Regulation of ovarian function by the TGF- β superfamily and follistatin

Shyr-Yeu Lin^{1,2}, John R. Morrison¹, David J. Phillips¹ and David M. de Kretser^{1*}

¹Centre for Molecular Reproduction and Endocrinology, Monash Institute of Reproduction and Development, Monash University, Melbourne, Victoria 3168, Australia; and ²Department of Obstetrics and Gynecology, Mackay Memorial Hospital, Taipei, Taiwan

The role of follistatin as an activin-binding protein has dominated the study of this molecule for the last 10 years. However, there is emerging evidence that follistatin has a role in modulating the biology of other members of the transforming growth factor β (TGF- β) superfamily. This review summarizes the current concepts encompassing follistatin biochemistry as well as molecules with which it is functionally associated. Moreover, the importance of the two follistatin isoforms (follistatin-288 and follistatin-315) is discussed with particular emphasis on the regulation of the ovary. In addition to activin, this review discusses the functions of other members of the TGF- β superfamily, for example growth differentiation factor 9 (GDF-9), bone morphogenetic protein 15 (BMP-15), BMP-6, BMP-4 and BMP-7, in the ovary, and the potential interactions between follistatin and these growth factors. The complex network of TGF- β superfamily growth factor members involved in the modulation of ovarian function and the interactions of follistatin with these proteins is highlighted.

The proteins inhibin, activin and follistatin were isolated from gonads by their ability to modulate pituitary FSH secretion. Activin stimulates FSH whereas inhibin and follistatin are inhibitory (Ling *et al.*, 1985; Robertson *et al.*, 1985; Ling *et al.*, 1986; Vale *et al.*, 1986; Robertson *et al.*, 1987; Ueno *et al.*, 1987). The inhibins and activins, members of the transforming growth factor β (TGF- β) superfamily, are known to play pivotal roles in developmental and reproductive processes. Follistatin, a glycosylated single-chain protein with no structural similarity to but functionally linked to members of the TGF- β superfamily, was also isolated by its suppression of FSH secretion by pituitary cells (Esch *et al.*, 1987; Robertson *et al.*, 1987). Subsequently, follistatin was shown to be an activin-binding protein (Nakamura *et al.*, 1990), which acts by binding and neutralizing the actions of the activins. Physiological studies have demonstrated that the inhibins function principally as reproductive hormones (DePaolo 1997; Mather *et al.*, 1997), whereas the activins have predominantly paracrine or autocrine functions (DePaolo, 1997; Mather *et al.*, 1997). In addition to inhibiting the actions of the activins, follistatin binds and regulates the function of other members of the TGF- β superfamily, thereby expanding its range of actions. This review will focus on the biochemical

characteristics of follistatin in relation to its influence on ovarian function.

TGF- β superfamily

The TGF- β superfamily of growth factors includes more than 30 structurally related mammalian proteins that have diverse functions during embryonic development and adult tissue homeostasis. They can be grouped into three families: the TGF- β family, the activin family, and the bone morphogenetic protein (BMP) family, although some TGF- β superfamily members may fall outside these three groupings.

Members of the TGF- β superfamily are synthesized as large precursor proteins that are composed of an amino-terminal signal sequence, a pro-domain and a mature domain (carboxyl-terminal domain) (Fig. 1a). The amino-terminal signal may direct the precursor to a secretory pathway. The variable pro-domain may facilitate folding, dimerization and regulation of TGF- β superfamily members (Kingsley, 1994). The signalling parts of the molecules are the hetero- or homodimers of the highly conserved carboxyl-terminal domain, most of which contain seven conserved cysteine residues. Six of these cysteines, forming three disulphide bonds within each monomer subunit, interact to build a structure termed a cystine knot (Fig. 1b). This knot consists of an eight-member ring constructed from two disulphide bonds, with a third disulphide bond

*Correspondence

Email: david.de.kretser@med.monash.edu.au

Fig. 1. Structure of transforming growth factor β (TGF- β) superfamily members. (a) A precursor protein of monomers of TGF- β superfamily members consists of a signal sequence, pro-domain and a mature domain (carboxyl-terminal domain). Cleavage at a dibasic site (RXXR motifs) releases a mature domain that contains seven highly conserved cysteine residues, forming three intra-monomeric disulphide bonds (C_I-C_{IV}, C_{II}-C_V and C_{III}-C_{VI}). (b) The cystine knot motif arises from the three intra-monomeric disulphide bonds by two (C_{II}-C_V and C_{III}-C_{VI}) of them building an eight-membered ring structure, through which the third (C_I-C_{IV}) passes. The remaining cysteine (labelled with *) forms an inter-monomeric disulphide bond with that of the other monomer into a dimer. (Based on Böttner *et al.* 2000.)

threaded through the central area of the ring (Daopin *et al.*, 1992; Schlunegger and Grutter, 1992; Böttner *et al.*, 2000). Moreover, this knot locks the base of several β -sheet strands together apparently contributing to the stabilization of TGF- β superfamily members in various conformations. The remaining cysteine residue in each monomer forms an additional disulphide bond that links two monomers into a dimer (Fig. 1b). This cysteine is missing in some members, for example growth differentiation factor 3 (GDF-3) and 9 (GDF-9). However, there are many hydrophobic contacts between the two monomer subunits that may promote dimerization even in the absence of a disulphide bond (Kingsley, 1994).

The inhibins are dimers of a unique α -subunit dimerized to either a β A or β B subunit, giving inhibin A (α - β A) or inhibin B (α - β B). Homo-dimerization or hetero-dimerization of either β A or β B subunits generates three activins, named activin A (β A- β A), activin B (β B- β B) and activin AB (β A- β B). Three additional β subunits (mammalian β C and β E, and *Xenopus* β D) have also been cloned (Oda *et al.*, 1995; Fang *et al.*, 1996; Schmitt *et al.*, 1996). Although their functions are yet to be determined, a study by Vejda *et al.* (2002) demonstrated that the liver is the major organ for production of β C and β E subunits and potential dimerization occurs between β A and β C subunits, β A and β E subunits, and β C and β E subunits. Moreover, co-transfection studies indicate that the β C subunit is unable to form a dimer with the inhibin α subunit (Mellor *et al.*, 2000).

