REPRODUCTION
REVIEW

Advances in human primordial follicle activation and premature ovarian insufficiency

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Abstract

In women, the non-growing population of follicles that comprise the ovarian reserve is determined at birth and serves as the reservoir for future fertility. This reserve of dormant, primordial follicles and the mechanisms controlling their selective activation which constitute the committing step into folliculogenesis are essential for determining fertility outcomes in women. Much of the available data on the mechanisms responsible for primordial follicle activation focuses on a selection of key molecular pathways, studied primarily in animal models, with findings often not synonymous in humans. The excessive induction of primordial follicle activation may cause the development of premature ovarian insufficiency (POI), a condition characterised by menopause before age 40 years. POI affects 1–2% of all women and is accompanied by additional health risks. Therefore, it is critical to further our understanding of primordial follicle activation in order to diagnose, treat and prevent premature infertility. Research in primordial follicle activation has focused on connecting new molecules to already established key signalling pathways, such as phosphatidylinositol 3-Kinase (PI3K) and mammalian target of rapamycin (mTOR). Additionally, other aspects of the ovarian environment, such as the function of the extracellular matrix, in contributing to primordial follicle activation have gained traction. Clinical applications are examining replication of this extracellular environment through the construction of biological matrices mimicking the 3D ovary, to support follicular growth through to ovulation. This review outlines the importance of the events leading to the establishment of the ovarian reserve and highlights the fundamental factors known to influence primordial follicle activation in humans presenting new horizons for female infertility treatment.

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Introduction

The total number of immature primordial follicles that reside in a woman's ovaries are established *in utero* from germ cell nests and is termed the ovarian reserve. Moreover, an estimation of a woman's ovarian reserve is considered a fundamental indicator of fertility. Of equal importance to fertility is the rate at which these immature primordial follicles are recruited for growth through a process known as 'activation'. Of these original primordial follicles, numbering between 500,000 and 1,000,000 in total at birth, only approximately 400 will fully mature into primary oocytes capable of being ovulated and fertilised during a woman's reproductive years (Hansen *et al.* 2008, Findlay *et al.* 2015). The overwhelming majority are fated to undergo atresia (ovarian-mediated cell death) (Baker 1963, Tilly 2001, Marcozzi *et al.* 2018).

Primordial follicle activation involves the recruitment of primordial follicles into folliculogenesis for the eventual selection of one oocyte for ovulation. As women age, the pool of primordial follicles, from which to source candidates for activation, plummets. When there are ~1000 primordial follicles remaining, this results in the cessation of fertility and the onset of menopause (Faddy *et al.* 1992, Hansen *et al.* 2008). A key aspect of our understanding of the depletion of the ovarian reserve is that primordial follicles cannot be regenerated or replaced. This concept has been contested by the identification of oogonial stem cells able to produce new oocytes within the mouse ovary, with emerging evidence for their presence in human ovaries (Clarkson *et al.* 2018), but there is still conjecture over a physiological role for these cells based on definitive evidence (reviewed in Horan & Williams 2017).

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Thus, the challenge for research is to elucidate the mechanisms controlling the depletion of the ovarian reserve via primordial follicle activation, so that we may provide solutions for those faced with the threat of early fertility loss, such as in the case of premature ovarian insufficiency (POI).

POI may also be referred to as premature ovarian failure, but for the purpose of this review, either term applies interchangeably. POI is an infertility condition diagnosed when menopause occurs prior to the age of 40, due to a significant reduction in, or absence of, a woman's pool of primordial follicles. This condition occurs globally in 1–2% of all women (Coulam *et al.* 1986). POI is defined as the premature cessation or absence of ovarian function and is characterised by amenorrhea, hypoestrogenism and an increase in gonadotrophin levels (Bachelot *et al.* 2009, Shelling 2010). A common cause of POI is an acceleration in the rate of primordial follicle activation, thereby resulting in a depletion of the ovarian reserve (Nelson 2009). However, the ovarian reserve may also be depleted via primordial follicle loss (Depalo *et al.* 2003) or from iatrogenic causes such as chemotherapeutics (Ben-Aharon & Shalgi 2012). Women often receive a diagnosis of POI when they are already in significant reproductive decline, and as a consequence of their diagnosis may face psychological impacts in addition to the physiological symptoms and infertility of this condition (Van Der Stege *et al.* 2008, Welt 2008). Long-term physical consequences of POI may be severe, even with traditional hormone replacement therapies to ameliorate the negative effects of a loss of ovarian hormonal support. Women with POI have an increased risk of cardiovascular disease, osteoporosis, urogenital atrophy and neurodegenerative disorders (reviewed in Podfigurna-Stopa *et al.* 2016). Thus, it is critical that we continue towards understanding the process of primordial follicle activation as a means of intervention to preserve the ovarian reserve.

Both the establishment of the ovarian reserve and the initial wave of primordial follicle activation occur *in utero,* prior to sexual maturity and gonadotrophic input, thus relying largely upon regulation by intraovarian factors (Lew 2019). Primordial follicle activation dictates the growth and development of the oocyte as well as the differentiation and proliferation of the surrounding somatic granulosa cells, both essential processes for the ultimate goal of ovulation. However, our understanding of the molecular and biochemical processes underpinning follicular activation in humans remains limited. Herein we review the current knowledge and explore future advances towards controlling primordial follicle recruitment and consequently preserving or prolonging female fertility. This is particularly pertinent for those women diagnosed with POI who experience accelerated activation and early depletion of the ovarian reserve.

