

# Mechanisms of fibroblast growth factor signaling in the ovarian follicle

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## Abstract

Fibroblast growth factors (FGFs) have been shown to alter growth and differentiation of reproductive tissues in a variety of species. Within the female reproductive tract, the effects of FGFs have been focused on the ovary, and the most studied one is FGF2, which stimulates granulosa cell proliferation and decreases differentiation (decreased steroidogenesis). Other FGFs have also been implicated in ovarian function, and this review summarizes the effects of members of two subfamilies on ovarian function; the FGF7 subfamily that also contains FGF10, and the FGF8 subfamily that also contains FGF18. There are data to suggest that FGF8 and FGF18 have distinct actions on granulosa cells, despite their apparent similar receptor binding properties. Studies of non-reproductive developmental biology also indicate that FGF8 is distinct from FGF18, and that FGF7 is also distinct from FGF10 despite similar receptor binding properties. In this review, the potential mechanisms of differential action of FGF7/FGF10 and FGF8/FGF18 during organogenesis will be reviewed and placed in the context of follicle development. A model is proposed in which FGF8 and FGF18 differentially activate receptors depending on the properties of the extracellular matrix in the follicle.

## Key Words

- ▶ reproduction
- ▶ ovary
- ▶ granulosa cell
- ▶ growth factor

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## Introduction

Development of the ovarian follicle from preantral to preovulatory stages is highly complex and involves multiple endocrine and paracrine signaling pathways. It is well known that the pituitary gonadotrophins are the main endocrine drivers of many stages of follicle development; however, it is becoming increasingly evident that several families of growth factors also play important roles within the follicle, including the insulin-like growth factor and transforming growth factor beta families (Knight & Glister 2006, Sudo *et al.* 2007). A further growth factor family with potential paracrine actions contains the fibroblast growth factors (FGFs). In mammals, this family consists of 18 secreted proteins (and four intracellular proteins called FGF homologous factors) that are grouped into subfamilies based on sequence homology (Itoh &

Ornitz 2004). It has been recognized for over 20 years that FGF2 acts on granulosa cells to promote cell proliferation and decrease apoptosis and steroidogenesis (Gospodarowicz & Bialecki 1979, Baird & Hsueh 1986, Lavranos *et al.* 1994, Vernon & Spicer 1994), and over the last decade several other FGFs have been implicated in ovarian function and follicle development, as recently reviewed (Chaves *et al.* 2012).

The expression of some FGF family members in the ovary is tissue specific and others are widely expressed; for example, FGF7 is expressed in theca cells but not granulosa cells or oocytes (Parrott *et al.* 1994), whereas FGFR2 is readily detected in granulosa, cumulus and theca cells, and the oocyte (see Table 1 for a summary of the known expression patterns of FGF and FGFR genes).

**Table 1** Localization of FGF and FGFR mRNA in antral ovarian follicles. Where a cell type is not listed, the relevant gene is not expressed

Gene	Cell type	Species	References <sup>a</sup>
FGF1	Theca > granulosa	Cattle	Berisha <i>et al.</i> (2004)
FGF2	Theca	Rat	Koos & Olson (1989)
FGF7	Theca	Cattle	Parrott <i>et al.</i> (1994)
FGF8	Oocyte	Mouse	Valve <i>et al.</i> (1997)
FGF9	Oocyte, theca, granulosa	Cattle	Buratini <i>et al.</i> (2005)
	Theca	Rat	Drummond <i>et al.</i> (2007)
FGF10	Granulosa	Cattle	Grado-Ahuir <i>et al.</i> (2011)
	Theca, oocyte	Cattle	Buratini <i>et al.</i> (2007)
FGF17	Oocytes >> granulosa, theca	Cattle	Machado <i>et al.</i> (2009)
FGF18	Theca	Cattle	Portela <i>et al.</i> (2010)
FGFR1b	Cumulus, oocyte	Cattle	Zhang & Ealy (2012)
FGFR1c	Cumulus, oocyte	Cattle	Zhang & Ealy (2012)
FGFR2b	Granulosa, cumulus, oocyte	Cattle	Berisha <i>et al.</i> (2004) and Zhang & Ealy (2012)
FGFR2c	Theca, granulosa, cumulus, oocyte	Cattle	Berisha <i>et al.</i> (2004) and Zhang & Ealy (2012)
FGFR3c	Theca, granulosa, cumulus	Cattle	Buratini <i>et al.</i> (2005)
FGFR4	Granulosa	Mouse	Puscheck <i>et al.</i> (1997)
	Theca	Cattle	Buratini <i>et al.</i> (2005)

<sup>a</sup>References are given for the first major report of expression pattern of each gene in the species indicated. Multiple species are given only where localization differs.

A restricted pattern of expression of a ligand raises the possibility of targeted signaling to neighboring cells, a classic example of which is mesenchymal to epithelial cell signaling by FGFs during embryo development. In the context of the ovarian follicle, theca cells are mesenchymal and granulosa cells are epithelial, therefore paracrine FGFs may play a role in follicle development. The purpose of this review is to summarize recent information on the potential role in the follicle of members of two FGF subfamilies involved in mesenchymal to epithelial cell signaling, the FGF7 and FGF8 families. Studies with reproductive and non-reproductive tissues have suggested that members of these subfamilies have divergent actions on their target tissues, and potential mechanisms for the actions of FGF7 and FGF8 family members in the ovary will be discussed.