In addition to the inhibins and activins, to date myostatin (also called GDF-8), BMP-4, BMP-7 and BMP-15 (also called GDF-9B) have all been identified as potential binding partners of follistatin. It is very likely that other members of the TGF- β superfamily that interact with follistatin will be reported in the future. The functions of the members that are associated with follistatin are discussed in the following sections.

Signalling pathways of the TGF- β superfamily

Members of the TGF- β superfamily use a common mechanism to signal to the nucleus. They bind to cell membrane receptors, which have an intracytoplasmic serine/threonine kinase domain, leading to the assembly of a receptor complex that phosphorylates proteins of the SMAD family. The SMADs then move into the nucleus, bind DNA and recruit the required transcriptional co-activators or co-repressors to control gene expression (Massagué, 1998; Massagué and Wotton, 2000) (Fig. 2). SMAD is a fusion of the names of two genes, the *Drosophila* gene *Mad* (*mothers against dpp*) and the *C. elegans* gene *sma* (*small body size*). The specific pairs of receptors to which the ligands of the TGF- β superfamily bind fall into two groups designated type I and type II receptors. Generally, each ligand has its own specific receptors; however, some receptors may be shared by different TGF- β ligands. When the ligand binds to the receptor, it brings together two type I receptors and two type II receptors to form an activated receptor complex. In this complex, the type I receptor becomes phosphorylated by the type II receptor and in turn phosphorylates the SMAD proteins that transmit the signal (Massagué, 1998; Massagué and Wotton, 2000).

SMADs are classified into three groups based on their roles in the signalling pathways: the receptor-regulated SMADs (R-SMADs), the common mediator SMAD (co-SMAD), and the inhibitory SMADs (I-SMADs). In vertebrates, the type I receptors for activin, TGF- β and nodal, signal through SMAD2 and SMAD3, whereas those for the BMPs, GDFs and anti-Müllerian hormone-Müllerian inhibiting substance (AMH-MIS) do so through SMAD1, SMAD5 and SMAD8. These SMADs are collectively called R-SMADs. The co-SMAD, SMAD4, is required to form a complex with the R-SMAD before moving into the nucleus. However, increased expression

Fig. 2. The basic transforming growth factor β (TGF- β) superfamily members SMAD pathway. After forming a ligand-receptor complex, the receptor I (RI), phosphorylated by the receptor II (RII), in turn phosphorylates R-SMAD (SMAD 2, 3 for TGF- β /activin; SMAD 1, 5, 8 for bone morphogenetic proteins (BMPs)). The further assembly of R-SMAD and Co-SMAD (SMAD 4) makes the resulting SMAD complex free to move into the nucleus and regulate the target genes of TGF- β superfamily members with some transcriptional co-activators or co-repressors.

of the I-SMADs, SMAD6 or SMAD7, inhibits TGF- β , activin and BMP signalling (Fig. 2). In addition, the signalling network of the TGF- β superfamily has become more complex, as recent studies revealed that the SMAD signalling system is also modulated by crosstalk with other kinase signalling cascades (Derynck *et al.*, 1998; Massagué and Wotton, 2000; Zimmerman and Padgett, 2000).

Structural characterization of follistatins

The follistatin gene

Follistatin protein is highly conserved among species with 97% amino acid homology between humans and mice (<http://www.ncbi.nlm.nih.gov/cgi-bin/UniGene/>).

The human follistatin gene localizes to chromosome 5q11.2 (<http://www.ncbi.nlm.nih.gov/LocusLink/>) and consists of six exons (Fig. 3). The first exon encodes the putative signal sequence, followed by four exons which encode four domains with the last three domains being very similar to each other. The last exon encodes for the 27 extra amino acids at the carboxyl terminal of the 344-residue precursor (Shimasaki *et al.*, 1988b).

Follistatin expression is tightly regulated during development (Feijen *et al.*, 1994; Patel *et al.*, 1999). To understand further the regulation of follistatin expression, several research groups studied the follistatin gene promoter. Characterization of the rat follistatin gene promoter identified three transcription start sites located at about 30 bp downstream of three distinct TATA-like

Fig. 3. Schematic representation of follistatin (FS) gene, alternative mRNA splicing and protein processing. Human follistatin gene 6 has six exons transcribed into the precursor follistatin-317 mRNA (pre FS-317 mRNA) and the precursor follistatin-344 mRNA (pre FS-344 mRNA) via alternative mRNA splicing. Both mRNA are then translated into pre FS-317 (a precursor of 317 amino acids of follistatin) and pre FS-344 (a precursor of 344 amino acids of follistatin), which subsequently become follistatin-288 (FS-288) and follistatin-315 (FS-315) by cleavage of signal peptide. FS-288 and FS-315 both have an N domain and FS domains I, II and III with an additional C domain in FS-315.

sequences. The promoter region revealed several DNA motifs for transcription factors, including Sp1, Ap-2, Ap-1, and a c-AMP-response element (CRE)-like sequence. de Groot *et al.* (2000) demonstrated that the murine follistatin promoter region has at least three distinct transcription initiation sites, which are each preceded by a TATA box with several consensus binding sites for transcription factors including Ap-1, Brachyury-T, CRE-binding protein (CREB), Sp1, Ap-2 and Tcf (de Groot *et al.*, 2000).

Follistatin isoforms from alternative pre-mRNA splicing

Molecular analysis of the various isoforms showed that follistatin is encoded by a single gene and the variety of molecular masses (31–39 kDa) arise from alternative splicing, glycosylation and proteolytic cleavage (Robertson *et al.*, 1987; Ueno *et al.*, 1987; Shimasaki *et al.*, 1988b). Alternative splicing occurs at the 3'-terminal of the gene between exon 5 and exon 6 (Fig. 3). The splicing out of intron 5, generating a stop codon following immediately the last amino acid of exon 5, leads to the termination of the coding sequence for a precursor of 317 amino acids (pre-follistatin 317), the COOH-terminal truncated

form. On the other hand, exon 6a is spliced out together with intron 5 to generate a precursor of 344 amino acids (pre-follistatin 344) (Shimasaki *et al.*, 1988a,b). Cleavage of the signal peptide (29 amino acids) generates the mature follistatin isoforms of 288 and 315 amino acids (follistatin-288 and follistatin-315). In addition, the majority of follistatin isolated from pig ovary is follistatin-303, which appears to be derived from follistatin-315 by proteolytic cleavage of the 12 C-terminal amino acids (Sugino *et al.*, 1993).

