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THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

ON..... [redacted] .....

..... [redacted] .

Sec. Research Graduate School Committee

AMENDMENTS

LIST OF ABBREVIATIONS: Abbreviation for interferon ã should read IFNã.

CHAPTER 1: General introduction

Page 48, line 2: Party should read partly.
Page 52, line 7: Inibin should read inhibin.
Page 54, line 17: Northen should read northern.

CHAPTER 2: Validation studies for the measurement of activin A and follistatin in biological fluids
Callibration of the activin A and follistatin assays: At the time this thesis was written, there were no WHO standards for the activin and follistatin ELISAs. The standards supplied to our laboratory by Prof Nigel Groome were calibrated against the suppliers controls and the absolute results obtained in our laboratory are comparable to data arising from other groups. However, differences between laboratories remain due to the lack of a consensus standard and controls for both assays. Since we were only interested in the overall profiles and relative changes of activin and follistatin in our sample groups, those differences in absolute values are not critical to the interpretation of the results. Potential future use of activin and follistatin as diagnostic markers will necessitate the formulation of WHO standards for the calibration of all assays.
Page 72, line 15: Streptavidin should read streptavidin alkaline phosphatase.
Figure 2.1: Graph depicts means ±SEM of pooled blood from five women.
Figure 2.2: Graph depicts means ±SEM of pooled blood from five women.
Figure 2.3: Graph depicts means ±SEM of pooled blood from five women.
Figure 2.4: Graph depicts percentage changes of activin A in pooled amniotic fluid from five women.
Figure 2.5 & 2.6: The y-axis refers to ng/ml.

CHAPTER 3: Maternal Serum total activin A and follistatin concentrations during late pregnancy
Figure 3.1: The assignation of bars have been changed.
Figure 3.3: Graph depicts means ± SEM of blood samples from eight women.
Figure 3.4: The graph displayed refers to figure 3.6. Displayed are means ± SEM from ten women.
Figure 3.5: The graph depicts means ± SEM.
Figure 3.6: The graph displayed refers to figure 3.4. Displayed are means ± SEM of blood from eight women.

CHAPTER 4: Activin A and follistatin in maternal serum during labour
Figure 4.1: The graph depicts means ±SEM .
Figure 4.2: The graph depicts means ±SEM.
Figure 4.3: The graph depicts means ± SEM.
Figure 4.4: The graph depicts means ± SEM.

CHAPTER 5: Maternal serum activin A and dysfunctional labour
Page 123, line 8: Insert "of" after evaluation.

CHAPTER 6: Activin and activin receptors in myometrium at term and during labour
Page 130: Molecular weight markers used range in size from 14 – 97 kDa.
Figure 6.1: Graph depicts means ± SEM.
Figure 6.2: The whole gel cannot be reproduced as the bands have faded too much to allow rescanning.
Figure 6.4: Myometrial samples from six non-pregnant, six pregnant and six labouring women were subjected to immunohistochemistry.

CHAPTER 7: Activin betaA-subunit and activin receptors in gestational tissues at term and after labour
Page 145, paragraph 7.2.5: Insert "antibody" after Receptor IB.
Figure 7.6: The whole immunoblot cannot be rescanned as the gels have faded too much.
Figure 7.7: The graph depicts means ± SEM.

CHAPTER 8: Maternal serum activin A and follistatin in association with severe intra-uterine growth restriction

Statistical analysis: As only three groups were compared, we believe that a t-test, after log transformation of the data, is satisfactory in this case. Post hoc analysis was not necessary as we compared between each group separately.

Table 8.2: In the last column (N of IUGR/PE pregnancies), on the 4th line, 15 should be replaced by 25.

CHAPTER 9: Activin A in maternal serum and gestational tissue from pre-eclamptic pregnancies

Page 171, line 16: Replace "of" with "or".

CHAPTER 10: General discussion

Page 180, line 18 (insert prior to "since"):

This raises the question whether abnormal labour with or without fetal oxygen deprivation may stimulate placental activin A secretion. We have shown that activin A levels in women with dysfunctional, prolonged labour who require oxytocin to augment contractions are normal. This is also the case for women who require instrumental deliveries for delay during 2nd stage of delivery or for women who are post-date. These findings are further supported by our results showing no relationship between maternal activin A levels and cord pH at delivery. It is important to note however, that in our study, blood samples were collected during labour, contrasting Petraglia's studies where blood samples were collected shortly after delivery (Petraglia et al, 1994a). Nevertheless, these data argue against feto-placental hypoxia and/or acidaemia underlying increased maternal serum activin A levels observed in association with emergency deliveries. An investigation into maternal serum activin A levels during the 2nd stage of labour would be interesting. However, **(continue line 18: "since").**

Page 181, line 3: Continue with:

The demonstration that fetal activin A levels are inversely related to umbilical artery pH lend further support to this hypothesis. Larger samples will however be required to substantiate this finding.

Page 184, line 2: Insert:

More recently, our findings were further supported by a similar study showing that maternal serum activin A concentrations are significantly elevated in fetal growth restricted pregnancies compared to normal, small-for-gestational age pregnancies (Bobrow et al, 2002). This study also demonstrated that the activin:follistatin ratio was raised in the severely growth restricted pregnancies. Does this observed increase of biologically active activin in the maternal circulation originate from the feto-placental unit or is it a reflection of maternal placental disorders? In a study investigating the relationship between hypertension/PE, fetal growth and maternal activin A, Keelan et al (2002) was unable to establish any differences in activin A levels between normal controls and women with growth restricted pregnancies. But although 67% of growth-restricted pregnancies in his study were below the 3rd percentile for growth, no information was given about their umbilical artery blood flow status, an important indicator of fetal well being. Keelan concludes that the observed increased maternal activin A concentrations in women with pre-eclampsia are due to maternal disease processes contributing to hypertension via the reported actions of activin A on endothelial cells. These few published studies highlight the need for further, larger studies with well-defined criteria for fetal growth and well-being.

Page 184, line 3: Change:"another pathological condition of pregnancy associated with chronic feto-placental hypoxia is pre-eclampsia" with " It is well known that pre-eclampsia is associated with chronic feto-placental hypoxia".

REFERENCES: Include:

Bobrow C.S., Holmes R.P., Muttukrishna S., Mohan A., Groome N., Murphy D.J., Soothill P.W. (2002). Maternal serum activin A, inhibin A, and follistatin in pregnancies with appropriately grown and small-for-gestational-age fetuses classified by umbilical artery Doppler ultrasound. *Am J Obstet Gynecol*, 186 (2), 283 – 287.

Keelan J.A., Rennae T., Schellenberg J., Groome N.P., Murray M.D., North R.A. (2002). Serum Activin A, Inhibin A, and Follistatin Concentrations in Preeclampsia or Small for Gestational Age Pregnancies. *Obstet Gynnecol*, 99 (2), 267 – 274.

dedicated to my father,

.....wish you could be here

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution, and contains no material previously published or written by any other person, except where due reference is made in the text of the thesis. The work described in this thesis was carried out exclusively by myself, unless otherwise indicated.

Michal Schneider-Kolsky

ACTIVIN A IN LATE HUMAN PREGNANCY AND PARTURITION

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of

Doctor of Philosophy

SUMMARY

During normal, healthy pregnancy maternal serum concentrations of activin A, a glycoprotein belonging to the TGFβ-family of proteins, increase 10- to 20 fold during the last trimester. Concentrations of activin A also increase in amniotic fluid and levels of activin A are increased in both maternal serum and amniotic fluid after labour. *In vitro* activin A can stimulate the release of several uterotonic factors such as prostaglandins and oxytocin. Taken together, these findings have led to the suggestion that activin A may be directly involved in regulating the onset of human parturition. The aim of the studies described in this thesis was to identify potential functions of activin A during pregnancy and parturition in the human by investigating in detail the profile of activin A and it's binding protein follistatin during the closing weeks of pregnancy and during labour and by examining the tissue content and localisation of activin βA-subunit and activin receptor proteins in gestational tissues in association with labour, as well as in pregnancies characterised by feto-placental hypoxia, such as pre-eclampsia and severe intra-uterine growth restriction. The results have shown that levels of activin A or follistatin do not alter during the last two to three weeks of pregnancy or during delivery, both spontaneous onset or induced delivery. Further, there was no association between activin βA-subunit, activin receptor protein localisation and labour in placenta and fetal membranes. In myometrium, no activin receptors were localised in the smooth muscle either before or during labour. However, activin receptors were localised to the endothelium of term placental and myometrial blood vessels suggesting that activin A may have a role modulating the function of these vessels during late pregnancy. Based on these findings and sheep studies demonstrating an association between fetal hypoxia and activin A, studies

were undertaken to investigate the possible relationship between activin A and acute hypoxia during labour, as well as chronic hypoxia in severely growth restricted fetuses and pregnancies with severe pre-eclampsia. The studies have revealed that the levels of maternal serum activin A are elevated significantly during both acute "stress" episodes encountered during labour and during chronic intra-uterine hypoxia observed in cases of severe intra-uterine growth restriction and pre-eclampsia. Preliminary data show that the fetus produces increased levels of activin A during hypoxic stress. Importantly, the results demonstrate that activin A, when measured in the maternal circulation, can distinguish between small-for-gestational age fetuses and severely growth restricted fetuses suggesting that activin A may be useful as a non-invasive marker of fetal well-being during mid-to late pregnancy and in the future may assist in the detection and management of compromised fetuses.

ACKNOWLEDGEMENTS

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Those last few weeks have been chaotic and I must give special thanks to Peter for assisting me with all sorts of administrative issues relating to my PhD.

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To all of you who have helped me recruit patients and collected blood samples for my studies: a huge thanks! A special thank you to Leslie & Donna.

Delivery Suite Staff

I could not have completed my work without the on-going help from all the nurses and clinicians at the delivery suite and in theatre. Thank you all. The vampire is checking out!

David Humphrey

A great deal of time was spent getting the images in this thesis to look right! Thanks David for your help and advice over the last several years – you are probably sick of looking at placentas.

and most importantly, my family

The biggest thanks must go to my family, my husband Gadi and our children, Dana, Elli and Jordie. You have had to put up with a lot, I know! Thank you for letting me study for such a long time. Thank you for letting me run out of the house at all hours of day and night, for letting me work on weekends and for putting up with me. You have been fantastic and without your support and love I don't think I would have been able to finish my thesis. **You are the best!** I promise to bake a decent leikach for shabbat.

.....Thank you also to my four-legged friends who have provided company night and day and kept me sane.

PUBLICATIONS AND PRESENTATIONS

LIST OF PUBLICATIONS ARISING FROM THESIS STUDIES

Euan M Wallace, **Michal Schneider-Kolsky** & Prema Thirunavurakasu (2000) Activin A, inhibin A, inhibin B and parturition: changes of maternal and cord serum levels according to mode of delivery. *Br J Obstet & Gynaecol* , **107**, 704.

Schneider-Kolsky ME, D'Antona D, Evans L, Taylor N, O'Connor A, Groome N, deKretser D, Wallace EM. (2000). Maternal serum total activin A and follistatin in pregnancy and parturition. *Br J Obstet & Gynaecol*, **107**, 995 – 1000.

Jenkin G, Ward J, Loose J, **Schneider-Kolsky ME**, Young R, Canny B, O'Connor A, deKretser D, Wallace EM. Physiological and regulatory roles of activin A in pregnancy. *Mol Cell Endocrinol* (accepted for publication).

Schneider-Kolsky ME, Manuelpillai U, Wallace EM. Activin A and activin receptors in human myometrium at term and during labour. *Br J Obstet & Gynaecol* (accepted for publication).

Manuelpilai U, **Schneider-Kolsky ME**, Dole A, Wallace EM. Activin A and activin receptors in gestational tissue from preclamptic pregnancies. *J Endocrinol* (accepted for publication).

Schneider-Kolsky ME, Manuelpillai U, Dole A, Wallace EM. Distribution of βA-subunit and activin receptors in gestational tissues during pregnancy, at term and after labour. *Placenta* (accepted for publications).

MANUSCRIPTS IN PREPARATION

Schneider-Kolsky ME, Baker L, Edwards A, Dole A, Wallace EM. Maternal serum activin A is elevated in association with severe intra-uterine growth restriction. *N Engl J Med.*

Schneider-Kolsky ME & Wallace EM. Umbilical arterial activin A in association with fetal hypoxia during delivery. *Lancet.*

Manuelpillai U, **Schneider-Kolsky ME,** Thirunavukarasu P, Waldron K, Wallace EM. Effect of hypoxia on placental activin A, inhibin A and follistatin. *Placenta.*

LIST OF ABSTRACTS PRESENTED AT CONFERENCES

Schneider-Kolsky ME, Baker L, Edwards A, Jenkin G, Dole A, Wallace EM. Maternal activin A is increased in intra uterine growth restricted but not small-for-gestational age pregnancies. Perinatal Society of Australia and New Zealand, 5th Annual Congress, 13 – 16 March 2001, Canberra, Australia.

Schneider-Kolsky ME, Manuelpillai U, Dole A, Wallace EM. Activin A and activin receptors in gestational tissues of normal and pre-eclamptic pregnancies. International workshop on Inhibins, Activins and Follistatins, 26 – 28 October 2000, Melbourne, Australia.

Jenkin G, Loose J, **Schneider-Kolsky ME**, Earley P, Hooper S, Young R, Canny B, O'Connor A, deKretser D, Wallace EM. Physiological and Regulatory Roles of Activin in Pregnancy. International workshop on Inhibins, Activins and Follistatin, 26 – 28 October 2000, Melbourne, Australia.

Manuelpillai U, **Schneider-Kolsky ME**, Thirunavakarasu P, Waldron K, Wallace EM. Effects of hypoxia on first trimester placental activin A and inhibin A synthesis. International workshop on Inhibins, Activins and Follistatins, 26 – 28 October 2000, Melbourne, Australia.

Schneider-Kolsky ME, Jenkin G, Groome NP, Wallace EM. Increased maternal serum activin A in association with intra-uterine growth restriction. 11th International Congress of Endocrinology, 29 October – 2 November 2000, Sydney, Australia

Manuelpillai U, **Schneider-Kolsky ME**, Thirunavukarasu P, Wallace EM. Hypoxia downregulates placental activin A and inhibin A synthesis. 11th International Congress of Endocrinology, 29 October – 2 November 2000, Sydney, Australia.

Euan M Wallace, **Schneider-Kolsky ME**, Thirunavukarasu P, Manuelpillai U. Ella Macknight Memorial Lecture. Inhibins in pregnancy: Transforming Growth Factors Transforming Care. The Royal Australian & New Zealand College of Obstetricians & Gynaecologists. Annual Scientific Meeting, 25 – 30 June 2000, Cairns, Australia.

Schneider-Kolsky ME, Manuelpillai U, Wallace EM. Activin A receptors and activin βA-subunit in human myometrium at term. Perinatal Society of Australia & New Zealand, 4th Annual Congress, 12 – 15 March 2000, Brisbane, Australia.

Manuelpillai U, **Schneider-Kolsky ME**, Wallace EM. Placental activin A and activin receptor levels in women with severe pre-eclampsia. Perinatal Society of Australia & New Zealand, 4th Annual Congress, 12 – 15 March 2000, Brisbane, Australia (Winner "Best 10 Posters" Award).

Schneider-Kolsky ME, Jenkin G, Wallace EM. Maternal serum activin A, follistatin and prostaglandins during spontaneous human labour. Perinatal Society of Australia & New Zealand, 3rd Annual Congress, 21 - 24 March 1999, Melbourne, Australia.

LIST OF ABBREVIATIONS

α_2M	Alpha$_2$macroglobulin
ACTH	Adrenocorticotropin-releasing hormone
BSA	Bovine serum albumin
C	Celsius
cAMP	cyclic AMP
cm	Centimeter
cpm	Counts per minute
CRH	Corticotropin-releasing factor
CSF-1	Colony-stimulating factor 1
dH$_2$O	Destilled water
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FS	Follistatin
FSH	Follicle stimulating hormone
g	Grams
GnRH	Gonadotropin-releasing hormone
h	Hours
hCG	Human chorionic gonadotrophin
HCL	Hydrochloric acid
HPA axis	Hypothalamic-pituitary-adrenal axis
hPL	Human placental lactogen
hr	Human recombinant
IL-1	Interleukin-1
IL-6	Interleukin-6
INFα	Interferon alpha
INFβ	Interferon beta
INGγ	Interferon gamma
Kd	Dissociation coefficient
KDa	Kilo Dalton
LAB	Labouring
LH	Luteinising hormone
LN	Liquid nitrogen
M	Molar concentration
mL	Milliliter
Mol wt	Molecular weight
MoM	Multiple of the median
mRNA	Messenger ribonucleic acid
ng	Nanograms
nM	Nannomolar
nm	Nanometer
NO	Nitric oxide
NP	Non-pregnant
OT	Oxytocin
O_2	Oxygen

P	Pregnant
PAPP-1	Pregnancy-associated plasma protein-A
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PE	Pre-eclampsia
pg	Picogram
PGDH	Prostaglandin dehydrogenase
PGE_2	Prostaglandin E_2
PGEM	Prostaglandin E-metabolites
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFM	Prostaglandin F-metabolites
PGHS	Prostaglandin endoperoxide H synthase
PGI_2	Prostacyclin
PIH	Pregnancy induced hypertension
pM	Pico molar
PT	Pre-term
RIA	Radioimmunoassay
rpm	Rotations per minute
RT PCR	Reverse transcriptase polymerase chain reaction
RT	Room temperature
SEM	Standard error of the mean
SGA	Small-for-gestational age
SP1	Schwangerschaftsprotein 1
TGFβ	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
TRIS	Tris(hydroxymethyl)methane
TXA2	Thromboxane A2
µl	Microliter
µM	Micromolar
IUGR	Intra-uterine growth restriction
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

FIGURES

TABLES

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 Discovery of the inhibin family of proteins

Experiments conducted during the 1920s and 1930s resulted in the prediction of a new group of proteins capable of modulating the secretion of follicle-stimulating hormone (FSH) from the anterior pituitary gland. Until that time it was accepted that only gonadal steroids were capable of influencing the release of FSH in a negative endocrine feedback manner. The first reported study which proposed the existence of such a novel substance came from Mottram and Cramer (1923) who observed that destroying the seminiferous tubules of the testis by exposure to radium, a treatmennt that left Leydig cell secretion of androgens intact, caused a marked hypertrophy of the anterior pituitary gland. A few years later McCullagh (1932) showed that castration induced hypertrophy of the pituitary and that this reaction was prevented by injections of a water-soluble testicular extract. Since steroid hormones are hydrophobic McCullagh concluded that an unknown protein regulator was present in the testis. He subsequently named this new hormone "inhibin". These observations initiated a wave of unsuccessful attempts to isolate and characterize inhibin (reviewed by de Jong 1979). However, despite repeated failures during the 1970's evidence for the existence of inhibin increased as several groups identified an FSH-inhibiting fraction in testis extracts (Keogh et al, 1976), fluid from the rete testis (Setchell and Jacks, 1974) and seminal plasma (Franchimont et al, 1972). The problems these groups had in isolating inhibin from testicular fluid (rete testis fluid) were related to the small initial sample volume available and to the rather low yield of inhibin present from these fluids, secondary to the high loss of protein after the purification steps. The

'breakthrough' however came when it was recognised that ovarian follicular fluid also contained high levels of inhibin (de Jong and Sharpe, 1976, Schwartz and Channing, 1977) and since follicular fluid was easily accessible in large volumes, this finally allowed the sequencing of the N-terminal amino acids of inhibin by the mid 1980s (Robertson et al, 1985, Ling et al, 1985, Miyamoto et al, 1985). Cloning and identification of the structure of inhibin was achieved shortly thereafter (Mason et al, 1985).

During the isolation and characterisation of inhibin, a functionally antagonistic but structurally related protein capable of stimulating the production and secretion of FSH *in vitro* was identified in porcine follicular fluid. It was named "activin" (Vale et al, 1986, Ying, 1988). At the same time as activin was identified it became clear that inhibin was not the only protein in follicular fluid which could inhibit FSH production. Two research groups independently isolated a structurally distinct substance, which had similar effects on FSH production in cultured rat anterior pituitary cells as inhibin (Ueno et al, 1987, Robertson et al, 1987). However, in contrast to pituitary cells treated with inhibin, the cells exposed to this substance, called follistatin, had significantly lower levels of intracellular FSH depletion suggesting that follistatin acted principally on the suppression of FSH release in contrast to inhibin which affected both FSH release and FSH synthesis (Ying, 1988). Using double-ligand blotting it was subsequently shown that follistatin was a binding protein that could bind to both inhibin and activin. However, whereas binding to inhibin had no effects on the actions of inhibin, binding of follistatin to activin neutralized the biological activity of activin (Shimonaka et al, 1991), thereby "mimicking" the FSH suppressing effects of inhibin. Thus, within a few years, the

confirmation of the existence of inhibin and the subsequent discovery of a family of inhibin-related proteins led rapidly to significant advancements in the understanding of those proteins and their regulation in the gonadal-pituitary axis and, as it quickly became apparent, their roles in many other organ systems. In particular, several investigators reported that inhibin and activin secretion was increased during pregnancy and in subsequent studies it was shown that the placenta was the major site of this production (Abe et al, 1990, Que et al, 1991, Tabei et al, 1991, Minami et al, 1992, Petraglia et al, 1991a, 1991c, 1992).

It is the focus of the studies in this thesis to investigate in detail the profiles and possible roles of activin A during human pregnancy and parturition. The general introduction will initially provide an overview of the structure, function and biological actions of activin A and follistatin, followed by a description of the anatomy and function of the gestational tissues during pregnancy and labour and lead into a review covering the current understanding of activin A during pregnancy and its proposed functions during parturition. Finally, the hypotheses and aims of this program of research will be stated.

1.2 Structure of inhibin and activin

Several laboratories began the task of purifying and characterising inhibin from bovine and porcine follicular fluid (Robertson et al, 1985, Miyamoto et al 1985, Ling et al, 1985, Rivier et al, 1985). Successful cloning of the proteins revealed that inhibin is a disulfide-linked heterodimer consisting of an α - and β subunit. Each subunit is encoded by separate genes. The α- and βB-subunit is localised on chromosome 2, whereas the βA-subunit is localised on chromosome 7 (Gaddy-Kurten, 1995). The

initial purification of inhibin from bovine follicular fluid revealed proteins with molecular weights (mol wt) of 58 kiloDaltons (kDa) and 32 kDa (Robertson et al, 1986). As was later shown, these proteins consist of a precursor α subunit linked to one of two homologous β subunits (mol. wt 14 kDA), named β_A and β_B (Ying 1988, Burger et al, 1988, de Kretser and Robertson, 1989, Vale et al, 1990). Proteolytic cleavage of the NH2-terminal segment of the 43 kDa precursor results in the smaller, biologically active "mature" 32 kDa isoform of inhibin (Mason et al, 1985, Forage et al, 1986, Robertson et al, 1986, McLachlan et al, 1986). Based on the dimerization between the α- and the two β-subunits there thus exists two isoforms of inhibin: inhibin A which is a heterodimer composed of the α chain linked to the β_A chain (α-β_A) and inhibin B (α-β_B). There is a 70% homology in amino acid sequences of the two β-subunits, as determined by sequencing of the N-terminal of the molecule (Ying, 1988). Between the α- and the β-subunits, the homology is 30% (Lockwood et al, 1998). Dimerization of the β-subunits generates the three known biologically active forms of activin: activin A (β_A-β_A), activin B (β_B-β_B), and activin AB (β_A-β_B). The structures of the inhibin and activin isoforms are shown in figure 1.1.

Recently, two new β-subunits were cloned from humans and Xenopus, β_C and β_D respectively (Hotten et al, 1995, Oda et al, 1995). Beta$_C$ and β_D-subunits show relatively high homology with the other β-subunits. The β_C-subunit has 51% homology to the β_A-subunits and 53% homology to the β_B-subunit. The homology between β_C and β_D-subunits itself is 63% (Ying et al, 1997).

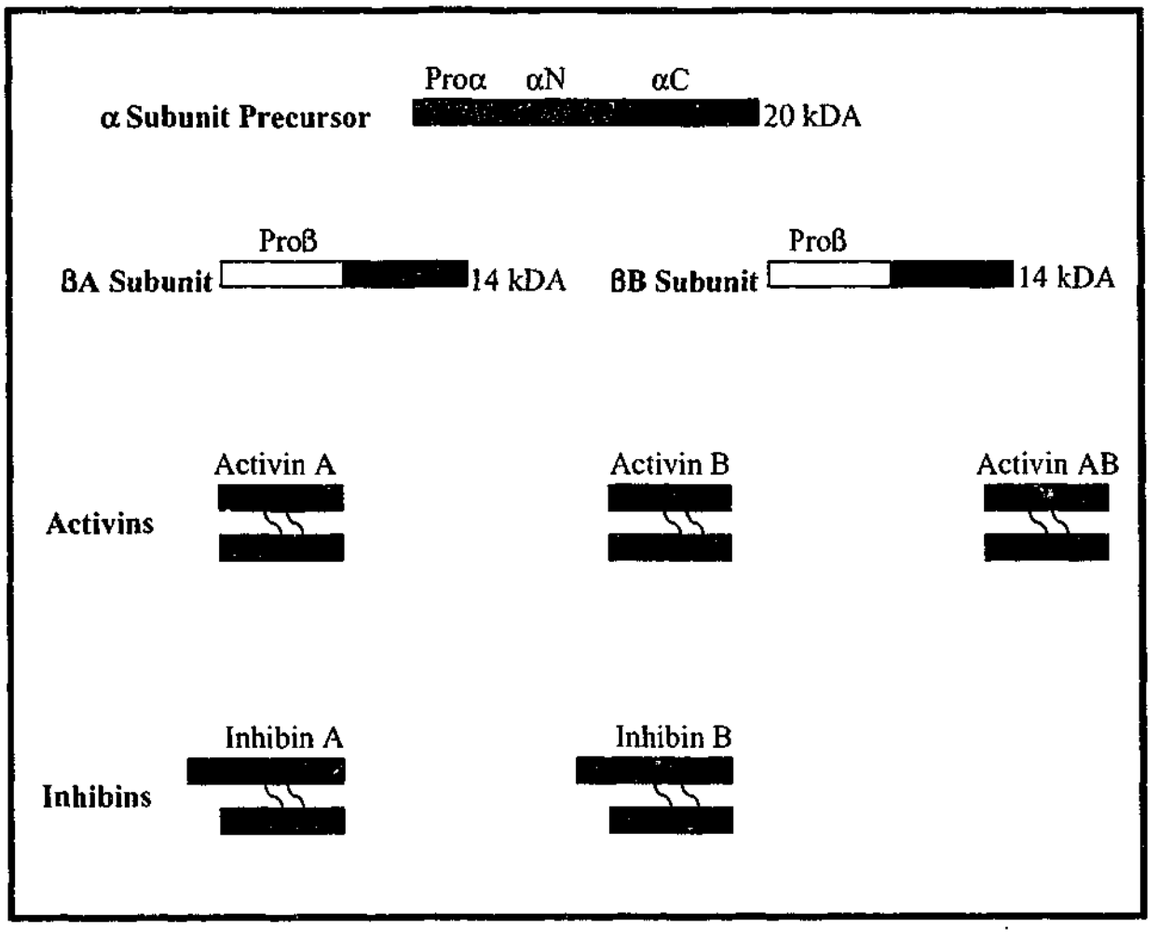

Figure 1.1. Structure of the inhibin and activin family of proteins.

Using monoclonal antibodies directed against the α- and β-subunit of 32 kDA inhibin, Miyamoto and co-workers (1986) identified six different species of inhibin with molecular weights of 120, 108, 88, 65, 55 and 32 kDA. Each form is believed to result from partial proteolytic cleavage of the α-subunit precursor. However, only the dimeric species are biologically active. Furthermore, Mason et al (1985) have shown that the large molecular weight forms of inhibin are not biologically active. It has been postulated that inhibin is released as high molecular weight forms and is then further processed via proteolytic cleavage into the biologically active form in either follicular fluid or in the peripheral circulation (Baird and Smith, 1993).

Unraveling the structure of the inhibin/activin-β dimer, it has become apparent that the β-subunit is identical to the β-subunit of the proteins belonging to the transforming growth factor-β (TGF-β) family. The inhibins and activins are hence classified as members of the TGF-β superfamily of proteins. Consistent with membership of a growth factor family, messenger RNA (mRNA) for the inhibin and activin subunit has been found not only in the ovary and testis but in a wide range of other tissues (Meunier et al, 1988) and it is now apparent that the inhibins and activins are involved in a diverse range of cellular processes including general cell proliferation and cell differentiation, embryogenesis (Roberts et al, 1991, Tuuri et al, 1994), folliculogenesis (Feng et al, 1993), spermatogenesis (Mather et al, 1990), erythropoesis (Eto et al, 1987, Broxmeyer et al, 1988), immune response (Ying and Becker, 1995), apoptosis (Nishihara et al, 1993, Hully et al, 1994) and cell death (Sewall et al, 1993), to name a few. It is likely that other actions and sites of action will continue to be identified as indeed the studies detailed later show.

Activin C has been proposed as a negative regulator of liver cell growth and has limited actions on mesoderm induction (Oda, 1995). Studies in rat testis, however, have shown that activin C is biologically inactive in this system (Mellor et al, 2000) and it is unclear wether activin C has biological activity in humans. However βC-subunits can form heterodimers with other known activin subunits and these heterodimers may have a significant impact upon the biological actions of activin A, activin B and activin AB. Although limited experiments with Xenopus embryos showed some effects of βD on body axis and expression of mesoderm markers, very little is currently known about their bioactivity (Ying et al, 1997). More recently, another beta-subunit has been identified, called β_E. The only information about β_E-

activin available to date indicates that this homodimer may be involved in liver inflammation or the hepatic acute phase response (O'Bryan et al, 2000). Further studies are needed to clarify the biology of these new activin forms and their potential roles in the human.

1.3 Structure and function of follistatin and α2-macroglobulin

As described above, one year after the isolation of activin, another inhibitor of FSH-release was identified in follicular fluid (Robertson et al, 1987, Ueno et al, 1987). This protein was structurally unrelated to both inhibin and activin but was capable of binding these proteins via the β-subunit (Shimonaka et al, 1991, Muttukrishna et al, 1995). The affinity of this protein, named follistatin, to activin was particularly high and it became apparent that by binding to activin, follistatin could inhibit the bioactivity of activin. In contrast, the biological activity of inhibin, at least *in vitro*, was not altered by being bound to follistatin.

Follistatin is a single chain glycoprotein encoded by a single gene located on chromosome 5 (Urbanek et al, 1999). Three size variants (FS-315, FS-303 and FS-288) have been purified, resulting from alternative splicing of the gene transcript and proteolytic cleavage (Inouye et al, 1991). The three isoforms have a molecular weight of 39, 35 and 31 kDa respectively, with a common NH_2-terminal sequence (Robertson et al, 1987, Nakamura et al, 1990, Kogawa et al, 1991a, de Kretser et al, 1994). FS-315 is converted to FS 303 by proteolytic cleavage and is the dominant follistatin form found in follicular fluid (Inouye et al, 1991). All variants of follistatin have the same binding affinity for activin (Kd = 540-680 pM, (Sugino et al, 1997). The activin-follistatin complex inhibits the biological activity of activin by interfering with the

presentation of activin to the type II receptors on the cell surface and also by facilitating lysosomal degradation of activin within the cell (Nakamura et al, 1990, de Winter et al, 1996, Hashimoto et al, 1997). Sugino et al 997) has shown that cell-associated follistatin 288 accelerates the internalization of activin into the cells thereby assisting in the clearance of activin from the system. Regardless of whether follistatin is in solution or bound to cell membrane, it appears to have equal potency with regard to the inhibition of activin binding to the type II receptor (de Winter et al, 1996). Circulating follistatin can exist in both activin-bound and free (unbound) forms. It is not known what functions the free forms of follistatin have although they may represent a mechanisms of inhibiting the actions of activin at distal sites.

In several tissues and cell lines, such as pituitary cells, activin and follistatin mRNA are co-expressed. It has thus been proposed that follistatin can modulate the actions of activin in an autocrine/paracrine fashion in these tissues. Follistatin also appears to have direct functions independent of its activin binding activities. Direct effects on rat granulosa cells and on neural induction by follistatin without co-affecting the actions of activin have been reported by Xiao & Findlay, (1991), Xiao et al (1992) and by Hemmati-Brivanlou et al (1994) respectively. It was further shown that follistatin was able to significantly reduce FSH receptor numbers and block the activity of activin in the rat granulosa cells (Minegishi et al, 1999, Knight and Glister, 2001).

In follicular fluid, follistatin is present in high concentrations and is probably the major binding protein for activin present in gonadal tissue. However, in serum, α_2-macroglobulin is the more abundant binding protein present (Vaughan and Vale, 1993) and is presumed to be involved in modulating the action of inhibin, activin and

follistatin in the peripheral circulation. The binding affinity of α2-macroglobulin to activin is much lower than the affinity of follistatin to activin (Vaughan and Vale, 1993).

Alpha$_2$-macroglobulin is a glycoprotein, which exists as two major species: a native stable form and a transformed, unstable form (Phillips et al, 1997). The transformed form binds to alpha$_2$-macroglobulin receptors and can be cleared from the circulation very rapidly. Alpha$_2$-macroglobulin is thus an important modulator of activin in serum being able to clear activin from the circulation, whereas the native form of alpha$_2$-macroglobulin can bind activin and maintain it's concentration in the blood (Phillips et al, 1997). Pertinent to the studies related in this thesis, α2-macroglobulin levels may increase significantly across pregnancy (Studd, 1972).

Similar to the inhibin/activin subunits, follistatin mRNA has been detected in many tissues, including the placenta and human fetus (Tuuri et al, 1994, Phillips et al, 1996). In the gonads, follistatin is present in the spermatogenic cells of the seminiferous tubules, in Leydig cells, in ovarian granulosa cells and secondary and Graafian follicles (Kogawa et al, 1991b). The epithelium of fallopian tubes and the smooth muscle layer of the uterine myometrium also strongly express follistatin (Kogawa et al, 1991b). During embryogenesis, follistatin mRNA is present in Xenopus (Tashiro et al, 1991). In the adult, follistatin is expressed in a diverse range of tissues and cells, including kidney (Shimasaki et al, 1989, Kogawa et al, 1991b), pancreas (Michel et al, 1990), the pituitary (Shimasaki et al, 1989), decidua (Kaiser et al, 1990), placenta and fetal membranes (de Kretser et al, 1994, Petraglia et al, 1994a,

1994b), in vascular endothelial cells (Kozian and Augustin, 1995, Michel et al, 1996) and is also present circulating in serum (Michel et al, 1998),

1.4 Activin Receptors

Similar to other members of the TGFβ family of proteins, activins signal by interacting with two types of structurally related receptors, called type I (~50 kDa) and type II receptors (~70 kDa) (Mathews and Vale, 1991, Mathews et al, 1992, Tsuchida et al, 1993, Lebrun et al, 1997). The activin type II receptors were first identified by expression cloning (Mathews and Vale, 1991), followed by cloning of a type I receptor in 1993 by Attisano and co-workers. The activin type I (actRI) and type II (actRII) receptors are characterized by a short extra-cellular ligand-binding domain, a single transmembrane domain and an intracellular domain with serine/threonine kinase activity (Tsuchida et al, 1993, Lebrun et al, 1997, Mathews and Vale, 1991, Mathews et al, 1992). The first step of ligand-receptor interaction involves binding of activin with high affinity to the extracellular domain of a type II receptor (Attisano et al, 1992, Esteve [illegible]993, Kondo et al, 1991, Mathews and Vale, 1991). Donaldson et al (1999) has shown that a soluble truncated extracellular domain of receptor type II, lacking the transmembrane and intracellular domains, is sufficient for high affinity binding of activin and thus binding of activin to a type II receptor does not require the transmembrane and intracellular components of the receptor. Following successful binding of activin to actRII, actRI (commonly referred to as ALK 4: activin like kinase 4) is recruited into the complex and phosphorylated (Tsuchida et al, 1993). The two receptor types are capable of both autophosphorylation and transphosphorylation (Tsuchida et al, 1993). It has been suggested that other as yet unknown substances are phosphorylated concurrently

thereby further stimulating or attenuating the signal (Gaddy-Kurten et al, 1995). The heteromeric complex composed of ligand – actRII – actRI then initiates signaling within the cell by directly phosphorylating a family of intracellular mediators, the pathway-specific Smad proteins, Smad2 and Smad3 (Massague, 1998). These two Smad proteins interact with the common partner Smad4, move from the cytoplasm to the nucleus, interact with other transscription factors and induce specific target genes. One example is the interaction of Smads with the transcription factor FoxH which activates the goosecoid promoter (Attisano et al, 2000). Figure 1.2 shows a diagram illustrating a proposed model for activin signalling.

