The fibroblast growth factor 8 family in the female reproductive tract

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Abstract

Several growth factor families have been shown to be involved in the function of the female reproductive tract. One subfamily of the fibroblast growth factor (FGF) superfamily, namely the FGF8 subfamily (including FGF17 and FGF18), has become important as Fgf8 has been described as an oocyte-derived factor essential for glycolysis in mouse cumulus cells and aberrant expression of *FGF18* has been described in ovarian and endometrial cancers. In this review, we describe the pattern of expression of these factors in normal ovaries and uteri in rodents, ruminants and humans, as well as the expression of their receptors and intracellular negative feedback regulators. Expression of these molecules in gynaecological cancers is also reviewed. The role of FGF8 and FGF18 in ovarian and uterine function is described, and potential differences between rodents and ruminants have been highlighted especially with respect to FGF18 signalling within the ovarian follicle. Finally, we identify major questions about the reproductive biology of FGFs that remain to be answered, including (1) the physiological concentrations within the ovary and uterus, (2) which cell types within the endometrial stroma and theca layer express FGFs and (3) which receptors are activated by FGF8 subfamily members in reproductive tissues.

Reproduction (2018) **155** R53–R62

Introduction

Among the various growth factor families, the FGF family is one of the largest families with significant expression profiles in the female reproductive tract and with potentially important roles to play in fertility. This family is composed of 18 secreted proteins that are grouped into subfamilies according to sequence homology (Itoh & Ornitz 2004), and members of each subfamily have similar receptor-binding characteristics. There are four tyrosine kinase FGF receptor (FGFR) genes, *FGFR1*, *FGFR2*, *FGFR3* and *FGFR4*, and alternative splicing gives rise to two variants of *FGFR1*, *FGFR2* and *FGFR3* proteins, commonly termed the 'b' and 'c' forms. These variants have markedly different ligand-binding properties (Zhang *et al.* 2006), which confers specificity for certain FGF ligands. In general, the 'b' splice variants are expressed in mesenchymal cells and the 'c' splice forms are expressed in epithelial cells, allowing for precise paracrine signalling between ligand–receptor pairs. The role of mesenchymal–epithelial signalling by FGFs in the ovarian follicle has previously been discussed (Price 2016).

Various FGFs are best known for their role in branching morphogenesis and cell proliferation, and their general biology and pathology have been detailed in several excellent reviews (Beenken & Mohammadi 2009,

Ornitz & Itoh 2015). Mice null for many members of the FGF superfamily die in utero or perinatally, which illustrates the critical role of these growth factors in embryogenesis (Ornitz & Itoh 2015). The impact of certain FGFs in adult tissue function is being appreciated, and they appear to be important for processes involving active tissue proliferation or regeneration, such as tumour development, wound repair, hair growth and ovarian follicle growth. The roles of a number of FGFs in the ovary has been reviewed (Chaves *et al.* 2012). The purpose of this review is to collate the available information about one specific subfamily of FGFs, the FGF8 subfamily and their receptors, in the female reproductive tract, with a focus on normal adult tissues, in order to highlight and discuss gaps in our understanding of these proteins.

The FGF8 subfamily

The FGF8 subfamily is of particular interest for reproductive biology and medicine. Indeed, the prototype ligand, FGF8, was first identified as an androgen-induced growth factor (its original name) secreted from a mammary carcinoma cell line (Tanaka *et al.* 1992). It has since been described in a number of breast and prostate cancer models (reviewed in Mattila & Härkönen 2007) and in ovarian tumours (Valve *et al.* 2000). The other two members of the mammalian FGF8 subfamily are

FGF17 and FGF18, and they have also been detected in prostate and breast cancer, respectively (Heer *et al.* 2004, Polnaszek *et al.* 2004, Mustacchi *et al.* 2013). The FGF8 subfamily is sometimes referred to as a syn-expression group, and although there are examples of where all three are expressed in the same tissue such as endothelial cells (Antoine *et al.* 2005, Chui *et al.* 2014), there are numerous examples where one or two members are not expressed; for example, *Fgf18* mRNA is abundant in adult mouse skin, whereas *Fgf8* and *Fgf17* mRNAs are essentially absent (Kawano *et al.* 2005).