### FGFs and mesenchymal-epithelial cell signaling

The structure and general function of FGFs has been well reviewed (Beenken & Mohammadi 2009, Ornitz & Itoh 2015). These ligands are grouped into subfamilies based on sequence homology and phylogeny (Itoh & Ornitz 2004), and members of the same subfamily have similar receptor binding properties. Most FGFs are considered to be paracrine factors although the FGF19 subfamily (FGF19, FGF21, and FGF23) are endocrine factors with roles to play in cholesterol, vitamin D, and phosphate homeostasis

(see Beenken & Mohammadi (2009) for review). Most FGF proteins possess signal peptides and are secreted through the conventional endoplasmic reticulum – Golgi pathway, although FGF1 and FGF2 are secreted through an unconventional pathway involving translocation through the cell membrane and binding to cell surface proteoglycans (Steringer *et al.* 2015). The association of paracrine FGFs to the extracellular matrix is of relevance here and will be discussed in more detail below.

The ligands are well conserved across mammalian species. It is worth noting at this point that FGF7, FGF10, FGF8, and FGF18 proteins are 95, 91, 98, and 100% homologous respectively between mice and cattle, the two species most commonly used in the studies described below.

The FGFs receptors (FGFR) are tyrosine kinase receptors that are derived from four main genes, *FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4*. Alternative splicing events result in two variants of FGFR1, FGFR2, and FGFR3 proteins commonly termed the 'b' and 'c' forms, and these variants have markedly different ligand binding properties. For example, members of the FGF7 family (FGF3, FGF7, FGF10, and FGF22) activate the 'b' splice forms of FGFR1 and FGFR2 but not the 'c' splice variants, whereas many other FGFs activate the 'c' splice variants to different degrees (Zhang *et al.* 2006). For a detailed discussion of the molecular basis for ligand specificity see (Belov & Mohammadi 2013). In general, 'b' splice variants of FGFR proteins are expressed in epithelial tissues

and 'c' splice variants are expressed in mesenchymal cells (Orr-Urtreger *et al.* 1993), which sets the scene for potential paracrine signaling between these two cell types.

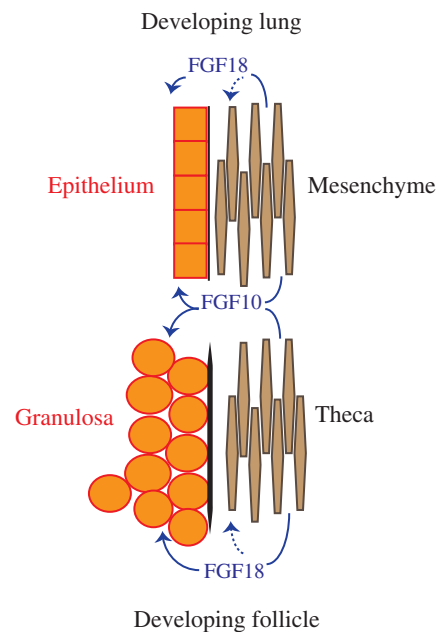
It has been recognized for some time that the biological activity of FGFs, as well as other growth factors, is dependent on interactions with the ECM (Kim *et al.* 2011). In order to activate their receptors, the paracrine FGFs need to bind to heparan sulfate (HS), which is a linear sulfated polysaccharide present within the ECM and on cell surfaces where it is linked to soluble (e.g. perlecan) or cell membrane bound proteins (e.g. syndecan, glypican) collectively known as HS proteoglycans (HSPG). Both FGF and FGFR bind to HS, which increases receptor binding affinity and stabilizes the FGF-FGFR complex (see Ornitz & Itoh (2015) for review). The paracrine FGFs are inactive when applied to HSPG deficient cell lines (Yayon *et al.* 1991, Spivak-Kroizman *et al.* 1994, Loo & Salmivirta 2002), and addition of heparin or HS restores biological activity. Mice null for key enzymes involved in elongation of HS chains fail to respond to FGF signaling (Shimokawa *et al.* 2011).

Mesenchymal-epithelial signaling by FGFs is most evident in organ development. In the developing limb, FGF10 secreted from the mesenchyme activates FGFR2b in the apical ectodermal ridge, which is a critical early step in formation of the limb bud. In the developing mouse lung, mesenchymal FGF10 activation of epithelial FGFR2b is essential for airway branching (Colvin *et al.* 2001), and mesenchymal FGF18 is essential for alveolar development, acting through epithelial FGFR2c (Usui *et al.* 2004). The pivotal early role of FGF10 signaling was demonstrated in FGFR2b knockout mice, which die at birth with severe defects of the limbs and lungs as well as salivary glands and other tissues (De Moerloose *et al.* 2000). Knockout of FGF10 or FGF18 in mice is also lethal and results in severe abnormalities in both skeletal and lung development (Itoh & Ornitz 2004).

An additional layer of fine control of organ development is exerted by important differences in biological activity of members of the same FGF subfamily. For example, FGF7 induces branching of epithelial limb buds whereas FGF10 induces bud elongation (Bellusci *et al.* 1997, Koyama *et al.* 2008, Makarenkova *et al.* 2009), and FGF18 stimulates expansion in the embryonic midbrain whereas FGF8 stimulates differentiation of the midbrain into cerebellum (Liu *et al.* 2003, Sato & Nakamura 2004). These differences will be explored further later in this review.