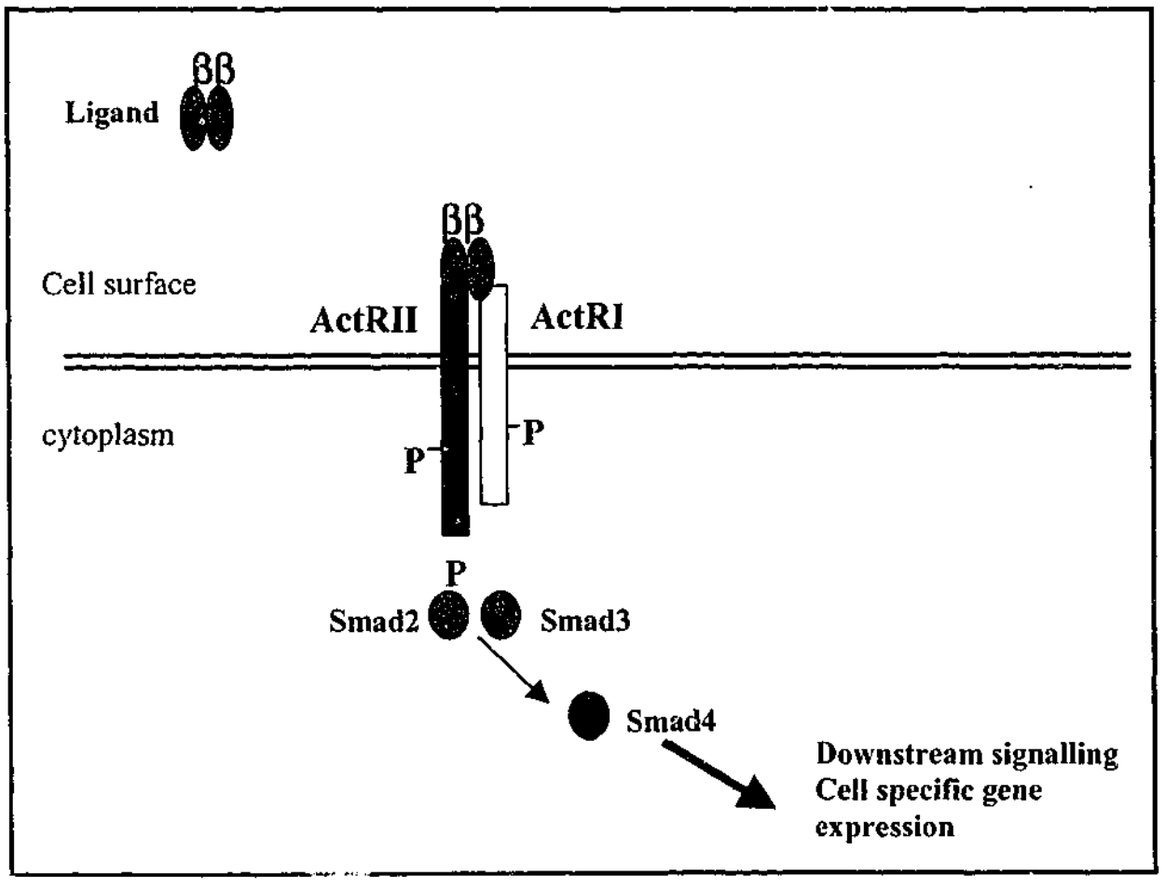

Figure 1.2. The activin receptor signaling pathway.

In addition to the ligand-receptor complex – Smad 2/3 interaction there are a number of further controls which can stimulate or inhibit the signaling pathways. For example, the antagonistic Smad proteins, Smad6 and Smad7, can block the activin signal by interfering with the receptor-Smad complex, and, further, follistatin can neutralize the activin signal by inhibiting activin–receptor complex formation on the cell surface. Inhibins can also bind to actRII and can, in some cell types, antagonize the actions of activin (Lebrun and Vale, 1997, Martens et al, 1997). This antagonism arises because, while inhibin occupies the actRII, it does not recruit the actRI. In addition, very recently, Lewis et al (2000) have shown that inhibins can signal by recruitment of a third TGF-β receptor type, the type III receptor, betaglycan. Betaglycan functions as an inhibin co-receptor with the activin type II receptor. Betaglycan binds with high affinity to the α-subunit of inhibin and can significantly increase the sensitivity of cell lines to inhibin and facilitate inhibin antagonism to activin.

As expected from the tissue distribution of inhibins and activin, receptors for activin have been detected in a wide range of tissues and cells. The majority of studies into the localisation of activin receptors have focused on mRNA expression of receptor type II forms. As expected, receptors type IIA and IIB have been found in the testis and the ovary where they are believed to allow autocrine regulation of male and female gonadal function (Feng et al, 1993, Roberts and Barth, 1994). Type II receptors have also been localised to other endocrine tissues, such as the pituitary and the brain (Roberts and Barth, 1994, Cameron et al, 1994), in smooth muscle cells (Roberts and Barth, 1994), salivary gland, neuronal tissue and ear, kidney and lung of developing rat embryos (Roberts and Barth, 1994), bone (Nagamine et al, 1998) and

bovine aortic endothelial cells (McCarthy and Bicknell, 1993, 1994, Ying and Zhang, 1995).

Pertinent to the studies reported in this thesis, receptors for activin have also been identified in trophoblast cells of first trimester and term placenta (Peng et al, 1993, Shinozaki et al, 1995) and in amnion (Petraglia et al, 1994a) suggesting autocrine and/or paracrine functions for activin in these gestational tissues. These findings have been the catalyst to a large number of studies into the potential roles of activin and inhibin during pregnancy. In contrast to receptors for activin, little is known to date, apart from the recent report of beta-glycan (Lewis et al, 2000), about the structure and function of receptors for inhibin.

1.5 Assays for inhibins and activins

1.5.1 Bioassays

In vivo bioassays

The effects on FSH stimulation were originally evaluated by assessing the effects of injections of human chorionic gonadotrophin (hCG) on reproductive organ weights under experimental conditions. The administration of gonadal or gonadally derived fluids to immature female rats resulted in a decrease in ovarian and uterine weights (Steelman & Pohley, 1953). This decrease in weight was believed to be due to a "suppressor" of FSH, but these experiments were difficult to interpret since the results were indirect and non-specific and furthermore, dependent upon the age of the animals used.

More direct methods of assessing inhibin-like activity on FSH levels employed castrated animals and subsequent injections with exogenous inhibin. Preference was given to ovariectomised adult rats since they had high starting levels of FSH and administration of inhibin-containing material resulted in the most effective response observed (de Jong, 1988). However, this approach required relatively large quantities of inhibin and the assay had low precision (Hudson et al, 1979).

In Vitro bioassays

These inadequacies led to the development of the more precise *in vitro* bioassays for the measurement of FSH inhibition. One type of assay employed whole pituitary glands. This method was simple to carry out and results were rapid (Davies et al, 1978). However, together with the development of a radioimmunoassay for FSH (Midgley et al, 1967), dispersed pituitary cells proved to be more sensitive, precise and reproducible than whole gland culture. These cells were obtained from male and female rats, hamsters, sheep or rabbits (reviewed by de Jong, 1988) dispersed, plated out and cultured for two to three days in the presence of fetal calf serum. The addition of oestrogen or progesterone was used by some laboratories to stimulate the release of FSH and LH from the cells (de Jong, 1988). Substances tested for inhibin-like activity were added and the cells incubated a further one to three days, the medium removed and assayed for FSH or LH activity. Alternatively, the decrease in pituitary cell content of FSH could be assessed (Scott et al, 1980). Inhibin containing preparations could also be added directly. Estimates of the amount of FSH which was released from the cells in response to various concentrations of inhibin-containing substances showed a dose-related suppression, while LH levels were unchanged. Problems with this assay related to various toxic and other non-specific substances which could be

present in the biological fluids or the medium and which could prevent the suppression of FSH, for example activins or binding proteins. Hence, the specificity of the dispersed pituitary cell assay was less than optimal. Overall, the sensitivity of this assay allowed estimation of inhibin concentrations in gonadal fluids, but not in the peripheral circulation (de Jong, 1988).

1.5.2 Radioimmunoassays

In 1985, inhibin (Robertson et al, 1985, Ling et al, 1985) and in 1986 activin (Vale et al, 1986) were purified, allowing the generation of antibodies against these peptides. Radioimmunoassays were developed in a number of laboratories over the following years (McLachlan et al, 1986, Ying et al, 1986, Vaughan et al, 1989), but of these first generation assays, the most widely utilised assay was the so-called "Monash assay", named after the institution of origin, Monash University (McLachlan et al, 1986). The Monash radioimmunoassay (RIA) used a rabbit polyclonal antibody (# 1989) raised against the 31 kDa inhibin with epitopes on the α-subunit. It was widely used and resulted in a large number of clinical investigations into male and female reproduction. However, as became apparent later (Schneyer et al, 1990), the Monash assay measured total α-subunit, dimeric inhibin and free, biologically inactive α-subunits. Several other groups attempted to measure dimeric inhibins using mono- and polyclonal antibodies in two-site immunoassays, but overall, these antibodies did not have high enough affinity to be specific and sensitive enough (Illingworth et al, 1991, Poncelet and Franchimont, 1994). Further limitations for these assays was high cross-reactivity between activin and inhibin due to the common β-subunit, cross-reactivity between various molecular weight forms of inhibin and activin and of course interference with free α-subunits and inactive bound forms. Attempts at an

immunoassay for activin has also resulted in limited success. A conventional RIA (Robertson et al, 1992) and several two-site EIA systems (Groome 1991, Wong et al, 1993) were tested in biological fluids but found to have very high interference by follistatin reducing the apparent concentration of activin by up to 70%.

1.5.3 Enzyme-linked immunosorbent assays (ELISA)

The most recent advances in the measurement of the inhibins and activins were made by the construction of ultrasensitive enzyme-linked immunosorbent assays using monoclonal antibodies against the α-subunit, βA-subunit, βB-subunit, pro-fraction of the α-subunit and follistatin (Groome et al, 1994, 1995, 1996, Knight et al, 1996, Evans et al, 1998). The sensitivity and specificity of these assays for inhibin A, inhibin B, activin A and activin AB was significantly increased by pre-treatment of the samples. Boiling with sodium dodecyl sulphate solution disrupts the bonds between activin and follistatin, and a further incubation step with hydrogen peroxide results in oxidisation of the methionine residues in the β-subunits (Knight and Muttukrishna, 1994). These two steps have allowed the measurements of the inhibin/activin superfamily of proteins in serum and tissues where the concentrations can be very low. The follistatin assay (Evans et al, 1998) uses a pair of monoclonal antibodies raised against follistatin 288 and measures total (bound and free) follistatin. Detailed descriptions of the method and performance characteristics of the activin A and follistatin assay used in the research reported in this thesis are given in chapter 2.

1.6 Structure and function of gestational tissues

1.6.1 The placenta

The human placenta is a haemochorial villous organ, which allows transfer of gases, nutrients and wastes to and from the fetus. Any abnormalities of placental development and function can significantly affect fetal development in utero and, as has recently been proposed, can also influence the health of the individual in adulthood (Barker et al, 1989, Byrne and Phillips, 2000).

Within a few days after fertilization finger-like projections of trophoblast cells begin to invade the endometrial stroma and the trophoblast differentiates into two layers: the inner cytotrophoblast and the outer syncytiotrophoblast layer, which are responsible for invasion of the endometrial epithelium. The cytotrophoblast layer gives rise to new syncytial cells, the chorionic villi and the amnion. The trophoblast cells immediately begin to secrete a range of hormones, growth factors and cytokines, which are important for the maintenance of pregnancy and necessary in inducing the metabolic adaptations of the fetus and the mother. The placenta, and especially the syncytiotrophoblast cells, synthesise a number of polypeptide and steroid hormones. The polypeptide hormones, such human chorionic gonadotrophin (hCG), and human placental lactogen (hPL), are involved in maintaining the corpus luteum in the first few weeks after fertilization, in suppressing the lymphocyte response from the maternal tissues to the invading trophoblast and in stimulating fetal growth and adrenal response for the production of fetal glucocorticoids and testosterone. The placenta also produces corticotropin-releasing hormone (CRH) and its binding protein corticotropin-releasing binding protein (CRH-BP). *In vitro* studies investigating placental CRH production have shown that the prostaglandins PGE_2 and $PGF_{2\alpha}$, as

well as oxytocin can modulate CRH production (Quartero and Fry, 1989, Benedetto et al, 1994) and that CRH can promote the dilatation of the feto-placental vessels (Clifton et al, 1994). It has thus been suggested that CRH may be an important regulator of human parturition (McLean and Smith, 2001).

In addition to the various proteins, the human syncitiotrophoblast is also responsible for the production of steroid hormones, progesterone and the oestrogens, oestrone, oestradiol and estriol. The synthesis of these hormones requires the interaction of maternal, fetal and placental tissues, since the enzymes necessary for the production of the steroid hormones are not all present in one organ system. Unlike most mammalian placentae, the human trophoblast cells do not express 17α-hydroxylase, an enzyme required for de novo oestrogen synthesis and important in the regulation of parturition in some animals such as the sheep. However, the human placenta is capable of increased conversion of C_{19} steroids such as androstenedione and testosterone to oestrone and oestradiol-17β. Steroid hormones, including progesterone have important functions, including the maintenance of pregnancy and the decidualisation of the endometrium (Khong & Pierce, 1987). Progesterone in particular is required for maintenance of uterine quiescence, inhibition of prolactin secretion and possibly excerts anaesthetic effects on the fetus (Thorburn and Challis, 1979).

The syncytiotrophoblast cells proliferate and invade the endometrial stroma. As the blastocyst penetrates the endometrium, maternal endometrial capillaries are eroded away by the syncytium resulting in the formation of intersyncytial spaces, also called "lacunae". These lacunae are already present at eight days after conception and later

form the intervillous space. Blood from the maternal circulation seeps into these lacunae and establish the beginnings of a uteroplacental circulation as early as 17 days after fertilization has occurred. Villi containing blood vessels are present by three weeks and after seven to eight weeks the first wave of invasion of the trophoblast is completed and a fully functioning placental circulation established (Blackburn and Loper, 1992). As the placenta develops further, the cytotrophoblast cells proliferate and differentiate into either syncytiotrophoblast cells on "floating" villi or into invasive cytotrophoblast cells. These villi are called "anchoring" villi as they invade and attach to the decidua (Cross, 1996). *In vitro* culture experiments of first trimester trophoblast cells have shown that the proliferation and differentiation of these cells is under complex regulation by a wide range of cytokines and growth factors produced by the mother, the fetus and also produced locally by the trophoblast and decidual cells. In particular, members of the TGFβ-family, TGFβ1 and TGFβ3 have been shown to suppress cytotrophoblast invasion *in vitro* (Caniggia et al, 1996), while the cytokine interleukin-1β (IL-1) promoted invasion of cytotrophoblst cells (Librach et al, 1994). Bass et al (1994) demonstrated upregulation of cytotrophoblast cells by epidermal growth factor (EGF) via paracrine regulation, while in another study, it was shown that the actions of EGF were gestational-age dependent, EGF being able to both up- and downregulate cytotrophoblast cell proliferation and differentiation (Maruo et al, 1992). The potent immune mediators interferon gamma (INFγ), interferon beta (INFβ) and interferon alpha (INFα) are all secreted by first trimester trophoblast cells (Paulsen et al, 1994, Bulmer et al, 1990). The receptor for INFγ was localised to cytotrophoblast and syncytiotrophoblast cells, but has also been localised to macrophages (Paulsen et al, 1994). A number of experiments have shown that the interferons have powerful immunological and antiviral effects, especially in early

pregnancy during trophoblast cell proliferation and remodeling of the endometrium (Hunt and Robertson, 1996). Placental growth is also mediated by insulin-like growth factor-II (IGF-II) and by platelet-derived growth factor (PDGF) (Fant et al, 1986, Holmgren et al, 1992).

The production of cytokines and growth factors by trophoblast cells can affect steroid and polypeptide hormone synthesis of the placenta. In vitro experiments have shown that EGF, fibroblast growth factor (FGF), interleukin-6 (IL-6), and colony-stimulating factor-1 (CSF-1) can stimulate hCG production, while IGF-1, EGF and IL-6 can stimulate placental lactogen (hPL) production, and IL-1 can stimulate aromatase activity in trophoblast cells (Mochizuki et al, 1998, Saito, 2000). TGFβ1, on the other hand, suppresses hCG and hPL secretion and inhibits trophoblast differentiation in a matrigel invasion assay (Graham et al, 1993). Activin βA-subunit and mRNA for activin receptor type II have been localised to first trimester cyto- and syncytiotrophoblast cells, as well as macrophages (Rabinovici et al, 1992, Peng et al, 1993) and were shown to regulate the differentiation of villous cytotrophoblast into the invasive extravillous phenotype *in vitro* (Caniggia et al, 1997). Further, activin A can influence placental endocrine function by stimulating hCG production from cultured human first trimester trophoblast cells (Steele et al, 1993).

Trophoblast invasion continues until the full thickness of the placenta is achieved by the fourth month of pregnancy. Thereafter, the placenta continues to grow by lengthening of existing villi, increase in the number and size of capillaries and extensions of new trophoblastic sprouts around the periphery of the tissue. These adaptations result in an increase in the surface area of the placenta, processes that lead

to increased functional efficiency and ensure that the demands of the growing fetus are maintained. The transfer efficiency from early to late pregnancy has, in fact, been estimated to rise six-fold (Blackburn and Loper, 1992). At term, the normal placenta weighs 500 to 600 g, is 15 to 20 cm in diameter and about three cm thick. The maternal surface is divided into 15 to 20 lobules, or cotyledons, separated by placental septae. The maternal surface of the placenta is shown in figure 1.3.

Figure 1.3. Maternal surface of a normal placenta at term

On the fetal surface, or chorionic plate, the placenta can be separated into the branches of primary stem villi that are supplied by the branches of the umbilical vessels (figure 1.4). The primary stem villi divide into secondary stem villi beneath the chorionic plate, running parallel under the surface and then branch downwards

into tertiary stem villi through the parenchyma of the placenta where terminal villi finally branch off. The structure of the mature placenta at term is shown in figure 1.5.

Figure 1.4. Fetal surface (chorionic plate) of the normal placenta at term

Nutrients and oxygen must pass through five layers of tissue, called the placental barrier, before entering the fetal circulation. From the maternal circulation, the first tissue layer is the microvillous membrane of the trophoblast, followed by the villous syncytiotrophoblast, the basal membrane of the trophoblast, the connective mesoderm tissue of the trophoblast and finally the vascular epithelium of the fetal blood vessels. Fetal blood reaches the placenta via two umbilical arterial vessels, which spiral

around the central umbilical vein. As the umbilical cord enters the chorionic plate, the umbilical arteries branch out radially into the villi. The arteries continue to divide and branch within the smallest villi thus establishing a dense network of arterio-venous exchange.

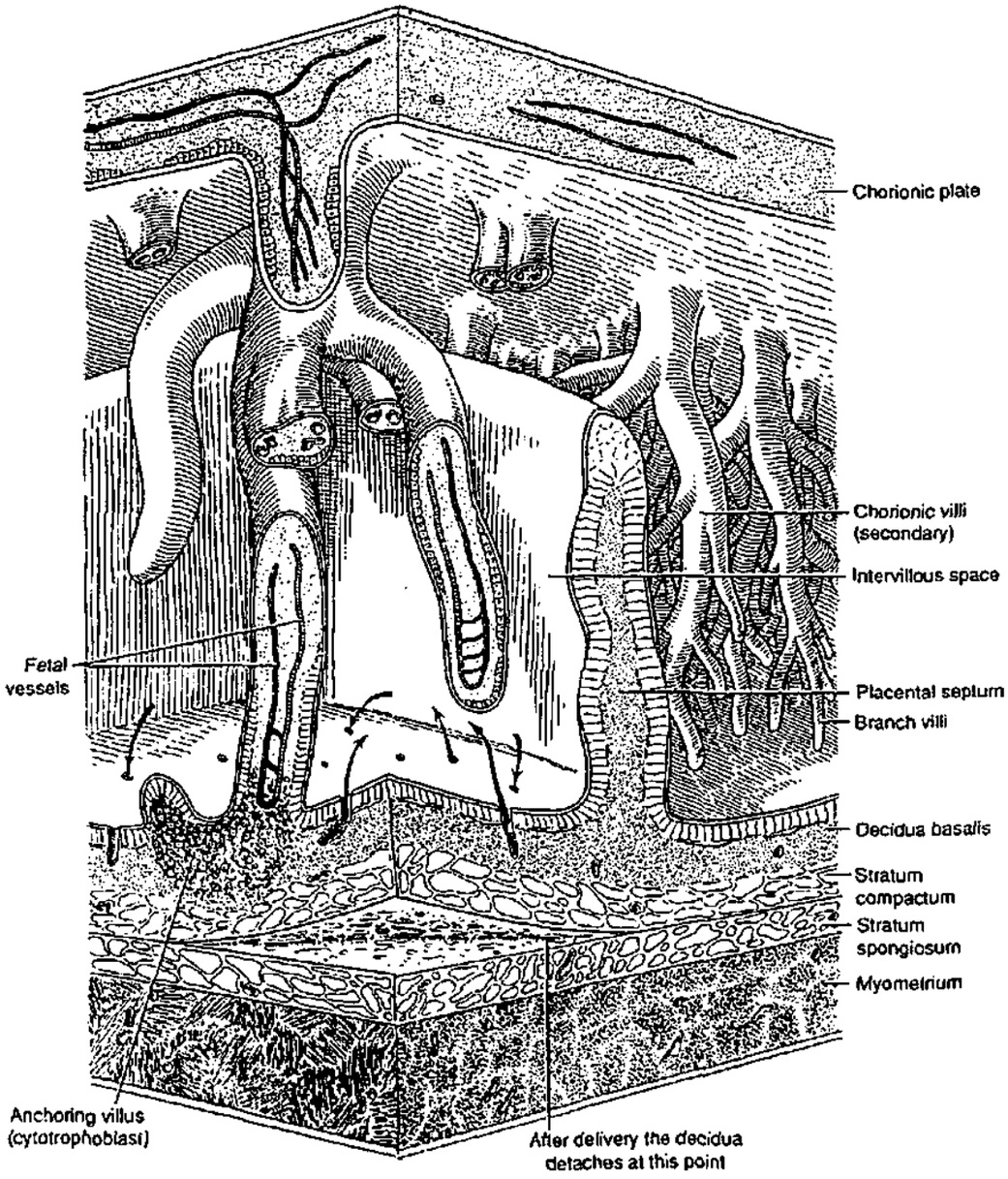

Figure 1.5. Cross-section through the mature placenta

1.6.2 The fetal membranes: amnion and chorion

The amnion and chorion consist of several layers each and are formed soon after fertilization. They grow continuously until week 28 of pregnancy, after which the membranes adapt to the growing fetus by stretching of the existing membranes (Lavery, 1987). The amnion consists of five layers. The innermost layer facing the fetus is the epithelial layer which is the site of significant fluid exchange and hormone and growth factor synthesis. The next layer is the basement layer, followed by the dense layer, a fibroblast layer and finally the mucus secreting spongy layer. The amnion is avascular and non-innervated.

The chorion is characterised by four layers of tissue: directly underlying the amniotic spongy layer is the cellular layer, under which lies the reticular layer, the pseudobasement membrane, and the layer composed of trophoblast cells is in close contact with the decidua capsularis. The chorion is not innervated but in contrast to the amnion, is well vascularised. Figure 1.6 shows a diagram outlining the layers of the amnion and chorion.

Arachidonic acid, the precursor of prostaglandins, such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which are important in the initiation of myometrial contractions (gap junction formation, increase of calcium concentrations in the cytoplasm of myometrial smooth muscle cells) and cervical ripening (Casey and MacDonald, 1986, 1988, Challis and Olson, 1994), is synthesised in the amnion (Lavery, 1987, Challis, 1988). The amniotic cells also secrete proteins and lipids, and modulate exchange of water, electrolytes and other solutes.

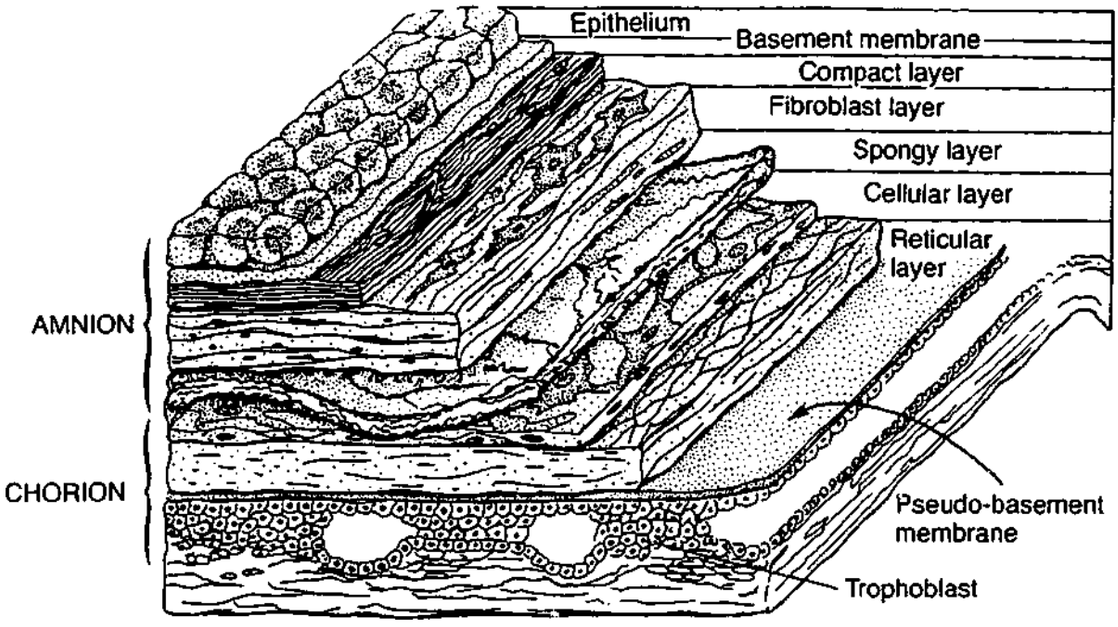

Figure 1.6: Crossection through the amnion and chorion at term

(Calter Blackburn Loper, 1992)

The chorion, in consort with the decidua, produce estrone which not only promotes local $PGF_{2\alpha}$ production but also downregulates production of prostaglandins inhibitory of myometrial contractility, such as prostacyclin (PGI_2), and promotes formation of oxytocin and prostaglandin receptors in readiness for labour (Challis and Olson, 1994). Both membranes also store phospholypase A_2, a lysosomal enzyme necessary to convert esterified arachidonic acid to its unesterified form which then becomes a precursor for prostaglandin synthesis (Blackburn and Loper, 1992).

Apart from providing a mechanisms for transport for nutrient, waste and oxygen exchange between the maternal and fetal circulation, the placenta also has metabolic, immunological, and endocrine functions throughout gestation, and, together with the fetal membranes is also involved in regulating the onset of parturition.

1.7 Regulation of parturition

The mechanisms which control the processes of parturition in the human are only party understood. Multiple endo-, para- and autocrine events in the fetus, the mother and the utero-placental unit must coordinate at the appropriate time to ensure the timely initiation of labour. The two major changes which are required are, firstly, the ripening and opening of the cervix, and, secondly, the conversion of a quiescent myometrium to an actively contracting organ (Weiss, 2000). The predominant hormones involved in these changes include the steroids oestrogen and progesterone, the prostaglandins PGE_2 and $PGF_{2\alpha}$, cytokines and fetally derived adrenal hormones, so- called "stress" hormones (Majzoub et al, 1999, Lockwood, 1999).

In some animals, such as rats, rabbits and sheep, the processes of labour are much better understood than in the human. In these species, systemic progesterone levels decline significantly while oestrogen levels increase in the peripheral circulation prior to labour leading to the loss of uterine quiescence. The changing ratio of progesterone to oestrogen is believed to be the trigger for the initiation of contractions. Uterine quiescence can be blocked in these animal species by ovariectomy and this response can be reversed by administration of exogenous progesterone (Csapo, 1956). However, in the human, maternal peripheral progesterone levels increase progressively up to the time of birth. Further, serum progesterone levels are not significantly different between women in labour and women not in labour at term (Weiss, 2000). Nevertheless, alterations in progesterone activity by binding to receptor and response elements locally within gestational tissues may be important steps in controlling parturition. This hypothesis is supported by clinical studies into mifepristone (RU 486), a progesterone antagonist, which is highly successful in

increasing myometrial responsiveness, cervical ripening and inducing contractions (Bygdeman et al, 2000). Progesterone also stimulates uterine nitric oxide (NO) synthase which is the major factor controlling myometrial quiescence and downregulates the production of prostaglandins and oxytocin receptors in the uterus (Garfield et al, 1998).

Concurrently to the progressively rising levels of progesterone, maternal plasma concentrations of unconjugated oestrogens increase steadily during pregnancy reaching peak levels at term (Albrecht & Pepe, 1990) but the sharp predelivery increase seen in the sheep does not occur. Thus, the typical increase in peripheral plasma oestrogen to progesterone ratio caused by progesterone withdrawal observed in some non-primate animal species during late pregnancy is not present in the human in the peripheral circulation. However, differences can be demonstrated in other fluids. In amniotic fluid, concentrations of 17 β-oestradiol rise significantly with pre-term and term labour and this increase is possibly due to fetally derived products released during labour (Mazor et al, 1994). Further, a predominance of oestrogen can also be effected locally rather than systemically by an increase in oestrogen receptors in target tissues. Indeed, such an effect has been demonstrated by Mitchell et al (1992). In fetal membranes and decidua, the relative abundance of oestrogen receptor mRNA was increased three- to fourfold with the onset of labour, while progesterone receptor mRNA remained unchanged. In primates, the conversion of androgens to oestrogens induces maternal endocrine and fetal membranes biochemical changes which results in increased myometrial activity and onset of contractions (Natanielsz et al, 1998). The local changes in tissue sensitivity to oestrogen are probably responsible for the release of a number of important uterotonic mediators of labour, such as the

prostaglandins, PGE_2 and $PGF_{2\alpha}$. Several studies have shown that oestrogen can stimulate the synthesis of the prostaglandins from gestational tissues and endometrium (Abel & Baird, 1980, Olson et al, 1983). The importance of prostaglandins for the intiation and progression of labour is well established. Rising levels of PGs have been reported with labour in maternal plasma and urine, as well as in amniotic fluid (Olson et al, 1995). In sheep, contractions of labour can be reduced and labour prolonged by blocking PG synthesis (Challis & Olson, 1994).

More recent work has focused on identifying the mechanisms which lead to the formation of PGs. Extracellular (Type II) phospholipase A_2 (PLA_2) which is involved in the formation of PGs from unesterified arachidonic acid, is present in human amnion, choriodecidua and placenta (Farrugia et al, 1993). Interestingly, PLA_2 is the only phospholipase that demonstrates increased immunoreactive and catalytic activity during labour. A similar profile has also been detected for another enzyme, prostaglandin endoperoxide H synthase, (PGHS), which controls the cyclooxygenase step during PG synthesis. Activity of PGHS increases during late pregnancy in the human amnion (Teixeira et al, 1994). PGHS exists as two forms transcribed from different genes, a constitutive form, termed PGHS-1, and an inducible form, called PGHS-2. Both are present in the amnion and decidua (Teixeira et al, 1994, Mitchell, 1994). During labour, messenger RNA levels of PGHS-2 are increased in the amniotic epithelial cells, whereas PGHS-1 levels remain unchanged (Hirst et al, 1995a & b). The increase of PGE_2 in amniotic fluid observed with labour has been attributed to the increased expression of PGHS-2 in the amnion. Another enzyme controlling PG formation is 15-hydroxyprostaglandin dehydrogenase (PGDH) which is regulated by progesterone and solely localised in fetal membranes to the chorionic trophoblast

(Challis et al, 2000). It has been proposed that PGDH acts as a metabolic barrier to the passage of unmetabolised PGs synthesised in the fetal membranes, thus preventing PGs from acting on the underlying decidua and myometrium (Okazaki et al, 1981, Cheung et al, 1992). Interestingly, women presenting with idiopathic pre-term labour were shown to have a deficiency of PDGH in the chorionic trophoblast, presumably allowing unmetabolised PGs to reach the myometrium and initiate contractions (Sangha et al, 1994).

Thus, localised changes of oestrogen and uterotonic agents, such as PGs and their metabolising enzymes probably account for some of the changes within gestational tissues which ultimately lead to myometrial smooth muscle contractions. However, a multitude of other factors play important roles along the highly complex set of event which result in parturition.

One factor recently proposed as a potentially important regulator of parturition is corticotropin-releasing factor (CRH). CRH is an hypothalamic hormone responsible for the control of adrenocorticotropin-releasing hormone (ACTH) which in turn is regulated via a feedback loop involving adrenal cortisol. During pregnancy, CRH is produced by the syncytiotrophoblast and CRH levels in maternal serum increase progressively to term whereas serum CRH binding protein levels fall prior to labour (Chrousos et al, 1998). It has been proposed that increasing levels of CRH lead to an intra-uterine environment which is conducive to parturition (Majzoub et al, 1999). *In vitro*, CRH is released from trophoblast cells and was shown to stimulate production of PGE_2 and $PGF_{2\alpha}$ from cultured placenta, amnion, chorion and decidual cells (Jones & Challis, 1989). However, the most critical role of hypothalamic and placental CRH

is probably in stimulating the fetal hypothalamic-pituitary-adrenal (HPA) axis under conditions of stress, such as hypoxaemia or infections. CRH levels increase in response to inflammatory cytokines released in response to inflammation (Petraglia et al, 1996). The fetal adrenal responds to these conditions by producing glucocorticoids which are required for the maturation of the fetal lung. CRH is currently being evaluated as a marker for women at risk of premature delivery (Giles et al, 1996).

1.8 Inibin/Activin and follistatin in gestational tissues during pregnancy

1.8.1 Inhibin

The placenta was identified as a source of inhibin in the 1980s (McLachlan et al, 1986, Mayo et al, 1986, de Krester et al, 1994). In 1987 Petraglia and colleagues reported the presence of both inhibin βA- and βB-subunits in extracts of term placental tissue. These studies coincided with the detection of elevated levels of bio- and immunoreactive inhibin in the serum of pregnant women compared to non-pregnant women (McLachlan et al, 1987, Abe et al, 1990, Tabei et al, 1991, Qu et al, 1991, Tovanabutra et al, 1993, Baird and Smith, 1993). These early studies utilised the Monash assay and due to the high cross-reactivity with free α-subunits, no information was available as to the contribution of dimeric inhibin, the biologically active form. It is now known that inhibin A is the major circulating form during pregnancy. Inhibin B levels are undetectable across gestation (Illingworth et al, 1996). Concentrations of plasma immuno-reactive inhibin A show a biphasic pattern, peaking at week nine to ten of gestation, decreasing again to plateau between week 15 to 30 and rising again to reach maximal levels at term (Muttukrishna et al, 1995, Fowler et al, 1998, Wallace et al, 1997). Bioactive inhibin A levels, in contrast, rise progressively throughout pregnancy, without the biphasic profile described for

immunoreactive inhibin A (Qu et al, 1991). The discrepancy between bio- and immunoactive inhibin has been attributed to the presence of activins or other proteins which can interfere with FSH secretion in cell cultures (Baird and Smith, 1993). In the early weeks of pregnancy, the corpus luteum is the predominant source of inhibin A with a switch of production to the feto-placental unit occurring during late first trimester of pregnancy (McLachlan et al, 1987, Yohkaichiya et al, 1991, Rombauts et al, 1996). Concentrations of circulating inhibin A fall sharply after delivery and in the studies of Qu and Thomas become undetectable after 24 hours suggesting that the feto-placental unit is the major source of circulating inhibin during pregnancy (Qu and Thomas, 1992, Qu and Thomas, 1998). The half-life of inhibin has been estimated at between 0.63 h and 0.76 h for bio- and immunoinhibin, respectively (Qu and Thomas, 1995).

