1 General Introduction

1.1 History of Follistatin:

Follistatin, a monomeric protein identified in 1987 (Robertson *et al.*, 1987; Ueno *et al.*, 1987), appears to have many roles in physiology with the possibility of some yet to be identified. Follistatin was first isolated from bovine ovarian follicular fluid on the basis of its first identified role in the suppression of FSH. Since it's discovery it has been linked with embryonic development, reproduction, the inflammatory system and wound repair. Its effects are due to its ability to modulate the action of activin and perhaps bone morphogenetic proteins, and it is also implicated in the actions of inhibins (Nakamura *et al.*, 1990; Dalkin *et al.*, 1998; Iemura *et al.*, 1998; Maguer-Satta *et al.*, 2003).

With its original discovery as a part of the reproductive axis it has become a focus of research and substantial effort investigating ovulation (Findlay, 1994), polycystic ovary syndrome (Dewailly, 1999), and pregnancy (Mercado *et al.*, 1993; Petraglia *et al.*, 1994b; D'Antona *et al.*, 2000). This project aims to further develop our knowledge and understanding of follistatin in pregnant women, particularly in labour and following delivery.

1.2 Structure of Follistatin:

The overall structure of follistatin (FS) is extremely well conserved retaining an 85% homology between all species and 95% within the mammalian pool (Shimasaki *et al.*, 1988a). The gene for follistatin has been localized to human chromosome 5, and is a small gene of only 6 kb. This gene contains 6 exons with an alternative splicing site allowing for production of 2 major species of follistatin (Shimasaki *et al.*, 1988a; Shimasaki *et al.*, 1988b) which encode a 344 amino acid and a 317 amino acid peptide.

The removal of the signal peptide sequence results in a mature polypeptide of length 315 (FS315) and 288 (FS288) amino acids. The FS288 is formed via the transcription of 5 exons from the gene whilst the FS315 is formed from transcription of all 6 exons (Shimasaki *et al.*, 1988a; Shimasaki *et al.*, 1988b; Wang *et al.*, 2000). There are a number of other follistatin isoforms formed from a variety of glycosylation and proteolytic cleavage events that can occur following translation. FS315 can be proteolytically cleaved at the C-terminal end to form the FS303 peptide (Esch *et al.*, 1987; Inouye *et al.*, 1991). mRNA for the two major follistatins shows that the relative abundance of mRNA for the FS288 is less than 5% of all follistatin, therefore the FS315 is thought to be the predominant form of follistatin (Esch *et al.*, 1987; Inouye *et al.*, 1991). Interestingly, the FS288 isoforms are 6-10 times more potent than the FS315 in their ability to suppress FSH (Inouye *et al.*, 1991). FS303 is found to have potency somewhere between that of FS288 and FS315, however most studies have been focused on the predominant FS288 and FS315 isoforms. Despite some differences, all follistatin isoforms have activin-binding characteristics (Wang *et al.*, 2000). Structurally, the follistatin molecule contains a signal sequence of 29 amino acid residues as well as five other distinct domains (Wang *et al.*, 2000) as shown in Figure 1-1.

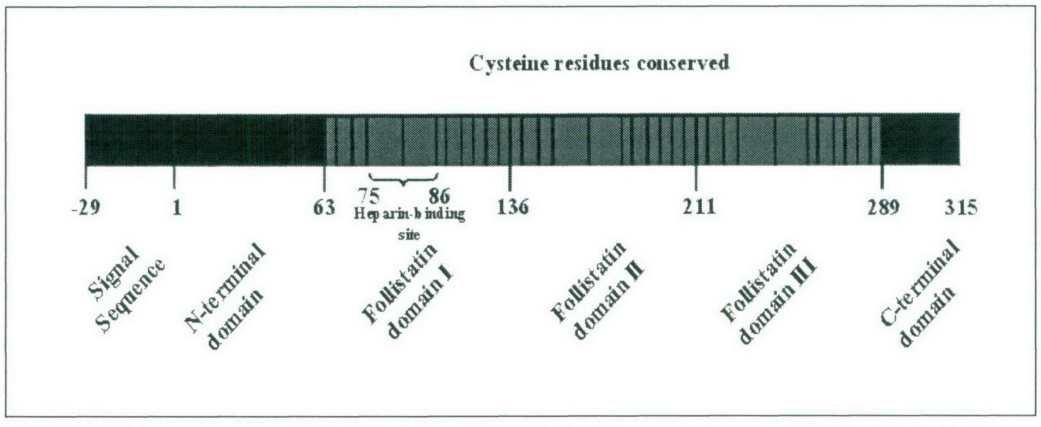

Figure 1-1 Structure of follistatin domains

The N-terminal domain is 63 amino acids long and is associated with the bioactivity of the follistatin molecule with insertion of mutations within this domain resulting in the loss of *in vitro* bioactivity (Wang *et al.*, 2000). The following three domains are between 73-75 amino acids long and are known as the follistatin module (Hohenester *et al.*, 1997; Wang *et al.*, 2000). The first of these domains contains a heparan sulfate-binding site that appears to enable the follistatin molecule to bind to cell surface proteoglycans (Wang *et al.*, 2000) and the significance of this will be discussed in Section 1.6. Each of the three follistatin domains contains 10 cysteine residues leaving follistatin rich in cysteine residues overall (Hohenester *et al.*, 1997; Wang *et al.*, 2000) and shares approximately 50% sequence homology (Shimasaki *et al.*, 1988a). The follistatin domains can be divided into two further sub-domains; sub-domain 1 is an elongated hairpin-like N-terminal region with the cysteine residues alignments resembling that of epidermal growth factor, whilst sub-domain 2 is a globular C-terminal containing a hydrophobic core resembling that of the Kazal family of protease inhibitors (Keutmann *et al.*, 2004).

The fifth domain (C-terminal domain) in follistatin is 27 amino acids long and is only found in the transcription product of all 6 exons. Therefore, it is only present in FS315 and other isoforms derived from this. This domain is highly acidic in its nature and isoforms containing the C terminal domain have a reduced affinity for heparan sulfate and therefore proteoglycans (Wang *et al.*, 2000).

1.3 Follistatin family of proteins:

Since follistatin's structure has been well characterised, other similar molecules have been added to the follistatin family of proteins. Membership to the family is not based on biological actions, but on the presence of the cysteine rich highly conserved follistatin

module (Mendis *et al.*, 1995; Eib & Martens, 1996). Although follistatin contains three of these modules, to obtain membership to the follistatin family only one module is needed. A schematic representation of the follistatin family of proteins to date is shown in Figure 1.2.

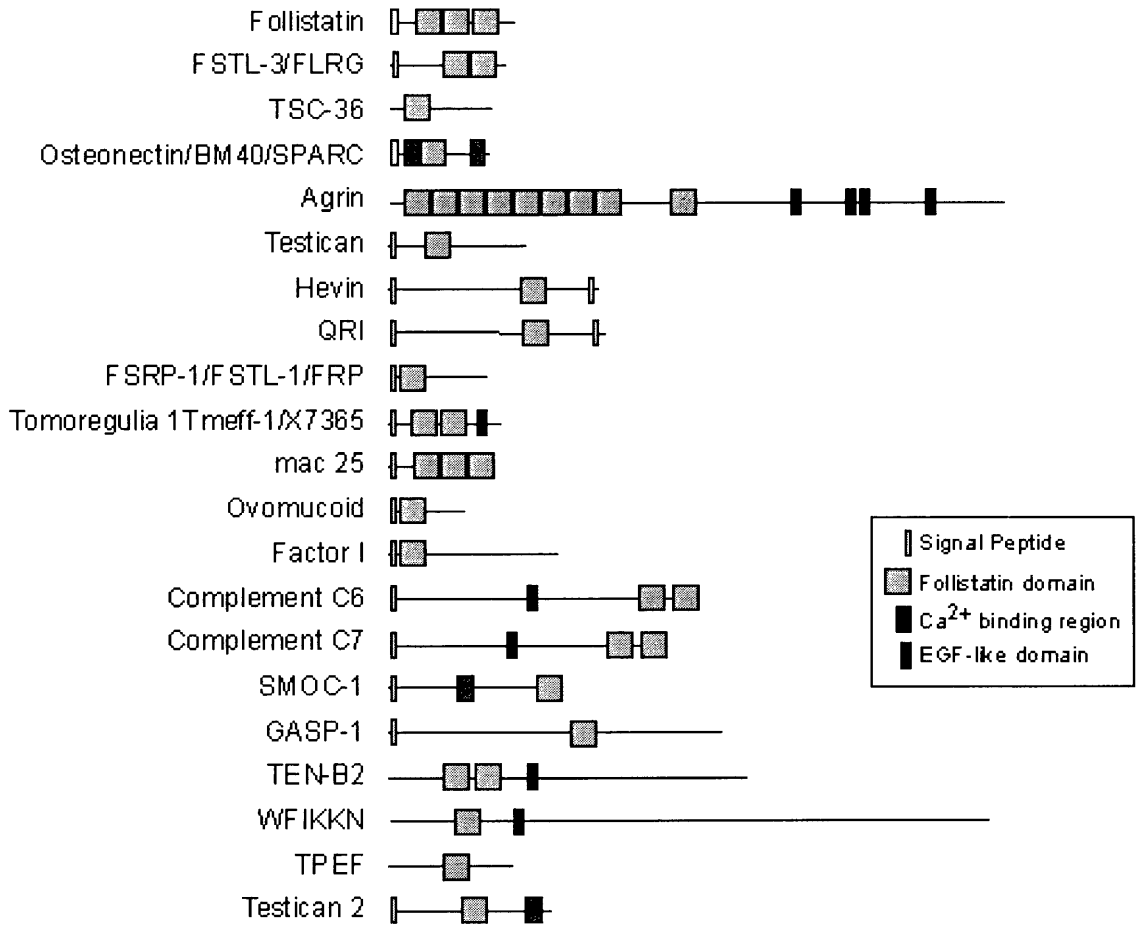

Figure 1-2 Representation of currently known follistatin family members. Modified from (Phillips & de Kretser, 1998; Horie *et al.* , 2000; Liang *et al.* , 2000; Tsuchida *et al.* , 2000; Glynne-Jones *et al.* , 2001; Trexler *et al.* , 2001; Vannahme *et al.* , 2002; Hill *et al.* , 2003; Vannahme *et al.* , 2003)

As research continues the number of members to the follistatin family continues to grow currently totalling 21 members. With the highly conserved nature of the follistatin domain, it is very likely immunoassays reported by various groups potentially have

additional cross-reactivities with other family members from the follistatin family. Despite each member of the follistatin family containing the follistatin module, until recently only follistatin was thought to be capable of binding members of the TGF-β superfamily (Tsuchida *et al.*, 2000). More recently a new gene has been identified that encodes an N-glycosylated protein with highly homologous sequences, particularly in the cysteine rich domains, to follistatin (Tsuchida *et al.*, 2000). This has been called the follistatin-related gene (FLRG) by the identifying group, (Tsuchida *et al.*, 2000) as well as follistatin-related protein (FSRP) by other groups, to emphasize the similarities in structure, sequence, and possibly function (Tortoriello *et al.*, 2001) and more recently follistatin like-3 (FSTL-3) to emphasize its similarities with follistatin (Schneyer *et al.*, 2003). FLRG/FSRP/FSTL-3 will be referred to in the remainder of this discussion as FSTL-3.

FSTL-3 contains two follistatin domains with the first FS domain being most similar in structure to the first FS domain of follistatin. The second domain of FSTL-3 is most similar to the structure of the second FS domain of follistatin (Tsuchida *et al.*, 2000). As well as missing the third domain found in follistatin, FSTL-3 does not contain the heparan sulfate-binding site found in the first FS domain (Tsuchida *et al.*, 2000). It shows similar characteristics to follistatin in binding both activin A and B with high affinity, although the affinity of FSTL-3 association with activin (estimated Kd -850pM) is not as high as the FS288 isoform (estimated Kd – 47pM) but similar to that of the FS315 isoform (estimated Kd – 430 pM) (Hashimoto *et al.*, 2000; Tsuchida *et al.*, 2000).