Obviously, lungs and limbs develop once in mammals, whereas the post-natal ovary is a site of constant

development of follicles from the resting pool of primordial follicles. These follicles consist of an immature oocyte surrounded by a single layer of squamous epithelial 'pre-granulosa' cells. To develop into growing follicles, the squamous pre-granulosa cells develop into cuboidal granulosa cells and proliferate, and the follicle acquires a layer of mesenchymal theca cells. As the antrum forms, cells of both epithelial and mesenchymal layers proliferate, and this is regulated in part by mesenchymal-epithelial communications (Knight & Glister 2006). Interestingly, certain features of FGF7 and FGF8 subfamily signaling in the follicle resemble those occurring during lung and limb development (Fig. 1), and while development of the preantral follicle would logically be most similar to development of the lung and limbs, there are very few studies of these FGFs in preantral follicles; mRNA and protein for both FGF7 and FGF10 have been reported in oocytes and granulosa cells of preantral follicles and in fetal ovaries in humans and cattle (Buratini *et al.* 2007, Abir *et al.* 2009, Oron *et al.* 2012, Castilho *et al.* 2014), and while *FGFR3c* mRNA was detected in secondary bovine



**Figure 1**

FGF10 and FGF18 signaling in the developing follicle parallels FGF signaling in embryonic tubular organs. In both structures, the mesenchyme secretes FGF10 which acts on FGFR2b expressed exclusively in epithelial cells. The mesenchyme also secretes FGF18 which acts on FGFR2c/FGFR3c/FGFR4 in epithelial cells (solid line) and may also be able to activate the same receptors in mesenchymal cells (dotted line), although definitive evidence is lacking.

follicles (Buratini *et al.* 2005), its cellular location has not been described.

There is much more information about the role of FGFs in mesenchymal-epithelial signaling in the antral follicle. Consistent with the pattern observed in organogenesis, *FGF10* is expressed predominantly in theca (mesenchymal) cells (Buratini *et al.* 2007) and *FGFR2b* mRNA is detected predominantly in granulosa (epithelial) cells (Berisha *et al.* 2004). Messenger RNA encoding FGF18 is also detected in theca cells (Portela *et al.* 2010) although expression of *FGFR2c* and *FGFR3c* is detectable in both granulosa and theca cells (Berisha *et al.* 2004, Buratini *et al.* 2005). This is not inconsistent with the developmental change of expression of *FGFR2c* in the embryonic mouse, for which mRNA is detected only in the epithelium of very early lung and limb buds (Oldridge *et al.* 1999, Usui *et al.* 2004) and becomes detectable also in the mesenchyme after embryonic day 15 (Usui *et al.* 2004). Whether a similar shift in the pattern of expression of *FGFR2c* and *FGFR3c* occurs during development of the ovary or development of preantral follicles is not known.

### The role of the FGF7 family in follicle development

Mesenchymal-epithelial signaling in the antral follicle was nicely demonstrated by Parrott *et al.* (1994) who first demonstrated that FGF7 is expressed in bovine theca but not in granulosa cells, and that it affects granulosa and not theca cells. These studies demonstrated the absence of functional *FGFR2b* in theca cells, which was later supported by PCR data (Berisha *et al.* 2004). Incubation of bovine or rat granulosa cells with FGF7 increased cell proliferation and decreased progesterone secretion and aromatase activity (Parrott & Skinner 1998). Theca cell FGF7 mRNA abundance was higher in large compared with small and medium sized bovine follicles in one study (Parrott & Skinner 1998), although a later study reported no affect of health status (based on follicular fluid oestradiol:progesterone ratio) on FGF7 mRNA levels (Buratini *et al.* 2007).

FGF10 has also been described in the antral follicle, and has a different pattern of expression compared with FGF7. Whereas FGF7 is restricted to the theca layer, FGF10 mRNA was identified in bovine theca cells and the oocyte (Buratini *et al.* 2007). It is interesting to note that both proteins have been detected in preantral follicles, which do not contain theca cells (Buratini *et al.* 2007, Abir *et al.* 2009, Oron *et al.* 2012, Castilho *et al.* 2014), thus a developmental switch in localization may occur once

follicles become antral. Further, whereas FGF7 mRNA levels were not altered by follicle health in bovine follicles, FGF10 mRNA levels were significantly higher in theca cells from less oestrogenic (atretic) compared with highly oestrogenic follicles (Buratini *et al.* 2007).

As with FGF7, the addition of FGF10 to granulosa cells *in vitro* inhibited steroid secretion (Buratini *et al.* 2007). More interestingly, the injection of FGF10 directly into a growing follicle in cattle *in vivo* caused follicle regression and lowered the abundance of granulosa cell *CYP19A1* mRNA (Gasperin *et al.* 2012).

The presence of *FGF10* mRNA in the oocyte raised the question of a role of oocyte derived FGF10 in cumulus cell function and oocyte maturation. This was first explored by Zhang *et al.* (2010) who demonstrated that addition of exogenous FGF10 to bovine cumulus oocyte complexes during *in vitro* maturation (IVM) increased cumulus expansion and the rate of blastocyst development, and that immunoneutralizing endogenous FGF10 reduced cumulus expansion and the rate of blastocyst development. This latter observation is compelling evidence of endogenous FGF10 signaling within the follicle. It has since been shown that exogenous FGF10 stimulates glucose uptake by cumulus cells and decreases the level of apoptosis in oocytes during IVM (Caixeta *et al.* 2013, Pomini Pinto *et al.* 2015). Whether FGF7 would have the same effect is unknown.