1.8.2 Activins and follistatin

1.8.2.1 Activins, activin receptors and follistatin in placenta

The placenta is the major source of activin during pregnancy. In first and second trimester placenta, activin βA- subunit has been detected by immunohistochemistry as early as week 5 of gestation in the cyto- and syncytiotrophoblast cell layers (Otani et al, 1998, Rabinovici et al, 1992). Hofbauer cells also stained positively for activin A in first and second trimester placental sections. At term, staining of activin βA-subunit was localised mainly to the syncytiotrophoblast cells with occasional staining of cytotrophobast cells (Petraglia et al, 1987, Que et al, 1992, Minami et al, 1992). Northern blot analysis has shown that mRNAs for the α-, βA- and βB-subunits are present in the placenta following a gestational-age related profile with the highest expression observed at term (Petraglia et al, 1991c). In first and second trimester there

was a predominance of α-subunit expression in placenta, while at term, βA-subunit expression was more intensive. Similar findings were provided by Meunier et al (1988) who observed that while the α-subunit is present in excess in a range of tissues (gonads, pituitary, brain and adrenal), in term placenta, the βA-subunit is present in excess compared to the other two subunits providing a possible explanation for the maximal levels of activin A observed in maternal serum at term. These findings suggest that the placenta produces mainly inhibin A early on in pregnancy and switches to a predominance of activin A towards term (Fowler et al, 1998).

Messenger RNA for activin receptors IIA (RIIA) and IIB (RIIB) have been localised to placenta of first, and third trimesters of pregnancy (Birdsall et al, 1997, Minami et al, 1992, Shinozaki et al, 1995, Rabinovici et al, 1992, Peng et al, 1993, Petraglia et al, 1994a, Petraglia et al, 1997b). *In situ* hybridisation demonstrated that the syncytiotrophoblast expressed receptor IIA and IIB mRNA at all stages of gestation. However, the most intense staining was detected in first trimester placental sections with less intense staining observed at term. Concurrently, expression of receptor IIA was low throughout gestation (Petraglia et al, 1994a). Shinozaki et al (1995), using Northen blot analysis, observed the same pattern of expression in the placental villi for receptors IIA and IIB and suggested a stage specific regulation of activin A in the placenta during gestation.

Trophoblast culture experiments have demonstrated that inhibin and activin are produced by first trimester and term trophoblast cell cultures and that activin, in consort with inhibin, can modulate hormonogenesis by the placenta in an auto- and paracrine fashion. Activin A stimulates the release of hCG from first trimester

trophoblast cells (Steele et al, 1993). The addition of activin A to cultured term cytotrophoblast cells stimulated a significant release of GnRH and progesterone and further augmented the release of hCG by GnRH (Petraglia et al, 1989). Inhibin, although unable to affect these placental hormones directly, could reverse the effects of activin. Activin A stimulates the release of PGE_2 from an amnion-derived cell line (Petraglia et al, 1993a) and from amnion explants (Keelan et al, 2000b). In a choriocarcinoma cell line, JEG-3 cells, activin A was able to stimulate progesterone production into the medium. Furthermore, activin A enhances the production of estradiol while in the presence of androstenedione. Both effects are inhibited by follistatin (Ni et al, 2000). Since neither activin nor follistatin were able to affect cell growth in this cell line, it was concluded that activin A is an autocrine regulator of steroid production.

Activin A has been detected in purified cytotrophoblast cells from first trimester placental tissue. When added to cultured first trimester trophoblast cells, activin A induces the early expression of matrix metalloproteinase (MMP) – 9 and –2, both of which are believed to aid the invasive properties of the villous cytotrophoblast (Caniggia et al, 1997). First trimester trophoblast cells do not repond to stimulation with GnRH, TGFβ, IL-1, dexamethasone or cyclic AMP (cAMP) (Rabinovici et al, 1992). However, at term, cAMP analogues increased the expression of activin βA-subunit in trophoblast cells (Tanimoto et al, 1992) providing some evidence that activin A is differentially regulated across gestation. Addition of oestrogens and progesterone to term placental cultures were shown to modulate the actions of activin on GnRH release (Petraglia et al, 1990) Immunohistochemical studies have shown that inhibin α-subunit, activin βA-subunit and GnRH are localised to the same cells

on the syncytiotrophoblast layer and inner cytotrophoblast cells (Petraglia et al, 1992) suggesting that these hormones form a paracrine/autocrine control mechanism within the placenta at term.

Follistatin has been detected in first trimester and term placenta by immunohistochemistry (Petraglia et al, 1994b). During pregnancy, the levels of total follistatin (follistatin bound to activin and free, unbound follistatin) in maternal serum increase markedly (Khoury et al, 1995, Wakatsuki et al, 1996, Sakamoto et al, 1996, Evans et al, 1998, O'Connor et al, 1999). Staining was localised to the syncytiotrophoblast layer, the same cells that stain for βA- and βB sub-units (Petraglia et al, 1991c, Petraglia et al, 1992). Follistatin has also been isolated in different isoforms from homogenates of term placenta using protein purification techniques (de Kretser et al, 1994, Yokoyama et al, 1995). Messenger RNA for follistatin has been detected in the placenta by RT PCR and protein in tissue lysates using ELISA (Petraglia et al, 1994a & b, Keelan et al, 1999). Placental cells, cultured *in vitro*, release follistatin but no stimulatory effects were demonstrated by addition of progesterone or forskolin into the medium suggesting that follistatin secretion is mediated by processes independent of intracellular cAMP (Petraglia et al, 1994b). When added to placental cultures, follistatin does not appear to directly regulate hCG or progesterone release, however, follistatin is able to completely reverse the release of hCG and progesterone induced by activin A (Petraglia et al, 1994b).

1.8.2.2 Activins, activin receptors and follistatin in fetal membranes and decidua

MessengerRNA for the α-, βA- and βB inhibin subunits and proteins themselves have been localised to the fetal membranes and the decidua (Petraglia et al, 1991c,

Petraglia et al, 1992, Petraglia et al, 1993a). In fetal membranes, the amniotic epithelial cells and the chorion were positive for activin βA-subunbit (Rabinovici et al, 1992). The amnion, together with the fetus, are a potential source of activin A in the amniotic fluid compartment. In first trimester amniotic fluid, dimeric inhibins or activin A are undetectable (Riley et al, 1998). In extraembryonic coelomic (EEC) fluid however, both inhibin A and inhibin B, as well as pro-αC and follistatin are present suggesting the chorion as a source of these proteins during early pregnancy prior to fusion of the chorion with the amnion (Riley et al, 1996, Riley et al, 1998). The absence of bioactive activin A in EEC (Riley et al, 1998) may be due to high levels of follistatin which acts to regulate the availability of activin A in this compartment.

Levels of activin A are measurable in amniotic fluid in the second trimester and levels increase progressively throughout the remainder of pregnancy (Riley et al 1998, Muttukrishna et al, 1999) to reach peak levels at term (Petraglia et al, 1993b). Amniotic fluid is largely composed of fetal urine and the changing concentrations of activin A in this compartment may reflect a fetal source. In fact, messenger RNA and protein for activin βA-subunit has been detected in fetal tissues, such as gonads, pituitary, adrenal glands and brain (Tuuri et al, 1994).

Petraglia et al (1993a) has localised immunoreactive βB-subunit to the amnion and to a lesser extent to the chorion. In the amnion, levels of βB-subunit mRNA were higher than inhibin-α- and βA-subunits. The low levels observed in the chorion may be due to the different embryological origins of these tissues. The amnion is derived from embryonic ectoderm, while the connective tissue of the chorion develops from

extraembryonic mesoderm. The presence of βB-subunit in fetal membranes was also reported in studies by Riley et al (2000). Activin B has been found in amniotic fluid and cord serum of the fetus but only at term prior to delivery (Petraglia et al, 1993b). The amniotic epithelium, together with fetal and some chorionic contributions, probably account for the activin B measured in amniotic fluid, but a placental source needs to be confirmed by further studies. Activin B could not be detected in medium of cultured trophoblast cells (Debieve et al, 2000) and activin B was also undetectable in maternal serum (Woodruff et al, 1997). However, more recently, using a monoclonal antibody directed against activin βB-subunit, activin B could be measured in serum of healthy pregnant women and localised to trophoblast cells by immunohistochemistry suggesting that, at least in part, the placenta can produce activin B (Vihko et al, 1998). It is currently unclear which gestational tissue is the main source of activin B and if the profile of activin B secretion changes throughout gestation.

Activin receptor type II has been detected in fetal membranes. The amniotic epithelium displayed an intense hybridisation signal to receptors IIA and especially to receptor IIB mRNA, with less intense signals detected in the chorion and decidua (Petraglia et al, 1994a, Petraglia et al, 1997b). No information is available on receptor type I mRNA and on receptor protein expression of either receptor type in amnion and chorion. However, the co-localisation of ligand and one of the two necessary receptor types necessary for signal transduction suggests that activin A may be regulated in a para- and/or autocrine fashion in these tissues as well as in placenta, decidua and the fetus.

Cytokines can stimulate the release of activin from fetal membranes *in vitro*. Keelan et al (2000a) have shown that the inflammatory mediators IL-1β and TNFα can stimulate a several-fold increase in activin A release from cultured amnion and choriodecidual explants. Lipopolysaccharides have a similar effect on choriodecidua but not on amnion with follistatin and inhibin A levels remaining unchanged by the stimulus. These results suggest that activin A can be released from the fetal membranes in the course of inflammatory processes as can often be observed during pre-term labour.

Follistatin has been localised to the fetal membranes by immunohistochemistry (Petraglia et al, 1994b). Immunoreactive follistatin stained the epithelial cells of the amnion and chorionic cells, as well decidual cells. Using reverse transcriptase polymerase chain reaction (RT PCR), mRNA for follistatin was also detected in these tissues (Petraglia et al, 1994b). Total (bound and free) follistatin was detected in amnion and choriodecidual lysates collected from tissues at term (Keelan et al, 1999). The co-localisation of follistatin with activin βA-subunit suggests a paracrine/autocrine mechanism for the regulation of activin by follistatin in these tissues.

Other gestational tissues are capable of producing activin A and follistatin during pregnancy. The decidua contains and produces inhibin-α-, βA- and βB-subunits during pregnancy (Petraglia et al, 1990). The mRNA expression of α- and βB-subunits increases as gestation advances, but βA-subunits levels decline towards term. It is not clear what roles activin A is playing in the decidua during pregnancy, but the strong expression observed in first trimester tissues may hint at a role in the regulation

of decidual cell differentiation and/or proliferation, possibly associated with tissue remodelling during implantation and placentation (Jones et al, 2000). Petraglia has also suggested a possible immunomodulatory role.

Messenger RNA for α-, βA- and βB-subunits have been found in a range of tissues in the developing embryo in the monkey, mouse, the rat and Xenopus (Roberts et al, 1991, Manova et al, 1992, van den Eiden-van Raaij, 1992, Webley et al, 1992, Dohrman et al, 1993, Albano et al, 1993, Roberts and Barth, 1994), as well as in human during mid-gestation (Tuuri et al, 1994). Tissues displaying the strongest expression of βA-subunit and follistatin in the human were neural tissue and muscular tissue, and less intense but distinct expression was seen in hemopoetic tissues, exocrine glandular tissue (kidney, pancreas, liver) and steroidogenic tissues (gonads, adrenal). Activin βA-subunit is expressed in the fetal adrenal gland. In cultured human fetal adrenal cells, activin A inhibits mitogenesis and enhances the release of ACTH-stimulated cortisol (Spencer et al, 1992). Furthermore, activin A and TGFβ have been shown to modulate the ACTH-induced synthesis of steroids in the fetal adrenal (Mesiano and Jaffe, 1997). It was concluded that the fetal adrenal may release activin A during times of stress such as during parturition thereby contributing to the overall concentrations of activin A in amniotic fluid, fetal serum and possibly maternal serum.

The findings suggest that activin and follistatin can regulate a range of organ systems during human embryogenesis and possibly also during parturition.

1.8.2.3 Activin and follistatin knockout studies

The activin and follistatin gene knockout mice have greatly increased our understanding of the actions of these proteins during pregnancy and fetal development. Transgenic mice carrying mutations in activin βA or follistatin die within 24 hours after birth. Both phenotypes have defects of the palate, conferring an inability to suckle and difficulties in breathing. They also have abnormal development of teeth and whiskers and are growth retarded (Matzuk et al, 1995, Jhaveri et al, 1998). The defects seen with follistatin-deficient mice are overall more widespread than the defects observed with activinβA-deficient animals suggesting that follistatin may be an important modulator of activin during embryo development. In mice with an overexpression of follistatin, offspring had varying degrees of reproductive tract abnormalities; depressed FSH levels and some were infertile (Guo et al, 1998). Matzuk et al (1995) generated mice with a null mutation in the RII gene. Although some of these mice had the skeletal and facial defects similar to those observed in activin-deficient mice, most lacked these abnormalities and developed into adults. However, their levels of FSH were depressed and they had defective reproductive performances. Interestingly, mice carrying a mutated α-subunit gene did not have developmental abnormalities. However, these mice later on developed gonadal stromal tumours and it was suggested that activin could function unopposed in the absence of inhibin resulting in subsequent tumour growth in the gonads (Matzuk et al, 1992). Mutations directed against the βB-subunit leads to pups with late developmental abnormalities of eyelid formation and in the female, increased perinatal mortality (Vassalli et al, 1994). Parturition is delayed and milk ejection impaired, processes which are normally stimulated by elevated OT prior to and during delivery. Since both activin subunits and oxytocin are localised to the same cells in

the hypothalamus (Sawchenko et al, 1988), it is possible that activin deficiency impacts on the timely and efficient release of OT thereby affecting the pathways of parturition. This hypothesis has been strengthened by experiments which showed that anti-activin antibodies can inhibit milk ejection (Plotsky et al, 1988) and that injections of activin into the hypothalamus increases the concentrations of OT in the peripheral circulation (Sawchenko et al, 1988).

1.9 Activin A and follistatin in maternal serum

The development of an ELISA system for the measurement of activin has facilitated the analysis of activin A in maternal serum (Knight et al, 1996). In normal pre-menopausal women, activin A is undetectable in serum (Woodruff and Mather, 1995). However, by eight weeks' gestation, maternal serum levels are easily detected. Comparison of levels of activin A in women who underwent a donor-insemination (no corpus luteum) with women who had normal spontaneous pregnancies, demonstrated no differences in profiles during the sampling period (week five to 12 of gestation) suggesting that activin A is a product of the placenta and not the corpus luteum (Birdsall et al, 1997). These data were supported by similar studies in early pregnancy (Lockwood et al, 1997, Muttukrishna et al, 1997b). During the remainder of pregnancy, concentrations of activin A increase progressively towards term, in particular during the third trimester (Muttukrishna et al, 1995, 1996, Woodruff et al, 1997). More recently, longitudinal prospective studies have observed a similar profile for total activin A (bound and free) (Fowler et al, 1998, O'Connor et al, 1999). Levels of activin A rise slightly until week 20 of gestation and by 36 weeks of gestation had increased to about six-fold. Similar to inhibin, levels of activin A decline sharply after

delivery and become undetectable by six hours postpartum (Petraglia et al, 1993b), consistent with a feto-placental origin of activin A.

The levels of follistatin in maternal serum display a similar trend. The concentrations of activin A and follistatin are highly correlated throughout pregnancy. Follistatin concentrations increased to a greater extent during the second trimester while activin A concentrations rise more steeply during the third trimester (O'Connor et al, 1999). This profile suggests a progressive increase of free, unbound activin A towards term as the ratio between total activin A to total follistatin is increased (Fowler et al, 1998).

1.10 Activin A/follistatin and the onset of labour

In maternal serum, concentrations of activin A are significantly elevated when measured immediately after spontaneous vaginal delivery or emergency Caesarean section and compared to gestation-matched women prior to labour onset (Petraglia et al, 1994a). In most cases the levels observed were elevated several fold shortly after delivery. In contrast, elective Caesarean section did not result in an increase of maternal serum activin A in any of the women studied. Woodruff et al (1997) followed seven pregnant women taking blood samples every two weeks until the last two weeks prior to delivery. In all but one woman, levels of activin A rose to peak levels at term. In one woman, activin A and follistatin levels remained undetectable throughout pregnancy and this woman subsequently required induction of labour using oxytocin. Follistatin levels were variable in the six women who delivered spontaneously. In three women, levels increased several fold in the final month of pregnancy, but fell sharply in the other three women to almost first trimester levels during the last two weeks before delivery.

In the rat, activin A appears to be capable of regulating the release of OT from the brain (Sawchenko et al, 1988). An intraventricular injection of activin A to male rats evokes a significant increase in circulating ACTH and CRH (Plotsky et al, 1991). The effect of activin A on OT, CRH, ACTH, all of which are important factors involved in human parturition, suggests that activin A may be involved in regulating the onset of parturition in the human.

The profile of activin A in amniotic fluid is similar to that observed in maternal serum of women undergoing labour. Women who underwent spontaneous labour at term had significantly higher concentrations of activin A in amniotic fluid than women undergoing an elective Caesarean section (no labour) (Petraglia et al, 1997b). Interestingly, the concentrations were highest in women who were undergoing pre-term labour and subsequent delivery, although activin A content in placenta was similar in non-labouring and labouring women at term (Keelan et al, 1999). Gallinelli et al (1996) observed that activin A is secreted in a pulsatile manner during pregnancy, similar to other placental neurohormones such as GnRH and CRH (Petraglia et al, 1994c). During pre-term labour and in women with gestational diseases such as diabetes, the pulse amplitude increased significantly when compared to non-labouring, healthy controls. Petraglia argues that the pulsatile pattern of serum activin A may be due to pulsatile release from trophoblast cells, but he also states that metabolic clearance rates and the presence of binding proteins, such as follistatin and α2-macroglobulin or other regulatory factors may affect the serum profile observed during the duration of the study. The significance of this study is as yet unclear.

Finally, using a rat model, Draper et al (1997) has shown that the maternal serum profile of activin A in the pregnant rat is identical to that in the human during gestation. In the rats, serum levels of activin A increased 22 fold by the last day of pregnancy. Furthermore, the study showed that radiolabelled ligand when injected into the pregnant rats bound preferentially to the myometrium with low or no binding to the endometrium. Inhibin did not bind to the myometrium. It was suggested that activin may be one of several molecules involved in stimulating myometrial contractions during pregnancy.

1.11 Activin A and follistatin in abnormal pregnancy

Circulating levels of activin A are elevated in a number of pregnancy pathologies. Activin A, as well as inhibin A, is elevated 10-fold in women with established pre-eclampsia (Muttukrishna et al, 1997a, Petraglia et al, 1995, Cuckle et al, 1998, Fraser et al, 1998). A correlation between activin A and the degree of proteinuria was also observed suggesting that activin A may be useful as a marker of disease severity (Laivuori et al, 1999). Activin A and inhibin A have been evaluated recently in a large study as biochemical predictors of pre-eclampsia. Both hormones were elevated prior to onset of disease (Muttukrishna et al, 2000a). Activin A was a better predictor of pre- eclampsia than inhibin but a large percentage of women (41 – 75% percent) could still not be detected using activin A alone. It was therefore suggested that activin A may be more useful in combination with measurements of other indices of the disease, similar to the triple or quadruple test used for detection of Down's syndrome. Larger cohort of women, especially high risk women, are needed to identify more accurately women at risk of early onset pre-eclampsia.

Activin A levels are significantly increased in pregnant women with hypertension (Silver et al, 1999). These findings contrast an earlier investigation which showed no difference in levels of activin A between normal, healthy and hypertensive pregnant women (Petraglia et al, 1995).

Pregnant women who develop diabetes have significantly elevated concentrations of maternal serum activin A. The levels decrease to normal range after insulin therapy (Petraglia et al, 1995). The ability of activin A to regulate insulin production was further demonstrated by culture experiments of human pancreatic cells (Florio et al, 2000). After addition of glucose, activin A was able to modulate the insulin secreting response of cultured pancreatic cells. However, a relationship between the degree of insulin sensitivity and activin A during pregnancy was not demonstrated (Laivuori et al, 1999).

Follistatin was not evaluated in these pregnancy abnormalities until very recently. D'Antona et al (2000) showed that, in pregnant women with pregnancy-induced hypertension, intra-uterine growth retardation or pre-eclampsia, levels of follistatin remain unchanged in the presence of elevated levels of activin A. These findings imply that more biologically free activin A is present in the peripheral circulation of women with certain pregnancy pathologies.

1.12 Regulation of activin A by inflammatory cytokines

In human first and second trimester placental sections, activin A has been detected in placental Hofbauer cells. Hofbauer cells are a subtype of macrophages and most commonly found during early pregnancy in the stromal tissue of the placental villi.

Their numbers decrease towards term and it has been suggested that these cells are involved in mediating the immune response between placental, fetal and maternal tissues during trophoblast invasion (Rabinovici et al, 1992).

A number of cytokines can influence activin A activity. In gestational tissues, interleukin-1β and tumour necrosis factor α are potent stimulators of activin A and have been proposed as possible regulators of pre-term, infection-based labour (Keelan et al, 1998). In turn, cell-mediated immune function of rat thymocytes (Hedger et al, 1989) and human monocyte differentiation into macrophages (Petraglia et al, 1991b, Yamada et al, 1992) can be modulated by both activins and inhibins and both may have para- and/or autocrine roles in the intra-uterine tissues during pre-term labour.

1.13 Activin A and follistatin regulation of vascular endothelial cells

Activin A and follistatin have direct effects on vascular endothelial cell activity. While activin A inhibits capillary endothelial cell growth in vitro (McCarthy and Bicknell, 1993), follistatin can counteract these effects and induces angiogenesis (Kozian et al, 1997). Activin A can also potentiate the proliferation and differentiation of erythroid progenitor cells *in vitro* (Yu et al, 1989) and in consort with erythropoietin stimulates differentiation of erythroid progenitor cells (Shiozaki et al, 1998, Broxmeyer et al, 1988). Injections of activin A to ovariectomized estrogen-treated rats induces a significant increase in the levels of circulating red blood cells and haemoglobin (Schwall et al, 1989).

The profile of activin A during pregnancy, together with its reported regulatory effects on important mediators of parturition, such as prostaglandins and oxytocin in

gestational tissues has lead to the suggestion that activin A may be a novel regulator of parturition in the human. The physiological actions of activin A in late pregnancy and during labour and its usefulness as a clinical marker of impending parturition need to be explored in detail and form the basis for the work described in this thesis.

1.14 Aims and Hypothesis

The aims of this thesis were:

1. To clarify the detailed ontogeny of serum activin A and follistatin during late human pregnancy (chapter 3).
2. To investigate the hypothesis that activin A is directly involved in the pathways of labour as reflected in changes in maternal serum (chapter 4).
3. To investigate the hypothesis that maternal and fetal serum activin A is associated with dysfunctional labour (chapter 5)
4. To investigate the hypothesis that activin A directly targets the myometrium during labour (chapter 6).
5. To identify βA-subunit and target sites (receptors) for activin A in placenta and fetal membranes and assess the association of activin A receptors and distribution with labour (chapter 7).
6. To evaluate the relationship between maternal serum activin A levels and chronic fetal hypoxia during mid and late pregnancy (chapter 8).
7. To evaluate the relationship between activin A and pre-eclampsia during mid and late pregnancy (chapter 9).

CHAPTER 2

VALIDATION STUDIES FOR THE MEASUREMENT OF ACTIVIN A AND FOLLISTATIN IN BIOLOGICAL FLUIDS

2.1 INTRODUCTION

The enzyme-linked immunoassays (ELISA) for activin A and follistatin described in section 1.5.3 (page 16) have been developed by NP Groome and his colleagues at the School of Biological Sciences, Oxford Brookes University, Oxford, UK (Knight et al, 1996, Evans et al, 1998) and are commercially available (Serotec, Oxford, UK). These assays have been validated on a range of biological fluids, including serum from cycling and pregnant women and found to be highly specific and sensitive being able to detect these proteins in very low concentrations in the peripheral circulation. These assays form the basis for the majority of studies outlined in this thesis. However, in order to achieve continously reproducible results over several years, it was important to assess the stability of activin A and follistatin under varying conditions in our laboratory to evaluate the possible alterations in observed concentrations of these proteins that might arise due to transport and storage.

The use of stored serum samples sometimes necessitates the freezing and thawing of the same samples on several occasions. The studies in this section were performed in order to assess whether repeated freezing and thawing had effects on the levels of activin A and follistatin. The stability of activin A in amniotic fluid was also examined and the usefulness of pooled amniotic fluid as a quality control in the assays tested.

2.2 MATERIALS & METHODS

2.2.1 Study 1: Stability of activin A and follistatin during storage

2.2.1.2 Patients

Five women in the third trimester of pregnancy were recruited for this study and all women gave informed consent. The women were healthy and had a normal, singleton pregnancy (weeks' 34 to 39 of gestation). Gestational age was calculated from either the first day of the last menstrual period or by an early pregnancy ultrasound. The studies were approved by the Monash Medical Centre Human Research and Ethics Committee.

2.2.1.2 Collection of blood samples

Blood samples were collected as serum or plasma. There are no differences in the concentrations of activin A or follistatin between serum and plasma (Graham Jenkin, personal communication).

A venous blood sample was collected by routine venepuncture from the five women. Each sample was divided into two aliquots. One aliquot was centrifuged at 4°C for 15 minutes at 3500 rpm and the other kept unseparated at room temperature (RT). The serum from the spun sample was then further divided into four sub-aliquots and one was stored at – 20 °C. The other three samples were left at RT overnight with subsequent frozen storage of one after 24 hours (– 20 °C), one at 48 hours and the other at 72 hours. The remaining sample was left for one week at RT. The same protocol was followed for the unseparated blood sample. The sample was aliquoted into four samples and, at 24 hours, 48 hours and 72 hours, one each was centrifuged

and the serum stored at – 20 °C. The last sample was left unseparated at RT for one week. Storage of follistatin was analysed as serum only.

2.2.1.3 Enzyme-linked immunosorbent assay (ELISA) for activin A

The assay protocol used was developed by Knight et al (1996) with some minor modification (Wallace et al, 1998). This specific two-site immunoassay measures total (free and bound) activin A in biological fluids. Immunoassay plates pre-coated with antibody (96 well; Nunc, Life Technologies, UK) and standards (partially purified from human follicular fluid) were purchased from Nigel P. Groome (School of Biological Sciences, Oxford Brookes University, Oxford, UK). The plates were coated by covalent coupling of a specific mouse monoclonal antibody, raised against a synthetic peptide corresponding to residues 82-114 of the βA-subunit (E4), to the plates. All late pregnancy serum samples and standards (range 78 pg/ml to 10000 pg/ml) were diluted in phosphate buffered saline (0.01M PBS) containing 5% bovine serum albumin (BSA, Sigma, Castle Hill, NSW, Australia) and 0.1 % (w/v) sodium azide. The assay included two pre-treatment steps, designed to improve the overall assay performance by reducing background binding and increasing sensitivity (Knight et al, 1996). The first pre-treatment step involved dilution of assay samples and standards in an equal volume of the denaturing agent sodium dodecyl sulphate (SDS, Amresco, Ohio, USA, 6 % in destilled water (dH_2O)). Samples were vortexed and then heated at 90 to 95 ° C for three minutes by placing of the rack of tubes into a container filled with recently boiled water. This step allows for the dissociation of activin A from binding proteins, such as follistatin and α_2-macroglobulin (α_2M), and the unfolding of the protein (Wallace et al 1998). After cooling to ambient temperature, the samples were treated with an oxidizing agent, hydrogen peroxide

(30% H_2O_2, 20µl/tube). Hydrogen peroxide converts methionine residues on the epitopes to their sulphoxide form to which the antibody binds with greater affinity (Knight & Muttukrishna, 1994). The tubes were vortexed and incubated again in a closed, moist container at RT for 30 minutes. Subsequent to these pretreatment steps, duplicate aliquots of samples and standards were added to the wells (100 µl/well) which had been pre-filled with 25 µl of assay diluent (0.1M Tris HCL, 0.15M NaCL with 5% triton X 100, 10% bovine serum albumin (BSA), 5% normal mouse serum, pH 7.5). The plates were incubated overnight at RT in a moist, closed container. The following day, the plates were washed ten times in wash buffer (0.05 M Tris-HCL buffer containing 0.15 mol NaCl, 0.05 % (v/v) Tween-20, in dH_2O, pH 7.5). Excess wash buffer was removed by repeatedly tapping the plates upside down onto paper towels. Fifty µl of biotinylated secondary antibody (B4, Nigel P. Groome) diluted 1:1200 in assay diluent, was then added to each well. The plates were incubated in a moist, closed container at RT for two hours and then washed in wash buffer ten times. Excess wash buffer was removed as described above and 50 µl of streptavidin (Nigel P. Groome, School of Biological Sciences, Oxford Brookes University, Oxford, UK) diluted 1:300 in assay diluent, added to each well. After one hour incubation at RT in the container, plates were washed again in wash buffer (10 times) and bound alkaline phosphatase quantified by means of a commercially available enzyme immunoassay amplification kit (ELISA Amplification System, Life Technologies, Mulgrave, Victoria, Australia). The reaction was stopped by addition of 0.3 M H_2SO_4 (50µl/well). Absorbancy was measured at 490 nm with a reference wavelength set at 620nm using a microplate reader (Emax, MAXline Microplate Readers, Molecular Devices, Sunnyvale, California, USA) with integrated software (Softmax; Molecular Devices). Internal controls were serially diluted, pooled mid-trimester amniotic fluid.

2.2.1.4 ELISA for follistatin

The ultra-sensitive two-site enzyme immunoassay for the detection of total (bound + free) follistatin in human serum has been developed and described by Evans et al, 1998. The assay employs a pair of mouse monoclonal antibodies directed against follistatin 288. Binding of activin to follistatin was disrupted by the use of a dissociating solution (Poncelet & Francimont, 1994). Immunoassay plates (96 well; Nunc, Life Technologies, UK) coated passively with specific mouse monoclonal antibodies raised against human recombinant follistatin 288 (hr-FS288) were purchased from NP Groome (School of Biological Science, Oxford Brookes University, Oxford, UK). The standard material used in the assay was hr-FS288 (NP Groome, range 19.53 pg/ml to 2500 pg/ml). Quality control consisted of serially diluted, pooled mid-trimester amniotic fluid. All standards, controls and samples were diluted in dissociating solution (84mmol sodium deoxycholate [BDH, Kilsyth, Victoria, Australia], 3.4 % (v/v) Tween-20 [Sigma, Castle Hill, NSW, Australia], 1 % (w/v) BSA [Sigma], 5 % (v/v) mouse serum in PBS). Duplicate 50µl amounts of standards, controls and samples were added to the wells. The plate was sealed and placed in a humidified container to incubate overnight at RT. The plate was then washed four times in wash buffer, banged dry and 50µl of antibody-alkaline phosphatase conjugate diluted in Tris conjugate buffer (1 % (w/v) BSA in 25mM Tris-HCL [BDH], pH 7.5, containing 0.15 M NaCl and 0.5 % (v/v) Tween 20) added to the wells. The plate was incubated for two hours at RT in the humidified container, washed again four times in wash buffer and banged to dryness. Alkaline phosphatase substrate (50µl) and amplifier solution (50µl) were subsequently added according to the manufacturer's instructions (ELISA Amplification System; Life Technologies,

Mulgrave, Victoria, Australia). The reaction was stopped by addition of 0.3 M H_2SO_4 (50µl/well) and the well absorbances read as described above for activin A (3.3.3.1).

2.2.1.5 Statistical analyses (study 1 & 2)

Activin A and follistatin levels were analysed by analysis of variance using ANOVA for repeated measures. Analyses of all studies were performed using Graphpad Prism software (Graphpad software, San Diego, CA, USA). Statistical significance was recognised when $P<0.05$.

2.2.1.6 Results

The limit of detection in the activin A assay, calculated from an entire plate of blanks (assay diluent only) and defined as the dose of activin A, which generated an absorbance value of three times the standard deviation of the blanks, was 72 pg/ml. Intra- and inter-assay coefficients of variation were 8% and 15% respectively. A minimum of three blanks was included in each plate, spread across the plate, to allow for "drift" of background absorbance across the plate. Cross-reactivity with inhibin A, inhibin B, and activin B was reported at less than 0.5 % and for pro-αC and follistatin less than 0.1% (Knight et al, 1996).

The limit of detection in the follistatin assay was 150 pg/ml and the intra- and inter-assay coefficients of variation were less than 5 %. The cross-reactivities with activin and dimeric inhibin isoforms have previously been reported at < 0.3%, while cross-reactivity with follistatin-315 was significant at 9.9% (Evans et al, 1998). Controls and serum samples gave dose response curves parallel to the standards.

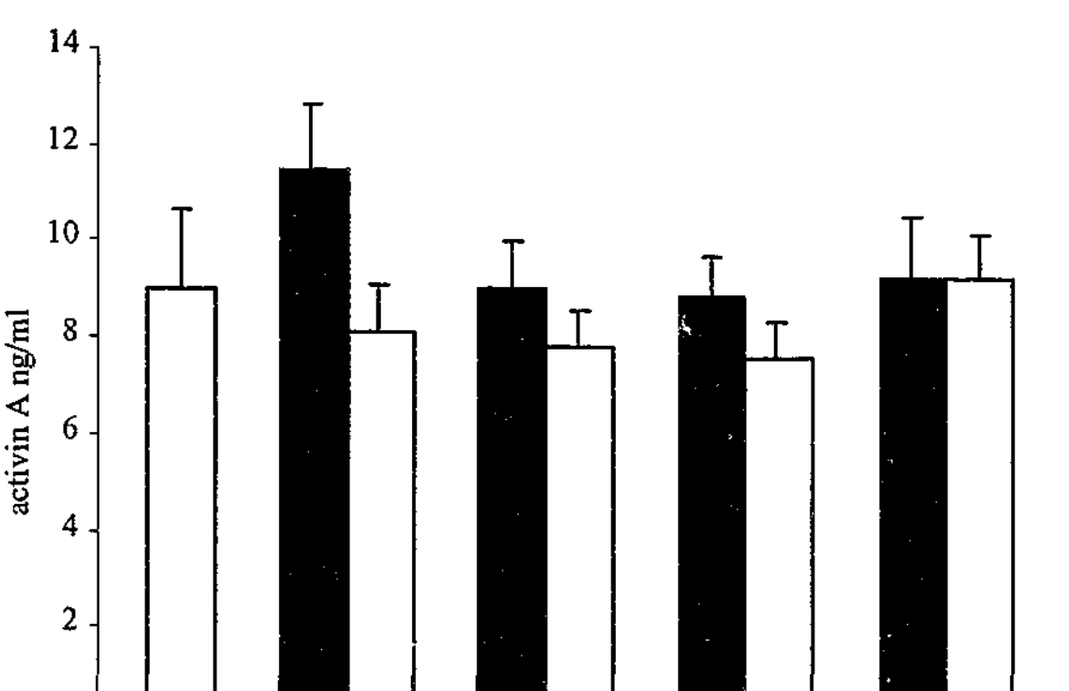

Figure 2.1 Changes in serum activin A (mean ± SEM) with storage at RT from five pregnant women as serum = □ , or as whole blood = ■.

Results for activin A in whole blood and serum according to storage are shown in figure 2.1. The equivalent results for follistatin in serum are shown in figure 2.2. The data are represented as the means ± standard error of the mean (SEM). The levels of activin A in serum and whole blood did not change significantly across the time period studied (P = 0.97 and 0.29 respectively). In contrast, levels of follistatin in serum changed significantly throughout the duration of storage (P = 0.03). By 48 hours after collection, levels had dropped from 5.235 ± 0.216 ng/ml to 3.624 ± 0.298 ng/ml, dropping further to 3.016 ± 0.153 ng/ml after 72 hours and remaining unchanged until day 7 after collection.

