EXPRESSION AND ROLE OF FIBROBLAST GROWTH FACTOR 9 (FGF9) IN BOVINE FOLLICULOGENESIS

By

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Abstract: In the ovaries, many members of the FGF family are involved in the regulation of folliculogenesis. One of these members, FGF9, has been characterized as an anti-differentiation factor for its effects on steroidogenesis of granulosa (GC) and theca cells (TC) of cattle, but whether endogenous production of FGF9 changes during follicular development of cattle was unknown. Also, in order to have a comprehensive view of how FGFs regulate folliculogenesis, information of how endogenous production of their receptors (FGFRs) is also required. The first objectives of our studies presented herein was to characterize *FGF9*, *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* mRNA abundance in GC and TC during development of dominant follicles in dairy cattle. Estrous cycles of non-lactating dairy cattle were synchronized, and ovaries were collected on either day 3-4 (n = 8) or day 5-6 (n = 8) post-ovulation for GC and TC mRNA extraction from small (1-5 mm), medium (5.1-8 mm) or large (8.1-18 mm) follicles for PCR analysis. In GC, *FGF9*, *FGFR1c*, *FGFR2c*, and *FGFR3c* mRNA abundance was greater ($P < 0.05$) in estrogen (E2)-inactive (i.e., concentrations of E2 < progesterone, P4) follicles than in large E2-active (i.e., concentrations of E2 > P4) follicles. In TC, *FGF9* mRNA abundance was greater ($P < 0.05$) in large E2-inactive follicles than in large E2-active follicles on day 3-4 post-ovulation whereas medium E2-inactive follicles have greater ($P < 0.05$) *FGFR1c* and *FGFR4* mRNA abundance than large E2-active and E2–inactive follicles on day 5-6 post-ovulation. In addition, *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* mRNA abundance was greater ($P < 0.05$) in medium E2-inactive follicles on day 5-6 post-ovulation than on day 3-4 post-ovulation. Following, we aimed to investigate effects of FGF9 on TC through the use of microarray (Affymetrix GeneChip Bovine Genome Arrays) and the bioinformatic tool Ingenuity Pathway Analysis. This software identified differentially expressed transcripts in 346 pathways in response to FGF9 in TC involved in functions such as cell cycle, proliferation, survival, and steroidogenesis. Taken together, these studies reinforce the importance of FGFs in folliculogenesis of cattle.

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CHAPTER I

INTRODUCTION

Fibroblast growth factors (FGFs) are polypeptides described to elicit a great variety of actions throughout the system of mammals during embryo development and adult life, including organogenesis, sex determination (Kim et al., 2006), angiogenesis (Cross and Claesson-Welsh, 2001; Robinson et al., 2008), and wound healing (Zheng et al., 2014). Also, aberrant FGF signaling has been associated with cancer. In fact, the involvement of FGF signaling in cancer incidence has been extensively studied in the last decades, showing the importance of these mitogens for health (Ornitz and Itoh, 2001; Gotoh, 2008; Nakao et al., 2013; Tanner and Grose, 2016). Moreover, there is an increasing body of studies throwing light on the functions of FGFs in the reproduction.

Currently, out of the 22 members of the mammalian FGF family, ten have been reported to be synthesized at different degrees in the oocyte, theca cells (TC), and granulosa cells (GC), playing autocrine, paracrine, and endocrine roles in the ovary (Drummond et al., 2007; Machado et al., 2009; Portela et al., 2010; Schreiber and Spicer, 2012; Castilho et al., 2015; Ferreira et al., 2016). These roles include regulation of steroidogenesis (Baird and Hsueh, 1986; Vernon and Spicer, 1994; Schreiber and Spicer, 2012), cell proliferation (Gospodarowicz et al., 1977a; Parrott et al., 1994; Spicer and Stewart, 1996; Schreiber et al., 2012), corpus luteum formation (Gospodarowicz et al., 1977b; Grazul-Bilska et al., 1995; Robinson et al., 2008), and atresia (Portela et al., 2015).

Among FGFs that regulate folliculogenesis of cattle, FGF9 stimulates GC proliferation while suppressing hormone-stimulated estradiol production (Schreiber and Spicer, 2012). Because estradiol production is critical for GC survival and differentiation of

dominant follicles (Knecht et al., 1985; Fortune et al., 2004), FGF9 appears to have an anti-differentiation function during ovarian follicular development of cattle. Whether endogenous production of FGF9 by ovarian follicular cells change during ovarian follicular growth and selection of cattle remains to be determined. Because FGF9 stimulates bovine TC proliferation while inhibiting steroidogenesis (Schreiber et al., 2012), studies to investigate FGF9 signaling mechanisms in TC of cattle are of great value for the understanding of how FGFs affect folliculogenesis in mono-ovulatory species. Advances in biotechnology and bioinformatics tools achieved in the last decades, such as the technology of cDNA microarray, which allows simultaneous measurement of thousands of gene transcripts (Schena et al., 1995), are critical for such an investigation. Nevertheless, interpretation of data generated by this tool is complex (Bowtell, 1999; Huang et al., 2009; Henderson-MacLennan et al., 2010) and would not be possible without the use of modern bioinformatics tools (Subramanian et al., 2005; Capriotti et al., 2012). Therefore, advances in bioinformatics technology for microarray-based genomic profiling provide an inestimable contribution to the advance in understanding the roles of various factors in reproductive biology.

To elicit their intracellular actions, FGFs must bind to high affinity single chain transmembrane tyrosine kinase receptors (FGFRs), which have different ligand-specificities and are encoded by four different genes in vertebrates (Itoh and Ornitz, 2004; Ornitz and Itoh, 2015): *FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4*. The diversity of these receptors is further increased by the occurrence of alternative mRNA splicing of the sequence of the immunoglobulin domain III of the genes *FGFR1*, *FGFR2*, and *FGFR3*, resulting in isoforms IIIb and IIIc, which are important for determining ligand-binding specificity (Givol and Yayon, 1992; Ornitz and Itoh, 2001; Itoh and Ornitz, 2004; Li et al., 2016). Due to the different ligand-binding specificities (Ornitz and Itoh, 2015), the localization of FGFRs in the different follicle compartments is of critical importance for the FGFs to exert their effects on ovarian physiology. Indeed, there is variation in the presence of isoforms of FGFRs in GC, TC, and oocytes (Berisha et al., 2004; Buratini et al., 2007; Machado et al., 2009; Zhang and

Ealy, 2012), contributing to the variation in effects of different FGFs observed in folliculogenesis of mammals. Some FGFRs change according to follicular fate (Berisha et al., 2004; Buratini et al., 2005a; Castilho et al., 2015), adding complexity to the roles of FGFs in ovarian follicular development. Therefore, studies about how endogenous production of some FGFRs by ovarian follicular cells change during selection of dominant follicles in a monotocous species such as cattle are very much needed for a comprehensive understanding of the roles of FGFs in folliculogenesis.

CHAPTER II

REVIEW OF LITERATURE

1. Fibroblast growth factors family of proteins and their receptors

Fibroblast growth factors (FGFs) constitute a large family of single chain polypeptide growth factors present in both vertebrates and invertebrates (Ornitz and Itoh, 2001; Dailey et al., 2005; Itoh and Ornitz, 2011; Li et al., 2016). The first members to be reported, FGF1 and FGF2, were designated acidic and basic FGF in view of their activity to stimulate fibroblast proliferation and their isoelectric point (Armelin, 1973; Gospodarowicz, 1974; Gambarini and Armelin, 1982). To date, 22 different members of the FGF family have been described in mammals (FGF 1-23, with no FGF15 identified in humans, and no FGF19 identified in rats and mice), most of them sharing highly conserved amino acid residues (Ornitz and Itoh, 2001; Itoh and Ornitz, 2011). These members can be categorized into several subgroups or subfamilies according to increased similarity in sequence, patterns of expression, function, and developmental and biochemical features, although each FGF still appears to have particular sites of expression (Ornitz and Itoh, 2001).

Currently, mammalian FGFs are classified into seven subfamilies containing two to four members each (Itoh and Ornitz, 2011; Li et al., 2016), as summarized in Table 1. These subfamilies can also be categorized into two general groups: the canonical FGFs, which comprise most of the members, and the non-canonical FGFs, which comprise members 11 to 14 (as reviewed by Li et al., 2016). The canonical FGFs exert autocrine, paracrine, and endocrine actions upon binding to high affinity FGF receptors (FGFRs) whereas the non-

canonical FGFs do not bind to FGFRs and act intracellularly through interaction with calcium and voltage-gated sodium channels (Wang et al., 2011; Hennessey e al., 2013; Li et al., 2016).

The majority of FGFs have an N-terminal signal peptide (Table 1) that facilitates secretion from cells through classical mechanisms (Ornitz & Itoh, 2001; Li et al., 2016). A second group of FGFs, including FGF1 (Prudovsky et al., 2003), FGF2 (Abraham et al., 1986; La Venuta et al., 2015; Steringer et al., 2015), FGF9 (Miyamoto et al., 1993; Miyakawa et al., 1999), FGF16 (Miyake et al., 1998), and FGF20 (Ohmachi et al., 2000), lacks a cleavable signal peptide and its members are secreted in a non-conventional manner, including through membrane pores, independently of the endoplasmic reticulum-Golgi pathway (for reviews, see Ornitz & Itoh, 2001; Li et al., 2016). Finally, a third group of FGFs (FGF 11 to FGF 14) lacks signal sequences and is believed to remain inside the cell (as reviewed by Ornitz and Itoh, 2001).

As mentioned, FGFs were first reported to promote fibroblast proliferation (Armelin, 1973; Gospodarowicz, 1974), but the functions of these polypeptides go much beyond of what the name implies. In fact, the name nowadays could be considered misleading, given that not all FGFs elicit activity in fibroblasts (Rubin et al., 1989; Powers et al., 2000; Li et al., 2016). FGFs have been detected in various tissues, playing several roles in development and metabolism, with some members acting exclusively during embryogenesis and others acting in both embryonic and adult tissues (Ornitz and Itoh, 2001). These growth factors can act to stimulate cell steroidogenesis (Johnson et al., 1980), proliferation (Gospodarowicz et al., 1977a; Murphy et al., 1990), survival (Gospodarowicz and Bialecki, 1978; Sievers et al., 1987), differentiation (Stemple et al., 1988; Dailey et al., 2005), and migration (Montesano et al., 1986), but they can also prevent cell proliferation (Oesterle et al., 2000) and inhibit differentiation (Joannes et al., 2016). Interestingly, the action of FGFs vary not just according to the cell type, but also to cell cycle stage, with different responses observed in immature and differentiating osteoblasts (Mansukhani et al., 2000).

The wide range of the functions of FGFs is influenced by the diversity of high affinity FGFRs and cofactors that regulate the FGF signaling complex. To date, four (FGFR1-FGFR4) receptors and several cofactors, including heparan sulfates (HS) and Klotho coreceptors have been identified (Givol and Yayon, 1992; Itoh and Ornitz, 2004; Dailey et al., 2005; Li et al., 2016). The FGFRs are tyrosine kinase receptors that have different ligand-specificities (table 2) and are encoded by four different genes in vertebrates (Itoh and Ornitz, 2004; Ornitz and Itoh, 2015). The extracellular ligand-binding portion of FGF receptors contains two or three immunoglobulin-like domains, and a heparin-binding domain (Plotnikov et al., 2000; Itoh and Ornitz, 2004). Immunoglobulin domains have a characteristic fold built up of a sandwich of two β sheets containing antiparallel strands linked by a disulphide bond. These domains have no enzymatic activity, but are important for determining ligand specificity (Dell and Williams, 1992; Barclay, 2003). Alternative mRNA splicing of the FGFR genes regulates the number (two or three) of immunoglobulin domains and dictates the sequence of the immunoglobulin domain III, resulting in isoforms IIIb and IIIc, which are important for determining ligand-binding specificity (Givol and Yayon, 1992; Ornitz and Itoh, 2001; Itoh and Ornitz, 2004). This splicing naturally occurs in the *FGFR1*, *FGFR2*, and *FGFR3* genes, but not in the *FGFR4* gene (as reviewed by Li et al., 2016).

The HS cofactors are components of proteoglycans on the cell surface and in the pericellular matrix that bind both FGF and FGFR to facilitate and stabilize their interaction (Yayon et al., 1991; Mohammadi et al., 2005; Li et al., 2016). For endocrine FGFs (including FGF19, FGF21, and FGF23), that have little affinity for HS, the transmembrane proteins Klotho/betaKlotho are important to facilitate binding to FGFRs (Goetz et al., 2007). Variation in sulfated domains of HS and in affinity to cofactors contribute to differences in FGF signaling activities in addition to FGFRs diversity (Guimond and Turnbull, 1999; Merry et al., 1999; Ye et al., 2001).

The binding of ligands to FGFRs causes receptor dimerization, transphosphorylation, and activation of the intracellular tyrosine kinase domain (Kelleher et al., 2013). Intracellular

tyrosine kinase takes part in signal transduction to the nucleus, stimulating mitogenesis (Radha et al., 1996; Ruetten and Thiemermann, 1997). Tyrosine autophosphorylation of receptors by tyrosine kinase is essential for the recruitment and activation by phosphorylation of many downstream cytoplasmic targets and subsequent initiation of dynamic signaling cascades (Dengjel et al., 2009; Goetz and Mohammadi, 2013). The main signaling pathways downstream of receptors for tyrosine kinase are: Ras/MAPK (mitogen-activated protein kinase) signaling cascades, which regulate cell proliferation; signaling pathways involving phospholipid second messengers via PLCγ (phospholipase C-γ) for regulation of cell motility; PI3K (phosphatidylinositol 3-kinase) for regulation of cell survival; and the STATs (signal transducers and activator of transcription) pathway (Dengjel et al., 2009; Goetz and Mohammadi, 2013). Nevertheless, for activation of the Ras/MAPK and the PI3K pathways to occur, the docking protein FRS2α needs to be phosphorylated in its tyrosine and serine/threonine residues, which occurs upon binding of FGF to its receptor (Kouhara et al., 1997; Gotoh, 2008; Ornitz and Itoh, 2015). The specificity of signal transduction at this initial stage is determined in great extent by the amino acid sequence surrounding the phosphorylated tyrosine residue (Dengjel et al., 2009).

2. Role of fibroblast growth factors on ovarian physiology

FGFs were first reported in the ovary in 1977 (Gospodarowicz et al., 1977b; Gospodarowicz et al., 1977c), when FGF1 was found to stimulate proliferation of bovine granulosa cells (GC) and theca cells (TC). From these pioneer studies to present, ten members of the FGF family have been described in the ovary of mammals, including FGF1 (Berisha et al., 2004), FGF2 (Berisha et al., 2000), FGF7 (Parrott and Skinner, 1998), FGF8 (Buratini et al., 2005a), FGF9 (Drummond et al., 2007; Schreiber and Spicer, 2012), FGF10 (Castilho et al., 2015), FGF16 (Ferreira et al., 2016), FGF17 (Machado et al., 2009), FGF18 (Portela et al., 2010), and FGF22 (Castilho et al., 2015), each with autocrine, paracrine, and endocrine

roles in the regulation of development of ovarian follicles (for reviews, see Chaves et al., 2012; Price, 2016).

Ovarian folliculogenesis is a highly regulated process by which the female oocyte develops within the somatic cells of the ovary, GC and TC, and matures into a fertilizable ovum (Eppig, 1991; Richards, 1994; Elvin and Matzuk, 1998; McGee and Hsueh, 2000; Ackert et al., 2001). Ovarian follicles develop through primordial, primary, and secondary stages before an antral cavity is formed (McGee and Hsueh, 2000). These preantral follicles remain in a dormant stage until primordial follicle activation occurs, followed by growth of both the oocyte and follicle somatic cells (Fortune et al., 2000). This process happens in the fetal life of domestic animals and primates and in early neonatal period of rodents and rabbits (Hirshfield, 1991; Van den Hurk et al., 1997; Fortune et al., 2000; Fortune, 2003).

The factors that stimulate development of preantral follicles are dependent on species and stage of follicles and include insulin, growth differentiation factor-9 (GDF-9), estradiol, insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), follicle stimulating hormone (FSH), and luteinizing hormone (LH), among others (McGee and Hsueh, 2000; Fortune, 2003; Hsueh et al., 2015). Nevertheless, the gonadotropins (FSH and LH) are unlikely to exert direct actions on primordial follicles because these structures lack gonadotropins receptors, so they start to be important beginning at the stage of primary follicles in some species, such as porcine and murine, and in small secondary follicles in other species, such as bovine (Hsueh et al., 1984; McGee and Hsueh, 2000; Fortune et al., 2003). Among the factors aforementioned, FGFs also have been reported to act in development of early stages of follicular development.

The production of FGFs within the ovary starts early in folliculogenesis: FGF2 was found in oocytes of immature bovine follicles (van Wezel et al., 1995); FGF7 has been reported in primordial, primary, and secondary follicles of cattle (Berisha et al., 2004; Buratini et al., 2007); FGF9 protein was found in the ovary of immature rats, located in

stromal cells and basement membranes surrounding GC as well as in TC precursors (Drummond et al., 2007); *FGF10* mRNA was detected in primordial, primary, and secondary follicles of cattle (Buratini et al., 2007). In addition, at least two members of the FGF family are reported to have an active role in preantral follicle development: FGF2 and FGF7. FGF2 was found to stimulate GC proliferation of preantral follicles in cattle (Wandji et al., 1996), transition of primordial follicles into primary follicles in rats (Nilsson, et al., 2001) and preantral follicle growth and survival in women (Wang et al., 2014) while FGF7 was found to stimulate preantral follicle development in rats (Hsueh et al., 2000; Kezele et al., 2005). These observations imply a role of FGFs in early folliculogenesis.

Members of the FGF family were also detected in antral follicles and in corpora lutea (Parrott and Skinner, 1998; Berisha et al., 2000; Castilho et al., 2008; Cho et al., 2008). After acquiring an antral cavity, a small group of follicles is recruited to initiate follicular growth towards ovulation (Bao and Garverick, 1998). Nevertheless, most of them undergo atretic degeneration while only a few of them will be selected to become the dominant follicles, which are larger than the subordinate follicles and will ovulate to release the mature oocyte for fertilization (Spicer and Echternkamp, 1986; McGee and Hsueh, 2000). The recruitment of a cohort of growing antral follicles and subsequent selection of dominant follicles is not only regulated by gonadotropic hormones, but also by factors produced by the oocyte and surrounding follicular cells (Eppig, 2001; Fortune et al., 2004). Hence, the presence of FGFs in antral follicles indicates their importance in all stages of folliculogenesis.

In antral follicles of cattle, FGF1, FGF2, FGF7, FGF18, and FGF22 are mainly produced by TC (Parrott and Skinner, 1998; Berisha et al., 2000; Berisha et al., 2004; Buratini et al., 2007; Portela et al., 2010; Castilho et al., 2015); FGF8 is produced by GC, TC, and oocytes (Buratini et al., 2005); FGF9 is produced in greater amounts in GC than in TC (Schreiber et al., 2012); FGF10 is produced by TC and oocytes (Buratini et al., 2007); FGF16 is produced by the oocyte (Ferreira et al., 2016); and FGF17 is detected mainly in oocytes, but also in GC (Machado et al., 2009). Interestingly, the production of some FGFs also

change according to the diameter of bovine antral follicles: *FGF2* and *FGF7* mRNA abundance, for example, is greater in large than in small follicles (Parrott and Skinner, 1998; Berisha et al., 2000; Berisha et al., 2004) while *FGF9* mRNA abundance is greater in small than in large follicles (Schreiber et al., 2012). Nevertheless, some FGFs such as FGF1 (Berisha et a., 2004; Berisha et al., 2006) and FGF22 (Castilho et al., 2015) do not change during antral follicular growth in cattle.

2.1 Role of fibroblast growth factors and gonadotropins on ovarian steroidogenesis

In response to gonadotropins (FSH and LH) secreted by the pituitary, the different compartments of the ovarian follicle interact in a highly integrated way to synthesize and secrete sex steroids (estrogens, progestins) in order to produce a fertilizable egg (Hsueh et al., 1984). Estradiol, the main estrogen produced in ovarian follicles, has direct effects on GC, stimulating cell proliferation, enhancing LH receptor formation and FSH-stimulated aromatase activity (Hsueh et al., 1984; Kessel et al., 1985), being therefore essential for development and survival of dominant follicles (Spicer and Echternkamp, 1986; Evans and Fortune, 1997; Robker and Richards, 1998). Besides providing an uterine environment to maintain pregnancy (Keyes and Wiltbank, 1988; Lonergan et al., 2007), progesterone also plays a role in the control of preovulatory follicle maturation: it increases close to the time of ovulation (Fortune and Hansel, 1995), regulates the time of release of prostaglandin F2α for luteolysis (Silvia et al., 1991; dos Santos et al., 2009), and regulates the release of LH and FSH by the pituitary, in addition to modulating pituitary responsiveness to gonadotropin-releasing hormone (GnRH) from the hypothalamus (Chang and Jaffe, 1978; Mahesh and Muldoon, 1987).

Follicular steroid production is crucial for maintenance and development of growing follicles and are dependent upon stimulation of FSH and LH, which regulate several key enzymes, such as cytochrome P450 side-chain cleavage enzyme (P450scc), cytochrome P450 17α- hydroxylase (17αOH), 3β-hydroxysteroid dehydrogenase (3β-HSD), and cytochrome P450 aromatase as well as the cholesterol transport protein, steroidogenic acute regulatory

protein (StAR) (Richards, 1994; Tian et al., 1995; Bao and Garverick, 1998; McGee and Hsueh, 2000; Logan et al., 2002; Orisaka et al., 2006). Each of these enzymes catalyzes a key step in the biosynthesis of steroids and their expression changes according to the type of cell and stage of follicle development (Tian et al., 1995; Bao and Garverick, 1998; Logan et al., 2002). As reviewed by Richards (1994), P450scc converts cholesterol into pregnenolone, 3β-HSD converts pregnenolone into progesterone, 17αOH converts progesterone to androstenedione, and aromatase converts androgens into estrogens.

LH plays a key role in determining the differentiation of TC in preovulatory follicles since, in these follicles, TC have high amounts of LH receptor and 17αOH and therefore synthesize great quantities of androgens (Richards, 1994; Tian et al., 1995; McGee and Hsueh, 2000). Androgens produced by TC are converted into estrogens by aromatase present in GC, and high concentrations of estrogens, in synergism with FSH, stimulate GC differentiation of large preovulatory follicles (Hsueh et al., 1984; Richards, 1994; McGee and Hsueh, 2000; Fortune et al., 2004). Moreover, in preovulatory follicles, FSH stimulates mRNA expression for P450scc, LH receptor, and StAR in GC, resulting in an increase in progesterone production by these cells (Orisaka et al., 2006).

FGFs diverge in their effects over steroidogenesis and subsequently over follicular survival. To date, from the ten members of the FGF family detected in the ovary, at least eight have effects on ovarian steroidogenesis: FGF1, FGF2, FGF7, FGF8, FGF9, FGF10, FGF17, and FGF18.

In cattle, FGF1 had no effect on progesterone production by GC (Schams et al., 2001). In contrast, FGF1 stimulates progesterone production by ovine luteal cells collected from day 5 of the estrous cycle, with no effects on luteal cells collected on later stages (days 10 and 15) of the estrous cycle (Grazul-Bilska et al., 1995). In pigs, FGF1 inhibited progesterone secretion by GC (Biswas et al., 1988), suggesting that differences exist among species in the ovarian cells in response to FGF1.

FGF2 effects on steroidogenesis have been extensively studied, indicating that FGF2 inhibits FSH-stimulated estradiol production and aromatase activity by rat (Baird and Hsueh, 1986; Yamoto et al., 1993) and bovine GC (Vernon and Spicer, 1994; Wandji et al., 1996). Also, FGF2 inhibits LH-stimulated progesterone and androstenedione production by bovine GC (Spicer and Stewart, 1996; Wandji et al., 1996). In contrast, FGF2 enhanced FSH-induced progesterone production by rat GC when FSH was used at suboptimal concentrations (Baird and Hsueh, 1986), and stimulated LH-induced production of both estradiol and progesterone by rat GC (Yamoto et al., 1993). These latter results imply that effects of FGF2 on steroidogenesis may change not only according to species, but also according to other factors such as the dose and type of gonadotropin used and developmental status of follicles when GC were collected.

FGF7 inhibits FSH-stimulated estradiol production and aromatase activity by bovine and rat GC and suppresses hCG-stimulated progesterone production by bovine GC (Parrott and Skinner, 1998) and human GC (Osuga et al., 2001).

FGF8 suppresses FSH-induced estradiol production by rat GC with no effects on production of progesterone and cAMP induced by FSH and forskolin (Miyoshi et al., 2010; Miyoshi et al., 2012).

FGF9 has divergent roles on steroidogenesis, depending on the species. In rats, progesterone production by GC was increased by FGF9 when stimulated by FSH, but not by LH (Drummond et al., 2007), implying that the FGF9 stimulatory effect on progesterone production may be dependent on degree of differentiation of GC. Drummond and colleagues (2007) also observed a stimulatory effect of FGF9 in mRNA levels for the steroidogenic enzymes StAR and P450scc in rat GC, with an inhibitory effect of a high dose of FGF9 on 3β-HSD mRNA in the presence of FSH. In pigs, FGF9 increased estradiol production by GC with inconsistent effects on progesterone production by GC, stimulating progesterone production in the presence of FSH, but inhibiting progesterone production in the presence of

FSH and IGF1 (Evans et al., 2014). In cattle, in contrast to what was observed for rats and pigs, FGF9 attenuated GC production of progesterone and estradiol stimulated by IGF-1 (Schreiber and Spicer, 2012). Schreiber and Spicer (2012) also showed that FGF9 decreased mRNA abundance for FSH receptor and the steroidogenic enzyme P450scc in GC of both small and large follicles with no effects on aromatase or StAR mRNA abundance. In bovine TC, FGF9 also had inhibitory effects on steroidogenesis, attenuating IGF-1 stimulated production of both androstenedione and progesterone and decreasing mRNA abundance for LH receptor and for P450scc enzyme and 17-α-hydroxylase in cells cultured with LH and IGF-1, with no effect on abundance of StAR mRNA (Schreiber et al., 2012).

FGF10 not only suppresses FSH-stimulated estradiol production by bovine GC (Buratini et al., 2007), but also decreased mRNA abundance for FSH receptor and for aromatase, with no effects on mRNA levels for STAR or the steroidogenic enzymes 3β-HSD and P450scc (Castilho et al., 2015).

FGF17 treatment decreased FSH-stimulated estradiol and progesterone secretion by bovine GC from small follicles in diameter (Machado et al., 2009).

FGF18 is a powerful suppressor of ovarian steroidogenesis in cattle (Portela et al., 2010), inhibiting FSH-stimulated estradiol and progesterone by GC in a dose-dependent manner as well as decreasing mRNA abundance for STAR and FSH receptor as well as decreasing aromatase, P450scc, 3β-HSD, and hydroxysteroid (17-β) dehydrogenase 1 mRNA abundance.

2.2 Role of fibroblast growth factors in proliferation of granulosa and theca cells

Members of the FGF family have been reported in many studies as powerful mitogens, acting in many organs and cell types to stimulate cell proliferation (for reviews, see Finch et al., 1989; Rodan et al., 1989; Igarashi et al., 1998; Skaletz-Rorowski et al., 1999; Boilly et al., 2000). These growth factors bind to tyrosine-kinase receptors to activate

Ras/MAPK signaling cascades to stimulate cell proliferation (Dailey et al., 2005; Dengjel et al., 2009), as well as organ development (Hogan, 1999; Metzger and Krasnow, 1999), wound healing (Wahl et al., 1989; Li et al., 2016), tumor formation (Ornitz and Itoh, 2001; Tanner and Grose, 2015), and more recently, ovarian folliculogenesis (Chaves et al., 2012; Price, 2016).

In ovarian follicular development, the compartments of the follicles, namely oocyte, GC, and TC, communicate in paracrine ways to stimulate somatic cell proliferation and follicular development. Presently, at least four members of the FGF family are known to stimulate bovine ovarian follicle cell proliferation: FGF1, FGF2, FGF7, and FGF9 (Gospodarowicz et al., 1977b; Gospodarowicz et al., 1977c; Gospodarowicz et al., 1985; Parrott et al., 1994; Spicer and Stewart, 1996; Schreiber and Spicer, 2012). In chickens, FGF1 and FGF2 stimulate both GC and TC proliferation whereas FGF5 and FGF7 have no effect (Roberts and Ellis, 1999), suggesting that species differences may exist in terms of which FGF is mitogenic in the follicle.

It has been proposed that oocytes carry out this intrafollicular conversation by secreting factors that affect GC morphology, which in turn affect the oocyte (Eppig, 2001; Matzuk et al., 2002) and theca interna (Liu et al., 2015). Indeed, among other factors, bone morphogenesis protein-15 (BMP-15) and GDF-9 are derived from mammalian oocytes to stimulate GC proliferation (Otsuka et al., 2000; Vitt et al. 2000; Spicer et al., 2006), TC proliferation, and theca interna development (Spicer et al., 2008; Liu et al., 2015). Within the FGF family, at least four members are currently known to be secreted by the oocyte of mammalian antral follicles, including FGF8, FGF10, FGF16, and FGF17 (Buratini et al., 2005a; Buratini et al., 2007; Sugiura et al., 2007; Machado et al., 2009; Santos-Biase et al., 2012; Ferreira et al., 2016), and only FGF8 appears to be stimulating GC proliferation, since it leads to expression of genes related to cell proliferation, such as MAPK3/1 and MAPK14, in GC (Jiang et al., 2013; Price, 2016). Future research is needed to verify if FGF8 is indeed

stimulating GC proliferation and if other FGFs are produced by the oocyte to stimulate proliferation of surrounding somatic cells.

Among FGFs that stimulate somatic cell proliferation of bovine ovarian antral follicles, FGF1, FGF2, and FGF7 are mainly produced by TC (Parrott and Skinner, 1998; Berisha et al., 2000; Berisha et al., 2004; Buratini et al., 2007), while the major producers of FGF9 are GC (Schreiber et al., 2012). FGF1, FGF2, and FGF9 stimulate both GC and TC proliferation in either autocrine or paracrine ways (Gospodarowicz et al., 1977b; Gospodarowicz et al., 1977c; Gospodarowicz et al., 1985; Spicer and Stewart, 1996; Schreiber and Spicer, 2012; Schreiber et al., 2012) whereas FGF7 stimulates GC proliferation in a paracrine way (Parrott et al., 1994).

2.3 Role of fibroblast growth factors in ovarian follicular fate

Besides stage of folliculogenesis and species, another important factor associated with the production of FGFs in antral follicles is follicular health (Buratini et al., 2007; Portela et al., 2010). It is now well accepted that antral follicles grow and develop until they reach one of two fates: ovulation or atresia, the latter occurring in the vast majority of the follicles (Hirshfield, 1988; Chun et al., 1994; Jolly et al., 1994; McGee and Hsueh, 2000). Follicular atresia is characterized as a degenerative process associated with apoptosis, occurring mainly in GC of many species, including rats (Hughes and Gorospe, 1991; Chun et al., 1995) and cattle (Rajakowski, 1960; Jolly et al., 1994; Evans et al., 2004). Ovarian follicles in all stages of growth and at any stage of life until the occurrence of reproductive senescence are susceptible to undergo atresia (Hughes and Gorospe, 1991). In cattle, an increase in the rate of atresia of large antral follicles is observed as the estrous cycle advances toward ovulation (Spicer and Echternkamp, 1986). In rats, a dramatic increase in numbers of atretic follicles appears just after ovulation (Hirshfield, 1988). Among other features, atretic follicles are characterized by pyknosis of the nuclei and fragmentation of DNA in GC, a decreased aromatase activity, and thus, estrogen production (Hughes and Gorospe, 1991; Jolly et al., 1994; Chun et al., 1994; van Wezel et al., 1999). Atretic follicles are also

characterized by TC with LH receptors uncoupled from adenylate cyclase, which are incapable of synthesizing steroids in response to LH, resulting in a reduction in theca androgen synthesis concomitant with an increased output of progesterone (McNatty et al., 1985) and TC hypertrophy (Rajakowski, 1960**;** Bukovsky et al., 1993). Therefore, large antral follicles that are selected to escape atresia to become dominant and ovulate have a high estrogen to progesterone ratio (Ireland and Roche, 1982; Spicer and Echternkamp, 1986). To date, members of the FGF family that appear to be associated with health of large antral follicles include FGF1 (Schams et al., 2009), FGF2 (Tilly et al., 1992; Lynch et al., 2000), FGF7 (Castilho et al., 2015), FGF8 (Sugiura et al., 2007; Miyoshi et al., 2010), FGF9 (Schreiber and Spicer, 2012), FGF10 (Buratini et al., 2007), FGF17 (Machado et al., 2009), and FGF18 (Portela et al., 2010). Depending on the species, these FGFs can stimulate survival or atresia of large follicles and their production change according to health of the follicles.

In cattle, FGF1 and FGF8 appear to be contributing to follicular survival. FGF1 stimulates bovine GC and TC proliferation (Gospodarowicz et al., 1977b; Gospodarowicz et al., 1977c) while polymorphisms of the *FGF8* gene were associated with the number of oocytes collected by ovum pick up (Santos-Biase et al., 2012), suggesting possible roles for this growth factor in follicular development.

As already mentioned in this review, FGF2, FGF7, FGF9, FGF10, FGF17, and FGF18 inhibit steroidogenic enzymes activity and FSH-stimulated estradiol production by GC in cattle (Vernon and Spicer, 1994; Parrott and Skinner, 1998; Buratini et al., 2007; Machado et al., 2009; Portela et al., 2010; Schreiber and Spicer, 2012; Castilho et al., 2015). Because estradiol production is important for GC survival and differentiation of dominant follicles (Knecht et al., 1985; Fortune et al., 2004), FGFs suppressing estradiol production may be inducing atresia or preventing differentiation of GC in cattle. In agreement with this idea is the fact that mRNA abundance for *FGF9*, *FGF10*, *FGF17*, and *FGF18* in bovine GC or TC is greater in subordinate or atretic follicles than in dominant follicles (Buratini et al.,

2007; Machado et al., 2009; Portela et al., 2010; Schreiber et al., 2012; Castilho et al., 2015). As mentioned above, FGF2, FGF7, and FGF9 stimulate bovine GC proliferation (Parrott et al., 1994; Spicer and Stewart, 1996; Schreiber and Spicer, 2012), and thus may act as anti-differentiation factors rather than stimulating follicular atresia in cattle. Although FGF10 does not stimulate GC proliferation (Buratini et al., 2007), FGF10 stimulates cumulus expansion and glucose uptake by the cumulus-oocyte complex in cattle (Caixeta et al., 2013), indicating that it may play a role in oocyte growth, maturation, and ovulation (Zuelke and Bracket, 1992; Chen et al., 1993; Downs et al., 1996; Sutton-McDowall et al., 2004). On the other hand, FGF18 induced regression of the dominant follicle when injected in vivo and increased cleaved caspase-3 in GC in vitro (Portela et al., 2015), which is a major downstream effector of apoptosis and serves a marker for GC apoptosis (Feranil et al., 2005), confirming the role for this protein in induction of atresia in bovine antral follicles.

In addition to their roles in folliculogenesis of cattle, FGFs have been shown to play roles in development of antral follicles of other species. *FGF1* and *FGF2* mRNA abundance is greater in TC of porcine periovulatory follicles (Schams et al., 2009), suggesting a positive role of these FGFs in follicular survival in this species. FGF2 seems to stimulate antral follicle survival of rats, since it stimulates the production of both estradiol and progesterone of rat GC (Yamoto et al., 1993) while inhibiting apoptosis of rat GC (Tilly et al., 1992). FGF8 appears to be important in antral follicle development in mice, since it acts to stimulate glycolysis of mouse cumulus cells, which is important to provide energy to the oocyte (Sugiura et al., 2007), but, in rat GC, FGF8 suppressed FSH-induced estradiol production (Miyoshi et al., 2010), what could imply a role in atresia in this species. Similarly, FGF9, as previously mentioned in this review, has stimulatory effects on steroidogenesis of murine (Drummond et al., 2007) and porcine GC (Evans et al., 2014), implying a role in antral follicle survival in these species.

2.4 Role of fibroblast growth factors in luteal function

Some FGFs appear also to influence formation and function of the corpus luteum. The corpus luteum is a transient endocrine gland in the adult ovary that forms from the ruptured follicle after ovulation and secretes progesterone to allow implantation of the early embryo in the endometrium and to provide a uterine environment that sustains pregnancy (as reviewed by Keyes and Wiltbank, 1988). From the 22 known members of the FGF family, at least FGF2, FGF7, FGF9, and/or FGF10 have been detected in corpus luteum of rats (Guthridge et al., 1992; Drummond et al., 2007) and cattle (van Wezel et al., 1995; Salli et al., 1998; Castilho et al., 2008).

In cattle and sheep, FGF1 seems to be contributing to early development of corpus luteum, stimulating luteal cell proliferation (Gospodarowicz et al., 1977b; Grazul-Bilska et al., 1995) and progesterone production of ovine luteal cells early (i.e., day 5) in the estrous cycle (Grazul-Bilska et al., 1995). Nevertheless, FGF1 may also be involved in luteolysis in cattle (Neuvians et al., 2004).

Similarly, FGF2 stimulates progesterone production by luteal cells from cattle (Miyamoto et al., 1992), sheep (Grazul-Bilska et al., 1995), and rats (Tamura et al., 1991). FGF2 has also been reported to stimulate angiogenesis (Cross and Claesson-Welsh, 2001; Robinson et al., 2008), a prominent characteristic of the corpus luteum formation (Keyes and Wiltbank, 1988), being present in vascular cells of early bovine corpus luteum (Schams et al., 1994) and in cytoplasm of endothelial cells of the capilary bed of theca interna and in larger vessels of the theca externa of mature bovine follicles (Berisha et al., 2000). In particular, vascular endothelial growth factor A (VEGFA) and FGF2 synergized to increase bovine luteal endothelial cell networks (Robinson et al., 2008), and intracellular injections of antibodies against either VEGFA or FGF2 markedly reduced corpus luteum volume and plasma progesterone concentrations (Yamashita et al., 2008). Therefore, formation and regulation of corpus luteum is another feature of FGFs in reproduction of mammals.

2.5 Role of fibroblast growth factor receptors on ovarian physiology

The FGFR is a single chain transmembrane tyrosine kinase with two or three immunoglobulin-like domains and a heparin-binding sequence in the extracellular ligand-binding portion (Ornitz and Itoh, 2001; Itoh and Ornitz, 2004; Li et al., 2016). As previously mentioned in this review, there are four distinct genes encoding for FGFRs (*FGF1-FGF4*) in vertebrates and mRNA alternative splicing occurs in the immunoglobulin-like domains III of the *FGFR1*, *FGFR2*, and *FGFR3* genes (but not of *FGFR4*), generating diversity of sequence and resulting in various isoforms (Itoh and Ornitz, 2004; Ornitz and Itoh, 2015; Li et al., 2016). The ligands of the FGF family have different affinities for these receptors (Itoh and Ornitz, 2004), which are determined by the immunoglobulin-like domains (Dell and Williams, 1992).

Because FGFRs have different ligand-binding specificities (see Table 2), the localization of FGFRs in the different follicle compartments is of fundamental importance for the FGFs to exert their effects on ovarian physiology. Of the ligands in this growth factor family that are produced in the ovary, the main receptors, according to ligand binding specificity, that would be expected to be present in order for cells to respond to them are: FGFR1c and FGFR3c for FGF1 and FGF2; FGFR2b followed by FGFR1b for FGF7, FGF10, and FGF22; FGFR3c followed by FGFR4 for FGF8, FGF17, and FGF18; and FGFR3c followed by FGFR2c for FGF9 and FGF16 (Ornitz and Itoh, 2015). Evidence clearly shows species differences exist in terms of which of these FGFRs exist in ovarian cells.

In cattle, *FGFR1c* mRNA, the preferred receptor for FGF1 and FGF2, has been detected in oocytes and in cumulus cells, increasing in response to FSH treatment (Zhang and Ealy, 2012). In chickens, *FGFR1* mRNA and protein are intensely expressed in GC and are critical for FGF1-induced GC proliferation (Lin et al., 2012). Therefore, FGFR1c in cattle and FGFR1 in chickens seem important for the actions of FGF1 in promoting follicular development. In rats, *FGFR1* mRNA was detected in ovaries during proestrus, estrus, and metestrus, but not during diestrus (Asakai et al., 1994). Interestingly, no FGFR1 immunostaining was detectable in human oocytes or GC (Ben-Haroush et al., 2005).

FGFR2b, the main receptor for FGF7, FGF10, and FGF22, is detected in GC and oocytes of cattle (Berisha et al., 2004; Buratini et al., 2007; Machado et al., 2009; Zhang and Ealy, 2012), while FGFR1b, the second most important receptor for these growth factors, is detected in bovine GC, TC, and oocytes (Zhang and Ealy, 2012; Castilho et al., 2015). As previously mentioned in this review, FGF7 and FGF10, produced by TC, decrease aromatase activity of GC (Parrot and Skinner, 1998; Castilho et al., 2015) and thus appear to play a role in anti-differentiation of these cells. Interestingly, *FGFR2b* and *FGFR1b* GC mRNA are both greater in future subordinate follicles than in future dominant follicles (Castilho et al., 2015), and this reinforces the idea that FGFs from this subfamily impede GC to differentiate and therefore may regulate follicle selection in cattle. In pigs, a polytocous species, different than in cattle, *FGFR2b* mRNA abundance is greater in TC than in GC, and greater in theca interna of large and preovulatory follicles than in those of small and medium follicles (Schams et al., 2009), suggesting a role for FGF7 and FGF10 in differentiation of TC in this species.

FGFR2c, the second main receptor for FGF9 and FGF16, has been detected in both bovine GC and TC, without changes in final follicular growth in cattle (Berisha et al., 2004). Little is currently known about the FGF16 functions in GC and TC, but FGF9, as stated earlier in this review, has effects on proliferation and steroidogenesis of both GC and TC in different sizes of antral follicles (Schreiber and Spicer, 2012; Schreiber et al., 2012) and may therefore rely on this receptor in all moments of folliculogenesis in cattle. In pigs, Schams and colleagues (2009) reported a greater *FGFR2c* mRNA in TC from large follicles with no differences for mRNA abundance of this isoform in GC among different sizes of follicles. In contrast, in a more recent study, Evans and coauthors (2014) reported a greater GC *FGFR2c* mRNA abundance in large than in small and medium follicles of gilts. Still, taken together, these observations imply a role for FGF9 in final maturation of ovarian follicles in pigs, and are in agreement with the observation that FGF9 stimulates estradiol production by GC in this species (Evans et al., 2014), as already mentioned in this review.