Function of follistatin as a binding protein

The actions of follistatin are principally explicable by its ability to bind and neutralize various members of the TGF- β superfamily. The activin–follistatin binding complex is generally considered to be composed of one activin and two follistatin molecules, whereas inhibin, containing one β -subunit, has only one binding site for follistatin, implying that follistatin binds to activin and inhibin through the common β subunits (Shimonaka *et al.*, 1991; de Winter *et al.*, 1996). However, it is unclear whether follistatin can prevent inhibin binding to the activin receptor. The published estimates for the affinity

(K_d value) of follistatin for activin ranges from 50 to 900 pmol l⁻¹, similar to that of activin for its receptors (from 100 to 400 pmol l⁻¹; Phillips and de Kretser, 1998), and explains why follistatin functions as a potent modulator of the actions of activin.

In addition, follistatin antagonizes the actions of BMP-4 in *Xenopus* embryos and mouse teratocarcinoma cells, and interacts directly with BMP-4 *in vitro* (Fainsod *et al.*, 1997). Follistatin also inhibits the effects of OP-1 (also known as BMP-7), if added at a 10-fold excess (Yamashita *et al.*, 1995). During the development of chick limbs, follistatin promotes the ability of BMP-7 to induce muscle growth but inhibits the ability of BMP-7 to induce apoptosis and muscle loss by flexible and reversible binding to BMP-7 (Amthor *et al.*, 2002). Iemura *et al.* (1998) demonstrated that in the early *Xenopus* embryo follistatin can inhibit the effects of BMP-2, -4 and -7 by direct binding to a complex of BMP and its receptor. Follistatin can also inhibit myostatin, another TGF- β family member that acts as a negative regulator of skeletal muscle mass, from binding to receptors (Lee and McPherron, 2001). Recent data indicate that follistatin binds and suppresses the actions of BMP-15 (Otsuka *et al.*, 2001a). Taken together, growing evidence indicates that follistatin not only functions as an activin-binding protein, but also interacts with other TGF- β family members, presumably through a similar binding mechanism.

Importantly, the C-terminal amino acid sequence of follistatin appears to be critical for follistatin binding to heparan sulphate proteoglycans, as the C-terminal truncated form, follistatin-288, shows much higher affinity for the rat granulosa cell surface (a K_d value of 2 nmol l⁻¹) than follistatin-303, whereas follistatin-315 has no affinity (Sugino *et al.*, 1993). Considering the strong interaction of activin with follistatin, the basement membranes of cells containing heparan sulphate proteoglycans are potentially major stores of both activin and follistatin. Supporting this concept is the rapid and robust release of activin A and follistatin by heparin in the circulation of patients undergoing cardiovascular procedures (Phillips *et al.*, 2000). Moreover, the association with cell surfaces promotes endocytotic degradation of the activin–follistatin complex in rat pituitary cells (Hashimoto *et al.*, 1997).

Follistatin also binds to α_2 -macroglobulin (Phillips *et al.*, 1997), but the significance of this property is still unclear. In addition, mRNA of a newly recognized BMP binding protein, Ep45, when microinjected into *Xenopus* embryos, blocks the ability of follistatin to suppress BMP activity, whereas it has no effect on the other BMP antagonists, chordin and noggin (Iemura *et al.*, 1999).

Differences between follistatin isoforms

The only difference in structure between follistatin-288 and follistatin-315 is the additional 27 amino acids

at the carboxyl terminal end of follistatin-315. Does this distinguish the functions of the isoforms in some important way? Why is this alternative mRNA splicing event for follistatin conserved in mammals (Shimasaki *et al.*, 1988a,b; Michel *et al.*, 1990)? Unfortunately, current knowledge on the difference between the follistatin isoforms is far from complete.

Are both follistatin isoforms distributed in similar amounts and at identical locations in tissues? For determination of the relative expression of pre-follistatin 344 mRNA and pre-follistatin 317 mRNA, S1-nuclease analysis of total RNA from rat tissues, including kidney, pancreas, uterus, muscle, lung, testis, cortex, thymus, pituitary, adrenal, heart and ovary, has demonstrated that pre-follistatin 317 mRNA is expressed at less than 5% of pre-follistatin 344 mRNA (Michel *et al.*, 1990). It is unclear whether the relative ratio between the amounts of follistatin-288 and follistatin-315 is finely regulated and crucial to normal function in diverse systems. Follistatin-288 is the predominant form present in human follicular fluid, whereas the main form in serum is follistatin-315 (Schneyer *et al.*, 1996). This finding, together with the fact that the cell surface and the extracellular matrix are rich in heparan sulphate proteoglycans to which follistatin-288 binds with much higher affinity than follistatin-315 (Sugino *et al.*, 1993), indicates that follistatin-288 is primarily a membrane-bound form of follistatin, whereas follistatin-315 is a circulating form. The distinct difference between the amounts of follistatin-288 and follistatin-315 in different locations implies different biological roles of the follistatin isoforms.