Early events within the ovary establish the ovarian reserve that sustains fertility

During human embryo sex determination, the primitive gonads are endowed with the primordial germ cells (Tam & Snow 1981) (Fig. 1). The germ cells migrate to the genital ridge of the primitive gonad, then rapidly proliferate until they number 5–6 million (Motta *et al.* 1997, Mamsen *et al.* 2011, Myers *et al.* 2014). This proliferation occurs rapidly and as cytokinesis is not wholly completed, clusters of germ cells (termed germ cell nests) connected via cytoplasmic bridges remain (reviewed by Pepling 2006). Germ cell nest breakdown directly precedes primordial follicle formation and is a major factor influencing the initial size of the ovarian reserve. During this process, pre-granulosa cells (flattened, squamous granulosa cells prior to differentiation into cuboidal granulosa cells) are primed to encapsulate the oocytes via signalling originating from oocytes and intracellular communication between other pre-granulosa cells (De Felici *et al.* 2005, Grive & Freiman 2015, Suzuki *et al.* 2015*a*). Only a small fraction of the original population of germ cells go on to form primordial follicles, while the remaining oocytes, estimated between one and two thirds, are targeted for coordinated degradation via classical apoptotic mechanisms (Albamonte *et al.* 2008). The role of autophagy in protecting germ cells from apoptosis has been established in mice (Rodrigues *et al.* 2009), with preliminary evidence this occurs in humans too (Sun *et al.* 2017, Zhou *et al.* 2019). The cause for such a substantial loss of oocytes is still unknown, but it is possible that a quality control mechanism exists through which faulty nuclei are lost, and healthy oocytes are preferentially encapsulated into primordial follicles (Tilly 2001, Sun *et al.* 2017). Additionally, a self-sacrifice mechanism previously established in *Drosophila* (de Cuevas *et al.* 1997), and later observed in mice may also be responsible for mammalian germ cell nest breakdown (Grive & Freiman 2015, Pepling 2016). In this mechanism, essential cellular factors are transported via cytoplasmic bridges from neighbouring germ cells within a cluster to the germ cells that will survive and become oocytes in primordial follicles, but this mechanism has not been confirmed in humans (Lei & Spradling 2016).

At the completion of germ cell nest breakdown, the oocytes are each surrounded by a layer of pre-granulosa cells and termed primordial follicles (Maheshwari & Fowler 2008). A surge of retinoic acid released from the mesonephros (primitive kidney) positioned adjacent to the immature ovaries, drives all primordial germ cells to enter meiosis, where they pause arrested at the diplotene stage of prophase I (Borum 1961, Peters 1969). Shortly before birth, primordial follicle activation commences with most follicles developing to the preantral follicle stage (Himelstein-Braw *et al.* 1976). It has been reported that in humans, some follicles will continue onto the antral stage prior to birth (Peters *et al.* 1978).

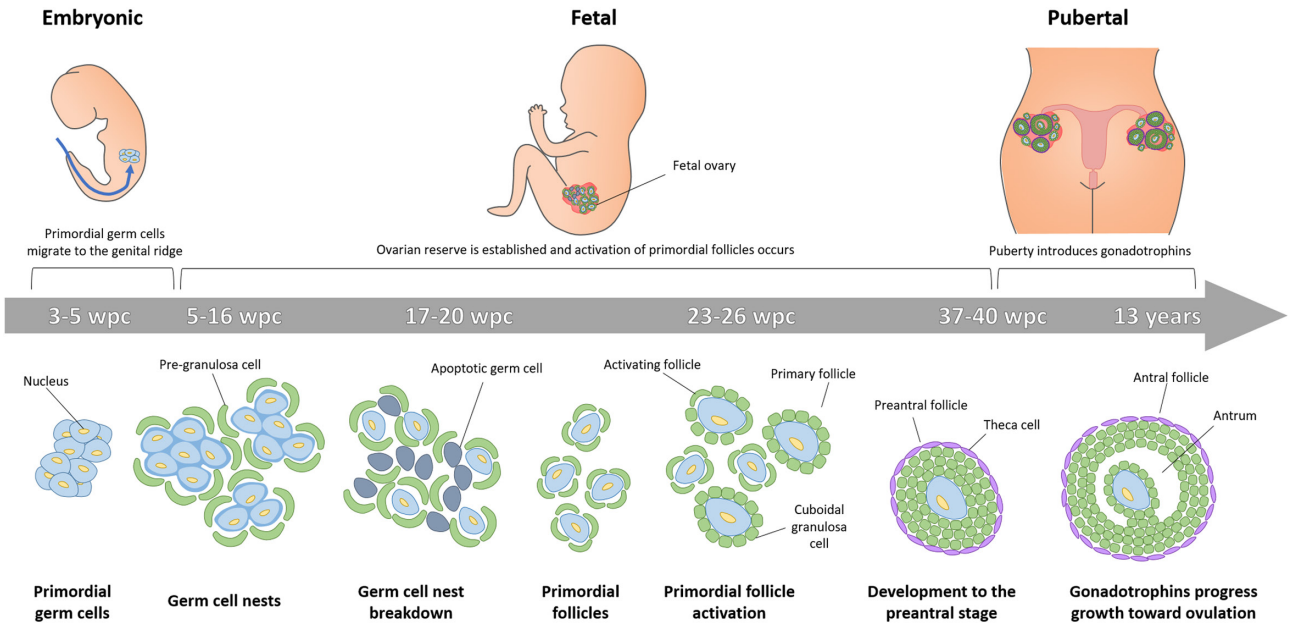

Figure 1 The development of the human ovarian reserve. The timeline of development of the ovarian reserve is depicted, with representations of the germ cells and somatic cells residing in the ovary, and the stage of life of the female at the point which this occurs. Beginning at 3–5 weeks post conception (wpc) in the embryo, the primordial germ cells migrate to the genital ridge. By 5–16 wpc in the foetus, the primordial germ cells proliferate rapidly and vastly increase in number, resulting in germ cell nests as cytokinesis is not wholly complete. The germ cell nests begin to break down at 17–20 wpc, with many germ cells undergoing apoptosis as the pre-granulosa cells start to encapsulate the germ cells to form primordial follicles. Primordial follicles undergo meiosis after a surge of retinoic acid from the mesenephros, and they remain arrested at prophase I of the cell cycle. From 23–26 wpc the first wave of primordial follicle activation commences, as the oocyte grows and granulosa cells grow and differentiate from their flattened, squamous shape to a cuboidal structure. At around the time of birth at 37–40 wpc, a small proportion of preantral and antral follicles are present. However, until puberty occurs at around 13 years of age, activated follicles can only grow until the early antral stage. At puberty, the input of gonadotrophin allows progression beyond early antral stage through to ovulation.

Increased gonadotrophic production at puberty enables successive follicle growth and ovulation (Dungan *et al.* 2006, Choi & Yoo 2013). Continued activation of primordial follicles occurs dynamically throughout the reproductive years until the onset of menopause. Primordial follicle activation constitutes the committing step into folliculogenesis, as the primordial follicles that are activated throughout a woman's life succumb to one of two fates; to be ovulated or destroyed.