Bovine FGFR3c, the main receptor for FGF8, FGF9, FGF16, FGF17, and FGF18 was detected in both GC and TC, but not in the oocyte, and increased in GC (but not in TC) of small healthy follicles and in response to FSH (Buratini et al. 2005a), implying a role for these FGFs in early stages of antral follicle development in cattle. In rats, FGFR3 protein levels were detected in oocytes, GC, TC, and corpora lutea (Drummond et al., 2007), suggesting an important role for ligands such as FGF9, an inducer of rat GC progesterone production (Drummond et al., 2007) in folliculogenesis of this species.

Bovine FGFR4, the second most important receptor for FGF8, FGF17, and FGF18, has been detected in TC, but not in GC or oocytes of antral follicles, being greater in small than in large follicles (Buratini et al., 2005a). Since FGF17 and FGF18 are apparently involved in follicular atresia, it is likely that they would be exerting this role in a receptor that is predominant in small antral follicles, since large follicles are being selected for dominance and ovulation. In mice, *FGFR4* mRNA has been detected in GC, but not in TC or oocytes, during most phases of folliculogenesis (Puscheck et al., 1997). In human follicles, FGFR4 immunostaining was most prevalent in oocytes but also existed in GC and stromal cells (Ben-Haroush et al., 2005).

As already mentioned in this review, the intraovarian production of FGFs start early in folliculogenesis. So, for these FGFs to have an effect, FGFRs must also need to be present. Indeed, mRNA encoding at least four FGFRs has currently been detected in fetal bovine preantral follicles, including FGFR1b, FGFR2b, FGFR3c, and FGFR4 (Buratini et al., 2005b; Castilho et al., 2014). In addition, in mice, *FGFR4* mRNA has been detected in GC of primary, secondary, and preantral follicles (Puscheck et al., 1997). Such observations confirm the importance of the FGF family in all stages of folliculogenesis in mammals.

3. Role of other growth factors on ovarian physiology

In addition to FGFs, aforementioned in this review, other growth factors are critical for folliculogenesis. Among these factors, members of the IGF system and the transforming

growth factor β family deserve special attention for their contribution in development of ovarian follicles.

3.1 IGF system

The growth factors IGF-1 and IGF-2 are single-chain polypeptides functionally and structurally related to insulin, first isolated from human plasma in the 1970s (Rinderknecht and Humbel, 1976; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978a; Zapf et al., 1984). The IGFs are mainly produced by the liver to be released in the circulation in order to stimulate endocrine actions in target cells, but are also locally produced in tissues to have a paracrine or autocrine actions (for review, see Sara and Hall, 1990). These actions include DNA and protein synthesis (Canalis, 1980; Zapf et al., 1984), cell proliferation (Frödin and Gammeltoft, 1994), decreased lipolysis (Zapf et al., 1978), increased glucose transport and oxidation (Schwartz and Goodman, 1976; Guler et al., 1987), and increased glycogenesis (Froesch et al., 1985; Parkes et al., 1986; Zapf et al., 1986) on various tissues. Because of their potent mitogenic effects, IGFs have also been reported in cancer of various tissues (Myal et al., 1984; Mattsson et al., 1986; Pollak et al., 1990; Halje et al., 2012).

For IGFs to exert their actions, they bind to two receptors, type 1 and type 2 IGF receptors (IGF-1 receptor and IGF-2 receptor, respectively), in various target tissues (Sara and Hall, 1990). The IGF-1 receptor is a glycoprotein consisting of two extracellular α-subunits linked via disulfide bonds with two transmembranal β-subunits with tyrosine-kinase activity, sharing a structural and functional similarity with the insulin receptor (Massagué and Czech, 1982; Morgan et al., 1986; LeRoith et al., 1995). The binding of the ligand to the extracellular subunits results in intracellular signal transduction by autophosphorylation of tyrosine residues within the intracellular subunits (Morgan et al., 1986). Distinct from the IGF-1 receptor, the IGF-2 receptor is monomeric and does not have intrinsic tyrosine kinase activity, but is phosphorylated on tyrosine residues (Laureys et al., 1988; Schmid, 1995). The IGF-1 receptor has a higher affinity for IGF-1 than for IGF-2, and a low affinity for insulin, while the IGF-2 receptor has a higher affinity for IGF-2 than for IGF-1, with no affinity for

insulin (Massagué and Czech, 1982; Schmid, 1995; Sara and Hall, 1990). Current evidence suggests that IGF-2 receptor is acting as a decoy receptor, inactivating IGF-2 in a similar way to IGFBPs (Spicer and Aad, 2007; Aad et al., 2013).

Effects of IGFs in the ovary of various species have been reported more than three decades ago (Savion et al., 1981; Baranao and Hammond, 1984). Since then, these polypeptides have been described to be important regulators of ovarian follicular cells proliferation, steroidogenesis, and selection of dominant follicles in various species (Adashi et al., 1991; Spicer and Echternkamp, 1995). In addition, synthesis of IGFs by ovarian follicular cells to stimulate autocrine and paracrine actions is under regulation of various hormones (Spicer and Echternkamp, 1995).

The roles of IGFs start early in folliculogenesis with mRNA expression for IGFs and IGF receptors being detected in different stages of preantral follicle development (Silva et al., 2009). In goats, IGF-1 and IGF-2 stimulated early antrum formation and oocyte growth, survival, and competence of prentral follicles (Zhou and Zhang, 2005; Magalhães-Padilha et al., 2012; Duarte et al., 2013). In mice, IGF-1 stimulated oocyte competence of preantral follicles in vitro (Jee et al., 2012). In buffalo, expression of IGF-2 and IGF receptors was detected in oocyte, GC, and TC of preantral follicles (Dubey et al., 2015). In primates, in vitro androgen increased IGF receptors in the oocytes of primordial follicles and increased the number of primary follicles, suggesting that the IGF system may be important for primordial follicle entry into the growth pool (Vendola et al., 1999). In humans, IGF-1 and IGF-1 receptor mRNA and protein have been detected in preantral follicles and IGF-1 stimulated initiation of preantral follicle growth (Stubbs et al., 2013). In cattle, IGF-1 receptor mRNA was detected in oocytes, GC and TC of preantral follicles, suggesting a role for the IGF system in early folliculogenesis (Amstrong et al., 2000; Amstrong et al., 2002).

In bovine antral follicles, *IGF-1* mRNA has been mainly detected in GC and *IGF-2* mRNA has been mainly detected in TC (Spicer et al., 1993; Yuan et al., 1998; Schams et al.,

2002) while IGF-1 receptor mRNA has been detected in GC, TC, and oocytes (Schams et al., 2002; Armstrong et al., 2002) and IGF-2 receptor mRNA has been detected in GC and TC (Spicer and Aad, 2007; Aad et al., 2013). Because IGF-2 has a greater expression than IGF-1 in the bovine ovary (Armstrong et al., 2000), it is currently believed that IGF-2 is the main intrafollicular IGF ligand, performing paracrine and autocrine actions while most of IGF-1 comes from the liver to elicit endocrine effects in this species (Velazquez et al., 2008). In rats, *IGF-1* mRNA is expressed in GC, but not in TC whereas *IGF-2* mRNA is present in TC but not GC (Oliver et al., 1989; Hernandez, 1995). In human small antral follicles, *IGF-1* and *IGF-2* mRNA detection was restricted to TC while IGF-1 receptor mRNA was detected exclusively in GC and IGF-2 receptor was detected in both GC and TC; in dominant follicles, no IGF-1 mRNA has been detected, IGF-2 mRNA has been detected exclusively in GC, IGF-1 receptor mRNA has been detected in GC, and IGF-2 receptor has been detected in both TC and GC (el-Roeiy et al., 1993). In porcine antral follicles, *IGF-1* mRNA has been detected in GC while *IGF-2* mRNA has been detected in TC (Hammond et al., 1985; Yuan et al., 1996).

Ovarian synthesis of IGFs and their receptors is under hormonal regulation. In cattle, FSH, LH, cortisol, insulin, and growth hormone (GH) alone and epidermal growth factor (EGF) or FGF2 together with insulin all decrease GC IGF-1 secretion in vitro (Spicer et al., 1993; Spicer and Chamberlain, 2000) while cortisol and tumor necrosis factor α (TNFα) decreased IGF-1 receptor synthesis by TC (Spicer and Chamberlain, 1998; Spicer, 2001). In rats, whole ovarian IGF-1 synthesis was decreased by FSH and increased by estrogen, IGF-2 production was decreased by estrogen, and IGF-1 receptor synthesis was increased by FSH (Hernandez, 1995; Zhou et al., 2013). In pigs, GH, FSH, and estradiol all increased GC IGF-1 secretion in vitro (Hsu and Hammond, 1987). In humans, FSH stimulates IGF-2 gene expression by GC (Baumgarten et al., 2015).

Mitogenic effects of IGFs in ovarian follicular cells are well established. Both ligands bind to IGF-1 receptors to stimulate cell proliferation (Schmid, 1995; Spicer and Aad, 2007). IGF-1 stimulates proliferation of both GC and TC of cattle (Savion et al., 1981; Spicer et al.,

1993; Stewart et al., 1995; Gutiérrez et al., 1997), pigs (Baranao and Hammond, 1984; May et al., 1988; May et al., 1992; Kolodziejczyk et al., 2003), and rats (Bley et al., 1992; Duleba et al., 1997; Duleba et al., 1999), and also stimulates proliferation of GC from sheep (Monniaux and Pisselet, 1992), and humans (Bergh et al., 1991; Yong et al., 1992). IGF-2 stimulates proliferation of GC of cattle (Spicer and Aad, 2007), humans (Di Blasio et al., 1994; Baumgarten et al., 2015), pigs (Baranao and Hammond, 1984), and rats (Adashi et al., 1985; Duleba et al., 1997).

Effects of IGFs in ovarian steroidogenesis have been extensively studied. In several mammalian species, these polypeptides have been described to amplify gonadotropins effects, stimulating steroidogenesis and steroidogenic enzymes in presence of FSH and LH (Adashi et al., 1991; Spicer and Echternkamp, 1995; Armstrong et al., 1996). In fact, IGF-1 is required for effects of FSH on the steroidogenis through the AKT signaling pathway in humans, mice, and rats GC (Zhou et al., 2013). In cattle, IGF-1 has been reported to enhance FSH-stimulated estradiol and LH-stimulated progesterone production by GC, also enhancing the expression of steroidogenic enzymes such as aromatase (Schams et al., 1988; Spicer et al., 1993; Gutiérrez et al., 1997; Spicer and Chamberlain, 1998; Spicer, 2001; Spicer and Aad, 2007), and to increase LH-stimulated progesterone and androstenedione production by TC (Stewart et al., 1995; Spicer and Chamberlain, 1998; Spicer, 2001). In pigs, IGF-1 enhances FSH-stimulated estradiol and progesterone production by GC (Baranao and Hammond, 1984; Kolodziejczyk et al., 2003; Ranzenigo et al., 2008). In rats, IGF-1 increases the FSH-supported estradiol and progesterone biosynthesis by GC, the FSH-mediated acquisition of LH receptors by GC, and the LH-stimulated progesterone by TC (Adashi et al., 1986; Adashi et al., 1991; Duleba et al., 1999; Zhou et al., 2013). In humans and sheep, IGF-1 also has a stimulatory effect in FSH-stimulated estradiol production (Bergh et al., 1991; Armstrong et al., 1996). Similar to IGF-1, IGF-2 enhances FSH-stimulated estradiol and progesterone biosynthesis and aromatase expression in bovine GC (Spicer and Aad, 2007), FSH-stimulated estradiol and progesterone production by human GC (Devoto et al., 1999; Baumgarten et al., 2015), and FSH-stimulated

progesterone production by porcine GC (Baranao and Hammond, 1984). These steroidogenic effects of IGF-2 are believed to be performed through the IGF-1 receptor (Spicer and Aad, 2007). Recently, it was suggested that changes in IGF-2 receptor abundance in GC may regulate ovulation rate in cattle (Aad et al., 2012).

Because of their stimulatory effects on steroidogenesis, IGFs are considered important for follicular selection for dominance in monotocous species such as cattle (Spicer, 2004; Fortune et al., 2004). Although, there are no differences in total IGF intrafollicular concentrations between dominant and subordinate follicles in cattle (Stewart et al., 1996), levels of insulin-like binding proteins (IGFBPs) change during follicular development in various species, including sheep and cattle (Monget et al., 1993; Spicer at al., 2001). The IGFBPs are important for storage and transportation of IGFs through circulation, being important regulators of the IGFs physiological action (Sara and Hall, 1990; Murphy, 1998). In the ovary, six IGFBPs (IGFBP-1 to IGFBP-6) have been identified in antral follicles of mammals (for reviews, see Spicer and Echternkamp, 1995; Fortune et al., 2004; Spicer, 2004). In cattle, the major forms of IFBPs produced by GC are IGFBP-2, IGFBP-5, and IGFBP-4 while the major forms produced by TC are IGFBP-3, IGFBP-2, IGFBP-5, and IGFBP-4 (Chamberlain and Spicer, 2001); in sheep, the major forms produced by GC are IGFBP-2, IGFBP-5, and IGFBP-1 while TC produces mainly IGFBP-4 and IGFBP-2 (Armstrong et al., 1996); in pigs, GC produce IGFBP -2 to -6 (Leighton et al., 1993). Some IGFBPs, specifically the low molecular weight IGFBP -2, -4, and -5 are increased in atretic follicles of sheep (Monget et al., 1993), cattle (de la Sota et al., 1996; Stewart et al., 1996; Spicer et al., 2001; Rivera and Fortune, 2003), mice (Wandji et al., 1998), and humans (San Roman and Magoffin, 1993; Wright et al., 2002). Moreover, IGFBP -2, -3, -4, and -5 and have inhibitory effects on IGF-stimulated steroidogenesis, binding IGFs and preventing them to bind to their receptors (Ui et al., 1989; Bicsak et al., 1990; Spicer and Chamberlain, 1999; Wright et al., 2002). Therefore, for follicular dominance to occur, levels of low molecular IGFBPs need to be reduced, which happens through the action of specific proteases such as

pregnancy-associated plasma protein A (Spicer et al., 2001; Rivera et al., 2001; Rivera and Fortune, 2003; Spicer, 2004).

In summary, the ovarian IGF system (IGF ligands, receptors, and binding proteins) is critical for follicular development and selection for dominance through mitogenic and steroidogenic activity. The bioavailability of IGF ligands, especially IGF-1, is more important than their total concentrations and levels of IGBPs are therefore suppressed in dominant follicles by specific proteases.

3.2 Transforming growth factor β family

The TGF-β superfamily is a large group of more than 35 polypeptides in vertebrates that regulate many aspects of cell proliferation and organogenesis, including development of gonads, heart, bones, and blood vessels. Members of this superfamily include TGF-βs, bone morphogenic proteins (BMPs), growth differentiation factors (GDFs), anti-Müllerian hormone (AMH), activins, and inhibins (for review, see Chang et al., 2002).

Members of the TGF-β superfamily possess homo- or hetero-dimers that bind to and activate two types of transmembrane serine/threonine kinase receptors, which then stimulate downstream regulatory SMAD proteins to migrate from the cytoplasm to the nucleus where they can act as transcriptional regulators. Activins and TGF-βs typically use different signal transduction pathways than BMPs. Activins and TGF-βs will first bind receptor type II, which will then recruit and phosphorylate receptor type I, which will in turn phosphorylate SMAD2 or SMAD3, allowing each to migrate to the nucleus and interact with SMAD4 and transcription factors to stimulate specific target genes; BMPs will bind to either type I or type II receptor to stimulate formation of a quaternary complex and then type I receptor will be phosphorylated by type II and will phosphorylate SMAD1, SMAD5, or SMAD8, allowing each to translocate to the nucleus to interact with SMAD4 and transcription factors in order to stimulate specific target genes (for reviews, see Heldin et al., 1997; Chang et al., 2002).

Many TGF-β superfamily members have been detected in the mammalian ovary, including TGF-α, TGF- β, inhibin, activin, follistatin, BMP-2, BMP-3, BMP-4, BMP-6, BMP-7, GDF-9, BMP-15, AMH (for reviews, see Ying, 1988; Knight and Glister, 2001; Durlinger et al., 2002a; Shimazaki et al., 2004; Drummond, 2005; Skinner, 2005; Mazerbourg and Hsueh, 2006). These polypeptides regulate folliculogenesis of various species through paracrine, autocrine, and endocrine actions, playing roles in ovarian steroidogenesis (Ying et al., 1986; Miró et al., 1991; Shukovski et al., 1991; Shimasaki et al., 1999; Campbell et al., 2012), follicular cell proliferation (Roberts and Skinner, 1991; Glister et al., 2004; Spicer et al., 2008; Kayani et al., 2009), and oocyte maturation (Alak et al., 1998).

3.2.1 Role of TGF-α and TGF- β on folliculogenesis

The 50 amino acid polypeptide TGF-α is structurally similar to epidermal growth factor (EGF) and able to bind to EGF receptors to elicit its effects in a variety of cells, including skin keratinocytes and epithelial cells in the brain, pituitary, and various embryonic tissues (Brachmann et al., 1989). In the ovary, *TGF-α* mRNA has been detected in rat GC (Yeh et al., 1993), in bovine TC (Skinner and Coffey, 1988; Lobb and Dorrington, 1992), in human TC (Lobb, 2009), and mainly in porcine GC, although small amounts were also detected in porcine TC (Singh and Armstrong, 1995).

The TGF-β polypeptide is a dimer of 112-amino acid subunits linked by disulfide bonds. In mammals, the family of TGF-β consists of at least four closely related isoforms: TGF-β1 (commonly referred as simply "TGF-β"), TGF-β2, TGF-β3, and TGF-β4 (for review, see Lyons and Moses, 1990). In the ovary of cattle, TGF-β1 has been detected in oocytes and GC while TGF-β2 and TGF-β3 have been detected in oocytes, GC, and TC with a stronger intensity than TGF-β1 in preantral and antral follicles (Nilsson et al., 2003). In sheep, TGF-β1 and TGF-β2 are restricted to TC (Juengel et al., 2004) and in humans, TGF-β1 has been detected in GC and TC of antral follicles (Lobb, 2009). In pigs, TC are the main source of TGF-β1 (May et al., 1996) and in rats, both TGF-β1 and TGF-β2 have been detected in GC

(Kim and Schomberg, 1989; Teerds and Dorrington, 1992), TC (Teerds and Dorrington, 1992), and oocytes (Teerds and Dorrington, 1992; Ergin et al., 2008), but the amounts of TGF-β1 are always greater than TGF-β2 in these cells (Teerds and Dorrington, 1992).

Although recognized for their mitotic effects in many tissues, TGFs have different actions in ovarian follicular cell proliferation according to the species. In cattle, TGFs have contrasting effects on GC proliferation: while TGF-α stimulates proliferation of GC, TGF-β inhibits GC proliferation by paracrine and autocrine actions (Lobb and Dorrington, 1992). In rats, TGF-β stimulates GC proliferation (Saragüeta et al., 2002) whereas both TGF-α and TGF-β increase TC proliferation (Pehlivan et al., 2001). In pigs, TGF-β has been described to inhibit EGF-stimulated GC proliferation, although the opposite has also been observed under diverse experimental circumstances (May et al., 1988; Mondschein et al., 1988). In humans, TGF-α stimulates, while TGF-β1 inhibits, GC from small antral follicles (Lobb, 2009).

The effects of TGF-α and TGF-β on steroidogenesis also vary according to the species. In cattle, TGF-α suppresses FSH-stimulated estradiol production by GC while both TGF-α and TGF-β suppress estradiol-stimulated progesterone production by TC (Roberts and Skinner, 1991; Legault et al., 1999) and EGF inhibited insulin-induced progesterone and androstenedione production by TC (Spicer and Stewart, 1996). Nevertheless, in the presence of estradiol, TGF-β increases progesterone production by TC, suggesting a distinct role for this polypeptide according to the stage of follicle development in cattle (Roberts and Skinner, 1991). In rats, both TGF-α and TGF-β enhance FSH-stimulated progesterone synthesis by GC (Yeh et al., 1993; Ke et al., 2004; Chen et al., 2008). In pigs, TGF-α stimulates estradiol production by GC from prepubertal females (Gangrade et al., 1991) whereas TGF-β inhibits FSH-induced progesterone production by porcine GC (Mondschein et al., 1988). In sheep, TGF-β suppresses progesterone production by GC (Juengel et al., 2004).

3.2.2 Role of activin, inhibin, and follistatin on folliculogenesis

Activins and inhibins are disulphide-linked dimeric glycoproteins: while inhibins are dimers of a α subunit linked to either a α A or a α B subunit to generate inhibin A (α-βA) or inhibin B (β-βB), activins are a result of dimerization of β subunits, which results in three forms of activin known as activin A (βA-βA), activin AB (βA-βB) and activin B (βB-βB). Follistatins are single chain glycosylated polypeptides rich in cysteine that bind to and suppress the effects of activin (for reviews, see Ying, 1988; Knight and Glister, 2001).

The first recognized role for activins, inhibins, and follistatin in reproduction was their ability to modulate the secretion of FSH from anterior pituitary gonadotropins in vitro. More specifically, inhibins and follistatin suppress FSH secretion while activins enhance FSH secretion (for reviews, see Ying, 1988; de Kretser and Robertson, 1989; Knight and Glister, 2001). In the 1980s, several groups isolated inhibins, activins, and follistatin from follicular fluid (Robertson et al., 1985; Ling et al., 1986; Ueno et al., 1987) and ovaries of farm animals (Henderson and Franchimont, 1981; Michel et al., 1989).

In the ovary of cattle, inhibins have been detected in mainly in GC, but also in TC (Henderson and Franchimont, 1981; Torney et al., 1989; Izadyar et al., 1998), activins have been detected in GC (Izadyar et al., 1998), and follistatin has been detected in GC and oocytes (Izadyar et al., 1998). In rats, inhibins have been detected in GC, TC, and oocytes (Erickson and Hsueh, 1978; Suzuki et al., 1987; Meunier et al., 1988; Ogawa et al., 1991), and activins and follistatin have been detected mainly in GC (Meunier, 1988; Kogawa et al., 1991; Nakatani et al., 1991), and follistatin has been detected in GC (Kogawa et al., 1991). In pigs, inhibins and follistatin have been detected in GC (Michel et al., 1989; Lindsell et al., 1995), and activins have been detected in in GC and TC (Van Den Hurk and Van De Pavert, 2001). In humans, inhibins have been detected in GC (Channing et al., 1984; Yamoto et al., 1992; Erämaa et al., 1995; Sidis et al., 1998), activins have been detected in GC (Sidis et al., 1998), and follistatin has been detected in GC and oocytes (Tuuri et al., 1994; Sidis et al., 1998).

Activins, inhibins, and follistatin play a distinct role in folliculogenesis of different species. In cattle, while activins have a suppressive effect on LH-stimulated progesterone production by GC and TC from antral follicles, follistatin increased LH-stimulated progesterone production on the same cell types (Shukovski et al., 1991; Shukovski et al., 1993), indicating that the actions of activin should be suppressed as the follicle approaches luteinization. In addition, inhibins suppress estradiol production by bovine GC (Jimenez-Krassel et al., 2003), showing an anti-differentiation action. In rats, activins act to increase responsiveness of GC to FSH, increasing FSH receptor concentrations, aromatase expression, progesterone secretion, estradiol production, and GC proliferation (Xiao et al., 1992; Li et al., 1995; Miró and Hiller, 1996) while follistatin suppresses activin-stimulated GC proliferation (Li et al., 1995) and inhibin increases proliferation of GC (Woodruff et al., 1990). In pigs, activin inhibits FSH-stimulated estradiol and progesterone synthesis (Chang et al., 1996). In humans, activin stimulates GC proliferation (Rabinovici et al., 1990), but inhibits FSH-stimulated estradiol and progesterone by GC while inhibin enhances FSH-stimulated estradiol production by GC and LH-stimulated progesterone production by TC (Alak et al., 1998; Gilling-Simth et al., 1997), and follistatin suppresses activin inhibitory effect on FSH-stimulated estradiol and progesterone synthesis by GC (Cataldo et al., 1994).

3.2.3 Role of oocyte-derived GDF-9, BMP-6, and BMP-15 on folliculogenesis

The communication between oocyte and surrounding somatic cells (GC and TC) is critical for folliculogenesis and it has been suggested that the oocyte carries this conversation (Matzuk et al., 2002). Among the members of the TGF-β superfamily that regulate ovarian folliculogenesis, GDF-9, BMP-15, and BMP-6 are produced mainly by oocytes (for review, see Elvin et al., 2000). Indeed, *GDF-9* mRNA and protein have been detected in the oocyte of primary, secondary and preantral follicles of rat (Hayashi et al., 1999; Jaatinen et al., 1999), in oocytes of human primary follicles (Aaltonen et al., 1999), in oocytes of mice at all stages of follicular development, except in primordial follicles (McGrath et al., 1995; Dube et al., 1998), and mainly in oocyte, but also in GC, of cattle during all stages of follicular

development (Bodensteiner et al., 1999; Spicer et al., 2008). BMP-15, which is structurally related to GDF-9, has a similar localization to GDF-9 in rats (Jaatinen et al., 1999), mice (Dube et al., 1998), and humans (Aaltonen et al., 1999), but has also been detected in oocytes and GC of antral follicles of rats (Erickson and Shimasaki, 2003) and exclusively in oocytes of bovine antral follicles (Pennetier et al., 2004). BMP-6 has been detected in oocytes of mice (Lyons et al., 1989) and in TC, GC, and oocytes from antral follicles of cattle (Glister et al., 2004; Kayani et al., 2009).

Knockout mice models indicate a greater importance for GDF-9 to fertility in comparison to BMP-15 and BMP-6, at least in the murine species: while GDF-9 knockout female mice are completely infertile and have no follicular development beyond the primary one layer follicle stage (Dong et al., 1996), BMP-15 deficient female mice are only subfertile, having reduced ovulation and defects in early follicle development (Yan et al., 2001), and BMP-6 deficient female mice are fertile (Solloway et al., 1998). Observations that GDF-9 deficient female mice have a limited oocyte development and have an impaired attachment of cumulus cells to the zona pelucida of oocytes may explain why this factor is critical in the early stages of follicular development (Carabatsos et al., 1998), since the communication between the oocyte and surrounding somatic cells is not working properly in this case. In addition, a recent study revealed that GDF9-induced Indian hedgehog homolog (IHH) production by GC is needed for TC development (Liu et al., 2015). Moreover, in later stages of folliculogenesis, GDF-9 stimulates progesterone production and the expression of enzymes involved in cumulus expansion by mice GC (Elvin et al., 1999).

In rats, GDF-9 promotes follicular survival and growth during the preantral to early antral transition, increasing estradiol and testosterone production by GC and therefore suppressing GC apoptosis and follicular atresia (Hayashi et al., 1999; Orisaka et al., 2006; Orisaka et al., 2009). In later stages, GDF-9 stimulates GC proliferation while it suppresses FSH-stimulated estradiol and progesterone production by GC from small antral follicles and preovulatory follicles of mice, therefore suppressing GC differentiation (Vitt et al., 2000).

BMP-15 also stimulates proliferation of GC from rat antral follicles while it suppresses FSH-stimulated progesterone production with no effects on FSH-stimulated estradiol (Otsuka et al., 2000; Otsuka et al., 2001a; Otsuka and Shimasaki, 2002), therefore acting as an anti-differentiation factor. Different than GDF-9 and BMP-15, BMP-6 does not stimulate mitogenic activity of rat GC, but acts like BMP-15 on steroidogenesis of GC, suppressing FSH-stimulated progesterone production without affecting estradiol production (Otsuka et al., 2001b).

In cattle, GDF-9 acts on TC from antral follicles, enhancing LH-stimulated TC proliferation in small follicles, but suppressing LH-stimulated TC proliferation in large follicles in the presence of IGF-1 (Spicer et al., 2008). Also, GDF-9 suppresses IGF-1 stimulation of both androstenedione and progesterone by TC from all sizes of antral follicles (Spicer et al., 2008). Similarly, GDF-9 stimulates proliferation of GC but inhibits IGF-1-induced steroidogenesis of GC from both small and large follicles of cattle (Spicer et al., 2006). These studies suggest that GDF-9 is acting as an anti-differentiation factor in cattle. On the other hand, when added to in vitro maturation medium of bovine cumulus-oocyte complexes, BMP-15 enhanced cumulus expansion, which is important for oocyte competence (Caixeta et al., 2013). Finally, oocyte-derived BMP-6 stimulates GC proliferation and estradiol production while suppressing progesterone production by both GC and TC from small bovine antral follicles (Glister et al., 2004; Kayani et al., 2009), thereby stimulating follicular survival.

3.2.4 Role of BMPs on folliculogenesis

In addition to BMPs derived from oocyte, BMP-2, BMP-3, BMP-4, and BMP-7 may also regulate the ovarian follicular development of mammals. These polypeptides regulate folliculogenesis of several species through modulation of steroidogenesis and mitosis of follicular ovarian somatic cells (for review, see Shimasaki et al., 2004). Among the BMPs produced by the ovary, BMP-2 has been detected mainly in GC of cattle (Kayani et al., 2009)

and rats (Erickson and Shimasaki, 2003), and in oocytes of pigs (Brankin et al., 2005a; Paradis et al., 2009). BMP-3 has been detected in TC of rats (Erickson and Shimasaki, 2003) and in GC of humans (Jaatinen et al., 1996) whereas BMP-4 has been detected in TC of cattle (Glister et al., 2004; Kayani et al., 2009) and rats (Shimasaki et al., 1999; Erickson and Shimasaki, 2003), and in the oocyte and GC of pigs (Zhu et al., 2008). BMP-7 has been detected in TC of cattle (Glister et al., 2004; Kayani et al., 2009) and rats (Shimasaki et al., 1999; Erickson and Shimazaki, 2003).

In cattle, BMP-2 functions as an autocrine factor during follicular dominance and ovulation in cattle, because it simulates estradiol production by GC and its expression in GC increases as antral follicles grow (Selvaraju et al., 2013). In pigs, BMP-2 suppresses progesterone production by GC and TC without affecting GC or TC proliferation (Brankin et al., 2005a; Brankin et al., 2005b).

Theca-derived BMP-4 and BMP-7 may also alter follicular development by increasing GC proliferation and modulating steroidogenesis. In rats, BMP-4 and BMP-7 increased FSH-stimulated estradiol production while suppressing FSH-stimulated progesterone production by GC (Shimasaki et al., 1999; Lee et al., 2001; Inagaki et al., 2009). Also in rats, BMP-4 promotes the primordial-to-primary follicle transition in neonatal rat ovaries (Nilsson and Skinner, 2003). In cattle, BMP-4 and BMP-7 inhibit steroidogenesis in GC (Glister et al., 2004; Spicer et al., 2006; Yamashita et al., 2011) and TC (Glister et al., 2005; Spicer et al., 2008). In humans, BMP-4 and BMP-7 appear to play a role in atresia, since both polypeptides decrease gap junction intercellular communication activity (Chang et al., 2013) while BMP-4 decreases basal estradiol production by GC (Khalaf et al., 2013).

3.2.5 Role of AMH on folliculogenesis

During fetal development, AMH, also called Müllerian inhibiting substance (MIS), is produced only by Sertoli cells, but ovarian follicles also produce this polypeptide after birth (Münsterberg and Lovell-Badge, 1991). The main ovarian follicular compartment producing

AMH in various species is GC: in cattle, *AMH* mRNA is expressed in GC of healthy antral follicles (Ireland et al., 2009), and in sheep, *AMH* mRNA is expressed exclusively in GC from secondary and antral follicles, decreasing as the follicles grow (Campbell et al., 2012). In mice, AMH protein has been detected in primary and early secondary follicles (Durlinger et al., 2002b), and in rats, *AMH* mRNA is expressed in GC from preantral and small antral follicles (Baarends et al., 1995). In humans, AMH protein has been detected in secondary, preantral, and small antral follicles (Weenen et al., 2004), and may be a valuable indicator of ovarian reserve in human assisted reproduction (La Marca et al., 2010; Tolikas et al., 2011).

In cattle, AMH is a biomarker for fertility, since GC from cows with high numbers of antral follicles per ovary have a greater AMH expression than those with numbers of antral follicles per ovary (Ireland et al., 2009), and abundance of GC *AMH* mRNA is greater in healthy than in atretic follicles (Rico et al., 2009). In contrast, knockdown of AMH bioactivity stimulates development of ovine antral follicles, suggesting an inhibitory role for AMH in modulating the response of both TC and GC to gonadotropins in sheep (Campbell et al., 2012). Furthermore, AMH suppresses FSH-stimulated estradiol production by GC in sheep (Campbell et al., 2012). In mice, AMH reduces numbers of growing primordial follicles (Durlinger et al., 2002) and reduces follicular diameter of preantral follicles in vitro (Durlinger et al., 2001), suggesting a negative regulation of folliculogenesis in this species. In humans, AMH reduces development of primordial follicles (Carlsoon et al., 2006).

4. Bioinformatics tools for microarray analysis

With the advent of the genome projects, our knowledge of the genomic sequences of humans and other organisms greatly increased (Quackenbush, 2001). Subsequently, a diversity of techniques has been developed to investigate the increasing body of information provided by the knowledge of the genome (Quackenbush, 2001). One of these techniques is cDNA microarray, which allows rapid quantification of expression levels of several genes in parallel (Schena et al., 1995; Schena, 1996).

The technology of cDNA microarray consists of reversing mRNA into cDNA labeled with fluorescent nucleotides, which then gets hybridized to a microarray under a glass cover slip and scanned for fluorescein emission following laser excitation (Schena et al., 1995; Schena, 1996). Differential expression measurements are carried out using a simultaneous, two-color hybridization approach of two biological samples of interest, which are mixed and hybridized to a single microarray for posterior scanning at two wave-lengths following independent excitation of the two fluors (Schena et al., 1995; Schena, 1996). Measurement of fluorescence intensity through scanning allows quantification of gene expression and, therefore, the direct comparison of gene expression levels between the samples (Schena et al., 1995; Schena, 1996; Quackenbush, 2001).

Identifying the various patterns in which a gene is expressed in a microarray and grouping genes into expression clusters provide clues into their biological function (Schena et al., 1995; Quackenbush, 2001). Nevertheless, interpretation of microarray expression data is not an exact science and can be quite challenging (Bowtell, 1999; Quackenbush, 2001; Khatri and Draghici, 2005; Huang et al., 2009; Henderson-MacLennan et al., 2010). Therefore, bioinformatics tools have becoming increasingly important as the technology for microarray-based genomic profiling has been progressing (Eisen et al., 1988; Bowtell, 1999; Subramanian et al., 2005; Capriotti et al., 2012).

Over the last few decades, bioinformatics approaches made it possible to find genes of pertinent biological functions among large lists of genes (Huang et al., 2009; Capriotti et al., 2012). In order to help researchers to find genes of interest in large lists, various high-throughput enrichment tools, including Onto-Express, GoMiner, and Database for Annotation, Visualization, and Integrated Discovery (DAVID), among many others, were developed (Khatri and Draghici, 2005; Huang et al., 2009). These bioinformatics enrichment tools identify groups of genes that share common biological function, chromosomal location, or regulatory elements (Subramanian et al., 2005; Huang et al., 2009). The theory behind this method is that clusters of genes with a common biological process, regulation, or at the same

chromosomal location (based on prior knowledge), should have an enriched potential as a relevant group by the high-throughput screening technologies (Subramanian et al., 2005; Huang et al., 2009). Therefore, instead of just comparing individual genes that were up- or down- regulated by a treatment or condition, bioinformatics enrichment tools give the researcher an opportunity to visualize how genes are being affected in a more global and logical manner.

The importance of enrichment tools such as DAVID can be observed in microarray research using Affymetrix gene chips to unveil altered biological functions in response to a pathological condition or a hormonal treatment, categorizing clusters of differentially expressed transcripts. For example, in farm animals, DAVID was used for identifying biological functions altered by IGF-1 treatment in cultured porcine GC (Grado-Ahuir et al., 2009) or to detect functional differences between bovine GC extracted from healthy or cystic antral follicles (Grado-Ahuir et al., 2009), providing valuable data to improve reproductive efficiency in pigs and cattle. Moreover, DAVID can be used to investigate biological functions altered by pathologies such as human prostatic cancer (Rowehl et al., 2008) or diabetic nephropathy (Cohen et al., 2008), providing a better understanding of causes and consequences of human diseases and contributing to a possible improvement in therapy efficiency.

Other valuable bioinformatics tool for the interpretation and management of large-scale genetic data sets is the pathway analysis software. Since biological processes commonly involve more than one pathway, this tool identifies networks of pathways altered by a treatment, biological condition, or different time points, allowing identification of biological functions and canonical signaling pathways, determining the enrichment of differentially expressed genes within groups of interconnected pathways and contributing to provide meaning to identified differences in gene expression (Werner, 2008; Henderson-MacLennan et al., 2010). Examples of pathway analysis software are Ingenuity Pathway Analysis (IPA),

GeneGo, BioBase, Genomatix, and Pathway Studio (Werner, 2008; Henderson-MacLennan et al., 2010).

The web-based pathway analysis software IPA facilitates a meaningful interpretation of data by allowing the identification of canonical pathways, mechanistic networks, upstream regulators, and downstream biological functions affected by diseases, treatments, or different time points (Krämer et al., 2013). Examples of the use of this software include the investigation of bovine ovarian follicular activation (Yang and Fortune, 2015), bovine oocyte maturation (Mamo et al., 2011), differences between ovarian follicular somatic cells (Hatzirodos et al., 2014a; 2014b) of healthy or atretic bovine antral follicles, and differences between microRNAs expression pattern in bovine GC of dominant or subordinate follicles (Wondim et al., 2014), which shows how the use of this bioinformatics tool has been emerging to help researchers to understand biological processes such as ovarian folliculogenesis.

Table 1. Characterization of FGFs subfamilies and their secretion from the cells.

FGF Subfamily	Signal peptide and secretion
FGF 1/ 2	Lack cleavable signal peptide, but are still secreted through non-conventional mechanisms
FGF 3/7/10/22	Have N-terminal signal peptide and are secreted through classical mechanisms
FGF 4/5/6	Have N-terminal signal peptide and are secreted through classical mechanisms
FGF 8/17/18	Have N-terminal signal peptide and are secreted through classical mechanisms
FGF 9/16/20	Lack cleavable signal peptide, but are still secreted through non-conventional mechanisms
FGF 11/12/13/14	Lack signal peptide and remain inside the cell
FGF 15/19/21/23	Have N-terminal signal peptide and are secreted through classical mechanisms

Adapted from: Itoh and Ornitz (2011); Li et al. (2016).

Table 2. Receptor affinities for different FGFs subfamilies.

FGF Subfamily	Receptor affinity
FGF 1/ 2	FGFR 1c, 3c > 2c, 1b, 4
FGF 3/7/10/22	FGFR 2b > 1b
FGF 4/5/6	FGFR 1c, 2c > 3c, 4
FGF 8/17/18	FGFR 3c > 4 > 2c > 1c > 3b
FGF 9/16/20	FGFR 3c > 2c > 1c, 3b > 4
FGF 11/12/13/14	Do not bind to FGFRs
FGF 15/19	FGFR 1c, 2c, 3c, 4
FGF 21	FGFR 1c, 3c
FGF 23	FGFR 1c, 3c, 4

Adapted from: Ornitz and Itoh (2015).

5. References

Aad, P. Y., S. E. Echternkamp, and L. J. Spicer. 2013. Possible role of IGF2 receptors in regulating selection of 2 dominant follicles in cattle selected for twin ovulations and births. Domest. Anim. Endocrinol. 45: 187-195.

Aad, P. Y., S. E. Echternkamp, D. D. Sypherd, N. B. Schreiber, and L. J. Spicer. 2012. The hedgehog system in ovarian follicles of cattle selected for twin ovulations and births: Evidence of a link between the IGF and hedgehog systems. Biol. Reprod. 87: 1-10.

Aaltonen, J., M. P. Laitinen, K. Vuojolainen, R. Jaatinen, N. Horelli-Kuitunen, L. Seppa, H. Louhio, T. Tuuri, J. Sjoberg, R. Butzow, O. Hovatta, L. Dale, and O. Ritvos. 1999. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9b are expressed in oocytes during early folliculogenesis. J. Clin. Endocrinol. Metab. 84: 2744-2750.

Abraham, J. A., A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Hjerrild, D. Gospodarowicz, and J. C. Fiddes. 1986. Nucleotide-sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth-factor. Science 233: 545-548.

Ackert, C. L., J. E. I. Gittens, M. J. O'Brien, J. J. Eppig, and G. M. Kidder. 2001. Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. Dev. Biol. 233: 258-270.

Adashi, E. Y., C. E. Resnick, A. J. Dercole, M. E. Svoboda, and J. J. Vanwyk. 1985. Insulin-like growth-factors as intraovarian regulators of granulosa-cell growth and function. Endocr. Rev. 6: 400-420.

Adashi, E. Y., C. E. Resnick, E. R. Hernandez, A. Hurwitz, C. T. Roberts, D. Leroith, and R. Rosenfeld. 1991. Insulin-like growth factor i as an intraovarian regulator: Basic and clinical implications. Ann. N. Y. Acad. Sci. 626: 161-168.

Adashi, E. Y., C. E. Resnick, M. E. Svoboda, and J. J. Vanwyk. 1986. Follicle-stimulating-hormone enhances somatomedin-c binding to cultured rat granulosa-cells - evidence for camp dependence. J. Biol. Chem. 261: 3923-3926.

Alak, B. M., S. Coskun, C. I. Friedman, E. A. Kennard, M. H. Kim, and D. B. Seifer. 1998. Activin a stimulates meiotic maturation of human oocytes and modulates granulosa cell steroidogenesis in vitro. Fertil. Steril. 70: 1126-1130.

Armelin, H. A. 1973. Pituitary extracts and steroid-hormones in control 3t3-cell growth. Proc. Natl. Acad. Sci. U S A 70: 2702-2706.

Armstrong, D. G., G. Baxter, C. O. Hogg, and K. J. Woad. 2002. Insulin-like growth factor (IGF) system in the oocyte and somatic cells of bovine preantral follicles. Reproduction 123: 789-797.