The FGF8 subfamily arose from a common ancestor found in early vertebrates around 550 million years ago (Popovici *et al.* 2005). The amino acid sequences of FGF8, FGF17 and FGF18 are highly conserved across species; there is 98% homology among humans, mice and cattle for FGF8 and FGF18, and between mouse and human for FGF17 although the currently available bovine FGF17 sequence differs somewhat from these two previous species (73% homology). Within species, there is approximately 60% homology between FGF8, FGF17 and FGF18. Fish and lower orders express a fourth member of the FGF8 family, *Fgf24*, but this gene was lost in the tetrapod lineage about 400 million years ago (Jovelin *et al.* 2010). The *FGF17* gene has been lost in frogs, fish and certain orders of birds including the chicken (Abramyan 2015). Owing to the importance of FGF8 and FGF18 in embryogenesis, it is not surprising that global knockout of *Fgf8* and of *Fgf18* causes embryonic or perinatal death in mice; *Fgf17* appears to be less important as null mice survive (although they have impaired brain development) (reviewed in Ornitz & Itoh 2015).

The FGF8 subfamily has very similar receptor-binding properties, as they activate the 'c' splice variants of FGFR1–3 and the non-spliced FGFR4. In addition, FGF8 undergoes alternative splicing to give rise to two main forms of biological relevance, FGF8a and FGF8b (Crossley & Martin 1995). In studies with a BaF3 cell line expressing specific FGFR splice variants, human recombinant FGF8b, FGF17 and FGF18 efficiently activated FGFR3c and FGFR4; the FGF8 subfamily members do not activate FGFR2c and FGFR1c to the same degree (Table 1) (Zhang *et al.* 2006). FGF8a appears to have only weak binding affinity to FGFRs (MacArthur *et al.* 1995a, Olsen *et al.* 2006).

Table 1 Relative receptor activating abilities of FGF8 subfamilies expressed relative to FGF1.

	FGFR1c	FGFR2c	FGFR3c	FGFR4
FGF8b	+++	+++	++++	++++
FGF17	+	+	++++	+++
FGF18	–	+	+++	++

Data derived from Zhang *et al.* (2006).

++++, more active than FGF1; +++, 51–100% activity of FGF1;

++, 30–50% activity of FGF1; +, 5–29% activity of FGF1;

–, <5% activity of FGF1.

Upon ligand binding, the intracellular tyrosine kinase domains of the receptors are phosphorylated and lead to activation of several intracellular signalling pathways including mitogen-activated protein kinase (MAPK), phospholipase C/protein kinase C, PI3K-AKT and signal transducer and activator of transcription (STAT), as has been extensively reviewed elsewhere (Ornitz & Itoh 2015). These typical pathways have been described in the reproductive system and various FGFs have been shown to stimulate MAPK, AKT and PKC pathways in granulosa cells (Peluso *et al.* 2001, Jiang *et al.* 2011).

These pathways converge in the nucleus to induce the expression of transcription factors including FOS and members of the NR4A, ETS and EGR families (Kwong *et al.* 2001, Lammi & Aarnisalo 2008, Jiang *et al.* 2013). Other FGF-response genes include negative feedback regulators of RTK activity, including members of the Sprouty (SPRY) family and interleukin 17 receptor D (IL17RD; also known as 'similar expression to FGF' SEF). These proteins act at different points along the MAPK signalling pathway (Ornitz & Itoh 2015).