Since the endowment of the ovarian reserve contains the potential for future fertility, and primordial follicle activation is responsible for regulating follicle progression beyond this point, it is important to consider how activation is so precisely controlled so that we may identify those women at risk of accelerated activation and POI and develop practices to preserve or prolong fertility.

Excessive activation of primordial follicles can lead to premature ovarian insufficiency

When the rate of primordial follicle activation is accelerated, and control over the size of the ovarian reserve is lost, POI can result (Kalantaridou *et al.* 1998). POI may be induced by aberrations in other vital ovarian processes which are outside the scope of the current review, including but not limited to meiosis, DNA repair and gonadotrophin control (Huhtaniemi *et al.* 2018). POI is largely idiopathic (50–70%) (Chapman *et al.* 2015), but known causes include iatrogenic factors (such as chemotherapeutics), genetic factors, environmental factors or autoimmunity. Autoimmunity is responsible for 10–30% of POI cases and is typically related to adrenal disease (Hoek *et al.* 1997, Ebrahimi & Akbari Asbagh 2015). Additional autoimmune diseases that may cause POI include Addison's disease, hypothyroidism, Whitaker syndrome and diabetes mellitus (Conway *et al.* 1996, Ebrahimi & Akbari Asbagh 2015, Komorowska 2016). Yet, the mechanism by which these diseases result in POI is not usually through excessive or uncontrolled primordial follicle activation but through follicular oocyte destruction (Persani *et al.* 2009 for a review). Thus, it is critical that the factors affecting primordial follicle activation be understood in order to identify those at risk of POI early so that fertility can be preserved.

Iatrogenic factors

Amongst the number of iatrogenic factors able to induce POI (radiation, surgery, physical damage to the ovary), it is chemotherapeutics that have been particularly linked

to changes in primordial follicle activation and/or the depletion of the ovarian reserve. Other studies have also linked damage to the vasculature of the ovarian cortex to depletion of primordial follicles (reviewed in Ben-Aharon & Shalgi 2012). In each of these scenarios, patients are at significant risk of developing POI, though it has not yet been confirmed if gonadotoxic treatment is due to accelerated activation or an increase in follicular atresia (Nguyen *et al.* 2019). Typically, chemotherapeutics target proliferating cells and depending on the dose, duration and treatment, granulosa cells are significantly compromised by these treatments, resulting in primordial follicle loss (Abir *et al.* 2008). After treatment with the alkylating agent cyclophosphamide, women have a 40% chance of developing POI, but the cellular mechanisms resulting in premature menopause remain unclear (Cox & Liu 2014). Human ovarian tissue sections cultured in the active metabolites of cyclophosphamide have a decreased primordial follicle population and a concomitant increase in developing follicles (Lande *et al.* 2017). However, in a combined *in vitro* treatment of chemotherapeutics (adriamycin, bleomycin, vinblastine and dacarbazine), the density of non-growing follicles in human ovarian tissue samples was increased (Mclaughlin *et al.* 2017).

For women undergoing chemotherapy, fertility preservation through cryopreservation of ovarian tissue, followed by transplantation or assisted reproductive technologies is practised, but further developments to ensure the widespread success of this procedure are still underway (Donnez & Dolmans 2017, Fisch & Abir 2018). New research in mice has identified another chemotherapeutic, dacarbazine, that contributes to primordial follicle depletion (Winship *et al.* 2018), which urges further research into its effect on the ovarian reserve in humans.

Genetic factors

Gene alterations, loss-of-function mutations, and whole chromosomal abnormalities, all demonstrate an ability to alter the rate of primordial follicle activation and thus affect female fertility. Abnormalities of the X chromosome such as deletions, duplications or complete ablation (i.e. Turner syndrome 45XO karyotype) contribute substantially to defects in ovarian development and later problems in fertility (Cordts *et al.* 2011). Some estimates suggest that up to 12% of cases of genetic POI are induced by errors within the X chromosome gene complement (Goswami & Conway 2005, Qin *et al.* 2014). While particular regions on the X chromosome have been identified as vulnerable with regard to the development of ovarian disorders such as POI, it appears that the complex interplay of these genes during folliculogenesis makes it difficult to define one particular causative agent (Chapman *et al.* 2015). Molecular and cytogenetic analyses on the types of genetic abnormalities present in POI patients have identified a 'critical' region on the long arm of the X chromosome (Xq13-27) that is frequently associated with this disease (reviewed in; Persani *et al.* 2009). Additionally, a number of genes have been associated with POI that regulate primordial follicle recruitment and the gonadotrophin-independent phase of follicular growth (reviewed in Huhtaniemi *et al.* 2018). Several of these have already been established in primordial follicle activation literature as discussed above and include PI3K/AKT/mTOR signalling, FOXL2 and TGFB signalling.

Despite the range of genetic factors known to cause a rapid depletion in available oocytes and the subsequent onset of POI, more genomic interrogation and cellular research is required to reveal the interactions between these factors and determine how they regulate the ovarian reserve through primordial follicle activation.

Environmental factors

Environmental factors with demonstrated impacts on fertility are numerous and are often linked to systemic effects not specifically related to the depletion of the ovarian reserve. However, there are multiple factors that have been directly linked to a decrease in the size of the primordial follicle pool or an increase in the rate of recruitment – both with implications for the development of POI.

Cigarette smoking has the capacity to directly affect the mammalian follicle reserve as evidenced through animal studies (Jurisicova *et al.* 2007, Gannon *et al.* 2012); yet, findings from human studies on primordial follicle populations affected by smoking remain inconsistent (Caserta *et al.* 2013, Peck *et al.* 2016). However, in a cohort of POI patients in Korea, cigarette smoking was strongly associated with an increased risk of the development of POI (Chang *et al.* 2007). In the human fetal ovary, maternal smoke exposure was found to activate aryl hydrocarbon receptor (AHR) and reduce germ cell proliferation, with concomitant implications for germ cell loss via downstream promotion of apoptosis (Mamsen *et al.* 2010, Anderson *et al.* 2014). However, further studies in humans are required to determine if AHR-driven depletion of the primordial follicle pool occurs as a direct result of exposure to cigarette smoke constituents.