Do both follistatin isoforms have similar affinity for activins or neutralizing ability for activins? Follistatin-288 is approximately 10-fold more potent than follistatin-315 in suppressing FSH secretion from the rat pituitary cells *in vitro* (Inouye *et al.*, 1991). Furthermore, all six molecular species of follistatin purified from pig ovaries have similar activin binding activity (K_d = 540–680 pmol l⁻¹). However, the COOH-terminal truncated form, follistatin-288, shows much higher affinity for the rat granulosa cell surface than follistatin-303, and follistatin-315 has no affinity. Follistatin-288 is more potent in suppressing pituitary FSH release than follistatin-303 and follistatin-315 (Sugino *et al.*, 1993), and another study using heparan sulphate purified from *Xenopus* embryos supports a higher affinity of follistatin-288 for heparan sulphate than that of follistatin-315 (Yamane *et al.*, 1998). Through its binding to ¹²⁵I-labelled activin A, follistatin-288 is able to accelerate the endocytotic internalization and lysosomal degradation of activin by pituitary cells (Hashimoto *et al.*, 1997). In contrast, follistatin-315 does not show this action. These observations together indicate that the difference between follistatin-288 and follistatin-315 in suppressing pituitary FSH secretion is due to their different capacities

to neutralize activin. However, Hashimoto *et al.* (2000), using surface plasmon resonance and affinity cross-linking, indicated an alternative explanation. First, the inhibitory effect of follistatin-288 on activin-induced transcriptional responses is more potent than that of follistatin-315 in the presence or absence of heparan sulphates. Second, follistatin-288 prevents activin from binding to its type II receptor more completely than does follistatin-315. Furthermore, there is a 10-fold difference in the K_d value of follistatin-288 and follistatin-315 for activin A. These observations led to the conclusion that the activin-neutralizing activity of follistatin isoforms is dependent on their affinity for activin.

Do both follistatin isoforms exert similar functions and effects in diverse tissues? Comprehensive studies addressing this particular question are lacking in the literature. In the rat pituitary cell *in vitro* system, follistatin-288 promotes proteolytic degradation, whereas activin–follistatin-315 complexes resist endocytotic and proteolytic degradation and are relatively more stable (Hashimoto *et al.*, 1997). Together with the different distribution and binding affinities of follistatin-288 and follistatin-315 (Michel *et al.*, 1990; Schneyer *et al.*, 1996; Hashimoto *et al.*, 2000), follistatin-315 may act as a reservoir for activin, to prevent activin from proteolysis locally and to help regulate the distribution, function and activity of activin in various tissues and bodily fluids (Delbaere *et al.*, 1999; McPherson *et al.*, 1999). Alternatively, follistatin-315 may bind and neutralize the actions of activin resulting from ‘over production’ at local sites. Whether there are mechanisms to permit the dissociation of activin from follistatin-315 to exert its biological effects remains unclear, as the binding of activins to follistatin is claimed to be almost irreversible (Schneyer *et al.*, 1996).

Follistatins in ovary

Folliculogenesis in mammals can be divided into two distinct periods: the gonadotrophin-independent (preantral) and gonadotrophin-dependent (antral or Graafian) periods. Responding to cyclic pituitary gonadotrophin secretion, the various follicular compartments interact in a highly integrated manner to secrete sex steroids and to produce a fertilizable ovum. Apart from being regulated by pituitary gonadotrophins, folliculogenesis is also modulated by various intraovarian growth factors, especially in the preantral stage. These growth factors include epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF- α , TGF- β , insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), GDF-9, BMPs, inhibins, activins and follistatins (Webb *et al.*, 1999; Elvin *et al.*, 2000). The role of the follistatins in ovarian function is related to the manner by which they function through interactions with some members of the TGF- β superfamily, that is activins, GDF-9 and BMPs.

Location and secretion of follistatin

Within the ovary, the granulosa cells in antral follicles and luteinized granulosa cells are the main sites that express follistatin mRNA and protein, whereas other structures, such as the theca cells, stroma and oocytes, appear to be devoid of follistatin mRNA (Shimasaki *et al.*, 1989; Roberts *et al.*, 1993; Braw-Tal, 1994; Tisdall *et al.*, 1994), although follistatin mRNA has been observed in human oocytes (Sidis *et al.*, 1998). However, follistatin protein was detected in the theca cell layers of human small antral follicles in the absence of follistatin mRNA expression, but then became undetectable with the development of a dominant follicle (Roberts *et al.*, 1993). Thus, the granulosa cells are the type of cell in the ovary responsible for producing and secreting follistatin in most species. It is important to note that the level of follistatin mRNA expression within the ovary depends on the developmental stages of follicles. Follistatin mRNA expression within the antral follicles increases as follicular maturation progresses and declines during the atretic process (Roberts *et al.*, 1993; Lindsell *et al.*, 1994). Moreover, the follistatin protein appears to be present in only the selected dominant follicles (Nakatani *et al.*, 1991). At the same time, primordial through to primary follicles do not express follistatin mRNA (Kogawa *et al.*, 1991; Braw-Tal, 1994; Tisdall *et al.*, 1994), implying that follistatin may not be involved in the initial stage of recruitment of follicles. Species differences occur, as the expression of follistatin is maintained in the corpus luteum in primates, whereas in other species, expression falls markedly after ovulation in the developing corpus luteum (Kogawa *et al.*, 1991; Roberts *et al.*, 1993; Wada *et al.*, 1996).

Follistatin expression in granulosa cells can be regulated through both cyclic AMP-dependent or protein kinase A (PKA) and protein kinase C (PKC) pathways (Miyanaga *et al.*, 1993; Lindsell *et al.*, 1994; Shukovski *et al.*, 1995; Tano *et al.*, 1995; Tuuri *et al.*, 1996). FSH, an activator of the PKA pathway, stimulates inhibin but not activin production, whereas GnRH, an activator of the PKC pathway, stimulates activin, and to a lesser extent, inhibin production. Both FSH and GnRH enhance follistatin protein production in an additive manner (Miyanaga *et al.*, 1993).

The extent of differentiation of granulosa cells may influence follistatin production in response to FSH, LH and activin A. FSH enhances follistatin production *in vitro* by undifferentiated and partially differentiated granulosa cells, but not by fully differentiated granulosa cells. In contrast, the only detectable effect of LH on follistatin production is on partially differentiated granulosa cells. Activin A promotes follistatin production by undifferentiated and partially differentiated granulosa cells, but suppresses follistatin production by fully differentiated granulosa cells (Shintani *et al.*, 1997). Moreover, an *in vivo* study in rats indicated that

Table 1. The antagonism between activin and follistatin in the ovary

	Granulosa cells	Numbers of FSH and LH receptors in granulosa cells	Steroidogenesis	Oocytes
Activin	↑ Differentiation ↓ Premature luteinization	↑	↑ E ₂ ↑ P ₄ in undifferentiated granulosa cells ↓ P ₄ in differentiated granulosa cells ↓ Androgen	↑ Meiotic and cytoplasmic maturation ↑ Developmental competence to form blastocysts
Follistatin	↑ Luteinization or atresia	↓	↓ E ₂ ↓ P ₄ in undifferentiated granulosa cells ↑ P ₄ in differentiated granulosa cells ↑ Androgen	↓ Meiotic and cytoplasmic maturation ↓ Developmental competence to form blastocysts