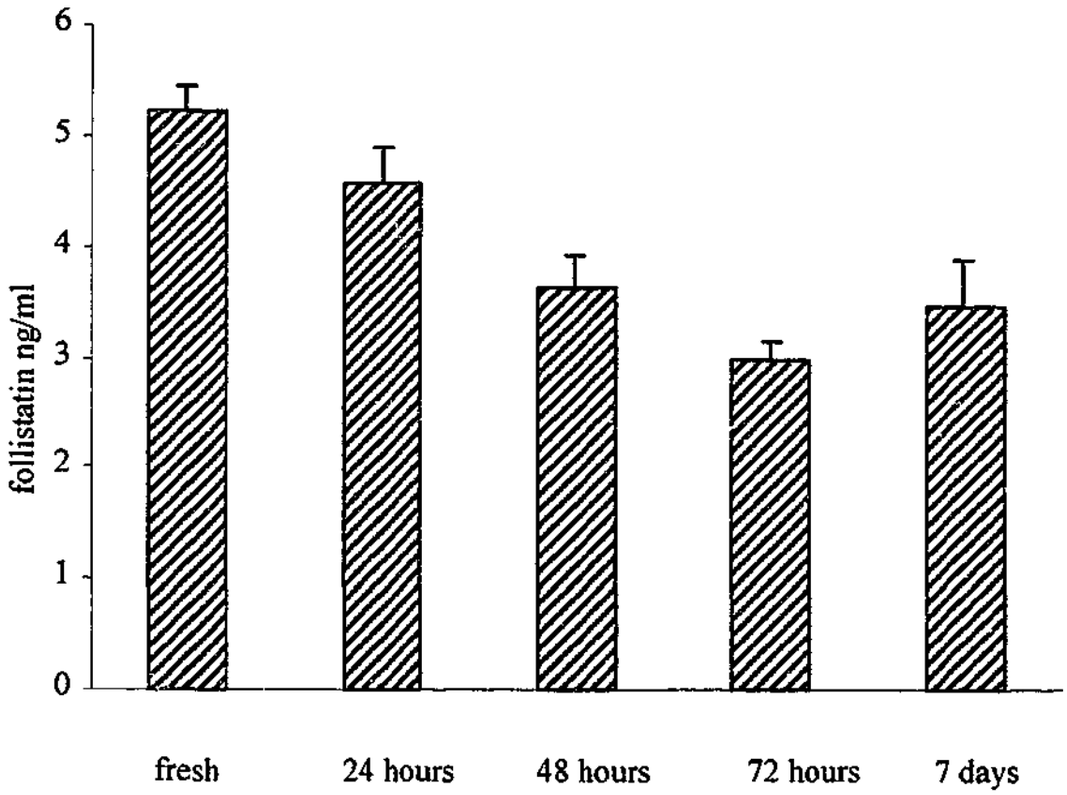

Figure 2.2 Changes in serum follistatin (mean ± SEM) with storage at RT from five pregnant women.

2.2.2 Study 2: Stability of serum activin A and follistatin after repeated freeze-thaw cycles

2.2.2.1 Patients

Five women undergoing an elective Caesarean section at term (38 to 40 weeks' gestation) for breech presentation or previous Caesarean section were recruited for this study and all women gave informed consent. The women were healthy and each had a normal, singleton pregnancy. Gestational age was calculated from either the first day of the last menstrual period or by an early pregnancy ultrasound. The studies

were approved by the Monash Medical Centre Human Research and Ethics Committee.

2.2.2.2 Collection of blood samples

A venous blood sample was collected by routine venepuncture immediately prior to surgery. The samples were centrifuged at 3500 rpm for 15 minutes at 4 °C. The serum was then aliquoted into four samples. All were frozen at – 20 °C. Three of the four samples were thawed 24 hours later and returned to the freezer. After 48 hours, two samples were thawed and frozen again. After 72 hours, one of the samples was thawed and returned to the freezer. The samples were therefore subjected to between 1 and 4 freeze-thaw cycles. On the following day, all samples were assayed in one batch for activin A and follistatin.

2.2.2.3 Collection of amniotic fluid

Amniotic fluid samples were collected during elective Caesarean section. All samples were pooled for the use as quality controls in the activin A assay. The samples were aliquoted, diluted either 1:2 or 1:8 in phosphate buffered saline (0.01M PBS) with 5% bovine serum albumin (BSA, Sigma, Castle Hill, NSW, Australia) and 0.1 % (w/v) sodium azide and subjected to repeated freeze-thaw cycles as described above for the serum samples. After the fourth freeze-thaw cycle, all samples were assayed in one batch for activin A.

2.2.2.4 ELISA for activin A and follistatin

The ELISA for measurements of total activin A has been described in section 2.2.1.3 and for follistatin in section 2.2.1.4.

2.2.2.5 Statistical analysis

The statistical analyses used in this study have been outlined in section 2.2.1.5.

2.2.2.6 Results

Results for stability of activin A and follistatin in serum after repeated freeze-thaw cycles are shown in figure 2.3 and the results for activin A in amniotic fluid are shown

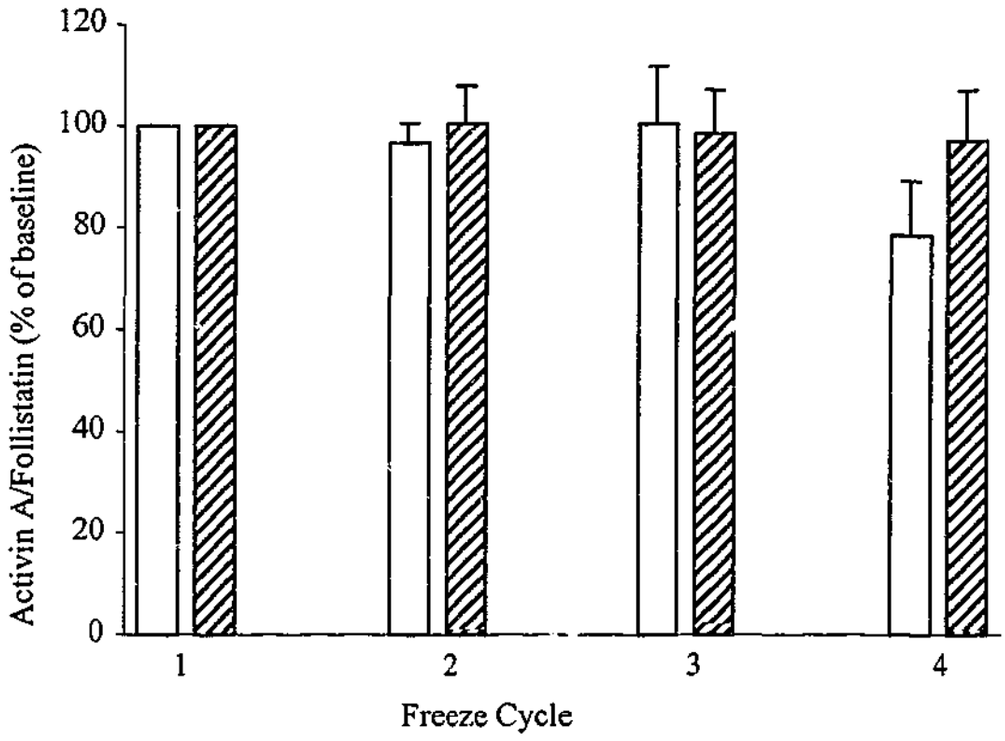

Figure 2.3 Stability of activin A and follistatin after repeated freeze-thaw cycles

Activin A = □, follistatin = ▨ .

in figure 2.4. Data are shown as percentage of the baseline samples (one freeze-thaw cycle). The levels of activin A observed in serum did not change after 3 freeze-thaw cycles. After the fourth cycle, the levels dropped to 78.6 % of baseline levels (P = 0.17). The levels of follistatin remained unchanged after 4 freeze-thaw cycles (P = 0.26). In contrast to the stability of activin A in serum, levels of activin A in amniotic fluid diluted 1:2 dropped sharply after 2 freeze-thaw cycles to 41% of baseline (P = 0.009) and remained unchanged thereafter regardless of further cycles. Activin A levels in amniotic fluid diluted 1:8 fell to 71% of baseline levels after 2 cycles, 85% after 3 cycles and 42% after 4 cycles of repeated freezing and thawing (P = 0.022).

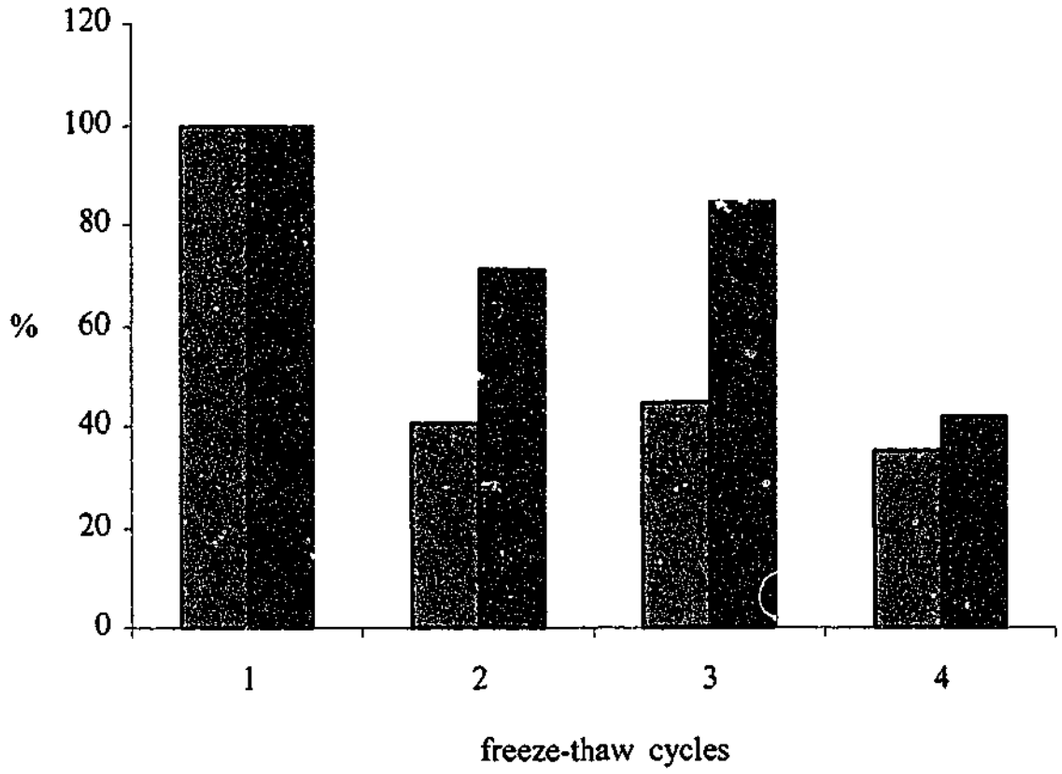

Figure 2.4 Stability of activin A in amniotic fluid after repeated freeze-thaw cycles.

Activin A diluted 1:2 in assay diluent = ■ , activin A diluted 1:8 in assay diluent = ■

2.2.3 Study 3: Validation of amniotic fluid as quality controls in ELISA

2.2.3.1 Introduction

This study was carried out in order to examine the suitability of amniotic fluid as quality controls for the activin A and follistatin assay.

2.2.3.2 Patients

The selection of patients for this study has been described in section 2.3.2.

2.2.3.3 Collection and processing of amniotic fluid

The method for collection of amniotic fluid has been outlined in section 2.3.3. Amniotic fluid was pooled and serially diluted in phosphate buffered saline (0.01M PBS) with 5% bovine serum albumin (BSA, Sigma, Castle Hill, NSW, Australia) and 0.1 % (w/v) sodium azide to give dilutions of 1:2 to 1:36. All samples were stored at -20°C.

2.2.3.4 ELISA for activin A and follistatin

The ELISA for measurements of total activin A has been described in section 2.2.1.3 and for follistatin in section 2.2.1.4.

2.2.3.5 Statistical analysis

The data was analysed using regression analysis (Pearson's correlation).

2.2.3.6 Results

The dose-response relationships between activin A and follistatin standards and serially diluted amniotic fluid are shown in figures 2.5 and 2.6 respectively. The dose-

response curve for serially diluted activin A and follistatin in amniotic fluid was significantly correlated to the standard curve ($r^2 = 0.86$ and $r^2 = 0.92$ respectively).

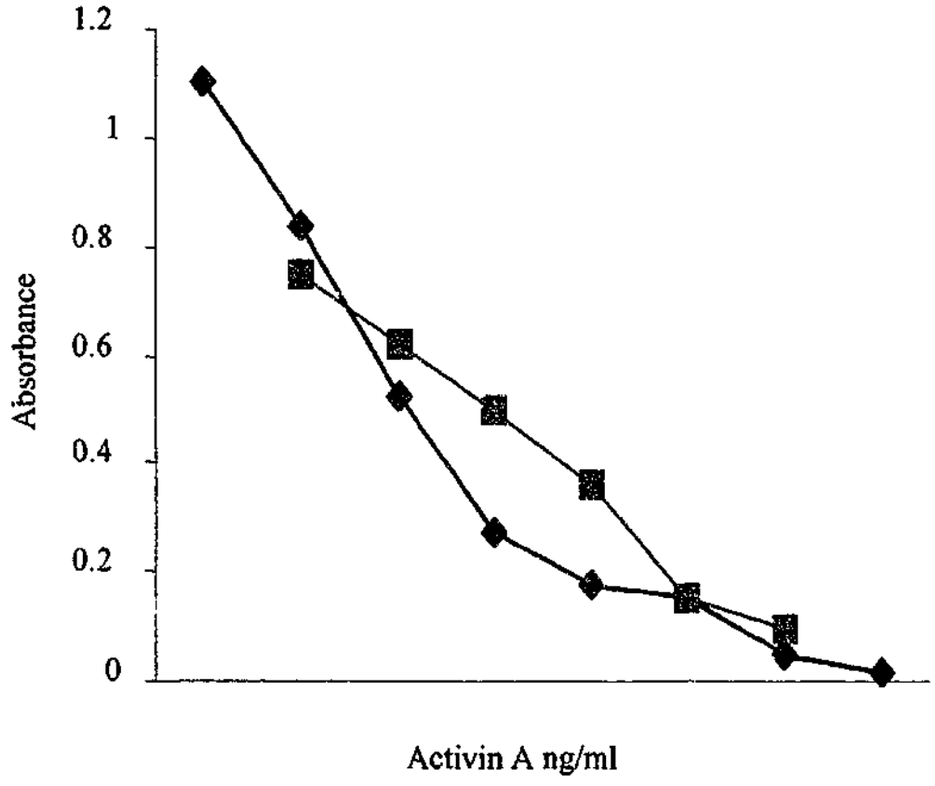

Figure 2.5 Dose-response relationship between activin A standard and serially diluted amniotic fluid. Standard curve = ■ , activin A in amniotic fluid = ■ .

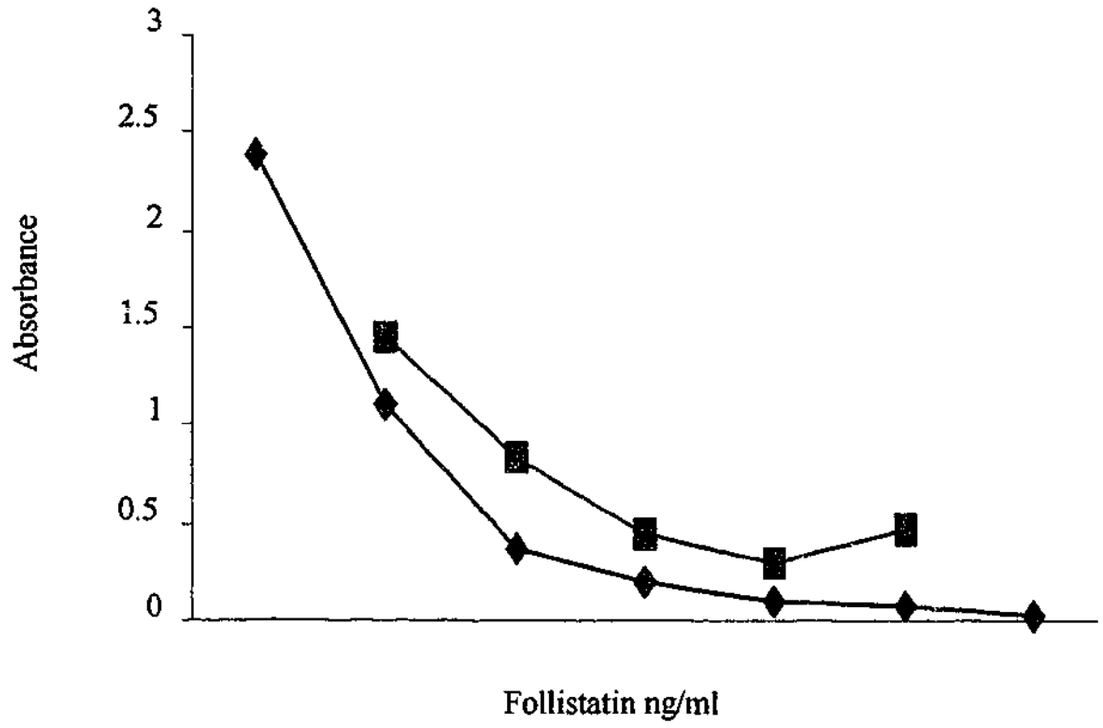

Figure 2.6 Dose-response relationship between follistatin standards and serially diluted amniotic fluid. Standard curve = ■, follistatin in amniotic fluid = ■

2.3 DISCUSSION

The data presented in this chapter has important implications for the use of the ELISA for activin A and follistatin under clinical conditions. The observed stability of both activin A and follistatin in serum under the conditions tested in our laboratory allows for the collection and storage of serum samples over extended periods of time without significant loss of either protein. The data clearly shows that activin A concentrations are highly correlated in serum and whole blood, and further, only minimal loss of activin A protein occurs within seven days of collection. However, serum follistatin levels dropped significantly after two days storage. Taken together, blood samples collected for analyses of activin A and follistatin can safely be stored at room

temperature (RT) for up to two days, or over a weekend. For measurements of activin A only, samples can be stored at RT for up to one week. The findings reported in this chapter further show, that no significant changes occur in the levels of activin A and follistatin in samples after repeated freezing and thawing, therefore permitting the repeated analysis of stored samples, including archived samples collected and analysed earlier.

Amniotic fluid when assayed for activin A and follistatin diluted out in parallel to the standards, but activin A levels in amniotic fluid fell significantly after repeated freezing and thawing. For the use of amniotic fluid as a quality control, all amniotic fluid samples were therefore collected on one day, pooled, then aliquoted and stored in one ml quantities at – 20 °C for use in subsequent assays. Thawed samples were discarded after completion of the assay.

The data presented here has provided important insights into the stability of activin A and follistatin proteins in blood samples. As a result, assay performance in our laboratory was highly reproducible and stable over several years and it also greatly facilitated the collection and storage of blood samples in the clinic and in labour ward.

CHAPTER 3

MATERNAL SERUM TOTAL ACTIVIN A AND FOLLISTATIN CONCENTRATIONS DURING LATE PREGNANCY

3.1 INTRODUCTION

Across the second and the third trimester of pregnancy, concentrations of activin A and its binding protein follistatin rise 10 to 20-fold in the peripheral maternal circulation reaching peak levels at term (Petraglia et al, 1993b, de Kretser et al, 1994, Yokoyama et al 1995, Fowler et al, 1998, O'Connor et al, 1999). The ratio between total activin A and total follistatin in the maternal circulation increases during the third trimester so that more unbound, biologically free activin A is present in the circulation towards the end of pregnancy (Wakatsuki et al, 1996, Woodruff et al, 1997, Fowler et al, 1998, Evans et al, 1998). Further, maternal serum levels appear to increase significantly in association with labour at term (Petraglia et al, 1994a), as well as with pre-term labour (Petraglia et al, 1995). Both activin A and follistatin were also shown to rise in amniotic fluid during pregnancy suggesting increasing release or synthesis by the fetus, the fetal membranes (amnion and chorion) or the uterus (Petraglia et al, 1993a, Muttukrishna et al, 1996, Wallace et al, 1997). Mirroring the profile of activin A observed in maternal serum and amniotic fluid, placental and fetal membrane mRNA levels increase during pregnancy and peak at term (Petraglia et al, 1990, Petraglia et al, 1991c). It has been suggested that activin A and follistatin are important regulators of the initiation of labour and the progressive rise seen in the peripheral circulation is typical of healthy women going into spontaneous labour (Fowler et al, 1998, O'Connor et al, 1999). This suggestion is supported by a study by

Woodruff et al (1997) who observed that one women who had no measurable levels of activin A or follistatin throughout pregnancy, did not go into spontaneous labour and subsequently required induction by oxytocin to initiate contractions.

However, all published observations on maternal serum activin A and follistatin during gestation are based on cross-sectional studies, include a very small group of women (Woodruff et al, 1997) or do not cover the days immediately prior to delivery (Petraglia et al, 1993b, Fowler et al, 1998, O'Connor et al, 1999). The detailed profile of maternal plasma activin A and follistatin during the last weeks of pregnancy and especially during the weeks immediately prior to delivery, is thus still unknown and there are no published data relating activin A and follistatin to the time of labour onset. Clearly defined longitudinal studies are thus needed, following maternal plasma levels of activin A and follistatin in pregnant women during late pregnancy until actual delivery. Such investigations may improve our understanding of the potential role of activin A during pregnancy and parturition. The aim of the studies described in this chapter was to assess the detailed profile of maternal plasma activin A and follistatin concentrations during the closing weeks of pregnancy until delivery.

3.2 MATERIALS AND METHODS

3.2.1 Patients

All women studied were normal and healthy. In each case, gestational age was calculated from the first day of their last menstrual period or by an early pregnancy ultrasound. All women had a normal singleton pregnancy. The studies were approved

by the Monash Medical Centre Human Research and Ethics Committee and each woman gave informed written consent.

3.2.2 Pregnancy studies

Three studies were undertaken investigating the detailed ontogeny of activin A and follistatin throughout pregnancy with special emphasis on the last weeks and days leading up to labour onset:

3.2.2.1 Cross-sectional pregnancy study:

A single venous blood sample was collected from 123 women at different stages of pregnancy, ranging from 8 - 42 weeks' gestation.

3.2.2.2 Longitudinal pregnancy study:

Venous blood was collected from eight women on a weekly basis from 30 weeks' gestation until delivery.

3.2.2.3 Detailed longitudinal pregnancy study:

Venous blood was collected from ten women three times a week from 37 weeks' gestation until delivery.

3.2.3 Collection and processing of blood samples

Blood was collected from a peripheral vein in the antecubital fossa (5-10ml), transported on ice to the laboratory and centrifuged (GLC-2B, Dupont Instruments, Sorvall) at 3500 rpm for 15 minutes at 4 °C. The serum was aspirated, aliqoted and stored at – 20°C until assayed.

3.2.4 ELISA for activin A and follistatin

The protocol for the ELISAs of activin A and follistatin has been described in chapter 2, section 2.2.1.3 and 2.2.1.4 respectively.

3.2.5 Statistical Analysis

Differences between the groups in the longitudinal studies (comparison between the levels of activin A across and within the sampling period up until delivery) were determined using Kruskal Wallis test. Differences in the cross sectional data were analysed using Mann-Whitney *U* test for non-parametric distribution. Correlation between hormones was analysed using Spearman Rank analysis. Significance was recognised when $P<0.05$.

3.3 RESULTS

3.3.1 Cross-sectional pregnancy study

The profile of activin A and follistatin throughout pregnancy is shown in figure 3.1. Levels of activin A were low and stable during the first and second trimester of pregnancy, increasing rapidly at the beginning of the third trimester such that at 28-31 weeks' gestation the mean ± SEM level was more than double that in the first trimester

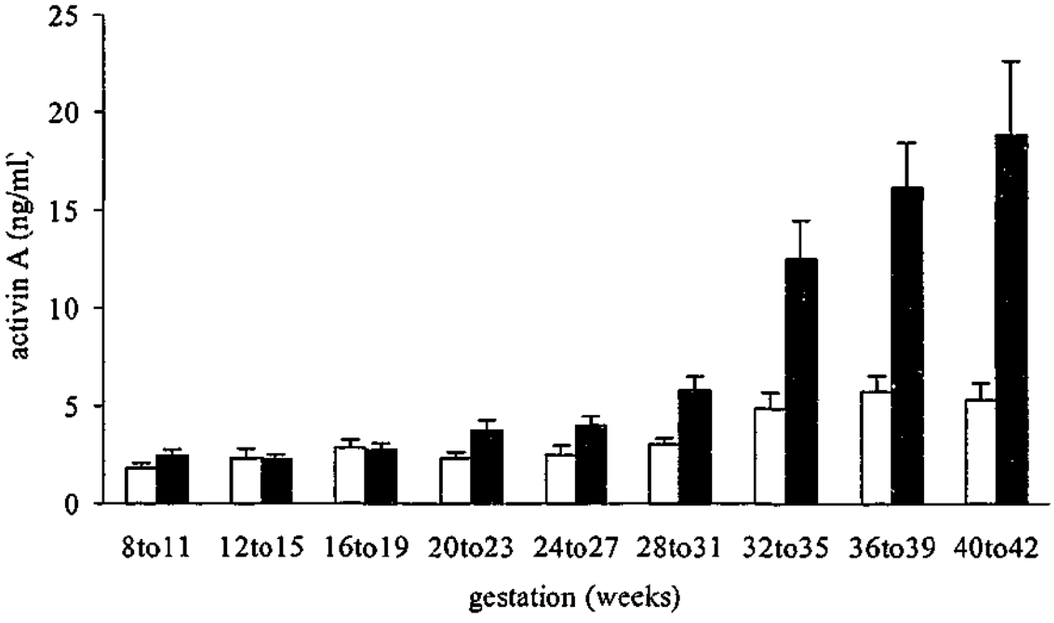

Figure 3.1. Profile of maternal serum activin A and follistatin during pregnancy

Activin A = ■, follistatin = ◘

(2.4 ± 0.3 ng/ml at 8-11 weeks vs 5.5 ± 0.8 ng/ml at 28-31 weeks (P< 0.001). By 32 – 35 weeks' gestation levels had again doubled (12.0 ± 1.8 ng/ml), reaching peak levels at term, between weeks' 37 to 41 of gestation (18.9 ± 3.8 ng/ml). Levels of follistatin also increased significantly during pregnancy (P=0.004). Mean levels at 8-11 weeks' gestation were 1.76 ± 0.27 ng/ml rising to 5.74 ± 0.8 ng/ml at term. The levels of activin A and follistatin were significantly correlated throughout gestation (r^2 = 0.92, P<0.0001, Spearman Rank correlation). The ratio of activin to follistatin increased from 1.27 in early pregnancy to 3.56 at term (P = 0.0087, figure 3.2). Because

measurements of activin A and follistatin were carried out in a random fashion across gestation, only the mean ratios are represented without error bars.

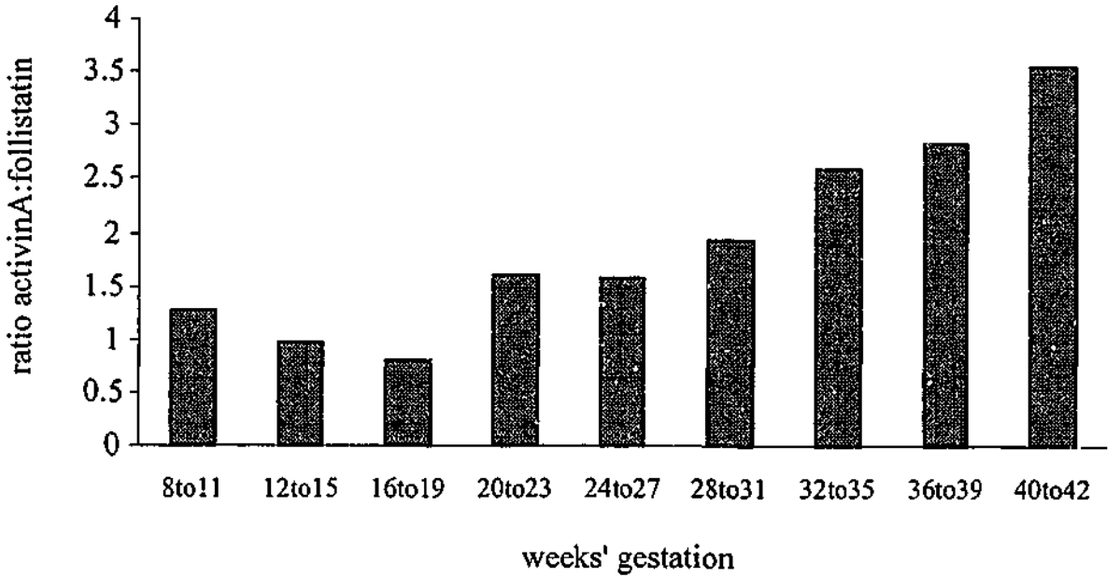

Figure 3.2 Ratio of activin A to follistatin during pregnancy.

3.3.2 Longitudinal pregnancy study: 30 weeks' gestation to delivery

The levels of activin A and follistatin in maternal serum were analysed and plotted relative to the day of spontaneous labour onset. Results are shown in figure 3.3. In all eight women, maternal serum activin A increased significantly over the period from 30 weeks' gestation until established labour at term (defined as the onset of contractions leading to delivery), ($P<0.0001$). However, peak levels (10.4 ± 2.0 ng/ml) were attained

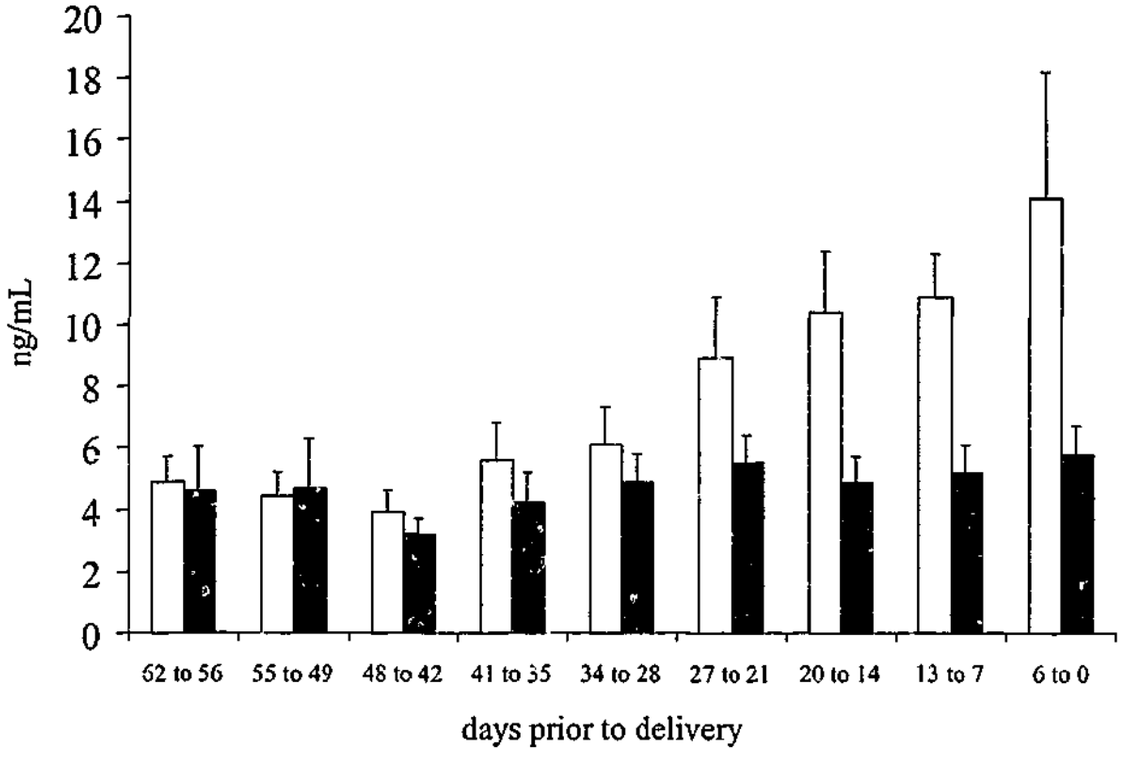

Figure 3.3 Levels of serum activin A and follistatin in a longitudinal study from week 30 to delivery. Activin A = □ , follistatin = ■ .

at 14-20 days prior to established labour and remained unchanged thereafter (P = 0.339). The levels of follistatin increased marginally throughout the time period investigated (P = 0.048). Mean ± SEM values of follistatin at 30 weeks' gestation were 4.6 ± 1.0 ng/ml and 5.8 ± 0.6 ng/ml at term. The ratio of activin A to follistatin, shown in figure 3.4, did not change significantly during the last three weeks of gestation (P = 0.22)

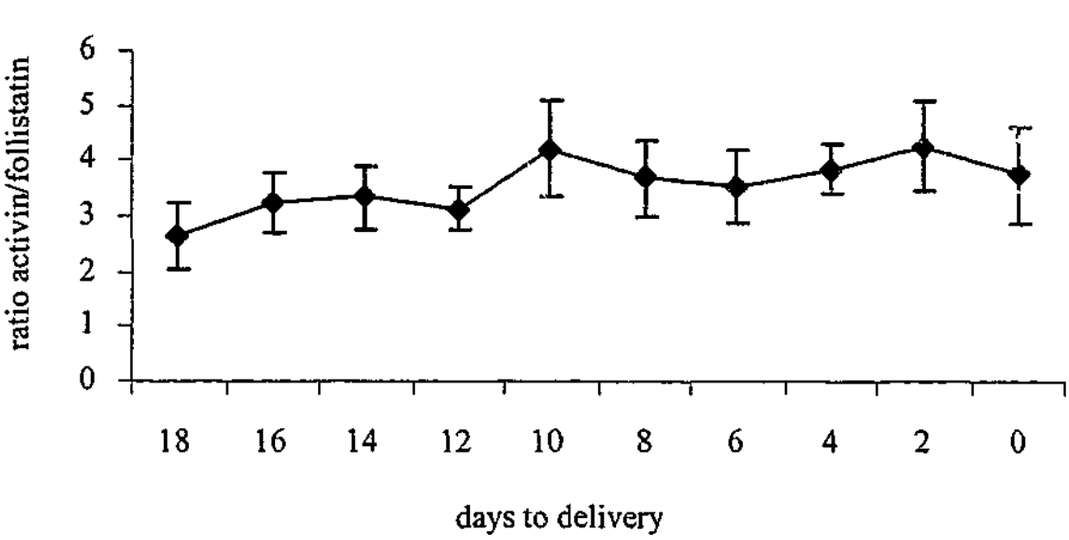

Figure 3.4 Ratio of activin A to follistatin from 30 weeks gestation until delivery

3.3.3 Detailed longitudinal pregnancy study: 37 weeks' gestation to delivery

The data are presented in figure 3.5. There were no significant changes in either activin A or follistatin during the last three weeks of pregnancy (P = 0.26 and 0.38 respectively). Mean maternal serum activin A levels were 16.0 ± 2.6 ng/ml at 37 weeks' gestation and 16.7 ± 3.5 ng/ml on the day of labour onset. The corresponding data for follistatin were 6.2 ± 1.4 ng/ml and 6.2 ± 0.5 ng/ml, respectively. The ratio of activin A to follistatin, shown in figure 3.6, did not change significantly during the course of this study (P = 0.35).

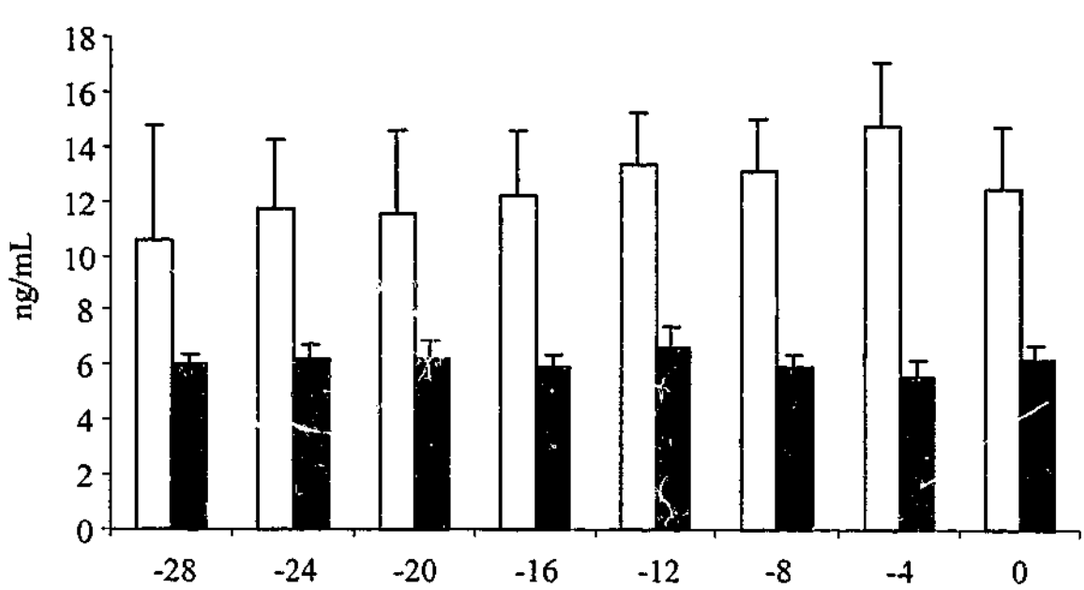

Figure 3.5 Total maternal serum levels of activin A and follistatin in a detailed longitudinal study in ten patients from 37 weeks gestation until delivery.

Activin A = □, follistatin = ■.