The effects of FGF7 and of FGF10 on the *in vitro* growth of preantral follicles have been explored by several laboratories. In rats, FGF7 stimulated development of primary follicles (McGee *et al.* 1999, Kezele *et al.* 2005) but did not do so in goats (Faustino *et al.* 2011), whereas FGF10 stimulated primary follicle development in goats (Chaves *et al.* 2010). Whether these discrepancies are related to different culture conditions or species is unclear.

Very little is known about the role of the two remaining members of the FGF7 family in the ovary. Messenger RNA encoding *FGF3* and *FGF22* has been detected in mouse oocytes (Zhong *et al.* 2006), although any potential action on follicular cells has not been reported.

### The role of the FGF8 family in follicle development

The FGF8 family consists of FGF17 and FGF18 in addition to the prototype FGF8. Messenger RNA encoding all three genes has been detected in mouse oocytes; in a microarray study, *Fgf8* and *Fgf18* were highly expressed in mouse oocytes whereas *Fgf17* was weakly expressed (Zhong *et al.*

2006). Although *Fgf8* mRNA was initially reported to be located exclusively in oocytes in the adult female mouse (Valve *et al.* 1997), another study reported transcripts in theca and granulosa cells in cattle (Schmitt *et al.* 1996, Zammit *et al.* 2002, Buratini *et al.* 2005). The main receptors for FGF8 subfamily members, FGFR3c and FGFR2c, have been localized to granulosa and theca cells in cattle (Berisha *et al.* 2004, Buratini *et al.* 2005).

Based on the presence of *FGF8* mRNA in the mouse oocyte, the role of this growth factor in cumulus function was explored. In the mouse COC, removal of the oocyte prevents cumulus expansion and inhibits glycolysis in the cumulus cell. The replacement of denuded oocytes to cumulus cells reverses this effect (Sugiura *et al.* 2007). In a search for the oocyte derived factors that influence cumulus glycolysis, Sugiura *et al.* (2007) cultured oocyte-tectomized cumulus cells with either the well known oocyte derived bone morphogenetic protein (BMP)-15 or with FGF8, and while neither alone had an effect on cumulus glycolysis, when added together they stimulated glycolysis to levels observed in the presence of oocytes.

Also in the mouse, FGF8 was shown to inhibit oestradiol secretion and *Cyp19a1* mRNA levels in granulosa cells, but had no effect on progesterone secretion (Miyoshi *et al.* 2010). Interestingly, single nucleotide polymorphisms have been detected in the bovine FGF8 gene that are correlated with the number of antral follicles (Santos-Biase *et al.* 2012), suggesting a potentially important role for FGF8 in regulating follicle activation or early development.

The effect of FGF17 and FGF18 on granulosa cells has been explored in cattle. *FGF17* mRNA was detected mainly in oocytes with trace amounts in granulosa and theca cells (Machado *et al.* 2009), which is not dissimilar to the pattern of expression of *FGF8* mRNA in this species. FGF17 protein was readily detected in oocytes and granulosa cells; addition of recombinant FGF17 to granulosa cells *in vitro* inhibited oestradiol and progesterone secretion (Machado *et al.* 2009). Similarly to FGF8, the restricted pattern of expression of FGF17 to the oocyte led to studies of a potential action in cumulus oocyte communication; addition of FGF17 increased the proportion of bovine COCs that fully expanded, but this did not improve the developmental competence of the oocyte (Machado *et al.* 2015).

The pattern of expression of FGF18 is more typical of the mesenchymal-epithelial signaling pathways of FGFs in general. Messenger RNA was not readily detected in the oocyte of cattle but is abundant in theca cells (Portela *et al.* 2010). Addition of recombinant FGF18 to bovine

granulosa cells *in vitro* inhibited oestradiol and progesterone secretion, and lowered abundance of mRNA encoding major estrogenic and progestagenic enzymes (Portela *et al.* 2010). Unlike the known actions of other FGFs in the ovary, however, FGF18 appears to be a proapoptotic factor. Abundance of *FGF18* mRNA and protein is higher in atretic compared with growing bovine follicles, and addition of FGF18 increased the amount of DNA laddering and caspase-3 activation in granulosa cells *in vitro* (Portela *et al.* 2010, 2015). The mechanisms of action of FGF18 in the follicle have not been fully elucidated, but it may act through a murine double minute 2 (MDM2) and p53 upregulated modulator of apoptosis (PUMA, also known as BBC3) pathway (Portela *et al.* 2015), although it has not yet been shown whether FGF18 alters p53 signaling. Most intriguingly, FGF18 does not result in the typical phosphorylation of MAPK3/1, but does phosphorylate MAPK14 (also known as p38); increased activity of MAPK14 has been linked to apoptosis in ovarian cells (Uma *et al.* 2003, Bu *et al.* 2006).