E₂: oestrogen; P₄: progesterone; ↑: increase or promote; ↓: decrease or inhibit.

the expression of follistatin in preovulatory follicles might be suppressed by the primary gonadotrophin surge during pro-oestrus (Ogawa *et al.*, 1994). Hence, the decrease of follistatin production in preovulatory follicles may occur before ovulation, possibly due to the primary gonadotrophin surge. Nevertheless, whether this phenomenon is crucial to the ovulation process needs further research. Other stimulators of ovarian follistatin *in vitro* include EGF and prostaglandin oestrogen (Lindsell *et al.*, 1993; Tano *et al.*, 1995; Tuuri and Ritovs, 1995; Shintani *et al.*, 1997). In addition, the stimulation of follistatin production by FSH can be suppressed by EGF but enhanced by activin (Michel *et al.*, 1992). Follistatin also antagonizes the action of activin on follistatin mRNA production (Tano *et al.*, 1995), indicating that a local regulatory loop may be present in the granulosa cells.

Effects of over-expression of follistatin on ovarian function

Follistatin-deficient mutants died soon after birth, making it difficult to study ovarian function in that model (Matzuk *et al.*, 1995). However, over-expression of mouse follistatin results in reproductive defects in transgenic mice (Guo *et al.*, 1998). In this study, a mouse metallothionein-I promoter was fused to a 5.1 kb genomic fragment, resulting in over-expression of mouse follistatin transgene in many tissues of several lines. In line 4, a highly expressing line, FSH concentrations were significantly suppressed and histological analysis showed small ovaries with a block in folliculogenesis. It seems likely that the decreased FSH results from the blocking of FSH stimulation by activin. It is unclear whether this phenotype represents purely the effects of FSH deficiency or whether there is a direct effect of the increased follistatin expression. Nevertheless, in lines 9 and 5, in which follistatin was moderately over-expressed and in which the FSH concentration was not suppressed, there was still some disruption in folliculogenesis. The phenotypes ranged from subfertility, a block at the stage of the early antral follicle, to a block at the stage of the

early primary follicle. Hence, the results also underline the importance of follistatin in ovarian function.

Interaction with activins

The distribution of follistatin and the inhibin-activin subunits in the ovary have provided insights into their actions (Table 1). Neither β A nor β B mRNA is detectable in oocytes, but the α subunit mRNA is weakly detectable in some human oocytes (Sidis *et al.*, 1998). The α subunit is present in granulosa cells and theca cells of antral follicles, and in luteinized granulosa cells, but not in luteinized theca cells (Roberts *et al.*, 1993; Mather *et al.*, 1997; Sidis *et al.*, 1998). The β A subunit mRNA and protein is observed in granulosa cells of all stages and theca cells of developing dominant follicles, whereas the β B subunit is found only in granulosa cells of small antral follicles. In addition, the transcripts for all four activin receptor subtypes (ActRIA, ActRIB, ActRIIA and ActRIIB) are detectable in oocytes, granulosa cells and theca cells. Thus, generally inhibins and activins appear to be produced by granulosa cells and exert both autocrine and paracrine actions. As granulosa cells are also the main source of follistatin in the ovary, these cells have the capacity to modulate the local actions of the activins and inhibins. The sites of localization indicate that follistatin and inhibin may play a role at the later stages of follicular development, whereas the activins exert their effects primarily at the early stages of follicular development (Roberts *et al.*, 1993; Lindsell *et al.*, 1994; Mather *et al.*, 1997; Sidis *et al.*, 1998) (Fig. 4). These studies also led to the hypothesis that the orderly transition from an activin-dominant to an inhibin-follistatin-dominant microenvironment is critical for dominant follicle development (Knight and Glister, 2001).

It is a generally accepted concept that activin, either alone or with FSH, exerts an autocrine effect on granulosa cells, promoting differentiation during the preantral and early antral stages of folliculogenesis and inhibiting premature luteinization at the later stages of antral follicle

Fig. 4. The effects of follistatin and transforming growth factor β (TGF- β) superfamily members on folliculogenesis. Activin facilitates the proliferation of granulosa cells and follicular growth from the stage of antral follicles, whereas it suppresses the formation of the corpus luteum. Follistatin plays its main role at the later stage of folliculogenesis and promotes the process of luteinization. Bone morphogenetic protein 7 (BMP-7) has similar effects to those of activin in folliculogenesis. BMP-15 and growth differentiation factor 9 (GDF-9) are both essential for the further growth of preantral follicles. In addition, BMP-15 is associated with ovulation.

development. Follistatin, on the other hand, modulates the function of granulosa cells in favour of luteinization or atresia by neutralizing the actions of activin and may also directly modulate progesterone metabolism by granulosa cells (Findlay, 1993; Li *et al.*, 1995; Miro and Hillier, 1996). However, the stimulatory action of activin on the proliferation of granulosa cells may be stage-specific (Yokota *et al.*, 1997; Liu *et al.*, 1998; Mizunuma *et al.*, 1999). Activin A has no effect on preantral follicles from adult mice and blocks FSH-stimulated follicular growth, although activin A enhances preantral follicular growth in immature mice (Yokota *et al.*, 1997). In addition, these authors showed that *in vitro* activin, secreted by secondary follicles, causes primary follicles to become dormant at the resting stage, an action reversed by follistatin (Mizunuma *et al.*, 1999). Together, these findings indicate that activin and follistatin may regulate the initiation of folliculogenesis and determine the size of the cohort of growing follicles.