The group identifying FSTL-3 found it to be expressed widely in mouse heart, lung, kidney and testis (Tsuchida *et al.*, 2000). Both FS and FSTL-3 show widespread distribution patterns throughout the body, however the distribution patterns and intensities are not the same and indicate different roles for each protein. Whilst follistatin

and follistatin-related protein both inhibit the actions of activin mounting evidence suggests that they differentially regulate activins and will be discussed in detail in Sections 1.6.1 and 1.6.2 (Sidis *et al.*, 2002; Sidis *et al.*, 2006).

Confirming the differences in distribution patterns and thus the increasing evidence for alternative regulation of growth factors by FS and FSTL-3 is work by Wankell and others (2001). Recently they have been working with a focus on skin repair in wounds (Wankell *et al.*, 2001a). This group showed the FS and FSTL-3 were expressed in differing concentrations and in different cell types of the skin. In wound repair, FS and FSTL-3 expression is controlled by a variety of growth factors such as EGF (epidermal growth factor), and KGF (keratinocyte growth factor). Upon stimulation differing patterns of expression of FS and FSTL-3 followed, indicating differential regulatory roles in keratinocytes and fibroblasts in wound repair (Wankell *et al.*, 2001a). Follistatin was localized to the epidermis and hair follicles whilst FSTL-3 was found in the basement membrane, around hair follicles, within sebaceous glands and around the blood vessels of the dermis (Wankell *et al.*, 2001a). During wound healing the induction of FSTL-3 by KGF, EGF and TGFβ1 was more rapid although weaker than that seen for follistatin (Wankell *et al.*, 2001a).

1.4 Distribution of Follistatin:

Follistatin mRNA and protein are found widely across the body, however, when looking at the distribution of follistatin, one must consider that the localization of FS with antisera does not necessarily correlate with follistatin production by that tissue. FS mRNA detection also does not necessarily correlate to protein translation by that cell type. Table 1.1 shows the known distribution of follistatin mRNA and protein in human tissues.

The focus of this project is the female reproductive tract and within this area follistatin is widespread. It has been localized within the ovary, granulosa and luteal cells are positive for follistatin mRNA as well as for the secreted protein (Nakatani *et al.*, 1991; Roberts *et al.*, 1993; Wada *et al.*, 1996). These cells as well as the theca cells and the oocyte also show immunostaining for follistatin (Nakatani *et al.*, 1991; Roberts *et al.*, 1993). The early corpus luteum contains both the mRNA and the protein (Nakatani *et al.*, 1991; Roberts *et al.*, 1993).

Table 1.1 Distribution of Follistatin mRNA and protein (Phillips & de Kretser, 1998)

Tissue	mRNA	Follistatin protein	Immunohistochemistry location
Reproductive tissues			
Ovary	Granulosa cells, early corpus luteum	Granulosa cells, early corpus luteum	Granulosa/luteal, theca cells/oocyte
Uterus	Detected	Myometrium	Myometrium
Pregnancy membranes	Decidua, placenta	Placenta, decidua, amnion chorion	
Testis	Germ cells, Sertoli cells	Germ cells, Sertoli cells Leydig cells	Leydig cells/spermatocytes spermatids
Endocrine glands			
Pituitary	Folliculostellate cells, somatotropes, gonadotropes	Somatotropes	Anterior lobe
Adrenal	Detected	Cortex	Cortex/zona glomerulosa, zona reticularis
Thyroid	Not known	Follicular cells	
Neural tissues			
Forebrain	Neocortex, olfactory region thalamus, hypothalamus	Neurons	
Cerebellum	Detected	Not known	
Spinal cord	Detected	Not known	
Glandular tissues			
Kidney	Collecting tubes	Proximal & distal tubules	Epithelium of proximal & distal tubes
Pancreas	Epithelium	Islets (B cells)	
Liver	Detected	Hepatocytes	Cords of hepatic cells
Stomach	Smooth muscle, epithelium	Not known	
Prostate	Basal epithelial cells, fibroblastic stroma	Epithelial cells, stroma	
Salivary gland	Detected	Not known	
Hematopoeitic tissues			
Bone	Osteoblasts, osteocytes	Chondrocytes, osteoblasts	
Spleen	Not detected	White pulp	White bulbs
Thymus	Detected	Not known	
Other organs			
Heart	Detected	Not known	
Blood vessels	Smooth muscle cells, endothelial cells	Endothelial cells, smooth muscle cells	
Lung	Epithelium	Not known	
Muscle	Detected	Detected	
Skin	Epithelium	Not known	
Eye	Detected	Not known	

The uterus has had mRNA detected in its tissues although the cellular location is still to be identified, though immunostaining reveals the follistatin protein is present in the myometrium (Kogawa *et al.*, 1991). In pregnancy, follistatin protein and mRNA is found

in the embryo, and mRNA within decidua, placenta and fetal membranes (Petraglia *et al.*, 1994b; Roberts & Barth, 1994; Schneider-Kolsky *et al.*, 2000; Jones *et al.*, 2002c; Jones *et al.*, 2006). The placenta, decidua, amnion and chorion all show follistatin protein distribution (Petraglia *et al.*, 1994b; Jones *et al.*, 2002c; Jones *et al.*, 2006).

1.5 Interacting Proteins: Activins, Inhibin, Bone Morphogenetic Proteins and Myostatin

Whilst follistatin has the ability to bind other members of the TGFβ superfamily this thesis will focus on reviewing only those in which follistatin has been shown to modulate their bioactivity, namely activins, inhibins, bone morphogenetic proteins and myostatin. Other members of the transforming growth factor β family include five different TGFβ isoforms, the group of bone morphogenetic proteins (BMP), maturation inducing steriod (MIS), growth differentiating factor (GDF), osteogenic protein (OP), myostatin and glial cell line derived neutrophic factor (GDNF) (Vale *et al.*, 1988; Kingsley, 1994). Ligands are grouped in this family through the C terminal domain, which contains a seven-cysteine region. There can be as little as 29% sequence similarity between TGFβ family members, although initial studies indicate that the three dimensional structure of this family will have similarity (Vale *et al.*, 1988; Kingsley, 1994).

1.5.1 Structure of Activins and Inhibins:

It is impossible to discuss follistatin in detail without a good understanding of both activin and inhibin. These two proteins form an integral part of follistatin's interactions within the body. Activin and inhibin are both members of the transforming growth factor β superfamily (Ling *et al.*, 1986; Vale *et al.*, 1986). These are both non-steroidal peptides formed by disulfide-bonded dimers (Ling *et al.*, 1986; Vale *et al.*, 1986).

Inhibin is a dimer of α and β sub-units whilst activin consists of a dimer of the inhibin β units (Ling *et al.*, 1985; Miyamoto *et al.*, 1985; Rivier *et al.*, 1985; Fukuda *et al.*, 1986; Robertson *et al.*, 1987). Initially three activins were identified, activin A, activin AB and activin B, however there have been three additional activin subunits identified β_C, β_D, β_E. β_C and β_E are mammalian whilst β_D is from the Xenopus (Oda *et al.*, 1995; Fang *et al.*, 1996; Bernard *et al.*, 2001).

Inhibin is made up of a dimer of β (β_A or β_B) and an α sub unit, therefore we can have both inhibin A and B (Miyamoto *et al.*, 1985). Inhibin has been shown to inhibit the production of follicle-stimulating hormone whilst activin has a stimulatory role (Miyamoto *et al.*, 1985; Vale *et al.*, 1986).

1.5.2 Structure of Bone Morphogenetic Proteins

Bone morphogenetic proteins also show high conservation between differing BMPs. The mature BMP protein is formed from a pre-cursor protein containing a number of hydrophobic residues and substantial pro-peptides. The mature protein is at the C-terminal end of the immature form. All BMPs have the 7 cysteine residues that are in identical locations to all other TGFβ family members. Six of these residues form the rigid structural cysteine knot whilst the seventh is involved in creating disulfide links between dimers. BMPs can form both homo and hetero-dimers *(Wozney* et al., *1988)*

1.5.3 Structure of Myostatin

A pre-cursor protein containing a signal sequence, N-terminal propeptide domain and the biologically active C-terminal domain is the immature structure of myostatin *(McPherron* et al., *1997)*. Following proteolytic processing the mature myostatin is formed between the propeptide domain and the C-terminal domain. Myostatin sequence has been highly conserved throughout evolution, with the sequence in the C-terminal domain being identical in human, rat, murine, porcine, turkey and chicken following

proteolytic processing. Functionally the gene also appears to be conserved across species *(McPherron* et al., *1997)*.

Both the immature and mature forms of myostatin form disulfide-linked dimers *(McPherron* et al., *1997)*. Unlike both activin and BMP's some regulation of myostatin occurs through the non-covalent binding of the mature peptide to its propeptide, preventing it binding to myostatin responsive cells *(Hill* et al., *2002)*.

1.5.4 Roles of Activin and Inhibins

The widespread distribution of both mRNA and the proteins of activins and inhibins at all stages of development has led to the suggestion that these proteins are in fact growth factors. Their distribution includes all stages of embryonic development through to maturity with tissues including pituitary, gonads, adrenals, thymus, bone marrow, brain, kidney, pancreas, placenta and fertilized and unfertilized ova. Activin has been implicated in cell growth and differentiation (Vale *et al.*, 1988; Petraglia, 1997); it appears to have both stimulatory as well as inhibitory roles dependent on the cell line. For example; activin inhibits the proliferation of hepatocytes and T-lymphocytes (DePaolo *et al.*, 1991; Yasuda *et al.*, 1993) whilst in foetal adrenal cells and ethryroid progenitor cells its actions are stimulatory (Shiozaki *et al.*, 1992; Caniggia *et al.*, 1997; Spencer *et al.*, 1999).

Activin binds to a series of activin receptors, discussed in more detail in Section 1.5.7, as well as being bound to follistatin. It is only unbound activin that is biologically active. The unbound activin is capable of binding to a complex array of receptors. This group of receptors transmits signals that are involved in a wide variety of physiological processes.

It should be said that almost all of the actions of activin are modulated via the molecule follistatin. Binding of follistatin to activin is a complex system in itself with still many questions to be answered. When FS288 and activin form a complex, the FS288/Act

complex, becomes bound to extracellular proteoglycans via the heparan sulfate-binding site of domain I in follistatin (Wang *et al.*, 2000). It appears that this complex is endocytosed via intracellular lysosomes (Hashimoto *et al.*, 1997; Hashimoto *et al.*, 2000). If however activin binds to FS315, the complex formed shows no affinity for the extracellular matrix. The role the FS315/Act complex plays in circulation is unknown, particularly as it appears to be the dominant form. It is possible that this complex remains in circulation in order to act as a signaling molecule to stimulate a systemic response, or it is circulation only as long as necessary to reach the pancreas or small intestine for protein degradation and modulation of activin's bioactivity.

1.5.5 Roles of Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMP) form part of a large sub-group of the transforming growth factor β superfamily (Hino *et al.*, 1996). Originally isolated from bone preparations, it was thought that their actions were confined to osteogenic activities. This group forms one of the largest members of the TGFβ superfamily with greater than twenty members (Hogan, 1996). The name bone morphogenetic protein is somewhat misleading as more current evidence suggests that BMPs have roles in cell proliferation, cell fate determination, apoptosis, differentiation and morphogenesis (Hogan, 1996). In vertebrates, BMPs are partially responsible for the development of almost all organs and tissues from the very first establishment of embryonic pattern (Hogan, 1996) through to a wide distribution of physiological roles. Of interest in this thesis is the role selected BMP play in the reproductive cycles of women. BMP-4 and 7 mRNA are present in theca, granulosa and oocyte cells. Concentrations of these proteins regulate the effects of FSH on estradiol and progesterone production (Shimasaki *et al.*, 1999). BMP-2, 3, 6, and 15 have all shown a variety of effects within the reproductive system in women (Huang *et al.*, 2001; Lee *et al.*, 2001; Otsuka *et al.*, 2001; Jaatinen *et al.*, 2002). BMPs have not

been previously described in human pregnancy and will be an interesting area for future studies.