Proapoptotic actions of FGFs are rare but other examples exist, including the observation that FGF18 inhibited intestinal crypt cell proliferation, while inhibition of FGFR3 increased cell proliferation (Arnaud-Dabernat *et al.* 2008). Further, an activating mutation of FGFR3c led to increased granulosa cell apoptosis in mice (Amsterdam *et al.* 2001). Collectively, these data suggest that FGF18 activates an apoptotic pathway through FGFR3c in granulosa as well as some other cell types.

The ability of FGF18 but not of FGF8 to increase apoptosis raises an intriguing question: how do two ligands that activate the same receptors have such different effects on the target cell? To explore this question, one needs to understand the mechanism of FGF signaling, which is reviewed in the next section.

### Intracellular FGF signaling in granulosa cells

The intracellular pathways used by FGFs have been elucidated in a variety of cell lines and have been well reviewed (Dailey *et al.* 2005, Cotton *et al.* 2008, Iwata & Hevner 2009). In brief, upon binding to the ligand, the activated FGF receptor dimerizes and the resulting conformational change in the receptor structure causes autophosphorylation of specific tyrosine residues. Two main branches of signaling are then activated: phosphorylation of MAPK via phospholipase C, and activation of phosphatidylinositol-3-kinase and subsequent Akt and protein kinase C (PKC) pathways. This results in expression of a number of early immediate response genes,

including the Sprouty (SPRY) family of negative regulators of tyrosine kinase receptors, and the nuclear orphan receptor 4A and ETS families of transcription factors.

Most of these pathways have been demonstrated to be active in granulosa cells, mainly using FGF2 as a 'typical' FGF ligand. In rat granulosa cells, FGF2 stimulated calcium signaling through a PKC dependent pathway (Peluso *et al.* 2001), and in bovine granulosa cells FGF2 stimulated MAPK3/1 and Akt phosphorylation (Jiang *et al.* 2011). In human granulosa lutein cells, FGF2 but not FGF4 or FGF10 increased *SPRY2* mRNA levels (Haimov-Kochman *et al.* 2005), whereas in bovine granulosa cells FGF2 and FGF4 stimulated *SPRY2* mRNA abundance (Jiang *et al.* 2011, Jiang & Price 2012). Several studies have demonstrated that FGF8 stimulates MAPK3/1 phosphorylation and *SPRY2* mRNA levels in rat, human and mouse granulosa/cumulus cells (Sugiura *et al.* 2009, Miyoshi *et al.* 2010, Jiang *et al.* 2013).

In a comparison of FGF8 and FGF18 signaling pathways in bovine granulosa cells, we demonstrated that FGF8 activates the typical FGF responses including phosphorylation of MAPK3/1 and rapid and transient expression of *SPRY2* and *NR4A2*, whereas FGF18 altered none of these targets (Jiang *et al.* 2013). Using a microarray approach, we identified additional early immediate response genes that were upregulated by FGF8 but not by FGF18, including *FOS* and *XIRP1*, and others that were stimulated by both FGF8 and FGF18, including *FOSL1* (Jiang *et al.* 2013). Perhaps the most interesting difference between FGF8 and FGF18 was the stimulation by FGF8 and marked inhibition by FGF18 of abundance of mRNA encoding the DNA damage response gene *GADD45B*. Decreased *GADD45B* expression has been linked to increased apoptosis in several cell types, and thus may be part of the mechanism used by FGF18 to increase apoptosis in granulosa cells. The known differences in the response of granulosa cells to FGF8 and FGF18 are summarized in Fig. 2.

### The basis for divergent signaling of related FGFs

Collectively, the literature suggests potential roles for FGF7 and FGF8 family members in preantral and/or antral follicle growth and development, and that some FGFs of the same subfamily that activate the same receptors have markedly different biological effects on their target cells. Such differences between closely related FGFs have been described during embryo development, and these are especially pertinent to the present discussion as they are

	FGF2	FGF8	FGF18
Oestradiol secretion	↓	↓	↓
Progesterone secretion	↓	—	↓
Apoptosis	—	NA	↑
Phospho MAPK3/1	↑	↑	—
Phospho MAPK14	↑	—	↑
<i>SPRY2</i> mRNA	↑	↑	—
<i>NR4A1</i> mRNA	↑	↑	—
<i>FOS</i> mRNA	NA	↑	—
<i>FOSL1</i> mRNA	NA	↑	↑
<i>GADD45B</i> mRNA	↑	↑	↓
<i>BBC3</i> mRNA	↑	NA	↑
<i>MDM2</i> mRNA	—	NA	↓

**Figure 2**

Schematic illustration of significant differences between the effects of FGF2, FGF8, and FGF18 on granulosa cell function (steroid secretion), health (apoptosis and abundance of *BBC3*, *GADD45B*, and *MDM2* mRNA), intracellular signaling (phosphorylation of MAPK3/1 and MAPK14), and abundance of mRNA encoding early immediate (*FOS*, *FOSL1*) and FGF response (*SPRY2*, *NR4A1*) genes. Horizontal lines indicate no effect; NA, data not available. Data from Miyoshi *et al.* (2010), Jiang *et al.* (2011, 2013) and Portela *et al.* (2015).