Expression of FGF8 family in the ovary

Ligands

In the adult mouse, mRNA encoding *Fgf8* was initially detected only in the testis and ovary by Northern blot (Lorenzi *et al.* 1995, MacArthur *et al.* 1995b), but has subsequently been detected in human prostate, kidney, heart and lung by PCR (Ghosh *et al.* 1996, Schmitt *et al.* 1996), in human and mouse cerebral cortex, human skin and intestine by immunohistochemistry (IHC) (Tanaka *et al.* 2001, Zammit *et al.* 2002) and in blood vessels by PCR (Antoine *et al.* 2005). Within the ovary, *Fgf8* mRNA was localised to the oocyte in mice by *in situ* hybridization (Valve *et al.* 1997) and PCR (Zhong *et al.* 2006), whereas mRNA levels appear low in oocytes, granulosa and theca cells in cattle (Buratini *et al.* 2005) and undetectable in normal human ovary (Valve *et al.* 2000). FGF8 protein has also been detected in human corpus luteum by IHC (Zammit *et al.* 2002).

Messenger RNA encoding FGF17 was detected in pooled mouse oocytes and in bovine oocytes by PCR (Zhong *et al.* 2006, Machado *et al.* 2009), although this gene appears to be weakly expressed: microarray data suggest that *Fgf17* mRNA abundance is close to background levels in mice (Zhong *et al.* 2006) and TaqMan probes failed to detect *FGF17* mRNA in single bovine oocytes (Ferreira *et al.* 2016). *FGF17* mRNA was also detected in bovine theca and granulosa cells but at levels lower than those seen in oocytes, and FGF17 protein was detected in oocytes and granulosa cells (Machado *et al.* 2009). Owing to the low level of expression of this gene in the ovary, the physiological relevance of this growth factor is unclear, and it may be dispensable in mammals as it is in birds.

Fgf18 mRNA was reported as one of the more highly expressed FGFs in mouse oocytes (Zhong *et al.* 2006) but curiously it was not detected in bovine oocytes; instead, *FGF18* mRNA was detected primarily in theca cells (Portela *et al.* 2010). FGF18 protein was detected by IHC in bovine theca, granulosa and luteal cells (Portela *et al.* 2010). A major caveat to IHC and immunoblot studies is the potential for antibodies to cross-react with other FGF8 subfamily members; unfortunately, such cross-reactivity data are not always provided by manufacturers and reactivity with species such as cattle is often predicted.

Thus, the available data suggest species differences in the pattern of expression of FGF8 subfamily members; there is convincing evidence for *Fgf8b* expression in mice but not so for cattle and humans and that FGF18 is an oocyte-derived factor in mice but of thecal origin in cattle. Studies of other species are required to understand better the diversity of expression patterns within the ovary.

Receptors

All four FGF receptor mRNAs have been detected in the ovary. Early studies using Northern blotting and *in situ* hybridization detected *Fgfr1* and *Fgfr2* mRNAs in rat theca and granulosa cells, and *Fgfr1* in the corpus luteum, whereas *Fgfr3* and *Fgfr4* mRNAs were not detected (Asakai *et al.* 1994). *Fgfr3* mRNA was also not detected by *in situ* hybridization in the mouse ovary (Puscheck *et al.* 1997), which suggests generally lower abundance of FGFR3 compared to FGFR1 and FGFR2. More recent PCR experiments demonstrated the presence of *FGFR3* mRNA in rat, mouse and human ovaries (Ben-Haroush *et al.* 2005, Drummond *et al.* 2007, Furukawa *et al.* 2014). *Fgfr2* protein was detected in theca, granulosa and luteal cells as well as in oocytes in rats, whereas *Fgfr3* protein was localized to the nucleus of granulosa cells (Drummond *et al.* 2007). FGFR1 and FGFR2 mRNAs and protein were detected in the parenchyma and vasculature of the sheep corpus luteum (Doraiswamy *et al.* 1998).

In mice, *Fgfr4* mRNA was detected in granulosa cells by *in situ* hybridization (Puscheck *et al.* 1997) and in human ovarian biopsy samples by PCR (Valve *et al.* 2000, Ben-Haroush *et al.* 2005). *FGFR4* mRNA was also detected in bovine theca cells and in buffalo granulosa and theca cells by PCR (Buratini *et al.* 2005, Mishra *et al.* 2016).

