Chapter 5

Follistatin in the Placenta

5.1 Introduction

5.1.1 Development of the Placenta

The maintenance of pregnancy is reliant upon the successful invasion and development of the placenta. The placenta derives from the adherence of the trophoblastic cells of the blastocyst to the luminal epithelium of the endometrium. This process appears to be mediated through the presence of heparin binding EGF-like growth factor, EGF receptors and heparan sulfate proteoglycans on both the trophoblastic and luminal epithelial cell surfaces (Leach *et al.*, 1999; Isaacs & Murphy, 2002; Chobotova *et al.*, 2005), providing binding for the adherence between the two cell types. This is known as the decidualisation reaction that allows for the invasive development of the placenta. Stromal cells enlarge and become more rounded in shape and begin production of a range of growth factors and cytokines that are thought to be integral to trophoblast invasion. Using these cytokines (particularly IL-15) the decidua recruits a large number of natural killer cells specific to the uterus (uNK) and associated macrophages (King *et al.*, 1998).

Members of the transforming growth factor β superfamily have been implicated in the angiogenesis, cell differentiation and re-modeling phases of the implantation process and are roles in which activin has been specifically implicated. Research by Jones *et al.* (2002) on cultured stromal cells has shown activin to promote decidualisation. Stromal cells produce both activin receptor and activin βA subunits in high concentrations with the onset of decidualisation (Jones *et al.*, 2002a). Early immunohistochemical studies have also shown activin A to be localized to areas of decidualisation (Otani *et al.*, 1998) Whilst activin βA and βB subunits are both dramatically up-regulated during decidualisation in both *in vitro* and *in vivo* studies, and appear to stimulate the decidualisation process, follistatin inhibits the decidualisation of stromal cells. (Jones *et*

al., 2002a; Tierney & Giudice, 2004). Although inhibin α subunits are present within the decidua, culture studies have shown that activin is produced preferentially with activin concentrations 1000-fold those of inhibin (Petraglia *et al.*, 1998).

Bone morphogenetic proteins are also thought to play a role in decidualisation, most particularly BMP-2, the expression of which mimics that of the spread of decidualisation. BMP-7 becomes localized to the stroma prior to implantation and remains in the sub-epithelial stroma subsequently (Ying & Zhao, 2001). Following decidual regression BMP-8a is found in the anti-mesometrial zone, while BMP-4 is found in the vasculature associated with the decidua (Ying & Zhao, 2001). A number of the binding proteins for BMP's (noggin, twisted gastrulation and dan/dante) have been localized to the implantation area, suggesting regulation of the action of BMP's is intrinsic to implantation (Ying & Zhao, 2001).

Prior to differentiation, the trophoblast cells of the blastocyst begin βhCG secretion which is positively stimulated by activin A, which in turn encourages differentiation in early gestation to form the cytotrophoblast. The cytotrophoblast cells are commonly known as placental trophoblast stem cells and differentiate into three types, all essential for appropriate placental formation; (i) extravillous anchoring trophoblast cell columns, (ii) invasive intermediate trophoblasts, and (iii) villous syncytiotrophoblasts (Kliman *et al.*, 1986). The trophoblast cell columns are made up of large mononuclear cells that express a distinct fibronectin, trophouteronectin (TUN), which appears to be involved in both attachment and invasion of the placenta to the uterus (Feinberg *et al.*, 1991; Kliman, 1993; Feinberg *et al.*, 1994).

Some of the cells within the trophoblast cell columns are known as invasive intermediate trophoblasts and as such are capable of extracellular matrix degradation, a necessity for trophoblast invasion. Known as intermediate trophoblasts, as their size is in between that of the cytotrophoblast and the syncytiotrophoblast, these cells express collegenases

and plasminogen activator inhibitor type 1, both of which would assist in their invasive task (Shih & Kurman, 2001; Singer *et al.*, 2002). A group of proteases, matrix metalloproteinase's (MMP), are essential for trophoblastic invasion. *In vitro* studies have shown that activin-stimulated decidualisation enhanced secretion of MMP-2 from cytotrophoblasts that promotes tip outgrowths of the trophoblast cell columns (Caniggia *et al.*, 1997). Additionally, alternate studies of invasion have shown that *in vitro* invasion is dependant on the expression of MMP-9 (Librach *et al.*, 1994). MMP's appear to be stimulated via activin A during decidualisation during the first 10 weeks of gestation whilst follistatin suppresses their actions from late in first trimester (Bearfield *et al.*, 2005). Throughout decidualisation macrophage inhibitory cytokine (MIC-1) is up-regulated and stimulates decidualisation, however it appears to act as a negative regulator of MMP- 2 and 9 during this process (Marjono *et al.*, 2003).

TGFβ also appear to have distinct roles in the cytotrophoblast outgrowth. TGFβ1 and β2 are the most abundant forms in the cytotrophoblasts but have somewhat different modes of action. TGFβ1 inhibits cell invasion by providing additional regulation of MMP's through targeting MMP inhibitors TIMP-1 and TIMP-2, along with reducing invasion through inhibition of hepatocyte growth factors. TGFβ2 in the decidua has anti-proliferative effects on the extravillous cells whilst TGFβ3 from immune cells inhibits trophoblast outgrowth and decreases MMP activity along with fibronectin deposition (Caniggia *et al.*, 1997). MIC-1 appears to also have a role in regulation of invasion through inhibition of growth and apoptosis thus reducing trophoblast invasion (Morrish *et al.*, 1991).

The differentiation of the cytotrophoblast cells into the syncytiotrophoblast cells is stimulated by increasing concentrations of both α and βhCG and occurs through a process of cellular fusion (Kliman *et al.*, 1986; Kao *et al.*, 1988). As opposed to the cytotrophoblast, the syncytiotrophoblast cells are multinucleated and have a large

number of endocrine functions. Activin A and inhibin are both found abundantly in the syncytiolysing trophoblast suggesting a role in this process (Debieve *et al.*, 2006). MIC-1 is found to stimulate cytotrophoblast cells into syncytiotrophoblast formation whilst TGFβ inhibits all formation of the syncytiotrophoblast (Morrish *et al.*, 1991; Morrish *et al.*, 2001). Interestingly, BMP-4 when added to embryonic stem cells stimulates production of βhCG suggesting it has a role in formation of syncytiotrophoblast (Xu, 2006).

The syncytiotrophoblast is the major site of placental endocrine function throughout pregnancy, producing a large number of both protein and steroid hormones.

5.1.2 Placental Structure

The human placenta is classified as a haemochorial placenta. Figure 6.1 shows the 6 layers of cell types separating the maternal and fetal circulation. However, as placenta develops further both the maternal endothelial and the maternal connective tissue become completely eroded in many areas so the maternal red blood cells are in direct contact with the fetal chorionic epithelium (Bloom & Fawcett, 1968; McGeady *et al.*, 2006). Once formed the placenta provides the developing embryo with all major nutrient and waste transportation until birth. Maternal blood is fountained into the placenta through uterine spiral arteries where it circulates to reach the epithelia of the chorionic villi. Fetal arteries work through the placental mass continuously branching until blood reaches capillary loops of chorionic villi. Blood vessels in the chorionic villi form an extensive surface area to facilitate maternal/fetal exchange with nutrients crossing cellular membranes via diffusion.