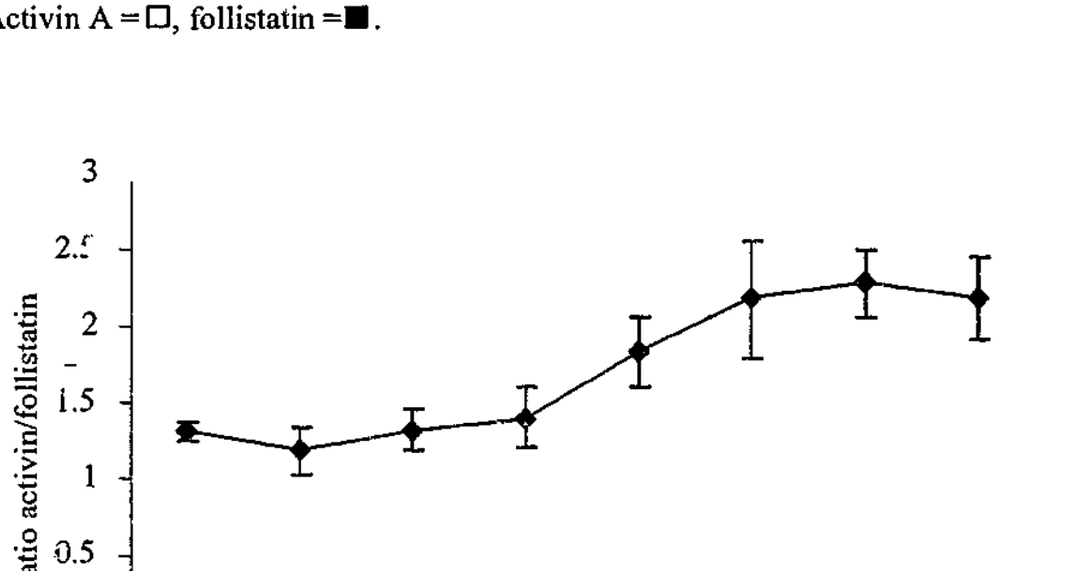

Figure 3.6 Ratio of serum activin to follistatin in a detailed longitudinal study from week 37 of gestation until delivery.

3.4 DISCUSSION

These studies are the first to examine the detailed ontogeny of activin A and follistatin in the maternal circulation in late pregnancy immediately preceding labour onset. The results presented here have provided new insights into the biology of these proteins in the weeks close to parturition.

Previous reports, using mainly cross-sectional studies, have shown that activin A levels rise in maternal serum towards term (Petraglia et al, 1993b, Muttukrishna et al, 1996, Woodruff et al, 1997, Fowler et al, 1998, O'Connor et al, 1999). However, these studies did not examine levels in detail proximate to labour. Woodruff et al (1997) reported that levels of activin A were undetectable in a woman throughout her entire pregnancy and this woman did subsequently not proceed into spontaneous labour requiring oxytocin to stimulate contractions. Furthermore, activin A has been shown to stimulate prostaglandin E_2 production from amnion-derived cells *in vitro* (Petraglia et al, 1993a), and GnRH and oxytocin from cultured trophoblast cells (Petraglia et al, 1989, Florio et al, 1996). Based on these combined observations, it has been suggested that activin A may be a candidate trigger for parturition. Indeed, mRNA expression of activin A is maximally elevated in amniotic fluid and fetal membranes at term compared to early pregnancy consistent with its increased production or accumulation in these compartments towards the closing stages of pregnancy (Petraglia et al, 1991c, Petraglia et al, 1997b). Further, levels of PGE_2 and $PGF_{2\alpha}$ which are synthesised by the fetal membranes are also maximal at term and during labour (Olson et al, 1983, Skinner & Challis, 1985). Petraglia et al (1994a) has shown that maternal serum levels of activin A are also significantly elevated after labour at term and at pre-term (Petraglia et al, 1995). Taken together, activin A has

subsequently been proposed as a para/autocrine regulator of PGE_2 and $PGF_{2\alpha}$ prior to and during labour.

The levels of follistatin in maternal serum rise modestly during the first trimester and increase more rapidly towards term. In our cross-sectional pregnancy study we have been able to confirm the previously reported pattern of follistatin by Petraglia et al, (1994b), Fowler et al (1998) and O'Connor et al (1999) and have shown that levels of biologically active (unbound) activin A are maximal at term. The absolute concentrations reported by some of these groups vary significantly from the levels observed in the studies reported here. This is due to the use of a radioimmunoassay to measure total follistatin (rather than an ELISA), as well as due to different standards employed for activin A and follistatin in different laboratories. The ratio of total activin A to total follistatin, as measured in our study in maternal serum, increased from a ratio of 1:1 in the first trimester to a ratio of 3.5:1 in favour of activin A when measured at 40 to 42 weeks' of gestation. In contrast to the activin:follistatin ratios described by Fowler et al (1998), the ratios presented here are about 2-fold higher across the whole sampling period. However, the reported levels of activin A were less than half of the levels described in this study whereas levels of follistatin were similar between Fowler's and this study. The low concentrations of activin A reported by Fowler may account for the low ratios of activin A to follistatin reported. Nevertheless, the profile for activin:follistatin ratio during the 36 weeks of pregnancy is similar in our studies and that of Fowler's study. Initially, ratios increase until about week 10 of pregnancy, dropping until about week 19 and thereafter, ratios increase progressively until term. These results imply that a large excess of biologically free

and therefore bioactive activin A exists at term, but the specific actions and functions of activin A remain to be explored.

The results of our two longitudinal studies (3.4.2 & 3.4.3) have provided a much more detailed maternal serum profile of activin A in the days leading up to labour. By adjusting the data relative to the day of spontaneous onset of labour for each woman (i.e. plotting the data "backwards" from the day of delivery) we have shown clearly that the concentrations of activin A in individual women remain unchanged in the last two to three weeks before the onset of parturition. Furthermore, neither study showed a change in the ratio of activin A to follistatin during this time. It is possible that another activin A-binding protein, α_2-macroglobulin, affects the bio-availability of activin A in serum (Phillips et al, 1997). We did not measure this protein in our studies. However, the affinity of activin A to α_2-macroglobulin is known to be much lower than that to follistatin, making significant changes to the profile of activin A unlikely. Nonetheless, there is no data available on the overall influence of α_2-macroglobulin on bioactivity of activin A during human pregnancy.

Although activin A levels do not change in late pregnancy in the maternal peripheral circulation, localised changes within the tissues may still occur. The decrease in progesterone and increase in oestrogen which are required for the initiation of labour, are not observed in the peripheral circulation in the human but occur locally within the gestational tissues (Weiss, 2000). The same may be true for activin A. It is possible that subtle changes in the secretion or activity of activin A may occur within the intra-uterine tissues where it may have auto- and paracrine actions without notably affecting the concentrations in the peripheral circulation. Petraglia et al (1997b) has

shown that the levels of activin A rise in amniotic fluid in term and preterm labour relative to non-labouring controls and activin receptor IIB expression is increased in the fetal membranes at term in association with labour. These observations suggest that the local production of activin A may be altered during labour, with the fetal membranes and possibly the fetus as potential sources of activin A. Indeed, it has been shown that activin A can stimulate PGE_2 production from an amnion-derived cell line (Petraglia et al, 1993a). Keelan et al (2000b), although unable to repeat Petraglia's results in amniotic cells, showed that amnion explants responded to activin A with an increase in PGE_2 in the medium. Thus, given the extensive evidence implicating fetal membrane prostaglandin secretion in the control of cervical ripening and myometrial contractility necessary for effective labour (Ellwood et al, 1980, Garfield et al, 1980, Brennnand et al, 1995) it is feasible that local rather than peripheral actions of activin A are important for the initiation of contractions. The changes in activin A and follistatin content in placenta, amnion and choriodecidua during labour are investigated in chapter 6.

In summary, when measured in the maternal peripheral circulation, activin A and follistatin levels do not change in the last weeks of pregnancy. These data are inconsistent with previous reports and do not support the hypothesis of activin A as a direct regulator of parturition. Nonetheless, the possibility that activin A is exerting local actions, particulary on myometrium or fetal membranes, has not been excluded. Furthermore, whether the profile of activin alters in the peripheral circulation during labour *per se* has not been adequately examined to date.

The possible association between maternal serum activin A and labour onset is explored further in chapter 4.

CHAPTER 4

ACTIVIN A AND FOLLISTATIN IN MATERNAL SERUM DURING LABOUR

4.1 INTRODUCTION

As was related in chapter 3, maternal serum activin A levels do not increase significantly during the last two to three weeks prior to labour onset. However, Petraglia et al (1994a) has reported the profile of activin A during labour showing that maternal serum activin A levels are higher in women after spontaneous labour than in those undergoing an elective Caesarean section at term. Further, women presenting in preterm labour had higher levels of maternal serum activin A compared to women in labour at term (Petraglia et al, 1995). The same investigators have also demonstrated that in amniotic fluid, collected by transabdominal amniocentesis, concentrations of activin are elevated following the initiation of labour than gestation-matched women not in labour (Petraglia et al, 1997b). In keeping with these observations and the serum data, levels of activin A protein were shown to be elevated in placental tissue after spontaneous labour compared to levels in placental tissue collected at term before labour, while follistatin concentrations were the same in non-labouring as in labouring tissues at term and pre-term (Keelan et al, 1999).

Further, the expression of activin βA-subunit mRNA was increased in amnion and chorion in association with both term and preterm labour (Petraglia et al, 1997b). *In vitro* studies too appear to lend support to the theory regarding a possible involvement of activin A in parturition. Apart from the stimulatory effect of activin A on PGE_2 secretion from amnion-derived cell lines and amniotic explants, activin A can

stimulate trophoblast cells to release OT (Florio et al, 1996). Both OT and PGE_2 have been implicated in facilitating uterine contractions and are used in clinical practice to initiate uterine activity (Hadi, 2000). It has also been shown that activin binding sites are present in the myometrium of pregnant and labouring rats, providing evidence in an animal model that activin A can target the uterus (Draper et al, 1997). However, recently a further study by Keelan et al (1999) showed that concentrations of activin A were lower in placentae from women undergoing pre-term delivery compared to those delivered at term and that, in amnion and choriodecidua, levels of activin A and follistatin were the same before and after term and pre-term labour (Keelan et al, 1999). The findings by Keelan's group contradict the observations noted by Petraglia and co-workers (Petraglia et al, 1997b) and question the suggestion postulated by Petrgalia that activin A has local effects in intra-uterine tissues during labour.

Importantly, the data providing evidence of an increased involvement of activin A during labour has been limited to date, as all studies in maternal serum and amniotic fluid conducted so far have used only very small groups of women or have been conducted at one time point only during or immediately after delivery.

Thus, in order to clarify the proposed association of activin A and follistatin with labour, a number of studies were designed with the aim of exploring changes in maternal serum activin A and follistatin throughout labour. Measurements of the prostaglandins PGE_2 and $PGF_{2\alpha}$ were carried out concurrently to assess a possible association between these and activin A.

4.2 MATERIALS AND METHODS

4.2.1 Patients

All women studied, except where noted, were normal and healthy and carried a singleton pregnancy. In each case, gestational age was calculated by either certain last menstrual period or by an early pregnancy ultrasound. The studies were approved by the Monash Medical Centre Human Research and Ethics Committee and each woman gave informed written consent.

4.2.2 Delivery studies

Four studies were undertaken with the aim of assessing the profile of activin A, follistatin and the prostaglandins E_2 and FM throughout delivery. All women who participated in these studies were at term (37 to 42 weeks' gestation).

*4.2.2.1 **Cross-sectional delivery study***: a single venous blood sample was collected from 36 women in established spontaneous labour, immediately after a vaginal examination performed routinely for cervical assessment and assayed for activin A, follistatin and prostaglandins E_2 and FM.

*4.2.2.2 **Longitudinal delivery study 1***: nine women undergoing induction of labour at term (37 to 42 completed weeks of gestation) by amniotomy with intravenous oxytocin gave venous blood samples immediately prior to amniotomy, every 30 minutes for 2 hours after amniotomy and 2 hourly thereafter for a total duration of 6 hours post-amniotomy. The indications for labour induction in the nine women were as follows: 2 for non-proteinuric pregnancy-induced hypertension (PIH), 2 for social

reasons, one for uncomplicated gestational diabetes at term and four for post-maturity (41-42 weeks' of gestation).

4.2.2.3 Longitudinal delivery study 2: ten women undergoing induction of labour by intra-vaginal PGE_2 gel (Dinoprostone, 2mg/3g, Pharmacia Upjohn, Rydalmere, NSW, Australia) gave venous blood samples immediately prior to induction, every 15 minutes for the first hour after commencement of induction and hourly thereafter for a total duration of 6 hours. The indications for induction were: four for diabetes, two for post-maturity (41 weeks' of gestation) and one each for hypertension, advanced maternal age, social reasons and ruptured membranes.

4.2.2.4 Elective Caesarean section study: a single venous blood sample was collected immediately prior to elective Caesarean section from 10 women at term (not in labour, 38 to 41 weeks of gestation) for measurements of activin A, follistatin and PGE_2 ad PGFM. The indications for delivery were: eight for previous Caesarean section and two for breech presentation.

4.2.2.5 Activin A clearance study: a single venous blood sample was collected from four healthy women (not in labour, 38 to 40 weeks of gestation) immediately prior to elective Caesarean section, and then at three hours , six hours, 12 hours, 18h and 24 hours after delivery. In all cases, the indication for surgery was a previous Caesarean section.

4.2.3 Collection of blood samples

All blood samples collected for measurements of activin A and follistatin were processed as described as in section 3.1.3.

For measurements of prostaglandin levels, blood (10ml) was collected in a plain tube containing indomethacin (0.001M, 10µl/ml). After centrifugation (as described in section 3.1.3, the serum was aspirated and aliquoted into two storage tubes. One was frozen immediately at – 20 ° C (for analysis of 13,14 dihydro, 15 keto prostaglandin $F_{2\alpha}$ (PGFM) the more stable circulating metabolite of $PGF_{2\alpha}$, the other (for analysis of PGE_2) was mixed with an equal volume of methyloximation reagent (8.2 % sodium acetate, 1 % methyloxyamine hydrochloride [Sigma, St.Louis, USA], in dH_2O) and left at RT overnight. The serum was stored at – 20°C.

4.2.4 Assays

4.2.4.1 ELISAs for activin A and follistatin

The assay used for measurements of activin A and follistatin in serum has been described previously in section 2.2.1.3 and 2.2.1.4 respectively.

4.2.4.2 Radioimmunoassay (RIA) for PGE_2

The assays were performed at the Department of Physiology, Monash University, with technical assistance by Jan Loose.

The radioimmunoassay used to detect PGE_2 in the serum samples has been described and validated for use in human pregnancy serum by Kelly et al (1986). The standards utilised in this assay were produced in-house. PGE_2 was methyloximated in an equal volume ethanol (1:1) using the same methyloximating reagent as for the serum samples, extracted and reconstituted in absolute ethanol in working dilutions of 2µM, 40nM and 2nM. Serial dilutions of pooled ovine serum served as quality controls. All

the samples, standards and quality controls were diluted in 0.1 M phosphate buffer (0.61 % $NaH_2PO_4.H_2O$, 0.87 % Na_2HPO_4, 0.9 % NaCl, 0.01 % NaN_3, 0.01 % gelatine, pH 7.0) and placed in tubes to make a total volume of 200 µl. Radiolabelled, methyloximated PGE_2 was used as tracer ((5,6,8,11,12,15(n)-^{3}H)-PGE_2 (Amersham/NEN, Australia), of which 100 µl was added to each tube. The working dilution of the tracer was 5000 cpm/100 µl. The antiserum directed against methyloximated PGE_2 (donated by Dr. R.I. Cox, CSRIO, Blacktown, NSW, Australia) was then added to each tube (100 µl) with the exception of the 'total counts' tubes. Bound tracer was separated from unbound tracer by the addition of 20mg/ml bovine gamma globulin (bγG, Calbiochem, Alexandria, NSW, Australia) diluted in PBS assay buffer, and 1 ml 22 % (w/v) polyethylene glycol 6000 (BDH Laboratory Supplies, Poole, Dorset, UK). All the tubes were vortexed and subsequently centrifuged (Beckman J6B, Beckman Instruments Inc., USA) at 2500 rpm for 15 minutes at 4°C. The supernatant was aspirated, the pellet resuspended in 50 µl absolute ethanol and 1 ml Ecosint A (National Diagnostics, Giorgia, USA) added to each tube. The tubes were capped, sonicated (Branson Ultrasonic Cleaner, Connecticut, USA) for 30 minutes and counted (Beckman LS 5000TA, Beckman Instruments Inc, USA). The intra- and interassay coefficient of variation of samples in the assay was 12.6% and 17% respectively. Crossreactivity with PGFM was 0.068. Sensitivity of the assay was 0.5 nM.

4.2.4.3 Radioimmunoassay for PGFM

The radioimmunoassay was developed in house (Burgess et al, 1990) and measures 13,14 dihydro-15-keto prostaglandin $F_2\alpha$, a stable metabolite of $PGF_2\alpha$. The assay can be applied directly to a range of biological fluids including serum. Native 13,14

dihydro-15-keto-$PGF_2\alpha$ (Upjohn Co., Kalamazoo, USA) was added to 200 µl of charcoal stripped plasma and used as standards. The quality control for this assay was pooled late gestation ovine serum. All samples, standards and quality controls were diluted in phosphate assay buffer (as for PGE_2) and 100 µl of tracer (13,14-dihydro-15-keto-[5,6,8,9,11,12,1(n)-^{3}H]-PGF2α, Amersham/NEN, Australia) added to each tube. The radioactivity of the tracer was 5000 cpm/100 µl. PGFM antiserum (100 µl, raised against PGFM and conjugated to glycerol in sheep, donated by Dr. R.I. Cox, CSRIO, Blacktown, NSW, Australia) was added to each tube. The tubes were then vortexed and incubated overnight at 4 ° C. On the following day, the tubes were placed on ice and a 50 µl aliquot of bovine gamma globulin (bγG), diluted at 20mg/ml in phosphate buffer, was added to all the tubes. Bound and unbound fractions of the PGFM conjugate were separated by the addition of 1 ml polyethylene glycol 6000 (30 % (w/v), BDH Laboratory Supplies, Poole, Dorset, UK). The tubes were vortexed and centrifuged at 3000 rpm at 4°C for 15 minutes. The supernatant was aspirated, the pellets resuspended in 50 µl destilled water and the tubes vortexed. Ecosint A (1.5 ml) was added to each tube, the tubes capped and sonicated for 30 minutes. The tubes were counted as described for the PGE_2 assay. The intra- and inter assay coefficient of variation of samples in the assays was 8.3 % and 12 % respectively. Crossreactivity with PGE_2 was less than 0.01. The sensitivity of the assay was 0.8 nM.

4.2.5 Statistical Analysis

Differences between the groups in the longitudinal studies were determined using Kruskall-Wallis test. The cross-sectional data were analysed using Mann-Whitney U test. Relationships between the hormones studied were assessed using Spearman's Rank Correlation (r^2). Significance was recognised when $P<0.05$.

4.3 RESULTS

4.3.1 Cross-sectional delivery study

The levels of activin A and follistatin during spontaneous labour are shown in figure 4.1. Thirty two of the 36 women had a normal vaginal delivery. Four women had an instrumental vaginal delivery for delay (>1 h) in second stage of labour. There were no differences between these two groups with respect to either activin A or follistatin (P = 0.99 and 0.5, respectively) allowing the results to be combined. The levels of activin A and follistatin remained stable from early labour until second stage.

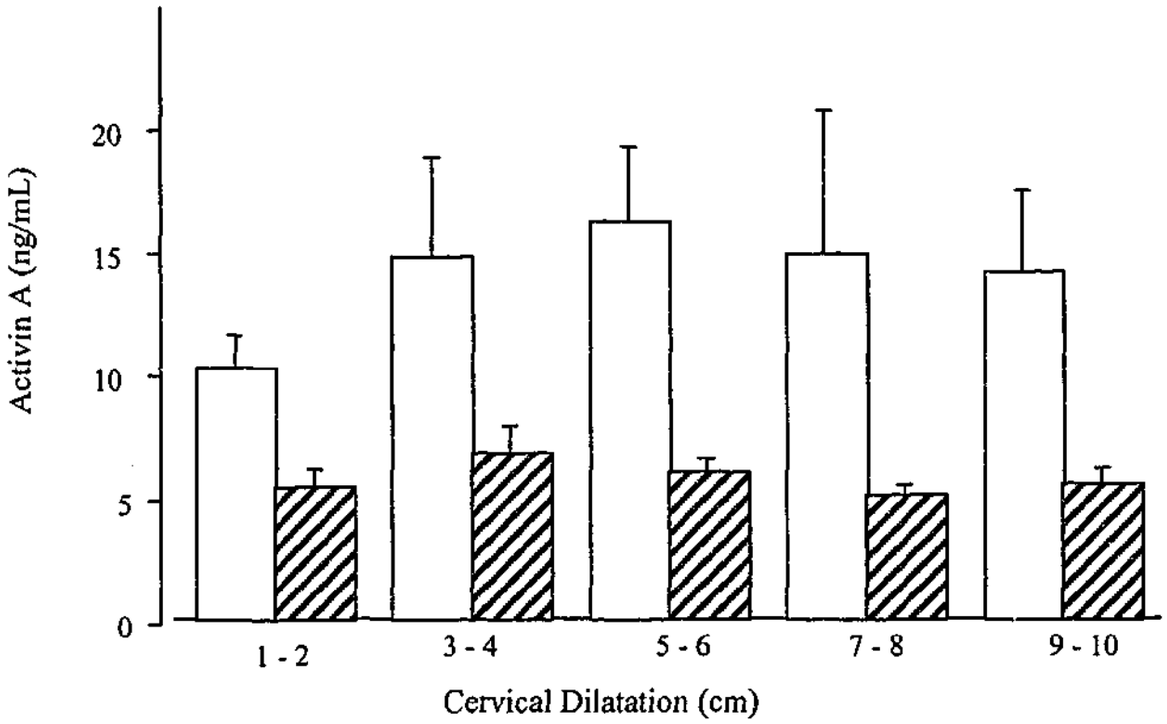

Figure 4.1 Profile of maternal serum activin A and follistatin serum concentrations during spontaneous labour in 36 women. Activin A = □, follistatin = ▨.

Results for the profiles of PGE_2 and PGFM at elective Caesarean section and during labour (cross-sectional delivery study) are shown in fig. 4.2. Levels of both prostaglandins PGE_2 and PGFM were undetectable in the women undergoing an elective Caesaeran section at term. However, in the women in established labour, these levels were increased dramatically to 15.08 ± 3.93 pg/mL and 39.43 ± 4.87 pg/mL, respectively in early labour (P = 0.02 and 0.01, respectively) with further rises towards full dilatation (19.3 pg/mL ± 4.5 and 47.04 pg/mL ± 10.6 respectively, P = 0.01 and 0.009, respectively). The correlation coefficient between PGE_2 and $PGF_{2\alpha}$ was 0.39. The correlation coefficients between activin A and PGE_2 and $PGF_{2\alpha}$ were –0.64 and –0.09 respectively, and between activin A and follistatin the correlation was 0.04.

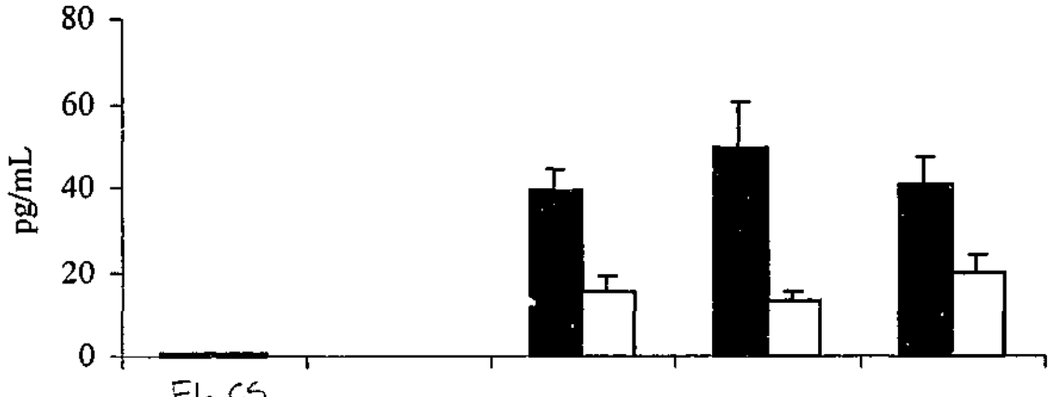

Figure 4.2. Maternal serum concentrations of PGE_2 and $PGF_{2\alpha}$ during elective Caesarean section and spontaneous labour at term in 36 women PGFM = ■ , PGE_2 = □

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4.3.2 Longitudinal delivery study

4.3.2.1 Study 1: Amniotomy

Six women required intravenous oxytocin, which was commenced two hours after amniotomy in the absence of spontaneous uterine activity, as per hospital protocol. Seven women delivered vaginally and two had a low cavity forceps delivery for delay (>1 hr) in second stage of labour. The infants were all normal and healthy at birth and had five minute Apgar scores of 9 or above. The mean amniotomy-delivery interval was 7 hrs 40 minutes ± 1 hr 50 minutes. The mean interval between onset of contractions to delivery was 4 hrs 11 minutes ± 1 hr 12 minutes.

The profile of activin A and follistatin before and after amniotomy is shown in figure 4.3. The concentrations of either protein did not change significantly throughout the sampling period (P = 0.35 and 0.78 for activin A and follistatin, respectively). There

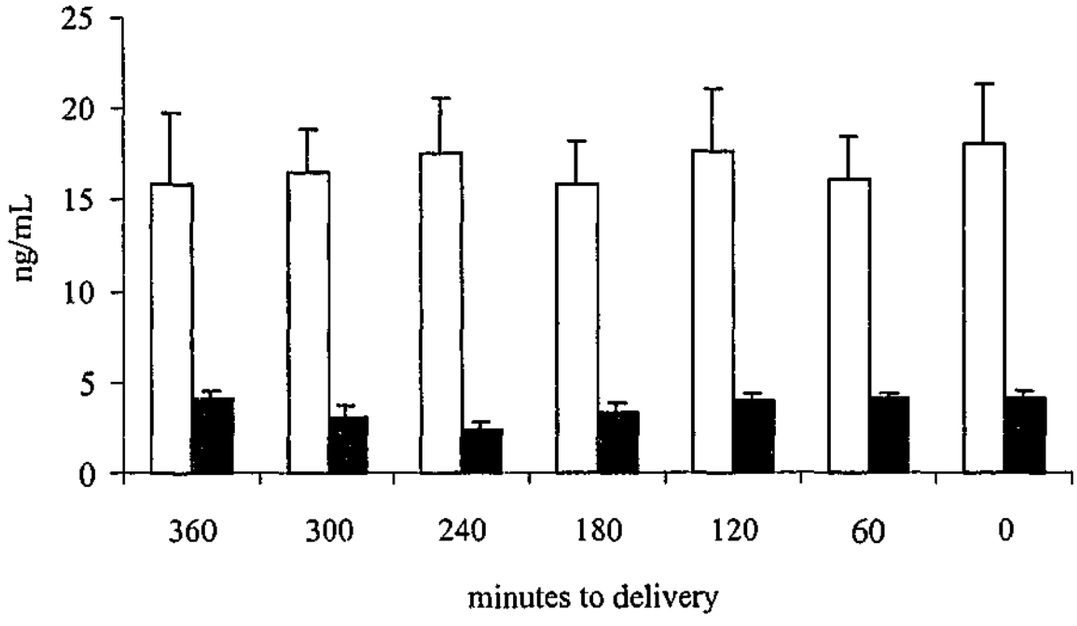


Figure 4.3 Maternal serum concentrations of activin A and follistatin after induction of labour by amniotomy. Activin A =□, follistatin = ■.

were no relationships between activin A or follistatin levels and the duration of labour or amniotomy to delivery interval. The highest levels of activin A were observed in the two women with pregnancy-induced hypertension (PIH), but the levels for these women did not change during labour.

4.3.2.2 Study 2: Induction of labour by vaginal PGE_2

All women received intravenous oxytocin, which was commenced two hours after induction by PGF_2 in the absence of spontaneous uterine activity, as per hospital protocol. 8 women delivered vaginally and 2 had a low cavity forceps delivery for delay (>1 hr) in second stage of labour. Nine of the ten infants born were normal and healthy at birth and had five minute Apgar scores of 9 or above. One infant had a five minute Apgar score of 8 but was otherwise healthy. The mean induction-delivery interval was 6 hrs 21 minutes ± 1 hr 24 minutes. The mean interval between onset of contractions to delivery was 5 hrs 55 minutes ± 1hr 38 minutes.

The profile of activin A and follistatin before and during prostaglandin induction is shown in figure 4.4. The concentrations of either protein did not change significantly throughout the sampling period ($P = 0.79$ and 0.43 for activin A and follistatin respectively). The correlation coefficient between activin A or follistatin was 0.0049.

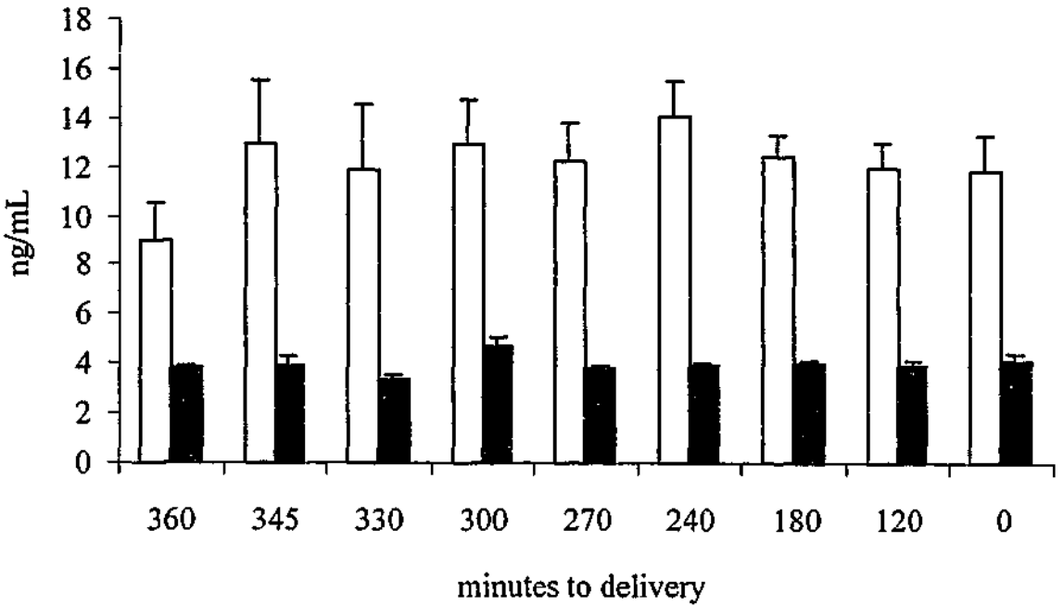


Figure 4.4. Maternal serum profiles of activin A and follistatin during induction of labour by vaginal PGE_2. Activin A = □, follistatin = ■.

4.3.2.3 Elective Caesarean section

Table 4.1 shows the levels of activin A and follistatin in the women undergoing elective Caesarean sections compared to the mean levels of samples collected from women undergoing spontaneous labour study. There was no statistical difference between these two groups (P = 0.23 and 0.84 respectively, Mann-Whitney U test).

Table 4.1 Total maternal serum activin A and follistatin at elective Caesarean section (n = 10) and during spontaneous vaginal delivery (n = 36).

Group	Activin A (ng/mL)	Follistatin (ng/mL)
Spontaneous labour	11.0 (0.93)	5.71 (0.67)
Elective Caesarean section	14.95 (2.8)	5.95 (0.67)

Results for the maternal serum activin A clearance study is shown in figure 4.5. The figure shows profiles for each of the four women studied. In three women, levels of activin A fell sharply within 3 hours of delivery. However, after 48 hours post delivery, levels in maternal serum were still readily detectable. In one woman, levels of activin did not decrease until 12 hours post delivery.

Figure 4.5. Clearance of activin A from maternal serum in four individual women after elective Caesarean section.

4.4 DISCUSSION

As previously discussed, the data on levels of activin A during labour have been conflicting and carried out in small groups of women. The data presented in this chapter are the first to clearly show that levels of maternal serum activin A do not change throughout the progression of labour. Furthermore, there is no apparent

change in the activin to follistatin ratio during the time periods under investigation suggesting that circulating levels of bioavailable activin A remain unchanged. While levels of activin A remained unchanged prior to and throughout either spontaneous or induced labour, serum levels of both prostaglandins, PGE_2 and PGFM, increased sharply from undetectable levels prior to labour onset to readily measurable levels very early on during labour, at a cervical dilatation of only one to two cms with further increases to full dilatation. The changes of prostaglandin levels observed in association with labour are consistent with previous findings (Johnston et al, 1993). The same investigators also showed that women with dysfunctional labour had significantly lower PGEM and PGFM levels and that the levels did not rise significantly with labour. It is well established that the fetal membranes are the principal source of the increased levels of peripheral prostaglandins seen with labour (Okazaki et al, 1981, Cheung & Challis, 1989). Cervical examination at term results in rapid synthesis of PGFM by the cervix (Mitchell et al, 1977, Ellwood et al, 1980, Bibby et al, 1979) and may have contributed to the concentrations of PGE_2 and PGFM measured in the serum of women in the cross-sectional delivery study. However, as the women in this study were in established labour prior to the vaginal examination (VE), it can be assumed that levels of PGs were already elevated when compared to non-labouring women at term (Johnston et al, 1993) and that the VE would have only changed the absolute levels measured, but not the overall profile. Further, since all blood samples were collected from the women in this group within 30 minutes of the VE, the effect of the VE would be similar for all women.

There is some indirect evidence that activin A may be involved in the initiation of labour. *In vitro* experiments have demonstrated that activin A can stimulate the

synthesis and/or release of prostaglandins from the amnion (Petraglia et al, 1993a, Keelan et al, 2000b). However, *in vivo*, in the peripheral circulation, no correlation between activin A and the prostaglandins levels could be demonstrated. Nevertheless, local changes in the concentration of activin A may occur within the intra-uterine tissues which may have downstream effects on other regulators of parturition. It is thus possible that the local tissue levels of activin A are elevated in gestational tissues with labour or that receptors are upregulated at term and during labour to increase the responsiveness of these tissues to activin A but that these changes were undetectable in the circulation. An investigation into these potential changes is described in chapter 7.

In three of the four women studied for activin A clearance after Caesarean section, activin A levels declined rapidly within 3 hours of delivery but then remained in the circulation in nanogram concentrations. These results contrast with earlier findings where levels of maternal serum activin A became undetectable within 6 hours after delivery. In our study, levels of activin A were easily detectable within 48 hours after delivery by Caesarean section. The levels at 48 hours post delivery were comparable to activin A levels in first trimester maternal serum. It is possible that these levels of activin A in serum are due to the surgical intervention which may be increased as a result of wound healing released from dermal tissues at the surgical site (Munz et al, 1999). In order to clarify these observations, clearance studies in normal vaginal deliveries need to be undertaken and compared with clearance after Caesarean section in the future. In one woman, levels of maternal serum activin A did not drop until 12 hours after delivery. This may have been caused by an infection or other complications after the delivery, however, clinical details on this patient were not

available on completion of this study. Nevertheless, it is obvious that circulating levels of activin A decline rapidly within a few hours after delivery further supporting the hypothesis that activin A is mainly produced by the feto-placental unit.

Apart from affecting local mediators within the fetal membranes, placenta and decidua, it also remains possible that activin A targets the myometrium to stimulate contractions towards the end of gestation. Draper et al (1997) has shown that in the rat, which has a peripheral activin A profile similar to the human, activin A has specific binding sites in the myometrium which are not competed for by inhibin. It is the aim of the work reported in chapter 6 to explore potential target sites of activin A in the human myometrium.

CHAPTER 5

MATERNAL SERUM ACTIVIN A AND DYSFUNCTIONAL LABOUR

5.1 INTRODUCTION

Contrary to previously published studies, the results of chapters 3 and 4 have shown that maternal serum levels of activin A are not acutely elevated prior to or during normal labour arguing against activin having a key role as an acute regulator of parturition. Importantly, in the studies described in chapter 3 and 4, all women underwent either an uneventful elective Caesarean section, had a normal vaginal delivery after spontaneous onset of labour or underwent an induction of labour by amniotomy or vaginal prostaglandin. However, as discussed before, Woodruff et al (1997) previously reported the case of a woman who had undetectable serum levels of activin A throughout pregnancy and who subsequently did not progress spontaneously into normal labour at term but required induction. On the basis of this single case it was suggested that activin may be associated with the timing of labour and also with the progression of labour, particularly in cases of dysfunctional labour. The studies reported in the previous chapters did not address the possible association between activin and dysfunctional labour and so the aim of the studies described here was to investigate maternal serum levels of activin A in women who experienced dysfunctional labour leading to instrumental delivery or emergency Caesarean section for "failure to progress." Concurrently, umbilical arterial blood samples were collected to assess fetally derived activin A in cases of normal and dysfunctional labour.