Splice variant-specific PCR demonstrated the presence of *FGFR1c*, *FGFR2c* and *FGFR3c* mRNAs in granulosa and theca cells of cattle (Berisha *et al.* 2004, Buratini *et al.* 2005, Mishra *et al.* 2016) and of *FGFR1c* and *FGFR2c* mRNAs in pig granulosa and theca cells (Schams *et al.* 2009, Evans *et al.* 2014, Furukawa *et al.* 2014). In human ovarian samples, *FGFR1c* and *FGFR2c* mRNAs were detected but *FGFR3c* mRNA was not

(Valve *et al.* 2000), whereas all 'c' splice forms and *FGFR4* mRNA was detected in commercially available human ovarian RNA (Cole *et al.* 2010). The abundance of FGFR mRNAs change with follicle development, for example, the abundance of *FGFR2c* mRNA in granulosa cells is highest in large compared to small follicles in pigs and buffalo (Evans *et al.* 2014, Mishra *et al.* 2016). A similar pattern has been described in theca cells in pigs (Schams *et al.* 2009), whereas no difference was noted in cattle (Berisha *et al.* 2004). Granulosa cell *FGFR3c* and *FGFR4* mRNA levels were highest in large follicles in cattle (Buratini *et al.* 2005) and buffalo (Mishra *et al.* 2016), respectively, compared with smaller follicles.

The previously mentioned data should be viewed with caution, as PCR studies of receptors and their splice variants give little indication of relative abundance of active receptor proteins on the cell surface. Unfortunately, some reports do not provide Cq values of target amplicons and some persist in measuring non-splice variant-specific targets. Work in this area is also hampered by the lack of splice-variant-specific antibodies.

Regulators

Of the Sprouty proteins, *SPRY2* mRNA was first detected in bovine granulosa cells (Robert *et al.* 2001) and subsequently detected in human granulosa-lutein cells and mouse cumulus cells (Haimov-Kochman *et al.* 2005, Sugiura *et al.* 2009). Haimov-Kochman and coworkers localized *SPRY2* protein to human granulosa-lutein cells by IHC but not to theca-lutein cells and to the stroma and granulosa cells of hCG-treated rats (Haimov-Kochman *et al.* 2005). Abundance of *SPRY1*, *SPRY2* and *SPRY4* mRNAs is increased by FGFs in bovine granulosa cells, including by FGF8 (Jiang *et al.* 2013); FGF18 appeared unable to stimulate *SPRY* mRNA levels. Messenger RNA encoding *SPRY3* has been detected in bovine granulosa cells but is not stimulated by FGFs (Jiang *et al.* 2011, 2013).

IL17RD protein and mRNA has been detected in mouse and human follicles, where they are found mostly in cumulus/granulosa cells and the oocyte but absent in luteal cells. Interleukin 17 receptor D proteins were not detected in theca cells of mice, but were detected in theca cells of human ovaries (Lutwak *et al.* 2014); the strength and importance of this apparent species difference remains to be determined.

Role of FGF8 subfamily in the ovary

The role of FGF8 as an oocyte-somatic cell signalling molecule was suggested by studies in mice demonstrating that two oocyte-derived proteins, FGF8b and BMP15, synergize to promote glycolysis in cumulus cells (Sugiura *et al.* 2007). Addition of FGF8b to rat granulosa-oocyte cocultures inhibited FSH-induced

oestradiol secretion and increased MAPK3/1 and MAPK8 phosphorylation (Miyoshi *et al.* 2010). Similarly, FGF8b increased MAPK3/1 phosphorylation in bovine granulosa cells (Jiang *et al.* 2013).