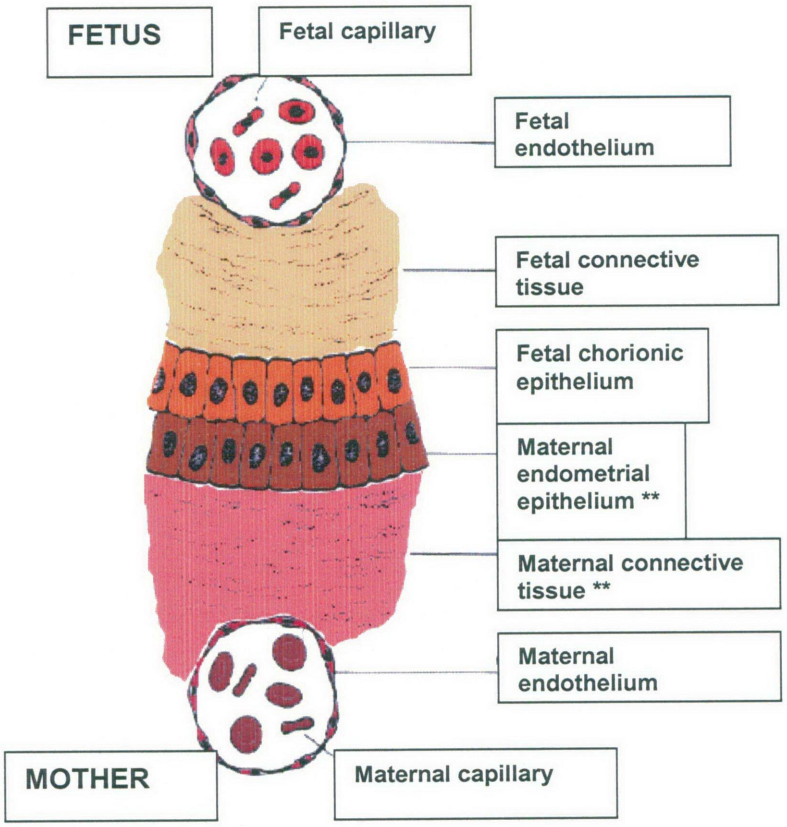

Figure 5-1 Cell types separating maternal and fetal circulation. ** Indicates cell layers that erodes through placental development across gestation.

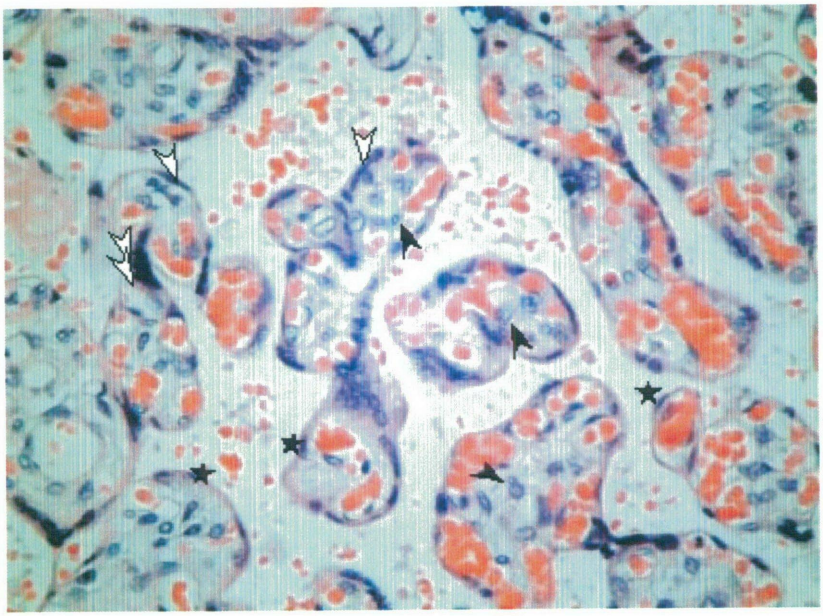

Figure 5-2 Chorionic Villi of the placenta (Haematoxylin and Eosin). Open arrow indicates syncytiotrophoblast cells, double open arrow indicates syncytiotrophoblast knots, closed arrow indicates Hofbauer cells and closed stars indicate cytotrophoblast cells.

At the cellular level, there are three main cell types within the chorionic villi of the placenta as shown in Figure 6-2, the cytotrophoblast, the syncytiotrophoblasts and the Hofbauer cells. The syncytiotrophoblast cells are known as the endocrine factory of the placenta and their functions will be dealt within detail in Section 6.1.3-Placental Endocrinology. Hofbauer cells are thought to function as placental macrophages whilst cytotrophoblasts are differentiating into the trophoblasts necessary for attachment and invasion.

5.1.3 Placental Endocrinology

In human pregnancy the anatomy of the placental interface is fully developed to support the embryo from 3-4 weeks gestation, however complete maternal blood flow through the placenta takes until 10-12 weeks gestation. The placenta has both an autocrine and paracrine role in pregnancy with the increased levels of hormones in maternal circulation probably due to placental production (Keelan *et al.*, 1999). Many of these placentally produced hormones, although chemically showing minor differences to their maternal counterparts, tend to mimic the actions of maternally derived hormones. For example, normal growth hormone (GH) is secreted from the anterior pituitary while growth hormone variant (GH-V) is from the placenta. Both molecules are 22kDa and share 93% homology. Both hormones bind to both the growth hormone receptor and the prolactin receptor, however both receptors bind GH-V with greater affinity (Ogren & Talamontes, 1994). Some of the endocrinology of the placental cell types is shown in Table 5.1.

Following stimulation of syncytiotrophoblast cells with hCG, these cells begin production of human placental lactogen (hPL), which is maintained throughout gestation and thought to have a role in lactation (Bhaumick *et al.*, 1992). hPL production by the syncytiotrophoblasts is stimulated by cAMP and EGF as well as by the production of high density lipoprotein (HDL) via the protein kinase C-dependant pathway (Lambot *et*

al., 2005). Prolactin, which is 67% homologous to hPL, is found within the syncytiotrophoblasts, however the mRNA for prolactin is found only within the decidual cells, suggesting the trophoblast cells actively uptake the protein (Jabbour & Critchley, 2001). Chorionic adrenocorticotropin (cACTH) is also found within the syncytiotrophoblasts and its release is stimulated by interleukin 1β (IL-1β). The role of cACTH is as yet unclear (Yagel *et al.*, 1989). Syncytiotrophoblast cells contain small quantities of parathyroid hormone related protein (PTH-rP) whilst the cytotrophoblast cells contain quite large amounts. It appears that the trophoblast cells contain PTH-rP in order to facilitate calcium transport form maternal to fetal circulations independent of maternal intervention (Emly *et al.*, 1994). The dual actions of the cytotrophoblast and the syncytiotrophoblast cells maintain a calcium concentration in the placenta equivalent to that found within the parathyroid (Dunne *et al.*, 1994; Farrugia *et al.*, 2000).

