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Seminal fluid effects on uterine receptivity to embryo implantation: transcriptomic strategies to define molecular mechanisms

Hon Y. Chan[A,*] and Sarah A. Robertson[A]

For full list of author affiliations and declarations see end of paper

***Correspondence to:**
Hon Y. Chan
The Robinson Research Institute and School of Biomedicine, University of Adelaide, Adelaide, SA 5005, Australia
Email: honyeung.chan@adelaide.edu.au

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ABSTRACT

Embryo implantation requires both a developmentally competent embryo and a receptive uterus. Impaired uterine receptivity is a common constraint on implantation success and reproductive outcome. Ovarian steroid hormones oestrogen and progesterone play a central role in establishing uterine receptivity, but other factors also contribute. One additional regulating factor is male partner seminal fluid. However, the full physiological impacts of seminal fluid on uterine receptivity and the specific molecular pathways involved are not yet completely defined. New advances in RNA-sequencing technologies provide a powerful means to examine how uterine tissues and cells respond to seminal fluid contact. Findings utilising sequencing technology provide strong cellular and molecular evidence in humans and mice that seminal fluid contact around the time of ovulation drives immune and vascular changes with potential to affect endometrial receptivity in the peri-implantation phase. This approach has led to the discovery of novel mediators and regulatory factors subsequently shown to facilitate embryo implantation in genetic mouse models, enabling functional validation. Here, we summarise the evidence from recent microarray and RNA-sequencing findings that seminal fluid contact can directly and indirectly impact the transcriptional state of endometrial tissue during the implantation window in mice and also in humans. Progress in elucidating the female reproductive tract response to seminal fluid will improve understanding of male partner effects on endometrial receptivity, and the knowledge gained will have practical applications for achieving healthy pregnancy and offspring outcomes.

Keywords: embryo implantation, endometrium, immune system, pre-implantation, seminal fluid, seminal plasma, sperm, uterine receptivity, uterus, vascular system.

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Introduction

Embryo implantation is a key step to establishing pregnancy, and is the paramount constraint limiting progression from conception to live birth in both unassisted and assisted reproduction. Both a developmentally competent embryo and a receptive endometrium are essential to implantation success. Receptivity is a transient state acquired after the peri-ovulatory phase endometrium undergoes major molecular and cellular changes that allow embryo apposition, attachment and subsequent invasion into the endometrial lining, setting the trajectory for robust placental development to support optimal fetal growth (Aplin and Ruane 2017; Lessey and Young 2019). Despite extensive research to define the biology of embryo implantation, it is still unclear why the rate of implantation failure is so high, with only one in three conceptions leading to viable pregnancy in humans (Chard 1991; Racowsky 2002). Moreover, pregnancy disorders including recurrent pregnancy loss, preeclampsia, fetal growth restriction, and spontaneous preterm birth are increasingly recognised to arise from disturbances to implantation and early placental development, and the molecular origins of these outcomes remain obscure (Norwitz 2006; Roberts *et al.* 2017; Rabaglino and Conrad 2019). To overcome infertility and prevent pregnancy disorders associated with impaired implantation, there is a need to better understand the underlying

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mechanisms that promote and constrain endometrial receptivity (Roberts *et al.* 2017; Walker *et al.* 2023).

Decades of research have shown that acquisition of receptivity is governed primarily by ovarian steroid hormones that elicit specific transcriptional states in endometrial epithelial and stromal cells, allowing embryos to attach and invade (Aplin 2000; Lessey and Young 2019). A critical action of these hormones is to drive adaptations in uterine immune cells and mediators that facilitate embryo attachment and invasion, and suppress activation of immune responses that inhibit placental development and function. Elevated progesterone in the luteal phase promotes adaptations in the cytokine profile and phenotype of endometrial immune cells to enable tolerance of the implanting embryo and assist in essential tissue remodelling changes (Lea and Sandra 2007; Salamonsen *et al.* 2007; Robertson *et al.* 2022).

In addition to hormones, seminal fluid factors contributed by the male partner at coitus are emerging as important in priming endometrial receptivity in many mammalian species. Extensive studies have described the impact of seminal fluid on female reproductive tract tissues including the uterus, cervix, oviduct and ovary, to exert direct and indirect effects on endometrial physiology and immunology (Robertson and Sharkey 2016; Schjenken and Robertson 2020). These changes can facilitate receptivity to implantation and placental development under permissive circumstances, or reduce receptivity when inhibitory cues are present, in both cases largely through effects on cells and mediators of the immune response (Schjenken and Robertson 2020).

In the hours following insemination in the peri-ovulatory phase, an inflammation-like response is elicited by soluble seminal plasma factors interacting with the estrogen-primed epithelial lining of the cervix or uterus – depending on the site of seminal fluid deposition, which varies according to the anatomy of different mammalian species (Schjenken and Robertson 2014). In mice, where the response is best studied, uterine epithelial cells activate a transcriptional response characterised by upregulated expression of genes encoding an array of pro-inflammatory cytokines, chemokines and microRNAs (Schjenken *et al.* 2015; Chan *et al.* 2021). This pulse of cytokine and chemokine signals leads to substantial molecular and cellular changes in the endometrium including the recruitment of various immune cell populations that, under favourable conditions, generate a state of maternal immune tolerance to male partner alloantigens (Robertson *et al.* 1996; Chan *et al.* 2021) and other changes including increased uterine vascularity (Faas *et al.* 2014), as well as pro-gestational effects in the ovary (Gangnuss *et al.* 2004) and oviduct (Bromfield *et al.* 2014). A comparable immune response to seminal fluid occurs in all mammalian species so far studied, to play a key role in the quality of maternal endometrial receptivity, which in turn influences the quality of pregnancy outcome (Chow *et al.* 2003; Bromfield *et al.* 2014; Schjenken and Robertson 2014).

The full range of physiological changes induced in the endometrium by seminal fluid and the specific molecular pathways and underlying molecular mechanisms are not yet completely defined, in large part because of the limitations of the various technical approaches employed in their evaluation. In light of this, the use of RNA-sequencing (RNA-seq) and related molecular profiling techniques have equipped researchers with capability to examine global gene expression patterns in endometrial tissue to shed light on exactly how seminal fluid exerts its impact on implantation biology. This has led to the discovery of novel factors pivotal to embryo implantation, and provided a basis for generation of gene-knockout mouse models to validate their physiological importance. Importantly, recent studies using RNA-seq provide new insight on the molecular mechanisms by which seminal fluid drives changes in the mouse and human uterus that maximise receptivity to embryo implantation. This review will summarise current understanding of the transcriptomic changes that occur after seminal fluid contact and their contribution to endometrial receptivity for successful pregnancy.