FGF7 vs FGF10, and FGF8 vs FGF18. Branching morphogenesis refers to the growth and branching of tubular structures in tissues such as lung, kidney, and salivary glands. In these tissues, FGF7 and FGF10 play distinct roles; FGF7 induces expansion or branching of epithelial buds whereas FGF10 induces bud elongation (Bellusci *et al.* 1997, Koyama *et al.* 2008, Makarenkova *et al.* 2009). For the FGF8 subfamily, FGF18 stimulates expansion in the embryonic midbrain whereas FGF8 transforms the midbrain into cerebellum (Liu *et al.* 2003, Sato & Nakamura 2004). The current literature suggests that these distinct biological activities may be directed by i) differential

intracellular signaling and/or ii) interactions between FGFs, FGFRs, and HSPGs.

### Differential intracellular signaling

Distinct signaling between FGF7 and FGF10 in HeLa cells transfected with FGFR2b results in FGF7 increasing cell proliferation whereas FGF10 promotes cell migration. This has been correlated with sustained phosphorylation of MAPK3/1 by FGF7 but transient (or weak) phosphorylation by FGF10 (Koyama *et al.* 2008, Francavilla *et al.* 2013), owing to a difference in the pattern of tyrosine phosphorylation in the kinase domain of FGFR2b (Francavilla *et al.* 2013). In the embryonic mouse kidney and submandibular gland, FGF7 but not FGF10 induced expression of the FGF target gene *Spry2* (Chi *et al.* 2004, Ohno *et al.* 2010), although other studies have shown increased *Spry2* mRNA levels following treatment with FGF10 (Hashimoto *et al.* 2012). In bovine granulosa cells, FGF10 results in a slow activation of MAPK3/1 without stimulating the expression of *SPRY2* mRNA levels (Jiang & Price 2012).

A similar dichotomy occurs for members of the FGF8 subfamily, albeit with an extra layer of complexity. Messenger RNA encoding FGF8 but not FGF18 undergoes alternative splicing to generate proteins with different N-termini (Crossley & Martin 1995). These splicing events result in a short form (FGF8a) and a longer form (FGF8b) among others, and it is recombinant FGF8b that has been used in studies of the ovary (Sugiura *et al.* 2007, Portela *et al.* 2015). FGF8a and FGF8b differ in their biological activities, as FGF8a induces expansion of the embryonic midbrain (in a manner similar to FGF18), whereas it is FGF8b that transforms the embryonic midbrain into a cerebellum (Liu *et al.* 2003, Sato & Nakamura 2004). FGF8b provokes a strong activation of MAPK3/1 in the midbrain, whereas FGF8a results in a lower level of MAPK3/1 phosphorylation (Sato & Nakamura 2004). Data from studies with bovine granulosa cells show that FGF8 results in a strong and transient phosphorylation of MAPK3/1 while addition of FGF18 provokes a very weak response (Jiang *et al.* 2013). The difference in the activities of FGF8a and FGF8b has been attributed to a single phenylalanine residue (F32) present in the N-terminus of FGF8b that allows strong binding with the receptor, and which is absent in FGF8a leading to a weak receptor binding complex (Olsen *et al.* 2006). However, this is not a satisfactory explanation of the difference between FGF8b and FGF18, as FGF18 contains the F32 residue present in FGF8b.

It is known that weak vs strong and transient vs sustained MAPK3/1 activation leads to distinct cell responses, driving cells toward proliferation or apoptosis in a cell context specific manner (Murphy *et al.* 2004, Glotin *et al.* 2006, Shaul & Seger 2007). This may thus account in part for the differential responses of granulosa cells to FGF8 and FGF18.

### Interactions between FGFs, FGFRs and HSPGs

FGF10 and FGF7 have been shown to bind to HSPGs (Bonneh-Barkay *et al.* 1997, Mongiat *et al.* 2000, Patel *et al.* 2007), and HS selectively alters the biological activity of FGF7/FGF10. In tissue explants, FGF7 and FGF10 have different binding affinities to the ECM, such that FGF7 diffuses readily through the ECM and reaches all cells in an explant, whereas FGF10 does not diffuse as well and reaches only those parts of an explant close to the source (Makarenkova *et al.* 2009). Addition of heparin to cultures of cell lines inhibits FGF7 mitogenic activity but stimulates that of FGF10 (Igarashi *et al.* 1998, Belleudi *et al.* 2007). Definitive evidence of the importance of HS in determining FGF7/FGF10 activity comes from a study in which the HS binding region FGF10 was mutated to that of FGF7 (R→V, see Fig. 3); one amino acid substitution converted FGF10 to a functional mimic of FGF7 (Makarenkova *et al.* 2009).

Sequence comparison between mouse and bovine proteins shows that the HS binding domain of FGF10 is fully conserved between species, whereas for FGF7 the

	Glycine box									
	G	X	X	B	B	G	X	X	T	
FGF7	K	G	I	P	V	R	G	K	K	T
FGF10	K	G	A	P	R	R	G	Q	K	T
FGF8b	K	G	R	P	R	K	G	S	K	T
FGF18	K	G	R	P	R	K	G	P	K	T

**Figure 3**

Sequence alignment of the HS binding domain ('glycine box') of paracrine FGFs 7, 10, 8b, and 18. Green boxes denote residues from the core of the binding domain; the yellow box denotes residues that switch biological activity between FGF7 and FGF10, and the blue box illustrates the sequence difference between FGF8b and FGF18. Sequence data from Olsen *et al.* (2006) and Makarenkova *et al.* (2009). X, any residue, B, basic residue.

bovine sequence differs slightly (I→V) but conserves the critical V.