Despite the interest in BMPs reproductive role, it should be noted that bone morphogenetic proteins are widely distributed across the body. Their distribution in a wide variety of tissues is similar to activin and inhibin. They can be found in embryonic tissues such as central nervous system, musculature and skeleton (Leong & Brickell, 1996) and a BMP signaling system operates in pituitary gonadotropes (Huang *et al.*, 2001).

1.5.6 Roles of Myostatin

Myostatin is expressed almost exclusively within skeletal muscle and plays an essential role in skeletal muscle growth *(McPherron* et al., *1997)*. Knockout mice studies have shown dramatic and widespread increases in skeletal muscle mass, through both hyperplasia and hypertrophy. Myostatin is expressed throughout embryonic development and is thought to be involved exclusively in inhibition of skeletal muscle development.

1.5.7 Activin and Inhibin Receptors

The complexity of the receptors for activin and inhibin has made interpretation of the pathways of actions by this family incredibly difficult to determine. Although some headway has been made there are still many areas in which further research is needed.

Activin receptors consist of transmembrane receptors with serine/threonine kinase activity. These receptors exist in two subgroups, namely a type II ligand-binding receptor (ActRII) and a type I signal-transducing receptor (ActRI) (Tuuri *et al.*, 1994). Activin initially binds to ActRII on the cell membrane. It must then complex with ActRI which becomes phosphorylated to initiate intracellular signaling. The activated ActRI then phosphorylates cytoplasmic Smad 2 or Smad 3. These molecules then complex

with Smad 4 (stimulatory) or Smad 6 or Smad 7 (inhibitory) to translocates to the nucleus for regulation of gene transcription (Massague, 1998).

Both types of receptors include the extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic domain. It is the cytoplasmic portion that contains the serine/threonine kinase activity. These receptors elicit cellular effects by interaction with activin receptor interacting proteins (ARIPs), which associate with specific Smad proteins (Iemura *et al.*, 1998; Tsuchida *et al.*, 2001; Korchynskyi & ten Dijke, 2002). The proposed pathways for activin action are discussed in detail in Section 1.6.

Type I receptors consist of two groups in humans, ActRI and ActRIB. ActRI is reported to not bind activin A but instead forms a signaling complex with the type II receptors (ten Dijke *et al.*, 1993). ActRIB performs very different actions from those of ActRI (Carcamo *et al.*, 1994). In humans there are two type II ligand- binding receptors, known as activin receptor type II (ActRII) and activin receptor type IIB (ActRIIB). The size and structural features of these two receptors is almost identical although the similarity at the amino acid level is only approximately 69% identical (Hilden *et al.*, 1994).

The ActRII receptor binds preferentially to activin A, activin B and inhibin A respectively (Mathews & Vale, 1991). The ActRIIB receptor also binds activin A. For intracellular signaling to occur activin requires the presence of both type I and II receptors on the cell surface.

This system of receptors becomes increasingly complicated when they are also able to bind a number of other members of the TGFβ superfamily. ActRII binds growth differentiation factor-5 (GDF-5) and BMP-7 whilst ActRIIB binds GDF-5 and BMP-2 and myostatin (Yamashita *et al.*, 1995). Some of the binding by these factors exhibit similar effects to binding with activin, although certainly not all. For example, one of the actions

of BMP-7 and activin when bound to ActRII includes erthyroid differentiation induction. However production of FSH in the pituitary is an effect seen only by activins binding to ActRII (Yamashita *et al.*, 1995).

1.5.8 Bone Morphogenetic Protein Receptors

Bone morphogenetic proteins bind to a group of transmembrane receptors bone morphogenetic receptors (BMP-R) with serine/threonine kinase activity as do both activins and inhibins (Leong & Brickell, 1996). In fact all members of the transforming growth factor β superfamily exert their biological effects via type I and type II receptors. Following binding to Type I and II receptors at the membrane and subsequent phosphorylation, intracellular Smad1, 5, and 8 are activated and bind to Smad 4. Once the complex with Smad 4 is formed it translocates to the nucleus and activates transcriptional factors in early BMP response genes.

Along with their BMP-R, BMP-7 and BMP-4 both bind to activin receptor-like kinase 3 and 6 (ALK-3 and ALK-6). These are both type I receptors that are found widely distributed in embryogenesis (Dewulf *et al.*, 1995). There are similarities in structure between activin receptors and BMP receptors as evidenced by the fact that ActRII binds BMP-7 and ActRIIB binds BMP-2 (Yamashita *et al.*, 1995). Given the vast number of BMPs, individual receptors will not be discussed further.

Follistatin interacts with many of the BMP group and inhibits all of their actions. Although follistatin binds with high affinity to BMP (Kd ~ 23 pM), it will preferentially bind activin (Kd ~ 47 pM) (Iemura *et al.*, 1998). It inhibits all actions of BMPs in early Xenopus embryo's (Iemura *et al.*, 1998), the mechanism of which will be discussed in early development in Section 1.7.2.

1.5.9 Myostatin Receptors

Myostatin, like inhibin, activin and BMPs, also binds to serine/threonine kinase receptors which then activate Smad proteins thus triggering signalling pathways. Myostatin binds both ActRIIA and ActRIIB with much higher affinity however to ActRIIB. The actions of myostatin are blocked either by both binding to follistatin or by binding to the propeptide which both inhibit binding to ActRIIB *(Lee & McPherron, 2001)*. Myostatin can be complexed in circulation with FSTL-3, or a newly identified member of the follistatin family, growth and differentiation factor-associated serum protein (GASP) in circulation that can both act as inhibitory agents *(Hill* et al., *2002; Hill* et al., *2003)*.

1.6 Follistatin and its binding

1.6.1 Binding to Activins, Inhibins, Bone Morphogenetic Proteins and Myostatin

Recent X-ray crystallographic studies on the activin A/follistatin (FS288) complex have confirmed previous work that suggested that follistatin binds to activin in a 2:1 ratio (Shimonaka *et al.*, 1991; de Winter *et al.*, 1996; Thompson *et al.*, 2005). Follistatin wraps around the activin protein with two main points of contact in a head to tail type formation shown in Figure 1-3 (Thompson *et al.*, 2005). Site 1 within the N-terminal domain wraps around the concave surface of activin's hydrophobic residues and one if its α helices (Thompson *et al.*, 2005). The first of the areas is in the 1-26 region, with the amino acids 3-5 being essential for binding activin and the second area in line with the a helix is region 44-59 with residues 46-47 being critical for activin binding (Wang *et al.*, 2000). Whilst activin binding occurs within the N-terminal domain, the presence of the N-terminal alone is insufficient to bind activin (Keutmann *et al.*, 2004). It also seems as if the hydrophobic interactions between the tryptophan residues in positions 4 and 36 and

activin are important components for follistatin/activin binding as when these are substituted with alanine there is a marked decrease in binding (Keutmann *et al.*, 2004).

Whilst initial studies suggested that the presence of the domains was necessary due to the cysteine residues performing some function in the binding of activin and the extremely conserved nature of these residues within other members of the follistatin family (Wang *et al.*, 2000), further work by Keutmann *et al.* (2004), suggests there are a number of other highly conserved key hydrophobic residues within the follistatin family. In all follistatin family members when cysteine residues within the domains are aligned there are four hydrophobic residues in domain 1 (Tyr-110, Leu-116, Leu-127 and Val-129) and two hydrophobic residues in domain 2 (Tyr-185 and Leu-191) that are also in alignment. Substitution of the first two of these residues in either one or both domains markedly reduces the activin binding (Keutmann *et al.*, 2004). They suggested that perhaps these hydrophobic residues have a purpose in maintaining structural conformation and the stability of the binding site for activin. Studies by Thompson *et al.* (2005), have confirmed this showing that activin binding at Site 2 is within the Kazal sub domain of FS domain 1 and FS domain 2. Site 2 interacts with the convex surface of activin and again the two molecules pack hydrophobic residues tightly together, along with a number of hydrogen bonds, and electrostatic interactions (Thompson *et al.*, 2005). Interestingly, the EGF sub domain of domain 1 does not come into contact with activin.

Follistatin domain 3 shows no points of contact with the activin molecule. Although there have been no other activin binding regions identified within the follistatin molecule, the N- terminal has markedly reduced follistatin binding without the presence of follistatin domains. Of the follistatin domains, domain 3 is non-essential for binding and can be removed with no loss of binding affinity. However, removing domain 2 reduces binding affinity and removing both domain 1 and 2 markedly so (Keutmann *et al.*, 2004). The N-

terminal and domains must be within the one molecule as when separated binding cannot occur, suggesting that the interactions between N-terminal and the first two follistatin domains are essential for activin interactions (Keutmann *et al.*, 2004). The order of the domains (ie: domain 1-domain 2-domain 3) also appears to be essential, as any alteration to this order results in a reduction in binding affinities for activin.

Previous studies have suggested that follistatin binding to activin prevention of activin binding to type II receptors (de Winter *et al.*, 1996; Iemura *et al.*, 1998; Fischer *et al.*, 2003), however the recent complex crystal structure study reveals that the complex inhibits activins actions by blocking both type I and type II receptors (Thompson *et al.*, 2005).

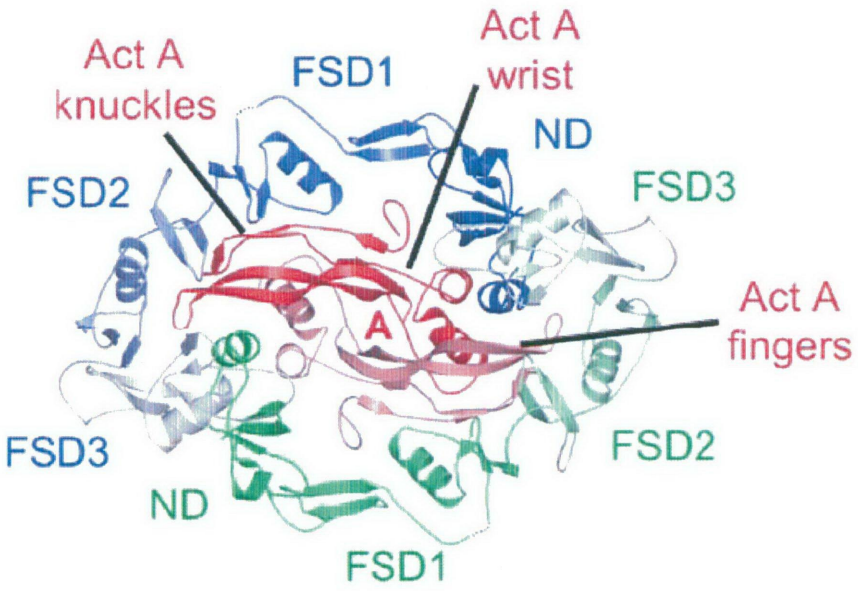

Figure 1-3 Head-to-tail formation of activin/follistatin complex. Activin is shown in red, whilst follistatin molecules are shown in blue and green. Modified from (Thompson *et al.* , 2005).

The 39 hydrophobic residues of activin are highly conserved within the activins, however 12 of these residues are conserved within the entire TGFβ superfamily. Follistatin is

able to bind to other members of the transforming growth factor β superfamily. That is, follistatin also widely inhibits the actions of bone morphogenetic proteins (Iemura *et al.*, 1998) and myostatin (Lee & McPherron, 2001). The affinity of follistatin for BMPs (Kd ~ 23 pM) is lower than that of follistatin for activin (Kd ~ 47 pM), whilst affinity for myostatin is between the two (Amthor *et al.*, 1996; Amthor *et al.*, 2002; Sidis *et al.*, 2006) . Follistatin appears to form a complex between BMP-4 and the BMP receptor, which suggests a different mechanism for the inhibitory actions on BMP compared to those on activin as shown in Figure 1.4 (Iemura *et al.*, 1998). Binding of follistatin to BMP is non-competitive and forms a trimeric complex between BMP receptor and BMP as shown in Figure 1.4 (Iemura *et al.*, 1998). BMPs are also regulated via the actions of chordin and noggin. The actions of both of these proteins are competitive and quite different from those of follistatin (Iemura *et al.*, 1998).