The acquisition of uterine receptivity

For successful embryo implantation to occur, the uterus must undergo major molecular and cellular changes in the endometrial lining in the period between ovulation and blastocyst-stage embryo development to enable the hatched blastocyst to attach and trophectoderm cells to invade (Aplin and Ruane 2017). While there are common elements and regulators of embryo implantation between mammalian species (McGowen *et al.* 2014), differences in endometrial biology between species undergoing a menstrual cycle (e.g. some primates including humans) compared to an oestrous cycle (e.g. most non-primate mammals including mice, rats and pigs) give rise to distinct mechanisms by which these species acquire receptivity. Given the mouse is a widely-used and valuable animal model in the study of embryo implantation (Maurya *et al.* 2021), this section will discuss the similarities and differences in the endometrial biology of humans and mice.

Endometrial receptivity in humans

In women, the structure and cellular composition of the endometrium changes primarily in response to fluctuations in ovarian steroid hormones oestrogen and progesterone across the approximately 28 day menstrual cycle (Aplin 2000; Maurya *et al.* 2021). The menstrual cycle is categorised into three stages: menstrual, proliferative, and secretory phase. Briefly, the action of progesterone on the oestrogen-primed endometrium causes endometrial epithelial and stromal cells to proliferate, and stromal cells to undergo a specific 'decidual' transformation. The differentiation of stromal cells

into decidual cells leads to production of pro-implantation factors allowing the endometrium to transiently enter a receptive state during the mid-secretory phase, generally known as the 'window of implantation' (Harper 1992; Evans *et al.* 2016). With a permissive molecular environment, embryos can appose to the endometrial surface epithelium, followed by firm attachment before invading into the stromal compartment, an event that further stimulates decidual transformation (Aplin 2000; Maurya *et al.* 2021). In a non-conception cycle, as the implantation window passes and the level of oestrogen and progesterone subsequently decline, decidualised tissues become senescent and are eliminated at menstruation.

A notable feature of the receptive endometrium is the abundant and highly specialised immune cell populations, which accumulate over the course of the estrogen-dominated proliferative phase and undergo phenotypic changes as progesterone progressively rises after ovulation. Macrophages, dendritic cells, uterine natural killer (uNK) cells and T cells are all enriched in the mid-secretory phase endometrium, coinciding with the window of implantation (Lee *et al.* 2015). There are extensive studies in animal models that show the necessity for each of these cell lineages in regulating the decidual response and stimulating trophoblast invasion, suppressing inflammation, preventing effector immunity, and eliciting vascular adaptations that facilitate placental development and function (Ashkar *et al.* 2003; Plaks *et al.* 2008; Care *et al.* 2013; Hosking *et al.* 2025). Many of the mechanisms by which this occurs have been delineated in mouse models (see below) and recapitulated in *ex vivo* models involving human cells and tissues (Robertson *et al.* 2022; Saito 2024).

To support the dynamic changes that precede receptivity, substantial remodelling is observed in the uterine vasculature and surrounding tissues. After blood vessels rupture to allow menstruation, abundant angiogenesis is required for vessel repair and to support rapid endometrial growth during the proliferative phase (Gambino *et al.* 2002), supported by enlargement of vascular capacity in the secretory phase (Zhang *et al.* 2019). Tightly regulated expression of the vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) family of proteins is implicated in this rapid vessel growth and tissue remodelling process (Plaisier *et al.* 2006). Appropriate vascular and tissue remodelling in the endometrium contribute to decidualisation, which in turn affects the complexity of vascular development and the extent of trophoblast invasion (Zambuto *et al.* 2024).

Endometrial receptivity in mice

Female mice undergo an oestrous cycle (approximately 4–5 days in duration) that is categorised into four stages, pro-oestrus, oestrus, metoestrus and dioestrus. Fluctuating oestrogen and progesterone levels drive cellular and morphological changes in the endometrium (Lee *et al.* 2007). Elevated oestrogen and progesterone levels lead to the proliferation of stromal cells and thickening of the endometrium at pro-oestrus, followed by ovulation at oestrus. In contrast to humans, female mice only accept the male for mating at oestrus. In non-conception cycles female mice then enter metestrus and apoptosis of stromal cells occurs, as oestrogen and progesterone levels decline. Senescent cells and tissues are reabsorbed into the uterus rather than being shed, thus no bleeding occurs. Another key difference between humans and mice is that female mice only acquire endometrial receptivity after mating, while women acquire receptivity every menstrual cycle. On day 4.0–4.5 post-coitum in mice (with day of copulatory plug detection denoted as day 0.5 post-coitum), the endometrium enters a receptive state that allows embryo attachment. Furthermore, while decidualisation of the stroma occurs spontaneously in women each cycle, this process is only initiated in the mouse after embryos make contact with the uterine epithelium (Rinkenberger *et al.* 1997).

Despite the between-species differences, vascular and immune changes in the endometrium are central cellular processes underpinning uterine receptivity in mice, as in humans. Our recent RNA-seq study comparing the endometrial transcriptome on day 3.5 post-coitum to oestrus, identified several immune-regulatory pathways amongst the top pathways activated, including Pathogen Induced Cytokine Storm Signalling Pathway, Tumour Microenvironment, and Leukocyte Extravasation Signalling (Chan *et al.* 2023). Pathways relevant to tissue and vascular remodelling such as Endocannabinoid Neuronal Synapse Pathway, Pulmonary Healing Signalling, and Neurovascular Coupling Signalling, were activated (Chan *et al.* 2023). Consistent with a role for increased uterine blood vessel permeability prior to implantation, angiogenic factors including VEGF and prostaglandin-endoperoxide synthase 2 (PTGS2; also known as cyclooxygenase-2 [COX2]), are elevated between ovulation and embryo implantation (Chan *et al.* 2023).