Activin stimulates the expansion of FSH and LH receptors on granulosa cells and follistatin antagonizes

these actions but has no direct effect (Xiao *et al.*, 1992; Tsuchiya *et al.*, 1999). Activin, at a higher dose (100 ng ml^{-1}), prevents FSH-induced downregulation of numbers of FSH receptors; however, at lower concentrations ($3\text{--}30 \text{ ng ml}^{-1}$) it enhances downregulation of numbers of FSH receptors by 20%. Surprisingly, follistatin alone prevents FSH-induced downregulation by increasing numbers of FSH receptors up to 40–50% (Xiao *et al.*, 1992), perhaps blocking the action of endogenous activin or other TGF- β family members. In rat granulosa cells, activin in the presence of FSH also induces LH receptor expansion significantly, and follistatin inhibits this effect in a dose-dependent manner (Tsuchiya *et al.*, 1999). In summary, activin plays a pivotal role in the acquisition of responsiveness to FSH by granulosa cells of preantral follicles, an essential step for their further development that can be facilitated by the low expression of follistatin in small follicles.

The actions of activin and follistatin on steroidogenesis in granulosa cells vary according to the extent of maturity of granulosa cells (Fig. 5). *In vitro* studies involving

Fig. 5. Schematic presentation of the effects of some members of the transforming growth factor β (TGF- β) superfamily and follistatin on steroidogenesis in granulosa cells. Follistatin antagonizes the actions of activin and bone morphogenetic protein 15 (BMP-15) on steroidogenesis in granulosa cells. E_2 : oestrogen; P_4 : progesterone; $\uparrow$: enhance or promote; $\downarrow$: suppress or inhibit; $\leftrightarrow$: no effect; $?$: no available mammalian data.

undifferentiated or partially differentiated granulosa cells have shown that activin enhances gonadotrophin-stimulated P450 aromatase activity and oestradiol production, regardless of the developmental stage. However, activin promotes gonadotrophin-stimulated progesterone production in undifferentiated granulosa cells, whereas it suppresses this effect in partially differentiated granulosa cells (Miro *et al.*, 1991; Shukovski *et al.*, 1991; Hillier and Miro, 1993). Furthermore, in fully differentiated granulosa cells, activin suppresses gonadotrophin-stimulated P450scc expression and progesterone production (Miro *et al.*, 1991; Cataldo *et al.*, 1994), effects that can be antagonized by follistatin (Xiao *et al.*, 1992; Cataldo *et al.*, 1994). These findings indicate that activin stimulates the early differentiation of granulosa cells and retards the late differentiation and luteinization, whereas follistatin antagonizes these actions potentiating luteinization in mature granulosa cells.

The main role of theca cells is to synthesize androgen substrates for oestradiol production by granulosa cells. In studies on theca cells in humans, rats and cows, activin attenuates LH-induced androgen production and suppresses the increase of androgen production stimulated by oestradiol, whereas follistatin reverses these effects (Hsueh *et al.*, 1987; Hillier, 1991; Wrathall and Knight, 1995). In contrast, activin induces and follistatin inhibits the proliferation of theca cells (Duleba *et al.*, 2001).

In vitro activin promotes and follistatin inhibits meiotic and cytoplasmic maturation of the oocytes (Alak *et al.*, 1996, 1998). Furthermore, activin potentiates the development of blastocysts from denuded or cumulus-enclosed bovine oocytes, whereas follistatin neutralizes these actions (Silva and Knight, 1998). Together, these findings indicate that activin and follistatin contribute to the regulation of oocyte maturation and competence.

Interaction of follistatin with other members of the TGF- β superfamily in the ovary

Compelling evidence indicates that follistatin can influence ovarian function by binding to other members of the TGF- β superfamily, especially BMPs (Yamashita *et al.*, 1995; Iemura *et al.*, 1998; Otsuka *et al.*, 2001a). Given the functions of GDF-9, BMP-15 (also known as GDF-9B), BMP-6, BMP-4 and BMP-7 in the ovary, follistatin potentially influences a number of the actions of these proteins (Table 2, Figs 4 and 5).

GDF-9, BMP-15 and BMP-6 show between 28 and 53% homology and are expressed in oocytes within the ovary (Lyons *et al.*, 1989; McGrath *et al.*, 1995; Dube *et al.*, 1998). Given the many actions of GDF-9 and BMP-15 on ovarian somatic cells, their role in follicular development is significant and confirms the bi-directional communication between oocytes and granulosa cells. In the ovaries of mice, cows and humans,

Table 2. Actions of other transforming growth factor β (TGF- β) superfamily members to which follistatin potentially binds in the ovary

	Folliculogenesis	Granulosa cell	Theca cell	Steroidogenesis	Interaction with follistatin
GDF-9	Deficiency in mouse: homozygous mutants $\rightarrow$ blocked at the stage of primary follicles; abnormal ultrastructural features of oocytes Induce <i>in vitro</i> cumulus expansion	$\uparrow$ Proliferation (FSH-independent) $\downarrow$ FSH-induced differentiation	Deficiency in mouse: unable to recruit the theca cell precursors	$\uparrow$ FSH-independent P ₄ and E ₂ production $\downarrow$ FSH-induced P ₄ and E ₂ production $\downarrow$ FSH-induced LH receptor production	?
BMP-15	Deficiency in sheep: homozygous mutants $\rightarrow$ blocked at the stage of primary follicles; heterozygous mutants $\rightarrow$ increased ovulation Deficiency in mouse: homozygous mutants $\rightarrow$ subfertile, decreased ovulation	$\uparrow$ Proliferation (FSH-independent)	?	$\downarrow$ FSH receptor production $\downarrow$ FSH-induced FSH and LH receptor production $\downarrow$ FSH-induced P ₄ production $\leftrightarrow$ FSH-induced E ₂ production	$\downarrow$ Effects of BMP-15 on the proliferation, FSH receptor and steroidogenesis
BMP-6	?	$\leftrightarrow$ Proliferation	?	$\downarrow$ FSH-induced P ₄ production $\leftrightarrow$ FSH-induced E ₂ production	?
BMP-4	?	?	?	$\uparrow$ FSH-induced E ₂ production	?
BMP-7	$\uparrow$ Follicular growth $\downarrow$ Ovulation and luteinization	$\uparrow$ Proliferation	?	$\downarrow$ FSH-induced P ₄ production $\uparrow$ FSH-induced E ₂ production	?

BMP: bone morphogenetic protein; E₂: oestrogen; GDF: growth differentiation factor; P₄: progesterone; $\uparrow$: enhance or promote; $\downarrow$: suppress or inhibit; $\leftrightarrow$: no effect; ?: no available mammalian data.