Within the syncytiotrophoblast, mRNA for chorionic somatomammotropin (GH-V) is found. This growth hormone variant shows minor differences from the adult growth hormone, probably due to an alternate splicing of the placental form (Sbracia *et al.*, 2004).

The placenta is also capable of producing a number of hypothalamic hormones, corticotropin releasing hormone (CRH), gonadotropin releasing hormone (GnRH), thyrotropin releasing hormone (TRH) and growth hormone releasing hormone (GHRH), of these very few have been localized to a precise tissue (Siler-Khodr, 1983; Petraglia *et al.*, 1989; Okamoto *et al.*, 1990; Petraglia *et al.*, 1992; McLean & Smith, 2001). CRH is found in the syncytiotrophoblasts and, although its role is unknown, increased concentrations are found with onset of preterm labour. It can be stimulated by increases in IL-1β concentrations. GnRH protein is found within the cytotrophoblast and secreted by cAMP pathway, all augmented under high estrogen concentrations.

Transforming growth factor β is found within the syncytiotrophoblast cells and believed to be implicated in the stimulation of trophoblasts to produce the uterine fibronectin, trophouteronectin (TUN), that is necessary for placental attachment and invasion (Jones *et al.*, 2006). Epidermal growth factor (EGF) and EGF receptors have also been localized to the syncytiotrophoblast and its cell surface. Increased concentrations of either TGFα or EGF can increase the mitotic rate of the cytotrophoblasts thus increasing placental size (Wright *et al.*, 2006).

The protein inhibin is found within the syncytiotrophoblast although its mRNA is only found within the cytotrophoblasts. The inhibin binding protein betaglycan has also been localized to the surface of the syncytiotrophoblast cells (Jones *et al.*, 2002c). EGF can stimulate the secretion of inhibin by cytotrophoblasts as can prostaglandins. Cytotrophoblast cells are thought to be the site of relaxin production. Pro-relaxin is localized to these cells and culture studies have demonstrated relaxin secretion (Hombach-Klonisch *et al.*, 2001).

The syncytiotrophoblast cells contain the enzymes necessary for side chain cleavage of cholesterol that results in progesterone. This process is up-regulated by cAMP and GnRH, whilst hCG suppresses (Bonenfant *et al.*, 2000). Estrogen cannot be produced in syncytiotrophoblast cells using either progesterone or cholesterol as starter as the appropriate enzymes are not there. However, the syncytiotrophoblasts can produce estrogens from fetal dehydroisoandrosterone from the fetal adrenal (Zbella *et al.*, 1986). This process is also up-regulated by cAMP but suppressed by insulin-like growth factor II and insulin.

Table 5-1 Some of the endocrinology of placental cells

Cell Type	Stimulated by	Produces	Effect
Trophoblast	• activin	• hCG	Differentiation into cytotrohoblasts
Cytotrophoblast	• EGF and prostaglandins	• Inhibin • Relaxin	
• Anchoring trophoblast		• Fibronectin / trophouteronectin (TUN)	Attachment and invasion
• Invasive trophoblast		• Collegenases • Plasminogen activator inhibitor type 1	Placental invasion
Syncytiotrophoblasts	• IL-1β • IL-1β • GnRH •	• hPL • cACTH • PTH-rP • GH-V • CRH • TGFβ • Progesterone derived from cholesterol • Estrogen derived from fetal dehydroisoandrosterone	• ?lactation • calcium transport • unknown but greatly increased in premature delivery • assist in trophouteronectin production

5.1.4 Follistatin, Activin and Inhibin in the Placenta

Homogenate studies by a variety of groups have shown that the placenta produces mRNAs that encode inhibins, activins and follistatin. de Kretser *et al.* (1994) suggested that inhibin A and B, activin A, AB, and B and three forms of follistatin were present in placental homogenate, whilst Yokoyama *et al.*, (1995) showed only follistatins and activin A (de Kretser *et al.*, 1994; Yokoyama *et al.*, 1995). While these two groups show some variation with activins, as far as follistatin goes, both have shown follistatin mRNA in the placenta, however which isoform is present has not been elucidated. However as both of these studies used placental homogenates, the cellular location of the follistatin mRNA was not determined.

It has been suggested, based on *in vitro* data, that the feto-placental unit is responsible for production and regulation of these peptide hormones. Unfortunately the regulation process *in vivo* is still very poorly understood. What is known, however, is that fetal and

trophoblast membranes produce activin type II and type IIB receptors as well as producing follistatins, inhibins and activins (Petraglia *et al.*, 1994b). The presence of the receptors alongside the peptides suggests an autocrine/paracine role in the feto-placental unit.

Previous immunohistochemical studies have localized activin A to amnion, chorion, trophoblasts, and maternal decidual cells (Qu & Thomas, 1995). mRNA to the subunit activin βA is expressed in the epithelial layer of the amnion and chorion (Petraglia *et al.*, 1994a; Petraglia *et al.*, 1994b). Figure 6-3 shows the localization of follistatin, activin and inhibin within the placental cells.

The amnion layer also contains mRNA for the activin type II and IIB receptors (Petraglia *et al.*, 1994b). Additional studies have shown activin receptors are also located in the endothelial cells of placental blood vessels (Jones *et al.*, 2002a). *In vitro* culture studies have shown that the addition of activin to cytotrophoblast cultures promotes the outgrowth of cytotrophoblast columns into the surrounding matrix, thus suggesting a role in endometrial invasion. The column outgrowths were accompanied by characteristic markers of invasion, such as cell division at the proximal end of the outgrowth, fibronectin production and production of factors such as human leukocyte antigen G and MMP-9 (Caniggia *et al.*, 1997). Addition of activin to cultured cytotrophoblast cells stimulates the production of both progesterone and hCG (Petraglia *et al.*, 1989; Petraglia *et al.*, 1992; Petraglia *et al.*, 1994b) . This effect is internally regulated by inhibin and also by the addition of follistatin to cultures, blocking all the effects of activin, again confirming follistatin and inhibin's roles as activin modulators (Caniggia *et al.*, 1997).