Experiments in mice have been instrumental in demonstrating how endometrial immune cells recognise and actively tolerate the implanting embryo to enable pregnancy progression (Erlebacher 2013). In particular, mouse studies demonstrate how immune cells called regulatory T cells (Treg cells) play an important role in establishing immune tolerance to enable peaceful coexistence of embryo-derived cells expressing paternally-inherited alloantigens, and the maternal immune response (Aluvihare *et al.* 2004; Shima *et al.* 2010; Samstein *et al.* 2012). To achieve an active state of tolerance before embryo implantation requires priming of Treg cells by paternal alloantigens present in seminal fluid, so that when those same alloantigens are encountered on trophoblasts of the invading blastocyst, effector immunity is not generated (Robertson *et al.* 2009; Guerin *et al.* 2011). In the context of favourable immune-regulatory signals provided by seminal plasma, $CD4^+$ T cells differentiate into Treg cells that are permissive and support trophoblast invasion, rather than effector T cells that perpetuate inflammation, impair placental development, and potentially terminate pregnancy (Robertson *et al.* 2009; Guerin *et al.* 2011). Treg cells interact

with uNK cells as well as dendritic cells and macrophages, and collectively these immune cells mediate tolerance and suppress inflammation for optimal placental development and pregnancy progression (Robertson *et al.* 2022). This leukocyte network also contributes to endometrial angiogenesis, and decidual vascular remodelling required for healthy placentation by secreting factors including VEGF, MMPs and tissue inhibitors of metalloproteinases (TIMPs) (Jing *et al.* 2023; Saito 2024; Hosking *et al.* 2025), and potentially also affects the decidual response through effects on dendritic cells and other regulators (Plaks *et al.* 2008).

Determinants of endometrial receptivity

A myriad of molecules contribute to specify uterine receptivity, many of which are controlled by female steroid hormones oestrogen and progesterone (Aplin 2000; Lessey and Young 2019; Maurya *et al.* 2021). Oestrogen facilitates the remodelling of the uterine epithelium by limiting cell proliferation and stimulating differentiation (Krege *et al.* 1998; Tan *et al.* 1999). The remodelling process is further mediated by progesterone, which drives molecular changes to the endometrial epithelium and in human, stromal cell decidualisation (Lydon *et al.* 1995; Brar *et al.* 1997). Androgenic sex hormones may fine tune endometrial receptivity through effects on decidualisation and regulation of endometrial receptivity genes (Gibson *et al.* 2016), but the mechanisms by which androgen modulates these events, and whether seminal fluid is a biologically important source of androgens, remain to be defined.

Ovarian hormones tightly regulate the spatial and temporal expression of signalling molecules including cytokines, chemokines and growth factors, which all play a key role in mediating uterine receptivity (Dimitriadis *et al.* 2005). Although the molecular profile defining a receptive human endometrium is still being resolved (Aplin and Stevens 2022), individual molecules underpinning the receptive state have been identified, many through the use of mouse models. Despite the differences in the implantation biology across species (Lee and DeMayo 2004), there are many common genes with conserved roles in implantation. For instance, leukemia inhibitory factor (LIF) and PTGS2 play an indispensable role in preparing the endometrium for implantation in humans and mice (Dimitriadis *et al.* 2000; Robb *et al.* 2002). In female mice with a null mutation for LIF, embryos develop normally but fail to implant due to decidualisation failure likely through impaired activation of signal transducer and activator of transcription 3 (STAT3) signalling (Chen *et al.* 2000). Similarly, PTGS2-null female mice exhibit impaired embryo attachment and decidualisation defects, leading to reduced fertility (Lim *et al.* 1997). Another critical molecule is VEGF, which promotes angiogenesis in the uterus to accompany implantation (Kim *et al.* 2013).

However, hormones do not fully explain the transition to receptivity as some endometrial genes that influence receptivity are not primarily controlled by female sex hormones. Some of these factors have been discussed by Cha and colleagues, including Msh homeobox 1 (MSX1) and Kruppel-like factor 5 (KLF5) (reviewed in Cha *et al.* (2012)). MSX1 is highly expressed in the mouse and human uterus during the acquisition of receptivity, but its expression then rapidly decreases and becomes undetectable once implantation commences (Daikoku *et al.* 2011). This finding suggests a key role in receptivity and explains the low implantation rate observed in female mice null in MSX1 (Nallasamy *et al.* 2012). KLF5 is another factor not regulated by ovarian steroid hormones. Female mice with uterine deletion of KLF5 display severely impaired fertility, as demonstrated by significantly reduced litter size and impaired decidualisation in female mice with KLF5 deficiency (Sun *et al.* 2012). The regulators of these molecules are still unclear, but their existence suggests that factors in addition to sex hormones contribute to the establishment of uterine receptivity.

Seminal fluid and uterine receptivity to embryo implantation

The male seminal fluid comprises two main components, spermatozoa and seminal plasma. Seminal plasma contains secretions from the epididymis and male accessory glands including the seminal vesicles, prostate and the bulbourethral glands (Aumüller and Riva 1992; Verze *et al.* 2016). This portion of semen has generally been seen as a transport medium for sperm to reach the oocyte for fertilisation. It acts to promote sperm survival and provide protection to sperm from oxidative stress (Garrido *et al.* 2004; Kawano *et al.* 2014; Araki *et al.* 2015). The soluble signalling factors within seminal plasma play a complex role in reproductive success by directly modulating the physiology of the female reproductive tract as shown in humans and many model species (Aumüller and Riva 1992; Maegawa *et al.* 2002; Sharkey *et al.* 2007; Schjenken and Robertson 2015). Focusing on mice and humans, the contribution of seminal fluid to implantation and the underlying molecular evidence will be discussed in this section.

Evidence of seminal fluid contact on improved implantation rate

Although the success of assisted reproductive technologies indicates seminal fluid is not essential for implantation and pregnancy progression, semen exposure to the female around the time of embryo transfer during *in vitro* fertilisation (IVF) is associated with an improved likelihood of pregnancy and fetal survival (Robertson and Sharkey 2016). In human IVF, fertilisation takes place outside the body and the embryo is later transferred into the uterus often without seminal fluid

exposure. A clinical studies in the 1980's first indicated that intravaginal seminal plasma administration at the time of embryo transfer can increase live birth rate after IVF (Bellinge *et al*. 1986). Subsequent studies showed that implantation rate was elevated in couples undergoing IVF when intercourse took place before or just after embryo transfer (Tremellen *et al*. 2000; Hou *et al*. 2023). Consistent with these findings, meta-analyses that combine results from seven or more randomised controlled trials confirm a clear beneficial role of seminal fluid exposure around the time of embryo transfer. A significant 24% improvement in clinical pregnancy rate was reported (Crawford *et al*. 2015; Saccone *et al*. 2019), indicating the likely significance of seminal fluid contact and its downstream impact on implantation. Whether intravaginal seminal fluid exposure also benefits live birth rates after IVF is the subject of ongoing investigation (Ata *et al*. 2018; Liffner *et al*. 2024).