5.2 MATERIALS AND METHODS

5.2.1 Patients

Sixty-four women were studied. Of those women, 37 gave both a maternal and an umbilical artery blood sample. Thirty-two other healthy women gave a cord blood sample only. All women were healthy, had normal, singleton pregnancies and were at term (38 to 41 weeks' gestation). In all cases the gestational age was calculated either by a known last menstrual period or by an early pregnancy ultrasound. The study was approved by the Monash Medical Centre Human Research and Ethics Committee and informed, written consent was obtained from each woman.

5.2.2 Collection of maternal serum and umbilical arterial serum samples

Women scheduled for an elective Caesarean section (N = 11) gave a blood sample immediately prior to surgery. Indications for the Caesarean section in this group included repeat surgery after previous Caesarean section (N = 8) or breech presentation (N = 3). All other women (N = 53) gave a blood sample during spontaneous onset of labour immediately after assessment of cervical dilatation. The median (range) cervical dilatation at time of blood sampling for all women studied was 5 cm (1 to 10). A blood sample from an umbilical artery was collected immediately after clamping of the cord at delivery. All samples were centrifuged at 3500 rpm for 15 minutes at 4°C and the serum stored at –20°C until assayed. The placental weight and birthweight of the fetus were recorded at the time of delivery.

5.2.3 ELISA for activin A

The ELISA for measurement of activin A in serum has been described in chapter 2, section 2.2.1.3.

5.2.4 Statistical analysis

Differences in the levels of maternal serum and cord artery activin A between groups were analysed using Students T-test after $\log_{10}$ transformation of the data. Differences between other birth parameters were analysed using Students T-test. Significance was established when $P<0.05$.

5.3 RESULTS

Outcome statistics for all four groups of women are shown in table 5.1. Of the 53 women undergoing spontaneous vaginal delivery, 30 had a normal, uncomplicated, vaginal delivery (NVD), 7 required delivery by an emergency Caesarean section for delay during first stage of labour (Em CS) and 16 required an instrumental delivery (by forceps or ventouse) for delay during second stage of labour (Instr. Del.). The median cervical dilatation at which blood samples were collected were 5 cm (range 1 – 10 cm) for normal, vaginal deliveries, 8 cm (range 2 – 10 cm) for instrumental deliveries and 7 cm (range 3 – 10 cm) for women requiring an emergency Caesarean sections.

Table 5.2 summarises the duration of labour (total, first stage and second stage) for each group. The total duration of labour for the women who had a normal vaginal delivery was significantly shorter than for the other two groups ($P = 0.04$ and 0.03 for instrumental delivery and emergency Caesarean section, respectively). The duration

of second stage was significantly shorter in normally delivering women compared to women requiring instrumental delivery (P = 0.01).

Table 5.1 Outcome for 64 women delivering at term. Values are represented as means with SEM in brackets.

Group	n	Birthweight (g)	Placental weight (g)	Gestation (weeks)	Activin A (ng/ml)
NVD	30	3400 (91)	642 (37)	39.2	11.2 (0.96)
Elect. CS	11	3459 (146)	627 (55)	38.6	11.1 (1.01)
Instr. Del.	16	3452 (137)	595 (42)	39.7	13 (1.06)
Em. CS	7	3729 (135)	695 (57)	40.2	21.2 (4.03)

Table 5.2. Duration of labour (mean ± SEM) in 64 women delivering at term.

	total duration	1.stage	2.stage
NVD	10h 18min (±1h 10min)	9h 40min(±1h 2min)	30min (±21min)
Instr. Del.	14h 48min (±2h 10min)	12h 47min (±2h 17min)	2h (±40min)
Em. CS	14h 52min (±2h 16min)	_	_

The mean (±SEM) levels of activin A (maternal serum) in each group are also shown in figure 5.1. There were no significant differences in maternal serum activin A levels between women who had a normal, vaginal delivery and women undergoing elective Caesarean section (P = 0.7) or women who had an instrumental delivery (P = 0.28).

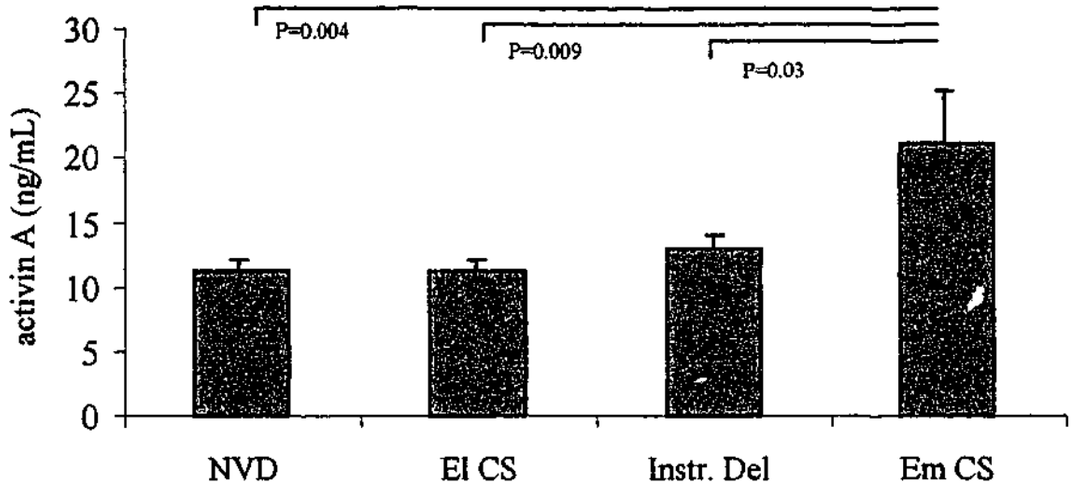


Figure 5.1 Total activin A (means ± SEM) in maternal serum of women according to mode of delivery. NVD = normal vaginal delivery, El CS = elective caesarean section, Instr. Del = instrumental delivery, Em CS = emergencu Caesarean section.

However, in women who had an emergency Caesarean delivery, the levels of activin A were significantly elevated compared to women delivering normally (P = 0.004), delivering instrumentally (P = 0.03) or women delivering by elective Caesarean section (P = 0.009).

Sixty-nine umbilical arterial cord blood samples were collected. Of these, 37 were from women who had had a normal, vaginal delivery, eight from women who had had an elective Caesarean section, indications: previous Caesarean section (n = 6) or breech presentation (n = 2), 11 from women who had required an instrumental delivery for delay during second stage of labour and 13 from women who underwent an emergency Caesarean section for delay during first stage of labour. The levels of umbilical arterial activin A did not differ significantly between any of the four groups

of women (El CS vs Instr. Del, NVD and EmCS, P = 0.77, 0.34 and 0.26 respectively) (Figure 5.2).

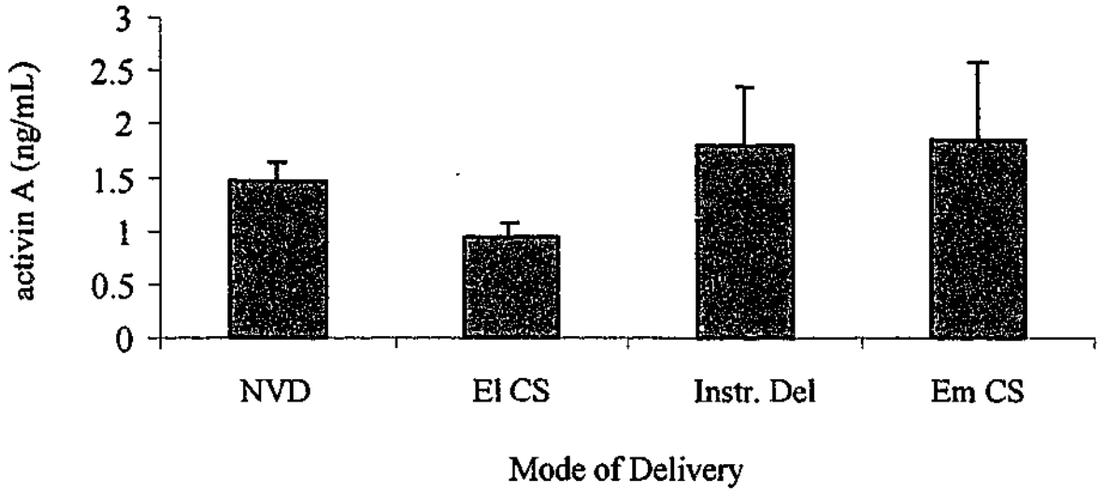


Figure 5.2 Levels of total activin A (means ± SEM) in umbilical arterial blood according to mode of delivery. (abbreviations as in figure 5.1).

There was no significant correlation between maternal serum and umbilical arterial activin A (figure 5.3) or umbilical arterial pH levels (figure 5.4) (r = 0.36, P = 0.22 and r = -0.34, P = 0.11, respectively). However, there was a significant correlation between arterial umbilical artery activin A and pH levels (figure 5.5) (r = -0.59, P = 0.0086)

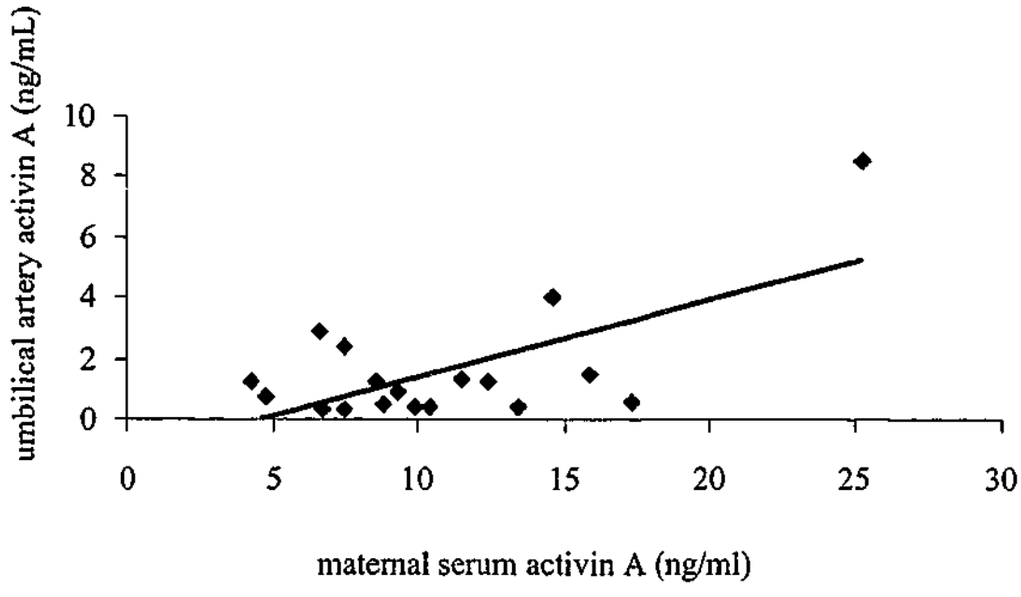


Figure 5.3 Correlation between maternal serum activin A and umbilical artery activin A concentrations at term. r = 0.36 (P = 0.22).

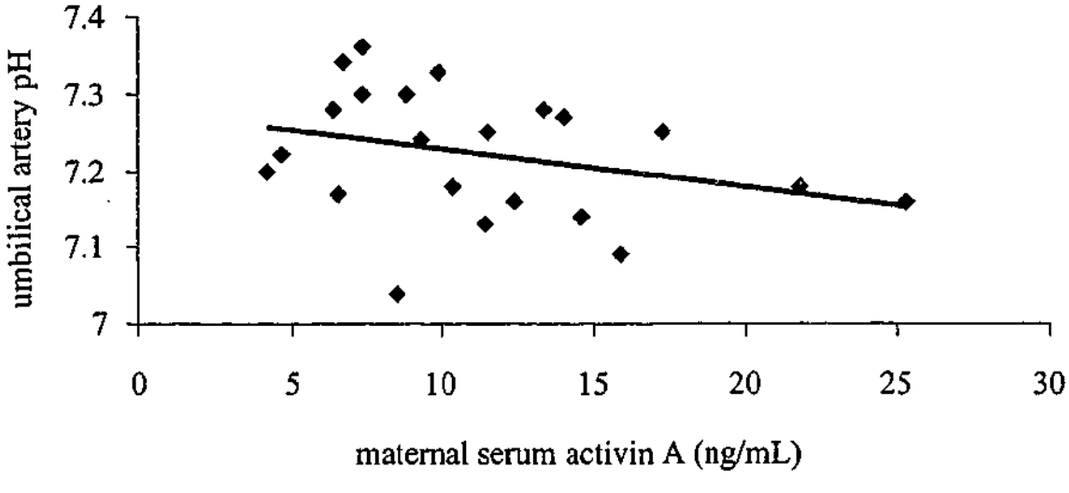


Figure 5.4 Correlation between maternal serum activin A and umbilical artery pH at term delivery. r = - 0.34 (P = 0.11).

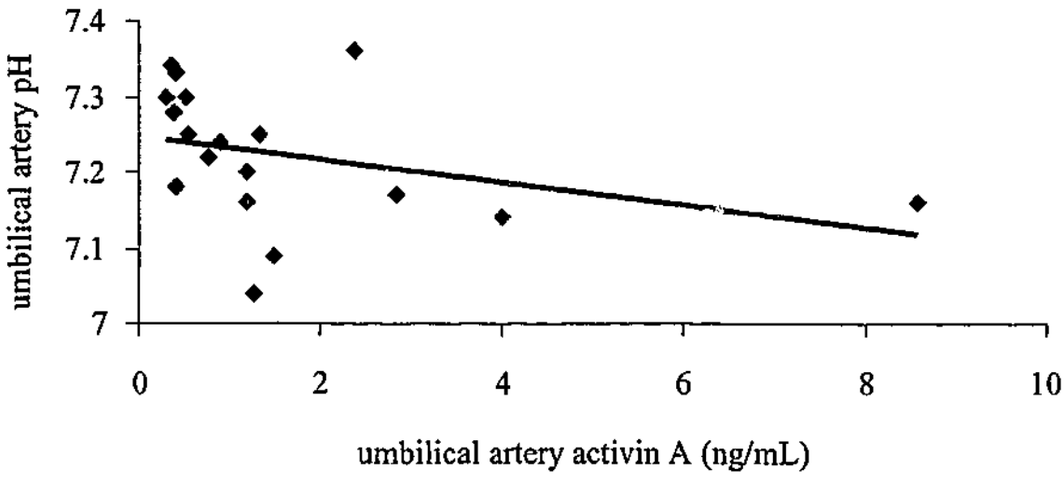


Figure 5.5 Correlation between umbilical artery activin A levels and umbilical artery pH at delivery at term. r = - 0.59 (P = 0.0086).

5.4 DISCUSSION

In earlier studies, maternal serum activin A was elevated in women after spontaneous term and pre-term delivery compared to women undergoing an elective Caesarean section (Petraglia et al, 1994a, Woodruff et al, 1997). In those studies, all women had normal, healthy pregnancies and deliveries and in all but one woman the characteristic increase in maternal serum activin A towards term was observed. The aim of the studies described in this chapter were to evaluate the potential correlation between maternal serum and umbilical artery activin A levels and dysfunctional labour. The levels of maternal serum activin A taken from women delivering before elective Caesarean section (no labour) and during normal vaginal delivery were similar and did not change with progression of labour. These results support the data obtained in chapter 4 which showed levels of activin A did not change prior to and during

spontaneous and induced labour in uncomplicated, normal deliveries. Women whose labour was significantly delayed during second stage had peripheral serum levels of activin A similar to the women with normal labour progression suggesting that levels of activin A are not associated with abnormal processes during the second stage of delivery. Difficulties during second stage of labour may be due to obstruction of fetal descent by the maternal pelvis, fetal malposition or abnormal development and may not reflect abnormalities of uterine activity. In contrast, delays during the first stage of labour are commonly caused by inefficient or in-coordinate contractile activity of the myometrium. Johnston et al (1993) have observed significantly lower concentrations of PGFM in serum of women with dysfunctional first stage of labour whereas concentrations of PGEM remained unchanged during normal or dysfunctional labour. These results are consistent with the understanding that PGFM is the major physiological uterotonic agent responsible for normal effective myometrial activity. In our study, women with delay during first stage of labour had significantly higher levels of serum activin A than women with normal, uncomplicated labours. There is no information on the interaction between $PGF_{2\alpha}$ and activin A *in vivo* or *in vitro*. In cultured amnion cells and explants, activin A stimulates release of PGE_2 into the medium (Petraglia et al, 1993a, Keelan et al, 2000b). The major roles of PGE_2 are not uterotonic but rather related to the physiological and pharmacological ripening of the cervix (Ellwood et al, 1980, Greer et al, 1990). It is unclear why the activin levels in the women requiring an emergency Caesarean section are increased. While PGFM levels were not measured in these women it is likely that levels were lower than in normal efficient labour (Johnston et al, 1993) and it is possible that, if activin stimulates $PGF_{2\alpha}$ release, the higher activin levels reflect an autoregulatory loop. On the other hand, activin may have no role in

the physiological regulation of PG release and labour progression. In light of the data already presented in chapters 3 and 4 it is thought that this latter explanation is more likely.

None of the women studied had abnormally low or even undetectable levels of peripheral activin A prior to commencement of the study and it is possible that such women would present with failure or delay of labour onset as has been described in one case by Woodruff et al (1997) rather than delay during labour progression itself. An evaluation this intriguing association would require random sampling prior to and follow up during labour in a large cohort of women, in particularly post-term women (at 42 weeks of gestation or later).

The source of the increased levels of maternal serum activin A observed in association with an emergency Caesarean section is unclear, but, apart from increased placental or fetal membrane output, the elevated levels in maternal serum may be due to elevated fetal production of activin A as reflected in levels of activin A in the umbilical artery. Interestingly, there were no statistical differences in cord arterial total activin A concentrations between the four groups of women studied, but the levels were highest in the group undergoing an emergency Caesarean section, followed closely by the group of infants delivered instrumentally. The levels recorded in both groups were almost double than those seen at elective Caesarean section. The increase of fetal activin A observed with instrumental delivery may be due to fetal stress episodes during delay of second stage of delivery. Indeed, the duration of second stage was significantly longer in women requiring an instrumental delivery compared to women with normal, uneventful deliveries. Similarly, umbilical artery activin A levels were

higher in the group of women with delay during first stage of labour. The observed increased secretion of activin A by the fetus may have contributed to the elevated levels of activin A measured in the maternal circulation. However, larger groups of subjects are needed to assess if the differences of fetally derived activin A between the groups are real. Based on our preliminary findings it appears that the fetus is producing increasing levels of activin A as a result of delay during first or second stage of labour. As will be discussed in chapters 8 and 9, activin secretion may be regulated by oxygenation and the higher activin levels in the emergency Caesarean section group which had the longest labours may reflect declining feto-placental oxygenation. Umbilical artery O2 and pH levels certainly decline with dysfunctional labour and a relationship between umbilical artery pH and activin was demonstrated. However, the correlation between umbilical artery pH and activin A was not significance when the sample with the highest recorded levels of activin A (8.5 ng/mL, pH = 7.16) was removed (r = -0.37). Larger sample sizes will need to be analysed to confirm whether the correlation between fetal production of activin A and umbilical artery pH is real.

The observed increase in fetally derived activin A may be either due to a direct release of activin A by the fetus due to hypoxia or indirectly as a result of fetal cortisol release due to stress experienced during delivery. *In vitro* experiments have shown that in cultured fetal adrenal cells βA-subunit mRNA expression is increased following stimulation with ACTH (Spencer et al, 1992). In sheep, hypoxic insults during pregnancy result in a significant increase in fetal and amniotic fluid activin A and PGFM and PGEM (Jenkin et al, 2001), although these increases were not observed in the maternal circulation. The lack of change in maternal plasma of the

sheep with intra-uterine hypoxia may be due to the different placental structure between sheep and human placenta. Based on the preliminary results presented here it is therefore possible that, in the human, changes in maternal serum activin A reflect episodes of acute intra-uterine hypoxia, the biochemical impacts of which too small to be detected by conventional fetal assessment during or after delivery. Indeed, all infants born were apparently healthy and, using Apgar score assessment and umbilical arterial blood pH analysis, no evidence of fetal distress was present in these infants.

The results of the studies presented in this chapter lend further support to the argument that activin A is not directly involved in regulating the progression of labour.

CHAPTER 6

ACTIVIN A AND ACTIVIN RECEPTORS IN MYOMETRIUM AT TERM AND DURING LABOUR

6.1 INTRODUCTION

As has been described and discussed in the previous chapters, concentrations of activin A in maternal serum and amniotic fluid have been observed by some investigators to be significantly elevated during term and pre-term labour compared to gestation-matched women not in labour (Petraglia et al, 1994a, Petraglia et al, 1997b), although the levels of activin A were not significantly different in placental lysates from women delivering vaginally and women delivering by elective Caesarean section at term (Keelan et al, 1999). Contrasting the data of Petraglia et al (1994a), the results of our studies in chapters three and four have shown that maternal serum levels of activin A are unchanged during the last two to three weeks prior to delivery, as well as during spontaneous and induced labour arguing against a direct role for activin A in the initiation of labour. Nevertheless, *in vitro* and *in vivo* observations have demonstrated that activin A can modulate a range of uterotonic peptides, all of which are either directly of indirectly capable of stimulating myometrial contractions. Activin A can modulate CRH and OT release from the brain (Plotsky et al, 1991, Sawchenko et al, 1988). CRH in turn, which is capable of stimulating myometrial activity, can enhance the production of PGs, OT and ACTH from gestational tissues (Jones et al, 1989, Petraglia et al, 1995) and also the myometrium (Benedetto et al, 1994), while OT is capable of stimulating PG production in the decidua (Hirst et al, 1993). PGE_2 and OT secretion was significantly increased by activin A from amnion and trophoblast cells respectively (Petraglia

et al, 1993a, Florio et al, 1996). Furthermore, activin βA-subunit expression and message for activin receptor type IIB were increased in fetal membranes after labour (Petraglia et al, 1997b).

In this regard, the recent demonstration, by radioligand binding studies, that activin A binds to the rat myometrium at term and during labour (Draper et al, 1997), offered evidence that activin A may be able to stimulate myometrial contractility directly, as well as via the established effectors such as the PGs, CRH and OT. This study was undertaken to determine whether activin βA-subunit and its receptors were also present in human myometrial smooth muscle at term and to investigate whether the distribution and/or the concentrations of these proteins altered in association with labour. The studies described here assessed activin A and follistatin content in myometrium, examined activin type I and type II receptor protein expression by Western analysis in myometrial tissue samples and isolated myometrial smooth muscle and microvascular cells, and also evaluated the localisation of activin receptor and βA-subunit protein by immunohistochemistry.

6.2 MATERIALS AND METHODS

6.2.1 Patients

Three groups of women were recruited to the study. The first group consisted of six pre-menopausal non-pregnant women who underwent a hysterectomy for benign gynaecological disease. None of these women received hormone treatment in the last three months prior to surgery. The second group consisted of six pregnant women undergoing an elective Caesarean section at term (37- 39 weeks' gestation) for malpresentation or previous Caesarean section. The third group comprised six women in established labour at term (39 – 41 weeks' gestation) who had an urgent Caesarean section for failure to progress in the first

stage. These women all received intravenous oxytocin for a median (range) duration of six hours 15 minutes (one hour 40 minutes to ten hours, ten minutes) and attained a median (range) cervical dilatation of 7cm (5 - 8 cm). All 12 pregnant women studied were otherwise healthy, on no medication and had had an uneventful, singleton pregnancy with a normal fetus. The study was approved by the Monash Medical Centre Human Research and Ethics Committee and each woman gave informed written consent.

6.2.2 Collection of myometrium

In the first group of women, a piece of myometrium ($1cm^2$) was collected immediately after removal of the uterus. In the other two groups, myometrial tissue (about 1 cm^2) was excised from the upper edge of the uterine incision during a lower uterine segment Caesarean section, after delivery of the baby and placenta. In all 18 cases tissue processing was identical. The tissue was rinsed with ice cold sterile PBS and sectioned into two pieces. One piece was snap frozen in liquid nitrogen and used for measuring activin A and activin receptor proteins. The other piece was fixed in 10% neutral buffered formalin for 12 to 16 hours and processed for standard immunohistochemistry.

6.2.3 Total Protein Assay

Frozen myometrial samples were homogenized in sterile PBS and centrifuged for 2 minutes at 10 000 rpm. The total protein concentration in the supernatant was measured by the bicinchoninic acid assay (BCA) following the manufacturer's instructions (Pierce, Illinois, USA).

6.2.4 ELISA for activin A and follistatin

The ELISA for measurement of activin A and follistatin has been described in section 2.2.1.3 and section 2.2.1.4 respectively.

6.2.5 Western hybridisation (Activin receptors type IA, IIA and IIB)

Fifteen microgram total protein was separated on denaturing gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the discontinuous system described by Laemmli (1970). The running gel (total per cent of acrylamide (w/v) T = 10%; total per cent of bisacrylamide out of total acrylamide (w/v) C = 3%) was prepared by dissolving 30% acrylamide (30% v/v) in 1.875 M Tris HCL (20% v/v, pH 8.8) and topped with dH_2O. The solution was degassed for approximately 20 minutes, and then 10% ammonium persulfate (APS, 25 µl/ml), 10% sodium dodecyl sulfate (SDS, 75 µl/ml) and Temed (5µl/ml) were added. The running gel was poured and left to set for at least one hour at RT. Thereafter, the stacking gel (T = 4%) was prepared by dissolving 30% acrylamide (12.5% v/v) in 1.25 M TrisHCL (10 % v/v, pH 6.8) topped with dH_2O. The solution was degassed for 20 minutes and then 10% SDS(100 µl/ml), Temed (10 µl/ml) and 10% APS (30 µl/ml) added. The gel was poured on top of the running gel, the combs positioned and the gel left to set at RT for one hour. The protein samples (15mg dissolved in PBS buffer to make a total volume of 15 µl) were mixed with an equivalent volume of 2x loading buffer (1.25 M TrisHCL buffer, 2-Mercaptoethanol, 87% glycerol, bromophenol blue in dH_20) and mixed for 10 seconds. The samples were placed in a boiling water bath for 5 minutes to denature the proteins, cooled on ice, centrifuged at 10 000 rpm for 2 minutes and subsequently loaded into the stacking gel. Samples were electrophoresed at 110 V until the dye front had reached the

lower edge of the running gel. The gel was carefully removed from the tank, "sandwiched" between several layers of filter paper and a nitrocellulose membrane (BioRad, Hercules, California, USA) and placed into the transfer cassette. The transfer tank was filled with transfer buffer (39 mM glycine, 48 mM Tris base (pH 9.2), 0.037% SDS, 20% v/v methanol) and samples transferred overnight at 15 V (0.1 A) between 10 to 15°C. On the following day, the membranes were recovered and non-specific binding was blocked in Tris buffered saline (TBST, 0.05 M Tris, pH 7.5, 0.15 M NaCl, 0.02% Tween 20, made up in dH_20) with 1% w/v gelatine on a shaker for one hour at room temperature. The membranes were washed twice in TBST for 10 minutes with gentle shaking and then separate membranes incubated with goat polyclonal anti-human activin receptor IA, IIA and IIB, (R&D Systems, Minneapolis, USA) respectively at concentrations of 0.3 μg/ml for each receptor for 60 minutes at room temperature with gentle shaking. The membranes were washed again as described above, followed by incubation with a horseradish peroxidase conjugated anti-goat secondary antibody (diluted 1:1000 in TBST, Zymed, San Francisco, CA, USA) with shaking for one hour at room temperature. After washing with TBS for 10 minutes, hybridisation was visualized using 4-chloro-1-naphthol (Sigma, St. Louis, USA). Relative levels of proteins were assessed by laser densitometric analysis (Quantity One, Bio Rad Laboratories, Hercules, CA, USA) performed on the Western blot relative to the marker bands (size range XX, Bio Rad Laboratories, Hercules, CA, USA). Endometrium collected during the secretory menstrual phase was prepared in the same way as the myometrial samples for Western and immunohistochemical analysis and used as a positive control (Otani et al, 1998).

6.2.6 Immunohistochemistry

Paraffin embedded sections (5 microns thick) of all myometrial and endometrial samples were analysed using a monoclonal mouse anti-human activin ßA-subunit antibody (NP Groome, Oxford Brookes University, UK), and polyclonal goat anti-human activin receptor type IA, IIA and IIB antibodies (R&D Systems, Minneapolis, USA). All procedures were performed at room temperature. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide in absolute methanol for 15 minutes, followed by blocking with the protein blocking agent PBA (Dako Corp., Carpinteria, USA) for 20 minutes. Primary antibodies were applied (2 µg/ml) and the sections incubated for 45 minutes. A biotinylated secondary antibody was subsequently applied ($LSAB^+$, Dako Corp., Carpinteria, USA) for 15 minutes. Reactions were visualized by addition of a streptavidin-horseradish peroxidase conjugate and 3-amino 9-ethylcarbazole (AEC) chromogen (Zymed, San Francisco, CA, USA). The sections were counterstained with Harris haematoxylin for 30 to 60 seconds. Sections were mounted with aqueous mounting medium and covered with a glass coverslip. Microvascular endothelial cell staining was performed on serial sections using the monoclonal mouse anti-human antigen CD34 (1:50 dilution, Serotec, Oxford, UK). Sections were incubated with primary antibody at 37°C for 45 minutes, followed by an alkaline phosphatase conjugated secondary antibody ($LSAB^+$, DAKO) for 15 minutes at room temperature. The sections were visualised using Vector Blue (Vector Laboratories, Burlingame, CA, USA) and mounted as described above. Negative controls for immunostaining were generated by substituting the primary antibodies with non-immune goat IgG (activin receptor) or non-immune mouse IgG1 (activin ßA subunit). Positive control tissue was human endometrium (Otani et al, 1998), subjected to the same treatment as the myometrium.

6.2.7 Western hybridisation of activin receptors in cultured myometrial smooth muscle and microvascular endothelial cells

The human myometrial cells used for this study were isolated and cultured by Ms Kristina Bucak of the Department of Obstetrics and Gynaecology, Monash University, Monash Medical Centre.

Single cell suspensions of myometrial microvascular endothelial cells and smooth muscle cells were obtained from myometrial tissue obtained from hysterectomy samples by dissociation with collagenase and DNAse as described by Gargett et al (2000). Briefly, microvascular endothelial cells were then separated from smooth muscle cells using *Ulex europeaus agglutinin-1* (UEA-1) coated Dynabeads (Dynal, Oslo, Norway) and cultured in M199 containing 15% human serum and 5% FCS in fibronectin (Gibco BRL, Gaithersburg, MD, USA) coated tissue culture flasks (Beckton Dickinson, Bedford, MA, USA). Western analysis was performed after second passage and further purification with UEA-1-coated Dynebeads (Gargett et al, 2000). The separated smooth muscle cells were cultured on uncoated plastic flasks in M199 containing 10% FCS for 5–7 days until confluent. The purity of myometrial microvascular endothelial and smooth muscle cell cultures were >99% as demonstrated by flow cytometry of CD31-immunostained cells (endothelial cell marker) and α-smooth muscle actin immunostaining (smooth muscle cell marker). Cells were subjected to Western analysis for the expression of activin receptors IA, IIA and IIB as described in section 6.2.5.

6.2.8 Statistical analysis

Differences in activin A and follistatin levels and activin receptor proteins between the three groups (non-pregnant, pregnant at term and labouring at term) were analysed using Student's t-test after $\log_{10}$ transformation of the raw data. Significance was recognized when $P<0.05$.

6.3 RESULTS

6.3.1 Activin A and follistatin content in myometrium

Activin A and follistatin was detected in all myometrial tissue lysates (figure 6.1). The total concentration of activin A was significantly higher in labouring myometrium compared to

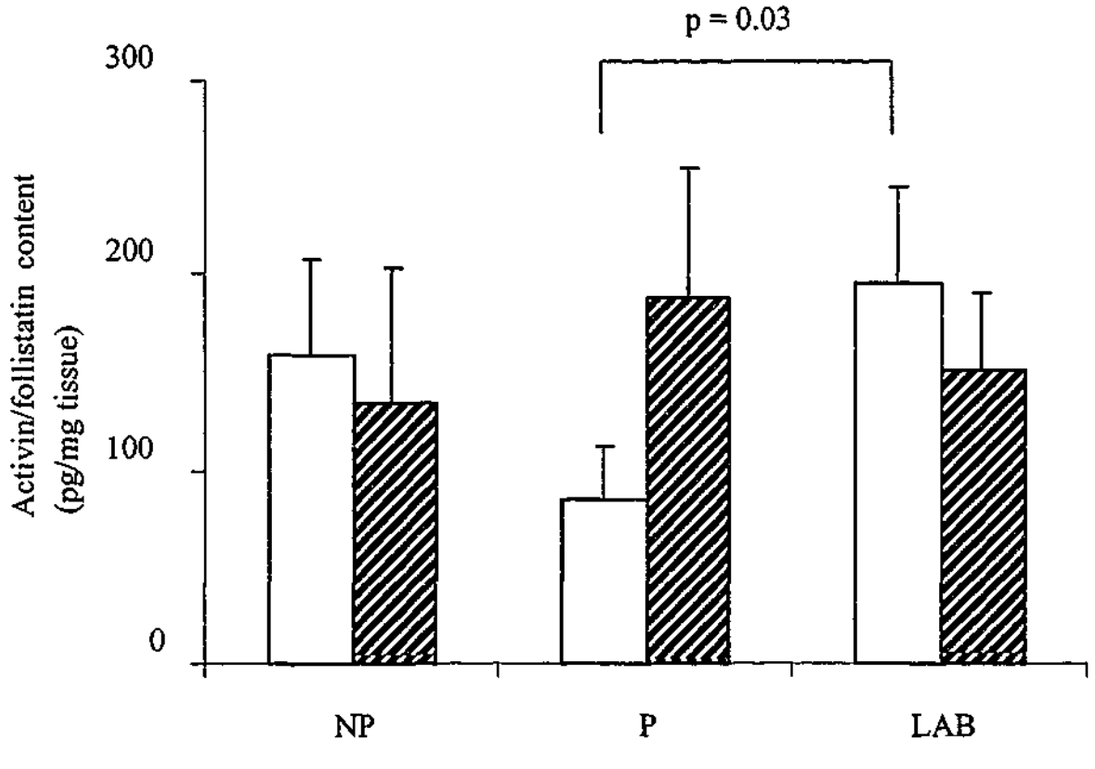


Figure 6.1. Concentrations of activin A (□) and follistatin (▨) in human myometrium from non-pregnant (NP), pregnant term (P) and labouring term (LAB) tissue,

gestation-matched non-labouring myometrium (194.9 ± 50.5 versus 84.3 ± 27.5 pg/mg/total protein, respectively, p = 0.03). The activin A content of non-pregnant myometrium was 158.6 ± 50.1 pg/mg/total protein, not statistically different from either of the two other groups. The levels of follistatin were not statistically different between the three groups (P = 0.09 between non-pregnant and pregnant myometrium, P = 0.2 between non-pregnant and labouring myometrium and P = 0.35 between pregnant and labouring myometrial tissue).

6.3.2 Western hybridisation of myometrial tissue

Incubation of Western blots with activin A receptor antibodies revealed distinct bands for RIA and RIIB with corresponding estimated sizes of 68 and 60 kDa (figure 6.2).