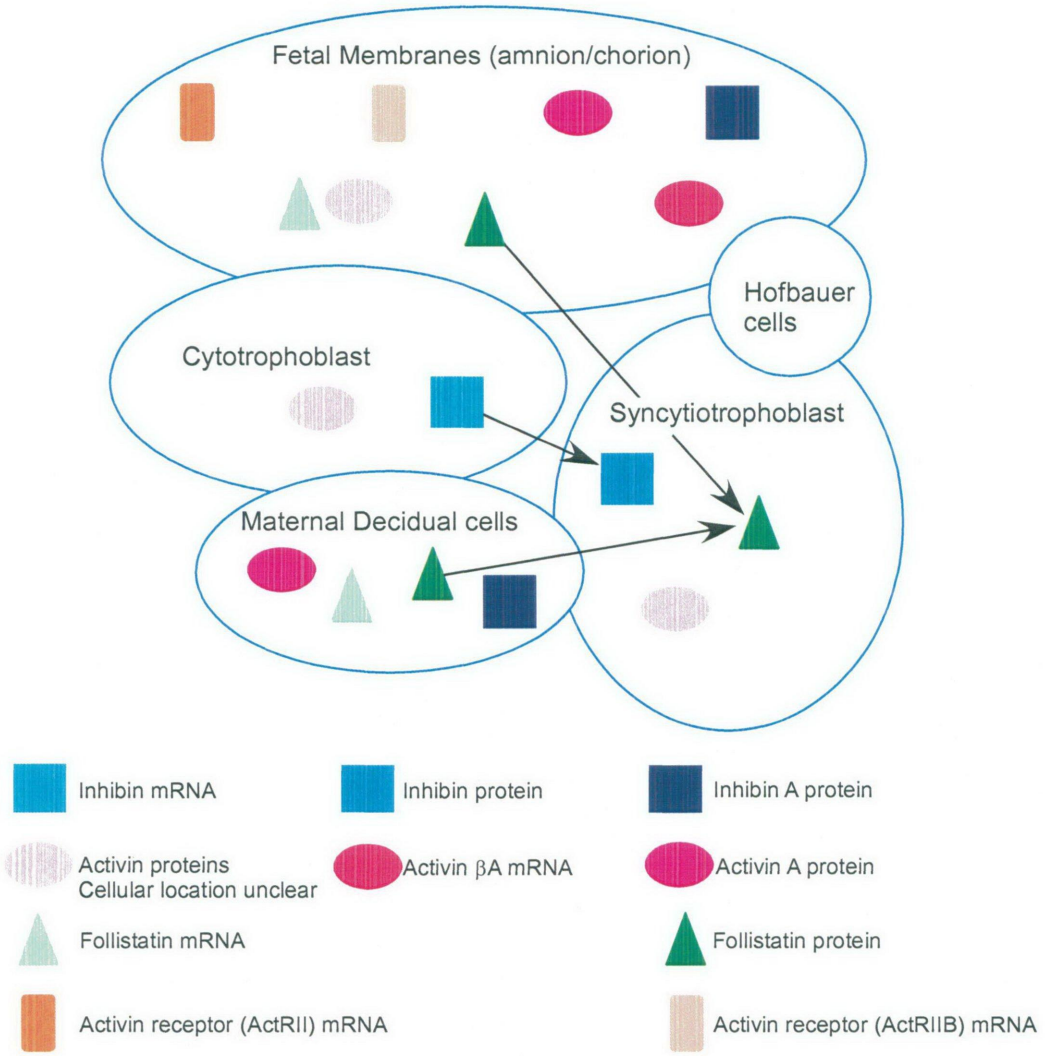

Figure 5-3 Localisation of follistatin, activin and inhibin protein and mRNA in placental cells (Petraglia *et al.* , 1994b; Petraglia, 1997; Keelan *et al.* , 1999; Jones *et al.* , 2002c)

Studies to localize follistatin have shown it to be in amnion epithelial and chorion cells as well as the syncytiotrophoblast, decidual and vascular endothelial cells (Petraglia *et al.*, 1994b; Jones *et al.*, 2002b). However, these studies used caesarean patients only, so that different follistatin expression due to vaginal labour has not been investigated. This study aims to determine the histological location of follistatin in the placenta in the spontaneous onset vaginal delivery patient. These results will then be compared to those of non-labouring patients (LSCS delivery) and patients undergoing induction procedures to instigate labour.

5.2 Methods and Materials

Details regarding the recruitment of patients, ethics approval, and assays have been previously described in Chapter 2 – General Materials and Methods.

5.2.1 Sample Collection

Fresh whole placenta were obtained following delivery of child and transported immediately to Pathology New England. Whole placentae were cut into two portions, the first of which was placed in phosphate buffered formalin until required. The second placental portion was cut into smaller wedges of tissue (approximately $5cm^3$) and frozen at –25° C until assayed.

Post delivery the placenta tissues were divided according to patient groups as previously outlined (Spontaneous onset vaginal delivery, Induced onset vaginal delivery and Non-labouring patients LSCS delivery). A total of 8 placentae were used for each group.

This study was approved via the New England Area Health service Clinical Research Group and Ethics Committee's as well as the University of New England Ethics Committee.

5.2.2 Immunohistochemistry method for follistatin:

Follistatin distribution in the placenta was determined using a immunohistochemistry techniques with the JMCK20 antibody. Complete antibody details have been discussed in Chapter 2, Section 2.3. Briefly, immunohistochemistry was conducted using specific chicken polyclonal antiserum (JMCK20) raised against follistatin peptide sequence AA 121-133. A microwave antigen retrieval step was performed prior to incubation with primary antibodies diluted to 0.01mg/ml. Localization was detected by sequential application of biotinylated rabbit anti-chicken IgG and alkaline phosphatase-conjugated streptavidin, and visualized with BCIP/NBT. A negative control was included for each

tissue section by substitution of the primary antibody with matched concentration of pre-immune chicken serum. This method has been taken from (Xia, 2001).

5.2.3 Determination of staining results:

In order to ensure the accuracy of both the cellular location and the intensity of staining all slides were reviewed by practicing clinical Pathologist Dr Vithia Chetty and Dr Lance Meng of Hunter New England Pathology.

5.3 Results:

5.3.1 Immunohistochemistry

Normal chorionic villi have been shown in Figure 5-2 with cell types within the chorionic villi labeled on image.

In Figures 5.4, 5.5 and 5.6 the chorionic villi of the three patient groups are displayed. The chorionic villi of spontaneous patients show positive staining for follistatin protein of the syncytiotrophoblasts and some minor staining of cytotrophoblasts and the Hofbauer cells. Induced patients are negative for syncytiotrophoblast staining although show minor staining of the cytotrophoblast cells and strong staining of the Hofbauer cells. LSCS patients show no staining of these three cell types. Figures 5.7, 5.8 and 5.9 also show the chorionic villi focusing particularly on the vascular endothelial cells surrounding capillaries. Both spontaneous and induced patients show strong staining around these vessels whilst LSCS is negative for follistatin.

The maternal decidual cells are shown in Figures 5.10, 5.11 and 5.12. Interestingly spontaneous and LSCS patients show extremely strong staining in these cells whilst the induced patient group have almost a complete absence of staining.

Fetal stromal tissue showed diffuse staining throughout and was similar between patient groups shown in Figure 5.13 and 5.14. Complete results are outlined in detail in Table 5.2.

Table 5-2 Comparative results for immunohistochemistry of placenta. Numerical values indicate the intensity of staining seen.