Phthalates are toxicants commonly used as plasticising agents and prone to leaching into the environment (Hannon & Flaws 2015). Human fetal ovaries exposed *in vitro* to mono-(2ethylhexyl) phthalate displayed altered lipid synthesis (Muczynski *et al.* 2012). Another phthalate, butyl benzyl phthalate has been demonstrated to decrease the viability of granulosa cells through AHR activation, which, as outlined above, is detrimental to follicular survival rates (Chen *et al.* 2012). These findings suggest ovarian dysfunction that may contribute to a loss in fertility as a consequence of phthalate exposure.

Bisphenol A (BPA) is another mass-produced toxicant that is widely present by virtue of its use in plastics for packaging and resins. BPA affects the ovaries as it has a similar molecular structure to estrogens and can bind to estrogen receptor alpha (Craig *et al.* 2011). Several animal studies have strongly linked BPA exposure to follicle depletion, and this effect is observed regardless of whether exposure occurs *in utero*, postnatally or during adulthood (reviewed in Richardson *et al.* 2014). Consequently, some countries have implemented bans on BPA use based on these animal studies, as human data on the reproductive consequences of BPA remain scarce (Richardson *et al.* 2014, Mathew & Mahalingaiah 2019). Coincidently, in a study of infertile women, those with higher than average urinary BPA were identified as having a decreased ovarian reserve, and another study explored IVF outcomes for women with higher serum BPA and observed lower pregnancy rates and higher association with miscarriage (Sugiura-Ogasawara *et al.* 2005, Lamb *et al.* 2008).

Cohorts, case studies and genome-wide association studies of POI have been instrumental in our understanding of human primordial follicle activation; yet, there are still substantial gaps in the knowledge of this process. Research is currently being focused towards extrapolating this genomic knowledge into further upstream or downstream influences in the molecular pathways implicated to contribute to the future treatment or even prevention of early depletion of the ovarian reserve.

Humans utilise the classical primordial follicle activation pathways differently

To achieve the preservation of female fertility, the mechanisms that control the size of the ovarian reserve, and the rate of primordial follicle recruitment must be determined. Both these events occur in the absence of gonadotrophic regulation and thus are likely to be wholly reliant on intrinsic signalling mechanisms. The complex network of signalling between the oocytes and the granulosa cells, and between neighbouring granulosa cells is critical in maintaining the size of the ovarian reserve and successive follicle development (Edson *et al.* 2009). Granulosa cells are vital for ensuring the development of the follicle, and their contribution whilst substantial is not yet fully elucidated. The status of granulosa cells is a substantial determinant of follicle survival as follicular atresia can occur if insufficient numbers of granulosa cells surround the oocyte or if these cells do not correctly transition to a cuboidal phenotype upon activation (Gougeon & Chainy 1987, Matsuda *et al.* 2012). There exists considerable literature on the signalling networks and molecules known to be involved in primordial follicle activation in model animal studies (see reviews Adhikari & Liu 2009, Kim 2012, Zhang & Liu 2015). For most of the factors discussed below, a regulatory role in primordial follicle recruitment was initially established using rodent 'loss-of-function' studies. In humans, an analogous phenotype to rodent studies demonstrating ovarian reserve depletion via accelerated primordial follicle activation is frequently observed in cases of POI (Fig. 2). The current hypothesis of primordial follicle activation in mice follows that mammalian target of rapamycin complex 1 (mTORC1) is activated in the flattened granulosa cells of primordial follicles, and then KIT ligand produced by activated granulosa cells then activates the oocyte via phosphatidylinositol 3-kinase (PI3K) signalling (Zhang *et al.* 2014). However, the model for primordial follicle activation remains unclear. The following section discusses the factors identified as playing a role in human primordial follicle activation and to what extent that role is similar to rodent literature (Table 1).

The PI3K/AKT/mTOR pathway

The phosphatidylinositol 3-kinase/AKT serine/threonine kinase/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is involved in cell survival, growth and migration in various tissues, via the modulation of transcription factors (Cantley 2002). In the mammalian ovary, the PI3K/AKT/mTOR pathway is essential for the regulation of primordial follicle activation, with multiple activators and suppressors identified (reviewed in Zhang & Liu 2015). The negative regulator, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), has been established in human primordial follicle granulosa cells at the protein and gene expression level (Goto *et al.* 2007, Makker *et al.* 2014, Zhang *et al.* 2018). In the ovaries, AKT is a prominent kinase in the PI3K/AKT/mTOR pathway and is expressed in both oocytes and granulosa cells of human follicles (Goto *et al.* 2007, Mclaughlin *et al.* 2014). AKT has a wide range of substrates with both direct and indirect roles in follicle activation (Cecconi *et al.* 2012). For women at risk of POI following chemotherapy, primordial follicle activation was stimulated via AKT promotion/PTEN inhibition in ovarian cortical fragments *in vitro* prior to transplantation, and this technique was able to achieve two live births thus far (Kawamura *et al.* 2013, Suzuki *et al.* 2015*b*, Zhai *et al.* 2016). However, the use of PTEN inhibition to initiate primordial follicle activation has been demonstrated to affect follicular survival (Lerer-Serfaty *et al.* 2013, Mclaughlin *et al.* 2014), while in an animal model, prevent DNA repair (Maidarti *et al.* 2019). Thus, this warrants further attention before the technique should be used routinely in human *in vitro* culture pre-transplantation.