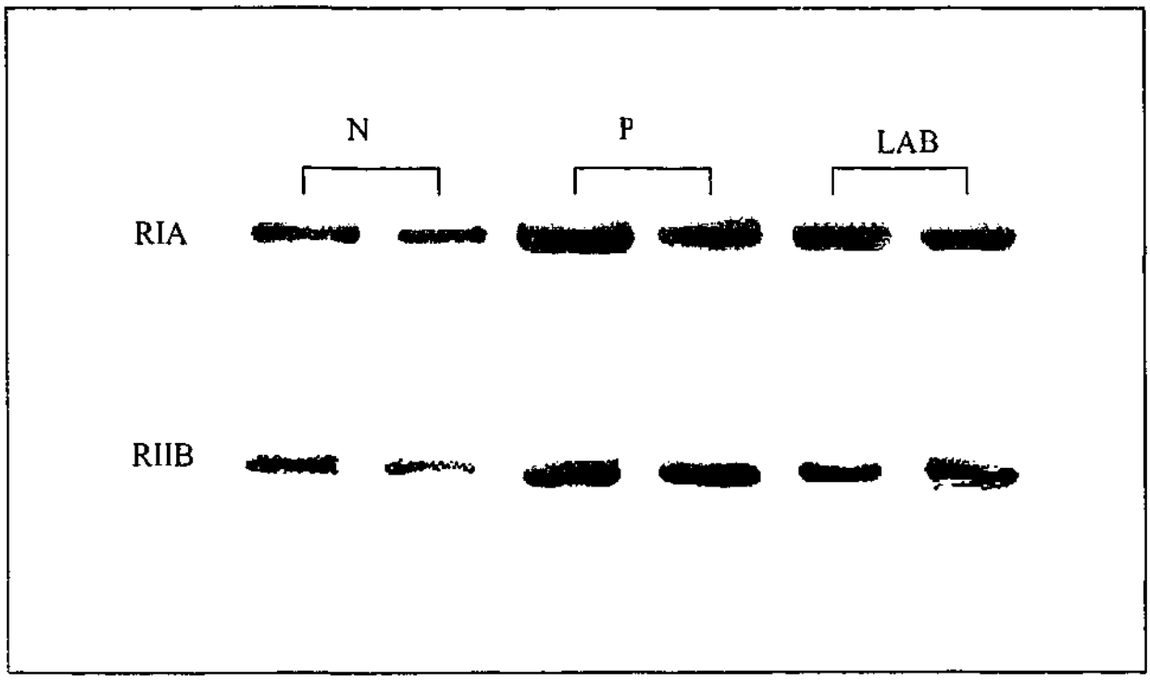


Figure 6.2. Activin receptor IA and IIB in non-pregnant (N), pregnant (P) and labouring (LAB) myometrium at term.

These two receptors were present in all of the three groups of tissues, as well as the positive control tissue, the endometrium. Relative levels of RIA and RIIB are shown in figure 6.3. Receptor type IIA (estimated size 62 kDA) was only very weakly expressed in all groups and it was not possible to perform densitometric analysis on these bands. Relative levels of

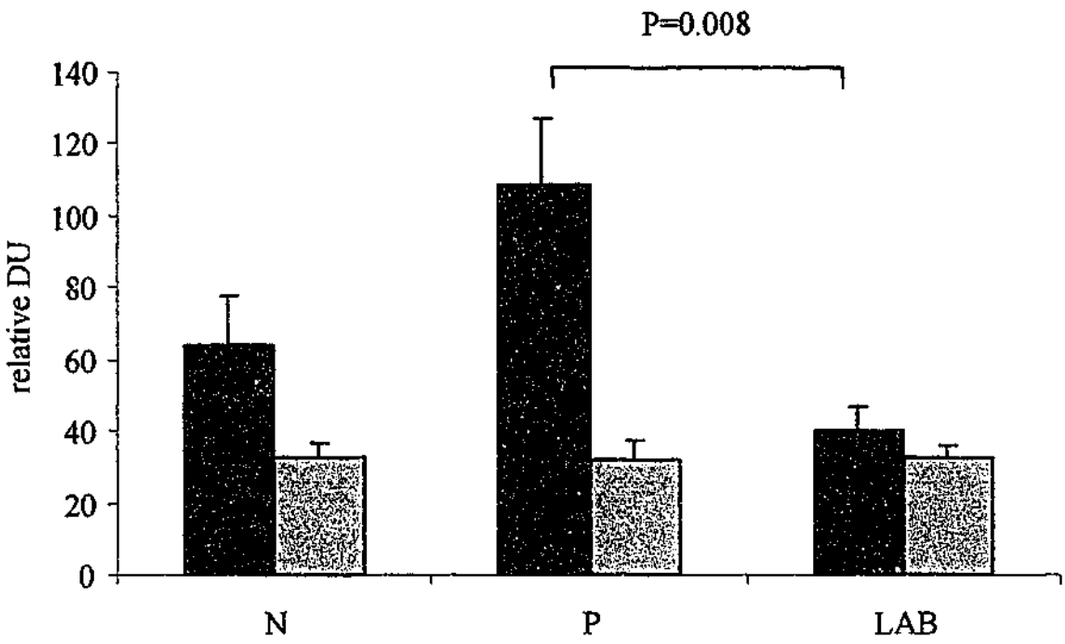


Figure 6.3. Relative densitometric analysis of activin receptors IA (■) and IIB (▣) in myometrial tissue from non-pregnant (N), pregnant (P) and labouring (LAB) women at term .

receptor IA were significantly lower in labouring myometrium compared to non-labouring myometrium (40.0 ± 6.8 versus 108.7 ± 18.1 relative densitometric units, respectively; P = 0.008). There was no statistical difference in the level of receptor IA expression between the other the groups (P = 0.07 between N and P, P = 0.3 between N and LAB). There were no significant differences in receptor IIB protein levels across the three groups of myometrial tissue (P = 0.7 between N and P, P = 0.93 between N and LAB, and P = 0.76 between P and LAB).

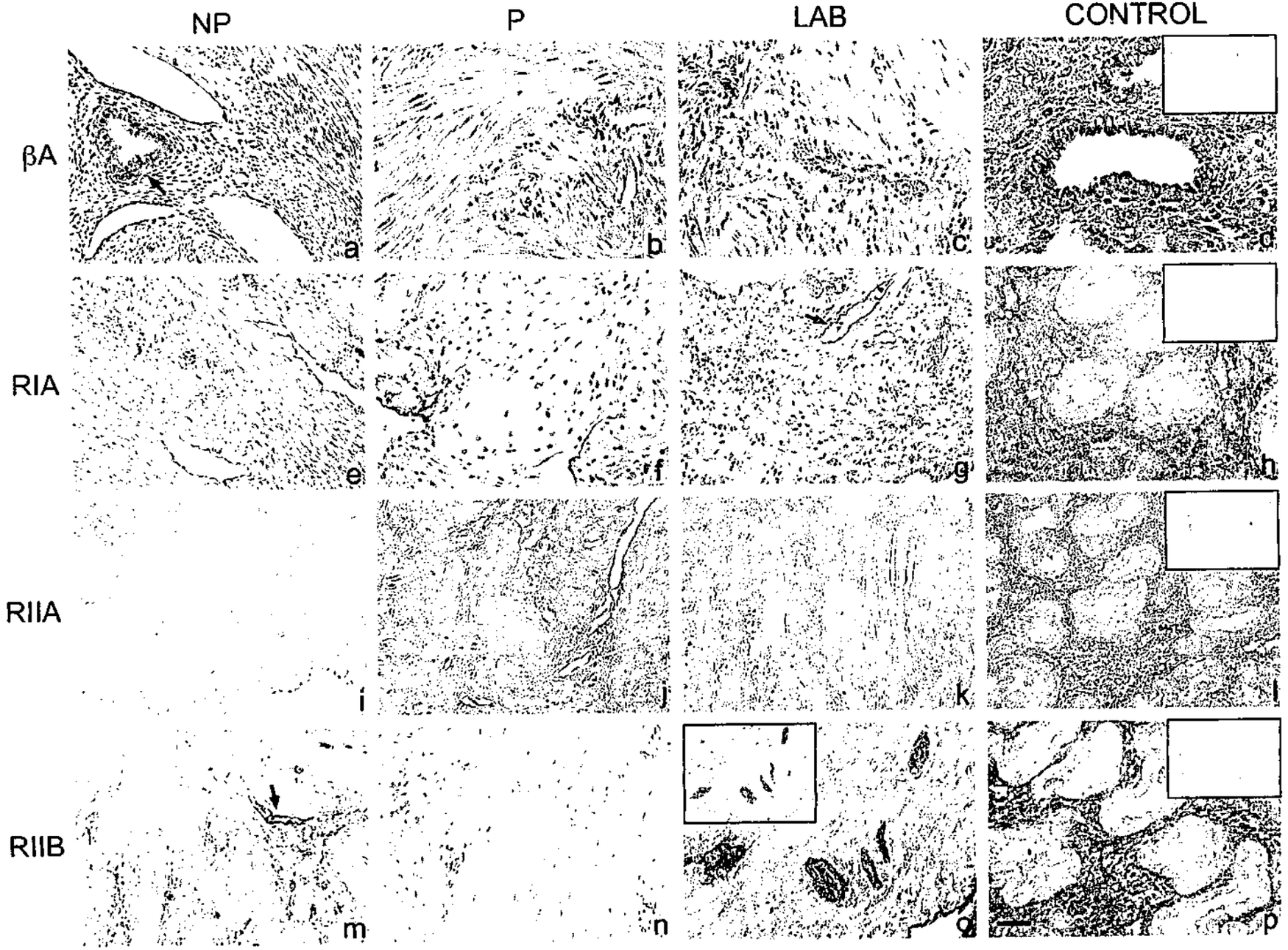
6.3.3 Immunohistochemistry

Figure 6.4 shows images for βA-subunit and all three activin receptors in the three groups of myometrial tissue analysed. The localization of ßA-subunit and activin receptors IA, IIA and IIB were similar in all three groups of tissues studied. None of the samples analysed showed positive staining for βA-subunit in the smooth muscle cells of the myometrium. Activin β_A-positive cells were localised to some vessels in all samples analysed (arrow 6.4a). The smooth muscle of the myometrium was negative for all receptors in the three groups studied. Intense staining was observed for receptor IIB in some of the myometrial blood vessels in samples from all three groups, especially in the labouring group (arrows, 6.4 g). Serial staining with CD34, a vascular endothelial cell specific antibody, confirmed localisation of activin receptors to vascular endothelial cells (6.4 o insert). Receptor IIA staining was weak in all samples. The corresponding positive controls (human endometrium from the secretory phase of the menstrual cycle) displayed intense staining in the glandular epithelium for the βA-subunit (6.4d) and distinct stromal staining for all three receptors (6.5 h, l and p). No staining was seen in negative control tissues (human endometrium, 6.4 d, h, l and p inserts).

6.3.4 Western hybridisation of isolated cell preparations

In keeping with the immunohistochemistry, Western hybridisation of isolated myometrial smooth muscle cells revealed no expression of any of the three activin receptor proteins. In

Figure 6.4. *Localisation of activin* βA-subunit and activin receptors IA, IIA and IIB in myometrium form non-pregnant (NP), pregnant (term, P) and labouring (term, LAB) women. The insert in panel 6.4 p is a serial section stained with CD34. Positive control sections for βA are depicted in 6.4 d, and for activin receptor IA, IIA and IIB in 6.4 h, l, and p respectively. The inserts show the corresponding negative controls. Scale bar = 50 μm.


NP
P
LAB
CONTROL
βA
RIA
RIIA
RIIB
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

contrast, isolated myometrial microvascular endothelial cells showed expression for activin receptor IA and IIB. However, the overall weak staining observed in the cellular extracts did not allow adequate picture quality after scanning and so could not be shown here. Expression of receptor protein IIA was not observed in these cells.

6.4 DISCUSSION

The identification of a new key regulator of parturition, particularly one with a direct action on the myometrium, would be both exciting and welcomed because it would offer the prospect of entirely novel therapies for both the suppression and induction of labour. Thus, when Draper and colleagues reported activin A binding to pregnant myometrium in the rat (Draper et al, 1997), the potential for activin to play such a key role, possibly via a direct action on myometrial smooth muscle cells, seemed all the more promising.

As described in chapter 1, activin requires the presence of both a type I and a type II receptor on the target cell surface for signal transduction to occur (Lebrun et al, 1997, Tsuchida et al, 1993). Initially, activin binds with high affinity to receptor type IIA or IIB on the cell membrane. Intracellular signaling pathways are initiated once a type I receptor is phosphorylated and recruited to the complex.

In this study, the initial findings that three activin receptors (IA, IIA and IIB) comprising both a type I and II receptor were present in the human myometrium, as detected by Western hydridisation, and that activin A was detected by ELISA in homogenised uterine tissue with the highest concentrations in labouring myometrium were also supportive of a role for activin A in stimulating myometrial activity. However, surprisingly the immunohistochemistry studies demonstrated that neither the activin receptors (IA, IIA and IIB) nor activin β_A-

subunit localised to human myometrial smooth muscle cells. Initially, this finding appeared at odds with the whole tissue receptor and ligand content data and certainly inconsistent with the hypothesis that activin has a direct action on human myometrium. However, the demonstration here that whole myometrial tissue contains activin A and the three receptors is explained by positive immunostaining for these four proteins within the tissue but with a vascular rather than myometrial smooth muscle cell localisation. Further, the co-localisation of their staining with that of a specific endothelial cell marker, CD34, and the detection of the IA and IIB receptors in purified myometrial microvascular endothelial cells but not in purified myometrial smooth muscle cells confirms that activin A and the activin receptors IA and IIB, and possibly IIA, are present in microvascular endothelial cells in myometrium. Others have shown that activin A receptors are present in endothelial cells in a range of tissues (McCarthy & Bicknell, 1993, 1994) and that activin A has potent inhibitory effects on the growth of cultured endothelial cells (McCarthy & Bicknell, 1993, 1994, Kozian et al, 1997). It is therefore possible that in the myometrium, as in other tissues, the function of activin A is as a regulator of vascular endothelial cell function and may have function which ultimately affect the blood flow to the smooth muscle of the myometrium, but this hypothesis requires further functional studies. In this regard it is interesting that maternal serum activin A levels are significantly increased in association with pre-eclampsia (Muttukrishna et al, 1997a), a condition characterised by endothelial cell damage and activation, and that we observed significant differences in the levels of activin receptor IA and activin A between labouring and non-labouring myometrium. What these apparent differences mean and indeed whether they are of any clinical relevance remains uncertain. Certainly, given the limited numbers of tissues studied here and the relatively insensitive quantification by Western immunoblotting, interpretation of these differences must be necessarily cautious. Nonetheless it is possible that these novel findings may reflect important vascular adaptations in

pregnancy and labour, perhaps in response to hypoxia (Jenkin et al, 2001), an association which is investigated in more detail in chapter 8.

In conclusion, the absence of receptors for activin A in the human smooth muscle of the myometrium at term and during labour argue strongly against the hypothesis that activin A can directly initiate or modulate myometrial contractions. However, these results do not address the possibility that activin A may stimulate contractions locally via other uterotonic mediators such as PGs and OT in the gestational tissues.

CHAPTER 7

ACTIVIN βA-SUBUNIT AND ACTIVIN RECEPTORS IN GESTATIONAL TISSUES AT TERM AND AFTER LABOUR

7.1 INTRODUCTION

In previous chapters it has been shown that activin A and follistatin concentrations in the peripheral circulation are not altered with labour and that the myometrial smooth muscle is not a target for activin A at term or during spontaneous onset of labour. Although the observed profile of activin A in the peripheral circulation would preclude activin A as a direct regulator of parturition, it is nonetheless possible that activin A, in consort with follistatin, has permissive roles in parturition and that activin A may act locally, within the placenta and/or the fetal membranes, to stimulate other modulators of parturition, such as the prostaglandins, CRH and OT, in a paracrine and/or autocrine fashion and also be involved in modulating blood flow in the myometrium. More information about localised labour-associated changes of activin A and its receptors within gestational tissues are thus needed in order to gain more insights into the actions of activin A during pregnancy and parturition.

Messenger RNA for the activin βA-subunit and activin receptors type II A and B have been localised to placental and chorion trophoblast as well as the amniotic epithelium at all stages of pregnancy (Meunier et al, 1988, Minami et al, 1992, Rabinovici et al, 1992, Peng et al, 1993, Petraglia et al, 1993a, Petraglia et al, 1994a, Shinozaki et al, 1995, Petraglia et al, 1997b, 1997c, Birdsall et al, 1997). These studies, describing mRNA expression, only show the potential of placenta and fetal membranes to produce activin or activin receptors but there

are no published reports of receptor protein localisation in gestational tissues, in particular of receptor type I.

Thus, the studies in this chapter were designed to further clarify local actions of activin A by investigating activin βA-subunit and receptor type I and type II protein expression and localisation in gestational tissues from term labouring and non-labouring pregnancies.

Also, since very little information is available on the function and actions of activin A in early pregnancy, first trimester gestational tissues have been included in this study.

7.2 MATERIALS AND METHODS

7.2.1 Study 1: Placental localisation of βA-subunit and activin receptors across pregnancy

7.2.1.1 Patients

For the localisation of βA-subunit and activin receptors IA, IB, IIA and IIB three groups of women were recruited into this study. Placental tissue was collected from women undergoing a surgical termination of pregnancy for psychosocial reasons in the first trimester at 8 – 12 weeks' gestation (Group 1, n = 8) and in the second trimester, at 14 – 17 weeks' gestation (Group 2, n = 7) and from women undergoing an elective Caesarean section at term (37 – 40 weeks' gestation) for malpresentation or a previous Caesarean section (Group 3, n = 8). All women were healthy and had singleton pregnancies.

7.2.2 Study 2: Activin βA-subunit, activin receptor expression and activin A and follistatin content in gestational tissues at term before and after labour.

7.2.2.1 Patients

For the localisation of βA-subunit and activin receptors IA,IB, IIA and IIB, sixteen women with normal healthy singleton pregnancies at term participated in the study. Eight women underwent an elective Caesarean section (for breech presentation or previous Caesarean section) and eight women had a normal vaginal delivery after spontaneous onset of labour. Both studies had the approval of the Monash Medical Centre Human Research and Ethics Committee and all women gave written, informed consent.

7.2.3 Tissue processing

Within 15 minutes of Caesarean or vaginal delivery, pieces of placental tissue (2 cm^2) were excised and rinsed in ice-cold PBS to remove excess blood and were then fixed in 10% neutral buffered formalin at 4 °C for 12 - 16 hours prior to being embedded in paraffin for immunohistochemistry.

For study 2, amnion and choriodecidua were also collected from women delivering at term, dissected free and processed as described above for immunohistochemistry. The fixation times for amnion and choriodecidua were 6 to 8 hours.

7.2.4 Total Protein Assay

Term tissues were thawed on ice, homogenized in ice-cold sterile PBS and centrifuged for 2 minutes at 10 000 rpm. The total protein concentration in the supernatant was measured by the bicinchoninic acid assay (BCA) following the manufacturer's instructions (Pierce, Illinois, USA).

7.2.5 Western hybridization

The procedure for Western hybridisation has been described in section 5.2.6. In this study, receptors IA, IB, IIA and IIB were analysed. Receptor IB was purchased from R&D Systems, Minneapolis, USA and applied in the same concentrations and following the same protocol as has been described previously for the other three activin receptors.

7.2.6 Immunohistochemistry

Paraffin embedded sections (5microns) of placenta and fetal membranes were analysed by standard immunohistochemistry for localisation of activin receptor IA, IB, IIA and IIB as described in chapter 6, section 6.2.6. Microvascular staining was analysed using CD34 as described in section 5.2.7. Endometrium from the secretory phase of the menstrual cycle was used as a positive and negative control and processed as described in chapter 6, section 6.2.6

7.2.7 ELISA for activin A and follistatin

The ELISAs utilised to measure activin A and follistatin in gestational tissues have been described in section 2.2.1.3 and 2.2.1.4 respectively.

7.2.8 Statistical analyses

Differences in activin A and follistatin content between the groups were analysed by Mann-Whitney-U test. Receptor protein expression was assessed by densitometric analysis using Quantity One software (BioRad, Hercules, CA, USA) and differences between the groups calculated using a Student t-test after $\log_{10}$ transformation. Significance was recognised when $P<0.05$.

7.3 RESULTS

7.3.1 Study 1. Localisation of βA-subunit and activin receptors in placenta across pregnancy

In first trimester placentae βA-subunit was localised to the syncytiotrophoblast and cytotrophoblast cells of the placental villi and to intermediate trophoblasts in cell columns (figure 7.1 a). All activin receptors (type I and type II, figure 7.1 b-e) were present in the syncytiotrophoblast of floating and anchoring villi with patchy staining observed in cytotrophoblast and intermediate trophoblasts. Occasional Hofbauer cells stained positive for βA-subunit and all activin receptors.

In second trimester placentae staining for βA-subunit was observed in syncytio- and cytotrophoblast, in the stroma and some Hofbauer cells (figure 7.1 f). All receptors were localised to the syncytiotrophoblast and to some cells of the cytotrophoblast amd occasional Hofbauer cells (figure 7.1 g-j). Overall, staining was less intense than that observed in first trimester.

The distribution of βA-subunit and activin receptors in term placenta is shown in figure 7.1 k-o and in figure 7.2. BetaA-subunit was detected in the syncytium, as well as in some isolated vascular endothelial cells of placental vessels (figure 7.2a, arrow) Occasional receptor staining was observed in the syncytiotrophoblast but receptors were primarily localised to the vascular endothelium of villous blood vessels (7.1 l - o, arrow, 7.2b). To confirm the localisation of receptors to vascular endothelial cells, serial sections were incubated with CD34 (Serotec, Oxford, UK), a vascular endothelium specific antibody. Vascular endothelial cells positive to both activin receptor and CD34 were identified in vessels of the placental villi (figure 7.3k - l, arrow). High power magnified images of βA-subunit in syncytiotrophoblast at term and activin receptor IA staining in the vascular endothelium of a placental villous at term are shown in figure 7.2 Overall, receptor staining intensity was highest for receptor IA, followed in staining intensity by receptor IB and receptor IIB, while receptor IIA was staining only very weakly.

7.3.2 Study 2. Activin βA-subunit, activin receptor localisation and activin A and follistatin content in gestational tissues at term before and after labour.

7.3.2.1 Immunohistochemistry

The immunohistochemical analysis detected no changes in the localisation or staining intensity of activin βA-subunit or activin receptors in placenta or fetal membranes before or after spontaneous labour onset (figure 7.3 and 7.4 respectively). Placental distribution of βA-subunit and activin receptors in term tissues has been described in section 7.3.1.

In the fetal membranes βA-subunit was stained most intensely in the amniotic epithelial cells (figure 7.4 a,f arrow). Weaker staining was also observed in the fibroblast and reticular layers and the chorionic trophoblast with adherent decidual cells before and after labour. No difference in staining intensity was observed between the two groups. The activin receptors in these tissues (figure 7.4 b-e and g-j) were localised primarily to the fibroblast and reticular layers and weak staining was present also in some cells of the chorionic trophoblast and decidua. Occasionally, a few isolated epithelial cells stained positive for the activin receptors studied (figure 7.4 g, arrow). In positive control tissue (human endometrium from the secretory phase of the menstrual cycle) staining for the βA-subunit localised to the glandular epithelium (see chapter 6, figure 6.4). In contrast, receptor type I and type II expression was localised to stromal cells in this tissue (figure 6.4 h,l and p).

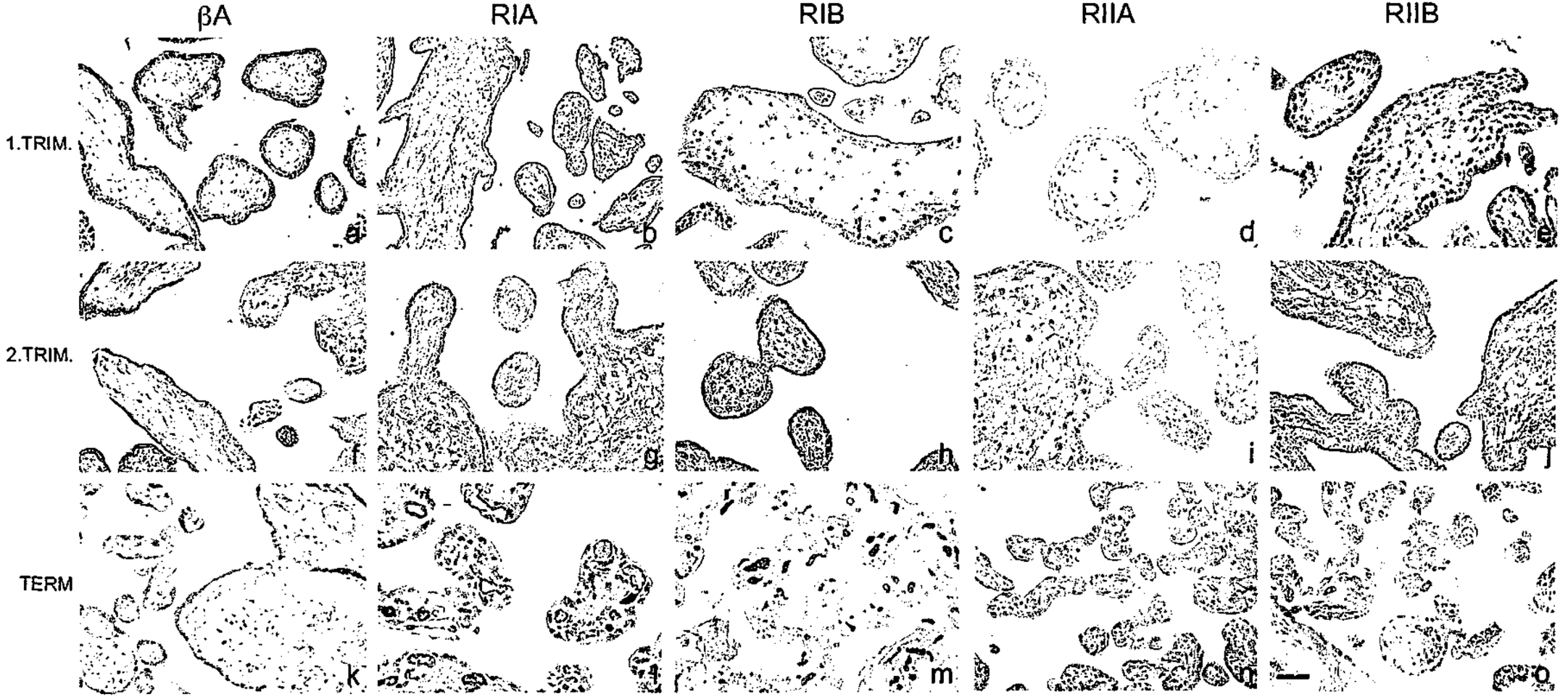


Figure 7.1. Activin βA-subunit and activin receptor localisation in first trimester (a-e), second trimester (f-j) and term placenta (k-o). Scale bar = 25 μm (a-k), scale bar = 50μm (l-o).

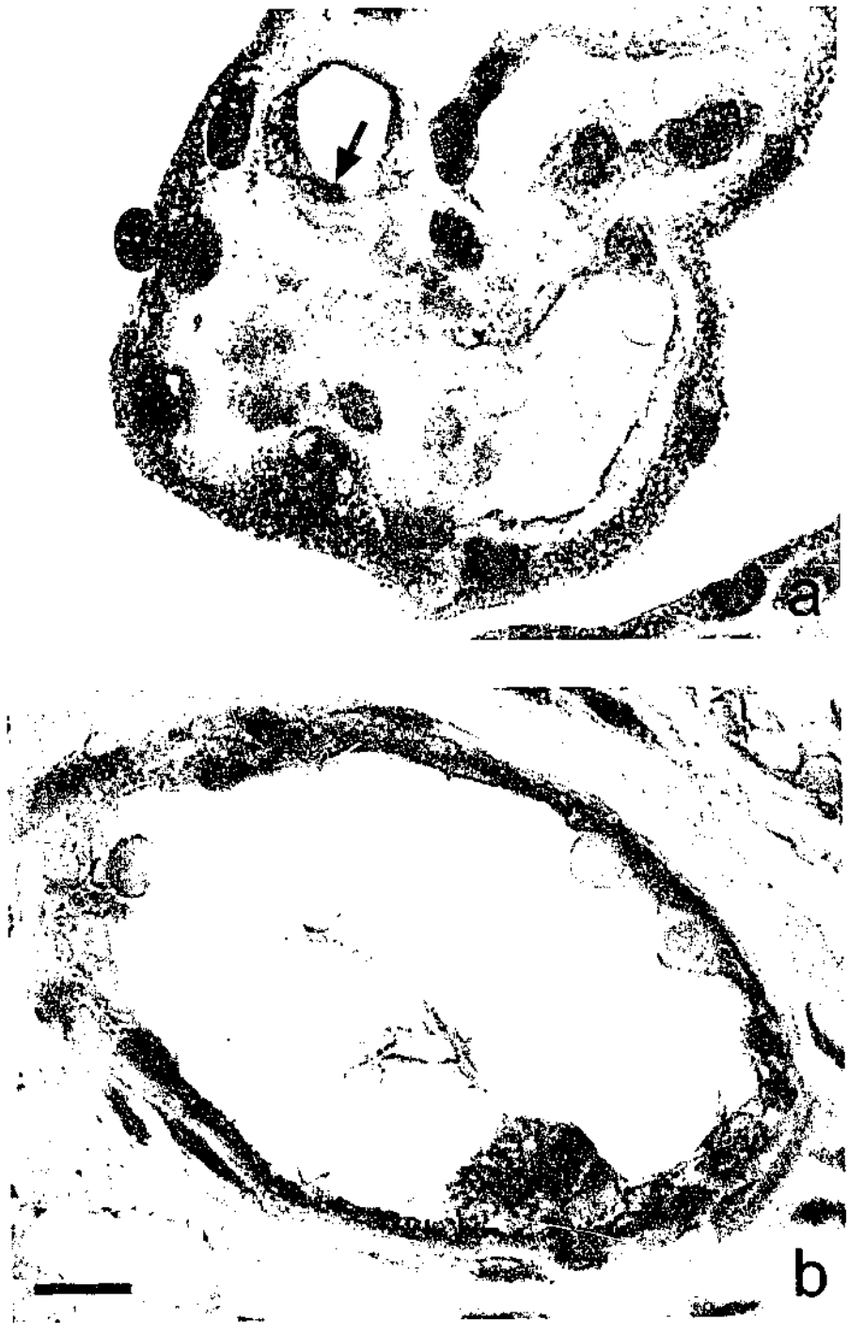


Figure 7.2. Activin βA-subunit (a) and activin receptor localisation (b) in term placenta. Scale bar = 10μm.

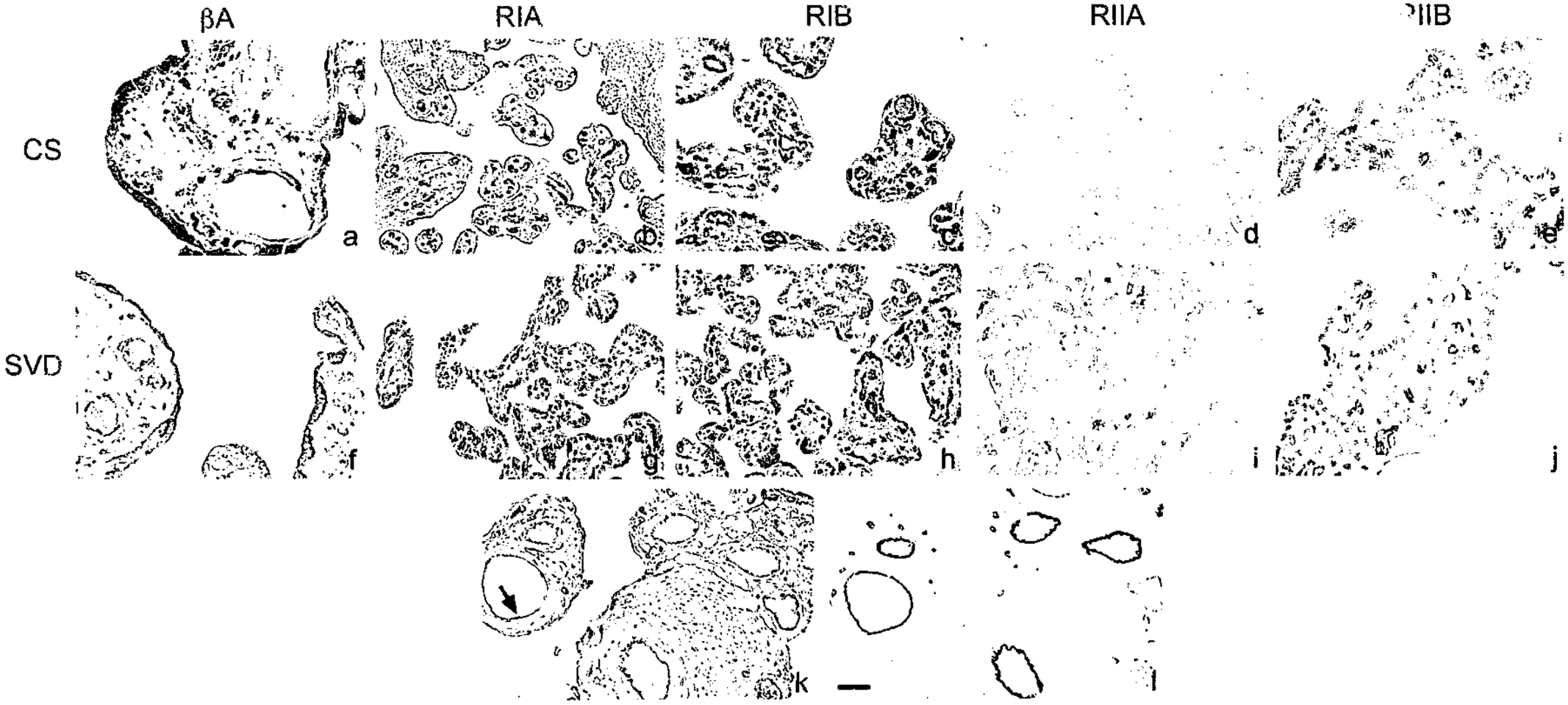


Figure 7.3. Activin βA-subunit and activin receptor localisation in placenta before labour (CS) and after labour (SVD). Figure 7.3k and 7.3 l shows serial sections for receptor 1A in endothelium of placental vessels (k, arrow) and CD34 (l). Scale bar (a,f,k,and l) = 25μm. Scale bar (b-j) 50 = μm.

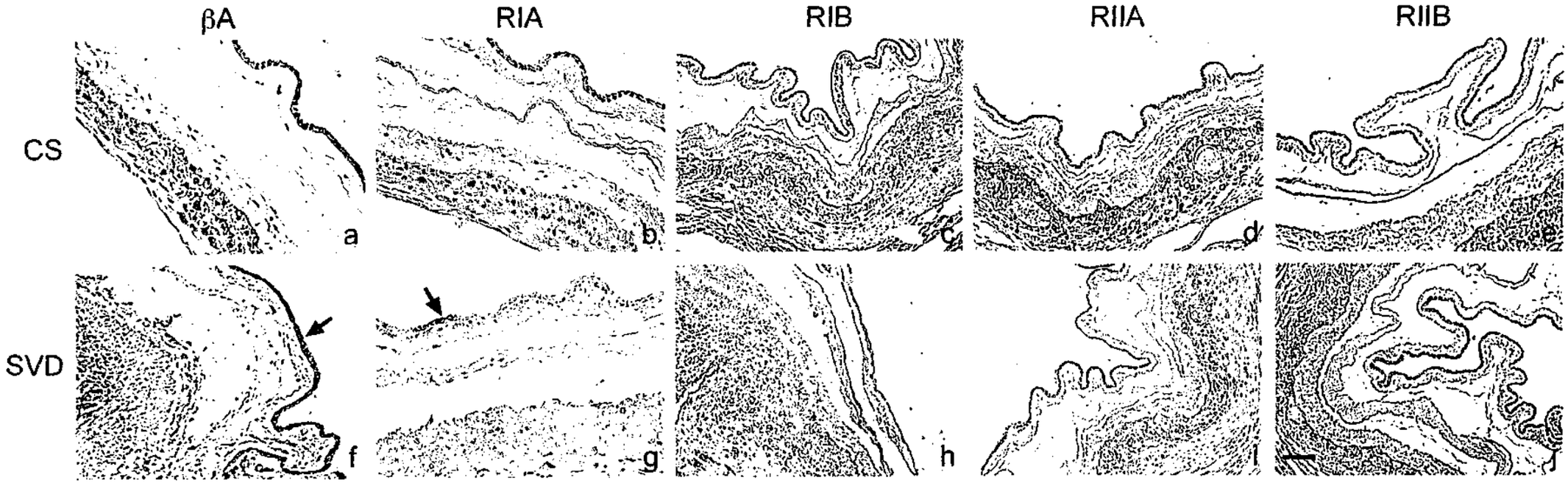


Figure 7.4. Activin βA-subunit and activin receptor localisation in fetal membranes before labour (CS) and after labour (SVD). Scale bar 25 = μm.

7.3.2.2 Activin A and follistatin content in placenta and fetal membranes before and after labour

Tissue content of activin A and follistatin in placentae, amnion and choriodecidua from women at term and from women after spontaneous labour is shown in figure 7.5. There were no differences in tissue content of either activin A (P = 0.6, 0.69, and 0.83 for placenta, amnion and choriodecidua respectively) or follistatin (P = 0.63, 0.28 and 0.93 for placenta, amnion and choriodecidua respectively) between non-labouring or labouring tissue. Weight for weight, levels of activin A in samples from elective Caesarean section and normal vaginal deliveries were higher in amnion and chorion compared to placenta (P = 0.001 and 0.0006 respectively), while levels of follistatin were significantly higher in choriodecidua compared to amnion (P = 0.0007) and placenta (P = 0.04).