Armstrong, D. G., C. G. Gutierrez, G. Baxter, A. L. Glazyrin, G. E. Mann, K. J. Woad, C. O. Hogg, and R. Webb. 2000. Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. J. Endocrinol. 165: 101-113.

Armstrong, D. G., C. O. Hogg, B. K. Campbell, and R. Webb. 1996. Insulin-like growth factor (IGF)-binding protein production by primary cultures of ovine granulosa and theca cells. The effects of IGF-I, gonadotropin, and follicle size. Biol. Reprod. 55: 1163-1171.

Asakai, R., S. Song, N. Itoh, T. Yamakuni, K. Tamura, and R. Okamoto. 1994. Differential gene-expression of fibroblast growth-factor receptor isoforms in rat ovary. Mol. Cell. Endocrinol. 104: 75-80.

Baarends, W. M., J. T. J. Uilenbroek, P. Kramer, J. W. Hoogerbrugge, E. C. M. Vanleeuwen, A. P. N. Themmen, and J. A. Grootegoed. 1995. Anti-mullerian hormone and anti-mullerian hormone type-II receptor messenger-ribonucleic-acid

expression in rat ovaries during postnatal-development, the estrous-cycle, and gonadotropin-induced follicle growth. Endocrinology 136: 4951-4962.

Baird, A., and A. J. Hsueh. 1986. Fibroblast growth factor as an intraovarian hormone: Differential regulation of steroidogenesis by an angiogenic factor. Regul. Pept. 16: 243-250.

Bao, B., and H. A. Garverick. 1998. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: A review. J. Anim. Sci. 76: 1903-1921.

Baranao, J. L. S., and J. M. Hammond. 1984. Comparative effects of insulin and insulin-like growth-factors on DNA-synthesis and differentiation of porcine granulosa-cells. Biochem. Biophys. Res. Commun. 124: 484-490.

Barclay, A. N. 2003. Membrane proteins with immunoglobulin-like domains--a master superfamily of interaction molecules. Semin. Immunol. 15: 215-223.

Baumgarten, S. C., S. M. Convissar, A. M. Zamah, M. A. Fierro, N. J. Winston, B. Scoccia, and C. Stocco. 2015. Fsh regulates IGF-2 expression in human granulosa cells in an akt-dependent manner. J. Clin. Endocrinol. Metab. 100: E1046-E1055.

Ben-Haroush, A., R. Abir, A. Ao, S. Jin, G. Kessler-Icekson, D. Feldberg, and B. Fisch. 2005. Expression of basic fibroblast growth factor and its receptors in human ovarian follicles from adults and fetuses. Fertil. Steril. 84: 1257-1268.

Bergh, C., J. H. Olsson, and T. Hillensjo. 1991. Effect of insulin-like growth factor-I on steroidogenesis in cultured human granulosa-cells. Acta Endocrinologica 125: 177-185.

Berisha, B., D. Schams, M. Kosmann, W. Amselgruber, and R. Einspanier. 2000. Expression and localisation of vascular endothelial growth factor and basic fibroblast

growth factor during the final growth of bovine ovarian follicles. J. Endocrinol. 167: 371-382.

Berisha, B., F. Sinowatz, and D. Schams. 2004. Expression and localization of fibroblast growth factor (FGF) family members during the final growth of bovine ovarian follicles. Mol. Reprod. Dev. 67: 162-171.

Berisha, B., H. Welter, T. Shimizu, A. Miyamoto, H. H. Meyer, and D. Schams. 2006. Expression of fibroblast growth factor 1 (FGF1) and FGF7 in mature follicles during the periovulatory period after GnRH in the cow. J. Reprod. Dev. 52: 307-313.

Bicsak, T. A., M. Shimonaka, M. Malkowski, and N. Ling. 1990. Insulin-like growth factor-binding protein (IGF-BP) inhibition of granulosa-cell function - effect on cyclic adenosine-3',5'-monophosphate, deoxyribonucleic-acid synthesis, and comparison with the effect of an IGF-I antibody. Endocrinology 126: 2184-2189.

Biswas, S. B., R. W. Hammond, and L. D. Anderson. 1988. Fibroblast growth-factors from bovine pituitary and human-placenta and their functions in the maturation of porcine granulosa-cells invitro. Endocrinology 123: 559-566.

Bley, M. A., J. C. Simon, A. G. Estevez, L. J. Deasua, and J. L. Baranao. 1992. Effect of follicle-stimulating-hormone on insulin-like growth factor-I-stimulated rat granulosa-cell deoxyribonucleic-acid synthesis. Endocrinology 131: 1223-1229.

Bodensteiner, K. J., C. M. Clay, C. L. Moeller, and H. R. Sawyer. 1999. Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. Biol. Reprod. 60: 381-386.

Boilly, B., A. S. Vercoutter-Edouart, H. Hondermarck, V. Nurcombe, and X. Le Bourhis. 2000. Fgf signals for cell proliferation and migration through different pathways. Cytokine Growth Factor Rev. 11: 295-302.

Bowtell, D. D. 1999. Options available--from start to finish--for obtaining expression data by microarray. Nat. Genet. 21: 25-32.

Brachmann, R., P. B. Lindquist, M. Nagashima, W. Kohr, T. Lipari, M. Napier, and R. Derynck. 1989. Transmembrane TGF-alpha precursors activate EGF TGF-alpha receptors. Cell 56: 691-700.

Brankin, V., R. L. Quinn, R. Webb, and M. G. Hunter. 2005a. Bmp-2 and-6 modulate porcine theca cell function alone and co-cultured with granulosa cells. Domest. Anim. Endocrinol. 29: 593-604.

Brankin, V., R. L. Quinn, R. Webb, and M. G. Hunter. 2005b. Evidence for a functional bone morphogenetic protein (BMP) system in the porcine ovary. Domest. Anim. Endocrinol. 28: 367-379.

Bukovsky, A., T. T. Chen, J. Wimalasena, and M. R. Caudle. 1993. Cellular-localization of luteinizing-hormone receptor immunoreactivity in the ovaries of immature, gonadotropin-primed and normal cycling rats. Biol. Reprod. 48: 1367-1382.

Buratini, J., V. F. Glapinski, I. C. Giometti, A. B. Teixeira, I. B. Costa, M. C. W. Avellar, C. M. Barros, and C. A. Price. 2005a. Expression of fibroblast growth factor-8 and its cognate receptors, fibroblast growth factor receptor (FGFr)-3c and-4, in fetal bovine preantral follicles. Mol. Reprod. Dev. 70: 255-261.

Buratini, J., M. G. L. Pinto, A. C. Castilho, R. L. Amorim, I. C. Giometti, V. M. Portela, E. S. Nicola, and C. A. Price. 2007. Expression and function of fibroblast growth factor 10 and its receptor, fibroblast growth factor receptor 213, in bovine follicles. Biol. Reprod. 77: 743-750.

Buratini, J., A. B. Teixeira, I. B. Costa, V. F. Glapinski, M. G. L. Pinto, I. C. Giometti, C. M. Barros, M. Cao, E. S. Nicola, and C. A. Price. 2005b. Expression of fibroblast

growth factor-8 and regulation of cognate receptors, fibroblast growth factor receptor-3c and-4, in bovine antral follicles. Reproduction 130: 343-350.

Caixeta, E. S., M. L. Sutton-McDowall, R. B. Gilchrist, J. G. Thompson, C. A. Price, M. F. Machado, P. F. Lima, and J. Buratini. 2013. Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes. Reproduction 146: 27-35.

Campbell, B. K., M. Clinton, and R. Webb. 2012. The role of anti-mullerian hormone (AMH) during follicle development in a monovulatory species (sheep). Endocrinology 153: 4533-4543.

Canalis, E. 1980. Effect of insulin-like growth factor-I on DNA and protein-synthesis in cultured rat calvaria. J. Clin. Invest. 66: 709-719.

Capriotti, E., N. L. Nehrt, M. G. Kann, and Y. Bromberg. 2012. Bioinformatics for personal genome interpretation. Brief Bioinform. 13: 495-512.

Carabatsos, M. J., J. Elvin, M. M. Matzuk, and D. F. Albertini. 1998. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev. Biol. 204: 373-384.

Carlsson, I. B., J. E. Scott, J. A. Visser, O. Ritvos, A. P. N. Themmen, and O. Hovatta. 2006. Anti-mullerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. Hum. Reprod. 21: 2223-2227.

Castilho, A. C., I. C. Giometti, B. Berisha, D. Schams, C. A. Price, R. L. Amorim, P. C. Papa, and J. Buratini. 2008. Expression of fibroblast growth factor 10 and its receptor, fibroblast growth factor receptor 2b, in the bovine corpus luteum. Mol. Reprod. Dev. 75: 940-945.

Castilho, A. C. S., R. B. da Silva, C. A. Price, M. F. Machado, R. L. Amorim, and J. Buratini. 2014. Expression of fibroblast growth factor 10 and cognate receptors in the developing bovine ovary. Theriogenology 81: 1268-1274.

Castilho, A. C. S. P., C.A.; Dalanezi, F.; Ereno, R.L.; Machado, M.F.; Barros, C.M.; Gasperin, B.G.; Gonçalves, P.B.D.; Buratini, J. 2015. Evidence that fibroblast growth factor 10 plays a role in follicle selection in cattle. Reprod. Fertil. Dev. doi:10.1071/RD15017 [Epub ahead of print].

Cataldo, N. A., J. Rabinovici, V. Y. Fujimoto, and R. B. Jaffe. 1994. Follistatin antagonizes the effects of activin-a on steroidogenesis in human luteinizing granulosa-cells. J. Clin. Endocrinol. Metab. 79: 272-277.

Chamberlain, C. S., and L. J. Spicer. 2001. Hormonal control of ovarian cell production of insulin-like growth factor binding proteins. Mol. Cell. Endocrinol. 182: 69-81.

Chang, H., C. W. Brown, and M. M. Matzuk. 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr. Rev. 23: 787-823.

Chang, H. M., J. C. Cheng, and P. C. K. Leung. 2013. Theca-derived BMP4 and BMP7 down-regulate connexin43 expression and decrease gap junction intercellular communication activity in immortalized human granulosa cells. J. Clin. Endocrinol. Metab. 98: E437-E445.

Chang, R. J., and R. B. Jaffe. 1978. Progesterone effects on gonadotropin release in women pretreated with estradiol. J. Clin. Endocrinol. Metab. 47: 119-125.

Chang, W. Y., F. Shidaifat, M. Uzumcu, and Y. C. Lin. 1996. Effects of transforming growth factor-beta(1) and activin-a on in vitro porcine granulosa cell steroidogenesis. Theriogenology 45: 1463-1472.

Channing, C. P., K. Tanabe, M. Chacon, and J. T. Tildon. 1984. Stimulatory effects of follicle-stimulating-hormone and luteinizing-hormone upon secretion of progesterone and inhibin activity by cultured infant human ovarian granulosa-cells. Fertil. Steril. 42: 598-605.

Chaves, R. N., M. H. T. de Matos, J. Buratini, and J. R. de Figueiredo. 2012. The fibroblast growth factor family: Involvement in the regulation of folliculogenesis. Reprod. Fertil. Dev. 24: 905-915.

Chen, L., P. T. Russell, and W. J. Larsen. 1993. Functional significance of cumulus expansion in the mouse: Roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Mol. Reprod. Dev. 34: 87-93.

Chen, Y. J., M. T. Lee, H. C. Yao, P. W. Hsiao, F. C. Ke, and J. J. Hwang. 2008. Crucial role of estrogen receptor-alpha interaction with transcription coregulators in follicle-stimulating hormone and transforming growth factor beta 1 up-regulation of steroidogenesis in rat ovarian granulosa cells. Endocrinology 149: 4658-4668.

Cho, J. H., T. Itoh, Y. Sendai, and H. Hoshi. 2008. Fibroblast growth factor 7 stimulates in vitro growth of oocytes originating from bovine early antral follicles. Mol. Reprod. Dev. 75: 1736-1743.

Chun, S. Y., H. Billig, J. L. Tilly, I. Furuta, A. Tsafriri, and A. J. W. Hsueh. 1994. Gonadotropin suppression of apoptosis in cultured preovulatory follicles - mediatory role of endogenous insulin-like growth-factor-I. Endocrinology 135: 1845-1853.

Chun, S. Y., K. M. Eisenhauer, M. Kubo, and A. J. Hsueh. 1995. Interleukin-1 beta suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. Endocrinology 136: 3120-3127.

Cohen, C. D., M. T. Lindenmeyer, F. Eichinger, A. Hahn, M. Seifert, A. G. Moll, H. Schmid, E. Kiss, E. Grone, H. J. Grone, M. Kretzler, T. Werner, and P. J. Nelson.

2008. Improved elucidation of biological processes linked to diabetic nephropathy by single probe-based microarray data analysis. PLoS One 3: e2937.

Cross, M. J., and L. Claesson-Welsh. 2001. FGF and VEGF function in angiogenesis: Signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol. Sci. 22: 201-207.

Dailey, L., D. Ambrosetti, A. Mansukhani, and C. Basilico. 2005. Mechanisms underlying differential responses to FGF signaling. Cytokine & Growth Factor Rev. 16: 233-247.

Dekretser, D. M., and D. M. Robertson. 1989. The isolation and physiology of inhibin and related proteins. Biol. Reprod. 40: 33-47.

delaSota, R. L., F. A. Simmen, T. Diaz, and W. W. Thatcher. 1996. Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. Biol. Reprod. 55: 803-812.

Dell, K. R., and L. T. Williams. 1992. A novel form of fibroblast growth factor receptor 2. Alternative splicing of the third immunoglobulin-like domain confers ligand binding specificity. J. Biol. Chem. 267: 21225-21229.

Dengjel, J., I. Kratchmarova, and B. Blagoev. 2009. Receptor tyrosine kinase signaling: A view from quantitative proteomics. Mol. Biosyst. 5: 1112-1121.

Devoto, L., L. K. Christenson, J. McAllister, A. Makrigiannakis, and J. F. Strauss. 1999. Insulin and insulin-like growth factor-I and -II modulate human granulosa-lutein cell steroidogenesis: Enhancement of steroidogenic acute regulatory protein (star) expression. Mol. Hum. Reprod. 5: 1003-1010.

Diblasio, A. M., P. Vigano, and A. Ferrari. 1994. Insulin-like growth factor-II stimulates human granulosa-luteal cell-proliferation in-vitro. Fertil. Steril. 61: 483-487.

Dong, J. W., D. F. Albertini, K. Nishimori, T. R. Kumar, N. F. Lu, and M. M. Matzuk. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383: 531-535.

dos Santos, R. M., M. D. Goissis, D. A. Fantini, C. M. Bertan, J. L. Vasconcelos, and M. Binelli. 2009. Elevated progesterone concentrations enhance prostaglandin f2alpha synthesis in dairy cows. Anim. Reprod. Sci. 114: 62-71.

Downs, S. M., P. G. Humpherson, K. L. Martin, and H. J. Leese. 1996. Glucose utilization during gonadotropin-induced meiotic maturation in cumulus cell-enclosed mouse oocytes. Mol. Reprod. Dev. 44: 121-131.

Drummond, A. E. 2005. TGF beta signalling in the development of ovarian function. Cell Tissue Res. 322: 107-115.

Drummond, A. E., M. Tellbach, M. Dyson, and J. K. Findlay. 2007. Fibroblast growth factor-9, a local regulator of ovarian function. Endocrinology 148: 3711-3721.

Duarte, A. B. G., V. R. Araujo, R. N. Chaves, G. M. da Silva, V. B. Luz, K. T. Haag, D. M. Magalhaes-Padilha, A. P. Almeida, C. H. Lobo, C. C. Campello, and J. R. de Figueiredo. 2013. Insulin-like growth factor II (IGF-II) and follicle stimulating hormone (FSH) combinations can improve the in vitro development of grown oocytes enclosed in caprine preantral follicles. Growth Hormone & IGF Research 23: 37-44.

Dube, J. L., P. Wang, J. Elvin, K. M. Lyons, A. J. Celeste, and M. M. Matzuk. 1998. The bone morphogenetic protein 15 gene is x-linked and expressed in oocytes. Mol. Endocrinol. 12: 1809-1817.

Dubey, P. K., A. Nath, V. Chandra, M. Sarkar, G. Saikumar, and G. T. Sharma. 2015. Expression of mRNA encoding IGF-I, IGF-II, type-I, and II IGF-receptors and IGF-binding proteins-1-4 during ovarian follicular development in buffalo (bubalus bubalis). Anim. Biotechnol. 26: 81-91.

Duleba, A. J., R. Z. Spaczynski, D. L. Olive, and H. R. Behrman. 1997. Effects of insulin and insulin-like growth factors on proliferation of rat ovarian theca-interstitial cells. Biol. Reprod. 56: 891-897.

Duleba, A. J., R. Z. Spaczynski, D. L. Olive, and H. R. Behrman. 1999. Divergent mechanisms regulate proliferation/survival and steroidogenesis of theca-interstitial cells. Mol Hum. Reprod. 5: 193-198.

Dupanloup, I., and H. Kaessmann. 2006. Evolutionary simulations to detect functional lineage-specific genes. Bioinformatics 22: 1815-1822.

Durlinger, A. L. L., M. J. G. Gruijters, P. Kramer, B. Karels, T. R. Kumar, M. M. Matzuk, U. M. Rose, F. H. de Jong, J. T. J. Uilenbroek, J. A. Grootegoed, and A. P. N. Themmen. 2001. Anti-mullerian hormone attenuates the effects of fsh on follicle development in the mouse ovary. Endocrinology 142: 4891-4899.

Durlinger, A. L. L., M. J. G. Gruijters, P. Kramer, B. Karels, H. A. Ingraham, M. W. Nachtigal, J. T. J. Uilenbroek, J. A. Grootegoed, and A. P. N. Themmen. 2002a. Anti-mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143: 1076-1084.

Durlinger, A. L. L., J. A. Visser, and A. P. N. Themmen. 2002b. Regulation of ovarian function: The role of anti-mullerian hormone. Reproduction 124: 601-609.

Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U S A 95: 14863-14868.

Elroeiy, A., X. H. Chen, V. J. Roberts, D. Leroith, C. T. Roberts, and S. S. C. Yen. 1993. Expression of insulin-like growth factor-I (IGF-I) and IGF-II and the IGF-I, IGF-II and insulin-receptor genes and localization of the gene-products in the human ovary. J. Clin. Endocrinol. Metab. 77: 1411-1418.

Elvin, J. A., A. T. Clark, P. Wang, N. M. Wolfman, and M. M. Matzuk. 1999. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol. Endocrinol. 13: 1035-1048.

Elvin, J. A., and M. M. Matzuk. 1998. Mouse models of ovarian failure. Reviews of Reproduction 3: 183-195.

Elvin, J. A., C. N. Yan, and M. M. Matzuk. 2000. Oocyte-expressed TGF-beta superfamily members in female fertility. Mol. Cell. Endocrinol. 159: 1-5.

Eppig, J. J. 1991. Intercommunication between mammalian oocytes and companion somatic-cells. Bioessays 13: 569-574.

Eppig, J. J. 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122: 829-838.

Eramaa, M., K. Hilden, T. Tuuri, and O. Ritvos. 1995. Regulation of inhibin activin sunbunit messenger ribonucleic-acids (messenger-RNAs) by activin-a and expression of activin receptor messenger-RNAs in cultured human granulosa-luteal cells. Endocrinology 136: 4382-4389.

Ergin, K., E. Gursoy, Y. B. Koca, H. Basaloglu, and K. Seyrek. 2008. Immunohistochemical detection of insulin-like growth factor-1, transforming growth factor-beta 2, basic fibroblast growth factor and epidermal growth factor-receptor expression in developing rat ovary. Cytokine 43: 209-214.

Erickson, G. F., and A. J. W. Hsueh. 1978. Secretion of inhibin by rat granulosa-cells invitro. Endocrinology 103: 1960-1963.

Erickson, G. F., and S. Shimasaki. 2003. The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reprod. Biol. Endocrinol. 1: 9.

Evans, A. C., and J. E. Fortune. 1997. Selection of the dominant follicle in cattle occurs in the absence of differences in the expression of messenger ribonucleic acid for gonadotropin receptors. Endocrinology 138: 2963-2971.

Evans, A. C., J. L. Ireland, M. E. Winn, P. Lonergan, G. W. Smith, P. M. Coussens, and J. J. Ireland. 2004. Identification of genes involved in apoptosis and dominant follicle development during follicular waves in cattle. Biol. Reprod. 70: 1475-1484.

Evans, J. R., N. B. Schreiber, J. A. Williams, and L. J. Spicer. 2014. Effects of fibroblast growth factor 9 on steroidogenesis and control of FGFR2IIIc mrna in porcine granulosa cells. J. Anim. Sci. 92: 511-519.

Feranil, J. B., N. Isobe, and T. Nakao. 2005. Apoptosis in the antral follicles of swamp buffalo and cattle ovary: Tunel and caspase-3 histochemistry. Reprod. Domest. Anim. 40: 111-116.

Ferreira, R. M. C., M.R.; Macabelli, C.H.; Rodrigues, C.A.; Ferraz, M.C.; Watanabe, Y.F.; Smith, L.C.; Meirelles, F.V.; Baruselli, P.S. 2016. The infertility of repeat-breeder cows during summer is associated with decreased mitochondrial DNA and increased expression of mitochondrial and apoptotic genes in oocytes. Biol. Reprod. 94: 1-10.

Finch, P. W., J. S. Rubin, T. Miki, D. Ron, and S. A. Aaronson. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. Science 245: 752-755.

Fortune, J. E. 2003. The early stages of follicular development: Activation of primordial follicles and growth of preantral follicles. Anim. Reprod. Sci. 78: 135-163.

Fortune, J. E., R. A. Cushman, C. M. Wahl, and S. Kito. 2000. The primordial to primary follicle transition. Mol. Cell. Endocrinol. 163: 53-60.

Fortune, J. E., and W. Hansel. 1985. Concentrations of steroids and gonadotropins in follicular fluid from normal heifers and heifers primed for superovulation. Biol. Reprod. 32: 1069-1079.

Fortune, J. E., G. M. Rivera, and M. Y. Yang. 2004. Follicular development: The role of the follicular microenvironment in selection of the dominant follicle. Anim. Reprod. Sci. 82-83: 109-126.

Frodin, M., and S. Gammeltoft. 1994. Insulin-like growth-factors act synergistically with basic fibroblast growth-factor and nerve growth-factor to promote chromaffin cell-proliferation. Proc. Natl. Acad. Sci. U S A 91: 1771-1775.

Froesch, E. R., C. Schmid, J. Schwander, and J. Zapf. 1985. Actions of insulin-like growth-factors. Annu. Rev. Physiol. 47: 443-467.

Gambarini, A. G., and H. A. Armelin. 1982. Purification and partial characterization of an acidic fibroblast growth-factor from bovine pituitary. J. Biol. Chem. 257: 9692-9697.

Gangrade, B. K., J. S. Davis, and J. V. May. 1991. A novel mechanism for the induction of aromatase in ovarian-cells invitro - role of transforming growth factor-alpha-induced protein tyrosine kinase. Endocrinology 129: 2790-2792.

GillingSmith, C., D. S. Willis, and S. Franks. 1997. Oestradiol feedback stimulation of androgen biosynthesis by human theca cells. Hum. Reprod. 12: 1621-1628.

Givol, D., and A. Yayon. 1992. Complexity of FGF receptors: Genetic basis for structural diversity and functional specificity. FASEB J 6: 3362-3369.

Glister, C., C. F. Kemp, and P. G. Knight. 2004. Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: Actions of BMP-4,-6 and-7 on granulosa cells and differential modulation of smad-1 phosphorylation by follistatin. Reproduction 127: 239-254.

Glister, C., S. L. Richards, and P. G. Knight. 2005. Bone morphogenetic proteins (BMP)-4,-6, and-7 potently suppress basal and luteinizing hormone-induced androgen production by bovine theca interna cells in primary culture: Could ovarian hyperandrogenic dysfunction be caused by a defect in thecal BMP signaling? Endocrinology 146: 1883-1892.

Goetz, R., A. Beenken, O. A. Ibrahimi, J. Kalinina, S. K. Olsen, A. V. Eliseenkova, C. Xu, T. A. Neubert, F. Zhang, R. J. Linhardt, X. Yu, K. E. White, T. Inagaki, S. A. Kliewer, M. Yamamoto, H. Kurosu, Y. Ogawa, M. Kuro-o, B. Lanske, M. S. Razzaque, and M. Mohammadi. 2007. Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. Mol. Cell. Biol. 27: 3417-3428.

Goetz, R., and M. Mohammadi. 2013. Exploring mechanisms of FGF signalling through the lens of structural biology. Nat. Rev. Mol. Cell. Biol. 14: 166-180.

Gospodarowicz, D. 1974. Localization of a fibroblast growth-factor and its effect alone and with hydrocortisone on 3t3 cell-growth. Nature 249: 123-127.

Gospodarowicz, D., C. R. Ill, and C. R. Birdwell. 1977a. Effects of fibroblast and epidermal growth-factors on ovarian cell-proliferation invitro .1. Characterization of response of granulosa-cells to FGF and EGF. Endocrinology 100: 1108-1120.

Gospodarowicz, D., C. R. Ill, and C. R. Birdwell. 1977b. Effects of fibroblast and epidermal growth-factors on ovarian cell-proliferation invitro. 2. Proliferative response of luteal cells to FGF but not EGF. Endocrinology 100: 1121-1128.

Gospodarowicz, D., C. R. Ill, P. J. Hornsby, and G. N. Gill. 1977c. Control of bovine adrenal cortical cell proliferation by fibroblast growth factor. Lack of effect of epidermal growth factor. Endocrinology 100: 1080-1089.

Gospodarowicz, D., and H. Bialecki. 1978. The effects of the epidermal and fibroblast growth factors on the replicative lifespan of cultured bovine granulosa cells. Endocrinology 103: 854-865.

Gospodarowicz, D., J. Cheng, G. M. Lui, A. Baird, F. Esch, and P. Bohlen. 1985. Corpus luteum angiogenic factor is related to fibroblast growth factor. Endocrinology 117: 2383-2391.

Gotoh, N. 2008. Regulation of growth factor signaling by frs2 family docking/scaffold adaptor proteins. Cancer Sci. 99: 1319-1325.

Grazul-Bilska, A. T., D. A. Redmer, A. Jablonka-Shariff, M. E. Biondini, and L. P. Reynolds. 1995. Proliferation and progesterone production of ovine luteal cells from several stages of the estrous cycle: Effects of fibroblast growth factors and luteinizing hormone. Can. J. Physiol. Pharmacol. 73: 491-500.

Guimond, S. E., and J. E. Turnbull. 1999. Fibroblast growth factor receptor signalling is dictated by specific heparan sulphate saccharides. Curr. Biol. 9: 1343-1346.

Guler, H. P., J. Zapf, and E. R. Froesch. 1987. Short-term metabolic effects of recombinant human insulin-like growth factor-I in healthy-adults. N. Engl. J. Med. 317: 137-140.

Guthridge, M., J. Bertolini, J. Cowling, and M. T. Hearn. 1992. Localization of bFGF mrna in cyclic rat ovary, diethylstilbesterol primed rat ovary, and cultured rat granulosa cells. Growth Factors 7: 15-25.

Gutierrez, C. G., B. K. Campbell, and R. Webb. 1997. Development of a long-term bovine granulosa cell culture system: Induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. Biol. Reprod. 56: 608-616.

Halje, M., M. Nordin, D. Bergman, and W. Engstrom. 2012. The effect of insulin-like growth factor II in the regulation of tumour cell growth in vitro and tumourigenesis in vivo. In Vivo 26: 519-526.

Hammond, J. M., J. L. S. Baranao, D. Skaleris, A. B. Knight, J. A. Romanus, and M. M. Rechler. 1985. Production of insulin-like growth-factors by ovarian granulosa-cells. Endocrinology 117: 2553-2555.

Hatzirodos, N., K. Hummitzsch, H. F. Irving-Rodgers, M. L. Harland, S. E. Morris, and R. J. Rodgers. 2014a. Transcriptome profiling of granulosa cells from bovine ovarian follicles during atresia. BMC Genomics 15: 15-40.

Hatzirodos, N., H. F. Irving-Rodgers, K. Hummitzsch, and R. J. Rodgers. 2014b. Transcriptome profiling of the theca interna from bovine ovarian follicles during atresia. PLoS One 9: e99706.

Hayashi, M., E. A. McGee, G. Min, C. Klein, U. M. Rose, M. van Duin, and A. J. W. Hsueh. 1999. Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. Endocrinology 140: 1236-1244.

Heldin, C. H., K. Miyazono, and P. tenDijke. 1997. Tgf-beta signalling from cell membrane to nucleus through smad proteins. Nature 390: 465-471.

Henderson, K. M., and P. Franchimont. 1981. Regulation of inhibin production by bovine ovarian-cells invitro. J. Reprod. Fertil. 63: 431-442.

Henderson-Maclennan, N. K., J. C. Papp, C. C. Talbot, Jr., E. R. McCabe, and A. P. Presson. 2010. Pathway analysis software: Annotation errors and solutions. Mol. Genet. Metab. 101: 134-140.

Hennessey, J. A., E. Q. Wei, and G. S. Pitt. 2013. Fibroblast growth factor homologous factors modulate cardiac calcium channels. Circ. Res. 113: 381-388.

Hernandez, E. R. 1995. Regulation of the genes for insulin-like growth-factor (IGF)-I and (IGF)-II and their receptors by steroids and gonadotropins in the ovary. J. Steroid Biochem. Mol. Biol. 53: 219-221.

Hirshfield, A. N. 1988. Size-frequency analysis of atresia in cycling rats. Biol. Reprod. 38: 1181-1188.

Hirshfield, A. N. 1991. Development of follicles in the mammalian ovary. Int. Rev. Cytol. 124: 43-101.

Hogan, B. L. 1999. Morphogenesis. Cell 96: 225-233.

Hsu, C. J., and J. M. Hammond. 1987. Concomitant effects of growth-hormone on secretion of insulin-like growth factor-I and progesterone by cultured porcine granulosa-cells. Endocrinology 121: 1343-1348.

Hsueh, A. J., E. Y. Adashi, P. B. Jones, and T. H. Welsh, Jr. 1984. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr. Rev. 5: 76-127.

Hsueh, A. J. W., K. Kawamura, Y. Cheng, and B. C. J. M. Fauser. 2015. Intraovarian control of early folliculogenesis. Endocr. Rev. 36: 1-24.

Hsueh, A. J. W., E. A. McGee, M. Hayashi, and S. Y. Hsu. 2000. Hormonal regulation of early follicle development in the rat ovary. Mol. Cell. Endocrinol. 163: 95-100.

Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37: 1-13.

Hughes, F. M., Jr., and W. C. Gorospe. 1991. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: Evidence for a potential mechanism underlying follicular atresia. Endocrinology 129: 2415-2422.

Igarashi, M., P. W. Finch, and S. A. Aaronson. 1998. Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). J. Biol. Chem. 273: 13230-13235.

Inagaki, K., F. Otsuka, T. Miyoshi, M. Yamashita, M. Takahashi, J. Goto, J. Suzuki, and H. Makino. 2009. P38-mitogen-activated protein kinase stimulated steroidogenesis in granulosa cell-oocyte cocultures: Role of bone morphogenetic proteins 2 and 4. Endocrinology 150: 1921-1930.

Ireland, J. J., and J. F. Roche. 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: Changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. Endocrinology 111: 2077-2086.

Ireland, J. J., A. E. Zielak-Steciwko, F. Jimenez-Krassel, J. Folger, A. Bettegowda, D. Scheetz, S. Walsh, F. Mossa, P. G. Knight, G. W. Smith, P. Lonergan, and A. C. O. Evans. 2009. Variation in the ovarian reserve is linked to alterations in intrafollicular estradiol production and ovarian biomarkers of follicular differentiation and oocyte quality in cattle. Biol. Reprod. 80: 954-964.

Itoh, N., and D. M. Ornitz. 2004. Evolution of the FGF and FGFr gene families. Trends Genet. 20: 563-569.

Itoh, N., and D. M. Ornitz. 2011. Fibroblast growth factors: From molecular evolution to roles in development, metabolism and disease. J. Biochem. 149: 121-130.

Izadyar, F., G. Dijkstra, H. T. A. Van Tol, A. J. M. van de Eljnden-Van Raaij, R. Van den Hurk, B. Colenbrander, and M. M. Bevers. 1998. Immunohistochemical localization and mrna expression of activin, inhibin, follistatin, and activin receptor in bovine cumulus-oocyte complexes during in vitro maturation. Mol. Reprod. Dev. 49: 186-195.

Jaatinen, R., M. P. Laitinen, K. Vuojolainen, J. Aaltonen, H. Louhio, K. Heikinheimo, E. Lehtonen, and O. Ritvos. 1999. Localization of growth differentiation factor-9 (GDF-9) mrna and protein in rat ovaries and cdna cloning of rat GDF-9 and its novel homolog GDF-9b. Mol. Cell. Endocrinol. 156: 189-193.

Jaatinen, R., V. Rosen, T. Tuuri, and O. Ritvos. 1996. Identification of ovarian granulosa cells as a novel site of expression for bone morphogenetic protein-3 (BMP-3/osteogenin) and regulation of BMP-3 messenger ribonucleic acids by chorionic gonadotropin in cultured human granulosa-luteal cells. J. Clin. Endocrinol. Metab. 81: 3877-3882.

Jee, B. C., J. H. Kim, H. Park da, H. Youm, C. S. Suh, and S. H. Kim. 2012. In vitro growth of mouse preantral follicles: Effect of animal age and stem cell factor/insulin-like growth factor supplementation. Clin. Exp. Reprod. Med. 39: 107-113.

Jiang, Z., H. M. Guerrero-Netro, J. L. Juengel, and C. A. Price. 2013. Divergence of intracellular signaling pathways and early response genes of two closely related fibroblast growth factors, FGF8 and FGF18, in bovine ovarian granulosa cells. Mol. Cell. Endocrinol. 375: 97-105.

Jimenez-Krassel, F., M. E. Winn, D. Burns, J. L. H. Ireland, and J. J. Ireland. 2003. Evidence for a negative intrafollicular role for inhibin in regulation of estradiol production by granulosa cells. Endocrinology 144: 1876-1886.

Joannes, A., S. Brayer, V. Besnard, J. Marchal-Somme, M. Jaillet, P. Mordant, H. Mal, R. Borie, B. Crestani, and A. A. Mailleux. 2016. FGF9 and FGF18 in idiopathic pulmonary fibrosis promote survival and migration and inhibit myofibroblast differentiation of human lung fibroblasts in vitro. Am. J. Physiol. Lung Cell Mol. Physiol. 310: L615-L629.

Johnson, C. C., W. E. Dawson, J. T. Turner, and J. H. Wyche. 1980. Regulation of rat ovarian cell growth and steroid secretion. J. Cell Biol. 86: 483-489.

Jolly, P. D., D. J. Tisdall, D. A. Heath, S. Lun, and K. P. McNatty. 1994. Apoptosis in bovine granulosa cells in relation to steroid synthesis, cyclic adenosine 3',5'-monophosphate response to follicle-stimulating hormone and luteinizing hormone, and follicular atresia. Biol. Reprod. 51: 934-944.

Juengel, J. L., A. H. Bibby, K. L. Reader, S. Lun, L. D. Quirke, L. J. Haydon, and K. P. McNatty. 2004. The role of transforming growth factor-beta (TGF-beta) during ovarian follicular development in sheep. Reprod. Biol. Endocrinol. 2: 78.

Kayani, A. R., C. Glister, and P. G. Knight. 2009. Evidence for an inhibitory role of bone morphogenetic protein(s) in the follicular-luteal transition in cattle. Reproduction 137: 67-78.

Ke, F. C., L. C. Chuang, M. T. Lee, Y. J. Chen, S. W. Lin, P. S. Wang, D. M. Stocco, and J. J. Hwang. 2004. The modulatory role of transforming growth factor beta 1 and androstenedione on follicle-stimulating hormone-induced gelatinase secretion and steroidogenesis in rat granulosa cells. Biol. Reprod. 70: 1292-1298.

Kelleher, F. C., H. O'Sullivan, E. Smyth, R. McDermott, and A. Viterbo. 2013. Fibroblast growth factor receptors, developmental corruption and malignant disease. Carcinogenesis 34: 2198-2205.

Kessel, B., Y. X. Liu, X. C. Jia, and A. J. Hsueh. 1985. Autocrine role of estrogens in the augmentation of luteinizing hormone receptor formation in cultured rat granulosa cells. Biol. Reprod. 32: 1038-1050.

Keyes, P. L., and M. C. Wiltbank. 1988. Endocrine regulation of the corpus luteum. Annu Rev Physiol 50: 465-482.

Kezele, P., E. E. Nilsson, and M. K. Skinner. 2005. Keratinocyte growth factor acts as a mesenchymal factor that promotes ovarian primordial to primary follicle transition. Biol. Reprod. 73: 967-973.

Khalaf, M., J. Morera, A. Bourret, Y. Reznik, C. Denoual, M. Herlicoviez, H. Mittre, and A. Benhaim. 2013. BMP system expression in gcs from polycystic ovary syndrome women and the in vitro effects of BMP4, BMP6, and BMP7 on GC steroidogenesis. Eur. J. Endocrinol. 168: 437-444.

Khatri, P., and S. Draghici. 2005. Ontological analysis of gene expression data: Current tools, limitations, and open problems. Bioinformatics 21: 3587-3595.

Kim, I. C., and D. W. Schomberg. 1989. The production of transforming growth factor-beta activity by rat granulosa-cell cultures. Endocrinology 124: 1345-1351.

Knecht, M., A. M. Brodie, and K. J. Catt. 1985. Aromatase inhibitors prevent granulosa cell differentiation: An obligatory role for estrogens in luteinizing hormone receptor expression. Endocrinology 117: 1156-1161.

Knight, P. G., and C. Glister. 2001. Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction 121: 503-512.

Kogawa, K., K. Ogawa, Y. Hayashi, T. Nakamura, K. Titani, and H. Sugino. 1991. Immunohistochemical localization of follistatin in rat-tissues. Endocrinologia Japonica 38: 383-391.

Kolodziejczyk, J., A. Gertler, H. Leibovich, J. Rzasa, and E. L. Gregoraszczuk. 2003. Synergistic action of growth hormone and insulin-like growth factor i (IGF-I) on proliferation and estradiol secretion in porcine granulosa and theca cells cultured alone or in coculture. Theriogenology 60: 559-570.

Kouhara, H., Y. R. Hadari, T. SpivakKroizman, J. Schilling, D. BarSagi, I. Lax, and J. Schlessinger. 1997. A lipid-anchored grb2-binding protein that links FGF-receptor activation to the ras/mapk signaling pathway. Cell 89: 693-702.

Kramer, A., J. Green, J. Pollard, Jr., and S. Tugendreich. 2014. Causal analysis approaches in ingenuity pathway analysis. Bioinformatics 30: 523-530.

La Marca, A., G. Sighinolfi, D. Radi, C. Argento, E. Baraldi, A. C. Artenisio, G. Stabile, and A. Volpe. 2010. Anti-mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (art). Hum. Reprod. Update 16: 113-130.

La Venuta, G., M. Zeitler, J. P. Steringer, H. M. Muller, and W. Nickel. 2015. The startling properties of fibroblast growth factor 2: How to exit mammalian cells without a signal peptide at hand. J. Biol. Chem. 290: 27015-27020.

Laureys, G., D. E. Barton, A. Ullrich, and U. Francke. 1988. Chromosomal mapping of the gene for the type-II insulin-like growth-factor receptor cation-independent mannose 6-phosphate receptor in man and mouse. Genomics 3: 224-229.

Lee, W. S., F. Otsuka, R. K. Moore, and S. Shimasaki. 2001. Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. Biol. Reprod. 65: 994-999.

Legault, S., J. L. Bailey, M. A. Fortier, P. Rouillier, and L. A. Guilbault. 1999. Intracellular regulation of estradiol and progesterone production by cultured bovine granulosa cells. Mol. Reprod. Dev. 54: 371-378.

Leighton, J. K., R. W. Grimes, S. Canning, and J. M. Hammond. 1993. Expression of the IGF system in primary and immortalized porcine ovarian granulosa-cells. Mol. Cell. Endocrinol. 97: 29-35.

Leroith, D., H. Werner, D. Beitnerjohnson, and C. T. Roberts. 1995. Molecular and cellular aspects of the insulin-like growth-factor-I receptor. Endocr. Rev. 16: 143-163.

Li, R. H., D. M. Phillips, and J. P. Mather. 1995. Activin promotes ovarian follicle development in-vitro. Endocrinology 136: 849-856.

Li, X. W., C.; Xiao, J.; McKeehan, W.L.; Wang, F. 2016. Fibroblast growth factors, old kids on the new block. Semin. Cell Dev. Biol. 53: 155-167.

Lin, J., Y. Jia, W. Zeng, Y. Mi, and C. Zhang. 2012. Basic FGF promotes proliferation of ovarian granulosa cells in the laying chickens via FGFR1 and PKC pathway. Reprod. Domest. Anim. 47: 135-142.

Lindsell, C. E., V. Misra, and B. D. Murphy. 1995. Follistatin has characteristics of a primary response gene in porcine granulosa-cells. Endocrine 3: 609-614.

Ling, N., S. Y. Ying, N. Ueno, S. Shimasaki, F. Esch, M. Hotta, and R. Guillemin. 1986. Pituitary FSH is released by a heterodimer of the beta-subunits from the 2 forms of inhibin. Nature 321: 779-782.

Liu, C., J. Peng, M. M. Matzuk, and H. H. C. Yao. 2015. Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells. Nat. Commun. 6: 6934.

Lobb, D. K. 2009. Expression and actions of transforming growth factors during human follicular development. Fertil. Steril. 92: 1080-1084.

Lobb, D. K., and J. Dorrington. 1992. Intraovarian regulation of follicular development. Anim. Reprod. Sci. 28: 343-354.

Logan, K. A., J. L. Juengel, and K. P. McNatty. 2002. Onset of steroidogenic enzyme gene expression during ovarian follicular development in sheep. Biol. Reprod. 66: 906-916.

Lonergan, P., A. Woods, T. Fair, F. Carter, D. Rizos, F. Ward, K. Quinn, and A. Evans. 2007. Effect of embryo source and recipient progesterone environment on embryo development in cattle. Reprod. Fertil. Dev. 19: 861-868.

Lynch, K., G. Fernandez, A. Pappalardo, and J. J. Peluso. 2000. Basic fibroblast growth factor inhibits apoptosis of spontaneously immortalized granulosa cells by regulating intracellular free calcium levels through a protein kinase cdelta-dependent pathway. Endocrinology 141: 4209-4217.