Although little is known about the regulation of FGF8 expression or secretion from oocytes, FSH has been shown to increase *Fgf8* mRNA levels from oocytes of cultured mouse follicles (Sánchez *et al.* 2010), whereas eCG priming *in vivo* decreased *Fgf8* mRNA abundance (Sánchez *et al.* 2011). Kit ligand (Kitl) stimulated oocyte *Fgf8* mRNA levels in rats (Miyoshi *et al.* 2012) and FGF8 was shown to increase *KITL* mRNA abundance in bovine cumulus cells (Lima *et al.* 2016), suggesting the presence of a feedforward loop between Fgf8 and Kitl. Interestingly, a G→C mutation in the bovine FGF8 gene has been associated with a reduction in the number of viable oocytes collected during ultrasound-guided ovum pick up in Nelore (*Bos indicus*) cows (Santos-Biase *et al.* 2012).

The effects of FGF17 and FGF18 on bovine granulosa cells have been reported, and both inhibit steroidogenesis (Machado *et al.* 2009, Portela *et al.* 2010). Addition of FGF17 to *in vitro* fertilization (IVF) medium enhanced cumulus expansion in cattle, and in combination with BMP15 increased the number of cells in the blastocyst inner cell mass (Machado *et al.* 2015). An atypical action of FGF18 was described, in that this ligand increased the proportion of apoptotic granulosa cells *in vitro*, possibly through a mechanism involving the intracellular death ligand BBC3 (also known as PUMA), and injection of FGF18 directly into a growing follicle *in vivo* resulted in atresia (Portela *et al.* 2010, 2015). While the potential signalling between oocytes and cumulus cells involving FGF8 may be common to rodents and ruminants, the involvement of theca-derived FGFs in rodents remains to be explored (Fig. 1).

Expression and role of FGF8 subfamily in the uterus

Within the uterus, FGF8 protein was detected in human endometrial glands and myometrium by IHC, although the endometrial stroma was negative, and in the oviductal epithelium (Zammit *et al.* 2002). *FGF18* mRNA was also detected in human endometrium (Yerlikaya *et al.* 2016) and in the mouse uterine stroma (Li *et al.* 2011).

Limited information is available to date about FGFR expression in the human uterus, although all FGFRs were detected in the placenta but not in the maternal decidua (Anteby *et al.* 2005). FGFR2 protein was subsequently detected predominantly in the human endometrial epithelium of the secretory phase and in trace amounts in the proliferative endometrium (Gatius *et al.* 2011) and FGFR1 and FGFR2 proteins were localized to the uterine epithelium in mice (Li *et al.* 2011). In pigs, the endometrium expresses *FGFR1c* and *FGFR2c* mRNAs, the latter in greater concentrations than the former, and

Figure 1 Model for the action of FGF8 subfamily members in the ovarian follicle. In both rodents and ruminants, FGF8 is expressed by (at least) the oocyte (Oo) and has been shown to stimulate *KITL* mRNA levels in bovine cumulus cells, and *Kitl* stimulated *Fgf8* mRNA in rodents; a feedforward loop likely exists between these two factors. Cumulus cell glycolysis is stimulated by Fgf8 and Bmp15 in mice, which has not yet been demonstrated in other species. In cattle, FGF18 is predominantly expressed by the theca cell layer and increases apoptosis in granulosa cells (GC). It is not clear whether FGF18 is secreted from thecal steroidogenic cells (S), fibroblasts (F) or endothelial cells (E), or whether *Fgf18* is expressed in rodent theca cells. Size of growth factor letters indicates approximate level of abundance.

FGFR2c mRNA levels were decreased by progesterone or oestradiol administration (Welter *et al.* 2004). Several FGFs are expressed by the ruminant endometrium, as well as *FGFR2c* (Okumu *et al.* 2014), although most studies have focused on FGF10 that signals to the conceptus, which expresses FGFRs (Chen *et al.* 2000, Ocón-Grove *et al.* 2008). Progesterone stimulated endometrial *FGF10* expression in sheep but not in cattle (Satterfield *et al.* 2008, Okumu *et al.* 2014); to date, no data are available on endometrial FGF8 subfamily members in ruminants.