The follistatin protein has a single chain structure that has no structural similarity to any of the members of the transforming growth factor - β super family (Kettel *et al.*, 1996). Follistatin has an extremely high affinity for activin with binding to activin by follistatin being almost irreversible with each activin subunit binding to one follistatin molecule, so that two follistatin molecules bind one activin dimer (Hashimoto *et al.*, 2000) as shown in Figure 1.3. Solid –phase ligand binding assays have shown that activin B is 10-fold less potent that activin A for binding to either FSTL-3 or follistatin (Schneyer *et al.*, 2003). Follistatin's affinity for AB has not been thoroughly compared to the other activins as yet, but early investigations of inhibins show inhibin and follistatin have a lower affinity for one another than any of the activins (Moore *et al.*, 1994). However, recent work on the follistatin isoforms has shown that each isoform for follistatin and FSTL-3 have different binding capacities for activin. Although activin does not bind directly to any other regions of follistatin, its binding in the N-terminal domain has a strong effect on both domain 3

and the C-terminal domain. Following binding with activin, these two domains are unable to hybridize with their own monoclonal directed antibodies suggesting that both of these regions undergo conformational changes following activin binding (Wang *et al.*, 2000). It is proposed that the longer carboxy-terminal that is not present in the shorter FS288 masks the 72-86 binding site. Follistatin can also bind both heparan and activin simultaneously indicating that there is little interaction between the N-terminal and domains 1 and 2 (Wang *et al.*, 2000).

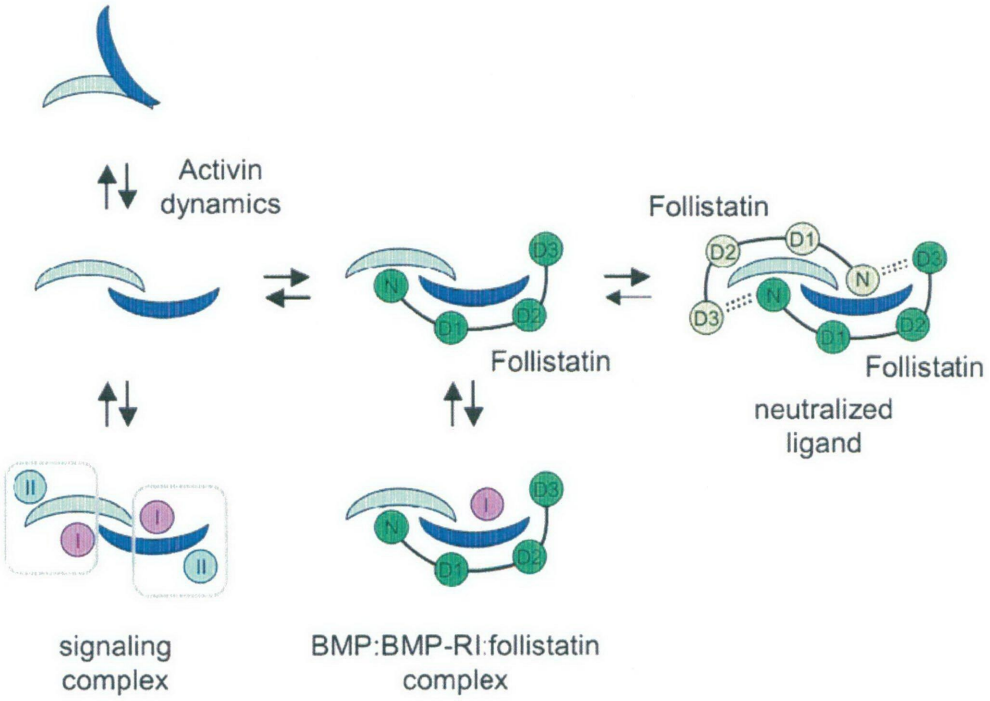

Figure 1-4 Differing modes of ligand complex formation and antagonism with follistatin. (Thompson *et al.* , 2005)

1.6.2 Binding to Cellular Membranes

As shown in Figure 1.1, within domain 1, the 75-86 region contains a heparin-binding sequence (HBS) with a strong affinity for heparan sulfate. Heparan sulfate proteoglycans are an important part of the extracellular matrix and are also involved in cell/cell and cell/extracellular matrix interactions. Whilst cellular proteoglycans have wide and varied

roles it is thought that heparan sulfate proteoglycans interact with growth factors and cytokines (Yamane *et al.*, 1998). Additionally it has been shown that they also have an influence on both the size, quantity and function of gap junctions that are important in cell to cell communication (Garfield & Hayashi, 1981; Ciray *et al.*, 1995; Iozzo, 1998).

Recent work by Sidis *et a*l. (2006), has examined the three major follistatin isoforms (FS288, FS303 and FS315), along with FSTL-3 in detail and determined that the binding ability of each of these proteins to the cell surfaces directly influences their ability to modulate activin, myostatin and BMPs (Sidis *et al.*, 2006). When each of the isoforms is examined in detail there are markedly different heparin binding abilities, with FS288 binding the most strongly to heparan sulfate, followed by FS303, FS315 and finally FSTL-3. These differences are due to both the presence of the heparin binding sequence in the follistatin domain 1 and the length of the acidic C-terminal tail. FS288 is missing the C-terminal encoded by the missing exon 6, but contains the HBS and thus forms strong bonding with the cell surface (Inouye *et al.*, 1992; Sumitomo *et al.*, 1995). However, whilst FS315 contains a HBS, it is formed from exons 1-6 and therefore contains a full length acidic tail which seems to interact with the basic residues of the HBS and limits the binding of FS315 with the cellular surface (Wang *et al.*, 2000). FS303 is formed by a proteolytic cleavage of the 315 protein and appears to bind heparan sulfate proteoglycans moderately (Sidis *et al.*, 2006). FSTL-3 has no binding sequences appropriate for heparin binding despite having two follistatin domains, and therefore has little to no ability to bind the cell surface (Sidis *et al.*, 2005) .

When assessing the activin binding affinities of the isoforms and FSTL-3, Sidis *et al.* (2006) found no significant differences in binding directly to soluble activin (Sidis *et al.*, 2006). However the ability to inhibit both endogenous and exogenous activin by the isoforms and FSTL-3 directly correlates with cell surface association, with FS288

inhibiting most strongly and FSTL-3 the most ineffective at activin inhibition (Sidis *et al.*, 2006). When FSTL-3 was linked to the cell surfaces via expression as a fusion protein its ability to suppress activin was similar to that seen by FS288 (Sidis *et al.*, 2006). Interestingly, FSTL-3 despite having the lowest bioactivity is expressed in the greatest concentrations in the HepG2 cells studied, whilst FS288 concentrations were lowest (Sidis *et al.*, 2006).

The extensive study by Sidis *et al.* (2006) in HepG2 and HEK cell lines also assessed all isoforms along with FSTL-3 for their ability to bind related TGFβ family ligands (Myostatin, BMP-2, BMP-4, BMP-6 and BMP-7) and compared binding affinities with activin. The three follistatin isoforms and FSTL-3 had great similarities in their binding potency to myostatin, BMP-6, BMP-7 to those with activin showing that the similarities in their TGFβ ligand binding specificities again relate to cell-surface interactions. All isoforms and FSTL-3 showed an inability to bind both BMP-2 and BMP-4. However, whilst FSTL-3 also showed a similar lack of affinity for BMP-2 and BMP-4, it also had limited binding to BMP-6 and BMP-7 as well. The work by Sidis *et al.* (2006) contradicts the previous work in Xenopus embryo's where follistatin (FS288) interacts with high affinity for BMP-2, 4 and -7 suggesting differences between species in embryogenesis (Iemura *et al.*, 1998; Yamamoto *et al.*, 2000). Interestingly, FSTL-3 has recently been shown to be the binding protein for myostatin in circulation (Hill *et al.*, 2002), despite this recent work which suggests its inhibitory effect on myostatin is approximately 5-fold less than that of FS288 (Sidis *et al.*, 2006).

The activin/follistatin (Act/FS) complex once formed binds to the proteoglycans on the cell surface. It has been proposed that when bound the Act/FS complex is endocytosed by the cellular lysosomal enzymes. In this way binding is irreversible as shown in Figure 1-5 (Hashimoto *et al.*, 1997). The FS288 protein forms the Act/FS complex that binds to

proteoglycans (Hashimoto *et al.*, 1997). It appears that cell surface affinity for the follistatins increases the inhibition of activin bioactivity, as expressing FSTL-3 attached to cell plasma membranes increased its ability to inhibit endogenous activin to that of FS288 (Sidis *et al.*, 2006). Therefore we see increasing inhibition with increased ability to interact with the cell surface (FSTL-3<FS315<FS303<FS288).

Binding activin to follistatin at the cell surface increases the rate of endocytic degradation of activin (Wankell *et al.*, 2001a). Recent work to elucidate the Smad pathway to signal transduction has shown that TGFβ members bound to type II receptor can be endocytosed into early endosomes (Itoh *et al.*, 2002; Panopoulou *et al.*, 2002; Itoh *et al.*, 2003; Lin *et al.*, 2004). When activin binds to the type II receptor it is endocytosed to early endosomes containing the Smad anchor for receptor activation (SARA) (Itoh *et al.*, 2002; Panopoulou *et al.*, 2002; Lin *et al.*, 2004). SARA is essential for the phosphorylation needed for Smad 2/3 to interact with Smad 4 that then translocates to the nucleus for activation of target genes. Early endosomes are eventually degraded through normal cellular metabolism. Theoretically, this pathway allows for FS288 to be endocytosed with activin into the cell along with acting as an activin antagonist at the receptor site to reduce activin signaling to limit activins bioactivity (Sidis *et al.*, 2006). Potentially this dual action, at cell surface and within early endosomes, explains the increased ability to suppress activin bioactivity by FS288.

The FS315 has the HBS masked and cannot present activin to the cell surface activin receptor. The Act/FS315 complex is therefore not bound and endocytosed within the cell and its fate is still unknown (Schneyer *et al.*, 2004b). However, it is clear that FS315 is the major form of activin binding protein in circulation (Schneyer *et al.*, 2004b).

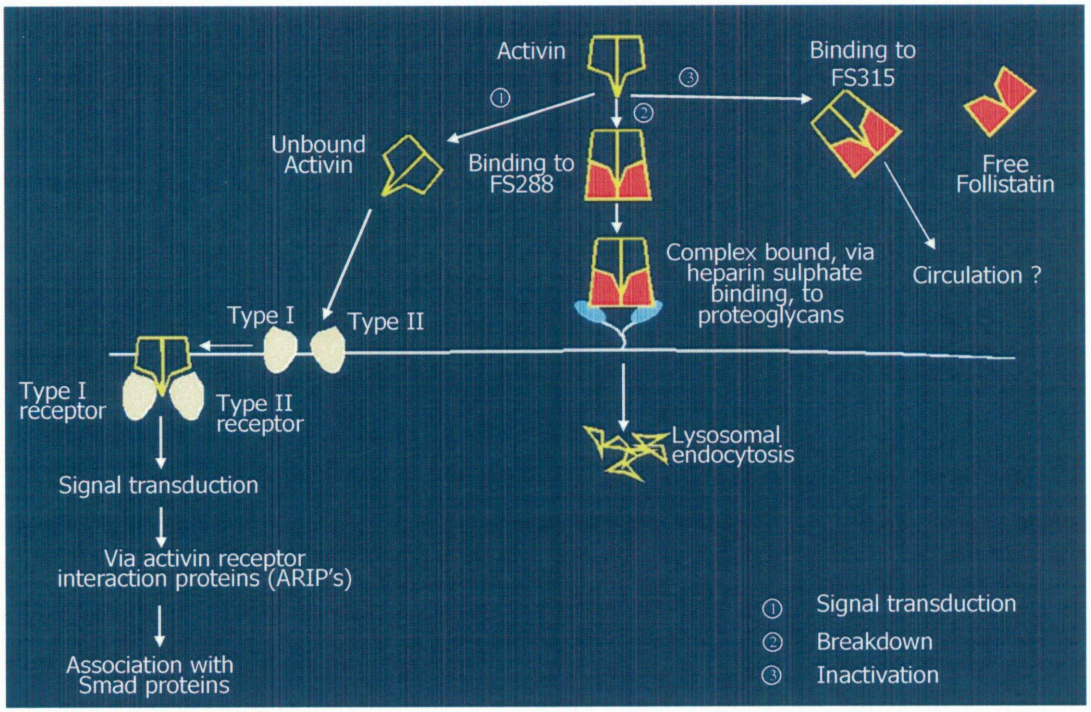

Figure 1-5 Proposed action pathways for activin (Hashimoto *et al.* , 1997) **and diagram modified from** (Phillips & de Kretser, 1998)

1.7 Known and proposed roles for follistatin:

Since the discovery of follistatin in 1987, studies have shown the widespread distribution of both mRNA and its protein, and the functionality of follistatin has been widely investigated. Whilst some of its roles seem clear, many are still under investigation and these results will greatly enhance our understanding of follistatin's physiological purpose. In the following sections, its main roles will be described briefly, however, this study has been designed to investigate follistatin in the onset of parturition and therefore not all aspects of the physiological actions of the protein can possibly be covered here.

