein (Hsp90) dimer, the cochaperone, p23, and one of four cochaperones which include a tetratricopeptide repeat (TPR) that binds to Hsp90 [8, 19, 20]. The immunophilin cochaperone, FKBP52-binding protein 4 (FKBP52), is one of these TPR-containing chaperones, binding both Hsp90 and PR, stabilizing the structure of the PR complex, thereby reinforcing P<sub>4</sub>-PR signaling [8, 19, 20]. Targeted deletion of FKBP52 attenuates uterine P<sub>4</sub>-PR signaling, but does not completely suppress it, because minimal binding of P<sub>4</sub> to PR is retained [8, 19, 20]. Excessive P<sub>4</sub> administration can strengthen PR signaling in the uterus on a CD1 background, a notable feature of FKBP52-null mice, which differentiates them from PR-null mice [20]. Moreover, FKBP52-null females on the CD1 background show normal ovulatory function with normal P<sub>4</sub> secretion [20]. Therefore, unlike PR-deficient mice, the CD1 FKBP52-deficient mice are very useful tools for exploring the molecular mechanisms of P<sub>4</sub>-PR signaling in the physiological processes of pregnancy after ovulation, including implantation, decidualization, and pregnancy maintenance. Previous investigations have demonstrated that FKBP52-null mice display decreased uterine responsiveness to P<sub>4</sub> and enhanced sensitivity to estrogen, which disturbs the proper regulation of endometrial PDS in the preimplantation period, thus ultimately inducing implantation failure [19]. However, these disorders of endometrial PDS and embryo implantation in the CD1 FKBP52 null mice can be totally recovered by modest supplementation of P<sub>4</sub> via silastic implants of P<sub>4</sub> [20], indicating that P<sub>4</sub>-PR signaling plays a crucial role in implantation. Consequently, FKBP52-null mice are a well-established unique animal model reflecting what is known as “P<sub>4</sub> resistance”, the diminished uterine responsiveness to P<sub>4</sub> which is reversed by P<sub>4</sub> supplementation in a genetic background-dependent manner (Fig. 1).

The regulation of appropriate balance between E<sub>2</sub> and P<sub>4</sub> signaling is a very sophisticated mechanism which defines uterine receptivity. In mice, a small rise in ovarian estrogen secretion just before implantation with preceding ovarian P<sub>4</sub> production strictly rules the “implantation window”, the time-limited acquisition of receptivity to embryo implantation in the uterus. Too much or too little E<sub>2</sub> results in opening failure of the implantation window [2–4, 21]. The causal connection between ovarian

Pregnancy Stages

P <sub>4</sub> -PR Signaling	Mouse models	Ovulation	Implantation	Decidualization	Pregnancy Maintenance	Term Delivery
		PR -/-	✗			
	PR -/- + P <sub>4</sub>	✗				
	FKBP52 -/-	○	✗			
	FKBP52 -/- + P <sub>4</sub> (minimum)	○	○	○	✗	
	FKBP52 -/- + P <sub>4</sub> (maximum)	○	○	○	○	○
	WT	○	○	○	○	○

Fig. 1. Differences of P<sub>4</sub>-PR signaling and pregnancy outcomes among the mouse models.