FGF signalling has been implicated in the control of uterine epithelial proliferation, as pharmacological inhibition of FGFR activity decreases proliferative activity in mice (Nallasamy *et al.* 2012). In order for implantation to occur, epithelial proliferation ceases under the control of a progesterone-induced basic helix-loop-helix transcription factor, Hand2, and loss of Hand2 increased the expression of several FGFs in the mouse uterus including *Fgf18* (Li *et al.* 2011). Another transcription factor, *Msx1*, is also critical for implantation

Figure 2 Current (mis)understanding of FGF8 family signalling in the uterus. Evidence suggests that in mice Fgf18 is secreted by the endometrial stroma (ES) and activates 'c' splice variants of Fgfr on the epithelium (EE) to promote proliferation and that this pathway is suppressed by progesterone to allow implantation. In ruminants (and other species), although it is believed that FGF10 activates the 'b' splice variants on the epithelium and the conceptus to facilitate implantation (grey text and lines), it is not yet known if FGF8 family members activate epithelial 'c' receptors to regulate proliferation. Myo, myometrium.

in mice, and loss of *Msx1* increased the expression of *Fgf18* and *Fgf10* among others (Nallasamy *et al.* 2012). Thus, in mice, the data suggest that progesterone signalling, through *Hand2* and potentially other factors such as *Msx1*, inhibits FGF expression in the uterine stroma leading to a reduction in FGF signalling to the uterine epithelium, which in turn reduces proliferation and allows the endometrium to become receptive to implantation. A hypothetical model of FGF8 subfamily signalling in rodents and ruminants is given in Fig. 2.

The negative RTK regulator *SPRY2* has been detected in human endometrial glands by IHC, and its abundance is higher in the secretory compared with the proliferative endometrium (Velasco *et al.* 2011). In humans, *SPRY4* and *IL17RD* proteins have been located to the endometrial epithelium without cyclical changes (Guo *et al.* 2014).

Gynaecological cancers

FGF8 mRNA and protein were detected in ovarian tumours and cancer cell lines by PCR and IHC (Valve *et al.* 2000). Levels of FGF18 protein have been proposed as a marker for poor prognosis of ovarian tumours (Wei *et al.* 2013, El-Gendi *et al.* 2016), and serum FGF18 protein concentrations were higher in postmenopausal ovarian cancer patients compared with healthy postmenopausal controls (Vathipadiekal *et al.* 2015). Moreover, a polymorphism in the 5' flanking region of *FGF18* was associated with improved response to platinum-based chemotherapy (Meng *et al.* 2013).

In endometrial cancer, abundance of *FGF18* mRNA was elevated and *HAND2* mRNA levels were decreased in endometrial adenocarcinomas compared to normal human proliferative endometria (Flannery *et al.* 2016). There is little evidence to date to suggest aberrant expression of FGF8 or FGF17 in endometrial cancers.

Alterations in FGFR gene structure and signalling have been associated with gynaecological cancers and have been reviewed elsewhere (Fearon *et al.* 2013); attention will be paid here only to events involving the FGF8 subfamily. Endometrial carcinoma has been associated with mutations that occur in FGFR2 (Pollock *et al.* 2007, Gatus *et al.* 2011), and a particularly prevalent mutation, Ser252Trp, alters ligand specificity of the receptor such that mutated FGFR2c is activated by FGFs that normally activate only 'b' splice variants (FGF7 and FGF10). Another study demonstrated that mutated FGFR2b is activated by ligands that normally activate the 'c' splice variants (Yu *et al.* 2000); therefore, it can be expected that FGF8 subfamily members may be able to activate the mutated FGFR2b, although this has not been tested.