Evidence from animal models point to a strong positive effect of seminal fluid contact on embryo implantation. One of the strengths of animal models is the potential to manipulate ejaculate composition through surgical means. For instance, seminal vesicle excision removes the majority of the seminal plasma content, causing ejaculation with only the sperm and epididymal fluid component. Our group showed that pregnancies sired by seminal vesicle-ablated (SVX) male mice displayed a reduced implantation rate (Bromfield *et al*. 2014). A similar reduction in implantation rate was observed in male golden hamsters when male accessory glands were surgically removed (O *et al*. 1988). Using male mice that were both SVX and vasectomised (SVX/VAS), we further showed that seminal fluid contact is required for upregulation of LIF at both the mRNA and protein level in the mouse endometrium (Schjenken *et al*. 2015). These findings are consistent with an observation of *LIF* upregulation in human epithelial and stromal cells after seminal plasma treatment in a dose-dependent manner (Gutsche *et al*. 2003). Given the indispensable role of LIF in successful implantation, this provides clear evidence that determinants of endometrial receptivity can be impacted by seminal fluid factors.

Seminal fluid components exerting biological effects in the female reproductive tract

Some of the key signalling molecules in seminal fluid have been characterised, while others remain to be identified. Broadly, they can be classified into permissive agents that promote receptivity and pregnancy immune tolerance, and inhibitory agents that impair receptivity and tolerance (Schjenken and Robertson 2020). These agents are largely derived from the seminal vesicle, prostate and epididymal tissues of the male reproductive system. They may be present in soluble form in seminal plasma (Robertson 2005), contained within extracellular vesicles (Kelly *et al*. 1991; Tannetta *et al*. 2014), or carried on the surface of spermatozoa, either as structures integral to the sperm plasma membrane or after sequestration from seminal plasma by spermatozoa after ejaculation (Tecle *et al*. 2019).

Permissive factor transforming growth factor beta (TGFB) is abundant in human and mouse seminal plasma (Nocera and Chu 1993; Tremellen *et al*. 1998). It binds to TGFB receptors expressed by human cervical epithelial cells (Sharkey *et al*. 2012*a*), and in mouse uterine epithelial cells (Tremellen *et al*. 1998; Schjenken *et al*. 2015). The TGFB-induced cytokines promote the development of tolerogenic dendritic cells (DCs) that in turn drive differentiation of Treg cells, so that male histocompatibility antigens delivered in the context of this cytokine are tolerated by the immune response (Ghiringhelli *et al*. 2005). Prostaglandins (PGs) are another group of soluble molecules present in human seminal fluid (Templeton *et al*. 1978) that boost a pro-tolerogenic environment in the uterus that favours implantation by inducing the expression of anti-inflammatory IL10 (Kelly *et al*. 1994, 1997; Kaliński *et al*. 1997).

Our recent studies in mice indicate that TLR4 expressed on uterine epithelial cells is activated by seminal fluid signals to contribute to immune tolerance. The unexpected significance of TLR4 was initially revealed by RNA-seq analysis of the endometrial changes induced by seminal fluid, and bioinformatic analysis of the upstream regulators of differentially expressed genes (Schjenken *et al*. 2015). We showed that genetic deficiency in TLR4 impairs cytokine expression during the inflammation-like response after mating and subsequent Treg cell generation and recruitment into the uterus (Chan *et al*. 2021). The identity of TLR4 ligands in semen likely include beta-defensins (Narciandi *et al*. 2014), heat shock proteins (Pilch and Mann 2006), and S100 proteins (Rego *et al*. 2014), but their exact contribution to immune changes in the female tract remain to be investigated.

In contrast, interferon-gamma (IFNG) in seminal plasma acts as a potent inhibitory factor in both mice and humans, as it suppresses the TGFB-induced inflammation-like response in uterine and cervical epithelial cells (Sharkey *et al*. 2018). IFNG can be elevated in men in association with reproductive tract infections, smoking, and other pro-inflammatory exposures (Sharkey *et al*. 2017). There is high variation in the content of different seminal plasma signalling agents between men and within men over time (Sharkey *et al*. 2016, 2017). Emerging meta-analyses support the interpretation that an appropriate balance of permissive and inhibitory factors in seminal fluid is instrumental in male fertility, even when sperm counts are within a normal range (Lyons *et al*. 2023). This is likely to be mediated through effects on the strength and quality of the female immune response to seminal fluid antigens, in turn impacting implantation and reproductive success.

The identity of the molecular structures utilised by sperm to engage directly with uterine epithelial cells in mice remains to be resolved. Application of upstream pathway analysis tools to predict regulators driving transcriptomic changes

induced by sperm indicated a high likelihood of TLR4 ligands, and experiments in *Tlr4* null mutant mice confirmed a requirement for this receptor in mediating effects of sperm ligation (Schjenken *et al.* 2021). Comparing pathways activated by whole semen to those activated by seminal plasma alone points to potential roles for additional factors including CD38, TNFSF12, SCAP, PPIF and IL5 – each of these now require further investigation, and transcriptomic approaches will no doubt assist in delineating the specific contribution of each of these components to the female tract response. In particular, CD38 shows promise as an important permissive regulator of seminal fluid-induced tolerance in both mouse and human systems (Kim *et al.* 2015), but since CD38 is synthesised by seminal vesicles, the extent to which it is delivered by sperm or in soluble form in seminal plasma is not resolved.