1.7.1 Roles suggested by Knockouts or Over-expressers

Loss of follistatin function (FS-) mice generally survive birth but die within hours of parturition, showing follistatin is a protein that is essential for life. FS- mice are retarded

in growth at term, surviving birth but fail to breathe showing that follistatin alone has a minor role in development (Matzuk *et al.*, 1995c). Many of the roles of follistatin are part of a complex system of feedback, stimulation and inhibition relating to activin, inhibin, BMPs and the variety of receptors involved with each of these molecules.

Overall, follistatin levels in the circulatory system remain fairly constant with the majority of follistatin bound to activin. Both the FS288 and the FS315 bind to activin and block its bioactivity (McConnell *et al.*, 1998). These follistatin levels are fairly unchanging with the following exceptions. Late pregnancy shows increases that will be discussed in detail In Chapter 4, whilst follistatin levels decrease with the onset of puberty (Kettel *et al.*, 1996). Further establishing a role for follistatin in the reproductive axis are mice with follistatin over-expression (FS+) genotype. These survive to adulthood, but have major abnormalities in the gonadal tissues although no other obvious defects (Guo *et al.*, 1998). Over-expresser males have defects in the Leydig cells and seminiferous tubules that render these FS+ mice infertile and FS+ females have underdeveloped ovaries and uteri (Guo *et al.*, 1998). Follicles of FS+ females have lesions in all follicles, which are only at the primary or secondary follicular stage of development (Guo *et al.*, 1998). Given that follistatin is known to suppress the levels of follicle stimulating hormone (FSH), it is surprising that very few of the FS+ mice have decreased levels of FSH concentrations (Guo *et al.*, 1998). Additional discussion of follistatin's actions within the reproductive axis of women appears in Section 1.7.9, and in Chapters 2, 3 and 4.

Matzuk *et al.* (1995) study of knockout mice shows, on histological examination, that the alveolar spaces are poorly expanded which is consistent with breathing failure, however there are no primary defects of the pulmonary system in the mice, only the diaphragm and intercostal muscles show a decreased muscle mass. In the musculo-skeletal system, defects to the thirteenth rib pair as well as the number of lumbar vertebrae

present were seen, as well as abnormal whisker, teeth and palate development (Matzuk *et al.*, 1995c). The FS- mice also have increased thickening of the granular and stratum corneum layers of the skin leading to very shiny taut skin.

Matzuk and colleagues (1995) determined that, although the phenotypic characteristics were not identical, there were some similarities seen between FS- mice and some mice with and without the TGFβ proteins. For example the TGFβ over-expresser had similar shiny taut skin to FS- mice, consistent with theories that follistatin modulates the actions of the TGFβ family. However the activin βA deficient mice had similar whisker and teeth defects and the BMP-5 mutant mice have defects in their thirteenth rib pair similar to FS- mice suggesting that these defects seen in both knockouts are due to the loss of the act/FS complex and BMP/FS complex rather than due to the actions of any of these proteins in isolation. These similarities suggest that follistatin is likely to be involved as a modulator for other members of the transforming growth factor β family, however some of the defects seen in FS- mice are not seen in activin A, B or ActRII deficiency mice (Vassalli *et al.*, 1994; Matzuk *et al.*, 1995a; Matzuk *et al.*, 1995b), suggesting some defects are unrelated to inhibition of activin.

1.7.2 Follistatin and Embryonic Development:

Following fertilization the blastula differentiates into the gastrula which develops into a three layered germ cell system in the mammal. These three layers are comprised of the ectoderm which eventually develops into the epidermis and melanocytes of the skin, as well as cells such as brain neurons. The mid-layer, the mesoderm, differentiates into tissue types such as red blood cells and cardiac, skeletal and smooth muscle, whilst the endoderm becomes the epithelial lining of the gastrointestinal tract and other organs including liver, pancreas and lungs.

Members of the TGFβ family are all potential mesoderm inducers and studies suggest that activin is perhaps the most potent. Follistatin shows the ability to suppress activin's mesoderm inducing activity in a dose dependant manner (Fukui *et al.*, 1993). A large number of studies across a variety of species have all shown activin to act as a mesoderm inducer (Slack, 1991; Nakamura *et al.*, 1992; Fukui *et al.*, 1993; Schulte-Merker *et al.*, 1994; Magnuson & Faust, 1995). Surprisingly, studies on early pre-implantation mice (6-9.5 days) show that activin is not present in the embryo during mesoderm formation, but the transcripts for both the βA and the βB inhibin subunits are specifically expressed in the areas of the deciduum tissue that encompasses the embryo at this time. The lack of inhibin subunit α suggests that the β subunits are activin rather than inhibin (Albano *et al.*, 1994a; Feijen *et al.*, 1994). However two different studies have shown quite different signal intensity for follistatin within the decidua. Albano *et al.* (1994) suggests follistatin is also present in the deciduum however its location is such that it is unlikely to be able to inhibit the functions of any decidual derived activin, therefore activin can still potentially act as a decidual mesoderm inducing factor. Whilst Feijen *et al.* (1994) suggest that the intensity of follistatin in the decidua is such that the embryo is unlikely to become exposed to any unbound activin. Feijin *et al.* (1994) do, however, suggest that that this variation may in fact be due to a follistatin isoform difference. Feijin *et al.* (1994) also discuss the possibility of an as yet undiscovered activin, as there are type IIB receptors present in the embryo at this time suggesting a role for activins.

It has also been suggested that activin has functions in addition to its mesoderm inducing properties. One such suggestion comes from the ability of activin to promote the mitogenesis of embryonic carcinoma cells (Hashimoto *et al.*, 1990). This particular cell line has similar properties to those of the epiblast, which undergoes rapid growth in

the post-implantation period. Activin derived from the decidua may be responsible for these actions, that being mesoderm patterning of the embryo (Albano *et al.*, 1994b).

Studies have shown that follistatin expression within the primitive streak itself is extremely high, as well as within the epiblast cells (Albano *et al.*, 1994b; Feijen *et al.*, 1994). Although this would suggest that activin could not possibly be involved in mesoderm induction, it may be that follistatin serves to reduce the spread of the mesoderm inducing signal within the streak. However if activin is involved in mesoderm patterning rather than induction, then the presence of follistatin in this area allows activin to remain active in the anterior portion of the embryo, rather that in the posterior where follistatin is present (Albano *et al.*, 1994b).

The studies by Albano *et al.* (1994), and Feijin *et al.* (1994), examined the expression pattern for follistatin within the deciduum and embryo over a number of days. These studies both suggested that follistatin has a role in development of the embryo that is quite distinct from that of activin. In early stages of streak development (6.5-7.5 days), follistatin expression is very high with no evidence of activins presence here. Follistatin expression declines in the streak at the early somite stage. Albano *et al.* (1994) suggest that the early somite stage may be a period of transition from control by external inducers to inducers within the streak/tailbud, so that follistatin's decline in this period suggests it is involved in the initial induction of the streak as an external inducer (Albano *et al.*, 1994b). Additionally, their study showed that follistatin expression increases again later in embryonic development (9.5 days in mouse) within the neuroectodermal tissues particularly the rhombomeres 2,4 and 6 of the hindbrain (Albano *et al.*, 1994b). They suggest that perhaps follistatin is involved in the segmentation of the hindbrain. Previous work on thecal cells has shown that follistatin can independently stimulate progesterone release, suggesting that follistatin functions independently in certain cell types

(Shukovski *et al.*, 1993). Potentially, the embryonic streak development may be another example of this paracrine activity.

From 10.5-12.5 days in the mouse fetus βA subunits are present within the mesenchymal structures, with other members of the TGFβ superfamily being expressed in adjacent tissues, particularly TGFβ2 and TGFβ3 (Feijen *et al.*, 1994). This finding suggests that the TGFβ members are regulating the activin β subunit expression within the mouse. Interestingly, the βB subunits were found within the central nervous system, particularly within the forebrain, hindbrain, and the lumen of the spinal cord (Feijen *et al.*, 1994).

However, within the oesophagus, pleuroperitoneal membrane, blood vessel mesenchyme and the intervertebral disc anlagen both the βA and the βB subunits are expressed together or in adjacent tissues, suggesting that activin A, B or AB may be present here (Feijen *et al.*, 1994). The Feijin *et al.* (1994) study also showed that the expression of activin receptors type IIA and IIB generally coincides or is adjacent to the sites of the β subunit expression.

Within human development, Tuuri and colleagues (1994) have shown strong evidence that the activin/follistatin complex is intimately involved in human fetal development, although its role is poorly understood (Tuuri *et al.*, 1994). Using Northern analyses they have shown that in mid-gestational fetuses activin, follistatin and activin type I, II, and IIB receptors are expressed in a wide variety of tissues of the fetus (Tuuri *et al.*, 1994). The levels of follistatin mRNA are expressed most highly in skeletal muscle, kidney and liver (Tuuri *et al.*, 1994). Moderate levels are seen in the cerebellum and follistatin is weakly expressed in the cerebrum, spinal cord, heart, stomach, smooth muscle, salivary glands and adrenal tissues (Tuuri *et al.*, 1994). Follistatin mRNA does not appear to be expressed in the bone marrow, spleen, thymus or pancreas (Tuuri *et al.*, 1994). Activin

expression was shown in similar tissues although the degree of expression was different with the highest levels detected in the cerebrum and heart muscle (Tuuri *et al.*, 1994). Also noteworthy, was the expression of activin in the bone marrow and spleen (Tuuri *et al.*, 1994). Activin receptors were also widely distributed throughout all tissues tested. Inhibin however, shows no expression except in the fetal testis and adrenal tissues (Tuuri *et al.*, 1994).

Given the breadth of activin and follistatin distribution within the mid-gestational fetus, there is strong evidence that this complex has a large role in fetal development, whilst it appears that inhibin is limited to a role restricted to the testis and adrenal tissues. Follistatin blocks the actions of both activin and BMP-4. Both of these molecules are mesoderm inducers, so that follistatin promotes the neural development of embryonic tissues (Chitnis & Kintner, 1995). It also appears that FS is involved in the segmentation of the hindbrain and somite development (Chitnis & Kintner, 1995), however the follistatin knockouts showed no significant abnormalities to the central and peripheral nervous system as previously discussed in Section 1.7.1 – Roles suggested by Knockouts and Overexpressors (Matzuk *et al.*, 1995c). Activin inhibits vascular endothelial cells (VEC) whilst FS blocks the activin inhibition of VEC's (Brown *et al.*, 1991). The potential is there for follistatin to have a role in vessel remodeling (Phillips & de Kretser, 1998).

1.7.3 Follistatin and Skin Development

Knockout studies have greatly aided our understanding of the role of activin and follistatin in skin morphogenesis and wound healing. Wound healing will be dealt with in Section 1.7.4.