The TSC1/mTORC1 subsection of the PI3K/AKT/mTOR pathway was implicated through mouse studies to have a function in driving primordial follicle activation via the differentiation and developmental fates of the

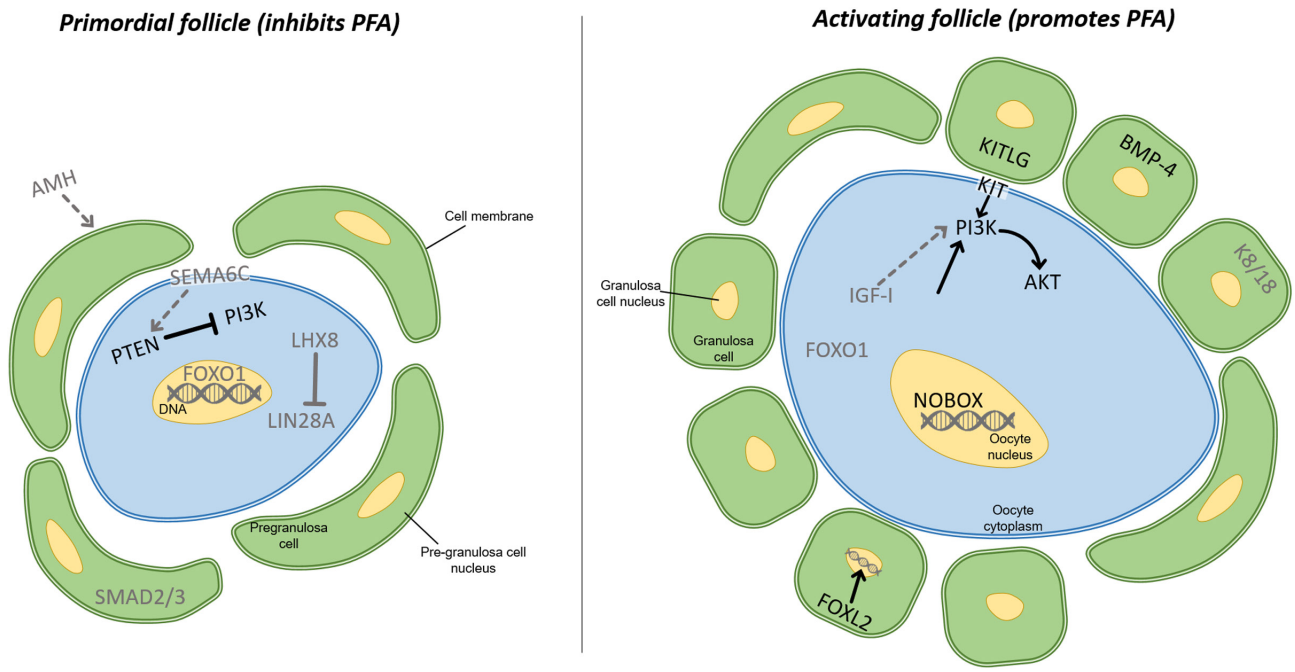

Figure 2 The inhibition and promotion of human primordial follicle activation. Schematics of the factors influencing the maintenance of primordial follicles (the inhibition of primordial follicle activation, PFA) and the activation of primordial follicles (promoting primordial follicle activation). The primordial follicle (left) is an oocyte (blue), surrounded by a single layer of flattened, pre-granulosa cells (green). Once the primordial follicle is activated (right), the oocyte begins to increase in size, and the granulosa cells proliferate and differentiate unto a cuboidal morphology. A number of molecules from classical primordial follicle activation pathways established in animal models, like PI3K/PTEN pathway, signal between oocytes and granulosa cells (black arrows indicate stimulation and interaction, blunt end arrow indicates inhibition) through the cell membrane (represented by double lines) where some proteins reside (i.e. SEMA6C and KIT). Additionally, a suite of transcription factors, like NOBOX, and FOXL2 have been established to interact within the nucleus (yellow) affecting the transcription of their target genes. Several factors have been speculated to influence primordial follicle activation (SEMA6C, K8/18), while others have demonstrated effects with no definitive influence (AMH) and these speculative or putative links are indicated by grey, dotted lines.

granulosa cells (Zhang *et al.* 2014), but additional evidence has shown that TSC1 in oocytes is dispensable for primordial follicle activation (Gorre *et al.* 2014). This suggests that mTORC1 is required for primordial follicle activation in the granulosa cells, but not within the oocyte. Within growing mouse oocytes, however, mTORC2 has been demonstrated to be an essential component of follicular development (Chen *et al.* 2015); yet, its role remains uncharacterised in human oocytes. A recent transcriptome study of human follicles identified an upregulation of the pathway's inhibitor *TSC1* in the oocytes of primary follicles when compared to primordial follicles (Zhang *et al.* 2018), which may suggest a similar role in primordial follicle activation, but whether this role functionally redundant as in animal models is yet to be determined. Furthermore, human follicles treated *in vitro* with an mTORC1 inhibitor exhibited a partial reduction in follicle growth and subsequent decrease in TSC1 mRNA (Grosbois & Demeestere 2018), thus providing supporting evidence for a role in primordial follicle activation.

Despite the PI3K/AKT/mTOR signalling pathways having extensive connections in both primordial follicle activation and the maintenance of quiescence, our fundamental understanding of these processes is still very limited, particularly in the human. Indeed, there are numerous other cellular mechanisms in addition to this pathway known to be involved in primordial follicle activation, and these include transcription factors, growth factors and cytokines.

Transcription factors

Transcription factors specific to the ovaries also contribute to the regulation of primordial follicle activation. A is a known suppressor of mammalian primordial follicle activation, an inducer of follicle atresia and a known substrate of AKT in the PI3K/AKT/mTOR pathway. Human studies using donor cells and tissue samples from women attending fertility clinics have primarily focused on FOXO3A in ovarian cancer, where it has roles in the apoptosis of follicles through AKT activation (Ding *et al.* 2015), granulosa apoptosis *in vitro* (Ono *et al.* 2014) and tumour progression as a positive regulator of p27 (Fei *et al.* 2009). However, aside from apoptosis in ovarian cancer, a defined role of FOXO3A in primordial follicle activation is lacking from human studies, despite evidence in mice that supports this transcription factor

Table 1 Comparison of factors influencing primordial follicle activation between human and animal models.