HS is also essential for FGF8 subfamily signaling, as addition of FGF8 to HS deficient CHO cells did not stimulate cell proliferation or phosphorylation of MAPK3/1, whereas these activities were evident upon co-treatment with heparin (Loo & Salmivirta 2002). Perlecan formed a ternary complex with FGF18 and FGFR3, and the presence of perlecan was essential for FGF18 induced proliferation of myeloid B cell lines (BaF32) expressing FGFR3 (Chuang *et al.* 2010). Whether sequence variation in the HS binding region accounts for different signaling between FGF8 and FGF18 has not been determined, although as they both possess the Arg residue that confers the biological activity of FGF10 (Fig. 3), this particular residue may not be a determinant of FGF8/FGF18 activity. The bovine and mouse FGF8 and FGF18 HS binding domains are fully conserved.

However, the presence or absence of HS is not the whole story, as the type of HS chain can also alter FGF biological activity. For example, perlecan derived from endothelial cells stimulated FGF18 induced proliferation of BaF32 cells to a much greater degree than did chondrocyte derived perlecan (Chuang *et al.* 2010). The type of HS also alters the ability of a FGF to activate a specific receptor, as short saccharide chains are sufficient to allow FGF1 to activate FGFR2b, whereas longer saccharide chains are required to allow FGF7 activation of FGFR2b (Ostrovsky *et al.* 2002). The ability of FGF10 to induce submandibular duct elongation or branching is dependent on the type of HS; 6-O-sulfated saccharides permit FGF10 induced elongation and the addition of 2-O-sulfated saccharides are required for branching (Patel *et al.* 2008).

FGF7 and FGF10 present a relatively simple situation in that they both activate one receptor. The situation is more complicated for FGF8 and FGF18, as they activate FGFR1c, FGFR2c, FGFR3c and FGFR4, and HS may alter affinity or activity of a specific ligand receptor pair; in mouse embryos, FGF8 binding to FGFR2c required 2-O- and 6-O-sulfated HS, whereas FGF8-FGFR3c binding required only 6-O-sulfated HS (Allen & Rapraeger 2003). Whether the same requirements apply for FGF18 binding to FGFR2c vs FGFR3c is not known. Developmental regulation of the type and degree of HS sulfation may permit a temporal control over FGF bioactivity; the complexity of 6-O-sulfation in embryonic brain HS changes during development and this may restrict FGF8 action to a much more defined period during development (Brickman *et al.* 1998, Ford-Perriss *et al.* 2002).

The two explanations described above are not mutually exclusive, as the mechanism by which HS alters FGF-FGFR signaling may include differential phosphorylation of tyrosine residues within the receptor kinase domain of the receptor. A link between HSPG and receptor activity was demonstrated for FGF2, which alone is able to induce phosphorylation of certain residues of FGFR1 in HS deficient CHO cells, whereas co-treatment with heparin is required for phosphorylation of additional residues that lead to PLC activation (Lundin *et al.* 2003).

Collectively, these studies point clearly to a modification of specific FGF-FGFR activities by proteoglycans during development. How does this impact FGF signaling in the follicle? Antral follicles contain numerous proteoglycans and granulosa cells produce HSPG (Yanagishita & Hascall 1983, McArthur *et al.* 2000). Theca cells are separated from granulosa cells by a basement membrane, which contains perlecan as well as other components, and the levels of these components change with follicle growth (Rodgers *et al.* 2003). In addition, a particular form of extracellular matrix (focimatrix) occurs as aggregates within the granulosa cell layer and which contains perlecan (Irving-Rodgers *et al.* 2004). Perlecan mRNA levels change during follicle development (Matti *et al.* 2010), and HSPG levels within the granulosa cell layer, likely in focimatrix, are significantly higher in atretic compared with healthy follicles (Huet *et al.* 1997, 1998, Irving-Rodgers *et al.* 2004, Matti *et al.* 2010). Further, follicle HSPG is sulfated and, at least in the cow, the quantity of certain sulfated HS derived saccharides change during follicle development and atresia (Hatzirodos *et al.* 2012). Therefore the potential for regulation of FGF signaling by follicular HSPGs exists.

How the ECM impacts FGF signaling in the follicle is unknown, but parallels can be drawn with the literature reviewed above. For example, it may be relatively easy for thecal FGF7 to cross the basement membrane and reach granulosa cells, but the passage of FGF10 may be restricted such that its influence is felt mainly by the layer of granulosa cells most closely associated with the basement membrane. Aggregates of focimatrix may play a role in regulating FGF10 or FGF18 bioactivity within the granulosa cell layer, and changes in sulfation of perlecan HS during follicle growth/atresia may diminish or enhance the potential pro-apoptotic actions of FGF18.