Some ovarian cancers are believed to arise from the surface epithelium, which expresses the epithelial 'b' splice variants of the FGFRs (Steele *et al.* 2001), and these splice forms of FGFR2 and FGFR3 are the predominant forms in ovarian cancer, along with FGFR1c (Cole *et al.* 2010). Ovarian cancer likely also arises from the oviduct (Kim *et al.* 2012), and limited information available suggests that the 'c' splice forms are expressed in the oviductal epithelium, at least in pigs (Wollenhaupt *et al.* 2004). Although FGFR2 mutations are considered rare in ovarian cancer, the Ser252Trp mutation seen in endometrial cancer has also been detected in the ovary (Byron *et al.* 2010), suggesting a loss of ligand specificity for FGF signalling in at least some ovarian cancers.

High levels of FGFR4 protein have been reported in serous ovarian carcinomas and were associated with poor patient survival (Zaid *et al.* 2013), although the ligand involved was suggested to be FGF19 (Hu & Cong 2015). On the other hand, a Gly388Arg mutation in FGFR4 has been reported in ovarian cancer and was associated with increased patient survival (Marmé *et al.* 2012).

In concert with the increase in FGF ligand expression in cancer, there is a decrease in expression of negative feedback regulators of RTK activity. Indeed, *SPRY1*, *SPRY2*, *SPRY4* and *IL17RD* mRNA levels were reported to be generally lower in ovarian tumours compared with normal human ovarian tissue (Zisman-Rozen *et al.* 2007, Masoumi-Moghaddam *et al.* 2015a,b). It seems likely that if FGF8 and FGF18 are major contributors to aberrant FGFR activity in cancer, the loss of endogenous negative feedback to the MAPK signalling cascade could exacerbate the oncogenic effects of FGFs within the female reproductive tract. It is perplexing that FGF18 is associated with tumour growth but is proapoptotic in non-cancerous granulosa cells. This may be because of altered receptor specificity or aberrant expression of co-factors or downstream regulators/pathways in tumours, and the causal relationship between FGF18 synthesis and tumour development has not been determined.

FGF8 signalling and microRNAs

MicroRNAs have emerged as regulators of gene expression and cell signalling, and although specific examples of FGF-microRNA interactions in the reproductive system have not been reported, interactions have been reported during morphogenesis of other organ systems. As an example, miR-130 and miR-133 have been shown to inhibit *Fgf8* mRNA levels in the embryonic chick heart (Lopez-Sanchez *et al.* 2015a,b), potentially by altering signalling through Fgfr1. In zebrafish, miR-9 inhibits FGF signalling in the developing brain and does so by binding directly to the 3'UTR of *Fgf8* mRNA (Leucht *et al.* 2008). In human endothelial cells, hsa-miR-505 inhibited FGF18 mRNA and protein levels and inhibited the activity of a luciferase reporter containing the 3'UTR of *FGF18* (Yang *et al.* 2014) suggesting that this microRNA interacts directly with *FGF18* mRNA. Another miRNA that interacts with the 3'UTR of *FGF18* is miR-195 (Wang *et al.* 2017). Whether these miRNAs are involved in reproductive physiology or medicine is just being explored, although miR-505 abundance was found to be downregulated in endometrial carcinoma (Chen *et al.* 2016), a tissue in which increased *FGF18* mRNA levels have been observed (see section on 'Gynaecological cancers').

Questions and challenges

There are many unresolved questions about the physiological role of FGFs in the reproductive as well as other organ systems. One fundamental question concerns the physiologically relevant concentrations of FGFs in biological fluids and tissues. Unlike the FGF19 subfamily, which are endocrine factors and secreted into the bloodstream, many other FGFs are paracrine factors and remain closely associated with

the extracellular matrix. Most is known about FGF2, which is exported to the cell surface and is not readily released into culture medium (Trudel *et al.* 2000); nevertheless, concentrations of FGF2 in human serum and follicular fluid (FF) have been reported to be 5–10 and 100–150 pg/mL, respectively (Hammadeh *et al.* 2003). Although some studies have reported biological effects of FGF2 on granulosa cells at 100 pg/mL (Vernon & Spicer 1994), most studies use doses of ≥ 1000 pg/mL (for example, Jiang *et al.* 2011), therefore determining the peri-cellular concentrations is important to establish whether experimental doses of FGF2 are physiologically relevant. The same reasoning applies to FGF18, for which the plasma FGF18 concentrations in healthy humans are around 160 pg/mL (Dr Michael Grusch, Medical University of Vienna, personal communication). If the plasma:FF ratio of 1:10 observed for FGF2 is valid also for FGF18, one can predict levels of 1.6 ng/mL in FF, which are 5-fold lower than concentrations used in most studies on the effects of FGF18.