Molecule	Species evidence	Role in primordial follicle activation	Cell type gene expressed	Cell type protein localised	References
PI3K		Promotes			
	Mouse		Oocyte	Oocyte	Adhikari & Liu (2009)
	Human		Oocyte	Oocyte	Makker *et al.* (2014), Sun *et al.* (2015), Zhang *et al.* (2018)
PTEN		Inhibits			
	Mouse		Oocyte	Oocyte	Adhikari & Liu (2009)
	Human		Oocyte	Oocyte	Goto *et al.* (2007)
AKT		Promotes			
	Mouse		Oocyte and granulosa	Oocyte and granulosa	Adhikari & Liu (2009)
	Human		Oocyte	Oocyte and granulosa	Goto *et al.* (2007), McLaughlin *et al.* (2014), Ernst *et al.* (2017)
FOXO3A					
	Mouse	Inhibits	Oocytes	Oocyte	Castrillon *et al.* (2003), John *et al.* (2008)
	Human	Unknown	Unknown	Unknown	Tarnawa *et al.* (2013)
mTORC1		Promotes			
	Mouse		Oocyte and granulosa	Oocyte and granulosa	Gorre *et al.* (2014), Zhang *et al.* (2014)
	Human		Oocyte and granulosa	Oocyte and granulosa	Ernst *et al.* (2017), Ernst *et al.* (2018)
mTORC2					
	Mouse	Promotes	Oocyte	Oocyte	Chen *et al.* (2015)
	Human	Unknown	Unknown	Unknown	
TSC1		Inhibits			
	Mouse		Oocyte and granulosa	Oocyte and granulosa	Gorre *et al.* (2014), Zhang *et al.* (2014)
	Human		Not specified	Not specified	Grosbois & Demeestere (2018), Zhang *et al.* (2018)
LHX8		Inhibits			
	Mouse		Oocyte	Oocyte	Pangas *et al.* (2006), Ren *et al.* (2015)
	Human		Oocyte	Not specified	Kristensen *et al.* (2015)
AMH					
	Rat and mouse	Inhibits	Granulosa	Granulosa	Baarends *et al.* (1995), Durlinger *et al.* (2002)
	Human	Unknown	Granulosa	Granulosa	Schmidt *et al.* (2005), Carlsson *et al.* (2006)
BMP-4		Promotes			
	Mouse		Granulosa and oocyte	granulosa and oocyte	Chang *et al.* (2002), Knight & Glister (2006)
	Human		Granulosa	Oocyte and Granulosa	Ikeda *et al.* (2016), Pierre *et al.* (2016)
BMP-15		Promotes			
	Mouse		Oocyte and granulosa	Oocyte and Granulosa	Chang *et al.* (2002), Knight & Glister (2006), Persani *et al.* (2014)
	Human		Oocyte and granulosa	Oocyte and granulosa	Margulis *et al.* (2009), Manavella *et al.* (2019)

as a key molecular regulator (Castrillon *et al.* 2003, John *et al.* 2008, Chang *et al.* 2015). While a few *FOXO3A* mutations have been identified in POI patients, the causative contribution these mutations may have on the condition requires investigation (Watkins *et al.* 2006), especially considering *FOXO3A* is not expressed in human primordial oocytes, unlike its mouse counterpart (Tarnawa *et al.* 2013). Thus, it is unlikely that FOXO3A maintains the quiescence of primordial follicles in humans as in mice.

The transcription factor Forkhead box L2 (FOXL2) is essential for squamous to cuboidal granulosa cell differentiation and subsequent formation of secondary follicles in animal models (Schmidt *et al.* 2004, Uda *et al.* 2004, Uhlenhaut & Treier 2006). Many studies in human cell lines, and in mice, have reported FOXL2 targets are also involved in apoptosis, differentiation and the cell cycle (as reviewed in Georges *et al.* 2014), further demonstrating the critical functions of this transcription factor. Notably, a study of human granulosa cells demonstrated that FOXL2 transcripts were less abundant in granulosa cells from primary follicles compared to granulosa cells from primordial follicles (Ernst *et al.* 2018), providing preliminary evidence that FOXL2 downregulation between these stages may coincide with a role in primordial follicle activation in humans. Further investigation of the role of this transcription factor in primordial follicle activation is warranted to confirm the role of FOXL2 in human primordial follicles.

A binding partner of FOXL2 is NOBOX, which is involved in folliculogenesis and the regulation of oocyte-specific gene expression (Huntriss *et al.* 2006, Bouilly *et al.* 2014). In mice, NOBOX is critical for early ovarian development and indirectly participates in

primordial follicle activation via its transcriptional targets (Rajkovic *et al.* 2004, Lechowska *et al.* 2011). Recently, NOBOX transcriptional targets in both humans and mice have been identified and include essential oocyte developmental factors such as growth differentiation factor 9 (GDF9) and octamer-binding transcription factor 4 (OCT4) (Choi & Rajkovic 2006, Bayne *et al.* 2015). Additionally, a novel, loss-of-function *NOBOX* mutation was identified in a POI patient (Li *et al.* 2017), providing further evidence of a key role for this gene in primordial follicle activation. These existing studies justify further investigation of the role that NOBOX has in human primordial follicles.

The LIM homeobox 8 (LHX8) transcription factor has long been associated with the suppression of mouse primordial follicle activation by blocking the expression of RNA-binding protein LIN28A, an upstream activator of the PI3K/AKT/mTOR pathway (Pangas *et al.* 2006, Ren *et al.* 2015). In humans, *Lhx8* transcripts were reported to have decreased expression in early primary follicle oocytes compared to primordial follicle oocytes, thus suggesting a role in the primordial to primary transition (Kristensen *et al.* 2015). This finding highlights the importance of identifying the extent of the roles of currently established pathways in primordial follicle activation.

Growth factors of the TGFB superfamily

The TGFB family of growth factors are involved in a range of cellular processes throughout the body, but in the ovaries specifically, they have roles in early ovarian development and follicle growth (Drummond 2005). Anti-Mullerian-inhibiting substance (AMH) is known, via animal models, to be expressed in the growing follicles, localising specifically to the granulosa cells, and has been established as a suppressor of primordial follicle activation (Visser & Themmen 2005). Corroborating studies in human ovaries have shown that when cultured *in vitro*, AMH could inhibit the proportion of primordial follicles being activated (Carlsson *et al.* 2006). However, conflicting evidence from a 4-week culture of human ovarian tissue supplemented with AMH demonstrated that significantly more follicles were activated to enter the growing phase (Schmidt *et al.* 2005). The mechanisms behind AMH's effect on the ovarian reserve remain unclear as even rodent primordial follicles do not express AMH receptors (Baarends *et al.* 1995, Durlinger *et al.* 2002). In mice, it has been established that *Amh* is a transcriptional target of FOXL2, and thus suggesting they operate in conjunction to maintain the ovarian reserve of primordial follicles (Park *et al.* 2014). Recent evidence in humans has identified that FOXL2 controls AMH indirectly through the transcriptional activation of steroidogenic factor-1, which is a regulator of AMH (Jin *et al.* 2016). Further research has established an inverse relationship whereby AMH is able to modulate the amount of FOXL2 in human granulosa cells (Sacchi *et al.* 2017).