Apart from soluble factors in seminal plasma and sperm, seminal fluid extracellular vesicles (SF-EVs) are an important component of seminal fluid and likely contribute to the effects of semen on endometrial receptivity. Seminal fluid carries abundant EV populations when compared to other bodily fluid including cerebrospinal fluid and blood plasma (Skalnikova *et al.* 2019). The SF-EV populations comprise secretion by the prostate, epididymis and seminal vesicles (Sahlén *et al.* 2010; Belleannée *et al.* 2013; Aalberts *et al.* 2014), all of which play a key supporting role in spermatozoa development and functions (Sullivan and Saez 2013; Tamessar *et al.* 2024). SF-EVs also have the potential to modulate female reproductive tract physiology, particularly the maternal immune and vascular changes required for normal pregnancy (see next section). While the precise identity of SF-EV bioactive molecules are unknown, several proteins carried by SF-EVs have well-demonstrated immune-regulatory properties, including CD38 which contributes to immune tolerance (Park *et al.* 2011; Kim *et al.* 2015) and CD48 which impairs NK cell degranulation (Tarazona *et al.* 2011).

Angiogenic factors present in seminal fluid may also exert effects on the endometrium. By examining the seminal vesicle transcriptome in the mouse, Skerrett-Byrne *et al.* (2021) demonstrated seminal vesicles synthesise angiogenic molecules including VEGF, fibroblast growth factor and angiopoietins, that are highly active in vascular and tissue remodelling processes such as VEGF Signalling, Integrin Signalling and Epithelial Adherens Junction Signalling. The amount of VEGFA in human seminal plasma is approximately 150,000 pg/mL – which is 1000 times higher than in blood plasma (Carlsson *et al.* 2016) – indicating clear potential for seminal fluid to directly modulate the uterine vasculature. Considering this together with the pro-angiogenic immune cell types recruited by seminal fluid and semen-mediated upregulation of *FGF2* and *VEGFA* gene expression in the endometrium (see below), there is a need to better delineate how these factors affect endometrial receptivity.

Transcriptomic evidence of seminal fluid effects on endometrial receptivity

The invention and development of microarray and RNA-seq technologies has allowed effects of seminal fluid on endometrial gene expression profiles to be determined, and enriched cellular functions elicited by seminal fluid exposure to be defined. The female response to seminal fluid immediately after mating has been well characterised in the mouse, human ectocervical cell lines and human endometrial epithelial and stromal cells by our group and others (Sharkey *et al.* 2007, 2012*b*; Chen *et al.* 2014; Schjenken *et al.* 2015).

In mice, comparison of matings involving intact and surgically manipulated males clearly demonstrate that seminal plasma is required to generate immune tolerance for pregnancy (Guerin *et al.* 2011). This is initiated when seminal fluid contacts epithelial cells lining the uterine lumen and elicits upregulation of cytokines including interleukin 6 (IL6), granulocyte-macrophage colony stimulating factor (GM-CSF), and tumour necrosis factor (TNF) (Robertson and Seamark 1990; Schjenken *et al.* 2015). RNA-seq and bioinformatics analyses revealed that following seminal fluid exposure, many immune response pathways including IL10 Signalling, IL6 Signalling and Acute Phase Response Signalling were enriched in the endometrium (Schjenken *et al.* 2015). TLR4 acts as a key receptor in the female tract mediating these signals from seminal fluid (Schjenken *et al.* 2015). TLR4 ligands therefore likely contribute to the recruitment of immune cell populations and the establishment of immune tolerance supporting embryo implantation (Chan *et al.* 2021).

In humans, recovery of cervical biopsies from women after seminal fluid contact showed seminal fluid induces a similar inflammation-like response to that described in mice and other species (Sharkey *et al.* 2012*b*). More recent studies employing endometrial biopsies show seminal fluid effects extend to the uterus in women (Catalini *et al.* 2024). The addition of diluted seminal plasma to ectocervical Ect1 cells *in vitro* elicited a similar upregulation of cytokine genes including *IL6, CSF2* and *CCL2* (Sharkey *et al.* 2007). *In vitro* experiments in Ect1 cells mirror the *in vivo* response to semen deposition after intercourse in the human cervix (Sharkey *et al.* 2012*b*), showing these cytokines each contribute to the inflammation-like response induced by semen contact in the cervix.

Although the female response to seminal fluid during the peri-conception period is well characterised, understanding of the contribution of semen to cellular and molecular changes beyond this timepoint, to affect endometrial receptivity acquisition, is limited. To consider whether seminal fluid plays a role in uterine receptivity, evidence of seminal fluid regulation of the vascular and immune changes in the endometrium at the cellular and molecular levels will be discussed.

Seminal fluid effects on endometrial receptivity mediated by immune cells and cytokines

A substantial body of evidence demonstrates the impact of seminal fluid contact on the female immune response prior to embryo implantation. This response is initiated when the sperm and acellular plasma components of semen interact with epithelial cells of the female tract to elicit a controlled inflammation-like response (Sharkey *et al.* 2007; Schjenken *et al.* 2021). Examining the endometrial transcriptome after seminal plasma treatment in human shows the activation of many immune pathways such as Leukocyte Migration and Chemokine Signalling Pathway (Chen *et al.* 2014; George *et al.* 2020; Catalini *et al.* 2024).

Studies in mice show that spermatozoa, as well as seminal plasma, influence the strength and quality of the endometrial inflammation-like response and the quality of the immune response at implantation. There is a substantial difference in the transcriptional profile of the mouse endometrium at 8 h after mating with intact versus vasectomised males, with more than 1000 differentially expressed genes in the presence of spermatozoa (Schjenken *et al.* 2021). Ingenuity Pathway Analysis revealed enrichment in immune pathways including IL6 Signalling, NF-κB Signalling and Dendritic Cell Maturation after contact with spermatozoa, indicating a novel role of spermatozoa in regulating the post-mating immune response to seminal fluid (Schjenken *et al.* 2021).

SF-EVs may also contribute to the regulation of the female immune response to seminal fluid. Evidence from human studies shows that purified prostasomes contributed to the immunosuppressive property of semen, potentially through modulating the phagocytosis activity of macrophages and neutrophils (Kelly *et al.* 1991; Skibinski *et al.* 1992). While the function of rodent SF-EVs in regulating the female response to semen is undefined, findings from livestock species are more extensive and have demonstrated the immunomodulatory property of SF-EVs. Similar to humans, EVs isolated from bovine seminal plasma also possess immunosuppressive properties and inhibit proliferation and phagocytosis of blood lymphocytes (Lazarevic *et al.* 1995). In pigs, exosomes isolated from boar semen modified the transcriptome of primary porcine endometrial epithelial cells, with 214 transcripts differentially regulated. Notably, bioinformatic analysis revealed the enrichment of immune-related functional terms, including immune response, inflammatory response and positive regulation of neutrophil chemotaxis (Bai *et al.* 2018).