## Concluding remarks and future directions

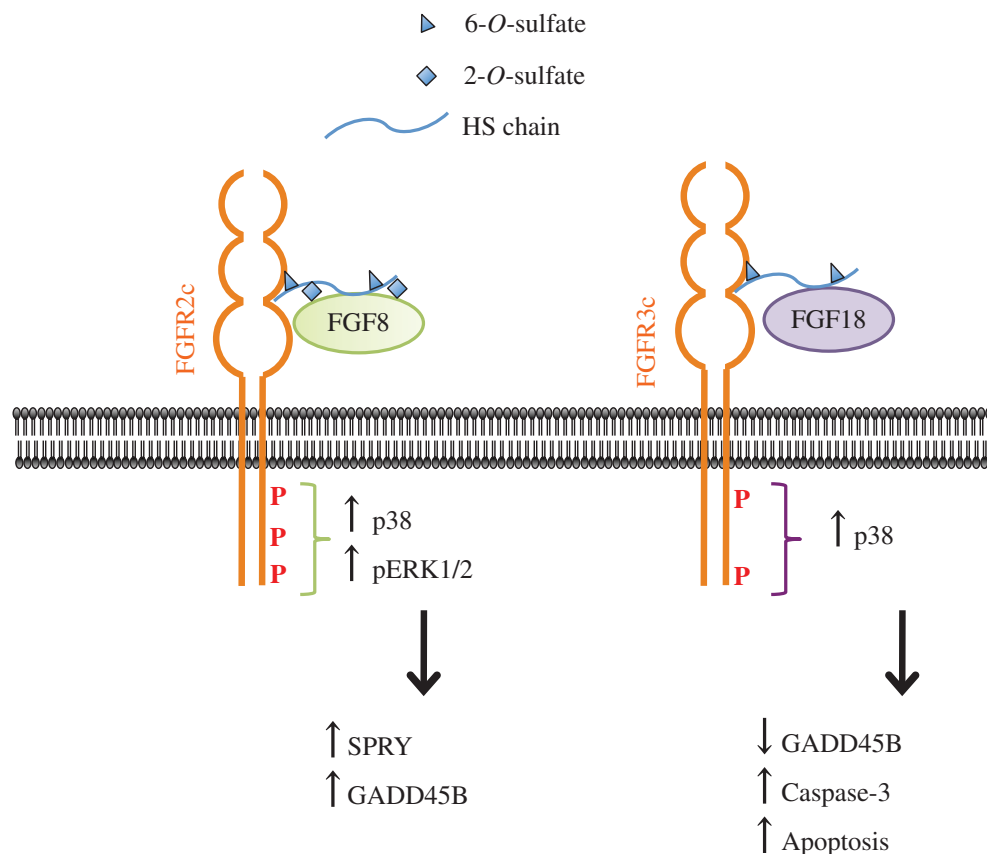
FGF signaling in developing tissues is highly regulated at multiple levels: gene transcription, alternative splicing,



activation of distinct intracellular pathways and the presence of specific sulfation patterns on target cell ECM proteins. In the ovary, FGF7 and FGF10 may have different effects on preantral follicle development, and FGF8 has markedly different effects on granulosa cells compared with FGF18. Although FGF7 and FGF10 bind to the same receptor, FGFR2b, they have different affinities for HS that causes a divergence of receptor phosphorylation events between these two ligands. Similarly for FGF8 subfamily members, observed differences of biological effects of FGF8 and FGF18 on granulosa cells may be related to the nature of sulfation of granulosa cell HSPGs. Extrapolating from studies in limb and lung development, a hypothetical model for the actions of FGF8 and FGF18 is presented in Fig. 4, in which FGF8 associates with 2-O- and 6-O-sulfated HS to phosphorylate multiple tyrosines in the kinase domain of the receptor, maybe predominantly FGFR2c leading to MAPK3/1 and MAPK14 activation (among

others) and the typical FGF response. In contrast, FGF18 may associate with mainly (or different) 6-O-sulfated HS and phosphorylate fewer or different tyrosine residues in FGFR3c, leading to activation of pro-apoptotic MAPK14 without the activation of anti-apoptotic MAPK3/1, which in turn drives the cell toward caspase-3 mediated apoptosis.

Further research is required i) to identify the specific 2-O- or 6-O-sulfated saccharides present in focimatrix or other components of the granulosa cell ECM, and whether these change during follicle development and atresia; ii) to determine which receptors are activated by FGF18 vs FGF8 in granulosa cells, FGFR2c or FGFR3c; iii) to identify the specific receptor phosphorylation sites activated by FGF8 and FGF18 in granulosa cells; and iv) identify the upstream intracellular events that direct FGF8/FGF18 signaling to MAPK3/1 and /or MAPK14. The cumulus cell should not be forgotten, as in the mouse *Fgf8* plays an important role in metabolism; further studies should



**Figure 4**

A hypothetical model of divergent signaling of FGF8 and FGF18 in granulosa cells, extrapolated from studies of branching morphogenesis. In this model, FGF8 associates with 2-O- and 6-O-sulfated HS to phosphorylate multiple tyrosines in the kinase domain of the receptor, predominantly FGFR2c, leading to MAPK3/1 (ERK 1/2) and MAPK14 (p38) activation (among others) and the typical FGF response. In contrast, FGF18 phosphorylates

fewer or different tyrosine residues, leading to activation of pro-apoptotic p38 without the activation of anti-apoptotic ERK1/2, which in turn drives the cell toward caspase-3 mediated apoptosis; this distinct signaling pathway may be associated with an affinity to mainly 6-O-sulfated HS and activation of predominantly FGFR3c.

explore the role of FGF18 in cumulus function. This information will shed new light on the role of FGF18 as an atypical pro-apoptotic FGF, and potentially lead to approaches to modify FGF18 activity and improve fertility in species such as cattle and humans.

#### 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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