GDF-9 and BMP-15 mRNA or protein are found in the oocytes of small primary follicles, but not in the primordial follicles (Elvin *et al.*, 2000). However, in cows and pigs, expression of GDF-9 is low in oocytes of primordial follicles (Bodensteiner *et al.*, 1999), and in humans, GDF-9 precedes the expression of BMP-15 (Aaltonen *et al.*, 1999). In all subsequent steps of folliculogenesis, both BMP-15 and GDF-9 are present (Elvin *et al.*, 2000). However, the interactions between follistatin and GDF-9 and BMP-15 would not occur in small follicles as follistatin is only expressed in granulosa cells of larger follicles.

Folliculogenesis in GDF-9-deficient mice does not proceed beyond the type 3b (primary) follicle (Dong *et al.*, 1996), despite increased serum FSH and LH concentrations. The granulosa cells cease proliferation and undergo apoptosis and are unable to recruit theca cell precursors to form a theca layer surrounding the follicle (Elvin *et al.*, 1999a). The GDF-9-deficient oocytes show perinuclear organelle aggregation, unusual peripheral Golgi complexes, fail to form cortical granules and show altered interconnections between granulosa cells and oocytes, together indicating abnormal cytoplasmic maturation of oocytes and granulosa cells. Northern blot analysis shows increased kit ligand expression and decreased inhibin-activin β B and follistatin expression in these GDF-9-deficient ovaries (Elvin *et al.*, 1999a), through mechanisms yet to be defined. Recombinant mouse GDF-9 (Elvin *et al.*, 1999b) induces hyaluronan synthase 2 (HAS2), cyclooxygenase 2 (COX-2), and steroidogenic acute regulator protein (StAR) mRNA synthesis but suppresses urokinase plasminogen activator (uPA) and LHR mRNA synthesis in cultured mouse granulosa cells. Consistent with the induction of StAR mRNA, recombinant GDF-9 enhances granulosa cell progesterone synthesis in the absence of FSH and, through its action on HAS2 and uPA, induces cumulus expansion of oocyctomized cumulus cell-oocyte complexes and the production of the hyaluronic acid-rich extracellular matrix during cumulus expansion. Treatment with recombinant GDF-9, but not FSH, can stimulate proliferation of cultured rat granulosa cells from both early antral and preovulatory follicles, probably by decreasing cAMP production (Vitt *et al.*, 2000), stimulating basal steroidogenesis in granulosa cells but attenuating FSH-stimulated progesterone and oestradiol production and LH receptor formation (Vitt *et al.*, 2000).

BMP-15 is a potent stimulator of FSH-independent granulosa cell proliferation (Otsuka *et al.*, 2000). Although it has no effect on steroidogenesis, BMP-15 alone suppresses FSH-induced progesterone production, but has no effect on oestradiol production (Otsuka *et al.*, 2000). Importantly, BMP-15 markedly suppresses both the basal and FSH-induced increases in the abundance of FSH receptor mRNA, indicating that this action may be the primary effect of BMP-15 in suppressing FSH action (Otsuka *et al.*, 2001b). The importance of BMP-

15 in folliculogenesis is supported by the block at the stage of primary follicles in BMP-15 homozygous sheep, features similar to the phenotype of GDF-9 deficiency (Galloway *et al.*, 2000). In contrast, BMP-15 heterozygous mutants of sheep have increased ovulation and multiple pregnancies. However, inactivation of the BMP-15 gene in mice (BMP-15^{-/-}) did not cause the same phenotype as that in sheep (Yan *et al.*, 2001), as the ovaries were grossly indistinguishable from either heterozygous (BMP-15^{+/-}) or wild-type ovaries. Nonetheless, BMP-15^{-/-} females were subfertile and had decreased ovulation and fertilization rates. These findings indicate that the dosage of intact BMP-15 and GDF-9 alleles influences the destiny of oocytes and subsequent fertility (Yan *et al.*, 2001).

Unlike BMP-15 and GDF-9, BMP-6, another oocyte-derived growth factor, has no effect on the mitogenic activity of rat granulosa cells (Otsuka *et al.*, 2001c), although it suppresses FSH-induced progesterone production but not oestradiol production. BMP-6 also exerts a similar action to BMP-15 by suppressing FSH-regulated progesterone and oestradiol production. This action probably occurs through the downregulation of adenylate cyclase activity, in contrast to BMP-15, which suppresses the basal FSH receptor expression (Otsuka *et al.*, 2001c).

Although there are no reports describing the interaction between follistatin and GDF-9 or BMP-6, follistatin binds BMP-15 in surface plasmon resonance studies (Otsuka *et al.*, 2001a) and inhibits BMP-15-induced thymidine uptake by rat granulosa cells *in vitro*, but is ineffective directly. Follistatin also reverses the suppressive effect of BMP-15 on FSH receptor expression and FSH-induced progesterone production. These data clearly broaden the roles of follistatin in the ovary and given the high structural identity between GDF-9 and BMP-15 make it very likely that follistatin also binds to GDF-9 and modulates its function.

Yamashita *et al.* (1995) reported the binding and neutralization of BMP-2, BMP-4, BMP-7 and 4/7 heterodimers by follistatin in *Xenopus* embryos, but no data are available for mammalian species. In contrast to GDF-9, BMP-15 and BMP-6, which are expressed by oocytes, the transcripts of BMP-4 and BMP-7 have been detected prominently in theca cells (Shimasaki *et al.*, 1999). *In situ* hybridization histochemistry identified strong mRNA labelling for BMP-4 and -7 in the theca cells and BMP receptor types IA, IB, and II in the granulosa cells and oocytes of most follicles in ovaries of normal cyclic rats, indicating potential paracrine mechanisms of BMP-4 and -7 within the ovary. Further *in vitro* studies showed that BMP-4 and -7 differentially modulated FSH actions in rat granulosa cells, enhancing FSH-induced oestradiol and suppressing progesterone production (Shimasaki *et al.*, 1999). In general, BMP-7 appears to promote follicular growth and development, while simultaneously suppressing ovulation and luteinization (Lee *et al.*, 2001).