Seminal fluid effects on antigen presenting cells

At the cellular level, antigen presenting cells macrophages and DCs are the most abundant leukocyte population recruited to the female tract after seminal fluid contact in women and mice (Robertson *et al.* 1996; Sharkey *et al.* 2012*b*). Pathway analyses of differentially expressed genes in human cervical cells after coitus revealed that Antigen Presentation and Immune Cell Trafficking were the most significant pathways activated by seminal fluid exposure (Sharkey *et al.* 2012*b*). Leukocyte Migration and Chemotaxis of Leukocytes were also activated in endometrial cells after seminal plasma treatment (Chen *et al.* 2014), in part driven by the upregulation of DC recruitment factors *CCL2* and *CCL7* (George *et al.* 2020). In fact, DCs recruited into the female tract after mating play a main role in establishing maternal immune tolerance. In response to immune-deviating factors carried by semen, DCs adapt to a more immature and tolerogenic subtype, tolerogenic DCs. Tolerogenic DCs enhance regulatory T (Treg) cell generation by secreting indoleamine 2,3-dioxygenase (IDO) that directly induces the differentiation of naive T cells into Treg cells (Blois *et al.* 2007). Besides playing a pro-tolerogenic role in the uterine environment, recent studies have shown that DCs also contribute to vascular changes during embryo implantation in mice. Their importance during early pregnancy was confirmed by Plaks and colleagues who demonstrated dysregulated vascular changes in the uterus after DC depletion on day 3.5 post-coitum, accompanied by compromised decidualisation during the implantation period (Plaks *et al.* 2008).

Seminal fluid effects on macrophages

Transcriptomic analyses of endometrial gene expression changes after seminal fluid contact in mice show substantial evidence of upregulation of macrophage-regulating genes such as *Csf1, Csf2, Csf3*, and *Ccl3*, and enriched pathways associated with macrophage recruitment (Schjenken *et al.* 2015, 2021). Histochemical analysis confirm recruitment of macrophages into the endometrium after mating in the mouse, pig and human (Robertson *et al.* 2000; Hudson Keenihan and Robertson 2004; O'Leary *et al.* 2004; Jiang *et al.* 2018). In mice, seminal fluid affects their recruitment into the ovary as well as the uterus (Gangnuss *et al.* 2004) and macrophage depletion during the peri-conception period led to implantation arrest associated with impaired development of the corpus luteum and progesterone secretion (Care *et al.* 2013). They contribute to maternal immune tolerance by secreting immunoregulatory cytokines TGFB and IL10 (Mantovani *et al.* 2004) and enhancing immunomodulation of T cells via the autocrine VEGF signalling pathway (Lai *et al.* 2019). Macrophages have also been implicated in maternal vascular and tissue remodelling by secreting VEGF and MMPs (Jeon *et al.* 2007; Newby 2016), factors known to be regulated by seminal plasma in humans and other species, including rodents and canines (Chow *et al.* 2003; Sharkey *et al.* 2012*a*; Schäfer-Somi *et al.* 2013). Importantly, macrophages can directly enhance uterine receptivity through the regulation of α(1,2)fucosyltransferase 2 (FUT2) in the endometrial epithelium in the mouse. FUT2 expression is required for embryo attachment to the uterus and is induced by LIF and IL1B (Jasper *et al.* 2011), further implicating seminal

fluid-mediated macrophage recruitment in endometrial receptivity.

Seminal fluid effects on neutrophils

Neutrophils are another key innate immune cell types recruited to the female tract depending on seminal fluid effects and sequencing studies have provided insight on the molecular pathways and regulators that control their recruitment and function. Following coitus, the neutrophil-specific chemokines elicited by soluble factors in seminal plasma, cause neutrophils to extravasate from uterine blood vessels, accumulate in the stromal tissue subjacent to the epithelial surface, and then traverse the uterine epithelium and enter the luminal space (Robertson *et al.* 1996). Through microarray experiments to examine global gene expression in the mouse endometrium induced by seminal fluid, we demonstrated that neutrophil recruitment factors *Cxcl1*, *Cxcl2* and *Cxcl5* were highly induced by seminal fluid contact, and spermatozoa were specifically implicated in their induction (Schjenken *et al.* 2015, 2021). Neutrophil recruitment into the endometrium depends on TLR4 activation by as yet unidentified ligands associated with spermatozoa, as $Ly6G^+$ neutrophils were 12-fold more abundant in the endometrium of intact-mated females than vasectomised-mated females (Schjenken *et al.* 2021). Their main role involves the clearance of superfluous sperm and cellular debris introduced at mating, and preferential sequestration of damaged sperm prevents them from accessing oocytes (Tecle *et al.* 2019). Recent studies have suggested a role in antigen presentation and potentially the activation of pro-angiogenic T cells (Nadkarni *et al.* 2016). Apart from their immune functions, neutrophils also facilitate ovulation and may also contribute to vascular changes in the female tract. In mice, neutrophil depletion in the peri-ovulatory phase impaired ovulation (Brännström *et al.* 1995), and at day 1.5 post-coitum led to decreased implantation rate and impaired placental structure, associated with reduced placental VEGF expression (Hebeda *et al.* 2022). Their likely angiogenic role is further demonstrated in women, where a subset of decidual neutrophils expresses high levels of angiogenic proteins including Arginase 1 (ARG1) and VEGF compared to peripheral blood neutrophils (Amsalem *et al.* 2014).

Seminal fluid effects on uterine natural killer cells

uNK cells are the most prominent innate immune cell type in the endometrium and have been indicated in implantation success (Agostinis *et al.* 2019). They have a key role in endometrial receptivity by virtue of their ability to modify the uterine vasculature, through secretion of angiogenic factors including VEGF and placental growth factor (Hanna *et al.* 2006; Kieckbusch *et al.* 2014). As well as assisting in vascular changes in the female tract, uNK cells maintain maternal immune tolerance, as demonstrated by their ability to suppress inflammation through antagonising immunogenic T helper-17 (Th-17) cells (Fu *et al.* 2013). uNK cells may act as a biosensor for embryo quality and regulate endometrial cell fate to allow or prevent implantation depending on molecular cues from the embryo (Kong *et al.* 2021).