The follistatin knockout mice show a taut shiny skin with epidermal hyperplasia (Matzuk *et al.*, 1995c), whilst activin βA subunit knockouts show alterations in the development of

the interfollicular epidermis (Matzuk *et al.*, 1996). Over-expressers of the activin βA subunit show keratinocyte hyperproliferation and the differentiation of the epidermis is altered (Munz *et al.*, 1999b). These mice show a thinner epidermis and a reduced density of the dermal matrix suggesting that activin does affect skin development. However the follistatin over-expressers show no alterations to keratinocyte proliferation and differentiation (Wankell *et al.*, 2001b).

Studies using a transgenic mice model, that has altered activin receptor signaling in the keratinocytes, shows that activin exerts some of its actions via the stromal cells in normal tissue granulation (Bamberger *et al.*, 2005). It seems essential for the activin receptor to be functional in both the keratinocytes and the stromal cells in skin morphogenesis (Bamberger *et al.*, 2005).

1.7.4 Role of Follistatin in Wound Healing

Knockouts and over-expressers for both follistatin and activin have elucidated clear roles for both activin and follistatin in wound repair. Follistatin over-expressers show a strong delay in wound re-epithelialization and the formation of granulation tissue (Wankell *et al.*, 2001b), suggesting biologically active activin is essential for wound repair. This is confirmed with activin over-expressers showing enhanced wound healing (Munz *et al.*, 1999b). These mice showed thinner layer of hyperproliferative epithelium and a reduction in the granulation tissue as well as abnormal collagen organisation in new wounds (Wankell *et al.*, 2001a; Wankell *et al.*, 2001b). When wound strength was tested these transgenic mice had significantly reduced wound breaking strength (Wankell *et al.*, 2001b). Follistatin mRNA has been localized to skin fibroblasts with the FS315 isoform being predominant (Kawakami *et al.*, 2001). Studies using the glucocorticoid dexamethasone suggest that follistatin mRNA and protein production are biphasically regulated by the glucocorticoid (Kawakami *et al.*, 2001), however the role this plays in

wound healing or morphogenesis is unclear. What is apparent however is that an increased level of follistatin has dire consequences in the healing of skin wounds.

Whilst it appears clear that activin has a role in skin healing at this stage it has not been studied in an activin βA knockout as these mice die shortly after birth. However, elevated activin βA mRNA along with protein were found in healing mouse skin wounds, and to a lesser extent βB mRNA (Hubner & Werner, 1996). The knockouts for activin receptors indicate that wound healing is also mediated via both the keratinocytes and stromal cells (Bamberger *et al.*, 2005).

Activin and follistatin appear to have involvement with the modulation of the acute phase reactions involved in the inflammatory response (Munz *et al.*, 1999a). This area of research began with the discovery that activins have the ability to activate T cells. The knowledge that follistatin levels in circulation increase dramatically following surgical trauma is also of interest. Follistatin increases in the first 8 hours, but drops back to basal levels by 24 hours (Wankell *et al.*, 2001a). Activins have been shown to suppress the production of interleukin-6 (IL-6) (some sites) and IL-1 which are both involved in stimulation of the liver to release the acute phase proteins (de Kretser *et al.*, 1999). Activin will stimulate the production of IL-6 from monocytes, (de Kretser *et al.*, 1999) whilst follistatin appears to peak somewhat later in the inflammatory process to modulate the actions of activin. Evidence collected in studies researching patients suffering meningitis and septicaemia show that follistatin is increased markedly in these conditions (Michel *et al.*, 1998; Michel *et al.*, 2000; Michel *et al.*, 2003). The origin of the large quantities of follistatin in circulation during this response is unknown. *In vitro* evidence suggests that possibly the endothelial cells are a source of follistatin as these cell groups respond by increasing follistatin levels when stimulated by inflammatory

agents (de Kretser *et al.*, 1999). The role of follistatin and activin in inflammation and wound healing will be discussed further in Section 1.7.5.

1.7.5 Inflammatory Response and Follistatin

One of the main aims of this study is to determine how follistatin interacts during the parturition process. In order to begin understanding parturition, we must first consider that many aspects of the onset of labour are similar to inflammatory events. Whilst we will spend more time reviewing the physiological and endocrine aspects of parturition in Chapter 4, it is essential to discuss the basic features of the inflammation process and the relationship to follistatin and activin.

Most *in vivo* studies on inflammation have utilized injections using microbial-derived substances from bacterial cell wall components such as lipopolysacharide (LPS) peptidoglycans, and lipoproteins to simulate a bacterial infection and study the immune responses. This innate immune response can be triggered by those mentioned above or bacterial DNA and double stranded RNA. Difficulty lies in finding animal models suitable for studying non-pathogenic inflammation, so that most of our understanding of inflammation comes from studies involving bacterial infection conditions, which may not truly reflect inflammatory response in chronic inflammatory diseases. Inflammation is stimulated by either a foreign body or by trauma with magnitude of the stimulus determining the extent of the inflammatory response mounted by the body.

The onset of parturition has long been discussed as a combination of endocrine and inflammatory factors. However non-pathogenic inflammatory mechanisms are poorly understood and therefore the inflammatory process in labour onset are yet to be elucidated. Therefore we must begin our study of inflammation in parturition via understanding bacterial onset of inflammation.

In recent years a group of receptors known as the Toll-like receptor (TLR) family has been studied for its role in the activation of the immune system (Hanada & Yoshimura, 2002). The TLR's are a group of at least 10 cell surface receptors that show specific binding actions to bacterial components and induce the activation of the immune response as shown by Table 1.2 (Silverman & Maniatis, 2001) and as indicated there are a number of TLR's whose precise function is yet to be established.

Table 1.2 Toll-like receptor function and the immune response

Toll-like Receptor	Stimulus
TLR2	Peptidoglycan + lipoprotein
TLR3	Double stranded RNA
TLR4	Lipopolysaccharide + lipoteichoic acids
TLR5	Bacterial flagellin
TLR9	Bacterial CpG DNA

In the presence of LPS, TLR-4 recognises and binds the LPS and then proceeds to activate a number of intracellular signaling pathways including the MyD88 signalling mechanism that stimulates the NFκB inducing pathways (Silverman & Maniatis, 2001). The induction of the NFκB path activates the transcription of the necessary pro-inflammatory cytokines. This activates the appropriate cytokines necessary to stimulate the immune response needed, depending on the type of antigen present.

Pro-inflammatory cytokines accelerate inflammation by either direct regulation of inflammation or via the induction of cellular adhesion molecules and/or induction of other cytokines from specific cell types. Before additional discussion of cytokines ensues it should be noted that, within the cytokine system, it extremely difficult to label specific functions to specific cytokines as the system demonstrates great redundancy. Each cytokine in the system can have many functions on various cell types but also multiple functions within the one cell type. This has made studies of specific functions of

individual cytokines extremely difficult, so that in this discussion on inflammation only the proteins directly related to the context of the study will be discussed in any detail.

Early inflammation is a non-specific response generating an increased blood flow to the site, increased vascular permeability, and the influx of neutrophils. Neutrophils are antigen non-specific but are effective in their phagocytic response. Early inflammation is assisted by the release of a number of pro-inflammatory cytokines, the most prominent being IL-1α, IL-1β, IL-6 and TNFα. Recent work has suggested that activin A is also stimulated by LPS in the early immune response, and data suggested that it is released shortly before TNFα and well before IL-6 (Jones *et al.*, 2000). In this study by Jones *et al* (2000) activin A showed a biphasic response to LPS injection in sheep with the first increases of activin A occurring at 50 minutes peaking at 1 hour and 3.75 hours and showing significant correlations with the fever response also observed. Whilst TNFα increases at 60 minutes and has a singular peak within 2 hours. IL-6 began increasing at 3 hours post stimulation with a peak at 5 hours and had returned to baseline levels by 24 hours. Follistatin begins rising slowly at 3 hours to peak at 8 hours post stimulation and remained elevated for more than 24 hours after the initial LPS stimulation. Follistatin was the only protein to remain high in circulation post 24 hours suggesting that its release is independent of activin and that follistatin has additional roles in the inflammatory response (Jones *et al.*, 2000).

In vitro studies using differing cell lines have all shown that TNFα and IL-1β stimulate activin A mRNA expression and/or protein production (Shao *et al.*, 1998). However, the Jones *et al.* (2000) study showed that *in vivo*, when the bioactivity of TNFα and IL-1 is blocked, the initial peak of activin is maintained and therefore occurs independently of TNFα and IL-1, whilst the subsequent activin peak was present although diminished when TNFα and IL-1 were blocked. Although the study by Jones *et al.* (2000) has shown

the *in vivo* nature of activin and follistatin release it has not elucidated the source of the rising concentrations of these proteins.

Following stimulation by LPS, Toll-like receptor 2 or 4 on macrophages and their immature counter-part monocytes are activated via the NFκB pathways and then flood the area along with antigen specific cells (T cells and B cells) (Brightbill *et al.*, 1999; Jones *et al.*, 2001). These stimulated macrophages or inflammatory cells express an increased amount of cell surface proteins and glycoproteins known as cell adhesion molecules (CAM). At a similar time point to macrophage stimulation, we find that the endothelial cells are also activated by the LPS triggered response of Toll-like receptor 2 and 4 (Faure *et al.*, 2001; Dauphinee & Karsan, 2006). Upon activation, these cells express adhesion molecule counter-receptors as well as a variety of chemokines. Chemokines are generally cytokines that have the ability to promote the migration of other effector cells involved in the inflammatory response.

Macrophages become attached to the vascular endothelial cells and are thus prevented from being removed from the site of inflammation. Interestingly, under certain conditions macrophages produce both activin and activin receptors (Eto *et al.*, 1987; Murata *et al.*, 1988; Funaba *et al.*, 1996). Also of interest is that vascular endothelial cells produce activin and activin A receptors, along with follistatin and inhibins (Michel *et al.*, 1996; Tannetta *et al.*, 2003; Maeshima *et al.*, 2004; Panopoulou *et al.*, 2005).

The extent of the inflammatory defence mounted is determined by the amount of immunomodulation by the anti-inflammatory cytokines. The main cytokines in this regulatory mechanism are IL-4, IL-10, IL-13, IL-16, IFNα, TGFβ, IL-1α, G-CSF, and soluble receptors for TNF or IL-6.

As inflammation is either suppressed or encouraged by its complex system of cytokine messengers, a consecutive acute phase response begins. This involves neurological,

endocrine and metabolic responses within the organism experiencing the inflammation and may take place on either a local or systemic level. The acute phase response is characterized by continued influx of inflammatory cells to the area as well as a number of biochemical changes. These changes involve a coordinated system of synthesis within the hepatocytes of the liver to produce the acute phase proteins (APP). The pro-inflammatory cytokines IL-1, IL-6 and TNFα have direct actions on the hepatocytes to encourage production of a number of acute phase proteins. Most commonly known and of greatest importance are C-reactive protein, fibrinogen, Mannose binding protein, and serum amyloid P. Each APP has a distinct role in inflammation and repair. C-reactive protein interacts with both B and T cells, is involved in the activation and opsonization of the complement system and binds to phosphorylcholine on cellular membranes. Fibrinogen assists in clotting and repair, whilst mannose binding protein recognizes the mannose linkages specific to bacteria and serum amyloid P is involved with the formation of IgG complexes.

Additionally, the pro-inflammatory cytokines IL-1, IL-6 and TNFα, have indirect actions on the brain causing the stimulation of CRH from the hypothalamus which in turn stimulates the anterior pituitary to produce ACTH. The production of ACTH then acts on the adrenals to stimulate the production of glucocorticosteroids.

The production of glucocorticoids is particularly important for the progression of the acute phase response. The synthesis of glucocorticoids is influenced by a number of cytokines but in turn they can influence the synthesis of a number of cytokines. The glucocorticoids function through binding to the glucocorticoid receptor which then forms a complex which is taken up into the cellular nucleus. Once within the cellular nucleus, the complex binds to the Glucocorticoid Response Elements (GRE) within the DNA of the cell and through the process of transrepression it prevents the transcription of a large

number of immune genes. Glucocorticoids act to suppress the cell-mediated immune response and inhibit the ongoing transcription of IL-1, 2, 3, 4, 5, 6, 7 and 8 as well as TNFγ. They also have the ability to act directly on the F_c receptor of macrophages thus suppressing phagocytosis by prevention of antigen detection by the macrophages. Whilst there are other actions of the glucocorticoids in the dampening down of the immune response these actions are not directly related to the core elements of this study and will therefore not be discussed further.