Labour group	Cell type	Intensity
Spontaneous (Refer to Figures 5.5, 5.7, 5.10, 5.13 and 5.14)	Hofbauer	1+
	Syncytiotrophoblast	2+
	Cytotrophoblast	1+
	Decidual cells –maternal interface	3+
	Fetal Interface	Diffuse
	Vascular endothelial cells	3+
Induced (Refer to Figures 5.5, 5.8, 5.11, 5.13 and 5.14)	Hofbauer	3+
	Syncytiotrophoblast	0
	Cytotrophoblast	1+
	Decidual cells - maternal interface	1+
	Fetal Interface	Diffuse
	Vascular endothelial cells	3+
Caesarean (Refer to Figures 5.6, 5.9, 5.12, 5.13 and 5.14)	Hofbauer	0
	Syncytiotrophoblast	0
	Cytotrophoblast	0
	Decidual cells – maternal interface	3+
	Fetal Interface	Diffuse
	Vascular endothelial cells	0

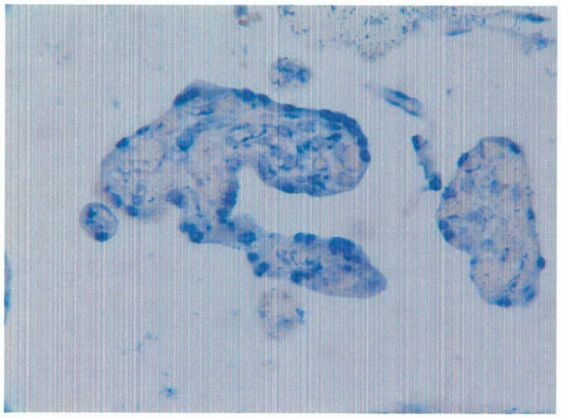

Figure 5-4 Chorionic villi -Spontaneous
Positive staining of the syncytiotrophoblasts is shown along with strong staining of the Hofbauer cells and minor staining of cytotrophoblasts.
(Field of view – 25μm)

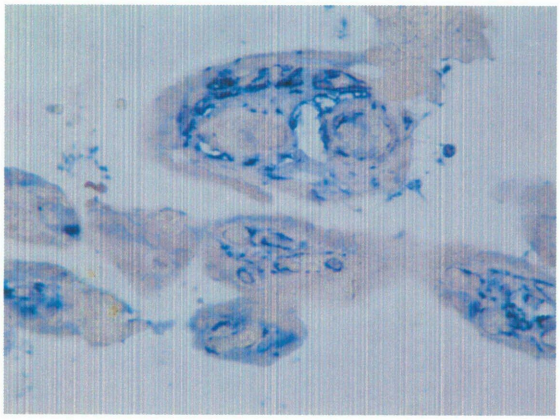

Figure 5-5 Chorionic villi -Induced
Some positive staining of the Hofbauer cells along with minor staining of the cytotrophoblast cells, however no staining of the syncytiotrophoblasts is seen.
(Field of view – 25μm)

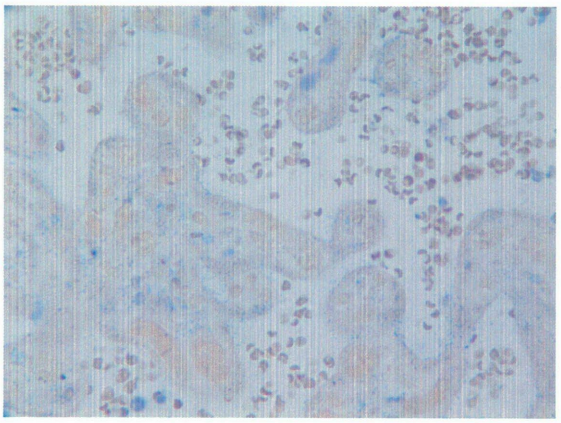

Figure 5-6 Chorionic villi – LSCS
No staining of syncytiotrophoblasts, cytotrophoblasts, and Hofbauer cells is seen.
(Field of view – 25μm)

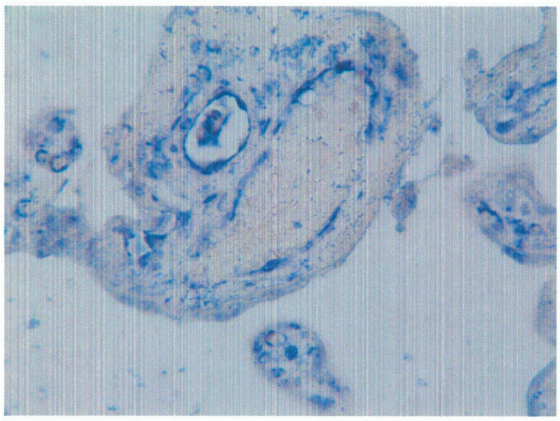

Figure 5-7 Vascular endothelial cells – Spontaneous
Positive staining of vascular endothelial cells
(Field of view – 25μm)

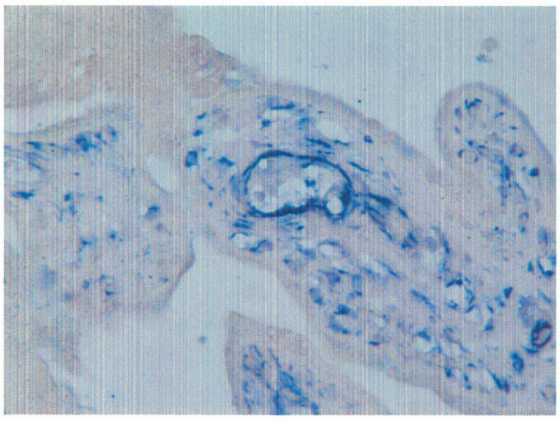

Figure 5-8 Vascular endothelial cells – Induced
Positive staining of vascular endothelial cells
(Field of view – 25μm)

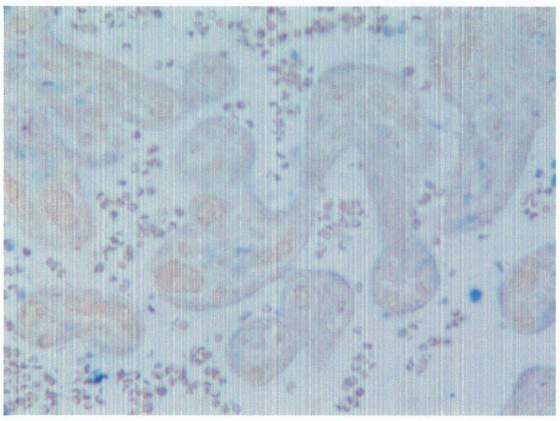

Figure 5-9 Vascular endothelial cells – LSCS
No staining of the vascular endothelial cells is seen
(Field of view – 25μm)

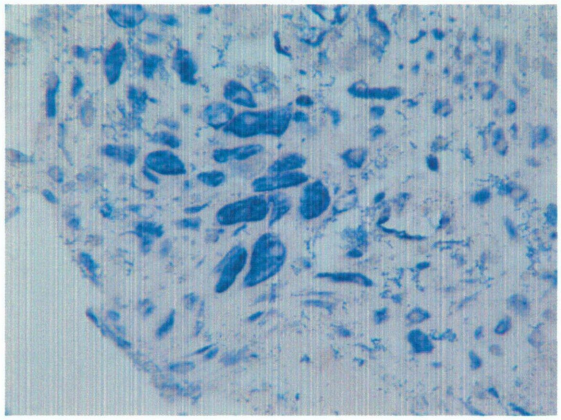

Figure 5-10 Maternal interface – Spontaneous
Strong staining of the decidual trophoblast cells of the maternal interface is evident
(Field of view – 25μm)