Transcriptomic analysis provides evidence of elevated NK cell chemokines such as *Cxcl10* and *Ccl19* in the mouse uterus after mating (Robertson *et al.* 2009; Guerin *et al.* 2011; Schjenken *et al.* 2021), but histochemical confirmation of effects of seminal fluid on uNK cells has not been conducted. There is convincing evidence of an effect of seminal fluid contact on uNK cells in human (Kimura *et al.* 2009). In that study, flow cytometry was utilised to analyse uNK cells in mid-secretory endometrial samples obtained from women who had sexual intercourse around the time of ovulation, and women who did not. uNK cells were markedly increased in women exposed to seminal fluid in the pre-ovulatory phase (Kimura *et al.* 2009).

Seminal fluid effects on T cells

T cells are essential immune cells that facilitates implantation and their activation, proliferation and recruitment into the uterus is strongly responsive to seminal fluid (Robertson *et al.* 2009; Guerin *et al.* 2011; Schjenken *et al.* 2021). In the mouse, they are expanded in lymph nodes draining the uterus and their recruitment into the endometrium becomes evident around day 3.5 post-coitum, the day before embryo implantation. Consequently, studies focusing on the female response in the first 12 h after seminal fluid contact do not detect transcriptomic evidence of T cell responses. Their activation and proliferation commence in the para-aortic lymph nodes around 24 h after seminal fluid contact, following antigen presentation by innate immune cells that traffic to lymph nodes during the inflammation-like response elicited by semen. The ensuing T cell response is biased towards Treg cells, which depart the lymph node and circulate via the blood to be recruited into the uterus. Critical chemokines involved in Treg cell recruitment include *Ccl19*, the uterine expression of which is upregulated by seminal plasma factors (Guerin *et al.* 2011). Once *in situ*, Treg cells promote maternal immune tolerance of the semi-allogeneic embryo, to suppress inflammation and effector immune responses that constrain fetal development (Shima *et al.* 2010; Robertson *et al.* 2018). The depletion of Treg cells during the pre-implantation period in mice causes implantation failure and later fetal loss (Aluvihare *et al.* 2004; Shima *et al.* 2010), accompanied by upregulation of pro-inflammatory cytokines and dysregulated uterine vascular remodelling (Care *et al.* 2018; Hosking *et al.* 2025), positioning Treg cell as a key player in early placental development.

We have shown that exposure to both sperm and seminal plasma fractions is necessary to drive maximal Treg cell proliferation and population expansion, as the increase in Treg cell number was negated after mating with vasectomised or

seminal vesicle excised males compared to intact males (Robertson *et al.* 2009; Guerin *et al.* 2011; Schjenken *et al.* 2021). Notably, a recent study has demonstrated that EVs isolated from human seminal plasma interact directly with T cells *in vitro*, and drive their differentiation into Treg cells by inducing FOXP3 and IL10 expression (Zhang *et al.* 2024). However, given that SF-EVs are unlikely to encounter maternal T cells *in vivo*, the physiological significance of this finding remains to be investigated.

Seminal fluid effects on endometrial receptivity mediated by regulators of vascular and tissue remodelling

There is evidence for an association between seminal fluid contact and uterine vascular development and remodelling associated with embryo implantation. In mice, seminal plasma contact induces expression of pro-angiogenic growth factor genes in the endometrium including *Vegfa* and *Tnf* (Schjenken *et al.* 2021). Matings without seminal plasma contact led to impaired placentation which is typically associated with uterine vascular dysfunction (Bromfield *et al.* 2014). Comparable vascular remodelling in the uterus was observed in females exposed to either full ejaculate or seminal plasma only (van der Heijden *et al.* 2005). Watkins *et al.* (2018) demonstrated paternal low-protein diet in male mice affected their seminal fluid signalling, such as that after mating, uterine blood vessels exhibited a reduced diameter and perimeter at implantation. At the molecular level, the spatial and temporal expression pattern of angiogenic and tissue-remodelling factors *Vegf* and *Mmp* in the golden hamster endometrium during the peri-implantation period was influenced by prior seminal plasma contact (Chow *et al.* 2003). In the pig, significant induction of *Vegf* expression and vascular bed development in the uterus was observed after seminal plasma infusion (Kaczmarek *et al.* 2013).

Seminal fluid may also elicit effects on the reproductive tract vasculature in humans. In an *in vitro* model system using HeLa cervical carcinoma cells, the addition of seminal plasma directly increased the expression of pro-angiogenic molecules including IL8 and growth regulated oncogene alpha (GRO) (Sales *et al.* 2012). The risk of preeclampsia is elevated in women without prior contact with the conceiving partner's seminal fluid, such as in women using donor spermatozoa or oocytes, or in women conceiving early in a sexual relationship (Serhal and Craft 1987; Smith *et al.* 1997; Wang *et al.* 2002; Schwarze *et al.* 2018). This observation raises the possibility that seminal fluid contact affects preeclampsia risk through effects on vascular changes in the endometrium, given insufficient vascular remodelling events during early pregnancy are a hallmark feature of this condition (Opichka *et al.* 2021).

Seminal fluid contact likely contributes to maternal vascular and tissue remodelling required for successful implantation by directly inducing angiogenic and tissue-remodelling factors. Our group has utilised microarray and demonstrated a likely contribution of seminal fluid to vascular mediators in cervical tissue at the site of semen deposition in humans. Cervical biopsies collected after seminal fluid exposure at coitus revealed key implantation factors LIF receptor (*LIFR*) and *PTGS2* were upregulated, compared to biopsies obtained after abstinence or coitus with condom use (Sharkey *et al.* 2012*b*). The upregulation of tissue remodelling factors *MMP1*, *MMP2*, *MMP3*, *MMP7* and *MMP10* was also evident (Sharkey *et al.* 2012*b*). A similar observation was made in ectocervical Ect1 cells, where genes in the VEGF Signalling Pathway including *PTGS2* and *VEGFA* were upregulated after 10% seminal plasma treatment (Sharkey *et al.* 2012*a*).

Whether these changes in the cervix extend to similar changes in the endometrium in women is unclear, but recent experiments point to this possibility. The use of microarray and RNA-seq to examine endometrial transcriptomes after seminal fluid contact supports this prospect. Chen and colleagues demonstrated that addition of 1% seminal plasma led to major changes to the transcriptome of primary human stromal fibroblasts, inducing angiogenic factors including *VEGFA*, *FGF2* and *LIF* (Chen *et al.* 2014). IPA further revealed the enrichment and activation of vascular pathways including Movement of Vascular Endothelial Cells and Chemotaxis Vascular Endothelial Cells (Chen *et al.* 2014).