Lyons, K. M., R. W. Pelton, and B. L. M. Hogan. 1989. Patterns of expression of murine vgr-1 and BMP-2a rna suggest that transforming growth factor-beta-like genes coordinately regulate aspects of embryonic-development. Genes & Development 3: 1657-1668.

Lyons, R. M., and H. L. Moses. 1990. Transforming growth-factors and the regulation of cell-proliferation. Eur. J. Biochem. 187: 467-473.

Machado, M. F., V. M. Portela, C. A. Price, I. B. Costa, P. Ripamonte, R. L. Amorim, and J. Buratini. 2009. Regulation and action of fibroblast growth factor 17 in bovine follicles. J. Endocrinol. 202: 347-353.

Magalhaes-Padilha, D. M., A. B. G. Duarte, V. R. Araujo, M. V. A. Saraiva, A. P. Almeida, G. Q. Rodrigues, M. H. T. Matos, C. C. Campello, J. R. Silva, M. O. Gastal, E. L. Gastal, and J. R. Figueiredo. 2012. Steady-state level of insulin-like growth factor-I (IGF-I) receptor mrna and the effect of IGF-I on the in vitro culture of caprine preantral follicles. Theriogenology 77: 206-213.

Mahesh, V. B., and T. G. Muldoon. 1987. Integration of the effects of estradiol and progesterone in the modulation of gonadotropin secretion. J. Steroid Biochem. 27: 665-675.

Mamo, S., F. Carter, P. Lonergan, C. L. Leal, A. Al Naib, P. McGettigan, J. P. Mehta, A. C. Evans, and T. Fair. 2011. Sequential analysis of global gene expression profiles in immature and in vitro matured bovine oocytes: Potential molecular markers of oocyte maturation. BMC Genomics 12: 151.

Mansukhani, A., P. Bellosta, M. Sahni, and C. Basilico. 2000. Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induces apoptosis in osteoblasts. J. Cell Biol. 149: 1297-1308.

Massague, J., and M. P. Czech. 1982. The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. J. Biol. Chem. 257: 5038-5045.

Mattsson, M. E. K., G. Enberg, A. I. Ruusala, K. Hall, and S. Pahlman. 1986. Mitogenic response of human SH-SY5Y neuroblastoma-cells to insulin-like growth factor-I and factor-II is dependent on the stage of differentiation. J. Cell Biol. 102: 1949-1954.

Matzuk, M. M., K. H. Burns, M. M. Viveiros, and J. J. Eppig. 2002. Intercellular communication in the mammalian ovary: Oocytes carry the conversation. Science 296: 2178-2180.

May, J. V., A. J. Bridge, E. D. Gotcher, and B. K. Gangrade. 1992. The regulation of porcine theca cell-proliferation invitro - synergistic actions of epidermal growth-factor and platelet-derived growth-factor. Endocrinology 131: 689-697.

May, J. V., J. P. Frost, and D. W. Schomberg. 1988. Differential-effects of epidermal growth-factor, somatomedin-c insulin-like growth factor-I, and transforming growth factor-beta on porcine granulosa-cell deoxyribonucleic-acid synthesis and cell-proliferation. Endocrinology 123: 168-179.

May, J. V., L. A. Stephenson, C. J. Turzcynski, H. W. Fong, Y. H. L. Mau, and J. S. Davis. 1996. Transforming growth factor beta expression in the porcine ovary: Evidence that theca cells are the major secretory source during antral follicle development. Biol. Reprod. 54: 485-496.

Mazerbourg, S., and A. J. W. Hsueh. 2006. Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands. Hum. Reprod. Update 12: 373-383.

McGee, E. A., and A. J. W. Hsueh. 2000. Initial and cyclic recruitment of ovarian follicles. Endocr. Rev. 21: 200-214.

Mcgrath, S. A., A. F. Esquela, and S. J. Lee. 1995. Oocyte-specific expression of growth/differentiation factor-9. Mol. Endocrinol. 9: 131-136.

McNatty, K. P., S. Lun, D. A. Heath, L. E. Kieboom, and K. M. Henderson. 1985. Influence of follicular atresia on LH-induced cAMP and steroid synthesis by bovine thecae interna. Mol. Cell. Endocrinol. 39: 209-215.

Merry, C. L. R., M. Lyon, J. A. Deakin, J. J. Hopwood, and J. T. Gallagher. 1999. Highly sensitive sequencing of the sulfated domains of heparan sulfate. J. Biol. Chem. 274: 18455-18462.

Metzger, R. J., and M. A. Krasnow. 1999. Genetic control of branching morphogenesis. Science 284: 1635-1639.

Meunier, H., S. B. Cajander, V. J. Roberts, C. Rivier, P. E. Sawchenko, A. J. W. Hsueh, and W. Vale. 1988. Rapid changes in the expression of inhibin alpha-subunits, beta-a-subunits, and beta-b-subunits in ovarian cell-types during the rat estrous-cycle. Mol. Endocrinol. 2: 1352-1363.

Michel, U., H. Jarry, M. Metten, and W. Wuttke. 1989. Inhibin production by porcine granulosa and luteal cells - development and biological validation of a ria. Acta Endocrinologica 120: 511-518.

Miro, F., and S. G. Hillier. 1996. Modulation of granulosa cell deoxyribonucleic acid synthesis and differentiation by activin. Endocrinology 137: 464-468.

Miro, F., C. D. Smyth, and S. G. Hillier. 1991. Development-related effects of recombinant activin on steroid-synthesis in rat granulosa-cells. Endocrinology 129: 3388-3394.

Miyakawa, K., K. Hatsuzawa, T. Kurokawa, M. Asada, T. Kuroiwa, and T. Imamura. 1999. A hydrophobic region locating at the center of fibroblast growth factor-9 is crucial for its secretion. J. Biol. Chem. 274: 29352-29357.

Miyake, A., M. Konishi, F. H. Martin, N. A. Hernday, K. Ozaki, S. Yamamoto, T. Mikami, T. Arakawa, and N. Itoh. 1998. Structure and expression of a novel member, FGF-16, of the fibroblast growth factor family. Biochem. Biophys. Res. Commun. 243: 148-152.

Miyamoto, A., K. Okuda, F. J. Schweigert, and D. Schams. 1992. Effects of basic fibroblast growth-factor, transforming growth-factor-beta and nerve growth-factor on the secretory function of the bovine corpus-luteum invitro. J. Endocrinol. 135: 103-114.

Miyamoto, M., K. Naruo, C. Seko, S. Matsumoto, T. Kondo, and T. Kurokawa. 1993. Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. Mol. Cell. Biol. 13: 4251-4259.

Miyoshi, T., F. Otsuka, E. Nakamura, K. Inagaki, K. Ogura-Ochi, N. Tsukamoto, M. Takeda, and H. Makino. 2012. Regulatory role of kit ligand-c-kit interaction and oocyte factors in steroidogenesis by rat granulosa cells. Mol. Cell. Endocrinol. 358: 18-26.

Miyoshi, T., F. Otsuka, M. Yamashita, K. Inagaki, E. Nakamura, N. Tsukamoto, M. Takeda, J. Suzuki, and H. Makino. 2010. Functional relationship between fibroblast growth factor-8 and bone morphogenetic proteins in regulating steroidogenesis by rat granulosa cells. Mol. Cell. Endocrinol. 325: 84-92.

Mohammadi, M., S. K. Olsen, and O. A. Ibrahimi. 2005. Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev 16: 107-137.

Mondschein, J. S., S. F. Canning, and J. M. Hammond. 1988. Effects of transforming growth factor-beta on the production of immunoreactive insulin-like growth factor-I and progesterone and on [h-3]thymidine incorporation in porcine granulosa-cell cultures. Endocrinology 123: 1970-1976.

Monget, P., D. Monniaux, C. Pisselet, and P. Durand. 1993. Changes in insulin-like growth factor-I (IGF-I), IGF-II, and their binding-proteins during growth and atresia of ovine ovarian follicles. Endocrinology 132: 1438-1446.

Monniaux, D., and C. Pisselet. 1992. Control of proliferation and differentiation of ovine granulosa-cells by insulin-like growth factor-I and follicle-stimulating-hormone invitro. Biol. Reprod. 46: 109-119.

Montesano, R., J. D. Vassalli, A. Baird, R. Guillemin, and L. Orci. 1986. Basic fibroblast growth factor induces angiogenesis in vitro. Proc. Natl. Acad. Sci. U S A 83: 7297-7301.

Morgan, D. O., K. Jarnagin, and R. A. Roth. 1986. Purification and characterization of the receptor for insulin-like growth factor I. Biochemistry 25: 5560-5564.

Munsterberg, A., and R. Lovellbadge. 1991. Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual-differentiation. Development 113: 613-624.

Murphy, L. J. 1998. Review - Insulin-like growth factor-binding proteins: Functional diversity or redundancy? J. Mol. Endocrinol. 21: 97-107.

Murphy, M., J. Drago, and P. F. Bartlett. 1990. Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells in vitro. J. Neurosci. Res. 25: 463-475.

Myal, Y., R. P. C. Shiu, B. Bhaumick, and M. Bala. 1984. Receptor-binding and growth-promoting activity of insulin-like growth-factors in human-breast cancer-cells (T-47D) in culture. Cancer Res. 44: 5486-5490.

Nakatani, A., S. Shimasaki, L. V. Depaolo, G. F. Erickson, and N. Ling. 1991. Cyclic changes in follistatin messenger-ribonucleic-acid and its protein in the rat ovary during the estrous-cycle. Endocrinology 129: 603-611.

Neuvians, T. P., B. Berisha, and D. Schams. 2004. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) expression during induced luteolysis in the bovine corpus luteum. Mol. Reprod. Dev. 67: 389-395.

Nilsson, E., J. A. Parrott, and M. K. Skinner. 2001. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. Mol. Cell. Endocrinol. 175: 123-130.

Nilsson, E. E., V. Doraiswamy, and M. K. Skinner. 2003. Transforming growth factor-beta isoform expression during bovine ovarian antral follicle development. Mol. Reprod. Dev. 66: 237-246.

Nilsson, E. E., and M. K. Skinner. 2003. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol. Reprod. 69: 1265-1272.

Oesterle, E. C., S. A. Bhave, and M. D. Coltrera. 2000. Basic fibroblast growth factor inhibits cell proliferation in cultured avian inner ear sensory epithelia. J. Comp. Neurol. 424: 307-326.

Ogawa, K., M. Kurohmaru, K. Shiota, M. Takahashi, T. Nishida, and Y. Hayashi. 1991. Histochemical-localization of inhibin and activin alpha, beta-a and beta-b subunits in rat gonads. J. Vet. Med. Sci. 53: 207-212.

Ohmachi, S., Y. Watanabe, T. Mikami, N. Kusu, T. Ibi, A. Akaike, and N. Itoh. 2000. FGF-20, a novel neurotrophic factor, preferentially expressed in the substantia nigra pars compacta of rat brain. Biochem. Biophys. Res. Commun. 277: 355-360.

Oliver, J. E., T. J. Aitman, J. F. Powell, C. A. Wilson, and R. N. Clayton. 1989. Insulin-like growth factor-I gene-expression in the rat ovary is confined to the granulosa-cells of developing follicles. Endocrinology 124: 2671-2679.

Orisaka, M., J. Y. Jiang, S. Orisaka, F. Kotsuji, and B. K. Tsang. 2009. Growth differentiation factor 9 promotes rat preantral follicle growth by up-regulating follicular androgen biosynthesis. Endocrinology 150: 2740-2748.

Orisaka, M., T. Mizutani, K. Tajima, S. Orisaka, K. Shukunami, K. Miyamoto, and F. Kotsuji. 2006. Effects of ovarian theca cells on granulosa cell differentiation during gonadotropin-independent follicular growth in cattle. Mol. Reprod. Dev. 73: 737-744.

Ornitz, D. M., and N. Itoh. 2001. Fibroblast growth factors. Genome Biology 2.

Ornitz, D. M., and N. Itoh. 2015. The fibroblast growth factor signaling pathway. Wiley Interdiscip. Rev. Dev. Biol. 4: 215-266.

Osuga, Y., K. Koga, O. Tsutsumi, T. Yano, K. Kugu, M. Momoeda, R. Okagaki, A. Suenaga, T. Fujiwara, A. Fujimoto, H. Matsumi, H. Hiroi, and Y. Taketani. 2001.

Evidence for the presence of keratinocyte growth factor (KGF) in human ovarian follicles. Endocr. J. 48: 161-166.

Otsuka, F., R. K. Moore, and S. Shimasaki. 2001a. Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J. Biol. Chem. 276: 32889-32895.

Otsuka, F., and S. Shimasaki. 2002. A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: Its role in regulating granulosa cell mitosis. Proc. Natl. Acad. Sci. U S A 99: 8060-8065.

Otsuka, F., S. Yamamoto, G. F. Erickson, and S. Shimasaki. 2001b. Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (fsh) action by suppressing fsh receptor expression. J. Biol. Chem. 276: 11387-11392.

Otsuka, F., Z. Yao, T. Lee, S. Yamamoto, G. F. Erickson, and S. Shimasaki. 2000. Bone morphogenetic protein-15. Identification of target cells and biological functions. J. Biol. Chem. 275: 39523-39528.

Paradis, F., S. Novak, G. K. Murdoch, M. K. Dyck, W. T. Dixon, and G. R. Foxcroft. 2009. Temporal regulation of BMP2, BMP6, BMP15, GDF9, BMPr1a, BMPr1b, BMPr2 and tgfbr1 mrna expression in the oocyte, granulosa and theca cells of developing preovulatory follicles in the pig. Reproduction 138: 115-129.

Parkes, J. L., R. R. Cardell, and G. Grieninger. 1986. Insulin-like growth-factors (IGF-I and IGF-II) mimic the effect of insulin on plasma-protein synthesis and glycogen deposition in cultured-hepatocytes. Biochem. Biophys. Res. Commun. 134: 427-435.

Parrott, J. A., and M. K. Skinner. 1998. Developmental and hormonal regulation of hepatocyte growth factor expression and action in the bovine ovarian follicle. Biol. Reprod. 59: 553-560.

Parrott, J. A., J. L. Vigne, B. Z. Chu, and M. K. Skinner. 1994. Mesenchymal-epithelial interactions in the ovarian follicle involve keratinocyte and hepatocyte growth factor production by thecal cells and their action on granulosa cells. Endocrinology 135: 569-575.

Pehlivan, T., A. Mansour, R. Z. Spaczynski, and A. J. Duleba. 2001. Effects of transforming growth factors-alpha and -beta on proliferation and apoptosis of rat theca-interstitial cells. J. Endocrinol. 170: 639-645.

Pennetier, S., S. Uzbekova, C. Perreau, P. Papillier, P. Mermillod, and R. Dalbies-Tran. 2004. Spatio-temporal expression of the germ cell marker genes mater, zar1, GDF9, BMP15, and vasa in adult bovine tissues, oocytes, and preimplantation embryos. Biol. Reprod. 71: 1359-1366.

Plotnikov, A. N., S. R. Hubbard, J. Schlessinger, and M. Mohammadi. 2000. Crystal structures of two FGF-FGFr complexes reveal the determinants of ligand-receptor specificity. Cell 101: 413-424.

Portela, V. M., E. Dirandeh, H. M. Guerrero-Netro, G. Zamberlam, M. H. Barreta, A. F. Goetten, and C. A. Price. 2015. The role of fibroblast growth factor-18 in follicular atresia in cattle. Biol. Reprod. 92: 1-8.

Portela, V. M., M. Machado, J. Buratini, G. Zamberlam, R. L. Amorim, P. Goncalves, and C. A. Price. 2010. Expression and function of fibroblast growth factor 18 in the ovarian follicle in cattle. Biol. Reprod. 83: 339-346.

Powers, C. J., S. W. McLeskey, and A. Wellstein. 2000. Fibroblast growth factors, their receptors and signaling. Endocr. Relat. Cancer 7: 165-197.

Price, C. A. 2016. Mechanisms of fibroblast growth factor signaling in the ovarian follicle. J. Endocrinol. 228: R31-R43.

Prudovsky, I., A. Mandinova, R. Soldi, C. Bagala, I. Graziani, M. Landriscina, F. Tarantini, M. Duarte, S. Bellum, H. Doherty, and T. Maciag. 2003. The non-classical export routes: FGF1 and IL-1 alpha point the way. J. Cell Sci. 116: 4871-4881.

Puscheck, E. E., Y. Patel, and D. A. Rappolee. 1997. Fibroblast growth factor receptor (FGFr)-4, but not FGFr-3 is expressed in the pregnant ovary. Mol. Cell. Endocrinol. 132: 169-176.

Quackenbush, J. 2001. Computational analysis of microarray data. Nat. Rev. Genet. 2: 418-427.

Rabinovici, J., S. J. Spencer, and R. B. Jaffe. 1990. Recombinant human activin-a promotes proliferation of human luteinized preovulatory granulosa-cells invitro. J. Clin. Endocrinol. Metab. 71: 1396-1398.

Radha, V., S. Nambirajan, and G. Swarup. 1996. Association of lyn tyrosine kinase with the nuclear matrix and cell-cycle-dependent changes in matrix-associated tyrosine kinase activity. Eur. J. Biochem. 236: 352-359.

Rajakoski, E. 1960. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, and left-right variations. Acta Endocrinologica 34: 7-68.

Ranzenigo, G., F. Caloni, F. Crernonesi, P. Y. Aad, and L. J. Spicer. 2008. Effects of fusarium mycotoxins on steroid production by porcine granulosa cells. Anim. Reprod. Sci. 107: 115-130.

Richards, J. S. 1994. Hormonal-control of gene-expression in the ovary. Endocr. Rev. 15: 725-751.

Rico, C., S. Fabre, C. Medigue, N. di Clemente, F. Clement, M. Bontoux, J. L. Touze, M. Dupont, E. Briant, B. Remy, J. F. Beckers, and D. Monniaux. 2009. Anti-mullerian

hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. Biol. Reprod. 80: 50-59.

Rinderknecht, E., and R. E. Humbel. 1976. Polypeptides with nonsuppressible insulin-like and cell-growth promoting activities in human-serum - isolation, chemical characterization, and some biological properties of forms 1 and 2. Proc. Natl. Acad. Sci. U S A 73: 2365-2369.

Rinderknecht, E., and R. E. Humbel. 1978a. Amino-acid sequence of human insulin-like growth factor-I and its structural homology with proinsulin. J. Biol. Chem. 253: 2769-2776.

Rinderknecht, E., and R. E. Humbel. 1978b. Primary structure of human insulin-like growth factor-II. Febs Letters 89: 283-286.

Rivera, G. M., Y. A. Chandrasekher, A. C. O. Evans, L. C. Giudice, and J. E. Fortune. 2001. A potential role for insulin-like growth factor binding protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle. Biol. Reprod. 65: 102-111.

Rivera, G. M., and J. E. Fortune. 2003. Selection of the dominant follicle and insulin-like growth factor (IGF)-binding proteins: Evidence that pregnancy-associated plasma protein a contributes to proteolysis of IGF-binding protein 5 in bovine follicular fluid. Endocrinology 144: 437-446.

Roberts, A. J., and M. K. Skinner. 1991. Transforming growth factor-alpha and factor-beta differentially regulate growth and steroidogenesis of bovine thecal cells during antral follicle development. Endocrinology 129: 2041-2048.

Roberts, R. D., and R. C. L. Ellis. 1999. Mitogenic effects of fibroblast growth factors on chicken granulosa and theca cells in vitro. Biol. Reprod. 61: 1387-1392.

Robertson, D. M., L. M. Foulds, L. Leversha, F. J. Morgan, M. T. W. Hearn, H. G. Burger, R. E. H. Wettenhall, and D. M. Dekretser. 1985. Isolation of inhibin from bovine follicular-fluid. Biochem. Biophys. Res. Commun. 126: 220-226.

Robinson, R. S., A. J. Hammond, G. E. Mann, and M. G. Hunter. 2008. A novel physiological culture system that mimics luteal angiogenesis. Reproduction 135: 405-413.

Robker, R. L., and J. S. Richards. 1998. Hormone-induced proliferation and differentiation of granulosa cells: A coordinated balance of the cell cycle regulators cyclin d2 and p27kip1. Mol. Endocrinol. 12: 924-940.

Rodan, S. B., G. Wesolowski, K. A. Thomas, K. Yoon, and G. A. Rodan. 1989. Effects of acidic and basic fibroblast growth factors on osteoblastic cells. Connect. Tissue Res. 20: 283-288.

Roman, G. A. S., and D. A. Magoffin. 1993. Insulin-like growth factor-binding proteins in healthy and atretic follicles during natural menstrual cycles. J. Clin. Endocrinol. Metab. 76: 625-632.

Rowehl, R. A., H. Crawford, A. Dufour, J. Ju, and G. I. Botchkina. 2008. Genomic analysis of prostate cancer stem cells isolated from a highly metastatic cell line. Cancer Genomics Proteomics 5: 301-310.

Rubin, J. S., H. Osada, P. W. Finch, W. G. Taylor, S. Rudikoff, and S. A. Aaronson. 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc. Natl. Acad. Sci. U S A 86: 802-806.

Ruetten, H., and C. Thiemermann. 1997. Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat: A possible role for protein tyrosine kinase. Br. J. Pharmacol. 122: 59-70.

Salilew-Wondim, D., I. Ahmad, S. Gebremedhn, S. Sahadevan, M. D. Hossain, F. Rings, M. Hoelker, E. Tholen, C. Neuhoff, C. Looft, K. Schellander, and D. Tesfaye. 2014. The expression pattern of micrornas in granulosa cells of subordinate and dominant follicles during the early luteal phase of the bovine estrous cycle. PLoS One 9: e106795.

Salli, U., F. F. Bartol, A. A. Wiley, B. J. Tarleton, and T. D. Braden. 1998. Keratinocyte growth factor expression by the bovine corpus luteum. Biol. Reprod. 59: 77-83.

Santos-Biase, W. K., F. H. Biase, J. Buratini, Jr., J. Balieiro, Y. F. Watanabe, M. F. Accorsi, C. R. Ferreira, P. Stranieri, A. R. Caetano, and F. V. Meirelles. 2012. Single nucleotide polymorphisms in the bovine genome are associated with the number of oocytes collected during ovum pick up. Anim. Reprod. Sci. 134: 141-149.

Sara, V. R., and K. Hall. 1990. Insulin-like growth-factors and their binding-proteins. Physiol. Rev. 70: 591-614.

Saragueta, P. E., G. M. Lanuza, and J. L. Baranao. 2002. Autocrine role of transforming growth factor beta 1 on rat granulosa cell proliferation. Biol. Reprod. 66: 1862-1868.

Savion, N., G. M. Lui, R. Laherty, and D. Gospodarowicz. 1981. Factors controlling proliferation and progesterone production by bovine granulosa-cells in serum-free medium. Endocrinology 109: 409-420.

Schams, D., W. Amselgruber, R. Einspanier, F. Sinowatz, and D. Gospodarowicz. 1994. Localization and tissue concentration of basic fibroblast growth-factor in the bovine corpus-luteum. Endocrine 2: 907-912.

Schams, D., B. Berisha, M. Kosmann, and W. M. Amselgruber. 2002. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. Domest. Anim. Endocrinol. 22: 51-72.

Schams, D., R. Koll, and C. H. Li. 1988. Insulin-like growth factor-I stimulates oxytocin and progesterone production by bovine granulosa-cells in culture. J. Endocrinol. 116: 97-100.

Schams, D., M. Kosmann, B. Berisha, W. M. Amselgruber, and A. Miyamoto. 2001. Stimulatory and synergistic effects of luteinising hormone and insulin like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulosa cells. Exp. Clin. Endocrinol. Diabetes 109: 155-162.

Schams, D., V. Steinberg, M. Steffl, H. H. Meyer, and B. Berisha. 2009. Expression and possible role of fibroblast growth factor family members in porcine antral follicles during final maturation. Reproduction 138: 141-149.

Schena, M. 1996. Genome analysis with gene expression microarrays. Bioessays 18: 427-431.

Schena, M., D. Shalon, R. W. Davis, and P. O. Brown. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270: 467-470.

Schmid, C. 1995. Insulin-like growth-factors. Cell Biol. Int. 19: 445-457.

Schreiber, N. B., and L. J. Spicer. 2012. Effects of fibroblast growth factor 9 (FGF9) on steroidogenesis and gene expression and control of FGF9 mRNA in bovine granulosa cells. Endocrinology 153: 4491-4501.

Schreiber, N. B., M. L. Totty, and L. J. Spicer. 2012. Expression and effect of fibroblast growth factor 9 in bovine theca cells. J. Endocrinol. 215: 167-175.

Schwartz, J., and H. M. Goodman. 1976. Comparison of delayed actions of growth-hormone and somatomedin on adipose-tissue metabolism. Endocrinology 98: 730-737.

Selvaraju, S., J. K. Folger, P. S. P. Gupta, J. J. Ireland, and G. W. Smith. 2013. Stage-specific expression and effect of bone morphogenetic protein 2 on bovine granulosa cell estradiol production: Regulation by cocaine and amphetamine regulated transcript. Domest. Anim. Endocrinol. 44: 115-120.

Shimasaki, S., R. K. Moore, F. Otsuka, and G. F. Erickson. 2004. The bone morphogenetic protein system in mammalian reproduction. Endocr. Rev. 25: 72-101.

Shimasaki, S., R. J. Zachow, D. M. Li, H. Kim, S. Iemura, N. Ueno, K. Sampath, R. J. Chang, and G. F. Erickson. 1999. A functional bone morphogenetic protein system in the ovary. Proc. Natl. Acad. Sci. U S A 96: 7282-7287.

Shukovski, L., M. Dyson, and J. K. Findlay. 1993. The effects of follistatin, activin and inhibin on steroidogenesis by bovine thecal cells. Mol. Cell. Endocrinol. 97: 19-27.

Shukovski, L., J. K. Findlay, and D. M. Robertson. 1991. The effect of follicle-stimulating hormone-suppressing protein or follistatin on luteinizing bovine granulosa-cells in vitro and its antagonistic effect on the action of activin. Endocrinology 129: 3395-3402.

Sidis, Y., T. Fujiwara, L. Leykin, K. Isaacson, T. Toth, and A. L. Schneyer. 1998. Characterization of inhibin/activin subunit, activin receptor, and follistatin messenger ribonucleic acid in human and mouse oocytes: Evidence for activin's paracrine signaling from granulosa cells to oocytes. Biol. Reprod. 59: 807-812.

Sievers, J., B. Hausmann, K. Unsicker, and M. Berry. 1987. Fibroblast growth factors promote the survival of adult rat retinal ganglion cells after transection of the optic nerve. Neurosci. Lett. 76: 157-162.

Silva, J. R. V., J. R. Figueiredo, and R. van den Hurk. 2009. Involvement of growth hormone (gh) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. Theriogenology 71: 1193-1208.

Silvia, W. J., G. S. Lewis, J. A. McCracken, W. W. Thatcher, and L. Wilson, Jr. 1991. Hormonal regulation of uterine secretion of prostaglandin f2 alpha during luteolysis in ruminants. Biol. Reprod. 45: 655-663.

Singh, B., and D. T. Armstrong. 1995. Transforming growth-factor-alpha gene-expression and peptide localization in porcine ovarian follicles. Biol. Reprod. 53: 1429-1435.

Skaletz-Rorowski, A., J. Waltenberger, J. G. Muller, E. Pawlus, K. Pinkernell, and G. Breithardt. 1999. Protein kinase c mediates basic fibroblast growth factor-Induced proliferation through mitogen-activated protein kinase in coronary smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 19: 1608-1614.

Skinner, M. K. 2005. Regulation of primordial follicle assembly and development. Hum. Reprod. Update 11: 461-471.

Skinner, M. K., and R. J. Coffey. 1988. Regulation of ovarian cell-growth through the local production of transforming growth factor-alpha by theca cells. Endocrinology 123: 2632-2638.

Solloway, M. J., A. T. Dudley, E. K. Bikoff, K. M. Lyons, B. L. M. Hogan, and E. J. Robertson. 1998. Mice lacking BMP6 function. Dev. Genet. 22: 321-339.

Spicer, L. J. 2001. Receptors for insulin-like growth factor-I and tumor necrosis factor-alpha are hormonally regulated in bovine granulosa and thecal cells. Anim. Reprod. Sci. 67: 45-58.

Spicer, L. J. 2004. Proteolytic degradation of insulin-like growth factor binding proteins by ovarian follicles: A control mechanism for selection of dominant follicles. Biol. Reprod. 70: 1223-1230.

Spicer, L. J., and P. Y. Aad. 2007. Insulin-like growth factor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: Role of follicle-stimulating hormone and IGF2 receptor. Biol. Reprod. 77: 18-27.

Spicer, L. J., P. Y. Aad, D. Allen, S. Mazerbourg, and A. J. Hsueh. 2006. Growth differentiation factor-9 has divergent effects on proliferation and steroidogenesis of bovine granulosa cells. J. Endocrinol. 189: 329-339.

Spicer, L. J., P. Y. Aad, D. T. Allen, S. Mazerbourg, A. H. Payne, and A. J. Hsueh. 2008. Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: Influence of follicle size on responses to GDF9. Biol. Reprod. 78: 243-253.

Spicer, L. J., E. Alpizar, and S. E. Echternkamp. 1993. Effects of insulin, insulin-like growth factor-I, and gonadotropins on bovine granulosa-cell proliferation, progesterone production, estradiol production, and (or) insulin-like growth factor-I production invitro. J. Anim. Sci. 71: 1232-1241.

Spicer, L. J., and C. S. Chamberlain. 1998. Influence of cortisol on insulin- and insulin-like growth factor 1 (IGF-1)-induced steroid production on IGF-1 receptors in cultured bovine granulosa cells and thecal cells. Endocrine 9: 153-161.

Spicer, L. J., and C. S. Chamberlain. 1999. Insulin-like growth factor binding protein-3: Its biological effect on bovine granulosa cells. Domest. Anim. Endocrinol. 16: 19-29.

Spicer, L. J., and C. S. Chamberlain. 2000. Production of insulin-like growth factor-I by granulosa cells but not thecal cells is hormonally responsive in cattle. J. Anim. Sci. 78: 2919-2926.

Spicer, L. J., C. S. Chamberlain, and G. L. Morgan. 2001. Proteolysis of insulin-like growth factor binding proteins during preovulatory follicular development in cattle. Domest. Anim. Endocrinol. 21: 1-15.

Spicer, L. J., and S. E. Echternkamp. 1986. Ovarian follicular growth, function and turnover in cattle: A review. J. Anim. Sci. 62: 428-451.

Spicer, L. J., and S. E. Echternkamp. 1995. The ovarian insulin and insulin-like growth-factor system with an emphasis on domestic-animals. Domest. Anim. Endocrinol. 12: 223-245.

Spicer, L. J., and R. E. Stewart. 1996. Interactions among basic fibroblast growth factor, epidermal growth factor, insulin, and insulin-like growth factor-I (IGF-I) on cell numbers and steroidogenesis of bovine thecal cells: Role of IGF-I receptors. Biol. Reprod. 54: 255-263.

Stemple, D. L., N. K. Mahanthappa, and D. J. Anderson. 1988. Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: A sequence of events in sympathetic development. Neuron 1: 517-525.

Steringer, J. P., H. M. Muller, and W. Nickel. 2015. Unconventional secretion of fibroblast growth factor 2--a novel type of protein translocation across membranes? J. Mol. Biol. 427: 1202-1210.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, and B. E. Keefer. 1995. Effects of insulin-like growth factor i and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: Involvement of glucose and receptors for insulin-like growth factor i and luteinizing hormone. J. Anim. Sci. 73: 3719-3731.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, B. E. Keefer, L. J. Dawson, G. L. Morgan, and S. E. Echternkamp. 1996. Levels of insulin-like growth factor (IGF) binding proteins, luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrous cycles. Endocrinology 137: 2842-2850.

Stubbs, S. A., L. J. Webber, J. Stark, S. Rice, R. Margara, S. Lavery, G. H. Trew, K. Hardy, and S. Franks. 2013. Role of insulin-like growth factors in initiation of follicle growth in normal and polycystic human ovaries. J. Clin. Endocrinol. Metab. 98: 3298-3305.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, and J. P. Mesirov. 2005. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U S A 102: 15545-15550.

Sugiura, K., Y. Q. Su, F. J. Diaz, S. A. Pangas, S. Sharma, K. Wigglesworth, M. J. O'Brien, M. M. Matzuk, S. Shimasaki, and J. J. Eppig. 2007. Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. Development 134: 2593-2603.

Sutton-McDowall, M. L., R. B. Gilchrist, and J. G. Thompson. 2004. Cumulus expansion and glucose utilisation by bovine cumulus-oocyte complexes during in vitro maturation: The influence of glucosamine and follicle-stimulating hormone. Reproduction 128: 313-319.

Suzuki, T., K. Miyamoto, Y. Hasegawa, Y. Abe, M. Ui, Y. Ibuki, and M. Igarashi. 1987. Regulation of inhibin production by rat granulosa-cells. Mol. Cell. Endocrinol. 54: 185-195.

Tamura, K., R. Asakai, and R. Okamoto. 1991. Basic fibroblast growth-factor in rat corpus-luteum stimulates prostaglandin-f2-alpha production. Biochem. Biophys. Res. Commun. 178: 393-399.

Tanner, Y., and R. P. Grose. 2016. Dysregulated FGF signalling in neoplastic disorders. Semin. Cell Dev. Biol. 53: 126-135.

Teerds, K. J., and J. H. Dorrington. 1992. Immunohistochemical localization of transforming growth factor-beta-1 and factor-beta-2 during follicular development in the adult-rat ovary. Mol. Cell. Endocrinol. 84: R7-R13.

Tian, X. C., A. K. Berndtson, and J. E. Fortune. 1995. Differentiation of bovine preovulatory follicles during the follicular phase is associated with increases in messenger ribonucleic acid for cytochrome p450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, and p450 17 alpha-hydroxylase, but not p450 aromatase. Endocrinology 136: 5102-5110.

Tilly, J. L., H. Billig, K. I. Kowalski, and A. J. Hsueh. 1992. Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase-dependent mechanism. Mol. Endocrinol. 6: 1942-1950.

Tolikas, A., E. Tsakos, S. Gerou, Y. Prapas, and A. Loufopoulos. 2011. Anti-mullerian hormone (AMH) levels in serum and follicular fluid as predictors of ovarian response in stimulated (IVF and ICSI) cycles. Hum. Fertil. 14: 246-253.

Torney, A. H., Y. M. Hodgson, R. Forage, and D. M. Dekretser. 1989. Cellular-localization of inhibin messenger-RNA in the bovine ovary by insitu hybridization. J. Reprod. Fertil. 86: 391-399.

Tuuri, T., M. Eramaa, K. Hilden, and O. Ritvos. 1994. Activin-binding protein follistatin messenger-ribonucleic-acid and secreted protein-levels are induced by chorionic-gonadotropin in cultured human granulosa-luteal cells. Endocrinology 135: 2196-2203.

Ueno, N., N. Ling, S. Y. Ying, F. Esch, S. Shimasaki, and R. Guillemin. 1987. Isolation and partial characterization of follistatin - a single-chain mr 35,000 monomeric protein

that inhibits the release of follicle-stimulating-hormone. Proc. Natl. Acad. Sci. U S A 84: 8282-8286.

Ui, M., M. Shimonaka, S. Shimasaki, and N. Ling. 1989. An insulin-like growth factor-binding protein in ovarian follicular-fluid blocks follicle-stimulating hormone-stimulated steroid-production by ovarian granulosa-cells. Endocrinology 125: 912-916.

van den Hurk, R., and S. A. van de Pavert. 2001. Localization of an activin/activin receptor system in the porcine ovary. Mol. Reprod. Dev. 60: 463-471.

Van Wezel, I. L., A. M. Dharmarajan, T. C. Lavranos, and R. J. Rodgers. 1999. Evidence for alternative pathways of granulosa cell heath in healthy and slightly atretic bovine antral follicles. Endocrinology 140: 2602-2612.

van Wezel, I. L. U., K.; Tilley, W.D.; Rodgers, R.J. 1995. Immunohistochemical localization of basic fibroblast growth factor in bovine ovarian follicles. Mol. Cell. Endocrinol. 115: 133-140.

vandenHurk, R., M. M. Bevers, and J. F. Beckers. 1997. In-vivo and in-vitro development of preantral follicles. Theriogenology 47: 73-82.

Velazquez, M. A., L. J. Spicer, and D. C. Wathes. 2008. The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. Domest. Anim. Endocrinol. 35: 325-342.

Vendola, K., J. Zhou, J. Wang, O. A. Famuyiwa, M. Bievre, and C. A. Bondy. 1999. Androgens promote oocyte insulin-like growth factor i expression and initiation of follicle development in the primate ovary. Biol. Reprod. 61: 353-357.

Vernon, R. K., and L. J. Spicer. 1994. Effects of basic fibroblast growth factor and heparin on follicle-stimulating hormone-induced steroidogenesis by bovine granulosa cells. J. Anim. Sci. 72: 2696-2702.

Vitt, U. A., M. Hayashi, C. Klein, and A. J. Hsueh. 2000a. Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. Biol. Reprod. 62: 370-377.

Vitt, U. A., M. Hayashi, C. Klein, and A. J. W. Hsueh. 2000b. Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. Biol. Reprod. 62: 370-377.

Wahl, S. M., H. Wong, and N. McCartney-Francis. 1989. Role of growth factors in inflammation and repair. J. Cell Biochem. 40: 193-199.

Wandji, S. A., J. J. Eppig, and J. E. Fortune. 1996. Fsh and growth factors affect the growth and endocrine function in vitro of granulosa cells of bovine preantral follicles. Theriogenology 45: 817-832.

Wandji, S. A., T. L. Wood, J. Crawford, S. W. Levison, and J. M. Hammond. 1998. Expression of mouse ovarian insulin growth factor system components during follicular development and atresia. Endocrinology 139: 5205-5214.

Wang, C. J., C. Wang, E. G. Hoch, and G. S. Pitt. 2011. Identification of novel interaction sites that determine specificity between fibroblast growth factor homologous factors and voltage-gated sodium channels. J. Biol. Chem. 286: 24253-24263.

Wang, T. R., L. Y. Yan, J. Yan, C. L. Lu, X. Xia, T. L. Yin, X. H. Zhu, J. M. Gao, T. Ding, W. H. Hu, H. Y. Guo, R. Li, and J. Qiao. 2014. Basic fibroblast growth factor

promotes the development of human ovarian early follicles during growth in vitro. Hum. Reprod. 29: 568-576.

Weenen, C., J. S. E. Laven, A. R. M. von Bergh, M. Cranfield, N. P. Groome, J. A. Visser, P. Kramer, B. C. J. M. Fauser, and A. P. N. Themmen. 2004. Anti-mullerian hormone expression pattern in the human ovary: Potential implications for initial and cyclic follicle recruitment. Mol. Hum. Reprod. 10: 77-83.

Werner, T. 2008. Bioinformatics applications for pathway analysis of microarray data. Curr. Opin. Biotechnol. 19: 50-54.

Woodruff, T. K., R. J. Lyon, S. E. Hansen, G. C. Rice, and J. P. Mather. 1990. Inhibin and activin locally regulate rat ovarian folliculogenesis. Endocrinology 127: 3196-3205.

Wright, R. J., J. M. P. Holly, R. Galea, M. Brincat, and H. D. Mason. 2002. Insulin-like growth factor (IGF)-independent effects of lgf binding protein-4 on human granulosa cell steroidogenesis. Biol. Reprod. 67: 776-781.

Xiao, S., D. M. Robertson, and J. K. Findlay. 1992. Effects of activin and follicle-stimulating-hormone (fsh)-suppressing protein follistatin on fsh receptors and differentiation of cultured rat granulosa-cells. Endocrinology 131: 1009-1016.

Yamashita, H., D. Kamada, K. Shirasuna, M. Matsui, T. Shimizu, K. Kida, B. Berisha, D. Schams, and A. Miyamoto. 2008. Effect of local neutralization of basic fibroblast growth factor or vascular endothelial growth factor by a specific antibody on the development of the corpus luteum in the cow. Mol. Reprod. Dev. 75: 1449-1456.

Yamashita, H., C. Murayama, R. Takasugi, A. Miyamoto, and T. Shimizu. 2011. BMP-4 suppresses progesterone production by inhibiting histone H3 acetylation of star in bovine granulosa cells in vitro. Mol. Cell. Biochem. 348: 183-190.

Yamoto, M., S. Minami, R. Nakano, and M. Kobayashi. 1992. Immunohistochemical localization of inhibin activin subunits in human ovarian follicles during the menstrual-cycle. J. Clin. Endocrinol. Metab. 74: 989-993.

Yamoto, M., T. Shikone, and R. Nakano. 1993. Opposite effects of basic fibroblast growth factor on gonadotropin-stimulated steroidogenesis in rat granulosa cells. Endocr. J. 40: 691-697.

Yan, C. N., P. Wang, J. DeMayo, F. J. DeMayo, J. A. Elvin, C. Carino, S. V. Prasad, S. S. Skinner, B. S. Dunbar, J. L. Dube, A. J. Celeste, and M. M. Matzuk. 2001. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol. Endocrinol. 15: 854-866.

Yang, M. Y., and J. E. Fortune. 2015. Changes in the transcriptome of bovine ovarian cortex during follicle activation in vitro. Physiol. Genomics 47: 600-611.

Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64: 841-848.

Ye, S., Y. D. Luo, W. Q. Lu, R. B. Jones, R. J. Linhardt, I. Capila, T. Toida, M. Kan, H. Pelletier, and W. L. McKeehan. 2001. Structural basis for interaction of FGF-1, FGF-2, and FGF-7 with different heparan sulfate motifs. Biochemistry 40: 14429-14439.

Yeh, J., G. Y. Lee, and E. Anderson. 1993. Presence of transforming growth factor-alpha messenger-ribonucleic-acid (messenger-RNA) and absence of epidermal growth-factor messenger-rna in rat ovarian granulosa-cells, and the effects of these factors on steroidogenesis invitro. Biol. Reprod. 48: 1071-1081.

Ying, S. Y. 1988. Inhibins, activins, and follistatins - gonadal proteins modulating the secretion of follicle-stimulating-hormone. Endocr. Rev. 9: 267-293.

Ying, S. Y., A. Becker, N. Ling, N. Ueno, and R. Guillemin. 1986. Inhibin and beta-type transforming growth-factor (TGF-beta) have opposite modulating effects on the follicle-stimulating-hormone (FSH)-induced aromatase-activity of cultured rat granulosa-cells. Biochem. Biophys. Res. Commun. 136: 969-975.