Further studies are required to clarify the interactions between follistatin and BMP-4 and -7 and to determine their physiological relevance within the mammalian ovary.

Interactions among members of the TGF- β superfamily

Although follistatin interacts with several members of the TGF- β superfamily, it is also important to note that interactions may occur between these growth factors in the control of ovarian function. These actions could occur at three levels. First, they may occur at the receptor level. As there is a high extent of identity of TGF- β superfamily members, it is likely that different members bind to the same receptor, resulting in competitive effects. For example, activin and BMP-7 bind to the same type II receptors, ActRII and ActRIIB (Macias-Silva *et al.*, 1998).

Second, these interactions may occur from crosstalk between the signalling pathways of TGF- β superfamily members. For example, signalling by activin and BMP-2/4 has been shown to interact antagonistically. In *Xenopus* ectodermic explants, activin induces dorsal-type mesoderm, whereas BMP-2/4 induces ventral mesoderm and blocks the dorsal mesoderm-inducing activity of activin. Investigation of the mechanisms underlying this phenomenon has revealed that this antagonism between activin and BMP-2/4 may be explained by intracellular competition for a limited pool of SMAD4, under certain physiological situations (Candia *et al.*, 1997). It has been proposed that SMAD4 is limiting in cells, hence the simultaneous activation of two signalling pathways could cause competitive effects. The outcome of this competition may dictate the ultimate signals. Another example is BAMBI (BMP and activin membrane-bound inhibitor; Onichtchouk *et al.*, 1999), which is induced by BMP signalling and inhibits not only BMP signalling, but also activin and TGF- β signalling.

Finally, these interplays may occur indirectly via the interactions of factors, the amounts of which are regulated by TGF- β superfamily members. In other words, some TGF- β superfamily members may not only interplay with each other directly, but also regulate the same factors, for example sex steroids (Miro *et al.*, 1991; Shukovski *et al.*, 1993; Alak *et al.*, 1998; Shimasaki *et al.*, 1999; Dooley *et al.*, 2000; Otsuka *et al.*, 2001c). Thus, the physiological activities of these TGF- β superfamily members arise from the net stimulatory or inhibitory inputs that impinge upon downstream targets, such as sex steroids. On the basis of the concepts mentioned here, follistatin functions in a multidimensional network, not just a simple linear system.

Conclusion

As follistatin can bind to activin and neutralize its activity (Nakamura *et al.*, 1990), and given the widespread actions of activin in regulating cellular homeostasis

(divide or die), differentiation (at fetal and adult stages) and hormonal homeostasis (the balance among hormones), follistatin plays a significant role in diverse areas of biology. In the area of reproduction, a wealth of evidence indicates that follistatin plays a significant role in the pituitary, ovary and testis in an autocrine or paracrine fashion, and that most of follistatin biology may be explained by its antagonism with activin. However, there is evidence to indicate that follistatin binds to other members of the TGF- β superfamily, modulating their functions in a manner analogous to activin. Hence, follistatin has been shown to be able to bind to BMPs and antagonize their effects in *Xenopus* embryos (Yamashita *et al.*, 1995; Fainsod *et al.*, 1997; Iemura *et al.*, 1998). Follistatin has also been shown to have the capacity to inhibit the action of myostatin, which is another TGF- β superfamily member and acts as a negative regulator of skeletal muscle mass (Lee and McPherron, 2001). In addition, follistatin is able to bind and suppress the activities of BMP-15 in the ovary (Otsuka *et al.*, 2001a). These findings open a new window of opportunity to assess the role of follistatin in ovarian functions. What is less clear is whether the role of follistatin in regulating the biology of the TGF- β superfamily ligands is simply dictated by the local concentration of follistatin. This appears unlikely given the complexity of the systems we have described. However, to date few regulators of follistatin function have been reported (Iemura *et al.*, 1999).

Amthor *et al.* (2002) showed that in the development of chick limbs, follistatin not only binds to BMP-7 in muscle, but also appears to be able to monitor and refine the concentration of BMP-7 in a way that promotes muscle growth. This finding also implicates some potential roles of follistatin in maintaining homeostasis in the body. Moreover, members of the TGF- β superfamily may act antagonistically or synergistically with each other. For example, BMP-3 is a negative regulator of bone density, opposing osteogenic activity displayed by other BMPs (Daluiski *et al.*, 2001); antagonistic actions of activin and BMP-2/4 exist during early embryogenesis of *Xenopus* (Kaufmann *et al.*, 1996; Candia *et al.*, 1997); and GDF-9 and BMP-15 exert their activities synergistically in the ovary (Yan *et al.*, 2001). Collectively, the findings indicating that follistatin could bind to other TGF- β superfamily ligands besides activin and the interplay that exists among these ligands, led to the idea that the role of follistatin in biology may be much more complex than initially predicted.

Activin and BMP-2,-4 are considered to function as morphogens during *Xenopus* development (Gurdon and Bourillot, 2001). Morphogens are defined as secreted signalling molecules that form a concentration gradient from a localized source and induce different cell fates of surrounding cells according to the local concentration of a morphogen perceived by the cells. Thus, morphogens can organize a field of surrounding

cells into patterns. However, these gradient-dependent activities of morphogens may be produced in another way (Dale and Wardle, 1999). The morphogens may be secreted locally in the entire field and a gradient of morphogen activity can be formed by the modulation of its activity by an inhibitor of the morphogen due to the long-range diffusion of the inhibitor from a localized source. In such a manner, follistatin may play a role as an inhibitor of morphogens that are members of the TGF- β superfamily. However, it is unclear whether this graded molecular information exists or plays a role in folliculogenesis. Some *in vitro* or *in vivo* experiments may be designed to answer this question.

Given that the vast majority of the results were generated from *in vitro* studies that may not mimic a physiological situation, to explore such a complex system in follistatin biology, it is necessary to establish appropriate *in vivo* models for research. For example, *in vivo* models that can separate the actions of the two isoforms of follistatin and also *in vivo* models that can manipulate the ratio of two follistatin isoforms would further enhance our understanding of follistatin biology. Moreover, *in vivo* models that can manipulate the levels of follistatin and other members of the TGF- β superfamily may be able to depict the networks in which follistatin functions.

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