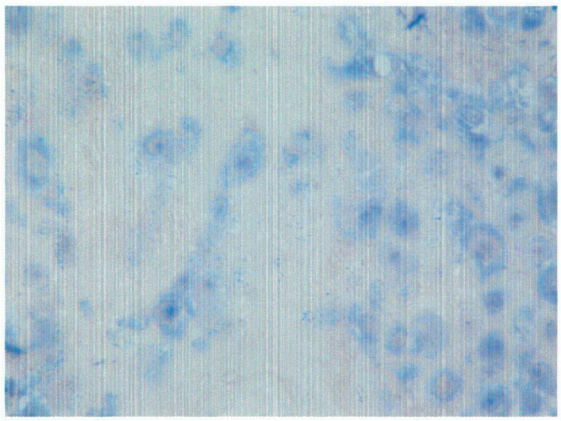

Figure 5-11 Maternal Interface - Induced
Non existent staining of the decidual cells of the maternal interface
(Field of view – 25μm)

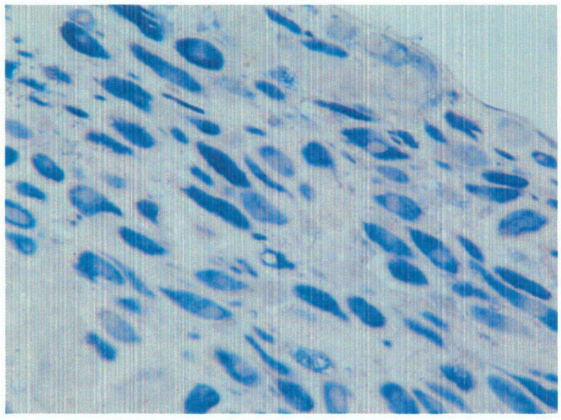

Figure 5-12 Maternal Interface - LSCS
Strong staining of decidual trophoblast of the maternal interface is shown
(Field of view – 25μm)

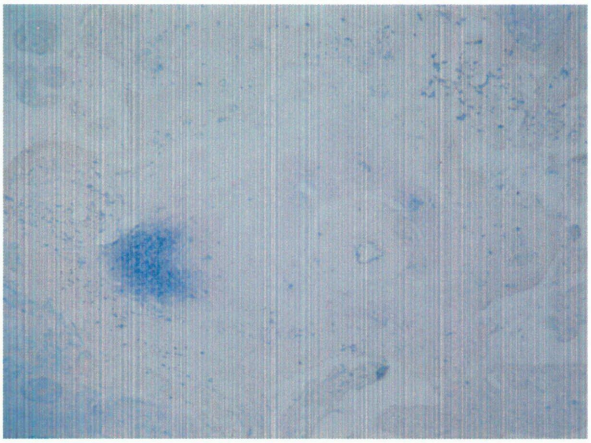

Figure 5-13 Fetal Interface – All groups showed diffuse staining of the fetal interface (10X)
(Field of view – 200μm)

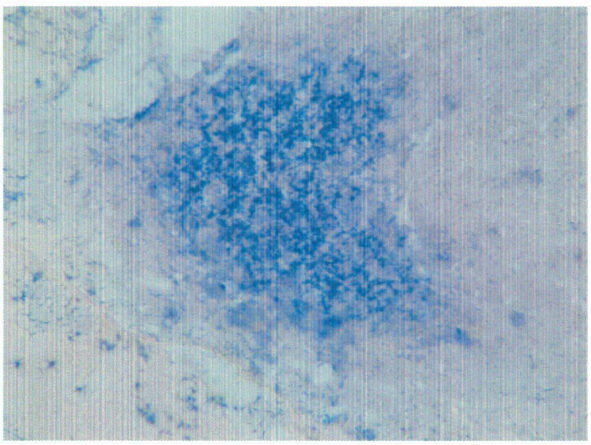

Figure 5-14 Fetal Interface – All groups showed diffuse staining of the fetal interface (40x)
(Field of view – 25μm)

5.4 Discussion:

Spontaneous patients are all term patients with no medical problems that had naturally begun labour and delivered a healthy child via a vaginal delivery. However, the induced group, were all induced as they had shown no inclination to begin the onset of labour despite being post dates. Inductions were performed using a prostin pessary to enhance the favourability of the cervix, and IV syntocin was used to begin uterine contractions, and if necessary, an amnioectomy was performed to rupture the membranes. Each of the women in this group had no medical complications and all offspring were healthy and delivered via a normal vaginal delivery. Anecdotally obstetric staff have discussed this group of women in some detail. The induced group have all failed to initiate a natural labour onset, and potentially lack the ability to do so. This group of women when induced, often fail to progress in labour and have higher rates of operative vaginal deliveries and increased rates of emergency C-sections. This suggests that these women have a physiological reason for fail to initiate parturition. The caesarean section group had no onset of labour and all had undergone a planned C-section, for either breech presentation, or have had a previous caesarean delivery. None of the group had any medical complications and all offspring were healthy. In summary to the best of our knowledge, we have studied one group that initiate and labour spontaneously, a second group who are unable to begin parturition and are chemically induced, and a third group who have the potential to labour but have been surgically apprehended prior to doing so.

Previous work on term placenta has shown follistatin mRNA to be localized to both the fetal membranes (amnion/chorion) and the maternal interface within the decidual cells (Petraglia *et al.*, 1994b). Studies have shown the protein to be expressed in both these cell types but also have localized follistatin protein to the syncytiotrophoblast cells of the

chorionic villi (Petraglia *et al.*, 1994b; Keelan *et al.*, 1999; Jones *et al.*, 2002b). However, none of these studies have considered the implications of the type of labour onset. It should also be noted that our antibody is distinctly different from these other groups, having been raised against an internal sequence of the follistatin protein present in all isoforms and should therefore recognize all follistatin variants. As shown in Chapter 3, the JMCK20 antibody is recognizing molecular weights of 20 kDa through to molecular weights of approximately 75 kDa. The data in Chapter 3 showed clear differences in the expression of these proteins between patient groups however, the predominant follistatin and the strongest differences appeared to be with the larger molecular weight proteins.

The questions to be asked when considering follistatin expression are, (i) how does the protein get there and (ii) what are the triggers for its expression within these cells. Our results confirm the presence of follistatin protein within the syncytiotrophoblast, however they suggest that the onset of labour is the trigger for its expression. We have shown that, depending on the type of labour undergone, the expression pattern of follistatin in the syncytiotrophoblast differs.