Yong, E. L., D. T. Baird, R. Yates, L. E. Reichert, and S. G. Hillier. 1992. Hormonal-regulation of the growth and steroidogenic function of human granulosa-cells. J. Clin. Endocrinol. Metab. 74: 842-849.

Yuan, W., B. Bao, H. A. Garverick, R. S. Youngquist, and M. C. Lucy. 1998. Follicular dominance in cattle is associated with divergent patterns of ovarian gene expression for insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein-2 in dominant and subordinate follicles. Domest. Anim. Endocrinol. 15: 55-63.

Yuan, W., M. C. Lucy, and M. F. Smith. 1996. Messenger ribonucleic acid for insulin-like growth factors-I and -II, insulin-like growth factor-binding protein-2, gonadotropin receptors, and steroidogenic enzymes in porcine follicles. Biol. Reprod. 55: 1045-1054.

Zapf, J., C. Hauri, M. Waldvogel, and E. R. Froesch. 1986. Acute metabolic effects and half-lives of intravenously administered insulin-like growth factor-I and factor-II in normal and hypophysectomized rats. J. Clin. Invest. 77: 1768-1775.

Zapf, J., C. Schmid, and E. R. Froesch. 1984. Biological and immunological properties of insulin-like growth factor-I, factor-II (IGF). Clin. Endocrinol. Metab. 13: 3-30.

Zapf, J., E. Schoenle, and E. R. Froesch. 1978. Insulin-like growth factor-I and factor-II - some biological actions and receptor-binding characteristics of 2 purified constituents of nonsuppressible insulin-like activity of human-serum. Eur. J. Biochem. 87: 285-296.

Zhang, K., and A. D. Ealy. 2012. Disruption of fibroblast growth factor receptor signaling in bovine cumulus-oocyte complexes during in vitro maturation reduces subsequent embryonic development. Domest. Anim. Endocrinol. 42: 230-238.

Zhou, H. M., and Y. Zhang. 2005. Effect of growth factors on in vitro development of caprine preantral follicle oocytes. Anim. Reprod. Sci. 90: 265-272.

Zhou, P., S. C. Baumgarten, Y. G. Wu, J. Bennett, N. Winston, J. Hirshfeld-Cytron, and C. Stocco. 2013. IGF-I signaling is essential for fsh stimulation of akt and steroidogenic genes in granulosa cells. Mol. Endocrinol. 27: 511-523.

Zhu, G. Y., B. G. Guo, D. K. Pan, Y. L. Mu, and S. T. Feng. 2008. Expression of bone morphogenetic proteins and receptors in porcine cumulus-oocyte complexes during in vitro maturation. Anim. Reprod. Sci. 104: 275-283.

Zuelke, K. A., and B. G. Brackett. 1992. Effects of luteinizing hormone on glucose metabolism in cumulus-enclosed bovine oocytes matured in vitro. Endocrinology 131: 2690-2696.

CHAPTER III

CHANGES IN FIBROBLAST GROWTH FACTOR 9 (FGF9) mRNA IN GRANULOSA AND THECA CELLS DURING OVARIAN FOLLICULAR GROWTH IN DAIRY CATTLE

1. Abstract

Fibroblast growth factor 9 (FGF9) has been suggested to act as an anti-differentiation factor in cattle by reducing steroidogenesis and increasing cell proliferation in granulosa (GC) and theca (TC) cells. The objective of this study was to characterize *FGF9* mRNA abundance in GC and TC during development of dominant follicles in dairy cattle. Estrous cycles of non-lactating dairy cattle were synchronized, and ovaries were collected on either day 3-4 (n = 8) or day 5-6 (n = 8) post-ovulation for GC and TC mRNA extraction from small (1-5 mm), medium (5.1-8 mm) or large (8.1-18 mm) follicles for PCR analysis. *FGF9* mRNA abundance was greater ($P < 0.05$) in GC than in TC. In GC, *FGF9* mRNA abundance was greater ($P < 0.05$) in small, medium and large estrogen (E2)-inactive (i.e., concentrations of E2 < progesterone, P4) follicles than in large E2-active (i.e., concentrations of E2 > P4) follicles at both early (day 3-4) and late (day 5-6) growing phases of first dominant follicle. Abundance of *FGF9* mRNA was greater ($P < 0.05$) in medium-sized follicles on day 5-6 than on day 3-4 post-ovulation. In TC, *FGF9* mRNA abundance was greater ($P < 0.05$) in large E2-inactive follicles than in large E2-active follicles on day 3-4 post-ovulation; no significant differences in TC *FGF9* mRNA existed among follicle types on day 5-6 post-ovulation. Correlations among levels of hormones and *FGF9* mRNA levels revealed significant negative correlations between GC *FGF9* mRNA abundance and follicular fluid E2 (r = -0.68), E2/P4 ratio (r = -0.58), and free insulin-like growth factor 1 (r = - 0.63). In summary, abundance of

FGF9 mRNA in GC and TC is greater in medium-sized follicles at late than at early growing phase of first dominant follicle, and is less in dominant E2-active than subordinate E2-inactive follicles, indicating that FGF9 signaling could contribute to normal follicle development and steroidogenesis in dairy cattle.

Key Words: Fibroblast growth factor-9 (FGF9), theca cell, granulosa cell, follicle growth, cattle.

2. Introduction

Fibroblast growth factors (FGFs) constitute a large family of polypeptide growth factors present in both vertebrates and invertebrates (Ornitz and Itoh, 2001). These growth factors and their receptors are distributed in various tissues and regulate development, metabolism and disease through intracrine, paracrine and endocrine mechanisms of action (Itoh and Ornitz, 2011). FGF family members have also been identified to play important roles in the reproduction of mammals including the human, caprine, ovine, bovine and murine species (for review see Chaves et al., 2012). To date, FGF1, FGF2, FGF7-9, FGF10, FGF17 and FGF18 have been identified in the ovary of humans (Oron et al., 2012), rodents (Drummond et al., 2007), and/or domestic animals (Berisha et al., 2004; Machado et al., 2009; Portela et al., 2010). Functions of FGF members in the ovary include regulation of granulosa cell (GC) steroidogenesis of cattle and pigs (Vernon and Spicer, 1994; Schreiber and Spicer, 2012; Evans et al., 2014), regulation of GC apoptosis and survival in rats (Tilly et al., 1992; Peluso et al., 2001) and cattle (Portela et al., 2010; Jiang and Price, 2012), control of bovine GC proliferation and differentiation (Berisha et al., 2004; Schreiber and Spicer, 2012), oocyte maturation in rats and cattle (LaPolt et al., 1990; Cho et al., 2008), and luteal development in cattle (Gabler et al., 2004; Woad et al., 2012).

Abundance of *FGF9* mRNA was first observed to be down-regulated in GC of cystic follicles of cattle compared with noncystic follicles in a microarray study, indicating that FGF9 may play a role in bovine follicular development (Grado-Ahuir et al., 2011).

Subsequently, abundance of *FGF9* mRNA was found to be greater in GC than in theca cells (TC) and greater in both GC and TC in small follicles (i.e., 1-5 mm) in comparison to large (i.e., ≥ 8 mm) follicles (Schreiber et al., 2012). Hormones known to be involved in follicular development (e.g., insulin-like growth factor 1; IGF1) also regulate *FGF9* mRNA in both GC (Schreiber and Spicer, 2012) and TC (Schreiber et al., 2012). Furthermore, FGF9 treatment stimulated bovine GC and TC proliferation while down-regulating hormone-stimulated steroidogenesis and steroidogenic enzyme gene expression, indicating a role for FGF9 as an anti-differentiation factor regulating folliculogenesis (Schreiber and Spicer, 2012; Schreiber et al., 2012). Increased free IGF1 may amplify FSH-induced estradiol (E2) production in the developing dominant follicle of cattle (Spicer, 2004). However, whether endogenous production of FGF9 by ovarian follicular cells changes during selection of dominant follicles in monotocous mammals such as cattle is still unknown. We hypothesized that *FGF9* mRNA decreases as follicles grow and differentiate. Therefore, the objective of this study was to determine if abundance of *FGF9* mRNA in GC and TC changes during growth of first-wave dominant follicles in dairy cattle exhibiting regular estrous cycles.

3. Materials and Methods

Animals and Experimental Design

Non-lactating Holstein cows (n = 18) were used for this experiment as previously described (Dentis et al., 2016). Briefly, estrous cycles were synchronized using two injections (im) of PGF2α (Lutalyse®, 25 mg) with an interval of 11 days. From the first injection of prostaglandin F2α to the occurrence of ovulation after the second injection, follicle development was monitored daily via ultrasonography using an Aloka 500V with a 7.5 MHz probe. Following ovulation, cows continued to be monitored with daily ultrasonography and were assigned to be ovariectomized either at d 3 to 4 (early growing phase of the first dominant follicle; n = 9 cows) or d 5 to 6 (late growing phase of the first dominant follicle; n = 9 cows). From the 18 cows used in the synchronization program, two failed (one from day 3

to 4 post-ovulation and one from day 5 to 6 post-ovulation groups) to ovulate and were excluded from this experiment. After each ovariectomy, ovaries were put on ice, and transported to the laboratory where diameters of all follicles ≥ 5 mm (surface diameter) in diameter were recorded, the numbers of all follicles ≥ 1 mm in diameter on the ovarian surface determined, and ovarian tissue and fluid collected (Dentis et al., 2016). The animal experimentation described in this report was approved by the Oklahoma State University Institutional Animal Care and Use Committee (Protocol No. AG106).

Cell and Follicular Fluid Collection

For GC sample collection, follicles were categorized by surface diameter as small (1-5 mm), medium (5.1-8 mm) or large (8.1-18 mm); TC samples were collected from only medium and large follicles. Follicular fluid (FFL) from medium and large follicles was aspirated individually and centrifuged to obtain GC; FFL from small follicles was pooled within each ovary and then centrifuged at 200 x g for 8 min to obtain GC as previously described (Stewart et al., 1996; Dentis et al., 2016). After centrifugation, FFL was aspirated and stored at -20 °C for measurement of E2 and progesterone (P4) via RIA. After collection of FFL, each medium and large follicle was bisected *in situ*, the inner wall was scraped, rinsed with Ham's F-12 to remove any remaining GC, and these GC were combined with GC collected from FFL as previously described (Schreiber and Spicer, 2012; Dentis et al., 2016). GC collected from small follicles were kept separate for each ovary. GC were lysed in 0.5 mL of TRIzol® reagent solution (Life Technologies, Inc., Grand Island, NY) and stored frozen at -80 °C until RNA extraction (see description below). TC were dissected from the bisected follicles and placed in 0.75 mL of TRIzol® reagent solution and homogenized for 2-3 min on ice using the Omni TH tissue homogenizer (Omni International Inc., Marietta, GA) with Omni Tip™ disposable generator probes as previously described (Aad et al., 2012; Aad et al., 2013).

RNA Extraction and Quantitative PCR (qPCR)

RNA was extracted from GC and TC as described elsewhere (Lagaly et al., 2008). Phase Lock Gel™ (5 Prime, Inc., Gaithersburg, MD) tubes were used to aid in the recovery of aqueous phase containing RNA. RNA samples were solubilized in diethylpyrocarbonate-treated water (Life Technologies, Inc.), quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and stored at -80 °C.

FGF9 primers and probe (supplied as 5' FAM reporter dye and a 3' TAMRA quencher dye; TaqMan TAMARA; Applied Biosystems Inc., Foster City, CA) for qPCR were designed using Primer Express software (Foster City, CA) as previously reported (Grado-Ahuir et al., 2011; Schreiber and Spicer, 2012). The *FGF9* mRNA (697 bp; Accession NM_001245926.1) forward primer was constructed between bp 387 and 408 with a Tm of 58°C and a sequence of 5'-TCTACCTCGGCATGAATGAGAA-3'. The reverse *FGF9* mRNA primer was constructed between bp 476 and 497 with a Tm of 57°C and a sequence of 5'- TGGAGGAGTACGTGTTGTACCA-3'. The *FGF9* mRNA probe was constructed between bp 421 and 444 with a Tm of 66°C and a sequence of 5'-TGGATCGGAAAAACTCACGCAAGA-3'. Relative mRNA abundance of target genes was quantified using fluorescent single-step qPCR using an ABI Prism® 7500 sequence detection system (Applied Biosystems). Target gene expression was normalized to constitutively expressed *18S* ribosomal RNA (*18S* rRNA; supplied as a VIC probe; TaqMan Ribosomal RNA Control Reagent, Applied Biosystems Inc.), which has been reported as a valid housekeeping gene for bovine GC and TC (Voge et al., 2004), and relative quantity of target gene mRNAs was expressed as $2^{-\Delta\Delta Ct}$ using the relative comparative threshold cycle (Ct) method as previously described (Livak and Schmittgen, 2001; Lagaly et al., 2008).

Radioimmunoassays (RIA) and IGF1 ELISA

Concentrations of P4 and E2 in FFL were determined by RIA as previously described (Stewart et al., 1996; Dentis et al., 2016). All samples were run in one assay for each of the

steroid RIA. The intra-assay coefficient of variation for P4 and E2 RIA averaged 11.6 % and 10.6 %, respectively.

Free IGF1 concentrations in FFL were determined in a subset of samples (n = 32) via a free IGF1 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol similar to previously described (Santiago et al., 2005; Spicer et al., 2005). If sample values were below the limit of detection of the assay, a 0.2 ng/mL value was assigned to that sample. All samples were quantified in one assay and the intra-assay coefficient of variation averaged 6.9 %.

FGF9 Western Blotting

Western blotting was utilized in an attempt to detect FGF9 protein in FFL. Protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma-Aldrich, St Louis, MO) at 1:30 and sample buffer (tris-hydrochloride buffer, 10% SDS, glycerol, bromophenol blue, and β–mercaptoethanol) at 1:3 were added to FFL samples. Recombinant human FGF9 (R&D systems Inc., Minneapolis, MN) was added to FFL (at a total amount of 2.5 ng) and PBS (at total amounts of 5 ng, 1 ng, and 0.1 ng) samples for evaluation of sensitivity of detection. Samples were boiled for 5 min, cooled down, centrifuged at 2000 x g for 1 min, and loaded into 4% stacking, 12% resolving SDS-PAGE mini gels. Precision Plus Protein™ Kaleidoscope™ Standards (Bio-Rad Laboratories, Inc., Hercules, CA) were added for monitoring electrophoretic separation and molecular weight sizing. Gels were ran at 120 V, for 80 min. Gels were transferred to nitrocellulose membranes via iBlot® Gel Transfer Device (Life Technologies, Inc., Carlsbad, California). After transfer, the nitrocellulose membranes were blocked in Tris-buffered saline (TBS) containing 5% (w/v) nonfat dry milk for 1 h at room temperature and then incubated with a FGF9 antibody (rabbit polyclonal antibody against human FGF9; Santa Cruz Biotechnology, Inc., Dallas, TX) at 1:250 overnight at 4°C. Membranes were washed with a mixture of Tris-buffered saline and Tween 20 (TBST) and incubated with secondary HRP-conjugated antibody (ImmunoPure HRP-

conjugated goat anti-rabbit IgG, Pierce Biotechnology, Rockford, IL) diluted in TBS containing 5% (w/v) nonfat dry milk at 1:50,000 for 1 h at room temperature on a rocking platform shaker. Membranes were then washed again with TBST, dried, and incubated with enhanced chemiluminescent (ECL) substrate (SuperSignal^{TM} West Femto maximum sensitivity substrate; Thermo Fisher Scientific, Inc., Waltham, MA) for 5 min at room temperature. Finally, membranes were developed via the FluorChem^{TM} E system Imager (ProteinSimple; San Jose, CA) on movie mode for 180 min chemiluminescence detection.

Statistical Analyses

The analysis of data aimed to determine if *FGF9* mRNA abundance differs in GC and TC at different periods of follicular development and to detect its relationship with steroidogenesis. Data were analyzed via factorial ANOVA with GLM procedures of SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC) and are presented as the least squares means (± SEM) of measurements. Main factors were days post-ovulation (early, day 3-4, and late, day 5-6, growing phase of the first dominant follicle), and follicle status based on size and follicle estrogenic status [large E2 active (E2>P4 concentrations), and small, medium, or large E2 inactive (E2<P4 concentrations) in the case of GC; and large E2 active and medium or large E2 inactive in the case of TC], and their various interactions. Some cows had two E2-active follicles on day 3-4 whereas some cows had no E2-active follicles on day 5-6. Also, if FFL samples were lost during collection, then E2-status could not be determined and gene expression data was not included in the analysis. For analysis of E2 and free IGF1 concentrations in the subset of FFL samples, main factors were days post-ovulation (early, day 3-4, and late, day 5-6, growing phase of the first dominant follicle), follicle status (n = 3) based on size (small EI, large EA or large EI) and their various interaction. To correct for heterogeneity of variance, abundance of *FGF9* mRNA was analyzed after transformation to natural log (x + 1). Mean differences were determined by Fisher's protected least significant differences test (Ott, 1977) only if significant main effects in the ANOVA were detected. To evaluate the relationships among variables measured, Pearson correlation coefficients were

generated using CORR procedure of SAS (SAS Institute). Significance was declared at $P < 0.05$.

4. Results

GC FGF9 mRNA Abundance

Averaged across follicle size groups, qPCR results indicated that *FGF9* mRNA abundance in GC was 6.2-fold greater ($P < 0.05$) than in TC, and that GC *FGF9* mRNA abundance was 2.9-fold greater ($P < 0.05$) at late (day 5-6 post-ovulation) than at early (day 3-4 post-ovulation) growing phase of the first dominant follicle (145 ± 46 and 49 ± 7, respectively). Abundance of *FGF9* mRNA was greater ($P < 0.01$) in large, medium, and small E2-inactive (E2/P4 ratio < 1) than in large E2-active (E2/P4 ratio > 1) follicles at both early (19-, 27- and 18-fold greater, respectively) and late (35-, 77- and 32-fold greater, respectively) growing phases of first dominant follicle (Figure 1A). Also, when E2-inactive follicles of same size were compared at different days post-ovulation, *FGF9* mRNA abundance was 3.7-fold greater ($P < 0.01$) in medium-sized follicles at late than at early growing phase of the first dominant follicle while no significant differences were detected between large or small E2-inactive follicles (Figure 1A). Abundance of *FGF9* mRNA in GC was 2.2–fold greater ($P < 0.01$) in E2-inactive medium- and small-sized follicles than in E2-inactive large-sized follicles at late growing phase (day 5-6) of the first dominant follicle, with no significant differences observed between other sizes at this phase or between any of the E2-inactive follicle sizes at day 3-4 post-ovulation (Figure 1A). Averaged across days, *FGF9* mRNA abundance in GC was several-fold greater ($P < 0.01$) in large (70.6 ± 14.1), medium (179.6 ± 38.6), and small (90.5 ± 21.1) E2-inactive than in large E2-active follicles (5.1 ± 1.0).

TC FGF9 mRNA Abundance

No significant interaction existed between days post-ovulation and follicle status for TC *FGF9* mRNA abundance. In contrast to what was observed in GC, averaged across follicle size groups there was a tendency ($P = 0.05$) for TC *FGF9* mRNA abundance to be greater in early (2.7 ± 3.1) than in late (1.6 ± 0.7) growing phase of the first dominant follicle. Abundance of *FGF9* mRNA in TC was 2.2-fold greater ($P < 0.05$) in large subordinate E2-inactive follicles than in large dominant E2-active follicles on day 3-4 post-ovulation (Figure 1B). No other significant differences were detected between subordinate E2-inactive and large dominant E2-active follicles on day 5-6 post-ovulation, or between large E2-inactive follicles and medium E2-inactive follicles. Abundance of *FGF9* mRNA in TC was greater in large subordinate E2-inactive follicles at day 3-4 than at day 5-6 post-ovulation, whereas no significant changes were observed in the other two groups of follicles (Figure 1B).

Follicle Size, E2, P4 and Free IGF1 Concentrations in Follicular Fluid

Follicle size and steroid concentrations in FFL have been reported for this study (Dentis et al., 2016). Briefly, diameter of large dominant E2-active, large subordinate E2-inactive, and medium E2-inactive follicles averaged 12.9 ± 0.5, 9.48 ± 0.36, and 6.37 ± 0.23 mm, respectively. Concentrations of E2 in FFL of large dominant E2-active, large subordinate E2-inactive, medium E2-inactive, and small E2-inactive follicles averaged 186.5 ± 29.5, 8.45 ± 3.7, 2.3 ± 0.8, and 2.0 ± 0.2 ng/ml, respectively. Concentrations of P4 in FFL did not differ ($P > 0.10$) among follicle groups and ranged between 61 ± 7 and 236 ± 42 ng/ml.

In a subset of these samples (n=32) representative of a range of steroid levels, concentrations of E2, P4 and free IGF1 in large dominant E2-active, large subordinate E2-inactive, and small E2-inactive follicles are shown in Figure 2. Large dominant E2-active follicles had greater ($P < 0.05$) free IGF1 (Figure 2A) and E2 (Figure 2B) but tended to have less ($P < 0.10$) P4 (Figure 2B) than large E2-inactive and small E2-inactive follicles.

Concentrations of free IGF1, E2 and P4 in large E2-inactive and small E2-inactive follicles did not differ ($P > 0.10$).

Correlations between FFL Concentrations of Hormones and *FGF9* mRNA Relative Abundance

Negative correlations existed between GC *FGF9* mRNA abundance and FFL E2 ($r = -0.68$, $P < 0.001$; Figure 3) and E2/P4 ratio ($r = -0.58$, $P < 0.001$; $n = 140$). In contrast, there was a weak positive correlation ($r = 0.18$, $P < 0.05$) between GC *FGF9* mRNA abundance and FFL P4. No significant correlation was observed between TC *FGF9* mRNA abundance and FFL P4 ($r = 0.06$, $P > .50$), but there was a weak negative correlation between TC *FGF9* mRNA abundance and FFL E2 ($r = -.26$, $P < 0.05$) and E2/P4 ratio ($r = -0.22$, $P = 0.05$; $n = 88$). Free IGF1 concentrations in FFL and GC *FGF9* mRNA abundance were negatively correlated ($r = -0.63$, $P < 0.001$; $n = 32$), whereas free IGF1 concentrations and E2 concentrations in FFL were positively correlated ($r = 0.85$, $P < 0.001$). There was no significant correlation between FFL concentrations of P4 and free IGF1 ($r = -0.19$, $P > 0.10$). The E2/P4 ratio was positively correlated with free IGF1 in FFL ($r = 0.63$, $P < 0.001$).

FGF9 Protein in FFL

Recombinant human FGF9 protein was detected via Western blotting at total amounts of 5 ng and 1 ng in 15 µL of PBS and 5 µL of sample buffer, and at a total amount of 2.5 ng in 5 µL FFL, 10 µL of PBS, and 5 µL of sample buffer (i.e., an equivalent sensitivity of 125 ng/mL of FFL). The sensitivity of the antibody, however, was not adequate to detect recombinant human FGF9 protein at a total amount of 0.1 ng in 15 µL of PBS and 5 µL of sample buffer or to detect endogenous FGF9 protein in either 10 µL of FFL, 5 µL of PBS, and 5 µL of sample buffer, or 5 µL of FFL, 10 µL of PBS, and 5 µL of sample buffer (Figure 4).

5. Discussion

FGF9 has been characterized as an anti-differentiation factor in folliculogenesis of cattle, stimulating both GC and TC proliferation while inhibiting steroidogenesis (Schreiber and Spicer, 2012; Schreiber et al., 2012). In the present study, we report for the first time that *FGF9* mRNA abundance in GC and TC changes according to E2 activity and size of follicles, and also during follicle development in cattle.

In the present study, follicles were collected at specific days of a synchronized cycle during the first follicular wave and thus represent a well-defined physiologic model. Consistent with what is expected from a factor that stimulates cells to proliferate while inhibiting their differentiation by decreasing E2 production (as described in Schreiber and Spicer, 2012), the abundance of *FGF9* mRNA in GC was less in large dominant E2-active follicles at early and late growing phases of the first follicular wave than in subordinate E2-inactive follicles of various sizes, indicating that FGF9 production likely decreases as the follicle becomes dominant and increased steroidogenesis is required for its further differentiation. In support of this latter suggestion, FFL E2 concentrations and E2:P4 ratio were negatively correlated with GC *FGF9* mRNA abundance in the present study. Alternatively, increased E2 may directly inhibit *FGF9* mRNA expression in GC, but this is unlikely because *in vivo*, diethylstilbesterol (DES) treatment had no significant effect on ovarian *FGF9* mRNA expression in rats (Drummond et al., 2007), and *in vitro*, E2 had no significant effect on bovine GC *FGF9* mRNA abundance (Schreiber and Spicer, 2012). The absence of any change in FFL P4 concentrations during significant changes in both GC and TC *FGF9* mRNA indicates that P4 likely does not regulate *FGF9* mRNA. Also in the present study, GC *FGF9* mRNA increased in medium E2-inactive follicles as the follicular wave advanced from early to late in the growing phase of the first dominant follicle resulting in a greater abundance of *FGF9* mRNA in medium than in large-sized follicles at day 5-6 post-ovulation. Perhaps FGF9 is important to assure that only one follicle will differentiate and dominate at the end of the first follicular wave in a monovulatory species such as the bovine. Previous studies in cattle found a greater abundance of *FGF18* mRNA in GC and TC of

subordinate versus dominant follicles (Portela et al., 2010), and a greater abundance of *FGF2* mRNA (Berisha et al., 2004) and *FGF10* mRNA (Buratini et al., 2007) in TC of estrogenic versus non-estrogenic antral follicles. In contrast, *FGF1* and *FGF7* mRNA abundance in GC and TC do not change during follicular development in cattle (Berisha et al., 2004) or pigs (Schams et al., 2009). Collectively, the previous and present studies indicate different developmental control of the various FGFs in GC and TC of follicles during folliculogenesis.

As mentioned, E2 does not directly affect *FGF9* mRNA abundance in GC (Schreiber and Spicer, 2012), suggesting that one or more hormone(s) or intraovarian factor(s) other than E2 is(are) the cause for lower abundance of *FGF9* mRNA in GC of large dominant EA follicles. One of these other intraovarian factors may be IGF1 because IGF1 decreases abundance of *FGF9* mRNA in GC from both small and large follicles (Schreiber and Spicer, 2012), and concentrations of free IGF1 in FFL increase in early dominant follicles (Fortune et al., 2001; Spicer, 2004) as well as in preovulatory dominant follicles (Santiago et al., 2005) in cattle. Also, the fact that IGF-1 suppresses Indian hedgehog production in TC (Spicer et al., 2009) and that sonic hedgehog (SHH) stimulates FGF9 production in the ovary (Schreiber and Spicer, 2012) and other tissues (Sun et al., 2000) indicate that hedgehog proteins may be important in FGF9 signaling in the ovary (Figure 5). In addition, we observed that wingless-type mouse mammary tumor virus integration site (WNT)-3A protein inhibited GC *FGF9* mRNA (Schreiber and Spicer, 2012). WNTs are secreted ligands that bind to frizzled (FZD) receptor proteins and subsequently transmit their intracellular signals (Grado-Ahuir et al., 2011; Castanon et al., 2012). Recent findings indicate that GC of dominant follicles have greater *FZD6* mRNA than subordinate follicles (Gupta et al., 2014) and that FSH increases mRNA abundance of one of FZD6 ligands, *WNT2,* in bovine granulosa cells (Castanon et al., 2012). Thus, we hypothesize that during dominant follicle development, increases in free IGF1 (Fortune et al., 2001; Spicer, 2004) and WNT signaling (Gupta et al., 2014) cause a decrease in GC *FGF9* mRNA (Figure 5). Further research will be required to verify this suggestion.

Similar to what was found for GC, there was a negative correlation between TC *FGF9* mRNA and E2/P4 ratio in FFL. Also, TC of large E2-inactive follicles had greater abundance of *FGF9* mRNA than large E2-active follicles, but only at day 3-4 post-ovulation. Hormones that regulate TC *FGF9* mRNA abundance have not been studied in any detail. Interestingly, factors (i.e., TNFα and WNT3A) that inhibit *FGF9* mRNA in GC (Schreiber and Spicer, 2012) and inhibit androstenedione production by TC (Spicer, 1998) were found to stimulate *FGF9* mRNA in TC (Schreiber et al., 2012). We further hypothesize that IGF1 and WNTs may act in a negative feedback loop within TC promoting FGF9 production to avoid premature differentiation of dominant follicles. Consistent with an opposing regulation of *FGF9* mRNA in TC versus GC is the general trend that *FGF9* mRNA was greater in TC and lower in GC at day 3-4 in comparison to day 5-6 post-ovulation in the present study. Further work will be required to elucidate the hormonal and developmental control of TC *FGF9* mRNA.

In the present study, regardless of follicle size, *FGF9* mRNA abundance was greater in GC than in TC; this is in agreement with a previous study (Schreiber et al., 2012) and indicates that GC is the main cell type responsible for ovarian follicle production of FGF9. Also, abundance of *FGF9* mRNA in GC varied more dramatically with follicle status than in TC, further suggesting a greater importance for GC in FGF9 production during folliculogenesis in cattle. Unfortunately, the two commercial ELISA kits available to measure FGF9 protein (i.e., bovine FGF9 ELISA, NovateinBio, Inc., Cambridge, MA; human FGF9 ELISA, R&D Systems, Minneapolis, MN) did not have adequate sensitivity to detect FGF9 protein in bovine FFL in the present study (Spicer and Schütz, unpublished results). Furthermore, attempts to use Western blotting to detect FGF9 in FFL were unsuccessful, most likely because of the small quantities of protein produced. Sensitivity of detection was 50 ng/mL in PBS and 125 ng/mL in FFL. Based on in vitro studies with half maximal inhibitory concentrations of FGF9 ranging between 2 and 12 ng/mL (Schreiber and Spicer, 2012; Schreiber et al., 2012), FGF9 concentrations in FFL would be near this range. Thus, an

estimation of sensitivity of Western blotting system would need to be increased by 5- to 50-fold. Additional developmental work will be required to ascertain changes in FFL FGF9 protein levels during follicular growth and atresia.

In summary, *FGF9* mRNA abundance in both GC and TC changed during development of dominant and subordinate follicles in cattle. Abundance of *FGF9* mRNA was greater in GC than in TC and the more dramatic change in *FGF9* mRNA in GC vs. TC indicate a greater importance for GC in ovarian FGF9 production during folliculogenesis in cattle. Dramatically less *FGF9* mRNA abundance in dominant E2-active follicles than in E2-inactive subordinate follicles is consistent with what is expected from an anti-differentiation factor. We postulate, as a framework for future studies and based on the experimental data presented in this paper, that FGF9 may play a role in follicular growth and atresia in cattle.

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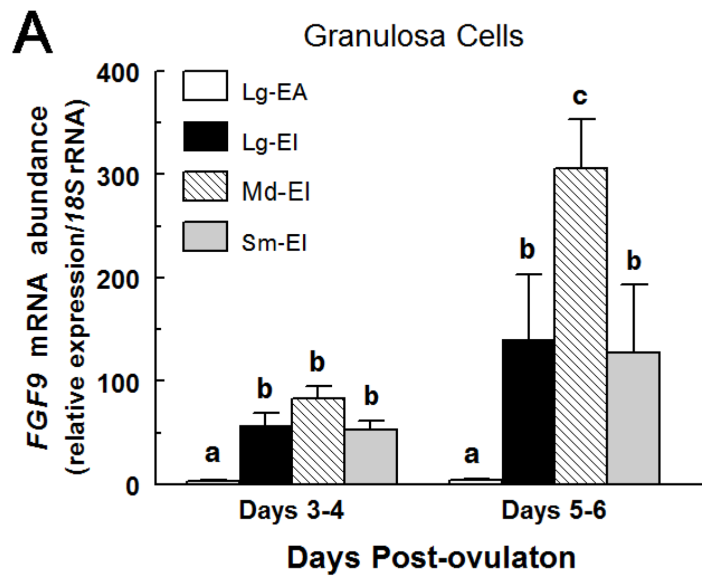
Fig. 1. Abundance of *FGF9* mRNA in granulosa and theca cells during follicular growth. Panel A: Effects of follicular size (Lg = Large; Md = Medium; Sm = Small) and E2 status (EA = estrogen active; EI = estrogen inactive) on *FGF9* mRNA in bovine granulosa cells on different days post-ovulation. [abc]Means without a common letter differ ($P < 0.05$); n = 10, 16, 36 and 16 for Lg-EA, Lg-EI, Md-EI and Sm-EI, respectively, for day 3-4; n = 5, 17, 27 and 14 for Lg-EA, Lg-EI, Md-EI and Sm-EI, respectively, for day 5-6. Panel B: Effect of follicular size (Lg = Large; Md = Medium) and E2 status (EA = estrogen active; EI = estrogen inactive) on abundance of *FGF9* mRNA in bovine theca cells on different days post-ovulation. [ab]Means without a common letter differ ($P < 0.05$); n = 9, 13 and 28 for Lg-EA, Lg-EI and Md-EI, respectively, for day 3-4; n = 4, 11 and 23 for Lg-EA, Lg-EI and Md-EI, respectively, for day 5-6. Values are normalized to constitutively expressed *18S* ribosomal RNA and are least squares means ± SEM.


A
Granulosa Cells
FGF9 mRNA abundance
(relative expression/18S rRNA)
400
300
200
100
0
Lg-EA
Lg-EI
Md-EI
Sm-EI
a
b
b
b
a
b
c
b
Days 3-4
Days 5-6
Days Post-ovulaton

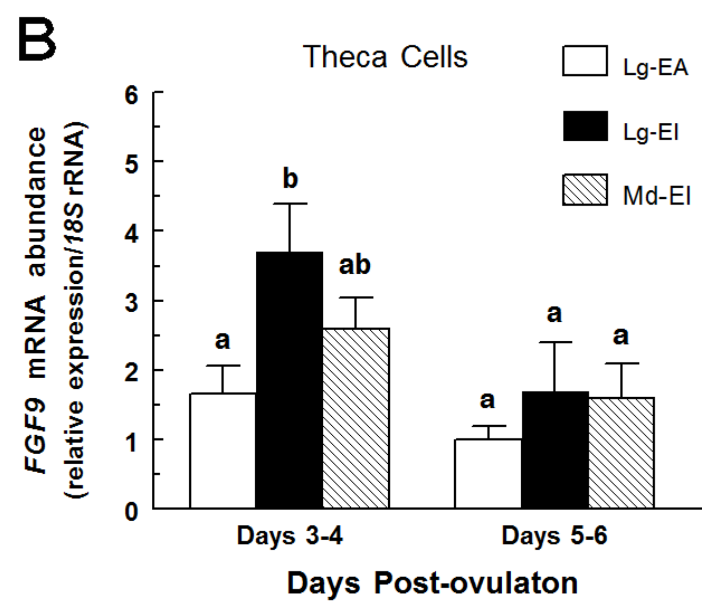
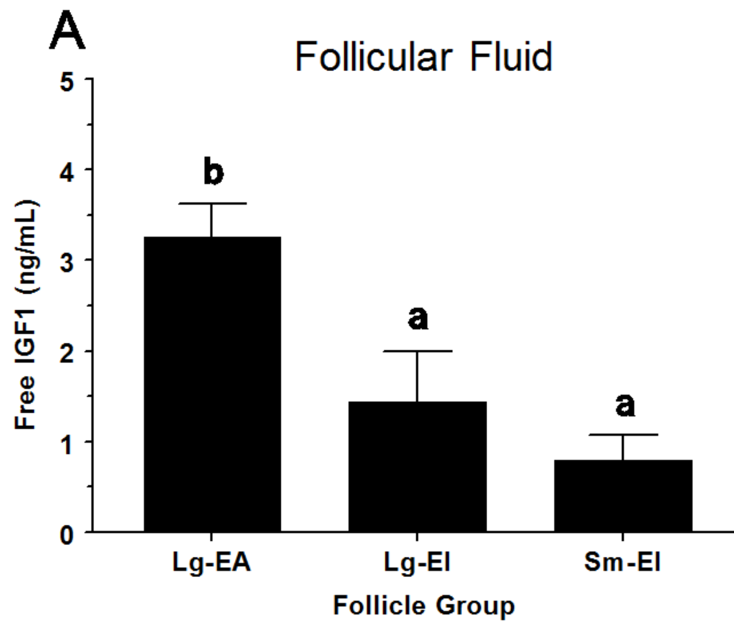

B
Theca Cells
FGF9 mRNA abundance
(relative expression/18S rRNA)
6
5
4
3
2
1
0
Lg-EA
Lg-EI
Md-EI
a
b
ab
a
a
a
Days 3-4
Days 5-6
Days Post-ovulaton

Fig. 2. Effects of follicular size (Lg = Large; Sm = Small) and E2 status (EA = estrogen active; EI = estrogen inactive) on concentrations of free IGF1 (Panel A), E2 and P4 (Panel B) in follicular fluid. [ab]Means (n = 6-18 follicles per group) without a common letter differ ($P < 0.05$); [cd]Means (n = 6-17 follicles per group) without a common letter differ ($P < 0.05$).


A
Follicular Fluid
Free IGF1 (ng/mL)
0
1
2
3
4
5
b
a
a
Lg-EA
Lg-EI
Sm-EI
Follicle Group

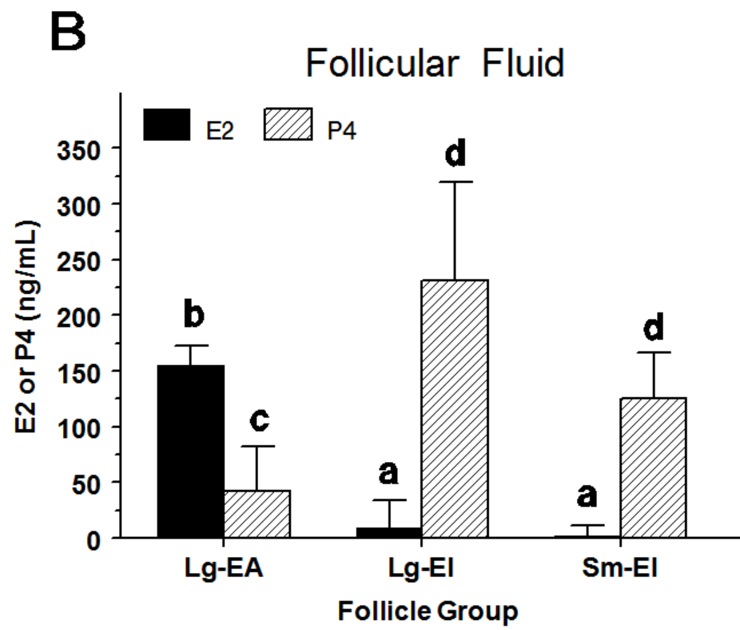

B
Follicular Fluid
E2
P4
E2 or P4 (ng/mL)
0
50
100
150
200
250
300
350
b
c
a
d
a
d
Lg-EA
Lg-EI
Sm-EI
Follicle Group

Fig. 3. Correlation between *FGF9* mRNA abundance in granulosa cells and concentrations of E2 in follicular fluid (FFL). The relationship between *FGF9* mRNA abundance and concentrations of E2 in FFL collected between days 3 and 6 post-ovulation is represented by the line (—). The line is a log-fit line.

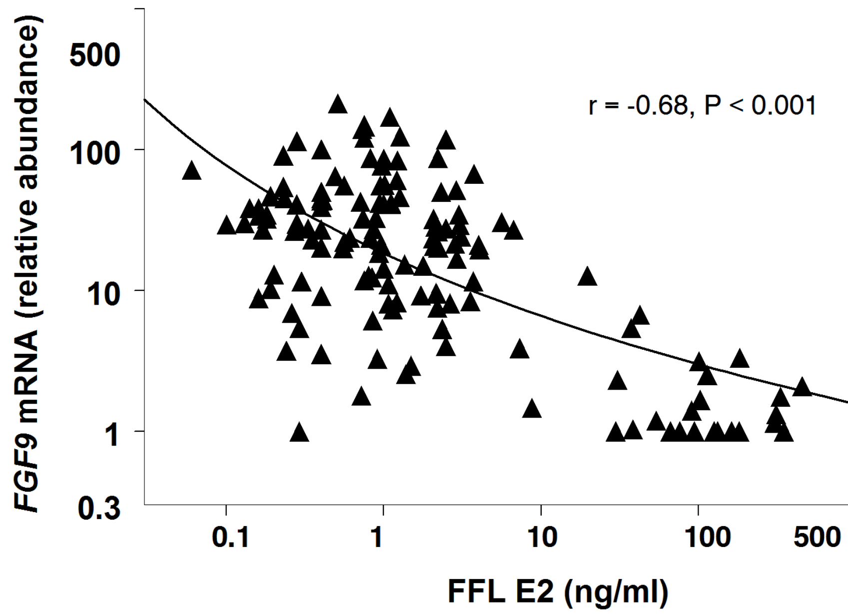


Fig. 4. Results from Western blotting to detect FGF9 protein in FFL. Lanes were loaded as following (from left to right): negative control (0 ng of recombinant human FGF9 in PBS); 5 ng of recombinant human FGF9 in 15 μL of PBS and 5 μL of sample buffer; 1 ng of recombinant human FGF9 in 15 μL of PBS and 5 μL of sample buffer; 0.1 ng of recombinant human FGF9 in in 15 μL of PBS and 5 μL of sample buffer; 2.5 ng of recombinant human FGF9 in 5 μL FFL, 10 μL of PBS, and 5 μL of sample buffer; 10 μL of FFL, 5 μL of PBS, and 5 μL of sample buffer; 5 μL of FFL, 10 μL of PBS, and 5 μL of sample buffer.