Bone morphogenetic proteins (BMPs) 4 and 15 may participate in early-stage folliculogenesis in humans, in line with several animal studies (see reviews Chang *et al.* 2002, Knight & Glister 2006, Persani *et al.* 2014). Preliminary studies in human gonadal cell culture models have identified BMP4 activity in both the somatic and germ cells during the establishment of the ovarian reserve via the promotion of primordial germ cell apoptosis and the differentiation of pre-granulosa cells (Childs *et al.* 2010, Bayne *et al.* 2016). Studies have also identified the involvement of BMP4 in facilitating the primordial to primary follicle transition in both mouse (Ding *et al.* 2013) and human ovary culture (Ikeda *et al.* 2016). *In vitro* treatment of cultured human GCs with BMP4 and/or 15 induced AMHR2 gene expression (Pierre *et al.* 2016) and this work built on that in sheep models (Estienne *et al.* 2015).

This section has provided an overview of the critical pathways, transcriptional regulators and growth factors that contribute to either the maintenance of the primordial follicle reserve or its activation and subsequent depletion in humans. Many of the factors traditionally associated with primordial follicle activation research have been studied extensively in rodent model systems, with a large portion as yet unstudied in humans or contributing inconsistencies between species. These contrasting studies highlight the importance of continued research in this field to determine if current animal-based research is consistent in humans or warrants the development of more suitable models.

Current research into primordial follicle activation

New insights into primordial follicle activation have arisen primarily from transcriptomic studies, in addition to conditional expression and knockdown studies in animals. Within this research, new factors are identified that link to already established pathways and events. Attention is also being focused towards how intraovarian factors derived from the granulosa cells and the extracellular matrix contribute to the activation status of the follicle.

A family of histone deacetylases, sirtuins, maintain homeostasis throughout the body, responding to changes in metabolism, inflammation and ageing (Vachharajani *et al.* 2016, Grabowska *et al.* 2017). Early work identified that sirtuin 1-null mice were infertile, with no exposition of the underpinning mechanism (Mcburney *et al.* 2003). The presence of sirtuins (SIRTs) has been confirmed in the ovaries of humans, including in the oocyte and granulosa cells, with little evidence, as yet, of their function and how they may contribute to the maintenance of the ovarian reserve or folliculogenesis (Tatone *et al.* 2018). However, SIRTs have recently been linked to the mTOR signalling pathway in rat ovaries,

capable of targeting the activation inhibitor FOXO3a in order to control primordial follicle activation in response to environmental cues like nutrient status (reviewed in Tatone *et al.* 2018). An upstream regulator of the PI3K/AKT/mTOR pathway, Semaphorin 6C (SEMA6C) (Fig. 2), has been newly identified to suppress primordial follicle activation in mice (Zhou *et al.* 2018). Semaphorins were traditionally characterised in the brain, but after links to extensive biological functions in other tissues, they were also found to be functional in human ovaries (Borgbo *et al.* 2013).

A recent study of the transcriptome of human primordial and primary follicles has revealed some novel proteins that may be associated with this developmental transition. Notably, an additional Forkhead transcription factor, *FOXO1*, which exhibited a transcriptional decrease during the primordial to primary transition (Ernst *et al.* 2017). This decrease was accompanied by a subsequent relocation of FOXO1 protein from the oocyte nucleus to cytoplasm indicating a similar role to the well-established function of FOXO3A. Members of the eukaryotic translation initiation factor 2 (EIF2) signalling pathway were also identified to be upregulated during the primordial to primary transition. In particular, the EIF4E gene was observed to increase at the transcript and protein level (Ernst *et al.* 2017). EIF4E traditionally facilitates the translation of stored mRNA in oocytes (Henderson *et al.* 2009).

There has also been growing interest in the TATA-binding proteins (TBPs) and the oocyte-specific TBP2. Emerging evidence in mice has identified the ontogeny of the expression patterns of this protein, which suggests it has a role in the primordial to primary transition – contributing to transcription status of the follicle (Schultz *et al.* 2018). However, studies in human POI cohorts reveal conflicting data, with overexpression observed in some cases, and other studies contend that TBPs do not contribute to POI (Tsuiko *et al.* 2016, Wang *et al.* 2016). A new potential marker of primordial follicles about to undergo activation is the expression status of keratin. The presence of the keratin 8/18 heterodimer (K8/K18) in granulosa cells was strongly correlated with survival status in addition to granulosa cells undergoing the squamous to cuboidal transition (Gaytan *et al.* 2018). Indeed, transcriptional silencing of K8/K18 using siRNA interference in human granulosa cell-like KGNs induced apoptosis (Trisdale *et al.* 2016). This evidence, while still in preliminary stages, prompts further investigation into the role of K8/18, and other typically epithelial-related proteins in granulosa cell physiology. Insulin-like growth factors (IGFs) are traditionally connected to proliferation and antiapoptotic signalling pathways in the ovaries (reviewed in Amutha & Rajkumar 2017) and have now been demonstrated to promote follicle growth via the PI3K/AKT/mTOR pathway in sheep (Bezerra *et al.* 2018), with further evidence demonstrating that transcripts from members of IGF1 signalling were differently expressed in human primordial and primary granulosa cells (Ernst *et al.* 2017, Steffensen *et al.* 2018). The cause for differential IGF1 expression between these two cell stages requires further investigation to determine if IGF1 expression is indeed linked to the activation of primordial follicles. However, the role of IGFs and whether they can modulate activation *in vivo* through androgens or PI3K signalling is yet to be validated.