E<sub>2</sub> secretion before implantation and uterine receptivity is still controversial in primates [22–24]. However, in humans, the principle of the “implantation window” is generally accepted [25], and there is evidence showing that heightened E<sub>2</sub>-estrogen receptor (ER) signaling disturbs the expression of some essential molecules during implantation, such as integrin, leading to a higher rate of implantation failure [26–29]. Implantation failure due to this aberrant hormonal signaling balance between E<sub>2</sub>-ER versus P<sub>4</sub>-PR is also observed in knockout mouse models other than FKBP52-deleted mice. Uterine specific deletion of the nuclear receptor co-activator 2 (Ncoa2) gene encoding steroid receptor co-activator 2 (SRC2) leads to implantation failure due to its absence, inhibiting the optimization of the PR function by Ncoa2 [32]. Ncoa2 is also expressed in the human endometrium, indicating its role in mediating P<sub>4</sub>-PR signaling [30–32]. Although *in vitro* studies have reported that nuclear receptor co-activator 6 (Ncoa6) interacts with ER $\alpha$  as a coactivator [33–36], an *in vivo* study showed that Ncoa6 does not act as a coactivator but promotes the ubiquitination and degradation of ER $\alpha$ , attenuating E<sub>2</sub>-ER signaling in the periimplantation period [37]. Uterine ablation of Ncoa6 causes accumulation of ER $\alpha$  and enhances E<sub>2</sub> sensitivity, leading to the disruption of the appropriate E<sub>2</sub>/P<sub>4</sub> signaling balance and thus implantation failure [37]. Interestingly, not only this imbalance of hormonal signaling but also implantation failure is rescued by treatment with the ER antagonist ICI-182780 [37]. Mice with uterine depletion of the signal transducer and activator of transcription 3 (Stat3), known as a downstream molecule of leukemia inhibitory factor (Lif) before implantation [38], also show implantation fail-

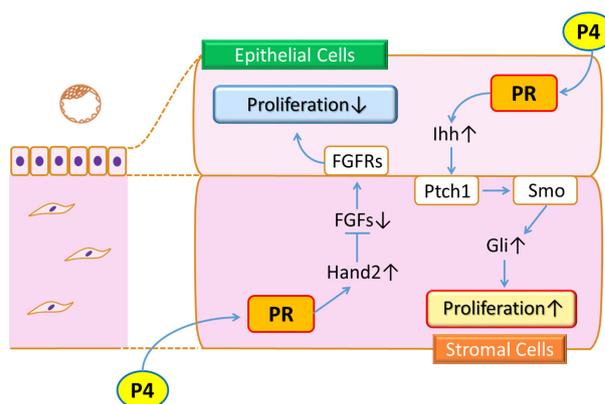
ure with greater influence of E<sub>2</sub>-ER than P<sub>4</sub>-PR signaling on the uterus in the preimplantation period [39]. However, the detailed mechanism of Stat3 and E<sub>2</sub>/P<sub>4</sub> signaling has not yet been fully elucidated.

Comprehensive investigations using other mouse models have also been conducted to clarify the downstream targets of P<sub>4</sub>-PR signaling. A microarray analysis of WT uteri with PR-antagonist RU486 treatment during the preimplantation phase revealed that heart and neural crest derivatives-expressed protein 2 (Hand2), one of basic helix-loop-helix transcription factors, is expressed in the endometrial stroma under the influence of P<sub>4</sub>-PR signaling, and inhibits epithelial proliferation through the suppression of fibroblast growth factor, while it does not contribute to stromal proliferation [40]. Indeed, uterine deletion of Hand2 leads to implantation failure, confirming that it is essential for embryo attachment [40]. In another study, a microarray analysis of PR-null uteri identified Indian hedgehog (Ihh), a hedgehog family molecule, as a downstream factor of PR, which is highly expressed in the uterine endometrial epithelium in WT mice just before implantation, and also in the human endometrium after treatment with progestin [41]. Ihh functions via its receptor Patched-1 (Ptch1) which is locally expressed in the endometrial stroma and induces stromal proliferation, thus conditioning the uterus for implantation [42–44]. The proposed downstream targets of Ihh signaling are transcriptional factor Gli proteins, and a nuclear receptor chicken ovalbumin upstream promoter-transcriptional factor (COUP-TFII). It has been suggested that Gli proteins contribute to stromal proliferation [42], and COUP-TFII modifies the balance between ER and PR signaling

which is necessary for implantation [45]. These findings show the presence of complicated but regulated interactions between the endometrial epithelium and stroma under the hormonal control (Fig. 2).