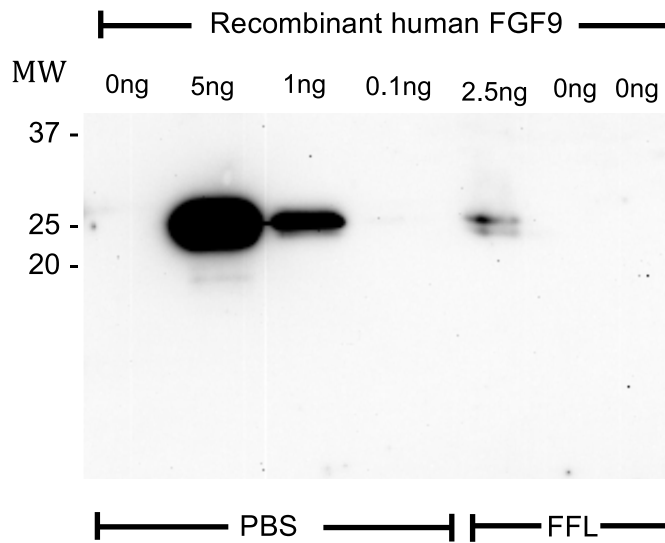
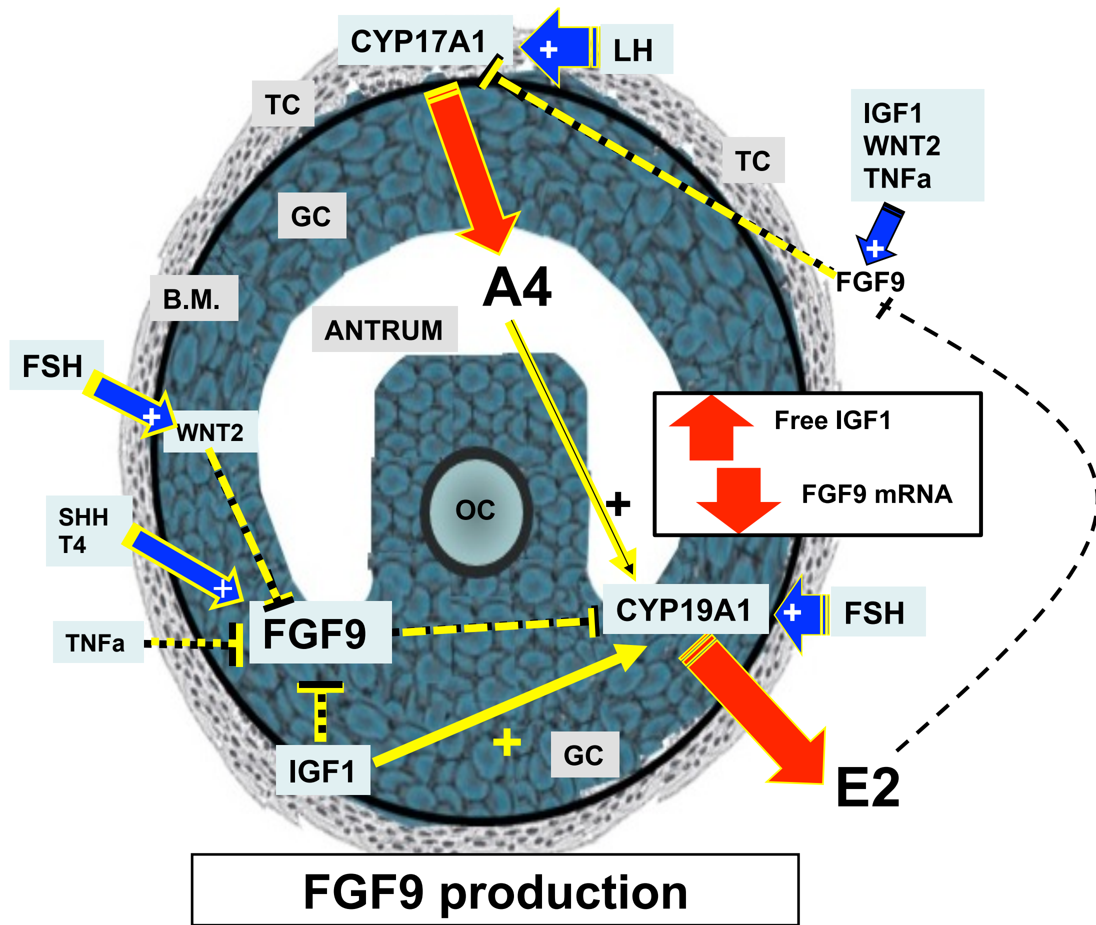


Fig. 5. Schematic model summarizing the hormonal regulation of FGF9 production by granulosa and theca cells. Dashed line with **T** at end indicates inhibitory effects; hormones by arrows with a "+" indicates stimulatory effects; hormones by arrows with a "-" indicates inhibitory effects. IGF1, FSH and LH are stimulatory to estradiol (E2) and androstenedione (A4) production via interaction with 17-hydroxylase (CYP17A1) and aromatase (CYP19A1). Increased WNT2 and free IGF1 decreases *FGF9* mRNA and this decreased FGF9 production causes a reduction in the FGF9 inhibition of E2 production by granulosa cells (GC) and A4 production by theca cells (TC), causing an overall increase in E2 production by the dominant follicle. SHH, Sonic hedgehog; T4, thyroxine; TNFa, tumor necrosis factor alpha; WNT2, wingless 2; B.M. = basement membrane.


CYP17A1
LH
TC
TC
IGF1
WNT2
TNFa
GC
FGF9
B.M.
A4
ANTRUM
FSH
WNT2
Free IGF1
FGF9 mRNA
OC
SHH
T4
+
CYP19A1
FSH
TNFa
FGF9
IGF1
GC
E2
FGF9 production

6. References

Aad, P. Y., S. E. Echternkamp, and L. J. Spicer. 2013. Possible role of IGF2 receptors in regulating selection of 2 dominant follicles in cattle selected for twin ovulations and births. Domest. Anim. Endocrinol. 45: 187-195.

Aad, P. Y., S. E. Echternkamp, D. D. Sypherd, N. B. Schreiber, and L. J. Spicer. 2012. The hedgehog system in ovarian follicles of cattle selected for twin ovulations and births: evidence of a link between the IGF and hedgehog systems. Biol. Reprod. 87: 1-10.

Berisha, B., F. Sinowatz, and D. Schams. 2004. Expression and localization of fibroblast growth factor (FGF) family members during the final growth of bovine ovarian follicles. Mol. Reprod. Dev. 67: 162-171.

Buratini, J., Jr., M. G. Pinto, A. C. Castilho, R. L. Amorim, I. C. Giometti, V. M. Portela, E. S. Nicola, and C. A. Price. 2007. Expression and function of fibroblast growth factor 10 and its receptor, fibroblast growth factor receptor 2B, in bovine follicles. Biol. Reprod. 77: 743-750.

Castanon, B. I., A. D. Stapp, C. A. Gifford, L. J. Spicer, D. M. Hallford, and J. A. Hernandez Gifford. 2012. Follicle-stimulating hormone regulation of estradiol production: possible involvement of WNT2 and beta-catenin in bovine granulosa cells. J. Anim. Sci. 90: 3789-3797.

Chaves, R. N., M. H. de Matos, J. Buratini, Jr., and J. R. de Figueiredo. 2012. The fibroblast growth factor family: involvement in the regulation of folliculogenesis. Reprod. Fertil. Dev. 24: 905-915.

Cho, J. H., T. Itoh, Y. Sendai, and H. Hoshi. 2008. Fibroblast growth factor 7 stimulates in vitro growth of oocytes originating from bovine early antral follicles. Mol. Reprod. Dev. 75: 1736-1743.

Dentis, J. L. Schreiber, N.B.; Gilliam, J.N.; Schutz, L.F.; Spicer, L.J. 2016. Changes in brain ribonuclease (BRB) mRNA in granulosa cells (GC) of dominant versus subordinate ovarian follicles of cattle and the regulation of *BRB* gene expression in bovine GC. Domest. Anim. Endocrinol. 55: 32-40.

Drummond, A. E., M. Tellbach, M. Dyson, and J. K. Findlay. 2007. Fibroblast growth factor-9, a local regulator of ovarian function. Endocrinology 148: 3711-3721.

Evans, J. R., N. B. Schreiber, J. A. Williams, and L. J. Spicer. 2014. Effects of fibroblast growth factor 9 on steroidogenesis and control of FGFR2IIIc mRNA in porcine granulosa cells. J. Anim. Sci. 92: 511-519.

Fortune, J. E., G. M. Rivera, A. C. Evans, and A. M. Turzillo. 2001. Differentiation of dominant versus subordinate follicles in cattle. Biol. Reprod. 65: 648-654.

Gabler, C., A. Plath-Gabler, G. J. Killian, B. Berisha, and D. Schams. 2004. Expression pattern of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) system members in bovine corpus luteum endothelial cells during treatment with FGF-2, VEGF or oestradiol. Reprod. Domest. Anim. = Zuchthygiene 39: 321-327.

Grado-Ahuir, J. A., P. Y. Aad, and L. J. Spicer. 2011. New insights into the pathogenesis of cystic follicles in cattle: microarray analysis of gene expression in granulosa cells. J. Anim. Sci. 89: 1769-1786.

Gupta, P. S., J. K. Folger, S. K. Rajput, L. Lv, J. Yao, J. J. Ireland, and G. W. Smith. 2014. Regulation and regulatory role of WNT signaling in potentiating FSH action during bovine dominant follicle selection. PloS One 9: e100201.

Itoh, N. and D. M. Ornitz. 2011. Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J. Biochem. 149: 121-130.

Jiang, Z. and C. A. Price. 2012. Differential actions of fibroblast growth factors on intracellular pathways and target gene expression in bovine ovarian granulosa cells. Reproduction 144: 625-632.

Lagaly, D. V., P. Y. Aad, J. A. Grado-Ahuir, L. B. Hulsey, and L. J. Spicer. 2008. Role of adiponectin in regulating ovarian theca and granulosa cell function. Mol. Cell. Endocrinol. 284: 38-45.

LaPolt, P. S., M. Yamoto, M. Veljkovic, C. Sincich, T. Ny, A. Tsafriri, and A. J. Hsueh. 1990. Basic fibroblast growth factor induction of granulosa cell tissue-type plasminogen activator expression and oocyte maturation: potential role as a paracrine ovarian hormone. Endocrinology 127: 2357-2363.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

Machado, M. F., V. M. Portela, C. A. Price, I. B. Costa, P. Ripamonte, R. L. Amorim, and J. Buratini, Jr. 2009. Regulation and action of fibroblast growth factor 17 in bovine follicles. J. Endocrinol. 202: 347-353.

Ornitz, D. M. and N. Itoh. 2001. Fibroblast growth factors. Genome Biol. 2: REVIEWS3005.

Oron, G., B. Fisch, X. Y. Zhang, R. Gabbay-Benziv, G. Kessler-Icekson, H. Krissi, A. Ben-Haroush, A. Ao, and R. Abir. 2012. Fibroblast growth factor 10 in human ovaries. Reprod. Biomed. Online 25: 396-401.

Ott, L. 1977. An introduction to statistical methods and data analysis. Duxbury Press, North Scituate, MA.

Peluso, J. J., A. Pappalardo, and G. Fernandez. 2001. Basic fibroblast growth factor maintains calcium homeostasis and granulosa cell viability by stimulating calcium efflux via a PKC delta-dependent pathway. Endocrinology 142: 4203-4211.

Portela, V. M., M. Machado, J. Buratini, Jr., G. Zamberlam, R. L. Amorim, P. Goncalves, and C. A. Price. 2010. Expression and function of fibroblast growth factor 18 in the ovarian follicle in cattle. Biol. Reprod. 83: 339-346.

Santiago, C.A., J. L. Voge, P. Y. Aad, D. T. Allen, D. R. Stein, J. R. Malayer, and L. J. Spicer. 2005. Pregnancy-associated plasma protein-A and insulin-like growth factor binding protein mRNAs in granulosa cells of dominant and subordinate follicles of preovulatory cattle. Domest. Anim. Endocrinol. 28: 46-63.

Schams, D., V. Steinberg, M. Steffl, H. H. Meyer, and B. Berisha. 2009. Expression and possible role of fibroblast growth factor family members in porcine antral follicles during final maturation. Reproduction 138: 141-149.

Schreiber, N. B., and L. J. Spicer. 2012. Effects of fibroblast growth factor 9 (FGF9) on steroidogenesis and gene expression and control of FGF9 mRNA in bovine granulosa cells. Endocrinology 153: 4491-4501.

Schreiber, N. B., M. L. Totty, and L. J. Spicer. 2012. Expression and effect of fibroblast growth factor 9 in bovine theca cells. J. Endocrinol. 215: 167-175.

Spicer, L. J. 1998. Tumor necrosis factor-α (TNF-α) inhibits steroidogenesis of bovine ovarian granulosa and thecal cells in vitro. Involvement of TNF-α receptors. Endocrine 8: 109-115.

Spicer, L. J. 2004. Proteolytic degradation of insulin-like growth factor binding proteins by ovarian follicles: a control mechanism for selection of dominant follicles. Biol. Reprod. 70: 1223-1230.

Spicer, L. J., C. A. Santiago, T. R. Davidson, T. S. Bridges, and C. S. Chamberlain. 2005. Follicular fluid concentrations of free insulin-like growth factor (IGF)-I during follicular development in mares. Domest. Anim. Endocrinol. 29: 573-581.

Spicer, L. J., S. Sudo, P. Y. Aad, L. S. Wang, S. Chun, I. Ben-Shlomo, C. Klein, and A. J. W. Hsueh. 2009. The hedgehog-patched signaling pathway and function in the mammalian ovary: a novel role for hedgehog proteins in stimulating proliferation and steroidogenesis of theca cells. Reproduction 138: 329-339.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, B. E. Keefer, L. J. Dawson, G. L. Morgan, and S. E. Echternkamp. 1996. Levels of insulin-like growth factor (IGF) binding proteins, luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrous cycles. Endocrinology 137: 2842-2850.

Sun, X., M. Lewandoski, E. N. Meyers, Y. Liu, R. E. Maxson Jr, and G. R. Martin. 2000. Conditional inactivation of *Fgf4* reveals complexity of signalling during limb bud development. Nat. Genet. 25: 83-86.

Tilly, J. L., H. Billig, K. I. Kowalski, and A. J. Hsueh. 1992. Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase-dependent mechanism. Mol. Endocrinol. 6: 1942-1950.

Vernon, R. K., and L. J. Spicer. 1994. Effects of basic fibroblast growth factor and heparin on follicle-stimulating hormone-induced steroidogenesis by bovine granulosa cells. J. Anim. Sci. 72: 2696-2702.

Voge, J. L., P. Y. Aad, C. A. T. Santiago, D. W. Goad, J. R. Malayer, D. Allen, and L. J. Spicer. 2004. Effect of insulin-like growth factors (IGF), FSH, and leptin on IGF-binding-protein mRNA expression in bovine granulosa and theca cells: quantitative detection by real-time PCR. Peptides 25: 2195-2203.

Woad, K. J., M. G. Hunter, G. E. Mann, M. Laird, A. J. Hammond, and R. S. Robinson. 2012. Fibroblast growth factor 2 is a key determinant of vascular sprouting during bovine luteal angiogenesis. Reproduction 143: 35-43.

CHAPTER IV

CHANGES IN FIBROBLAST GROWTH FACTOR RECEPTORS-1c, -2c, -3c, AND -4 mRNA DURING OVARIAN FOLLICULAR GROWTH IN CATTLE

1. Abstract

Fibroblast growth factors (FGFs) regulate folliculogenesis of several mammalian species, including cattle, through autocrine, paracrine, and endocrine mechanisms. The diversity of roles played by FGFs is influenced by the nature of the ligands and the diversity of high affinity fibroblast growth factors receptors (FGFRs). There are four distinct genes encoding for FGFRs (*FGFR1-FGFR4*) in vertebrates and the occurrence of mRNA splicing in the immunoglobulin-like domain III generates diversity of sequence and results in various isoforms of *FGFR1*, *FGFR2*, and *FGFR3* genes (but not of *FGFR4*). Because FGFRs have different ligand-specificities, the localization of FGFRs in the different compartments of bovine antral follicles is of fundamental importance for the FGFs to exert their effects in the ovary. Hence, the objective of this study was to determine if FGFR1c, FGFR2c, FGFR3c, and FGFR4 change according to follicular size, steroidogenic status, and days post-ovulation during growth of first-wave dominant follicles in cattle exhibiting a regular estrous cycle. Estrous cycles of non-lactating dairy cattle were synchronized, and ovaries were collected on either day 3-4 (n = 8) or day 5-6 (n = 8) post-ovulation for GC and TC mRNA extraction from small (1-5 mm), medium (5.1-8 mm) or large (8.1-18 mm) follicles for real time-PCR analysis. In GC, *FGFR1c* and *FGFR2c* mRNA relative abundance was greater ($P < 0.01$) in estrogen (E2)-inactive (i.e., concentrations of E2 < progesterone, P4) follicles of all sizes than in GC from large E2-active follicles (i.e., concentrations of E2 < P4), *FGFR3c* mRNA relative abundance was greater ($P < 0.05$) in large and medium E2-inactive than in large E2-active follicles, and *FGFR4* mRNA abundance did not differ among different groups of

follicles. In TC, medium E2-inactive follicles had greater ($P < 0.05$) *FGFR1c* and *FGFR4* mRNA abundance than large E2-active and E2–inactive follicles at the late growing phase of the first dominant follicle whereas *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* mRNA abundance was greater ($P < 0.05$) in medium E2-inactive follicles at late (day 5-6 post-ovulation) than at early (day 3-4 post-ovulation) growing phase of first dominant follicle. Taken together, the findings that *FGFR1c*, *FGFR2c*, and *FGFR3c* mRNA abundance was less in GC of E2-active follicles and was greater in TC of medium inactive follicles at late than at early growing phase of first dominant follicle for all FGFRs isoforms tested support an anti-differentiation role for FGFs and their FGFRs as well as support the idea that changes in FGFs and their receptors regulate selection of dominant follicles in cattle.

Key Words: Fibroblast growth factor receptors (FGFRs), FGFR1c, FGFR2c, FGFR3c, FGFR4, theca cell, granulosa cell, cattle.

2. Introduction

Ovarian folliculogenesis is a tightly regulated process where the somatic cells of the follicle, granulosa (GC) and theca (TC) cells, communicate in a coordinated way with the oocyte for both follicular and oocyte growth and maturation (Eppig, 1991; Elvin and Matzuk, 1998; Fortune et al., 2004). Among other growth factors that control folliculogenesis, the fibroblast growth factors (FGFs) have been emerging as important regulators of ovarian function, playing autocrine, paracrine, and endocrine roles in the regulation of development of ovarian follicles (for reviews, see Chaves et al., 2012; Price, 2016). These polypeptides belong to a family of 22 members in mammals (Ornitz and Itoh, 2001; Itoh and Ornitz, 2011), and, to date, ten members have been detected in the ovary: FGF1, 2, 7-10, 16-18, 22. In antral follicles of cattle, FGF1, FGF2, FGF7, FGF18, and FGF22 are mainly produced by TC (Parrott and Skinner, 1998; Berisha et al., 2000; Berisha et al., 2004; Buratini et al., 2007; Portela et al., 2010; Castilho et al., 2015); FGF8 is produced by GC, TC, and oocytes (Buratini et al., 2005); FGF9 is produced in greater amounts in GC than in TC (Schreiber et

al., 2012); FGF10 is produced by TC and oocytes (Buratini et al., 2007); FGF16 is produced by the oocyte (Ferreira et al., 2016); and FGF17 is detected mainly in oocytes, but also in GC (Machado et al., 2009).

In cattle, FGFs play diverse roles in ovarian function. To date, at least four members of this polypeptide family stimulate proliferation of somatic cells of ovarian follicles: FGF1, FGF2, FGF7, and FGF9 stimulate GC and/or TC proliferation (Gospodarowicz et al., 1977a; Gospodarowicz et al., 1977b; Gospodarowicz et al., 1985; Parrott et al., 1994; Spicer and Stewart, 1996; Schreiber and Spicer, 2012; Schreiber et al., 2012). In addition, FGFs regulate steroidogenesis: FGF2, FGF7, and FGF18 inhibit FSH-stimulated estradiol (E2) and LH-stimulated progesterone (P4) production by GC (Vernon and Spicer, 1994; Spicer and Stewart, 1996; Wandji et al., 1996; Parrott and Skinner, 1998; Machado et al., 2009; Portela et al., 2010); FGF9 attenuates insulin-like growth factor 1-stimulated E2 and P4 production by GC (Schreiber and Spicer, 2012) and suppresses insulin-like growth factor 1-stimulated P4 production by TC (Schreiber et al., 2012).

In order to exert their actions in the ovary, FGFs need to bind to high affinity receptors (FGFRs). The FGFR is a single chain transmembrane tyrosine kinase with two or three immunoglobulin-like domains and a heparin-binding domain in the extracellular ligand-binding portion (Plotnikov et al., 2000; Ornitz and Itoh, 2001; Itoh and Ornitz, 2004; Li et al., 2016). There are four distinct genes encoding for FGFRs (*FGFR1-FGFR4*) in vertebrates and mRNA alternative splicing occurs in the immunoglobulin-like domain III of the *FGFR1*, *FGFR2*, and *FGFR3* genes (but not of *FGFR4*), generating diversity of sequence and resulting in various isoforms (Itoh and Ornitz, 2004; Ornitz and Itoh, 2015; Li et al., 2016). The ligands of the FGF family have different affinities for these receptors (Itoh and Ornitz, 2004), which are determined by the immunoglobulin-like domains (Dell and Williams, 1992). According to ligand binding specificity, the preferred receptors for FGFs produced in the ovary are: FGFR1c for FGF1 and FGF2; FGFR2b for FGF7, FGF10, and FGF22; FGFR3c for FGF1, FGF2, FGF8, FGF9, FGF16, FGF17, and FGF18 (Ornitz and Itoh, 2015). In

addition, FGFR1b is the second preferred for FGF7, FGF10, and FGF22; FGFR2c is the second preferred for FGF9 and FGF16; and FGFR4 is the second preferred for FGF8, FGF17, and FGF18 (Ornitz and Itoh, 2015).

Since FGFRs have different ligand-specificities, the localization of FGFRs in the different compartments of bovine antral follicles is of fundamental importance for the FGFs effects: FGFR1b has been detected in GC, TC, and oocytes (Zhang and Ealy, 2012; Castilho et al., 2015); FGFR1c and FGFR2b have been detected in GC and oocytes (Berisha et al., 2004; Buratini et al., 2007; Machado et al., 2009; Zhang and Ealy, 2012); FGFR2c and FGFR3c have been detected in both GC and TC (Berisha et al., 2004; Buratini et al., 2005); FGFR4 has been detected exclusively in TC (Buratini et al., 2005). In addition, some FGFRs change according to follicular fate in cattle: FGFR1b and FGFR2b GC mRNA abundance is greater in future subordinate follicles than in future dominant follicles (Berisha et al., 2004; Castilho et al., 2015); FGFR3c mRNA abundance is greater in GC (but not in TC) of small healthy follicles and in response to FSH (Buratini et al. 2005); FGFR4 mRNA abundance is greater in small than in large follicles (Buratini et al., 2005). Nevertheless, information of how endogenous production of FGFRs by ovarian follicular cells change during selection of dominant follicles in cattle needs further clarification. Hence, the objective of this study was to determine if mRNA abundance of FGFR1c, FGFR2c, FGFR3c, and FGFR4 in GC and TC changes during growth of first-wave dominant follicles in cattle exhibiting regular estrous cycle.

3. Material and Methods

Animals and Experimental Design

Non-lactating Holstein cows (n = 16), culled for non-reproductive reasons from Oklahoma State University herd, were used for this experiment as previously described (Dentis et al., 2016). Briefly, estrous cycles were synchronized using two injections (i.m.) of prostaglandin $F_{2\alpha}$ (Lutalyse®, 25 mg) with an interval of 11 days, after which, follicle

development was monitored daily via ultrasonography using an Aloka 500V with a 7.5 MHz probe. Following ovulation, cows were assigned to be ovariectomized either at day 3-4 (early growing phase of the first dominant follicle; n = 8 cows) or day 5-6 post-ovulation (late growing phase of the first dominant follicle; n = 8 cows). After each ovariectomy, ovaries were put on ice, and transported to the laboratory where diameters of all follicles ≥ 5 mm (surface diameter) in diameter were recorded, and ovarian tissue and fluid collected (Dentis et al., 2016). The animal experimentation described in this report was approved by the Oklahoma State University Institutional Animal Care and Use Committee (Protocol No. AG106).

Cell and Follicular Fluid Collection

For GC sample collection, follicles were categorized by surface diameter as small (1-5 mm), medium (5.1-8 mm) or large (8.1-18 mm); TC samples were collected from only medium and large follicles. Follicular fluid (FFL) from medium and large follicles was aspirated individually and centrifuged to obtain GC, and FFL from small follicles was pooled within each ovary and then centrifuged to obtain GC as previously described (Stewart et al., 1996; Dentis et al., 2016). After centrifugation, FFL was aspirated and stored at -20 °C for measurement of E2 and P4 via RIA. After collection of FFL, each medium and large follicle was bisected *in situ*, the inner wall was scraped, rinsed with Ham's F-12 to remove any remaining GC, and these GC were combined with GC collected from FFL as previously described (Schreiber and Spicer, 2012; Dentis et al., 2016). GC collected from small follicles were kept separate for each ovary. GC were lysed in 0.5 mL of TRIzol® reagent solution (Life Technologies, Inc., Grand Island, NY) and stored frozen at -80 °C until RNA extraction (see description below). TC were dissected from the bisected follicles and placed in 0.75 mL of TRIzol Reagent and homogenized for 2-3 min on ice using the Omni TH tissue homogenizer (Omni International Inc., Marietta, GA) with Omni Tip™ disposable generator probes as previously described (Aad et al., 2012).

RNA Extraction and Quantitative PCR (qPCR)

Ovarian GC and TC mRNA was isolated as described elsewhere (Voge et al., 2004). RNA samples were solubilized in diethylpyrocarbonate-treated water (Life Technologies), quantitated at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and stored at -80 °C.

Primers and probes for *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* (supplied as 5' FAM reporter dye and a 3' TAMRA quencher dye; TaqMan TAMARA; Applied Biosystems Inc., Foster City, CA) for quantitative qPCR (Table 1) were designed using Primer Express software (Foster City, CA) as previously reported (Grado-Ahuir et al., 2011). A "highly similar sequences" BLAST query search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted for each primer and probe to ensure specificity of the designed primers and probes. Relative mRNA abundance of target genes was quantified using fluorescent quantitative single-step RT-PCR using a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Target gene expression was normalized to constitutively expressed *18S* ribosomal RNA (*18S* rRNA; supplied as a VIC probe; TaqMan Ribosomal RNA Control Reagent, Applied Biosystems Inc.) and relative quantity of target gene mRNAs was expressed as $2^{-\Delta\Delta Ct}$ using the relative comparative threshold cycle (Ct) method as previously described (Livak and Schmittgen, 2001; Voge et al., 2004; Lagaly et al., 2008).

Radioimmunoassays (RIA)

Concentrations of P4 and E2 in FFL were determined by RIA as previously described (Stewart et al., 1996; Dentis et al., 2016). All samples were run in one assay for each of the steroid RIA. The intra-assay coefficient of variation for P4 and E2 RIA was 11.6 % and 10.6 %, respectively.

Statistical Analyses

Through analysis of these data, we sought to determine if *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* mRNA abundance varies in GC and TC at different periods of follicular development and according to follicular size and estrogenic status. Data were

analyzed via factorial ANOVA with GLM procedures of SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC) and are presented as the least squares means (± SEM) of measurements. Main factors were days post-ovulation (early, day 3-4, and late, day 5-6, growing phase of the first dominant follicle), and follicle status based on size (small, medium, or large in the case of GC, and medium or large in the case of TC) and estrogenic status (E2 active: E2>P4 concentrations in FFL or E2 inactive: E2<P4 concentrations in FFL), and their interaction. Some cows had two E2-active follicles on day 3-4 whereas some cows had no E2-active follicles on day 5-6. Also, if FFL samples were lost during collection, then E2-status could not be determined and gene expression data was not included in the analysis. To correct for heterogeneity of variance, target gene abundance was analyzed after transformation to natural log (x + 1). Mean differences were determined by Fisher's protected least significant differences test (Ott, 1977) only if significant main effects in the ANOVA were detected. To evaluate the relationships among variables measured, Pearson correlation coefficients were generated using CORR procedure of SAS (SAS Institute). Because of the wide range and heterogeneous variances of the variables measured, log-transformed variables were correlated among each other. Significance was declared at $P < 0.05$.

4. Results

Follicle size, E2, and P4 Concentrations in follicular fluid

Follicle size and steroid concentrations in FFL have been reported for this study (Dentis et al., 2016). Briefly, diameter of large dominant E2-active, large subordinate E2-inactive, and medium E2-inactive follicles averaged 12.9 ± 0.5, 9.48 ± 0.36, and 6.37 ± 0.23 mm, respectively. Concentrations of E2 in FFL of large dominant E2-active, large subordinate E2-inactive, medium E2-inactive, and small E2-inactive follicles averaged 186.5 ± 29.5, 8.45 ± 3.7, 2.3 ± 0.8, and 2.0 ± 0.2 ng/ml, respectively. Concentrations of P4 in FFL did not differ ($P > 0.10$) among follicle groups and ranged between 61 ± 7 and 236 ± 42 ng/ml.

GC *FGFR1c* mRNA Relative Abundance

Abundance of *FGFR1c* mRNA, the main receptor for FGF1 and FGF2, was significantly affected by follicle group, but not by days post-ovulation or their interaction. Specifically, *FGFR1c* mRNA abundance was greater ($P < 0.01$) in large, medium, and small E2-inactive (E2/P4 ratio < 1) than in large E2-active (E2/P4 ratio > 1) follicles (4.3-, 6.1-, and 4.2-fold, respectively) and was 1.4–fold greater ($P < 0.05$) in medium E2-inactive than in large and small E2-inactive follicles (Fig. 1A). No other significant differences were detected among follicles of different sizes and steroidogenic status.

GC *FGFR2c* mRNA Relative Abundance

Abundance of *FGFR2c* mRNA, the second main receptor for FGF9 and FGF16, was significantly affected by follicle group, but not by days post-ovulation or their interaction (Fig. 1B). Specifically, *FGFR2c* mRNA abundance was greater ($P < 0.01$) in large, medium, and small E2-inactive than in large E2-active follicles (7.5-, 10.4-, and 4.9-fold, respectively). No other significant differences were detected among follicles of different sizes and steroidogenic status.

GC *FGFR3c* mRNA Relative Abundance

Abundance of *FGFR3c* mRNA, the main receptor for FGF8, FGF9, FGF16, FGF17, and FGF18, was significantly affected by follicle group, but not by days post-ovulation or their interaction (Fig. 1C). Specifically, *FGFR3c* mRNA abundance was greater ($P < 0.05$) in large and medium E2-inactive than in large E2-active follicles (3.3- and 3.7-fold, respectively) and tended to be greater ($P = 0.06$) in small E2-inactive than in large E2-active follicles (Fig. 1C). No other significant differences were detected among follicles of different sizes and steroidogenic status.

GC *FGFR4* mRNA Relative Abundance

Abundance of *FGFR4* mRNA was not different (*P* > 0.10) among follicles of different sizes and steroidogenic status or days post-ovulation (Fig. 1D).

TC *FGFR1c* mRNA Relative Abundance

Abundance of *FGFR1c* mRNA in TC was affected (*P* < 0.05) by follicle group, days post-ovulation, and their interaction. Specifically, *FGFR1c* mRNA abundance was (2.7- and 1.7-fold, respectively) greater (*P* < 0.05) in medium E2-inactive than in large E2-active and small E2–inactive follicles at late growing phase of first dominant follicle (Fig. 2A). Moreover, *FGFR1c* mRNA abundance was 2–fold greater in medium E2-inactive at late than at early growing phase of first dominant follicle. No significant differences in *FGFR1c* mRNA abundance were detected between large E2-active, large E2-inactive and medium E2-inactive follicles at early growing phase of first dominant follicle.

TC *FGFR2c* mRNA Relative Abundance

Abundance of *FGFR2c* mRNA tended (*P* < 0.07) to be affected by follicle group x days post-ovulation such that *FGFR2c* mRNA abundance was 1.9–fold greater (*P* < 0.05) in medium E2-inactive follicles at late than at early growing phase of first dominant follicle (Fig. 2B). No other significant differences were detected among follicles of different sizes and estrogen activity at early or late growing phases of first dominant follicle.

TC *FGFR3c* mRNA Relative Abundance

Abundance of *FGFR3c* mRNA tended (*P* < 0.10) to be affected by the follicle group by days post-ovulation interaction such that *FGFR3c* mRNA abundance was 2.7–fold greater (*P* < 0.05) in medium E2-inactive than in large E2-inactive follicles at late growing phase of first dominant follicle (Fig. 2C). In addition, *FGFR3c* mRNA abundance was 1.8–fold greater (*P* < 0.01) in medium E2-inactive follicles at late days post-ovulation than at early days post-ovulation. No other significant differences were detected between follicles of different sizes at early or late growing phases of first dominant follicle.

TC *FGFR4* mRNA Relative Abundance

Abundance of *FGFR4* tended ($P < 0.09$) to be affected by the follicle group by days post-ovulation interaction such that *FGFR4* mRNA abundance was 1.4–fold greater ($P < 0.05$) in medium E2-inactive at late days post-ovulation than at early days post-ovulation (Fig. 2D). In addition, *FGFR4* mRNA abundance was (5.2- and 1.7-fold, respectively) greater ($P < 0.05$) in medium E2-inactive than in large E2-active and E2–inactive follicles at late growing phase of first dominant follicle. No other significant differences were detected between follicles of different sizes at early or late growing phases of first dominant follicle.

Correlations among Follicular Size, FFL Levels of Steroids, and FGFRs mRNA Relative Abundance

In GC, negative correlations existed between follicular size and *FGFR1c* (r = -0.30, $P < 0.01$, n = 141) and *FGFR2c* mRNA abundance (r = - 0.23, $P < 0.01$, n = 142). In TC, a negative correlation existed between follicular size and *FGFR4* (r = -0.23, $P < 0.05$, n = 82). No significant correlations were observed between follicular size and *FGFR3c* or *FGFR4* mRNA abundance in GC or between follicular size and *FGFR1c*, *FGFR2c*, or *FGFR3c* mRNA abundance in TC.

In GC, correlations between FFL concentrations of E2 and FGFRs mRNA abundance were negative for all FGFR isoforms. Specifically, a negative correlation existed between FFL E2 concentrations and *FGFR1c* (r = -0.20, $P < 0.05$, n = 136) and *FGFR2c* (r = -0.17, $P < 0.01$, n = 137) mRNA abundance. Also, log-transformed values of *FGFR3c* (r = -0.24, $P < 0.05$, n = 135) and *FGFR4* (r = -0.22, $P < 0.05$, n = 138) mRNA abundance were negatively correlated to levels of E2 and log-transformed values of levels of E2 in FFL, respectively. In TC, a negative correlation existed between FFL E2 concentrations and *FGFR1c* (r = -0.25, $P < 0.05$, n = 79) and between log-transformed values of *FGFR4* (r = -0.30, $P < 0.05$, n = 78) mRNA abundance and FFL E2 concentrations whereas no significant correlations were observed between FFL concentrations of E2 and *FGFR2c* or *FGFR3c* mRNA abundance.

In GC, positive correlations were detected between FFL concentrations of P4 and log-transformed *FGFR1c* (r = 0.27, $P < 0.01$, n = 127), *FGFR2c* (r = 0.21, $P < 0.05$, n = 129), and log-transformed *FGFR3c* (r = 0.26, $P < 0.01$, n = 127) mRNA relative abundance. In TC, log-transformed values of levels of P4 in FFL were correlated with log-transformed values of *FGFR1c* (r = 0.35, $P < 0.05$, n = 74) and *FGFR4* (r = 0.24, $P < 0.05$, n = 73) mRNA relative abundance. No significant correlations were detected between FFL concentrations of P4 and GC *FGFR4* mRNA abundance or TC *FGFR2c* and *FGFR3c* mRNA abundance.

In GC, negative correlations were detected between *FGFR1c* (r = - 0.22, $P < 0.05$, n = 125), log-transformed *FGFR2c* (r = - 0.54, $P < 0.01$, n = 127), or log-transformed *FGFR3c* (r = - 0.29, $P < 0.01$, n = 125) mRNA relative abundance and E2/P4 concentrations ratio in FFL. In TC, log-transformed value of *FGFR1c* mRNA abundance was negatively correlated with E2/P4 concentrations ratio in FFL (r = -0.30, $P < 0.01$, n = 73). No significant correlations were detected between FFL E2/P4 concentrations ratio and GC *FGFR4* mRNA abundance and between FFL E2/P4 concentrations ratio and TC *FGFR2c, FGFR3c,* or *FGFR4* mRNA abundance.

5. Discussion

Actions of FGFs on the ovary were first reported in the seventies (Gospodarowicz et al., 1977a; Gospodarowicz et al., 1977b), when FGF1 was found to stimulate proliferation of bovine granulosa and luteal cells. To date, ten members of the FGF family have been shown to regulate ovarian folliculogenesis, binding to FGFRs to elicit GC and TC proliferation and steroidogenesis (for reviews, see Chaves et al., 2012; Li, 2016). The diversity of roles played by FGFs is influenced by the nature of the ligands and the diversity of high affinity FGFRs and cofactors that regulate the FGF signaling complex (Givol and Yayon, 1992; Itoh and Ornitz, 2004; Dailey et al., 2005; Li et al., 2016). In the present study, *FGFR1c* and *FGFR2c* mRNA abundance was greater in GC from E2-inactive follicles of all sizes than in GC from large E2-active follicles whereas *FGFR3c* mRNA abundance was greater in GC from large

and medium E2-inactive, and tended to be greater in GC from small E2-inactive than in large E2-active follicles. In addition, TC from medium E2-inactive follicles had greater *FGFR1c* mRNA abundance than TC from large E2-active follicles while *FGFR1c*, *FGFR2c*, and *FGFR3c* mRNA abundance was greater in TC from medium E2-inactive follicles at late than at early growing phase of first dominant follicle. Furthermore, abundance *FGFR1c*, *FGFR2c*, and *FGFR3c* mRNA in GC was negatively correlated with FFL E2 concentrations and E2/P4 ratio and positively correlated with P4 concentrations whereas *FGFR1c* mRNA abundance in TC was also negatively correlated with FFL E2 concentrations and E2/P4 ratio and positively correlated with FFL P4 concentrations. Because a follicle's estrogenic status can be used to assess the health of follicles, large E2-active follicles are considered as those selected to escape atresia and become dominant (Ireland and Roche, 1982; Spicer and Echternkamp, 1986), and the present results indicate that FGFR1c, FGFR2c, and FGFR3c are produced in greater amounts in GC and TC from subordinate than from dominant follicles, implying a pro-atretic or an anti-differentiation role for these receptors. The fact that relative abundance of mRNA for *FGFR1c* and *FGFR2c* in GC is negatively correlated with size and E2/P4 ratio reinforces this idea.

Relative abundance of GC *FGFR4* mRNA was not different among follicles of different estrogenic status and sizes, but it was greater in TC from medium E2-inactive follicles at days 5 to 6 post-ovulation than at days 3 to 4 post-ovulation. In addition, *FGFR4* mRNA abundance in TC was negatively correlated with size and with FFL E2 concentrations and positively correlated with FFL P4 concentrations. Since transcripts for FGFR4 only changed in TC, but not in GC, of different groups of follicles across days, it is likely to suppose that the action of the ligands that bind to FGFR4 may be more regulated in TC than in GC. This is in agreement to previous observations (Buratini et al., 2005a) where *FGFR4* mRNA was only detected in TC, but not in GC or oocytes, from bovine antral follicles. Moreover, the fact that *FGFR4* mRNA is increasing in E2-inactive follicles as the time of follicular dominance approaches is an indication that this receptor would be playing a role in

preventing differentiation of bovine antral follicles. This is also in agreement with previous findings (Buratini et al., 2005a), where transcripts for FGFR4 were greater in TC from small than from large antral follicles of cattle.

Interestingly, many ligands that preferentially bind to FGFR1c, FGFR2c, FGFR3c, and FGFR4 appear to be critical regulators of large follicle differentiation and atresia. For example, FGF2 (preferentially binding to FGFR1c and FGFR3c), FGF9 (preferentially binding to FGFR3c followed by FGFR2c), and FGF17 and FGF18 (preferentially binding to FGFR3c followed by FGFR4) inhibit steroidogenic enzyme activity and FSH-stimulated E2 production by GC in cattle (Vernon and Spicer, 1994; Machado et al., 2009; Portela et al., 2010; Schreiber and Spicer, 2012). Because E2 is important for GC survival and differentiation of dominant follicles (Knecht et al., 1985; Fortune et al., 2004), FGFs suppressing E2 production may be playing a role in inducing atresia or preventing differentiation of GC in cattle. The fact that some of the ligands that preferentially bind to FGFR3c, including FGF9, FGF17, and FGF18, have a greater mRNA abundance in subordinate or atretic antral follicles than in dominant follicles in cattle (Machado et al., 2009; Portela et al., 2010; Schreiber et al., 2012) reinforces this idea. Furthermore, of the ligands mentioned above, FGF18 induced regression of the dominant follicle when injected in vivo and increased cleaved caspase-3 in GC in vitro (Portela et al., 2015), which is a major downstream effector of apoptosis and serves a marker for GC apoptosis (Feranil et al., 2005), confirming the role for this polypeptide in induction of atresia in bovine antral follicles.

It is noteworthy that some members of the FGF family that preferentially bind to FGFR1c and/or FGFR3c are mitogenic factors of ovarian follicle somatic cells of cattle. Specifically, FGF1 (Gospodarowicz et al., 1977a) stimulate GC proliferation whereas FGF2 (Gospodarowicz et al., 1985; Spicer and Stewart, 1996), and FGF9 (Schreiber and Spicer, 2012; Schreiber et al., 2012) stimulate both GC and TC proliferation. In addition, FGF8 appears to be stimulating GC proliferation, since it leads to expression of genes related to cell proliferation, such as MAPK3/1 and MAPK14, in GC (Jiang et al., 2013; Price, 2016). Hence,

FGFR1c and FGFR3c appear to be playing a positive role in early development of bovine antral follicles.

In summary, *FGFR1c*, *FGFR2c*, *FGFR3c* had greater mRNA abundance in GC of medium, large, and/or small E2-inactive follicles than in large E2-active follicles and *FGFR1c*, *FGFR2c*, *FGFR3c*, *FGFR4* mRNA abundance was greater in TC of medium E2-inactive follicles at late than at early growing phase of first dominant follicle. Furthermore, *FGFR1c* and *FGFR4* mRNA relative abundance was greater in TC of medium E2-inactive follicles than large E2-active and E2-inactive follicles at late than at early growing phase of first dominant follicle. Taken together with the fact that several of their ligands are produced in greater amounts by subordinate than by dominant follicles and inhibit E2 production, the present findings suggest a role for FGFs and their FGFRs as anti-differentiation factors of follicular somatic cells, avoiding the selection of multiple follicles to become dominant in a mono-ovulatory species such as cattle.