The extracellular matrix within the ovary is known to be essential for granulosa cell survival and proliferation, and as such is an important consideration in ovarian developmental abnormalities (Berkholtz *et al.* 2006). An appropriately rigid ovarian extracellular environment may be a necessary requirement for follicle survival and has been implicated in the induction of conditions such as POI via the loss of factors essential for maintaining stromal thickness, like FOXL2 (Woodruff & Shea 2011). A recent finding in cat ovarian studies has identified a potential link to primordial follicle activation within a group of extracellular matrix enzymes, the matrix metalloproteinases (MMPs) (Fujihara *et al.* 2018). The activity of MMP9 was stimulated *in vitro* via a high dose of retinoic acid, which led to an increase in the number of follicles undergoing primordial follicle activation (Fujihara *et al.* 2018). Indeed, other growth factors involved in primordial follicle activation (including TGFB superfamily members) are capable of binding to ECM components (Smith *et al.* 1999), and thus, the availability of these growth factors may be able to be regulated by the extracellular matrix and ensure the survival of primordial follicles. Recent evidence in the mouse has linked the expression of other TGFB signalling pathway members, the downstream SMAD2/3, to the inhibition of primordial follicle activation by preventing granulosa cell proliferation (Hardy *et al.* 2018). While current laboratory research is focused on dissecting primordial follicle activation pathways, in clinical research, the challenges lie in diagnostics and controlling the growth and maturation of captured primordial follicles.

Future clinical directions towards harnessing the primordial follicle

By understanding the factors controlling primordial follicle recruitment, we aim to provide POI patients with targeted intervention to prolong their reproductive lifespan. The development and validation of markers and tests that will enable practitioners to identify at risk women are crucial, and with new advancements in technology, there is hope for novel diagnostics and treatments in the coming decades. The technique of optical coherence tomography imaging, used commonly in ophthalmology, was utilised to assess accurately (when compared to histological controls) cortical ovarian tissue sections of chemotherapy patients (Wang *et al.* 2015), indicating its future potential as a non-invasive method for primordial follicle assessment.

A technique for the preservation of fertility still regarded as experimental, but used by clinicians nonetheless, particularly in patients undergoing chemotherapeutic treatment or who have a reduced ovarian reserve, is the surgical removal of small fragment(s) of the ovarian cortex, where primordial follicles reside (Oktay *et al.* 1997, Mclaughlin *et al.* 2015). Intact whole ovaries can also be removed and preserved to excise fragments for later use (Gellert *et al.* 2018). Fragments can be cryopreserved until such time that they are transplanted back into the patient, with follicle activation stimulated *in vivo* (post transplantation), and this strategy has now been successful in producing over 130 live births (Demeestere *et al.* 2015, Donnez & Dolmans 2017, Shapira *et al.* 2018). However, the revascularisation of this transplanted cortical tissue remains a limiting factor in treating infertility. Despite these limitations, promising evidence has emerged through the use of engineered endothelial cells expressing AMH. In a mouse model, the co-transplantation of these engineered epithelial cells with cryopreserved tissue has revealed both the promotion of quiescence in primordial follicles and increased perfusion (Man *et al.* 2018). Alternate efforts to promote angiogenesis of transplanted human ovarian tissue using a mouse model has also been achieved via the assistance of adipose tissue-derived stem cells. The adipose-derived stem cells were preseeded onto the grafting site prior to transplantation of human ovarian tissue and were able to differentiate into human blood vessels and support ovarian survival (Manavella *et al.* 2019).

Ovarian cortical tissue fragments may also be used to grow follicles *in vitro* via an 'artificial ovary' – typically a biological matrix of materials like fibrin, collagen and alginate (Telfer & Fauser 2016, Kallen *et al.* 2018). These models are able to elicit patterns of hormonal fluctuations and growth of human follicles to the antral stage in a manner closely resembling those observed *in vivo* (Skory *et al.* 2015). Ovarian cortical tissue fragments can be directly placed within the matrix and cultured to grow mature follicles (Laronda *et al.* 2014). Primordial follicles can also be isolated from the tissue before being placed in the artificial ovary for activation; they may also be activated *in vitro* prior to being placed in the matrix (Mclaughlin *et al.* 2011, Chiti *et al.* 2017). Alternatively, primary or secondary follicles can be removed from the tissue fragment and cultured successfully in a hydrogel matrix, with ovulation observed in mice, and a small number of meiotically competent metaphase II stage oocytes achieved in human follicles after IVM (Skory *et al.* 2015, Xiao *et al.* 2015). While these methods are still in early development, it is hoped that they will maximise the survival and retention of primordial follicles obtained from patients for future *in vitro* maturation and subsequent IVF.

Despite these novel developments, the fact remains that *in vitro* control over the activation of primordial follicles and future developmental competency is yet to be realised in human oocytes, and this is fundamentally linked to our limited understanding of the process of primordial follicle activation. Ovarian cortical tissue culture usually leads to mass spontaneous, uncontrolled primordial follicle activation, and thus future challenges lie in advancing the culture media and 3D support structures to include the necessary inhibitors to allow the timing of activation to occur in an appropriate and controlled manner (reviewed in Bertoldo *et al.* 2018). This mass activation that occurs *in vitro* has recently been tied to disruptions in Hippo signalling caused by cortex fragmentation, specifically by the movement of Hippo pathway effector, yes-associated protein (YAP), into the nucleus of granulosa cells in humans and mice. The translocation of YAP subsequently introduced growth factors and apoptosis inhibitors, which resulted in follicle growth, indicating a positive influence on primordial follicle activation (Grosbois & Demeestere 2018). This activity was subsequently found to be mediated via AKT of the PI3K/AKT/mTOR pathway (Hu *et al.* 2019), thus demonstrating roles for Hippo-yap signalling in regulating primordial follicle activation, and new potential targets for future drug developments *in vitro* fertility preservation.

Conclusion

The committing step of primordial follicle activation and the regulated depletion of the ovarian reserve remain barriers to current attempts to preserve fertility, particularly in cases of POI. Previous studies have focused on dissecting intraovarian pathways involved in the growth and differentiation of the follicle. However, the reliance on animal models has resulted in some limitations, with findings in human studies not always synonymous. While the aetiology of POI is complex and inducible by internal and external factors, future research into controlling the rate of activation may provide strategies for early diagnosis or prevention. The clinical need for solutions to maintain the primordial follicle pool, particularly in cases where girls and young women must undergo chemotherapy, requires a greater focus in human studies, coupled with the development of robust modelling systems such as those discussed in this review. Enhancing the knowledge of primordial follicle activation, and the factors that facilitate the entry to this process will not only improve outcomes for those at risk of premature fertility loss but may provide the key to preventing these conditions altogether.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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