### Regulation of P<sub>4</sub>-PR Signaling via microRNA during Implantation

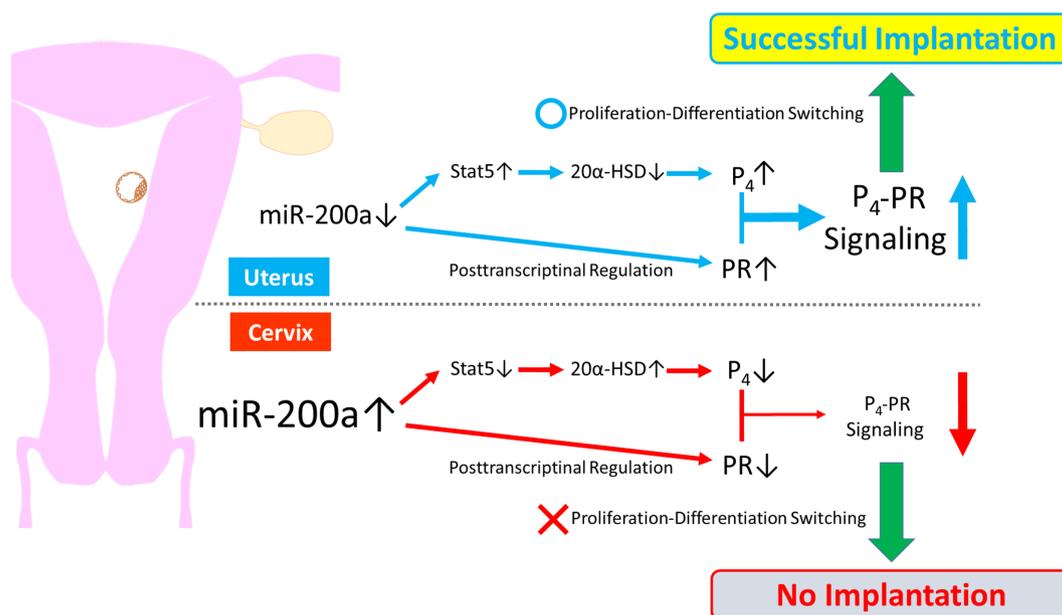
As mentioned above, endometrial PDS, epithelial differentiation and stromal proliferation are initiated in the receptive uterus, and this change is considered to be essential for implantation success as well as being an index of uterine receptivity to the embryo. Endometrial PDS is dependent on P<sub>4</sub>-PR signaling, which is shown in FKBP52 null uteri with P<sub>4</sub> treatment [19, 20]. We recently discovered that endometrial PDS occurs in a spatial manner, between the uterus and cervix [16]. Under normal conditions, blastocysts implant in the uterus, not the cervix. In mice, the uterus shows PDS which is countered by RU486, while the cervix does exhibit any changes in proliferation or differentiation in neither the epithelium nor the stroma. Similarly in human tissues, the uterus presents dynamic proliferation-differentiation switching between the proliferative phase and the secretory phase, in contrast to the cervix which shows no significant changes in the proliferation status [16]. These findings suggest different mechanisms of regulation of P<sub>4</sub>-PR signaling between the uterus and cervix, and it has been postulated that the reduced P<sub>4</sub>-PR signaling in the cervix might prevent embryo implantation there. Therefore, comparing molecular signals between the uterus and cervix might help us to identify the essential factors involving P<sub>4</sub>-PR signaling behind implantation. Interestingly, we found that P<sub>4</sub>-PR signaling in the cervix is down-regulated by microRNA (miR)-200a in two different pathways. First, heightened miR-200a in the cervix directly reduces PR protein levels by post-transcriptional regulation [16]. Then, miR-200a induces up-regulation of 20 $\alpha$ -hydroxysteroid dehydrogenase, the P<sub>4</sub>-metabolizing enzyme, through down-regulation of Stat5, as previously reported [46], leading to the local metabolism of P<sub>4</sub> in the cervix. Moreover, we recently found that miR-200a expression levels are low in the receptive uterus compared to the pre-receptive one (unpublished observation), indicating that the reduction of miR-200a induces the heightened uterine P<sub>4</sub>-PR signaling which contributes to successful implantation (Fig. 3). Our findings indicate that epigenetic regulation of P<sub>4</sub>-PR signaling is involved in the mechanisms behind embryo implantation.