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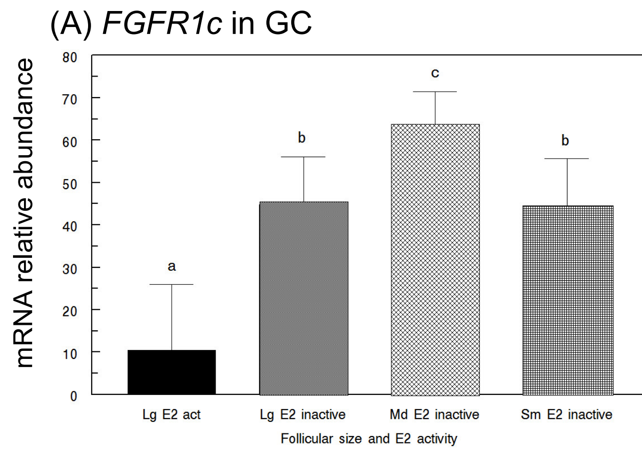
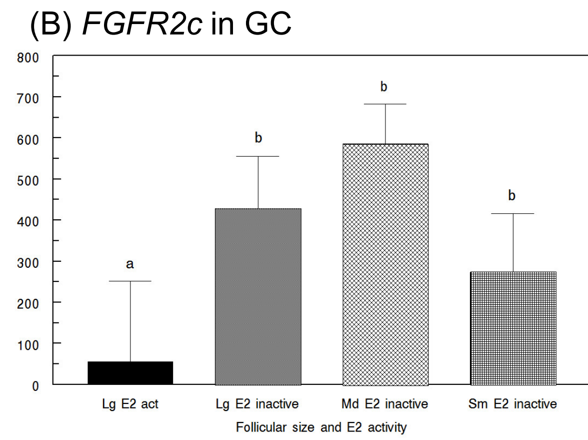
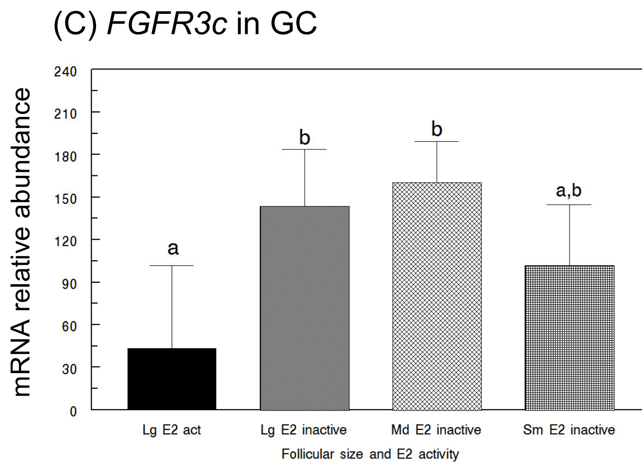
Table 1. Information about primers and probes.

Gene	Oligo	Sequence	Accession	Tm (°C)
FGFR1c	FWD	AGGTGAACGGGAGTAAGATTGG	XM_010820329.2	56.5
	REV	GTGCAGCACCTCCATCTCTTT		57.6
	Probe	TCTTGAAGACGGCCGCCGGAGTTAACACCA		63.3
FGFR2c	FWD	GTTCCAATGCGGAAGTGCTG	XM_010820096.2	57.1
	REV	GTTTTGGCAGGACAGTGAGC		56.8
	Probe	AGGCGGATGCTGGCGAGTATATTTGTAAGG		63.9
FGFR3c	FWD	TAACACCACCGACAAGGAGC	XM_014478386.1	57.2
	REV	CCACGCAGAGTGATGGGAAA		57.6
	Probe	TGCGCAATGTCACCTTTGAGGACG		62.0
FGFR4	FWD	CACTGCCCCCCAGAGCTATAC	XM_005209123.2	59.5
	REV	AGGACCTTGTCCAGTGCCTCTA		59.6
	Probe	AGCACCCTCTCAGAGGCCCACTTTCA		65.3

Fig. 1. Effects of follicular size (Lg = Large; Md = Medium; Sm = Small) and E2 status (EA = estrogen active; EI = estrogen inactive) on *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* mRNA relative abundance in bovine granulosa cells. Panel A: Effects of follicular size and E2 status on *FGF1c* mRNA in bovine granulosa cells (GC); n = 16, 33, 64, and 29 and for Lg-EA, Lg-EI, Md-EI and Sm-EI, respectively. Panel B: Effects of follicular size and E2 status on *FGF2c* mRNA in bovine GC; n = 16, 33, 62, and 28 for Lg-EA, Lg-EI, Md-EI and Sm-EI, respectively. Panel C: Effects of follicular size and E2 status on *FGF3c* mRNA in bovine GC; n = 16, 32, 61, and 28 for Lg-EA, Lg-EI, Md-EI and Sm-EI, respectively. Panel D: Effects of follicular size and E2 status on *FGF4* mRNA in bovine GC; n = 15, 28, 61, and 29 for Lg-EA, Lg-EI, Md-EI and Sm-EI, respectively. Values are normalized to constitutively expressed *18S* ribosomal RNA and are least squares means ± pooled SEM of values averaged across day 3 to 4 and day 5 to 6 post-ovulation. [abc]Within a panel, means without a common letter differ ($P < 0.05$).


(A) FGFR1c in GC
mRNA relative abundance
80
70
60
50
40
30
20
10
0
a
b
c
b
Lg E2 act
Lg E2 inactive
Md E2 inactive
Sm E2 inactive
Follicular size and E2 activity


(B) FGFR2c in GC
800
700
600
500
400
300
200
100
0
a
b
b
b
Lg E2 act
Lg E2 inactive
Md E2 inactive
Sm E2 inactive
Follicular size and E2 activity


(C) FGFR3c in GC
mRNA relative abundance
240
210
180
150
120
90
60
30
0
a
b
b
a,b
Lg E2 act
Lg E2 inactive
Md E2 inactive
Sm E2 inactive
Follicular size and E2 activity

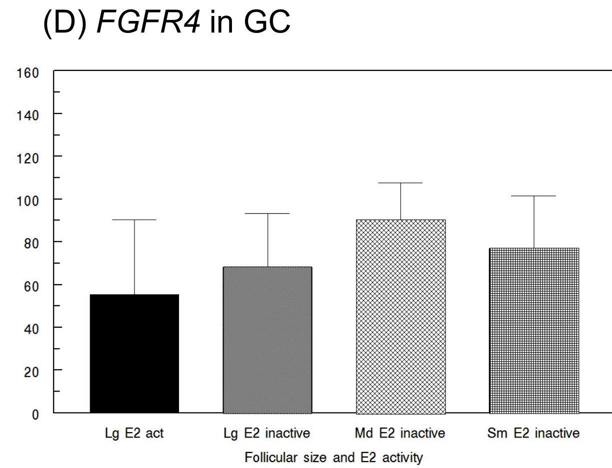
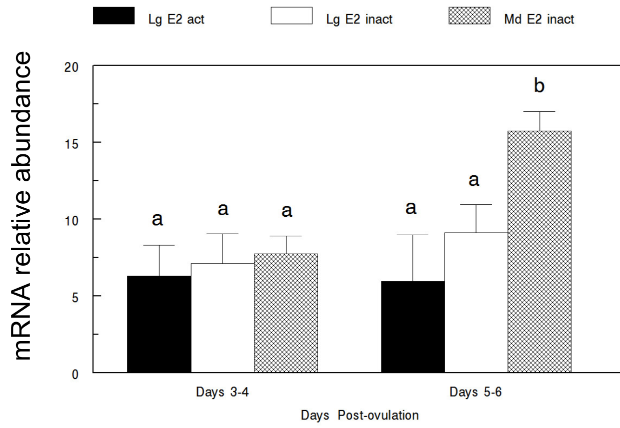
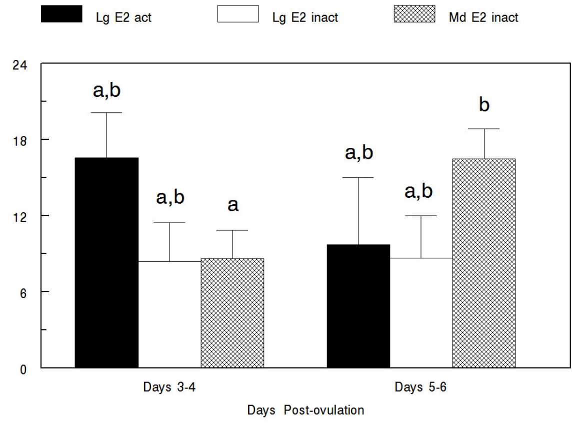
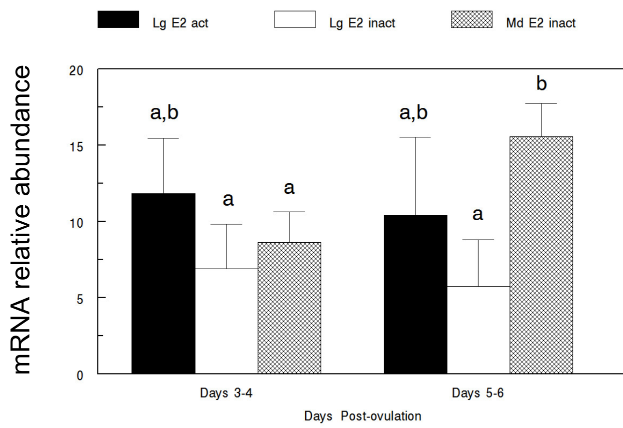
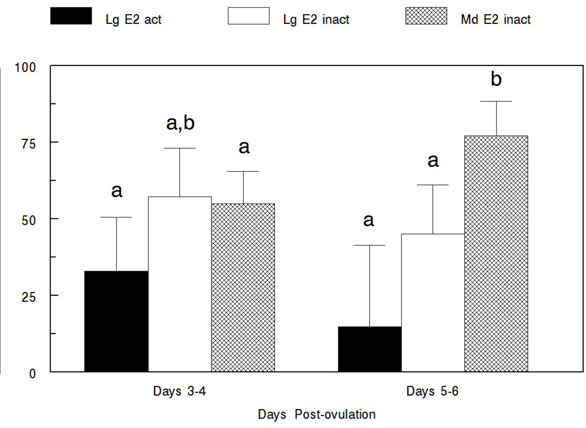

(D) FGFR4 in GC
160
140
120
100
80
60
40
20
0
Lg E2 act
Lg E2 inactive
Md E2 inactive
Sm E2 inactive
Follicular size and E2 activity

Fig. 2. Effects of follicular size (Lg = Large; Md = Medium; Sm = Small) and E2 status (EA = estrogen active; EI = estrogen inactive) on *FGFR1c*, *FGFR2c*, *FGFR3c*, and *FGFR4* mRNA abundance in bovine theca cells (TC) on different days post-ovulation. Panel A: Effects of follicular size and E2 status on *FGF1c* mRNA in bovine TC; n = 9, 10, and 27 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 3-4; n = 4, 11, and 22 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 5-6. Panel B: Effects of follicular size and E2 status on *FGFR2c* mRNA in bovine TC; n = 9, 12, and 23 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 3-4; n = 4, 10, and 20 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 5-6. Panel C: Effects of follicular size and E2 status on *FGFR3c* mRNA in bovine TC; n = 8, 12, and 26 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 3-4; n = 4, 11, and 22 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 5-6. Panel D: Effects of follicular size and E2 status on *FGFR4* mRNA in bovine TC; n = 9, 11, and 25 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 3-4; n = 4, 11, and 22 for Lg-EA, Lg-EI, and Md-EI, respectively, for day 5-6. Values are normalized to constitutively expressed *18S* ribosomal RNA and are least squares means ± pooled SEM. [ab]Within a panel, means without a common letter differ ($P < 0.05$).


(A) FGFR1c in TC
Lg E2 act
Lg E2 inact
Md E2 inact
mRNA relative abundance
0
5
10
15
20
a
a
a
a
a
b
Days 3-4
Days 5-6
Days Post-ovulation


(B) FGFR2c in TC
Lg E2 act
Lg E2 inact
Md E2 inact
0
6
12
18
24
a,b
a,b
a
a,b
a,b
b
Days 3-4
Days 5-6
Days Post-ovulation


(C) FGFR3c in TC
Lg E2 act
Lg E2 inact
Md E2 inact
mRNA relative abundance
0
5
10
15
20
a,b
a
a
a,b
a
b
Days 3-4
Days 5-6
Days Post-ovulation


(D) FGFR4 in TC
Lg E2 act
Lg E2 inact
Md E2 inact
0
25
50
75
100
a
a,b
a
a
a
b
Days 3-4
Days 5-6
Days Post-ovulation

6. References

Aad, P. Y., S. E. Echternkamp, D. D. Sypherd, N. B. Schreiber, and L. J. Spicer. 2012. The hedgehog system in ovarian follicles of cattle selected for twin ovulations and births: Evidence of a link between the IGF and hedgehog systems. Biol. Reprod. 87: 1-10.

Berisha, B., D. Schams, M. Kosmann, W. Amselgruber, and R. Einspanier. 2000. Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during the final growth of bovine ovarian follicles. J. Endocrinol. 167: 371-382.

Berisha, B., F. Sinowatz, and D. Schams. 2004. Expression and localization of fibroblast growth factor (FGF) family members during the final growth of bovine ovarian follicles. Mol. Reprod. Dev. 67: 162-171.

Buratini, J., M. G. L. Pinto, A. C. Castilho, R. L. Amorim, I. C. Giometti, V. M. Portela, E. S. Nicola, and C. A. Price. 2007. Expression and function of fibroblast growth factor 10 and its receptor, fibroblast growth factor receptor 2B, in bovine follicles. Biol. Reprod. 77: 743-750.

Buratini, J., A. B. Teixeira, I. B. Costa, V. F. Glapinski, M. G. L. Pinto, I. C. Giometti, C. M. Barros, M. Cao, E. S. Nicola, and C. A. Price. 2005. Expression of fibroblast growth factor-8 and regulation of cognate receptors, fibroblast growth factor receptor-3c and-4, in bovine antral follicles. Reproduction 130: 343-350.

Castilho, A. C. S. P., C.A.; Dalanezi, F.; Ereno, R.L.; Machado, M.F.; Barros, C.M.; Gasperin, B.G.; Gonçalves, P.B.D.; Buratini, J. . 2015. Evidence that fibroblast growth factor 10 plays a role in follicle selection in cattle. Reprod. Fertil. Dev. 2015 doi:10.1071/RD15017 [Epub ahead of print].

Chaves, R. N., M. H. T. de Matos, J. Buratini, and J. R. de Figueiredo. 2012. The fibroblast growth factor family: Involvement in the regulation of folliculogenesis. Reprod. Fertil. Dev. 24: 905-915.

Dailey, L., D. Ambrosetti, A. Mansukhani, and C. Basilico. 2005. Mechanisms underlying differential responses to FGF signaling. Cytokine & Growth Factor Rev. 16: 233-247.

Dell, K. R., and L. T. Williams. 1992. A novel form of fibroblast growth factor receptor 2. Alternative splicing of the third immunoglobulin-like domain confers ligand binding specificity. J. Biol. Chem. 267: 21225-21229.

Dentis, J. L. Schreiber, N.B.; Gilliam, J.N.; Schutz, L.F.; Spicer, L.J. 2016. Changes in brain ribonuclease (BRB) mRNA in granulosa cells (GC) of dominant versus subordinate ovarian follicles of cattle and the regulation of *BRB* gene expression in bovine GC. Domest. Anim. Endocrinol. 55: 32-40.

Elvin, J. A., and M. M. Matzuk. 1998. Mouse models of ovarian failure. Rev. Reprod. 3: 183-195.

Eppig, J. J. 1991. Intercommunication between mammalian oocytes and companion somatic-cells. Bioessays 13: 569-574.

Feranil, J. B., N. Isobe, and T. Nakao. 2005. Apoptosis in the antral follicles of swamp buffalo and cattle ovary: Tunel and caspase-3 histochemistry. Reprod. Domest. Anim. 40: 111-116.

Ferreira, R. M. C., M.R.; Macabelli, C.H.; Rodrigues, C.A.; Ferraz, M.C.; Watanabe, Y.F.; Smith, L.C.; Meirelles, F.V.; Baruselli, P.S. 2016. The infertility of repeat-breeder cows during summer is associated with decreased mitochondrial DNA and increased expression of mitochondrial and apoptotic genes in oocytes. Biol. Reprod. 94: 1-10.

Fortune, J. E., G. M. Rivera, and M. Y. Yang. 2004. Follicular development: The role of the follicular microenvironment in selection of the dominant follicle. Anim. Reprod. Sci. 82-83: 109-126.

Givol, D., and A. Yayon. 1992. Complexity of fgf receptors: Genetic basis for structural diversity and functional specificity. FASEB J. 6: 3362-3369.

Gospodarowicz, D., J. Cheng, G. M. Lui, A. Baird, F. Esch, and P. Bohlen. 1985. Corpus luteum angiogenic factor is related to fibroblast growth factor. Endocrinology 117: 2383-2391.

Gospodarowicz, D., C. R. Ill, and C. R. Birdwell. 1977a. Effects of fibroblast and epidermal growth-factors on ovarian cell-proliferation in vitro .1. Characterization of response of granulosa-cells to FGF and EGF. Endocrinology 100: 1108-1120.

Gospodarowicz, D., C. R. Ill, and C. R. Birdwell. 1977b. Effects of fibroblast and epidermal growth-factors on ovarian cell-proliferation invitro. 2. Proliferative response of luteal cells to FGF but not EGF. Endocrinology 100: 1121-1128.

Grado-Ahuir, J. A., P. Y. Aad, and L. J. Spicer. 2011. New insights into the pathogenesis of cystic follicles in cattle: microarray analysis of gene expression in granulosa cells. J. Anim. Sci. 89:1769-1786.

Ireland, J. J., and J. F. Roche. 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: Changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. Endocrinology 111: 2077-2086.

Itoh, N., and D. M. Ornitz. 2004. Evolution of the FGF and FGFR gene families. Trends Genet. 20: 563-569.

Itoh, N., and D. M. Ornitz. 2011. Fibroblast growth factors: From molecular evolution to roles in development, metabolism and disease. J. Biochem. 149: 121-130.

Jiang, Z., H. M. Guerrero-Netro, J. L. Juengel, and C. A. Price. 2013. Divergence of intracellular signaling pathways and early response genes of two closely related

fibroblast growth factors, fgf8 and fgf18, in bovine ovarian granulosa cells. Mol. Cell. Endocrinol. 375: 97-105.

Knecht, M., A. M. Brodie, and K. J. Catt. 1985. Aromatase inhibitors prevent granulosa cell differentiation: An obligatory role for estrogens in luteinizing hormone receptor expression. Endocrinology 117: 1156-1161.

Lagaly, D. V., P. Y. Aad, J. A. Grado-Ahuir, L. B. Hulsey, and L. J. Spicer. 2008. Role of adiponectin in regulating ovarian theca and granulosa cell function. Mol. Cell. Endocrinol. 284: 38-45.

Li, X. W., C.; Xiao, J.; McKeehan, W.L.; Wang, F. 2016. Fibroblast growth factors, old kids on the new block. Semin. Cell. Dev. Biol. 53: 155-167.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

Machado, M. F., V. M. Portela, C. A. Price, I. B. Costa, P. Ripamonte, R. L. Amorim, and J. Buratini. 2009. Regulation and action of fibroblast growth factor 17 in bovine follicles. J. Endocrinol. 202: 347-353.

Ornitz, D. M. and N. Itoh. 2001. Fibroblast growth factors. Genome Biol. 2: REVIEWS3005.

Ornitz, D. M., and N. Itoh. 2015. The fibroblast growth factor signaling pathway. Wiley Interdiscip. Rev. Dev. Biol. 4: 215-266.

Ott, L. 1977. An introduction to statistical methods and data analysis Duxbury Press, North Scituate, MA.

Parrott, J. A., and M. K. Skinner. 1998. Developmental and hormonal regulation of hepatocyte growth factor expression and action in the bovine ovarian follicle. Biol. Reprod. 59: 553-560.

Parrott, J. A., J. L. Vigne, B. Z. Chu, and M. K. Skinner. 1994. Mesenchymal-epithelial interactions in the ovarian follicle involve keratinocyte and hepatocyte growth factor production by thecal cells and their action on granulosa cells. Endocrinology 135: 569-575.

Plotnikov, A. N., S. R. Hubbard, J. Schlessinger, and M. Mohammadi. 2000. Crystal structures of two fgf-fgfr complexes reveal the determinants of ligand-receptor specificity. Cell 101: 413-424.

Portela, V. M., E. Dirandeh, H. M. Guerrero-Netro, G. Zamberlam, M. H. Barreta, A. F. Goetten, and C. A. Price. 2015. The role of fibroblast growth factor-18 in follicular atresia in cattle. Biol. Reprod. 92: 1-8.

Portela, V. M., M. Machado, J. Buratini, G. Zamberlam, R. L. Amorim, P. Goncalves, and C. A. Price. 2010. Expression and function of fibroblast growth factor 18 in the ovarian follicle in cattle. Biol. Reprod. 83: 339-346.

Price, C. A. 2016. Mechanisms of fibroblast growth factor signaling in the ovarian follicle. J Endocrinol.

Schreiber, N. B., and L. J. Spicer. 2012. Effects of fibroblast growth factor 9 (FGF9) on steroidogenesis and gene expression and control of FGF9 mRNA in bovine granulosa cells. Endocrinology 153: 4491-4501.

Schreiber, N. B., M. L. Totty, and L. J. Spicer. 2012. Expression and effect of fibroblast growth factor 9 in bovine theca cells. J. Endocrinol. 215: 167-175.

Spicer, L. J., and S. E. Echternkamp. 1986. Ovarian follicular growth, function and turnover in cattle: A review. J. Anim. Sci. 62: 428-451.

Spicer, L. J., and R. E. Stewart. 1996. Interactions among basic fibroblast growth factor, epidermal growth factor, insulin, and insulin-like growth factor-I (IGF-I) on cell

numbers and steroidogenesis of bovine thecal cells: Role of IGF-I receptors. Biol. Reprod. 54: 255-263.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, B. E. Keefer, L. J. Dawson, G. L. Morgan, and S. E. Echternkamp. 1996. Levels of insulin-like growth factor (IGF) binding proteins, luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrous cycles. Endocrinology 137: 2842-2850.

Vernon, R. K., and L. J. Spicer. 1994. Effects of basic fibroblast growth factor and heparin on follicle-stimulating hormone-induced steroidogenesis by bovine granulosa cells. J. Anim. Sci. 72: 2696-2702.

Voge, J. L., C. A. Santiago, P. Y. Aad, D. W. Goad, J. R. Malayer, and L. J. Spicer. 2004. Quantification of insulin-like growth factor binding protein mRNA using real-time PCR in bovine granulosa and theca cells: effect of estradiol, insulin, and gonadotropins. Domest. Anim. Endocrinol. 26: 241-258.

Wandji, S. A., J. J. Eppig, and J. E. Fortune. 1996. FSH and growth factors affect the growth and endocrine function in vitro of granulosa cells of bovine preantral follicles. Theriogenology 45: 817-832.

Zhang, K., and A. D. Ealy. 2012. Disruption of fibroblast growth factor receptor signaling in bovine cumulus-oocyte complexes during in vitro maturation reduces subsequent embryonic development. Domest. Anim. Endocrinol. 42: 230-238.

CHAPTER V

TRANSCRIPTOME PROFILING OF BOVINE THECA CELLS TREATED WITH FIBROBLAST GROWTH FACTOR 9

1. Abstract

Fibroblast growth factor 9 (FGF9), a member of a large family of single chain polypeptide factors, controls several functions in many different tissues of mammals. In the ovaries of cattle, FGF9 appears to be acting as an anti-differentiation factor, stimulating in vitro proliferation of granulosa (GC) and theca (TC) cells while suppressing steroidogenesis of these cells in the presence of insulin-like growth factor 1 (IGF-1). Also, *FGF9* mRNA abundance in GC and TC changes according to the size and estrogenic status of follicles and days post-ovulation in cattle. Nevertheless, information about signaling mechanisms activated by FGF9 in TC is somewhat lacking. Therefore, the objective of this study was to investigate how bovine TC from large ovarian follicles respond to exogenous FGF9 in vitro. In order to detect differentially expressed transcripts by TC following FGF9 in vitro treatment, microarray technology was utilized. Ovaries were obtained from beef heifers at a local abattoir, TC were isolated from large antral follicles (8.1-22 mm in surface diameter), and cultured in basal medium containing 10% FCS for 48 h prior to treatment with or without 30 ng/mL of FGF9 for 24 h in the presence of LH and IGF-1. Following treatment, total RNA was extracted from TC and processed for microarray. A total of eight chips (Affymetrix GeneChip Bovine Genome Arrays) were hybridized with RNA extracted from four biological replicates of the same number of TC pools in a paired design for the two treatments (FGF9 or control). Affymetrix GeneChip Operating Software was used to quantitate each GeneChip® and summary intensities for each probe were loaded into DNA-Chip Analyzer (dChip) for analysis. Paired t-tests were calculated using dChip to evaluate significant differences

between treatments. Analysis of hybridized GeneChip Bovine Genome arrays comparing FGF9-treated TC with control TC identified 355 differentially expressed transcripts, with 164 elements up-regulated and 191 elements down-regulated by FGF9. The QIAGEN's Ingenuity® Pathway Analysis was utilized to investigate how FGF9 treatment affects molecular pathways, biological functions, and the connection between molecules in bovine TC. The IPA software identified differentially expressed transcripts in 346 pathways in response to FGF9 in TC involved in functions such as cell cycle, growth, development, proliferation, death and survival, cellular assembly and organization, and steroidogenesis. Networks of differentially expressed transcripts unveiled interesting relationships among genes related to cellular growth and proliferation and to steroidogenesis. Overall, genes, pathways, and networks identified in this study painted an interesting picture of how FGF9 regulates folliculogenesis, providing novel candidates for further investigation of FGF9 functions in the ovary of cattle.

Keywords: cattle; cell proliferation; Fibroblast growth factor-9 (FGF9); microarray; steroidogenesis; theca cell.

2. Introduction

Fibroblast growth factors (FGFs) constitute a large family of single chain polypeptide factors present in both vertebrates and invertebrates (Ornitz and Itoh, 2001; Itoh and Ornitz, 2011; Li et al., 2016). Currently, 22 different members have been reported in mammals (FGF1 to 23), binding to high affinity receptors and several cofactors to play a variety of roles in various tissues (Givol and Yayon, 1992; Itoh and Ornitz, 2004; Dailey e al., 2005).

Fibroblast growth factor 9 (FGF9) was originally isolated from human glioma cells and characterized as a mitogenic factor (Miyamoto et al., 1993). In the last two decades, research has shown that this polypeptide plays diverse roles in many different tissues, including heart (Lavine et al., 2005; Singla et al., 2015), cartilage (Weksler et al., 1999), liver (Antoine et al., 2007), intestine (Geske et al., 2008), and reproductive (Colvin et al., 2001;

Drummond et al., 2007) and nervous systems (Reuss et al., 2000; Meier et al., 2014). In these tissues, FGF9 binds to FGF receptors (FGFR1c, FGFR2c, FGFR3c, FGFR3b, and FGFR4) to activate specific tyrosine residues and downstream intracellular signaling pathways, including the RAS-MAPK, PI3K-AKT, PLCγ, and STAT pathways, which regulate cell proliferation, survival, metabolism, and differentiation (Ornitz and Itoh, 2015). In fact, FGF9 has been shown to regulate sex determination (Kim et al., 2006), steroidogenesis (Schreiber and Spicer, 2012; Schreiber et al., 2012; Lai et al., 2014), tissue development (Harada et al., 2009), wound healing (Zheng et al., 2014), and even mood disorders (Aurbach et al., 2015). Moreover, expression of FGF9 has been related to brain (Todo et al., 1998) and gastric (Sun et al., 2015) cancers.

In the ovaries, FGF9 was first described to be present in murine corpora lutea, stromal, and theca cells (TC) and was observed to stimulate progesterone (P4) production by granulosa cells (GC) in a paracrine way (Drummond et al., 2007). In porcine GC, FGF9 in the presence of insulin-like growth factor 1 (IGF-1) stimulated cell proliferation and steroidogenesis (Evans et al., 2014). In cattle, FGF9 has been suggested to be an anti-differentiation factor by stimulating in vitro proliferation of TC and GC while suppressing P4 production by TC and GC and estradiol (E2) production by GC in the presence of IGF-1 (Schreiber and Spicer, 2012; Schreiber et al., 2012). Abundance of FGF9 mRNA in GC and TC is hormonally regulated (Schreiber and Spicer, 2012; Schreiber et al., 2012) and changes according to the size and estrogenic status of follicles and days post-ovulation in cattle (Schütz et al., 2016, unpublished data). Hence, it seems clear that FGF9 is an important regulator of ovarian function in mammals, but its role may differ between monotocous and polytocous animals. Nevertheless, detailed information about signaling mechanisms activated by FGF9 in TC is lacking.

The technology of microarray is a powerful tool for one to investigate how a specific cell type reacts to certain stimuli, enabling the simultaneous measurement of thousands of gene transcripts (Schena et al., 1995). Therefore, we utilized this resource to investigate how

bovine TC from large ovarian follicles respond to exogenous FGF9 in vitro. Unveiling signaling pathways activated by FGF9 in TC may provide valuable information to understand molecular aspects of cell proliferation, steroidogenesis, and apoptosis in a mono-ovulatory species such as cattle.

3. Material and Methods

Reagents and Hormones

The reagents used in cell culture were Ham's F-12 (F12), DMEM, gentamicin, glutamine, sodium bicarbonate, trypan blue, deoxyribonuclease (DNase), and collagenase from Sigma-Aldrich Chemical Co. (St. Louis, MO), and fetal calf serum (FCS) from Equitech-Bio, Inc. (Kerrville, TX). The hormones used in cell culture were recombinant human FGF9 and IGF-1 (R&D Systems, Minneapolis, MN; all carrier-free), and ovine LH (NIDDK-oLH-26; activity: 1.0× NIH-LH-S1 U/mg) from the National Hormone & Pituitary Program (Torrance, CA).

Cell Collection and In Vitro Culture

Ovaries were obtained from beef heifers at a local abattoir and transported to the laboratory in 0.9% saline with 1% streptomycin/penicillin on ice. TC were isolated from large antral follicles (8.1-22 mm in surface diameter) with adequate vascularity and moderately transparent follicular fluid as previously described (Stewart et al., 1995; Lagaly et al., 2008). Briefly, follicles were bisected, GC were scraped free from the theca interna and the theca interna tissue was removed via microdissection and enzymatically digested for 1 h at 37 °C on a rocking platform. Non-digested thecal tissue was removed via filtration through a 149 µm mesh screen (Gelman Sciences, Ann Arbor, MI, USA). TC were then centrifuged at 50 x g for 7 min, washed twice in medium (1:1 DMEM and F12 containing 2.0 mM glutamine, 0.12 mM gentamicin, and 38.5 mM sodium bicarbonate), and resuspended in serum-free medium containing collagenase and DNase at 1.25 and 0.5 mg/mL, respectively, to prevent clumping of cells before plating (Lagaly et al., 2008).

Viability of TC was determined by trypan blue exclusion test and viable cells were transferred to Falcon 24-well multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) with medium containing 10% FCS. Cells were cultured at 38.5 °C in a humidified 95% air and 5% CO_2 environment for the first 48 h with a medium change at 24 h. Following, TC were washed twice in serum-free medium and cultured in 1 mL serum-free medium containing 15 ng/mL of LH and 15 ng/mL of IGF-1 with or without 30 ng/mL of FGF9 for 24 h.

RNA Extraction, Microarray and Statistical Analyses

Following treatment, TC were lysed with 0.5 mL of TRIzol® Reagent (Life Technologies Inc., Gaithersburg, MD) and total RNA was extracted as previously described (Lagaly et al., 2008; Grado-Ahuir et al., 2009). Affymetrix GeneChip Bovine Genome Arrays (Affymetrix, Santa Clara, CA) were used for the microarray as previously described (Grado-Ahuir et al., 2011). This particular array is designed to monitor expression of approximately 23,000 bovine transcripts through 24,072 probe sets. A total of eight chips were hybridized with RNA extracted from four biological replicates of the same number of TC pools in a paired design for the two treatments (FGF9 or control). Each pool of TC was generated from 5 to 7 large follicles. The processing of RNA, including RNA purification and hybridization of microarray slides, was performed by the University of Tulsa Microarray Core Facility. Affymetrix GeneChip Operating Software (GCOS ver. 1.1.1, Affymetrix, Santa Clara, CA) was used to quantitate each GeneChip®. Summary intensities for each probe were loaded into DNA-Chip Analyzer (dChip), version 1.3, for normalization, standardization, and analysis. Paired t-tests were calculated using dChip to evaluate significant differences between treatments as previously described (Grado-Ahuir et al., 2009; Grado-Ahuir et al., 2011).

Microarray Functional Data Analysis

To explore the biological knowledge associated with the statistically significant probe sets from the microarray chips in addition to the annotation produced along with statistical

comparisons in dChip, the QIAGEN's Ingenuity® Pathway Analysis (IPA®; QIAGEN Redwood City, http://www.qiagen.com/ingenuity) was utilized. The analyses were performed to investigate how FGF9 treatment affects molecular pathways, biological functions, and the connection between molecules in bovine TC.

4. Results

Analysis of hybridized GeneChip Bovine Genome arrays comparing FGF9-treated TC with control identified 355 differentially expressed transcripts ($p < 0.05$ according to t-tests and at least 1.3-fold change), with 164 elements up-regulated and 191 elements down-regulated. The IPA software utilized a total of 345 differentially expressed transcripts in its analysis. The 20 genes found to be the most up- and down-regulated among the differentially expressed genes are summarized in Table 1 and Table 2, respectively, in order of fold-change.

Identification of Molecular Canonical Pathways Altered by FGF9 Treatment

The IPA software identified differentially expressed transcripts in 346 pathways in response to FGF9 in TC. The top ten canonical pathways affected by FGF9 (based on p-value on negative log scale) are shown in Fig. 1. The main functions associated with the top altered pathways include cellular assembly and organization, and cell cycle, growth, development, proliferation, death and survival. In addition to these, it is noteworthy to mention that FGF9 treatment affected transcripts for genes in pathways related to ovarian steroidogenesis, including: Wnt/β-catenin signaling, IGF-1 signaling, PI3K/AKT signaling, TGF-β signaling, EGF signaling, and pregnenolone biosynthesis.

Identification of Biological Functions Altered by FGF9 Treatment

The IPA identified 500 annotated biological functions for the differentially expressed transcripts altered by FGF9 in vitro treatment in bovine TC. The top ten identified biological functions predicted to be increased by FGF9 in TC are shown in Fig. 2 in order of activation z-scores (z-scores measure the correlation between relationship direction and gene

expression, but only z-scores greater than 2 or smaller than -2 are considered significant for predicted activation state).

Because FGF9 has been reported to stimulate bovine TC proliferation at the same time that it suppresses steroidogenesis (Schreiber et al., 2012), special attention was given to functions related to cell fate, including cell proliferation, survival, differentiation, death, and apoptosis among the altered identified biological functions (Fig. 3). Nevertheless, although cell proliferation, survival, and differentiation functions all had a positive activation z-score, only cell proliferation and cell survival were predicted to be increased by FGF9 in TC (i.e., with z-scores greater than 2).

Identification of Molecular Networks Affected by FGF9 Treatment

Seeking to understand how molecules affected by FGF9 connect to each other and relate to specific functions, we analyzed the networks generated by IPA. The differentially expressed transcripts affected by FGF9 were categorized in 21 different networks. The top 10 networks according to p-score are shown in Fig. 4.

In order to assess how FGF9 treatment may affect ovarian function, special attention was given to networks involving cell proliferation and steroidogenesis, two actions already reported to be affected by FGF9 in bovine TC (Schreiber et al., 2012). These networks are shown in Figs. 5 and 6, respectively. For cell proliferation, up-regulated ($p < 0.05$) transcripts were *CCND1*, *FZD5*, *MYB*, *CASP3*, *FRMD4A*, *ACKR3*, *JADE2*, *RBPJ*, *HAUS8*, *ARRDC2*, *DENND2A*, *H2AFZ*, while down-regulated ($p < 0.05$) transcripts were *HLA-DRA*, *AARSD1*, *MLF1*, *JADE3*, *UBE2D4*, *PPP2R2B*, *NQO1*, *HMOX1*, *AGTR1*, *RNF150*, *SOD*, *KCNAB1*. For steroidogenesis, up-regulated genes ($p < 0.05$) by FGF9 were: *TFPI*, *VLDR*, *SLC38A2*, *ACSL4*, *PRKCA*, *ARG2*, *PER2* while down-regulated genes ($p < 0.05$) by FGF9 were: *COL14A1*, *OGN*, *IGF2*, *SORT1*, *AGTR1*, *GPIIB-IIIA*, *CYP2C9*, *STAR*, *CYP11A1*, *SIRT3*, *RIMS1*, *ARID5B;* frizzled molecules were either down-regulated (*SRFP2*, *FZD2*, *FZD4*) or up-regulated (*FZD5*).

5. Discussion

FGF9 is a powerful mitogen that stimulates cell proliferation in many tissues and has been implicated in many types of cancer (Todo et al., 1998; Hendrix et al., 2006; Sun et al., 2015). In the ovary, FGF9 has been reported to stimulate proliferation of both GC and TC (Schreiber and Spicer, 2012; Schreiber et al., 2012). Hence, it is no surprise that, based on Z-activation scores, cell proliferation was the biological function most affected by FGF9 in the present analysis (Fig. 2), with 157 out of 346 differentially expressed transcripts related with this function.

The fact that the gene encoding for cyclin D1 (*CCND1*), a protein required for progression of the G1 phase of the cell cycle (Baldin et al., 1993) that has been previously detected in proliferating murine TC, but not GC (Robker and Richards, 1998), was the most up-regulated transcript by FGF9 treatment in the present microarray further supports the importance of FGF9 in stimulating TC proliferation. In addition**,** two other genes among the top 20 up-regulated transcripts by FGF9 (*FZD5* and *MYB*) are related to cell proliferation (as shown in Table 1 and Fig. 5). In fact, *FZD5* encodes the protein Frizzled family receptor 5, a cell-surface receptor that interacts with Wnt proteins to stimulate cell proliferation of human endometrial adenocarcinoma cells (Carmon and Loose, 2008) and bovine TC (Spicer, 2016), while *MYB* (v-myb myeloblastosis viral oncogene homolog) encodes the protein c-Myb, a transcription factor that stimulates cell proliferation while suppressing apoptosis and differentiation of a variety of cell types, including human myeloid leukemia cells (Anfossi et al., 1989; Lyon et al., 1994) and human breast cancer cells (Drabsch et al., 2010). The protein c-Myb is a positive regulator of *CCND1* in human liver cancer (Zhang et al., 2012) and breast carcinoma (Mitra et al., 2016) cells. Bartunek and colleagues (2002) reported that MYB and FGF2 cooperate to sustain avian hematopoietic cell proliferation while preventing them to differentiate into red blood cells. Hence, FGF9 appears to be stimulating the progression of the G1 phase of the cell cycle for bovine TC proliferation through the Wnt signaling pathway and the regulation of *CCND1*. Interestingly, WNT3A, a member of the Wnt family of ligands

has been reported to increase *FGF9* mRNA abundance in bovine TC (Schreiber et al., 2012), reinforcing the association between FGF9 and the Wnt signaling pathway.

Another intriguing observation from the cell proliferation Network generated by IPA (Fig. 5) is the fact that transcripts for caspase-3 (*CASP3*) were increased in TC in response to FGF9. Indeed, although CASP3 is a major downstream effector of apoptosis in GC of atretic follicles, CASP3 immunostaining has also been reported in TC of healthy follicles (Boone and Tsang, 1998). Reports imply that CASP3 may be involved in selective destruction of organelles and may be enhancing cell survival and proliferation in certain situations (Perfettini and Kroemer, 2003; Kuranaga, 2011). Interestingly, CASP3 cleaves the cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$), a mammalian regulator of the cell cycle that induces G1 arrest, thus stimulating lymphoid cell proliferation (Frost et al., 2001). Because $p27^{Kip1}$ decreases during TC proliferation and increases during TC terminal differentiation (Robker and Richards, 1998), the present data indicate that, like with lymphoid cells, CASP3 may be stimulating bovine TC proliferation by preventing $p27^{Kip1}$ to arrest cells at G1. A lack of change in $p27^{Kip1}$ mRNA following FGF9 treatment supports a post-transcriptional regulation of $p27^{Kip1}$. Furthermore, *MIR221*, one of the top up-regulated genes in the present study, has been reported to down-regulate $p27^{Kip1}$, via prevention of translation, in human hepatocellular carcinoma (Fornari et al., 2008).

It is also noteworthy that the genes *HMOX1* and *NQO1* were down-regulated by FGF9, as shown in the cell proliferation Network (Fig. 5). These genes are antioxidant and cytoprotective, being activated by the transcription activator NRF2 in response to oxidative stress in order to protect the cells (Attucks et al., 2014). Not by coincidence, NRF2-mediated oxidative stress response is among the top canonical pathways affected by FGF9 in the present study (Fig. 1). Reactive oxygen species (ROS) are constantly produced during metabolic processes and it is now well accepted that ROS at high concentrations are cytotoxic, but, at moderate concentrations, ROS play a role in signal transduction processes, including cell proliferation (Hu et al., 2005; Zhang et al., 2016). In the ovary, high levels of

ROS have been shown to stimulate the initiation of apoptosis in GC of antral follicles (Devine et al., 2012; Zhang et al., 2016). Nevertheless, in theca-interstitial cells, generation of ROS is required to maintain cell proliferation, and a reduction in the level of ROS inhibits cell proliferation (Duleba et al., 2004). Hence, the reduction in transcripts for HMOX1 and NQO1, factors that reduce ROS concentrations, by FGF9 observed in the present study suggests that ROS may be important for bovine TC proliferation. This idea is further supported by the fact that ROS mediate FGF2 biological effects in chondrocytes (as reviewed by Sainz et al., 2012). In addition, Cui and co-authors (2015) recently reported that NQO1 is frequently up-regulated in human ovarian carcinomas, supporting the idea that controlled regulation of NQO1 may play an important role in ovarian function.