We suggest that the differences in expression pattern at the maternal interface in the two labouring groups, (spontaneous versus induced) is due to the aberration that determines whether or not a women will go into natural labour. The lack of follistatin within the maternal interface (decidual cells) of induced patients may be the reason that this group fails to initiate a natural onset of labour, whilst both our other groups can naturally labour. That is, we know that our spontaneous patients have laboured naturally, whilst our C-section patients have the potential to do so, and have merely been interrupted from the natural onset due to a presentation issue associated with the fetal position.

It must be noted that these two groups of women (spontaneous and induced) differ considerably in the type of triggers for uterine contraction and therefore placental changes close to delivery. Our spontaneous patients naturally go into labour, and therefore all of the natural triggers of labour occur whilst the induced patients had the uterus forced into contractions suing syntocin thus completely overriding any natural processes. Interestingly, the positive staining for follistatin seen in the fetal tissues is fairly diffuse and appears to be localized to the fetal stromal cells, regardless of the type of labour onset.

The positive follistatin staining in the syncytiotrophoblast of spontaneous patients suggests this may be the source of the rising plasma follistatin seen in this group, as discussed in Chapter 4, however, this seems unlikely as plasma follistatin concentrations remain high in maternal circulation in the early post-partum phase when the placental circulation to the mother has been cut by the placenta's removal. We also propose that the follistatin seen in the syncytiotrophoblasts of this patient group may be the source of the FS288 mRNA that has only been identified in spontaneous patients as shown in Chapter 3. However, our studies in sheep (shown in Chapter 3, Figure 3.1) suggest that follistatin drops in the early postpartum and then subsequently increases to reach maximal concentrations in the later postpartum period. It is possible that this drop immediately postpartum is being masked by rising systemic follistatin in the later postpartum period. A larger cohort of patients and more regular sampling across the postpartum and for a longer period may help elucidate this.

The complete absence of follistatin staining in the syncytiotrophoblast cells of the induced patient group suggests that an induction completely overrides the natural progression of labour, where we see such definite staining of these cell types. There is also the possibility that the differences in staining may be due to variations in labour

lengths (6.5 versus 4.5 hours respectively), however we would have expected a lower level of staining in the same cell types rather than the complete absence of staining. It is our theory that there is an additional endocrine signal that occurs prior to the rising plasma follistatin that stimulates the secretion of follistatin protein from either the fetal or the maternal compartments. This signal may well be one of the cytokines involved in the inflammatory processes necessary for the cervical ripening that precedes labour onset or perhaps a change in activin. However, data in Chapter 4 suggests that neither TNFα nor activin is responsible for the increased concentrations demonstrated in parturition.

Whilst the mechanism for follistatin protein expression in the major endocrine cell of the placenta, the syncytiotrophoblast, is unclear; what is certain, is that follistatin acts in a either/or both an autocrine and a paracrine manner within the placenta. Interestingly the syncytiotrophoblast cells contain the protein of inhibin, activin and follistatin but previous studies have failed to show mRNA for any of this group of proteins, suggesting the same for both activin and inhibin. At this point it is impossible to determine whether the follistatin we visualize within the syncytiotrophoblast cells of the placenta has originated from the maternal interface in the decidual cells or from the fetal amnion/chorion membranes.

Similarities in follistatin expression are clear between the induced and spontaneous patient groups with both groups having some staining of Hofbauer cells, cytotrophoblast cells and vascular endothelial cells. Possibly the larger molecular weight proteins discussed in Chapter 3 are those seen here. Both induced and spontaneous patients show a 76 kDa heparan binding follistatin like molecule and a 52 kDa glycosylated heparan binding protein in common. Potentially these uncharacterized proteins are responsible for the similarities we see in the staining of these patient groups, however,

extensive work is needed to truly elucidate the actions of these larger molecular weight proteins.

These immunohistochemistry images show clear differences between the type of labour onset and the localization of the follistatin protein. However, it still fails to determine where the mRNA for the follistatin isoform production is localized to and whether it changes depending on the type of labour undergone. Additional work using *in situ* hybridization would be extremely beneficial in determining this.

Chapter 6

General Discussion

Parturition involves the complex interplay of both the inflammatory and endocrine systems and much research has been directed at finding the initiator for its onset. Follistatin has been widely investigated in reproduction and in particular, due to increases in maternal serum across gestation, for its role in the onset of parturition. The high homology between species, the multiple isoforms and the variability between assays have made follistatin an incredibly complex protein to understand. Previous work on follistatin has suggested that follistatin isoforms are compartmentalized and are differentially regulated in folliculogenesis suggesting each isoform has a distinct physiological role, however this has not been investigated in pregnancy and parturition (Schneyer *et al.*, 2004b; Glister *et al.*, 2006).

This present study aimed to determine if follistatin alters in maternal and fetal serum when the onset of labour differs through investigating spontaneous labour, induced labour and elective caesarean delivery in women. Focus has also been placed on localization of follistatin protein in placental tissues and determination of mRNA isoform differences between patient groups. In addition, the effect of parity, labour length and fetal gender on follistatin has been investigated in both maternal and fetal serum.

This study has been the first to show that follistatin isoform mRNA expression in the placenta is different depending on the type of labour onset. Patients who begin labour spontaneously show FS288 mRNA and FS315 mRNA in the placenta whilst those who undergo an induction or an elective caesarean only show FS315 mRNA. The role and the localization of this mRNA is unclear, as previous mRNA studies have not differentiated between the follistatin isoforms and *in situ* hybridisation research has shown follistatin mRNA to be present in both the maternal decidua and fetal membranes

(Petraglia *et al.*, 1994b; Jones *et al.*, 2002c). However Western Blot analysis show spontaneous patients have proteins bands of 29 and 31kDa, whilst induced and caesarean delivery groups have only the 31kDa band present, confirming the mRNA data.

The results obtained during studies on heparan binding and glycosylated variants of follistatin isoforms have proved difficult to interpret. Our antibody has shown that the predominant follistatin isoforms/follistatin-like proteins of the placenta are at higher molecular weights than expected. Whilst molecular weights of 65 kDa and 78 kDA have been previously reported in human CSF, these proteins have not been characterised and therefore it is difficult to know if we are seeing a novel follistatin-like protein or merely a posttranslational variant of known follistatins (Michel *et al.*, 1996). However it is clear that as the predominant placental follistatin, these larger molecular weight proteins have distinct roles to play in parturition. Our antibody (JMCK20) recognizes an amino acid sequence within follistatin domain 1, part of which overlaps the hydrophobic residues of the activin binding site, suggesting that these larger molecules have the potential to bind activin. These molecules are likely to be very important molecules in reproduction, particularly parturition.

Spontaneous labour patients show no heparin binding follistatin proteins, despite having mRNA for the heparin binding FS288 isoform. Induced (71, 65 and 52 kDa) and LSCS (68, 60 and 52 kDa) patients both show a number of heparin binding follistatins with some bands identical between groups. The presence of heparin binding follistatins in these patient groups suggest that these heparin binding follistatins are essential for uterine quiescence through pregnancy and are perhaps involved in sequestering activin to the cell membranes via heparan sulfate proteoglycans.