An exciting recent study compared the transcriptome of endometrial biopsies after seminal plasma or placebo application before IVF treatment. In these samples, KEGG pathway analysis revealed VEGF Signalling Pathway, Hedgehog Signalling Pathway and Cell Adhesion Molecules were amongst the pathways positively enriched by seminal plasma (Catalini *et al.* 2024). Furthermore, *ex vivo* experiments showed seminal plasma treatment of stromal fibroblasts led to a major shift in the transcriptome profile including the upregulation of *FGF2*, *LIF* and *PTGS2*, together with increased endometrial stromal fibroblast decidualisation, in a steroid hormone-independent manner (George *et al.* 2020).

As well as seminal plasma, there is evidence that spermatozoa carried in semen also contribute to regulating vascular mediators in the female reproductive tract. In mice, mating with intact males upregulated *Vegf* expression in the endometrium within 8 h post-coitum, while *Vegf* expression was not induced in females mated with vasectomised males (i.e. without spermatozoa exposure) (Schjenken *et al.* 2021). In humans, incubating spermatozoa from fertile men with endometrial cells led to the upregulation of genes including *VEGF*, *LIF* and *LIFR* (Ajdary *et al.* 2021), all of which are key to successful implantation.

The endometrial transcriptome has been shown to undergo major changes after co-incubation with high density SF-EVs. High-density SF-EVs in human semen are proposed to regulate

endometrial receptivity through increasing endometrial *LIF* expression, as demonstrated in an *in vitro* model utilising Ishikawa endometrial epithelial cells (Wang *et al.* 2024). A total of 1274 differentially expressed genes were identified after high-density SF-EV treatment and pointed to enriched angiogenic functions and pathways including Focal Adhesion and Adherens Junction (Wang *et al.* 2024). Indeed, SF-EVs enhanced adhesion of model blastocysts to Ishikawa cells, in a LIF-dependent manner (Wang *et al.* 2024). Whether non-transformed, primary endometrial cells respond similarly remains to be investigated, but it is plausible as SF-EV treatment increased prolactin production in primary human endometrial cells and enhanced decidualisation (Rodriguez-Caro *et al.* 2019), a process that involves substantial vascular changes. Interestingly, exosomes isolated from men with sperm abnormalities failed to upregulate *LIF* and *VEGF* expression in primary endometrial epithelial cells compared to those isolated from fertile men (Gholipour *et al.* 2023). These findings collectively suggest that seminal fluid has potential to enhance endometrial receptivity through effects on the uterine vasculature.

Summary and future directions

Failure to acquire optimal endometrial receptivity is a major constraint on reproductive success. There is now strong evidence that seminal fluid contact attenuates endometrial tissue composition and function, particularly through effects on the immune and vascular compartments, to contribute to immune tolerance and robust placental development. The application of RNA-seq and related genomic approaches has provided a powerful opportunity to examine the global transcriptomic changes induced by seminal fluid and their physiological regulation from both the female and male side of the interaction. The impact of immunoregulatory factors in seminal fluid on the maternal immune system is now well characterised, and the differential influence of permissive components TGFB and TLR4 ligands, and inhibitory factors such as IFNG, is being clarified. How seminal fluid signals might differentially modify the uterine vasculature remains ill-defined. However, factors such as VEGFA potentially exert a direct impact, and immune cells with a pro-angiogenic phenotype recruited in response to semen contact likely contribute.

A knowledge gap of considerable interest for future research is the nature of the physiological and pathophysiological mechanisms by which male partner exposures impact the composition of seminal fluid, and the impact this exerts on the endometrial receptivity of the female partner. Transcriptomic approaches along with proteomic strategies will be important for defining how various conditions and exposures affect seminal vesicle secretions, to in turn affect female tract receptivity. For example, experiments to analyse the effects of exposure to the reproductive toxin acrylamide on seminal vesicle gene expression in mice have revealed substantial changes in response to this insult, through pathways relating to inflammation and immune response regulation, and the cell stress response (Skerrett-Byrne *et al.* 2021). Upstream regulator analysis identified several signalling pathways, including GM-CSF, IFNG and Fas cell surface death receptor signalling, by which the seminal vesicle transcriptome can be modified (Skerrett-Byrne *et al.* 2021). Similar approaches will help decipher how a range of other insults and exposures – such as infection, heat, and nutritional stress – impact secretions from the seminal vesicle and other male reproductive organs. Environmental modulation of seminal plasma composition may turn out to be an important mechanism by which female fertility is modulated.

The examples set forth in this review demonstrate how RNA-seq and related transcriptomic approaches will be essential to resolving these questions. The molecular evidence from bulk RNA-seq and earlier microarray studies has been invaluable to understanding the influence of semen on the endometrium. It remains challenging to delineate the mechanisms by which semen affects these changes in women given the ethical and practical challenges of studying seminal fluid effects *in vivo*. In order to demonstrate the complex interaction between seminal fluid and the female tract, mouse models that allow the manipulation of seminal fluid content will remain important tools. While there are caveats to the extent to which discoveries made in mice are applicable to humans because of species-specific differences, there are many common pathways and cellular changes linking seminal fluid contact with endometrial receptivity, and conserved seminal fluid molecules implicated in driving these changes.

One major limitation of bulk RNA-seq is the lack of capability to distinguish contributions of individual cell populations to the transcriptomic changes identified, which limits a comprehensive understanding of endometrial biology. This is particularly problematic for minor cell populations, such as many immune cells that have major impacts on tissue function despite their rarity. Different cell types may also have opposing gene expression profiles and mask genuine variation of genes of interest. These limitations presumably contribute to the striking lack of agreement between studies striving to identify the molecular features distinguishing a receptive endometrium (Aplin and Stevens 2022). The availability of new methodologies, specifically single cell-sequencing and spatial transcriptomic analysis, will allow us to link individual cell types in the endometrium to specific cellular functions and events, at high spatial resolution. Similarly, the identification of novel upstream factors in seminal fluid that drive these changes will ultimately allow development of diagnostic tools to expand understanding of male fertility status and better understand male partner contributions to implantation success.

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Author affiliation

[A]The Robinson Research Institute and School of Biomedicine, University of Adelaide, Adelaide, SA 5005, Australia.