FGF9 has also been reported as a regulator of steroidogenesis by murine, bovine, and porcine follicular somatic cells (Drummond et al., 2007; Schreiber and Spicer, 2012; Schreiber et al., 2012; Evans et al., 2014). In bovine GC, FGF9 decreases IGF-1 plus FSH-induced E2, P4, and pregnenolone production while decreasing mRNA abundance for the steroidogenic enzyme CYP11A1 and for the FSH receptor (FSHR) of both small (1-5 mm) and large (8-22 mm) antral follicles (Schreiber and Spicer, 2012). In bovine TC, FGF9 decreases IGF-1 plus LH-induced P4 and androstenedione (A4) production while decreasing mRNA abundance for the steroidogenic enzymes CYP11A1 and CYP17A1 and for the LH receptor (LHCGR) of large antral follicles (Schreiber et al., 2012). Therefore, the fact that CYP11A1 is among the top down-regulated genes is not surprising (Table 2). In addition, other genes related to steroidogenesis of TC were down-regulated by FGF9 in this study (Fig. 6), including the genes encoding for steroidogenic acute regulatory protein (STAR), insulin-like growth factor 2 (IGF2), angiotensin II receptor, type I (AGTR1), frizzled class receptor 4 (FZD4), and cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9). STAR is responsible for mediating the transport of cholesterol to mitochondria (Lin et al., 1995); IGF2, AGTR1 and FZD4 appear to be important to P4 production, since both IGF2 (Spicer et al., 2004) and angiotensin II (Acosta et al., 1999) have been reported to stimulate P4 release

while FZD4 knockout mice have lower P4 production (Hsieh et al., 2005); CYP2C9 is involved in catabolism of P4 (Yamazaki and Shimada, 1997). Hence, these observations suggest that FGF9 affects several genes that may act in concert to suppress P4 and A4 production by bovine TC. The fact that STAR was down-regulated in this study is in contrast to a previous report by our group in FGF9-treated TC and requires further investigation (Schreiber et al., 2012). Finally, the observation that the frizzled proteins encoding genes *FZD2*, *FZD4*, and *SRFP2* were down-regulated in the steroidogenesis Network suggests that these genes can be also linking the Wnt signaling pathway with steroidogenesis in TC, but this hypothesis also requires further investigation. Recent studies support this idea by showing that WNT5A increases androstenedione production by bovine TC (Spicer, 2016).

Steroidogenesis by TC and GC of large antral follicles is crucial for follicular differentiation (Knecht et al., 1984; Evans and Fortune, 1997; McGee and Hsueh, 2000; Young and McNeilly, 2010). Since FGF9 decreases synthesis of P4 and A4 and reduces mRNA abundance for both *LHCGR* and *CYP17A1* (Schreiber et al., 2012), critical factors for TC differentiation in preovulatory follicles (Richards, 1994; McGee and Hsueh, 2000), it seems likely to suppose that FGF9 acts as an anti-differentiation factor of TC of large antral follicles, at least in cattle. This idea is further reinforced by the observation that abundance of bovine *FGF9* mRNA is greater in TC of all sizes of E2-inactive follicles (i.e., future atretic follicles) than in large E2-active follicles (i.e., dominant follicles) at an early growing phase of the first dominant follicle (Schütz et al., 2016). Thus, the fact that FGF9 up-regulated *CCND1* and *MYB*, factors that suppress cell differentiation while stimulating cell proliferation (Lyon et al., 1994; Robker and Richards, 1998), together with the observation that FGF9 was not predicted to increase TC differentiation (Fig. 3) in the current study corroborates the idea that FGF9 is stimulating bovine TC proliferation rather than differentiation.

In summary, the current microarray study allowed the identification of 345 differentially expressed transcripts in bovine TC following in vitro FGF9 treatment, several of them related to cell proliferation, survival, and steroidogenesis. The data presented herein

provide an interesting picture of how FGF9 promotes TC proliferation while suppressing cell differentiation. The investigation of the candidate genes and pathways unraveled here by IPA will not only provide an insight of how FGF9 plays a role in folliculogenesis in cattle, but will also help to unveil how this growth factor affects pathological conditions such as cystic ovarian follicles and ovarian cancer.

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Table 1. 20 most up-regulated genes by FGF9 treatment in bovine TC.

	Probe set	Gene symbol	Gene Name	Fold change	P-value
1	Bt.16538.2.A1_at	*CCND1*	Cyclin D1	4.96	0.0000569
2	Bt.12927.1.S1_at	*HAS2*	hyaluronan synthase 2	3.90	0.000803649
3	Bt.1817.1.S1_at	*ETV1*	Ets variant 1	3.49	0.00142088
4	Bt.996.1.S1_at	---	transcribed locus	3.25	0.00080151
5	Bt.19826.1.A1_at	---	transcribed locus	3.23	0.0210203
6	Bt.6983.1.S1_at	*SOX9*	SRY (Sex determining region Y)-box 9	3.16	0.0377339
7	Bt.29956.1.A1_at	*BMP2*	bone morphogenetic protein 2	2.98	0.00193277
8	Bt.10212.1.S1_at	*PHLDA1*	Pleckstrin homology-like domain, family A, member 1	2.97	0.000000737
9	Bt.3051.1.S1_at	---	Transcribed locus	2.92	0.0000611
10	Bt.72.1.S1_at	*MMP1*	matrix metallopeptidase 1 (interstitial collagenase)	2.90	0.00506371
11	Bt.16100.1.S1_at	*FZD5*	Frizzled family receptor 5	2.81	0.000317748
12	Bt.303.1.S1_at	*P2RY1*	purinergic receptor P2Y, G-protein coupled, 1	2.74	0.0194217
13	Bt.13073.1.S1_at	*SERPINB2*	serpin peptidase inhibitor, clade B (ovalbumin), member 2	2.63	0.0182733
14	Bt.568.1.S1_at	*IBSP*	integrin-binding sialoprotein	2.52	0.0337404
15	Bt.10648.1.S1_at	*RRM2*	ribonucleotide reductase M2	2.43	0.0240124
16	Bt.11109.1.S1_at	*MIR221*	microRNA mir-221	2.43	0.0000000273
17	Bt.12781.1.S1_at	*MYB*	v-myb myeloblastosis viral oncogene homolog (avian)	2.40	0.0397893
18	Bt.17951.1.A1_at	---	Transcribed locus	2.36	0.0257273
19	Bt.5618.1.A1_at	*CNTNAP2*	contactin associated protein-like 2	2.33	0.000372504
20	Bt.20507.1.S1_at	*CYP26B1*	cytochrome P450, family 26, subfamily B, polypeptide 1	2.31	0.00301863

Table 2. 20 most down-regulated genes by FGF9 treatment in bovine TC.

	Probe set	Gene symbol	Gene Name	Fold change	P-value
1	Bt.4939.1.S1_at	*SFRP2*	secreted frizzled-related protein 2	-4.09	0.00392234
2	Bt.7190.1.S1_at	*CYP11A1*	cytochrome P450, family 11, subfamily A, polypeptide 1	-4.09	0.0236564
3	Bt.13482.1.S1_at	*NOV*	nephroblastoma overexpressed	-3.25	0.0116386
4	Bt.13855.2.S1_at	*ADAMDEC1*	ADAM-like, decysin 1	-3.25	0.00490709
5	Bt.11656.1.S1_at	*CGN*	cingulin	-3.06	0.000463423
6	Bt.19661.1.S1_at	*PPP2R2B*	protein phosphatase 2, regulatory subunit B, beta	-3.01	0.000310913
7	Bt.20574.1.S1_at	*AQP11*	aquaporin 11	-2.99	0.00102593
8	Bt.10021.1.S1_at	*MYL7*	myosin, light chain 7, regulatory	-2.72	0.0261566
9	Bt.19322.1.A1_at	*COL4A6*	collagen, type IV, alpha 6	-2.69	0.0213395
10	Bt.5341.1.S1_at	*OGN*	osteoglycin	-2.68	0.00729922
11	Bt.23113.1.A1_at	---	transcribed locus	-2.66	0.0000184
12	Bt.22768.1.A1_at	*SVEP1*	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	-2.65	0.0126702
13	Bt.24211.1.A1_at	*ASPN*	asporin	-2.61	0.00459269
14	Bt.1745.1.S1_at	*KRT18*	keratin 18	-2.59	0.00608511
15	Bt.7042.1.S1_at	*NPVF*	neuropeptide VF precursor	-2.58	0.00210952
16	Bt.16495.1.A1_at	*COL4A5*	collagen, type IV, alpha 5	-2.56	0.0140642
17	Bt.5530.1.S1_at	*DHRS3*	dehydrogenase/reductase (SDR family) member 3	-2.49	0.00124302
18	Bt.27645.1.A1_at	*ISM1*	isthmin 1 homolog (zebrafish)	-2.46	0.00446444
19	Bt.3885.4.S1_X_at	*CLCA3P*	chloride channel accessory 3 (pseudogene)	-2.36	0.00487499
20	Bt.12685.1.S1_at	*MYH11*	myosin, heavy chain 11, smooth muscle	-2.30	0.0294988

Fig. 1. Top 10 canonical pathways altered by FGF9 in bovine TC identified by IPA. Numbers to the right on the bars indicate the number of differentially expressed transcripts in each pathway.

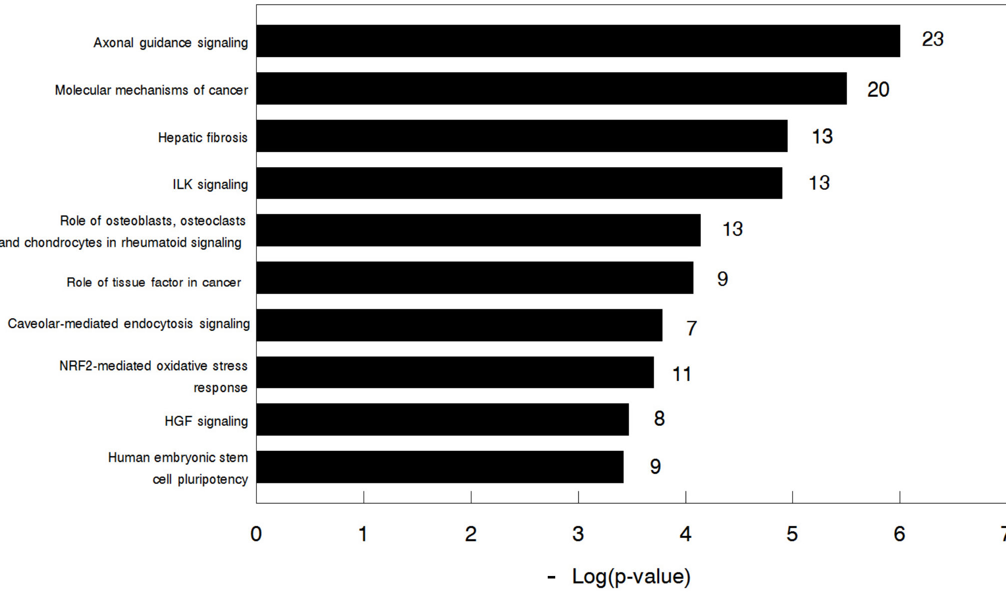


Fig. 2. Top 10 biological functions for the differentially expressed transcripts altered by FGF9 treatment in bovine TC identified by IPA. Numbers to the right on the bars indicate the number of differentially expressed transcripts in each pathway.

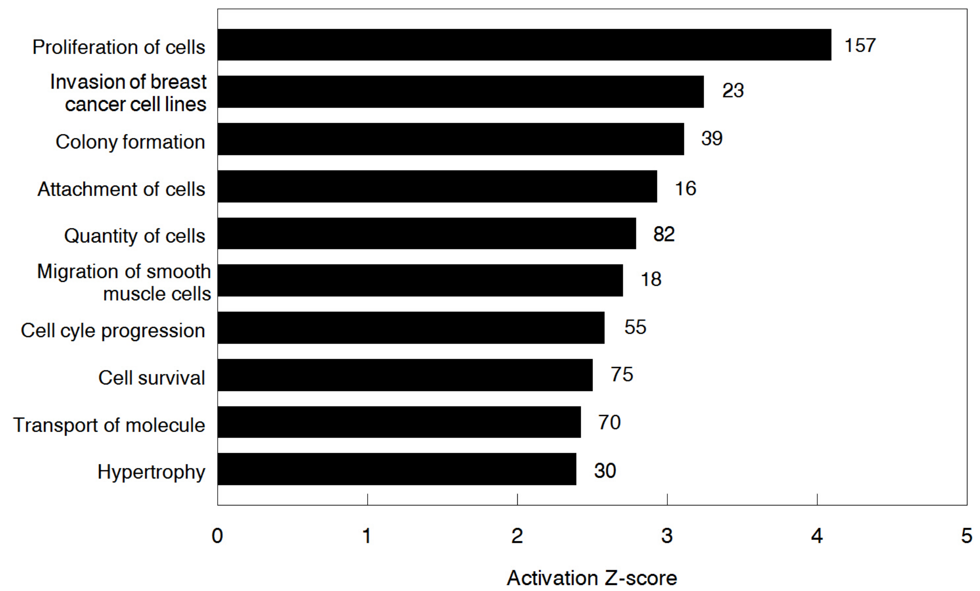


Fig. 3. Biological functions related to cell fate altered by FGF9 in bovine TC identified by IPA. Among these functions, based on activation z-score, only proliferation of cell and cell survival were predicted to be significantly increased in response to FGF9 in TC. The numbers on the top of each bar indicate the number of differentially expressed transcripts in each pathway.

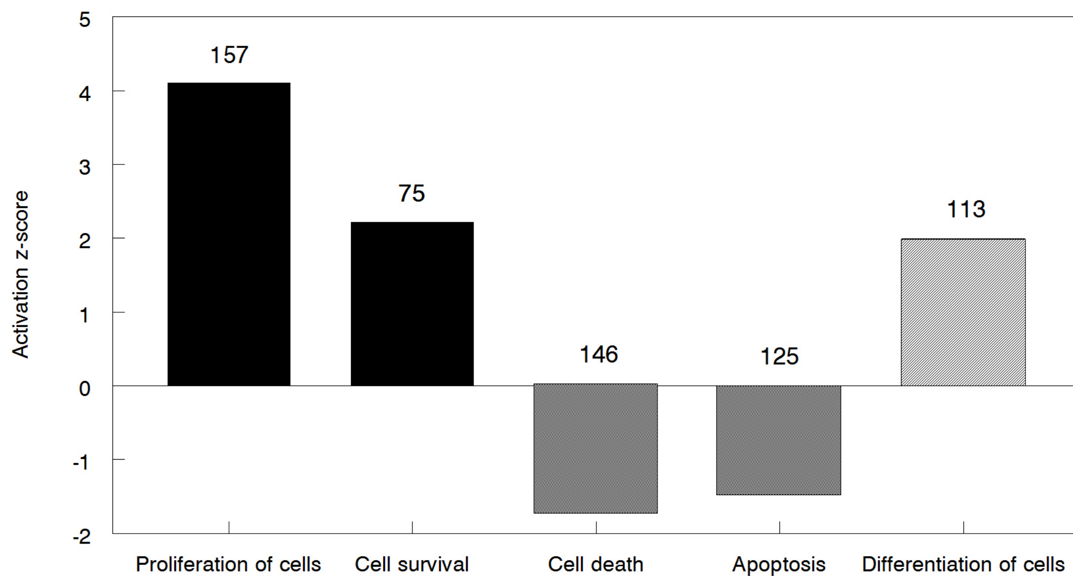


Fig. 4. Top 10 networks generated by IPA according to score. The greater the area in the graph, the higher the score of the network.

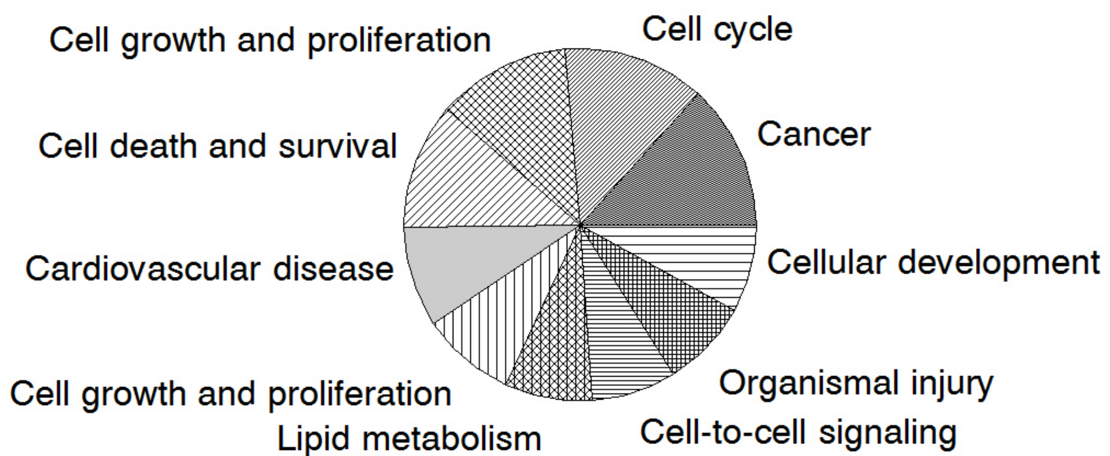


Fig. 5. Network of differentially expressed transcripts related to cellular growth and proliferation (Network 3). The bold lines show a direct association among molecules while dashed lines show an indirect association among the molecules. Different shapes of molecules indicate different functions. Significantly up-regulated genes ($p < 0.05$) by FGF9 in this network were: *CCND1*, *FZD5*, *MYB*, *CASP3*, *FRMD4A*, *ACKR3*, *JADE2*, *RBPJ*, *HAUS8*, *ARRDC2*, *DENND2A*, *H2AFZ*; significantly down-regulated genes ($p < 0.05$) by FGF9 in this network were: *HLA-DRA*, *AARSD1*, *MLF1*, *JADE3*, *UBE2D4*, *PPP2R2B*, *NQO1*, *HMOX1*, *AGTR1*, *RNF150*, *SOD*, *KCNAB1*.

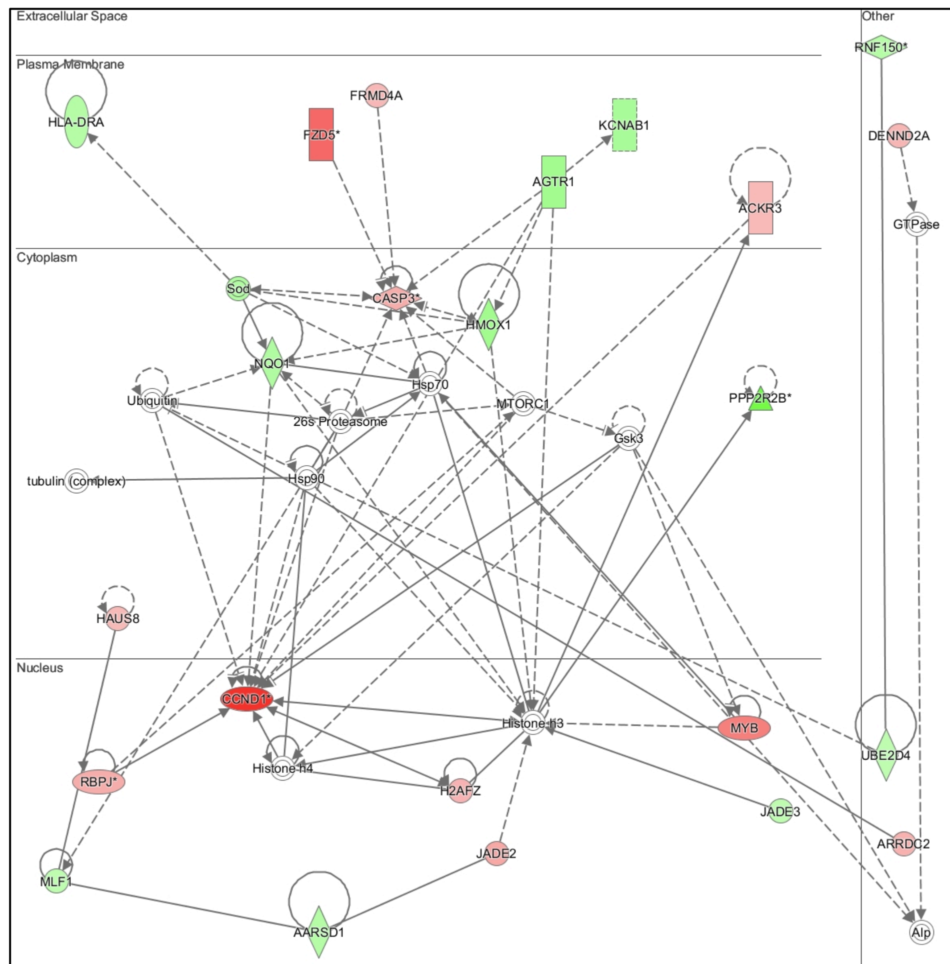
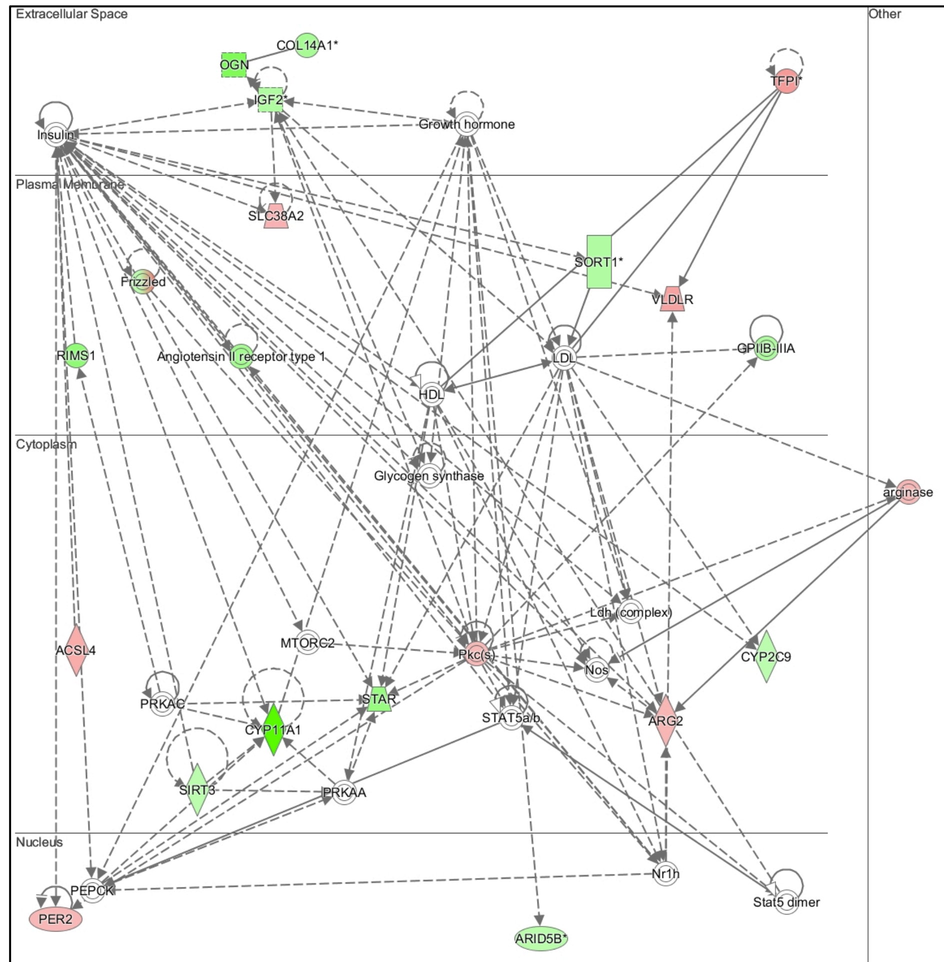


Fig. 6. Network of differentially expressed transcripts related to steroidogenesis (Network 15: Lipid metabolism, molecular transport, small molecule biochemistry). The bold lines show a direct association among molecules while dashed lines show an indirect association among the molecules. Different shapes of molecules indicate different functions. Significantly up-regulated genes ($p < 0.05$) by FGF9 in this network were: *TFPI*, *VLDR*, *SLC38A2*, *ACSL4*, *PRKCA*, *ARG2*, *PER2*; significantly down-regulated genes ($p < 0.05$) by FGF9 in this network were: *COL14A1*, *OGN*, *IGF2*, *SORT1*, *AGTR1*, *GPIIB-IIIA*, *CYP2C9*, *STAR*, *CYP11A1*, *SIRT3*, *RIMS1*, *ARID5B*. Frizzled molecules were either down-regulated (*SRFP2*, *FZD2*, *FZD4*) or up-regulated (*FZD5*).



6. References

Acosta, T. J., B. Berisha, T. Ozawa, K. Sato, D. Schams, and A. Miyamoto. 1999. Evidence for a local endothelin-angiotensin-atrial natriuretic peptide systemin bovine mature follicles in vitro: effects on steroid hormones and prostaglandin secretion. Biol. Reprod. 61: 1419-1425.

Anfossi, G., A. M. Gewirtz, and B. Calabretta. 1989. An oligomer complementary to c-myb-encoded mRNA inhibits proliferation of human myeloid leukemia cell lines. Proc. Natl. Acad. Sci. U S A 86: 3379-3383.

Antoine, M., W. Wirz, C. G. Tag, A. M. Gressner, M. Marvituna, M. Wycislo, C. Hellerbrand, and P. Kiefer. 2007. Expression and function of fibroblast growth factor (FGF) 9 in hepatic stellate cells and its role in toxic liver injury. Biochem. Biophys. Res. Commun. 361: 335-341.

Attucks, O. C., K. J. Jasmer, M. Hannink, J. Kassis, Z. Zhong, S. Gupta, S. F. Victory, M. Guzel, D. R. Polisetti, R. Andrews, A. M. Mjalli, and M. J. Kostura. 2014. Induction of heme oxygenase I (HMOX1) by HPP-4382: a novel modulator of Bach1 activity. PLoS One 9: e101044.

Aurbach, E. L., E. G. Inui, C. A. Turner, M. H. Hagenauer, K. E. Prater, J. Z. Li, D. Absher, N. Shah, P. Blandino, Jr., W. E. Bunney, R. M. Myers, J. D. Barchas, A. F. Schatzberg, S. J. Watson, Jr., and H. Akil. 2015. Fibroblast growth factor 9 is a novel modulator of negative affect. Proc. Natl. Acad. Sci. U S A 112: 11953-11958.

Baldin, V., J. Lukas, M. J. Marcote, M. Pagano, and G. Draetta. 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. 7: 812-821.

Barak, H., S. H. Huh, S. Chen, C. Jeanpierre, J. Martinovic, M. Parisot, C. Bole-Feysot, P. Nitschke, R. Salomon, C. Antignac, D. M. Ornitz, and R. Kopan. 2012. FGF9 and

FGF20 maintain the stemness of nephron progenitors in mice and man. Dev. Cell. 22: 1191-1207.

Bartunek, P., P. Pajer, V. Karafiat, G. Blendinger, M. Dvorak, and M. Zenke. 2002. bFGF signaling and v-Myb cooperate in sustained growth of primitive erythroid progenitors. Oncogene 21: 400-410.

Boone, D. L., and B. K. Tsang. 1998. Caspase-3 in the rat ovary: localization and possible role in follicular atresia and luteal regression. Biol. Reprod. 58: 1533-1539.

Carmon, K. S., and D. S. Loose. 2008. Secreted frizzled-related protein 4 regulates two Wnt7a signaling pathways and inhibits proliferation in endometrial cancer cells. Mol. Cancer Res. 6: 1017-1028.

Colvin, J. S., R. P. Green, J. Schmahl, B. Capel, and D. M. Ornitz. 2001a. Male-to-female sex reversal in mice lacking fibroblast growth factor 9. Cell 104: 875-889.

Colvin, J. S., A. C. White, S. J. Pratt, and D. M. Ornitz. 2001b. Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme. Development 128: 2095-2106.

Cui, X., L. Li, G. Yan, K. Meng, Z. Lin, Y. Nan, G. Jin, and C. Li. 2015. High expression of NQO1 is associated with poor prognosis in serous ovarian carcinoma. BMC Cancer 15: 244.

Dailey, L., D. Ambrosetti, A. Mansukhani, and C. Basilico. 2005. Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev. 16: 233-247.

Devine, P. J., S. D. Perreault, and U. Luderer. 2012. Roles of reactive oxygen species and antioxidants in ovarian toxicity. Biol. Reprod. 86: 1-10.

Dichmann, D. S., C. P. Miller, J. Jensen, R. Scott Heller, and P. Serup. 2003. Expression and misexpression of members of the FGF and TGFbeta families of growth factors in the developing mouse pancreas. Dev. Dyn. 226: 663-674.

Drabsch, Y., R. G. Robert, and T. J. Gonda. 2010. MYB suppresses differentiation and apoptosis of human breast cancer cells. Breast Cancer Res. 12: R55.

Drummond, A. E., M. Tellbach, M. Dyson, and J. K. Findlay. 2007. Fibroblast growth factor-9, a local regulator of ovarian function. Endocrinology 148: 3711-3721.

Duleba, A. J., N. Foyouzi, M. Karaca, T. Pehlivan, J. Kwintkiewicz, and H. R. Behrman. 2004. Proliferation of ovarian theca-interstitial cells is modulated by antioxidants and oxidative stress. Hum. Reprod. 19: 1519-1524.

Evans, A. C., and J. E. Fortune. 1997. Selection of the dominant follicle in cattle occurs in the absence of differences in the expression of messenger ribonucleic acid for gonadotropin receptors. Endocrinology 138: 2963-2971.

Evans, J. R., N. B. Schreiber, J. A. Williams, and L. J. Spicer. 2014. Effects of fibroblast growth factor 9 on steroidogenesis and control of FGFR2IIIc mRNA in porcine granulosa cells. J. Anim. Sci. 92: 511-519.

Fornari, F., L. Gramantieri, M. Ferracin, A. Veronese, S. Sabbioni, G. A. Calin, G. L. Grazi, C. Giovannini, C. M. Croce, L. Bolondi, and M. Negrini. 2008. MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene 27: 5651-5661.

Frost, V., S. Al-Mehairi, and A. J. Sinclair. 2001. Exploitation of a non-apoptotic caspase to regulate the abundance of the cdkI p27(KIP1) in transformed lymphoid cells. Oncogene 20: 2737-2748.

Geske, M. J., X. Zhang, K. K. Patel, D. M. Ornitz, and T. S. Stappenbeck. 2008. Fgf9 signaling regulates small intestinal elongation and mesenchymal development. Development 135: 2959-2968.

Givol, D., and A. Yayon. 1992. Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. FASEB J 6: 3362-3369.

Grado-Ahuir, J. A., P. Y. Aad, G. Ranzenigo, F. Caloni, F. Cremonesi, and L. J. Spicer. 2009. Microarray analysis of insulin-like growth factor-I-induced changes in messenger ribonucleic acid expression in cultured porcine granulosa cells: possible role of insulin-like growth factor-I in angiogenesis. J. Anim. Sci. 87: 1921-1933.

Grado-Ahuir, J. A., P. Y. Aad, and L. J. Spicer. 2011. New insights into the pathogenesis of cystic follicles in cattle: Microarray analysis of gene expression in granulosa cells. J. Anim. Sci. 89: 1769-1786.

Harada, M., H. Murakami, A. Okawa, N. Okimoto, S. Hiraoka, T. Nakahara, R. Akasaka, Y. Shiraishi, N. Futatsugi, Y. Mizutani-Koseki, A. Kuroiwa, M. Shirouzu, S. Yokoyama, M. Taiji, S. Iseki, D. M. Ornitz, and H. Koseki. 2009. FGF9 monomer-dimer equilibrium regulates extracellular matrix affinity and tissue diffusion. Nat. Genet. 41: 289-298.

Hendrix, N. D., R. Wu, R. Kuick, D. R. Schwartz, E. R. Fearon, and K. R. Cho. 2006. Fibroblast growth factor 9 has oncogenic activity and is a downstream target of Wnt signaling in ovarian endometrioid adenocarcinomas. Cancer Res. 66: 1354-1362.

Hsieh, M., D. Boerboom, M. Shimada, Y. Lo, A. F. Parlow, U. F. Luhmann, W. Berger, and J. S. Richards. 2005. Mice null for Frizzled4 (Fzd4-/-) are infertile and exhibit impaired corpora lutea formation and function. Biol. Reprod. 73: 1135-1146.

Hu, Y., D. G. Rosen, Y. Zhou, L. Feng, G. Yang, J. Liu, and P. Huang. 2005. Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: role in cell proliferation and response to oxidative stress. J. Biol. Chem. 280: 39485-39492.

Itoh, N., and D. M. Ornitz. 2004. Evolution of the Fgf and Fgfr gene families. Trends Genet. 20: 563-569.

Itoh, N., and D. M. Ornitz. 2011. Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J. Biochem. 149: 121-130.

Kim, Y., A. Kobayashi, R. Sekido, L. DiNapoli, J. Brennan, M. C. Chaboissier, F. Poulat, R. R. Behringer, R. Lovell-Badge, and B. Capel. 2006. Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. PLoS Biol. 4: e187.

Knecht, M., J. M. Darbon, T. Ranta, A. J. Baukal, and K. J. Catt. 1984. Estrogens enhance the adenosine 3',5'-monophosphate-mediated induction of follicle-stimulating hormone and luteinizing hormone receptors in rat granulosa cells. Endocrinology 115: 41-49.

Kuranaga, E. 2011. Caspase signaling in animal development. Dev. Growth Differ. 53: 137-148.

Lai, M. S., Y. S. Cheng, P. R. Chen, S. J. Tsai, and B. M. Huang. 2014. Fibroblast growth factor 9 activates akt and MAPK pathways to stimulate steroidogenesis in mouse leydig cells. PLoS One 9: e90243.

Lavine, K. J., K. Yu, A. C. White, X. Zhang, C. Smith, J. Partanen, and D. M. Ornitz. 2005. Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. Dev. Cell 8: 85-95.

Li, X., C. Wang, J. Xiao, W. L. McKeehan, and F. Wang. 2016. Fibroblast growth factors, old kids on the new block. Semin. Cell. Dev. Biol. 53: 155-167.

Lin, D., T. Sugawara, J. F. Strauss, 3rd, B. J. Clark, D. M. Stocco, P. Saenger, A. Rogol, and W. L. Miller. 1995. Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 267: 1828-1831.

Lyon, J., C. Robinson, and R. Watson. 1994. The role of Myb proteins in normal and neoplastic cell proliferation. Crit. Rev. Oncog. 5: 373-388.

McGee, E. A., and A. J. Hsueh. 2000. Initial and cyclic recruitment of ovarian follicles. Endocr. Rev. 21: 200-214.

Meier, F., F. Giesert, S. Delic, T. Faus-Kessler, F. Matheus, A. Simeone, S. M. Holter, R. Kuhn, D. M. Weisenhorn, W. Wurst, and N. Prakash. 2014. FGF/FGFR2 signaling regulates the generation and correct positioning of Bergmann glia cells in the developing mouse cerebellum. PLoS One 9: e101124.

Mitra, P., R. M. Yang, J. Sutton, R. G. Ramsay, and T. J. Gonda. 2016. CDK9 inhibitors selectively target estrogen receptor-positive breast cancer cells through combined inhibition of MYB and MCL-1 expression. Oncotarget 7: 9069-9083.

Miyamoto, M., K. Naruo, C. Seko, S. Matsumoto, T. Kondo, and T. Kurokawa. 1993. Molecular-Cloning of a Novel Cytokine Cdna-Encoding the 9th Member of the Fibroblast Growth-Factor Family, Which Has a Unique Secretion Property. Mol. Cell. Biol. 13: 4251-4259.

Ornitz, D. M., and N. Itoh. 2001. Fibroblast growth factors. Genome Biol. 2: REVIEWS3005.

Ornitz, D. M., and N. Itoh. 2015. The Fibroblast Growth Factor signaling pathway. Wiley Interdiscip. Rev. Dev. Biol. 4: 215-266.

Perfettini, J. L., and G. Kroemer. 2003. Caspase activation is not death. Nat. Immunol. 4: 308-310.

Reuss, B., M. Hertel, S. Werner, and K. Unsicker. 2000. Fibroblast growth factors-5 and -9 distinctly regulate expression and function of the gap junction protein connexin43 in cultured astroglial cells from different brain regions. Glia 30: 231-241.

Richards, J. S. 1994. Hormonal control of gene expression in the ovary. Endocr. Rev. 15: 725-751.

Robinson, D., A. Hasharoni, A. Oganesian, L. J. Sandell, A. Yayon, and Z. Nevo. 2001. Role of FGF9 and FGF receptor 3 in osteochondroma formation. Orthopedics 24: 783-787.

Robker, R. L., and J. S. Richards. 1998. Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. Mol. Endocrinol. 12: 924-940.

Sainz, R. M., F. Lombo, and J. C. Mayo. 2012. Radical decisions in cancer: redox control of cell growth and death. Cancers (Basel) 4: 442-474.

Schena, M., D. Shalon, R. W. Davis, and P. O. Brown. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270: 467-470.

Schreiber, N. B., and L. J. Spicer. 2012. Effects of fibroblast growth factor 9 (FGF9) on steroidogenesis and gene expression and control of FGF9 mRNA in bovine granulosa cells. Endocrinology 153: 4491-4501.

Schreiber, N. B., M. L. Totty, and L. J. Spicer. 2012. Expression and effect of fibroblast growth factor 9 in bovine theca cells. J. Endocrinol. 215: 167-175.

Singla, D. K., R. D. Singla, L. S. Abdelli, and C. Glass. 2015. Fibroblast growth factor-9 enhances M2 macrophage differentiation and attenuates adverse cardiac remodeling in the infarcted diabetic heart. PLoS One 10: e0120739.

Spicer, L. J., J. L. Voge, and D. T. Allen. 2004. Insulin-like growth factor-II stimulates steroidogenesis in cultured bovine thecal cells. Mol. Cell. Endocrinol. 227: 1-7.

Spicer, L.J. Wingless-type mouse mammary tumor virus integration site (WNT) regulation of ovarian theca cells of cattle. J. Anim. Sci. 94 (E-Suppl. 3): (in press), 2016.

Stewart, R. E., L. J. Spicer, T. D. Hamilton, and B. E. Keefer. 1995. Effects of insulin-like growth factor I and insulin on proliferation and on basal and luteinizing hormone-induced steroidogenesis of bovine thecal cells: involvement of glucose and receptors for insulin-like growth factor I and luteinizing hormone. J. Anim. Sci. 73: 3719-3731.

Sun, C., H. Fukui, K. Hara, X. Zhang, Y. Kitayama, H. Eda, T. Tomita, T. Oshima, S. Kikuchi, J. Watari, M. Sasako, and H. Miwa. 2015. FGF9 from cancer-associated fibroblasts is a possible mediator of invasion and anti-apoptosis of gastric cancer cells. BMC Cancer 15: 333.

Todo, T., T. Kondo, T. Kirino, A. Asai, E. F. Adams, S. Nakamura, K. Ikeda, and T. Kurokawa. 1998. Expression and growth stimulatory effect of fibroblast growth factor 9 in human brain tumors. Neurosurgery 43: 337-346.

Weksler, N. B., G. P. Lunstrum, E. S. Reid, and W. A. Horton. 1999. Differential effects of fibroblast growth factor (FGF) 9 and FGF2 on proliferation, differentiation and terminal differentiation of chondrocytic cells in vitro. Biochem. J. 342 Pt 3: 677-682.

Yamazaki, H., and T. Shimada. 1997. Progesterone and testosterone hydroxylation by cytochromes P450 2C19, 2C9, and 3A4 in human liver microsomes. Arch. Biochem. Biophys. 346: 161-169.

Young, J. M., and A. S. McNeilly. 2010. Theca: the forgotten cell of the ovarian follicle. Reproduction 140: 489-504.

Zhang, J., N. Luo, Y. Luo, Z. Peng, T. Zhang, and S. Li. 2012. microRNA-150 inhibits human CD133-positive liver cancer stem cells through negative regulation of the transcription factor c-Myb. Int. J. Oncol. 40: 747-756.

Zhang, J. Q., B. W. Gao, J. Wang, Q. L. Ren, J. F. Chen, Q. Ma, Z. J. Zhang, and B. S. Xing. 2016. Critical Role of FoxO1 in Granulosa Cell Apoptosis Caused by Oxidative Stress and Protective Effects of Grape Seed Procyanidin B2. Oxid. Med. Cell Longev. 2016: 6147345.

Zheng, Z., H. Y. Kang, S. Lee, S. W. Kang, B. Goo, and S. B. Cho. 2014. Up-regulation of fibroblast growth factor (FGF) 9 expression and FGF-WNT/beta-catenin signaling in laser-induced wound healing. Wound Repair Regen. 22: 660-665.

CHAPTER VI

SUMMARY AND CONCLUSION

Several factors produced by the ovarian follicular somatic cells, granulosa (GC) and theca cells (TC), are known to regulate follicular development, including the fibroblast growth factors (FGFs). Among these, FGF9 has been characterized as an anti-differentiation factor of bovine follicles, stimulating both GC and TC proliferation while suppressing hormone-stimulated steroidogenesis. Nevertheless, whether production of FGF9 by ovarian follicular cells changes during folliculogenesis is unknown. Therefore, we investigated changes in *FGF9* mRNA abundance in bovine GC and TC during development of first dominant follicle. Our findings reinforce the idea that FGF9 is acting as an anti-differentiation factor, since its mRNA abundance was greater in subordinate than in large dominant follicles. In addition, seeking to have a comprehensive view of the role of FGFs in folliculogenesis, we investigated how transcripts for selected FGF receptors (FGFR1c, FGFR2c, FGFR3c, and FGFR4) change in GC and TC during development of the first dominant follicle of cattle. We found that mRNA for these FGFRs are also greater in subordinate than in large dominant follicles, suggesting that, similar to FGF9, FGFRs may contribute to follicular selection in cattle. Finally, we investigated how FGF9 affects biological functions of TC through the use of microarray technology and bioinformatics tools such as Ingenuity Pathway Analysis. We were able to identify pathways and novel genes affected by FGF9 that are involved in

steroidogenesis, cell proliferation, and survival. Taken together, these findings support the idea that FGF9 regulates selection of the dominant follicle in cattle. Possible applications of this research include using FGF9 in protocols of superovulation and the manipulation of follicular waves in cattle in order to improve reproductive efficiency. Furthermore, genes and pathways unveiled by microarray could be targeted in research for therapy of ovarian cancer caused by dysregulated FGF9 signaling.

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