Precise cell localization in non-epithelial cell compartments remains to be clarified. Endometrial stroma and the follicular theca layer contain many mesenchymal cell types and the contribution of each to FGF production is not clear. Specifically, FGF18 is possibly produced by fibroblasts or endothelial cells within the endometrial stroma as it has been detected in these cell types of other tissues (Kapoun *et al.* 2004, Antoine *et al.* 2005). This may also hold for the theca cell layer, as expression has not, to our knowledge, been demonstrated in steroidogenic cells. It should also be noted that in human umbilical vein endothelial cell lines the expression of FGF18 is regulated by hsa-miR-505, as is the expression of high-mobility group box 1 (HMGB1) (Yang *et al.* 2014), and HMGB1 has been implicated in preeclampsia (Nadeau-Vallée *et al.* 2016). As FGF18 administration caused dilatation in the rat cerebral vasculature (Ellsworth *et al.* 2003), it would be worth investigating if FGF18 plays a role in preeclampsia.

A further critical question that remains largely unanswered concerns receptor presence and specificity. The landmark papers that identified which receptors are activated by specific ligands was performed with a mouse pre-B cell line (BaF3) transfected with individual FGFRs (Ornitz *et al.* 1996, Zhang *et al.* 2006), but few attempts have been made to confirm that the same pattern of receptor activation occurs in primary cells. Measuring receptor abundance by PCR is also problematic, as mRNA abundance does not always reflect levels of active protein. As an example, human neutrophils express *FGFR1*, *FGFR2* and *FGFR4* mRNA but only FGFR2 protein exists as a transmembrane receptor – FGFR1 and FGFR4 proteins were localized to an intracellular compartment by confocal immunofluorescence (Haddad *et al.* 2011). In rat granulosa cells, Fgfr2 protein was localised to the cytoplasm by IHC, and therefore, potentially at the cell surface, whereas Fgfr3 protein was restricted to the

nucleus (Drummond *et al.* 2007); whether this reflects receptors that have been internalized after activation or a non-functional form of receptor is unknown, but it should be remembered that Fgfr3 is not an abundant receptor in the ovary (see above). By using specific receptor-blocking antibodies, Fortin and coworkers demonstrated with rat oligodendrocytes that FGF8 does not activate FGFR1 (Fortin *et al.* 2005), whereas it does in BaF3 cells (Zhang *et al.* 2006). Thus, the extent to which a given FGF activates specific receptors may be dependent on cell context (cell type, species, stage of differentiation).

Finally, the question of potential species differences should be addressed. Minor differences in receptor splice variant mRNA levels may not be physiologically relevant but some other differences stand out. These include the robust expression of Fgf8b exclusively in mouse oocytes but weaker expression throughout the follicle in cattle, the localization of *Fgf18* mRNA to the oocyte in mice but robust expression of *FGF18* in theca cells of cattle and the expression of *Il17rd* mRNA in granulosa cells and oocytes of mice but in theca cells in humans. These patterns must be verified in a greater range of species before any conclusions can be drawn, but the glaring difference in ovulation rate between mice and humans/cattle suggest that FGFs may play a role in determining the number of follicles that develop to the preovulatory stage.

Declaration of interest

C A P is an Editor-in-Chief of Reproduction.

Funding

Work from the authors' laboratory included in this review has been funded by the Natural Sciences and Engineering Research Council of Canada (NSERC).

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Received 31 August 2017

First decision 28 September 2017

Revised manuscript received 12 October 2017

Accepted 6 November 2017