**Fig. 2.** Crosstalk between epithelium and stroma downstream of P<sub>4</sub>-PR signaling. Fibroblast growth factors, FGFs; fibroblast growth factor receptors, FGFR; heart and neural crest derivatives-expressed protein 2, Hand2; Indian hedgehog, Ihh; progesterone, P<sub>4</sub>; progesterone receptor, PR; Patched-1, Ptch1; Smoothed, Smo.

### Decidualization and P<sub>4</sub>-PR Signaling

Successful implantation is essential for subsequent gestational events, but the process of decidualization following embryo attachment is also important for pregnancy success. In mice, decidualization occurs in the stroma cells surrounding the implanting blastocyst, where stromal cells undergo extensive proliferation and differentiation into specialized cell types called decidual cells, which eventually envelope the embryo in the antimesometrial bed [2–4]. In humans, decidualization with polyploidy is also drastically accelerated once embryo attachment occurs [3]. Embryo attachment is a molecular and mechanical stimulus for the recipient endometrium, and in mice, decidualization is mechanically inducible with artificial stimulation. As an experimental mouse model of artificial decidualization, the uterine lumen scratched by a needle or injected with oil after E<sub>2</sub> and P<sub>4</sub> treatment in ovariectomized WT mice dramatically increases the size and weight of the uterine horn [11, 20]. In contrast, PR-null uteri do not show these changes [11]. These results suggest the involvement of P<sub>4</sub>-PR signaling in decidual formation, and are backed by the results of studies using FKBP52-null mice. P<sub>4</sub> supplementation in CD1 FKBP52-null mice normally induces decidualization in response to embryo attachment, although it can only partially rescue artificial decidualization [20], indicating that P<sub>4</sub>-PR signaling is an essential component of decidualization. However, mechanical stimulus alone is insufficient for decidualization, which also requires without the molecular crosstalk between the implanting em-



**Fig. 3.** Regulation of P<sub>4</sub>-PR signaling by microRNA-200a in the uterus and cervix. MicroRNA-200a, miR-200a; 20 $\alpha$ -hydroxysteroid dehydrogenase, 20 $\alpha$ -HSD.

bryo and the uterus. Female mice lacking homeobox A10 (Hoxa10), an abdominal B-like Hox gene, exhibit infertility, the cause of which is mainly decidualization failure, although implantation failure is also involved [47, 48]. In Hoxa10-null uteri, stromal cell proliferation in response to P<sub>4</sub> is severely compromised, implying Hoxa10 has a critical role in mediating the effect of P<sub>4</sub>-PR signaling during implantation, especially in decidualization [47, 48].

### P<sub>4</sub>-PR Signaling Contributes to Pregnancy Maintenance

Although treatment with a modest amount of P<sub>4</sub> by silastic implant can rescue implantation failure in FKBP52-null mice [19, 20], it results in spontaneous abortion and full-term delivery is not achieved [20]. However, term delivery can be accomplished by treatment with daily injections of 2 mg of P<sub>4</sub> to raise the blood level of P<sub>4</sub>. Based on these findings, the concept of pregnancy-stage-specific demands of P<sub>4</sub>-PR signaling has been proposed [20]. A variety of molecules are regulated under the influence of P<sub>4</sub>-PR signaling, and clarification of their detailed functions may reveal how P<sub>4</sub> protects embryos from resorption. Our data obtained from a proteomic analysis shows that galectin-1, a glycan-binding protein, is induced in the endometrial stroma under the influence of P<sub>4</sub>. Treatment with galectin-1 remarkably decreases heightened rates of resorption in FKBP52 null females with a small amount