Recent work on the human hepatocytes cell line has shown that activin can suppress the actions of IL-6 and inhibit the proliferation of the HepG2 cells (Russell *et al.*, 1999). When IL-1β was added to activin this caused a greater inhibition of the proliferation, whilst the addition of follistatin to the cultures blocked these actions (Russell *et al.*, 1999). When activin alone was added to the hepatocytes it stimulated the production of follistatin which could be suppressed by both IL-6 and IL-1β (Russell *et al.*, 1999). This study suggests that activin acting in conjunction with IL-6 and IL-1β co-ordinates the production of the acute phase proteins in the liver.

The role of the inflammatory response in parturition will be discussed in Chapter 4, Sections 4.2.

1.7.6 Follistatin and Liver

The liver plays a large number of roles in the physiology of an organism. In the fetus it produces the bulk of red blood cells (RBC) until the 32 week gestation when the bone marrow is mature enough for production. In the mammal, it functions to excrete bile, metabolise both carbohydrates and lipids, produce coagulation factors, metabolise drugs and break down hemoglobin for recycling through the system. Within the liver it is clear that both follistatin and activin have a role to play in some of these functions. As discussed earlier in Section 1.4, mRNA for follistatin has been localized to hepatocytes

of the liver along with the protein and activin subunits also being localised to this organ (Schwall *et al.*, 1993; Yasuda *et al.*, 1993). Some of this has been discussed in Section 1.7.5, where studies have shown involvement in the cytokine triggers of the acute phase response.

Studies have shown that activin A both *in vivo* and *in vitro* rat hepatocytes inhibits the initiation of DNA synthesis (Yasuda *et al.*, 1993) and treatment of hepatocyte cells with activin induces apoptosis (Schwall *et al.*, 1993). Additional work on mutant mice has shown activin to signal through the Act RII receptor in the hepatocytes to cause degeneration of the hepatocytes (Matzuk *et al.*, 1994; Coerver *et al.*, 1996; Guo *et al.*, 1998). The administration of follistatin inhibits the actions of activin in the liver and reduces hepatocyte death, thereby accelerating liver regeneration (Kogure *et al.*, 1995; Kogure *et al.*, 1996; Kogure *et al.*, 1998; Kogure *et al.*, 2000).

These studies along with those outlined in Section 1.7.5, show clear indications that the follistatin/activin system has definite roles within the liver in tissue regeneration and in modulation of the acute phase components of inflammation.

1.7.7 Follistatin and the Male Reproductive Tract

Follistatin has been widely investigated within the male reproductive tract and results suggest that it has distinct functions both in spermatogenesis as well as in the seminal fluid ejaculate. As this study is designed to focus on female reproduction, follistatin's role in male reproduction will only be reviewed briefly. The testis is responsible for both sperm production and the synthesis of testosterone, androstenedione, and dihydroandrosterone. The testis has two main cell types which are responsible for these functions, the Sertoli cells and the Leydig cells.

The Sertoli cells nourish and develop the germ cells and follistatin mRNA has been found in the developing spermatogonia, spermatids and spermatocytes, however it is

found in a stage specific manner (Kaipia *et al.*, 1992; Majdic *et al.*, 1997; Meinhardt *et al.*, 1998). Follistatin protein is also found within the cytoplasm of the Sertoli cells (Anderson *et al.*, 1998). Interestingly, this is only found in the adult whilst in fetal Sertoli cells there is no follistatin mRNA or protein (Majdic *et al.*, 1997). However, in both the fetus and adult testis, mRNA for the activin βA subunit and the βB and α subunit is present. *In vitro* studies have shown Sertoli cells can produce activin A, which stimulates germ cell proliferation, whilst the addition of follistatin appears to modulate some of these actions (Mather *et al.*, 1993; de Winter *et al.*, 1996). The presence of both βB along with α subunits suggests that Sertoli cells are the main producer of inhibin B and activin A in the testis (Anderson *et al.*, 1998).

Leydig cells function to produce the androgens of the testis and also contain follistatin protein, βA, βB and the α subunits for activin A and inhibin production (Kaipia *et al.*, 1992). However, as per the Sertoli cells, there is no follistatin protein or mRNA seen in the fetus (Majdic *et al.*, 1997; Meinhardt *et al.*, 1998).

Seminal fluid contains follistatin at concentrations 100-fold those seen in matched plasma, however no differences in this concentration were noted in studies involving vasectomised and non-vasectomised patients suggesting that the testis is not the source of this follistatin (Anderson *et al.*, 1998). Whilst for activin clear differences were seen between the patient groups indicating that the activin in the seminal fluid is derived from testicular cells, probably from the Sertoli cells (Anderson *et al.*, 1998). The prostate contains both mRNA and protein for follistatin which is localized in the basal epithelial cells and fibroblastic stroma suggesting it is the source of seminal fluid follistatin (Thomas *et al.*, 1997).

The combination of these studies clearly indicate that follistatin and activin have functions within in the male reproductive tract in spermatogenesis and the high levels of

follistatin found in the seminal fluid suggest a possible role for follistatin within the female reproductive tract and fertility (Anderson *et al.*, 1998).

1.7.8 Follistatin and the Female Reproductive Tract

Follistatin was first isolated for its ability to inhibit the production of the pituitary produced follicle-stimulating hormone (FSH) (Ueno *et al.*, 1987). Follistatin has shown no ability to suppress or influence luteinizing hormone (LH) that is also released from the pituitary (Ying *et al.*, 1987). Its ability to inhibit FSH production has since been attributed to its interactions with activin.

Generally speaking, circulating follistatin levels are higher in normal cycling and post-menopausal women compared to pubescent females. However all of these groups have significantly higher levels of circulating follistatin than men (Kettel *et al.*, 1996). Interestingly, circulating inhibin levels change cyclically with menstruation whereas both activin and follistatin show no significant changes in serum levels (see Figure 1.6) (McLachlan *et al.*, 1987). Also noteworthy is that the two different inhibins, inhibin A and inhibin B, follow different cyclical patterns (Groome *et al.*, 1994; Muttukrishna *et al.*, 1994; Sehested *et al.*, 2000). In women inhibin A peaks at the LH surges midcycle, whilst in sheep, rats and heifers variations across oestrus between species were noted (Fahy *et al.*, 1995; Woodruff *et al.*, 1996; Knight *et al.*, 1998; Bleach *et al.*, 2001). It appears that inhibins and FSH operate in an endocrine negative feedback system. This cycle begins prior to the onset of ovulation in pubescent girls and continues throughout the reproductive lifespan until post-menopause, when women do not exhibit this cyclic pattern of inhibin and FSH secretion (Stouffer *et al.*, 1994).

Both total and free activins show no changes during the menstrual cycle in women (Demura *et al.*, 1993; Knight, 1996; Knight & Glister, 2001) however, studies in rats

have shown free activin peaks at the same time as the pre-ovulatory FSH surge in proestrus and a second peak at oestrus when the second wave of FSH occurs (Besecke *et al.*, 1997).

Data across menstruation has been contradictory with the study by Gilfillan and Robertson (1994) showing total serum follistatin concentrations were higher in the follicular phase than in the luteal phase, whilst others have shown total follistatin to remain unchanged throughout the menstrual cycle (Gilfillan & Robertson, 1994; Khoury *et al.*, 1995; Evans *et al.*, 1998; McConnell *et al.*, 1998). In sheep follistatin concentrations also show discrepancies between assays with Klein *et al.* (1993) showing increases in follistatin rise significantly during the luteal phase whilst McFarlane *et al.* (2002) showed no significant changes to follistatin across oestrus.

As follistatin mediates the bioactivity of activin through binding and inactivation of activin, the levels of bound and free follistatin in circulation are of interest. Like the male circulation, the current view is that the levels of both free and bound follistatin remain unchanged in women through the menstrual cycle. There have been varying reports of the levels of unbound follistatin. Levels vary between 10% and 24% of total follistatin circulating freely (McConnell *et al.*, 1998). These low levels of free follistatin in circulation indicate that follistatin does not play an endocrine role in the body, however it does appear to feature heavily in the hypothalamic-pituitary-gonadal axis. In contrast to circulation, the levels of free follistatin are substantial in ovarian follicular fluid and within the anterior pituitary (McConnell *et al.*, 1998). The concentration of follistatin in the follicular fluid has been reported as 3 to 150 times greater that serum levels of follistatin (Khoury *et al.*, 1995). Potentially these tissues are the source of free follistatin, which then binds to activin in the circulatory system (McConnell *et al.*, 1998).

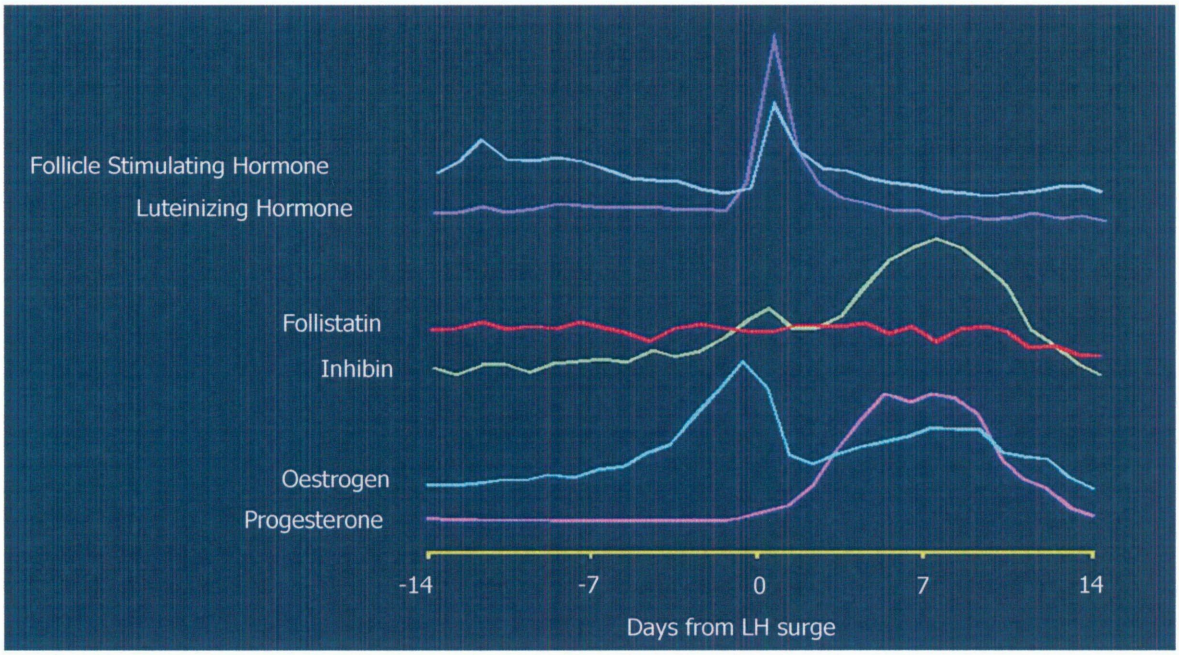

Figure 1-6 Follistatin across the menstrual cycle. Modified from (Kettel *et al.* , 1996)

Within the ovary however, follistatin shows cyclical changes with folliculogenensis suggesting an autocrine/paracrine role in this process. Follistatin is synthesised and secreted by the granulosa cells (Khoury *et al.*, 1995). In the corpus luteum, FS mRNA is present in the luteinised granulosa cells in small amounts (Nakatani *et al.*, 1991; Tisdall *et al.*, 1992; Roberts *et al.*, 1993). There is no evidence of FS mRNA in theca, stroma or the oocyte (Nakatani *et al.*, 1991; Tisdall *et al.*, 1992; Roberts *et al.*, 1993). The primordial follicles contain no evidence of FS protein or FS mRNA indicating that FS plays no role in the early stages of primordial growth. Antral follicles containing more than two layers of granulosa cells are the site of highest FS mRNA expression in the ovary, particularly within the granulosa cells (Schneyer *et al.*, 2000). The levels of FS mRNA increase with size of the antral follicle particularly once the follicle has established dominance (Nakatani *et al.*, 1991; Roberts *et al.*, 1993). Noteworthy however is that circulating levels of follistatin do not reflect the ovarian activity of women (Khoury *et al.*,

1995). Normal cycling women, and those women being treated with exogenous gonadotropins in IVF treatment, showed no significant differences in their circulating levels of follistatin despite the IVF patients having far greater numbers of developing antral follicles (Khoury *et al.*, 1995). In fact the only significant differences in circulating FS is found in pregnant women whose antral follicles fail to develop beyond the small antral stage (Khoury *et al.*, 1995).