Studies involving glycosylated follistatin proteins also show the predominance of high molecular weight proteins in placental tissues of all groups, however there are no clear

differences between each group. The minor differences between groups suggest that the degree of glycosylation of the follistatin protein has some role in parturition however it remains unclear. Interestingly, induced patients are the only group to show the traditionally sized isoform of 31 kDa, indicating it is important in this type of labour. Whilst these results of heparin binding and glycosylated variants have proved interesting, until antibodies specific to each isoform, and the identity of the high molecular weight isoforms characterised, and antibodies have been developed and the isoforms specifically localised within the placenta the exact role these play will remain undetermined. Purification and sequence analysis of the larger molecular weight proteins will also be necessary to determine if the protein bands seen are posttranslational modifications or novel follistatin-like proteins. When the JMCK20 follistatin antibody was designed and again more recently, the peptide sequence was reviewed in Genebank databases and found no known overlaps of this peptide with any other known proteins.

Immunohistochemistry studies using the same antibody as above showed that spontaneous patients were the only patient group to show follistatin protein staining in the syncytiotrophoblast cells of the placenta. However as discussed previously, the antibody used detects both FS288 and FS315, along with much larger follistatin proteins, so the individual isoform protein localization within the placenta remains difficult to determine. Previous research has also shown mRNA for follistatin is not present in syncytiotrophoblasts, so the source and the triggers for follistatin presence in these cells are unclear (Petraglia *et al.*, 1994b; Jones *et al.*, 2002c).

Spontaneous and LSCS patients also express intense staining of the maternal decidual cells while induced patients show no staining of the maternal decidual cells. Previous studies have shown that mRNA for follistatin is within these cells, however both previous mRNA studies used placentae obtained from caesarean section (Petraglia *et al.*, 1994b;

Jones *et al.*, 2002c), so it may be possible that women requiring an induction fail to have the mRNA for follistatin in the maternal decidua. Both labour groups show intense staining of the vascular endothelial cells however induced patients show no staining of syncytiotrophoblasts. LSCS patients show strong staining of the decidua however they show no staining of syncytiotrophoblast or the vascular endothelial cells.

We propose that the reason some women fail to advance into parturition is due to a lack of follistatin protein in the maternal decidua, and that through the natural onset of labour, follistatin protein becomes expressed in the syncytiotrophoblast cells. Obstetric anecdotes tell of these women who fail to come into labour and need chemical induction. These women are often incredibly difficult to induce into myometrial contractions – some patients taking days of prostin pessaries, and many hours of syntocin IV. Sadly, this group of women have very high rates of operative vaginal deliveries and emergency caesarean procedures. In subsequent pregnancies these women become even more unlikely to be chemically induced and each in progressive pregnancy the risk of emergency operative procedure increases dramatically. We suggest that follistatin may act directly on heparan sulfate proteoglycans to assist with uterine re-modelling or that it may act in partnership by binding with activin. However, dual staining of activin and follistatin of placental tissues is necessary to determine if activin have any role to play.

It should be noted that researchers have previously shown positive follistatin protein staining in the syncytiotrophoblast cells of LSCS patients (Petraglia *et al.*, 1994b), however a different antibody was used to the one used in this present study and therefore we make the assumption that each study is measuring either a differing isoforms or a related follistatin-like proteins. The presence of follistatin in the vascular endothelial cells in both labouring groups suggests that the myometrial contractions of labour are possibly the stimulus for its expression as no staining is seen in LSCS delivery patients.

As discussed in earlier chapters, follistatin has the ability to interact with the heparan sulfate proteoglycans that appear to be essential for development and expansion of gap junctions in the myometrium. Research has shown that in patients with reduced expression of the heparan sulphate proteoglycan, syndecan 3, have a very prolonged labour (Cluff *et al.*, 2006). Potentially the staining of follistatin protein we see in the maternal decidua, adjacent to the myometrium, provides the necessary interactions to allow myometrial matrix remodeling essential for parturition. We therefore propose that a reduced follistatin within the placenta would also lead to prolonged labour.

The antibody used to determine follistatin in serum (#204) is different from the one used in both Western Blot and immunohistochemistry studies (JMCK20). In maternal serum we see follistatin rising dramatically in spontaneous patients from very early in the labour process to reach concentrations that are 3-fold antenatal concentrations. These spontaneous onset patients also show an increased maternal serum follistatin throughout labour and the early postpartum period when compared to other delivery groups. Whilst induced patients do show some minor changes in follistatin they are almost unchanged from the antenatal period. Interestingly, both patient groups show increases in activin however these molar concentrations approximately 9-fold below that of follistatin (0.2 moles/ml versus 1.8 mole/ml respectively). When we consider that follistatin binds activin in a 2:1 ratio there is still substantial follistatin unaccounted for. Although both proteins are increasing in early labour, with such large differences in concentrations it seems unlikely that follistatin is acting as a mere activin modulator. Results suggest that it may have individual effects in parturition onset and progression. Induced patients do show higher activin than spontaneous patients, as anticipated due their reduced follistatin concentrations.

It seems clear that a natural labour onset needs follistatin to increase in circulation however the purpose of this increase is unclear. Previous studies suggest that FS315 is

the follistatin in the circulatory system, so we assume the same is true in parturition, and that the concentration increases we observe are due to FS315 (Hashimoto *et al.*, 1997; Schneyer *et al.*, 2004b).

Despite this current study being one of the largest studies on follistatin during parturition in women it is clear that an even larger cohort of women is required. Our studies reviewing follistatin concentrations along with fetal gender and labour length show that follistatin is clearly influenced by both of these parameters. It is also clear that the type of labour onset and delivery also influence follistatin results. In order to fully characterise the profile of follistatin in parturition, sufficient patient numbers are required so that gender and labour length can be assessed according to labour onset rather than grouping both induced and spontaneous onset together. As shown by previous studies, follistatin results in parturition are highly variable between women (Woodruff *et al.*, 1997), and indicate additional unknown variables are required for follistatin concentration assessment, potentially patient, fetal or placental body mass.

In summary, the present study has shown follistatin clearly plays an important role throughout the labouring period. Larger molecular weight follistatin-like proteins or isoforms are the predominant protein in the placenta however future purification and isolation studies are required to elucidate their character and roles. Both heparan binding and glycosylation of follistatins alter with labour onset and delivery and we suggest these are essential for proteoglycan interactions needed for gap junction formation along with uterine remodeling in the postpartum period. Follistatin protein localization within the placenta is influenced by the type of labour onset and may have roles in initiation of uterine myometrial contractions. Follistatin in maternal serum is up-regulated throughout a natural labour, however its concentrations are influenced by fetal gender, suggesting a maternal immune response to fetal gender. Labour length also

results in alterations to follistatin in both maternal and fetal serum. This study has shown that the changes to follistatin concentrations across parturition are distinct from changes to activin and the acute phase response. This work suggests that future studies focussed on follistatin in parturition particularly, the larger molecular weight follistatin-like proteins, and using larger patient cohort would be particularly interesting.