of P<sub>4</sub> treatment [49, 50]. These results indicate that galectin-1 plays a crucial role in pregnancy maintenance as a downstream molecule of P<sub>4</sub>-PR signaling. A previous study has reported that galectin-1 induces apoptosis of Th1 cells and Th2 bias which is associated with immune tolerance [51], and female mice with galectin-1-deficiency show increased rates of stress-induced spontaneous abortion [52]. The consensus hypothesis is that galectin-1 partially supersedes P<sub>4</sub> functions in coordinating the creation of an immunological environment in the uterus which is capable of sustaining a normal pregnancy. Further investigations utilizing FKBP52-null females may reveal other candidate downstream molecules of P<sub>4</sub>-PR signaling that are essential for the maintenance pregnancy.

### Conclusions

#### *Future prospects in the study of P<sub>4</sub>-PR signaling*

The number of babies born after the treatments using assisted reproductive technology is increasing along with the rise in the age of initial gestation and advances in techniques of *in vitro* fertilization [53]. In order to improve fertility rates, a lot of problems need to be overcome, for example, recurrent miscarriage despite the quality of transplanted embryos [4, 54]. Implantation failure is one of the major causes of unexplained infertility, and also the most puzzling issue, since there are no effective

tive treatments. Several molecules working within a very limited time are involved in the formation of the implantation window, and basic research is required to detail the mechanisms of implantation failure and to establish effective treatments for it. One possible mechanism of implantation failure is “P<sub>4</sub> resistance”, as observed in FKBP52-null mice [8, 19, 20]. P<sub>4</sub> resistance can be recovered by treatment with P<sub>4</sub> in these knockout mice in a genetic background-dependent manner. P<sub>4</sub> treatment for implantation failure in humans is also common in fertility clinics, and statistical data also support its effectiveness in the treatment of patients with luteal insufficiency [10]. However, P<sub>4</sub> supplementation does not help many infertile patients suffering from implantation failure. Moreover, current therapies cannot cure patients with severe P<sub>4</sub> resistance. The comparison of P<sub>4</sub>-PR signaling between different genetic backgrounds of FKBP52-deficient mice might help to elucidate the causes of P<sub>4</sub> resistance in humans, and further investigations are required. The FKBP52-null mouse is a well-designed experimental model which reflects the influence of P<sub>4</sub> resistance on early pregnancy loss.

Not only implantation failure but also preterm birth is a major concern in current female reproductive health. Scarce animal models faithfully mimic aspects of human prematurity, because murine parturition is initiated by luteolysis and a decrease in the circulating P<sub>4</sub> level, which does not occur in humans [1]. Notably, mice with conditional deletion of the tumor suppressor gene, p53, and mice carrying hypomorphic alleles of hydroxyprostaglandin dehydrogenase 15 are useful models of spontaneous preterm labor with increased amounts of prostaglandin F<sub>2α</sub> instead of P<sub>4</sub> withdrawal before parturition [55] [56]. These models will help us to explore signaling pathways other than P<sub>4</sub>-PR signaling, and at the same time, study the effect of P<sub>4</sub> resistance in preterm delivery. In fact, lipopolysaccharide -induced preterm delivery in p53 conditional knockout mice cannot be rescued by P<sub>4</sub> treatment alone, it requires concomitant treatment with P<sub>4</sub> and a mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin. In addition, randomized clinical control trials have revealed that progestin supplementation for women with cervical shortening or with a history of preterm birth, which are risk factors of prematurity, significantly reduces the incidence of preterm delivery [57, 58]. In those studies, however, some patients had preterm birth even after progestin treatment, suggesting that P<sub>4</sub> resistance is involved as a cause of preterm birth. Better appreciation of pregnancy from the viewpoint of P<sub>4</sub> functions will be of great help in developing novel approaches to preterm birth as well as infertility and contraception.

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