In the uterus follistatin mRNA is also expressed as to whether it follows a cyclical pattern similar to that within the ovary is unknown by researchers at this stage. There is evidence in rats that the levels of its expression are influenced by progesterone stimulation (Mercado *et al.*, 1993). Follistatin mRNA expression increases 3-fold between pro-oestrus and oestrus whilst pentobarbitol treatment to block the progesterone level increase, halted the increase in uterine FS mRNA expression. The rise in FS mRNA could be restored through treatment with exogenous progesterone (Mercado *et al.*, 1993). This potentially indicates a role for progesterone in the regulation of uterine expression of follistatin (Mercado *et al.*, 1993).

1.7.9 Follistatin and Disease States

As our understanding of some of the roles of follistatin has developed, a number of researchers are beginning to examine particular disease states and determine if follistatin has a role to play in the onset of these conditions. While there are many more areas of disease in which follistatin is being studied this will only be a brief outline of some diseases currently being investigated.

1.7.9.1 Liver Disease

Within the liver it appears clear that follistatin and activin are involved in liver homeostasis. Activin A induces cell death and inhibits the synthesis of DNA in the hepatocytes whilst follistatin modulates the actions of activin but also induces DNA

synthesis of liver (Kogure *et al.*, 2000). Recent work by Rossmanith *et al.* (2002), has shown that follistatin concentrations were elevated in approximately 40% of liver tumours in rat and mice when compared to surrounding tissue concentrations (Rossmanith *et al.*, 2002). They suggest that the elevated follistatin is caused by a response of the malignant cells to activin inhibitory actions. In studies of cirrhotic liver patients, activin, follistatin and TGFα were all elevated and both activin and follistatin positively correlated with alkaline phosphatase and GGT, common biochemical markers of liver disease (Yuen *et al.*, 2002).

1.7.9.2 Prostate Disease

In the healthy prostate, follistatin is localized to the basal and stromal epithelial cells. Activins are also found in the normal prostate and function to induce apoptosis of the prostate cells and thus inhibit cancer growth (Wang *et al.*, 1999a; Wang *et al.*, 1999b). However in cancer we see both follistatin and activin co-localised to the cells and authors suggest that follistatin has increased due to a resistance to activin. This was confirmd when prostate carcinoma cultures suppressed cell proliferation upon activin addition and enhanced epithelial cell proliferation with follistatin (Wang *et al.*, 1999a). These studies clearly show that the activin-follistatin system has a definite role in prostate cancer.

1.7.9.3 Skin Disease

Studies on a variety of skin conditions suggest that the activin-follistatin regulatory system also functions here to maintain healthy skin. Studies on both melanocytes and melanoma cells show that treatment with activin results in the activation of the Smad pathways to signaling. Activin functions to induce cell apoptosis in melanocytes which can be prevented by the treatment with follistatin. This same study also showed that

follistatin is secreted by melanoma suggesting a role in melanoma onset (Stove *et al.*, 2004).

Alopecia is the term used for diminution of visible hair. Whilst there are a number of different types of alopecia, current treatment has limited success. The identification of genes involved in the phases of hair growth – anagen, exogen, catagen and telogen, and hair morphogenesis may perhaps lead to the development of new treatment options. Follistatin, activin and BMP's have all been linked to the morphogenesis of the hair. BMP-2 is a repressor of the formation of placodes (hair follicle pre-cursors), whilst it appears that follistatin acts to determine the shape of the follicle and activin activates the dermis to begin anagen (Cotsarelis & Millar, 2001). These studies show that follistatin and activin function within the skin to maintain structure and hair growth.

1.7.9.4 Ovarian Disease

Follistatin has been studied in a wide variety of reproductive diseases and studies are showing that it has definite roles in Polycystic Ovary Syndrome (PCOS) that leads to polycystic ovaries, decreased fertility, obesity, and insulin resistance. Genetic linkage studies have shown that the gene with the most significant linkage to PCOS was the gene for follistatin. Mice over-expressing follistatin show suppressed serum FSH and arrested ovarian folliculogenesis, such as that exhibited by PCOS patients (Guo *et al.*, 1998). With these symptoms we would also expect to see that overexpression of follistatin would lead to an increase in the production of androgens from the ovary and dysfunction to insulin release (Urbanek *et al.*, 1999; Urbanek *et al.*, 2000). This work suggests that follistatin may become a marker or treatment target for women with PCOS.

In ovarian tumors, activin induces proliferation and follistatin inhibits the actions of activin in the proliferation of epithelial ovarian carcinomata (Welt *et al.*, 1997). Welt *et al.* (1997), showed that activin was secreted in 24 out of 35 ovarian epithelial tumours. This group

showed that in epithelial tumours of the ovary, mRNA for the βA subunit is expressed and, whilst inhibin and follistatin are secreted, activin protein is secreted in higher quantities, approximately 10-20 fold that of follistatin (Welt *et al.*, 1997). Both of the activin receptors, type I and II mRNA, were also found in all of the tumours cell lines. They also found that in some cases of ovarian cancer, serum plasma activin reflected the patient's tumour secretion (Welt *et al.*, 1997). Whilst the mechanisms for activin/follistatin part in the tumourogenesis are yet to be determined it appears clear that the pair act as growth factors, which potentially can lead to diagnostic or treatment targets in this disease.

1.8 Project Aim

This project aims to investigate the role that follistatin plays within the female reproductive tract, with the particular focus period being at the onset of parturition. Follistatin protein will be analysed in maternal serum, fetal serum and localized within the placental tissue. It will be studied in conjunction with a number of reproductive hormones, progesterone, estradiol and prolactin, along with some marker of inflammation, cortisol, activin, C-reactive protein, TNFα and IL-6. Follistatin mRNA for both of the major follistatin isoforms will be investigated within the placenta. All of these studies will utilize patient groups consisting of women who have undergone a spontaneous onset and delivery, those who have undergone an induction, (with no medical complications), and those patients having an elective caesarian section with no labour onset or medical complications. In this way we hope to elucidate clear roles for follistatin in the onset of parturition in women.

Chapter 2

General Methods and Materials

2.1.1 Patient recruitment and groups for analysis

Patients of the Tamworth Base Hospital, Hunter New England Area Health Service were recruited into the study. The mean age of women used in the study was 27.8 years (range of 18.8 to 42.7 years). Previous term pregnancies of recruited patients ranged from 0-4. All women recruited to the study were in good health at the time of recruitment. The gestation of the women was calculated from a definite last menstrual period date or by pediatric judgment post-delivery. The mean gestation of women in the study at term was 39.1 weeks with a range of 32 to 44 weeks. Using a cohort of 48 women who delivered by the end of the study they were divided into 2 major groups with some subsets. Table 2-1 outlines patient groups using the type of delivery as criteria for patient separation.

Table 2-1 Characteristics of patient groups using type of delivery as criteria for inclusion

Characteristics	Vaginal delivery	Caesarian section (LSCS)
No. of deliveries	51	10
Gestation (weeks)	40.5	39.7
Age (years)	28.5	30.1
Parity (previous term pregnancies)	1.18	1.63
Fetal Sex ratio (Male:Female)	0.77 (17:22)	1.25 (5:4)

Patients undergoing vaginal delivery were further separated according to spontaneous or induced onset of labour as shown in Table 2-2. Patients were induced by operative rupture of membranes and IV syntocin administered until the delivery.

Table 2-2 Characteristics of vaginal delivery patients using type of onset as criteria for inclusion

Characteristics	Spontaneous Onset	Induced Onset
No. of deliveries	33	18
Gestation (weeks)	39.3	38.6
Age (years)	27.6	26.7
Parity (previous term pregnancies)	1.4	0.46
Length of labour (hours)	6.5	4.5

Serum results were also analysed using the sex of the child. Due to limited patient numbers in order to maximize the number of samples to consider sex differences and hormones we have grouped all vaginally delivered patients together. Details of these groups are listed in Chapter 4.

2.1.2 Sample Collection

Serum -
Samples collected from patients have been grouped according to:

- Antenatal (ANTE) (Average gestation of samples – 35 weeks)
- Early Labour <3cm vaginal dilation (Lab1)
- Late Labour >3cm vaginal dilation (Lab2)
- Early Post Partum 0-3 hours post delivery (Post1)
- Late Post Partum >3 hours post delivery (Post2)

Blood samples (5ml) collected into 5ml vacuette Z serum separator clot activator tubes. These samples were centrifuged at 4000 rpm for 10 minutes and immediately frozen at -15°C and stored until assayed.

Cord Blood-

Blood samples (5ml) collected into 5ml vacuette Z serum separator clot activator tubes tubes. These samples were centrifuged at 4000rpm for 10 minutes and immediately frozen at -15°C and stored until assayed.

Placenta-

Immunohistochemistry studies

Immediately following delivery (whether vaginal or LSCS) the placenta was refrigerated at 4°C until collection by pathology staff. 3 cm cubed sections of placenta were then taken and placed in phosphate buffered formalin until studied using immunohistochemistry.

Follistatin Isoform studies

Immediately following delivery of the placenta, a 3 cm cubed section of placenta was taken and snap frozen in liquid nitrogen and stored at -25°C until needed for homogenate studies. Placentae being used for RNA studies had ~200ug of tissue sectioned and placed into RNA*later* solution (Ambion Inc, 2130 Woodward St, Austin, Texas, USA) until required for RNA isolation.

2.1.3 Antibodies for Follistatin studies

Two different antibodies have been used in this thesis. #204 has been used in Chapter 4 and 5 in an RIA assay for analysis of human sera. JMCK20 has been used in Chapters 3 and 6 in immunohistochemistry and Western Blotting analysis.

Antibody #204

This antibody uses a rabbit anti-serum raised against purified 35kDa bovine follistatin.

Antibody JMCK20

This antibody uses a chicken anti-serum that has been raised against a peptide corresponding to amino acids 121-133 of follistatin. Work by Saito *et al.* (1991) suggested that the amino acid region of 123-134 of follistatin recognized both the long and short isoforms for follistatin (Saito *et al.*, 1991). This sequence has a high degree of conservation between species thereby making it an ideal choice for use across animal

studies. The sequence of this peptide is KEQPELEVQYQG, and Genebank searching at the time of antibody development, and again more recently showed no overlaps of this peptide with other proteins. Antibody JMCK20 recognises the sequence 121-133 within Follistatin domain 1 (See Figure 1-1) at the C-terminal end of this domain. This region of the domain does not interfere with the heparin binding sequence (75-86) of the follistatin domain.

2.1.4 Ethics:

Experimental work from Chapters 3-5 was approved by the New England Area Health Service Clinical Research Group and Ethics Committees, as well as by the University of New England Ethics Committee. Studies from Chapter 6 were approved by the Hunter New England Human Research Ethics Committee (Approval number DB118) in accordance with the protocols for the National Health and Medical Research Council and the University of New England Ethics Committee.

2.1.5 Image Processing

All agarose gel images were visualized under ultraviolet light and photographed using a computer linked Polaroid Camera and images for this thesis were edited using Canvas 8 Build 437 (2001), Deneba Systems Inc. All Western Blot membranes and SDS-PAGE Electrophoresis gels were scanned using PIXMA MP150 Scanner/Printer and edited using Paintshop Pro 5 Version 5.01, Jasc Software Inc. Immunohistochemical sections were digitally photographed using Nikon Coolpix (950) and edited in Paintshop Pro 5 Version 5.01, Jasc Software Inc.