7.3.2.3 Activin receptor expression in placenta and fetal membranes before and after labour

Figure 7.6 shows representative blots (Western hybridisation) probed for activin receptors type IA, IB, IIA and IIB with estimated sizes of immunoreactive proteins of 68, 69, 62 and 60 kD respectively in placenta, amnion and choriodecidua, before and after spontaneous labour. All receptors were present in the tissues analysed. In amnion, receptor IIA expression was too weak to allow densitometric scanning, a result consistent with the immunohistochemical analysis described above. All four receptors were detected in the control tissue (endometrium from the secretory phase of the cycle, see figure 6.4).

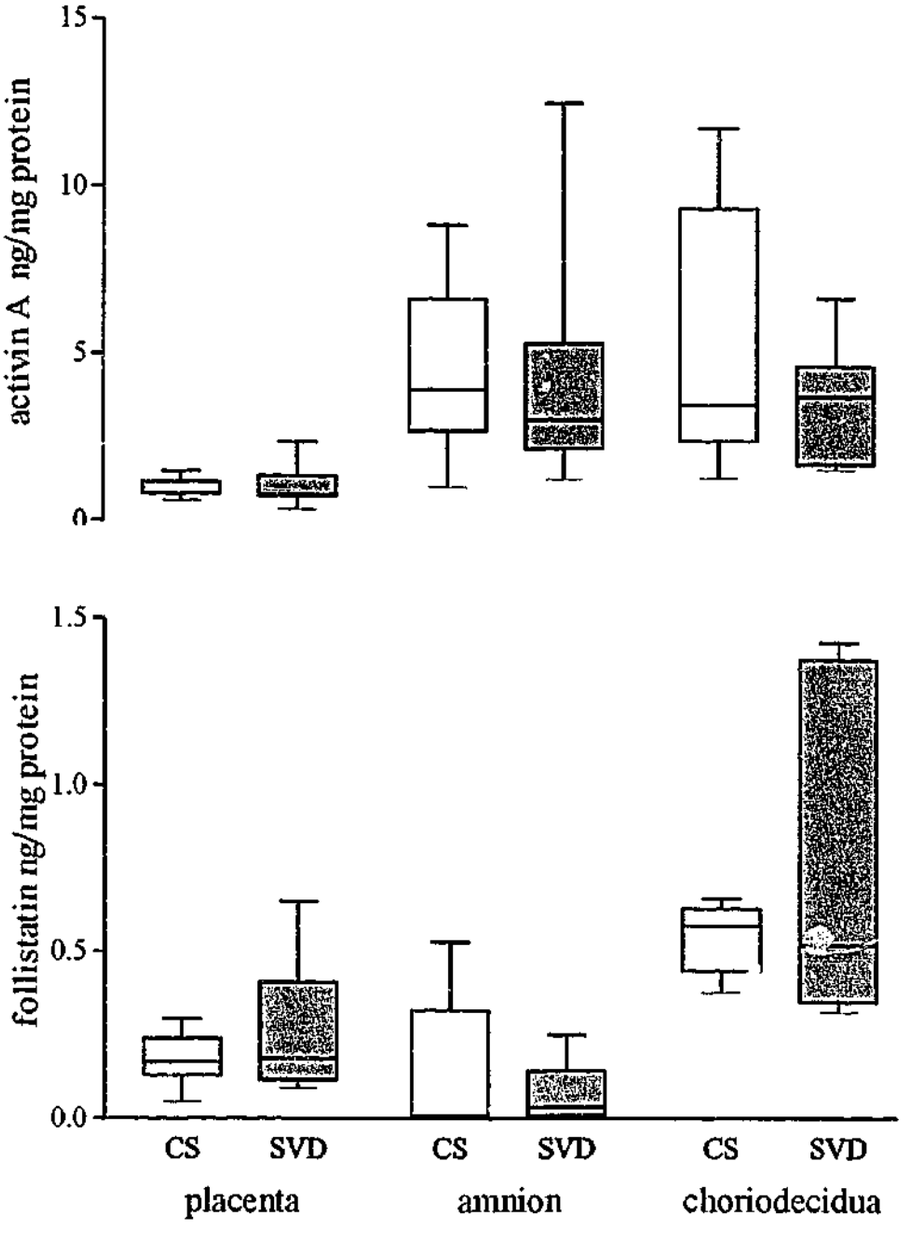


Figure 7.5. Concentration of activin A and follistatin in term placenta, amnion and choriodecidua collected after elective Caesarean section (CS) or spontaneous vaginal delivery (SVD). Lines shown denote medians, boxes denote the 25th and 75th percentile and upper and lower error bars denote the 95% confidence intervals.

A densitometric analysis of the relative levels of all receptors is shown in figure 7.7. There were no differences between the labouring and non-labouring tissues with the exception of receptor IA which was modestly elevated in non-labouring chorion compared to labouring chorion ($p = 0.02$).

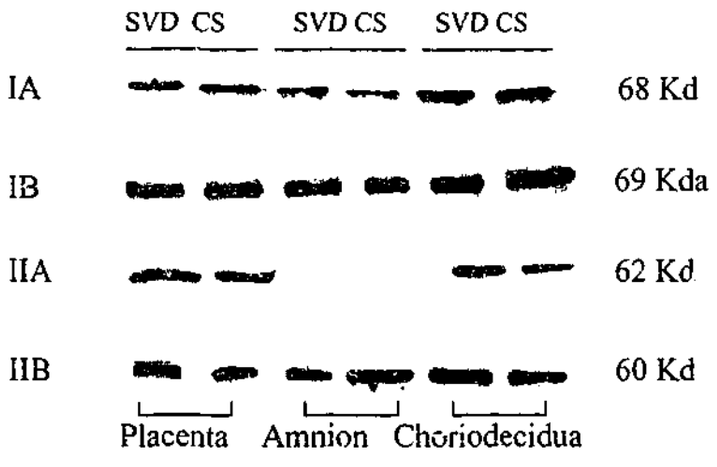


Figure 7.6. Western hybridization of activin receptors IA, IB, IIA and IIB in term placenta, amnion and choriodecidua collected after elective Caesarean section (CS) and spontaneous vaginal delivery (SVD).

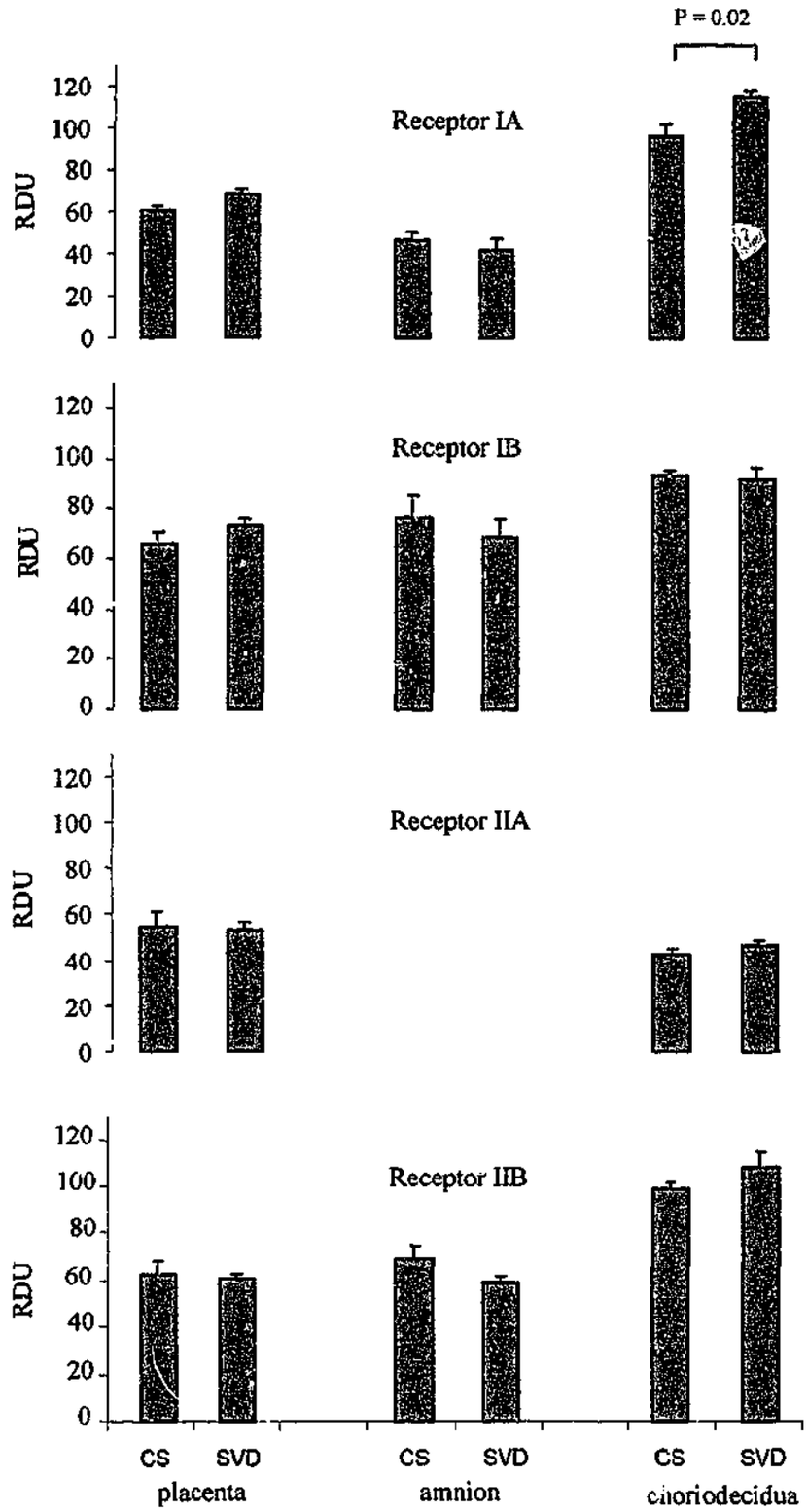


Figure 7.7 Relative densitometric analysis of activin receptors IA, IB, IIA and IIB in placenta, amnion and choriodecidua before labour (CS) and after spontaneous vaginal delivery(SVD).

7.4 DISCUSSION

While it is clear that the feto-placental unit is the major source of circulating activin in human pregnancy it remains much less clear what physiological role(s) activin may have in pregnancy. In particular, whether activin has a role in the initiation and/or regulation of parturition remains uncertain although such a role looks less likely from the studies reported in this thesis. The studies reported here have investigated the distribution of activin βA-subunit and activin type I and type II receptor proteins in the placenta throughout pregnancy and after labour, and in the fetal membranes at term before and after labour. While previous studies have related βA-subunit localisation in the placenta (Meunier et al, 1988, Rabinovici et al, 1992, Petraglia et al 1993a) existing reports relating the distribution of activin receptors have been limited to messenger RNA expression using Northern analysis and *in-situ* hybridisation, rather than protein studies, or have reported only the type II receptor (Peng et al, 1993a, Petraglia et al, 1997b, 1997c, Shinozaki et al, 1995, Peng et al, 2000). The data demonstrating protein expression and localisation for both type I and type II activin receptors throughout pregnancy and in association with labour is novel and may offer useful insights into possible local actions of activin in the placenta and fetal membranes.

In the placenta, the findings agree with those of others showing strong βA-subunit and receptor staining in the syncytiotrophoblast and cytotrophoblast of the first and second trimester placental villi (Rabinovici et al, 1992, Minami et al, 1992, Shinozaki et al, 1995). This pattern of staining for activin and its receptors would be consistent with activin playing a role in trophoblast invasion in early pregnancy, as previously suggested by the *in vitro* observation that activin A can stimulate cytotrophoblast cells of first trimester chorionic villous explants to differentiate into intermediate or invasive phenotypes (Caniggia et al, 1997). Support for such a role for activin A is also afforded by evidence that other members

of the TGF-ß superfamily, such as TGFß1 and TGFβ3 can modulate trophoblast invasion, at least *in vitro* (Caniggia et al, 2000, Getsios et al, 1998), although further functional studies are clearly required to provide more direct evidence.

In contrast to the observations made in placenta in early pregnancy, in the term placenta the activin βA-subunit was localised to vascular endothelial cells of villous blood vessels as well as to syncytiotrophoblast cells. Whether the staining in the endothelial cells represents secretion or receptor-bound ligand is currently not clear. However, all four receptor proteins localised to the vascular endothelium rather than to trophoblast and these observations suggest that, with advancing gestation the main target sites for activin A change from the trophoblast to endothelial cells. Activin A has been previously localised to endothelial cells elsewhere: in bovine aorta and in human umbilical vein (McCarthy and Bicknell, 1993). Furthermore, activin A, in consort with its binding protein follistatin, can modulate endothelial cell functions such as vascular endothelial cell proliferation and angiogenesis (McCarthy & Bicknell, 1993, 1994, Kozian et al, 1997). It is therefore possible that the very high levels of maternal serum activin A observed in late pregnancy underlie important vascular adaptations, either systemically or, more likely, locally within the placenta. Indeed, the rapid rise in circulating activin A in the third trimester of pregnancy occurs at a time of declining fetal oxygenation and pH (Gregg & Weiner, 1993) and it is possible that in late pregnancy activin A has a role in modulating feto-placental perfusion. Consistent with this is the observation that induced acute feto-placental hypoxia in the sheep is associated with a rapid rise in activin A and that this in turn may stimulate the production of PGE_2, a potent vasodilator (Jenkin et al, 2001). Hypoxia is also known to acutely up-regulate activin A in the neonatal and adult brain (Wu et al, 1999; Lai et al, 1996) and very high levels of activin A are observed in women with pre-eclampsia, a condition characterised by chronic feto-placental hypoxia (Muttukrishna et al, 1997a, D'Antona et al, 2000). Clearly, further functional studies

are required to define whether activin A has an important role in the regulation of fetoplacental perfusion.

As discussed above, several earlier studies have reported elevated maternal serum and amniotic fluid concentrations of activin A in women in labour, particularly preterm labour compared to non-labouring women (Petraglia et al, 1993b, Petraglia et al, 1997b). Immunolocalisation of activin or its receptors was not reported in those studies but in light of our findings discussed here it is feasible that those pregnancies were complicated by feto-placental hypoxia – a common finding in preterm deliveries. Indeed, in otherwise uncomplicated preterm deliveries placental activin A levels are not elevated (Keelan et al, 1999).

Nonetheless, the possibility still exists that changes in the distribution and/or production of activin or activin receptors within gestational tissues might be a mechanism whereby activin could exert effects. On the contrary, we found no changes in activin A, or follistatin content in either placenta, amnion or choriodecidua in association with labour. These results are broadly in agreement with a previous study (Keelan et al 1999) except that in that earlier study the authors found a modest increase in placental activin A with term labour. Furthermore, we have shown that there are no differences in either the localisation or staining intensity of activin or its receptors in placenta and fetal membranes collected after labour compared to those pre labour. These observations would support the conclusion, derived from activin A levels in the circulation reported earlier (chapters 3,4 and 6), that activin A does not play an acute role in labour.

The findings that levels of receptor IIA in placenta and chorion were lower than those of other receptors and very weak, by immunohistochemistry or Western hybridisation, in amnion are also broadly in agreement with a previous study of receptor mRNA levels (Petraglia et al 1994a). Together, these findings imply that receptor IIA is not involved in activin signal transduction in the amnion. However, in contrast to the *in situ* localisation of receptor mRNA to the amniotic epithelium (Petraglia *et al* 1994a) the study presented here could only detect very weak staining for activin receptor IIA protein in amniotic epithelium. Immunostaining for receptor types IA, IB and IIB was localised to the underlying mesenchyme and chorionic trophoblast cells and also to some epithelial cells. Interestingly, the amniotic mesenchyme is known to be an important source of intrauterine prostaglandins. Prostaglandins, key regulators of uterine activity, are synthesised from arachadonic acid by prostaglandin endoperoxide H synthase (PGHS) which is localised to amniotic epithelial cells and amniotic mesenchyme with maximal levels detected at term (Gibb and Sun, 1996, Hirst et al, 1995a, 1995b). The co-localisation of PGHS, especially PGHS II, the inducible form, with activin receptors in amniotic mesenchyme allows us to postulate a novel pathway involving activin A in the regulation of amniotic prostaglandins during late pregnancy and labour. Support for this hypothesis has been provided by Petraglia and co-workers (1993a), who showed that activin A stimulates the release of prostaglandin E_2 from a cultured amnion-derived cell line and by Keelan et al (2000b) who demonstrated that activin A can modulate PGE_2 production from amniotic explants. However, in contrast to PGHS II which is elevated after labour (Hirst et al, 1995b), activin A levels and activin receptor expression do not change with the onset of labour.

The relatively high levels of follistatin observed in the chorion may reflect an important anti-activin barrier protecting against the actions of activin, in a manner similar to the anti-prostaglandin barrier that is afforded by chorionic prostaglandin dehydrogenase (PGDH) (Challis & Olson, 1994). However, unlike PGDH which is down-regulated in association with labour, no changes in follistatin content was observed in association with labour. Nonetheless, it would be interesting to investigate chorionic concentrations of follistatin in early and mid pregnancy.

In conclusion, the content and localisation studies reported here do not support a direct role for activin in the initiation or regulation of labour but instead suggest that activin may play a role in early pregnancy trophoblast function, in late pregnancy placental perfusion and local regulation of uterotonic peptide release from fetal membranes, such as PGs. Further functional studies would be required to provide more direct evidence for these suggestions.

CHAPTER 8

MATERNAL SERUM ACTIVIN A AND FOLLISTATIN IN ASSOCIATION WITH SEVERE INTRA-UTERINE GROWTH RESTRICTION

8.1 INTRODUCTION

The studies described in chapter 5 have shown that maternal serum levels of activin A were significantly elevated in women undergoing an emergency Caesarean section for delay during first stage after spontaneous onset of labour. Although not reaching significance, levels of activin A were elevated concurrently in the umbilical arterial vessel of fetuses delivered by an emergency Caesarean section or by vaginal instrumental delivery due to delay during second stage of labour. These observations together with the hypoxic sheep-model studies described by Jenkin et al (2001), suggested that maternal serum activin A may be increased as a consequence of intra-uterine hypoxia. Indeed, the increasing levels of maternal serum activin A observed in late pregnancy (see chapter3, Fowler et al, 1998, O'Connor et al, 1999) may be a reflection of decreasing fetal oxygenation. In addition, in pregnancy pathologies associated with feto-placental oxygenation, such as pre-eclampsia, maternal serum levels of activin A have been shown to be significantly increased (Petraglia et al, 1995, Muttukrishna, 1997a, see chapter 9). We were therefore interested to investigate levels of activin A in pregnancies complicated by intra-uterine fetal growth restriction (IUGR) the most common cause of which is impaired placentation resulting in chronic feto-placental hypoxia and increased perinatal morbidity and morrality (Nicolaides et al, 1988, Alfirevic and Neilson, 1995).

The aim of the study described in this chapter was to describe the maternal serum profile of activin A in pregnancies complicated by severe IUGR.

8.2. MATERIALS AND METHODS

8.2.1 Patients

A total of 57 women with a singleton pregnancy were recruited to the study. In all cases, the women had been referred to the Maternal-Fetal-Medicine (MFM) Unit at Monash Medical Centre for assessment of fetal size because of a clinical diagnosis of small for gestation (SGA), based upon the measurement of the symphyseo-fundal height in the clinic. In each case fetal size was assessed on ultrasound using an ATL HDI 3000 ultrasound machine (Advanced Technology Laboratories, Dee Why, NSW, Australia) with a 3.5MHz or 4-2MHz curved array probe. These assessments were undertaken as part of routine clinical care by the medical and nursing staff of the MFM Unit. In each case, fetal growth restriction was diagnosed when estimated fetal weight was below the 10th percentile of the normal range for an Australian population (Guaran et al, 1994). The control group comprised 123 women with a normal healthy singleton pregnancy, as described in Study 3.1.2.1, Chapter 3. The study was approved by the Monash Medical Centre Human Research and Ethics Committee and all women gave informed consent.

8.2.2 Collection of blood samples

In each case, a venous blood sample was collected from the women attending the MFM Unit at the time a diagnosis of SGA was confirmed. The samples were immediately centrifuged at 3500 rpm for 15 minutes at 4 °C and the serum stored at – 20 °C.

8.2.3 Classification of SGA, IUGR

After delivery, the case-records of each women were reviewed independently by two MFM physicians (Associate Professor Euan Wallace and Dr Andrew Edwards), blinded to the activin and follistatin results. In this review, each pregnancy was classified as having either a normal constitutionally small fetus (small for gestational age, SGA), a fetus with intrauterine fetal growth restriction (IUGR) or a fetus with IUGR and in whom the mother had pre-eclampsia (PE-IUGR) based upon the clinical features of the mother and the antenatal fetal surveillance (eg evidence of feto-placental compromise such as umbilical artery absent or reversed end-diastolic flow, abnormal biophysical surveillance, or fall of fetal growth on serial ultrasound investigations).

8.2.4 ELISA for activin A and follistatin

The ELISA for the measurement of activin A and follistatin in serum has been described in section 2.2.1.3. and 2.2.1.4. respectively.

8.2.5 Statistical analysis

To allow for correction of gestational ages across gestation, all data were expressed as multiples of the normal median (MoM) for week of pregnancy. Differences between the groups were analysed using Students T-test after $\log_{10}$ transformation of the data. Significance was recognised when $P < 0.05$.

8.3 RESULTS

There were 82 normal singleton pregnancies sampled at 23 – 40 weeks' gestation as the control group (Group 1). Of the 57 women with a fetus confirmed with biometry below the 10^{th} percentile for gestation, 16 had a SGA fetus (group 2), 17 had a fetus with IUGR (group 3) and 24 women had a fetus with IUGR in association with maternal pre-eclampsia (group 4). The median gestation at diagnosis and delivery for each group was 37and 37, 36 and 37, 27 and 27 for groups 2, 3 and 4, respectively. The mean ± SEM birthweights for each group were 3402g ± 55g, 2447g ± 55g, 1736 g ± 180g and 985.5g ± 164g, respectively for groups 1, 2, 3 and 4. The median (95% confidence intervals, CI) multiple of the normal median (MoM) activin A and follistatin in the four are shown in figure 8.1 and table 8.1. The levels of activin A did not differ between controls and group 2 (SGA), (P = 0.63). However, levels of activin A were significantly elevated in the groups 3 (IUGR) and 4 (PE-IUGR), (P = 0.0027 and 0.0001, respectively) compared to gestation-matched controls. Activin A levels were significantly elevated in group 3 compared to the group 2 (P = 0.0027).

Levels of follistatin were significantly elevated in all three groups of women compared to normal controls (group 2, P = 0.001; group 3, P = 0.004; group 4, P = 0.003). Levels of follistatin were similar between the women with IUGR and the women with IUGR/PE (P = 0.54).

The predictive value of activin A as a marker of intra-uterine fetal compromise in IUGR is shown in table 8.2.

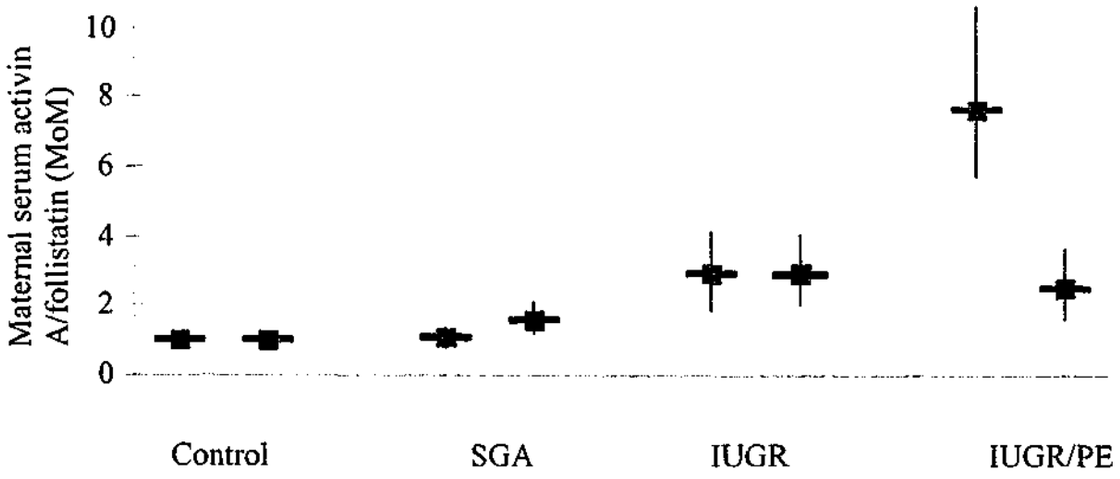


Figure 8.1 Levels of activin A (■) and follistatin (■) (MoMs and 95% confidence intervals) in normal pregnancies (control), SGA (group 2), IUGR (group 3) and IUGR/PE (group 4).

Table 8.1 Multiple of the medians (MoMs) and 95% confidence intervals (CI) in normal, healthy pregnancies (control, N=123), small-for-gestational-age fetuses (SGA, N=16)), intra-uterine growth restricted fetuses (IUGR, N=17) and fetuses with IUGR and maternal pre-eclampsia (PE, N=24).

	Control	**SGA**	**IUGR**	**IUGR/PE**
Activin A				
MoM	**1**	**1.12**	**3**	**7.7**
- 95%CI	0.91	0.72	1.84	5.73
+95%CI	1.11	1.39	4.11	10.62
Follistatin				
MoM	**1**	**1.6**	**2.91**	**2.5**
- 95%CI	0.95	1.17	2.04	1.65
+95%CI	1.04	2.13	4.07	3.63

Table 8.2 IUGR detection and screen positive rates for maternal serum activin A at various cut-off thresholds.

MoM	No (%) of SGA Pregnancies	No (%) of IUGR Pregnancies	No (%) of IUGR/PE Pregnancies
0.5	14(88)	17(100)	25(100)
1.0	8(50)	17(100)	25(100)
1.5	3(20)	17(100)	25(100)
2.0	1(7)	15 (88)	15(100)
2.5	0(0)	12 (71)	22 (88)
3.0	0(0)	9(53)	22 (88)
3.5	0(0)	9(53)	19 (76)
4.0	0(0)	8(47)	18 (72)
4.5	0(0)	7(41)	18 (72)

8.4 DISCUSSION

The evaluation of fetal well-being in a clinically small fetus confirmed by ultrasound is fundamental to the timely delivery of the fetus with IUGR who is at increased risk of perinatal loss and morbidity (Karsdorp, et al, 1994). Indeed, the use of umbilical artery Doppler ultrasonography in such pregnancies has been shown to significantly improve perinatal survival without increases in delivery intervention (Neilson and Alfirevic, Cochrane Reviews 2001). However, in the third trimester of pregnancy the Doppler umbilical artery flow velocity waveform may be normal even in pregnancies with IUGR and the management of the small ($<10^{th}$ percentile on ultrasound) fetus continues to involve frequent repetitive surveillance to allow discernment between SGA and IUGR pregnancies. Such management not only engenders parental anxiety it is also costly. The potential of a non-invasive marker that could differentiate between

the SGA and IUGR pregnancies at the time of presentation is therefore clearly considerable.

This is the first investigation of maternal serum activin A in pregnancies complicated by fetal SGA and IUGR. The study described here has shown that maternal serum activin A is significantly elevated in women with an IUGR fetus but not in women with an SGA fetus. This is potentially a clinically important finding since it is often difficult to distinguish between these two groups based on Doppler ultrasonography and or biophysical assessment alone at the time of presentation. Underlying causes of severe IUGR may include abnormalities of feto-placental exchange as a result of abnormal villous function and abnormalities of uteroplacental exchange due to incomplete trophoblast invasion, placental vascular anomalies, infarctions or vasculopathy (Harrington and Campbell, 1993). In severe cases complicated by absent- or reversed end-diastolic flow, the terminal villi, which are the primary sites of gas- and nutrient exchange, are reduced in volume (Jackson et al, 1995) or malformed (Krebs et al, 1996) leading to a reduction in the availability of gas and nutrients to the fetus. Such conditions are commonly associated with chronic fetal hypoxia. An association between abnormal placental vascular function and elevated maternal serum levels of activin A have previously been made in women with severe pre-eclampsia (Muttukrishna et al, 1997a, 2000a). Further, activin A has binding sites on the vascular endothelial cell surface and can inhibit the proliferation of vascular endothelial cells (McCarthy and Bicknell, 1993, 1994). Results of our studies described in chapter 7 have also shown that activin receptors are present on vascular endothelium of placental vessels at term. Taken together, these studies support a role for activin A in the regulation of vascular endothelium in the placenta. It is thus

feasible to suggest that activin A levels are altered when placental function is impaired, as can be the case in severely growth-restricted fetuses. During episodes of acute fetal hypoxia, such as those encountered during dysfunctional labour, levels of maternal serum and umbilical artery activin A are elevated (see results chapter 5). Chronic duration of hypoxia may thus be the underlying cause of the increased concentrations of activin A observed in the maternal circulation

Interestingly, the levels of follistatin were increased significantly in all groups, including the small-for-gestational age fetuses. However, the largest increase observed occurred in the group with severe IUGR and IUGR/PE. These results contrast with the findings of D'Antona et al (2000) who did not detect changes of maternal serum follistatin in women with pre-eclampsia. Follistatin was earlier shown to stimulate vascular endothelial cell growth and induce angiogenesis (Kozian et al, 1997). It is possible that in circumstances where placental vascular function is impaired, follistatin levels are elevated to stimulate compensatory vessel growth. Further studies investigating βA-subunit, activin receptor and follistatin expression and immunohistochemical localisation in placenta from severely growth-restricted fetuses, as well as activin A and follistatin levels in the umbilical arterial vessel of severely growth-restricted fetuses may provide further clues as to the regulation of activin and follistatin in cases where placental function is disturbed.

Larger scale studies are needed to confirm the preliminary data reported here but clearly these data offer the exciting possibility that maternal serum activin A may be able to direct fetal surveillance in pregnancies where the fetus is small.

CHAPTER 9

ACTIVIN A IN MATERNAL SERUM AND GESTATIONAL TISSUE FROM PRE-ECLAMPTIC PREGNANCIES

9.1 INTRODUCTION

In chapters 5 and 8 the potential link between *in utero* hypoxia and activin A in both maternal serum and umbilical arterial serum was discussed. As has been demonstrated, both compartments show increases in concentrations of activin A under acute or chronic hypoxic conditions during pregnancy or delivery. The possibility that the production of activin A is associated with reduced intervillous blood flow stimulated the investigation of the profile of activin A in women with severe pre-eclampsia, a condition characterised by abnormal trophoblast invasion leading to placental hypoxia and dysfunction (Conrad and Benyo, 1997). Previous studies have shown that in maternal serum, levels of activin A are significantly higher in pre-elamptic pregnancies than in gestation matched normal pregnancies (Muttukrishna et al, 1997a, 2000a, D'Antona et al, 2000). The underlying causes of this increase are not known. Pre-eclampsia is characterised by impaired renal function (Blackburn and Loper, 1992) and it has been suggested that the elevated levels of activin A are due to reduced clearance from the circulation. However, maternal serum levels of activin A are elevated well before the onset of renal involvement or hypertension (Muttukrishna et al, 2000a) arguing against impaired renal clearance as a major cause of the observed high levels of activin A and supporting the suggestion that activin A levels are elevated because of increased placental production. The aim of this study was to investigate not only maternal serum levels of activin A in women with severe pre-eclampsia, but also to measure activin A content in the gestational tissues, assess the

correlation between the tissue and serum levels of activin A and compare these with normal, gestation matched pregnancies.

9.2 MATERIALS AND METHODS

9.2.1 Patients

Severe pre-eclampsia was diagnosed according to International Society for the Study of Hypertension in Pregnancy criteria (Perry and Beevers, 1994). Twenty-three women with severe pre-eclampsia were recruited either preterm (29 – 35 weeks' gestation, n = 11) or at term (37 - 40 weeks' gestation, n = 12). The control group consisted of healthy women with a normal, singleton pregnancy delivered at term (37 – 40 weeks' gestation, n = 62). Placenta and fetal membranes were collected from eight preterm pre-eclamptic (PT PE) women, from ten term pre-eclamptic (T PE) women and from ten normal vaginal deliveries at term (T C). There was no obvious intra-uterine growth restriction in any of the pre-eclamptic pregnancies, as defined by birth weight >10th percentile for gestation (Guaran et al, 1994). All women were delivered by Caesarean section prior to labour. In all cases the gestational age was calculated either by the last certain menstrual period of by an early pregnancy ultrasound. The study was approved by the Monash Medical Centre Human Research and Ethics Committee and informed, written consent was obtained from each patient.

9.2.2 Collection of maternal serum

The collection, processing and storage of maternal blood samples has been described in chapter 3, section 3.1.3.

9.2.3 Collection of gestational tissue

The collection, processing and storage of placental, amnion and choriodecidual samples has been described in chapter 7, section 7.2.3.

9.2.4 ELISA of activin A

The measurement of activin A in serum and tissue samples has been described in chapter 2, section 2.2.1.3.

9.2.5 Protein assay

The assay used to measure total protein concentration in the tissue samples has been described in chapter 6, section 6.2.3.

9.2.6 Statistical analysis

To correct for gestational related differences, maternal serum activin A levels were expressed as multiples of the normal median (MoM) derived from normal, healthy controls. Differences in serum and tissue activin A content were assessed by the Mann-Whitney U test. Correlation between serum and tissue activin A concentrations were evaluated using Pearson's linear correlation coefficient (n = 18). Significance was attributed when $P<0.05$.

9.3 RESULTS

The levels of activin A in maternal serum from women with severe pre-eclampsia (n = 23) at preterm and term are shown in figure 9.1. Levels of activin A were significantly elevated in these women compared to normal, healthy women. The median (95% CI) activin A MoMs were 3.5 (2.6-6.1) vs 1.0 (0.9-1.1) for pre-eclamptic and control

women respectively (P < 0.0001). Levels of activin A in pre-eclamptic serum collected preterm (29 – 34 weeks') were not statistically different from those collected from women at term (37 – 40 weeks', P = 0.46).

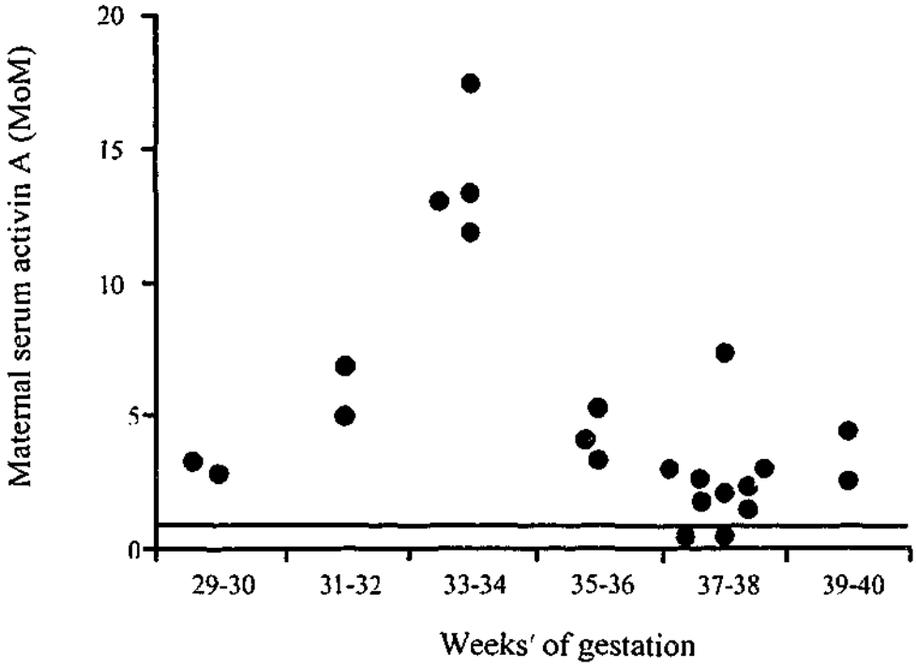


Figure 9.1. Total activin A levels in maternal serum of women with pre-eclampsia. Levels are expressed as multiples of the median (MoM). The horizontal line represents the median value of controls.

Activin A was detected in all placental, amnion and choriodecidual tissue lysates (Figure 9.2). The concentration of activin A in placental samples was significantly higher in PT PE and T PE tissue compared to that observed in control tissue (P = 0.001 and 0.0005 respectively). Among the pre-eclamptic tissue samples, samples from PT PE placentae were significantly elevated compared to T PE tissue (P = 0.002). In the amnion, however, the content of activin A was significantly reduced in both pre-eclamptic groups compared to control (PT PE: P = 0.005, T PE: P = 0.014)

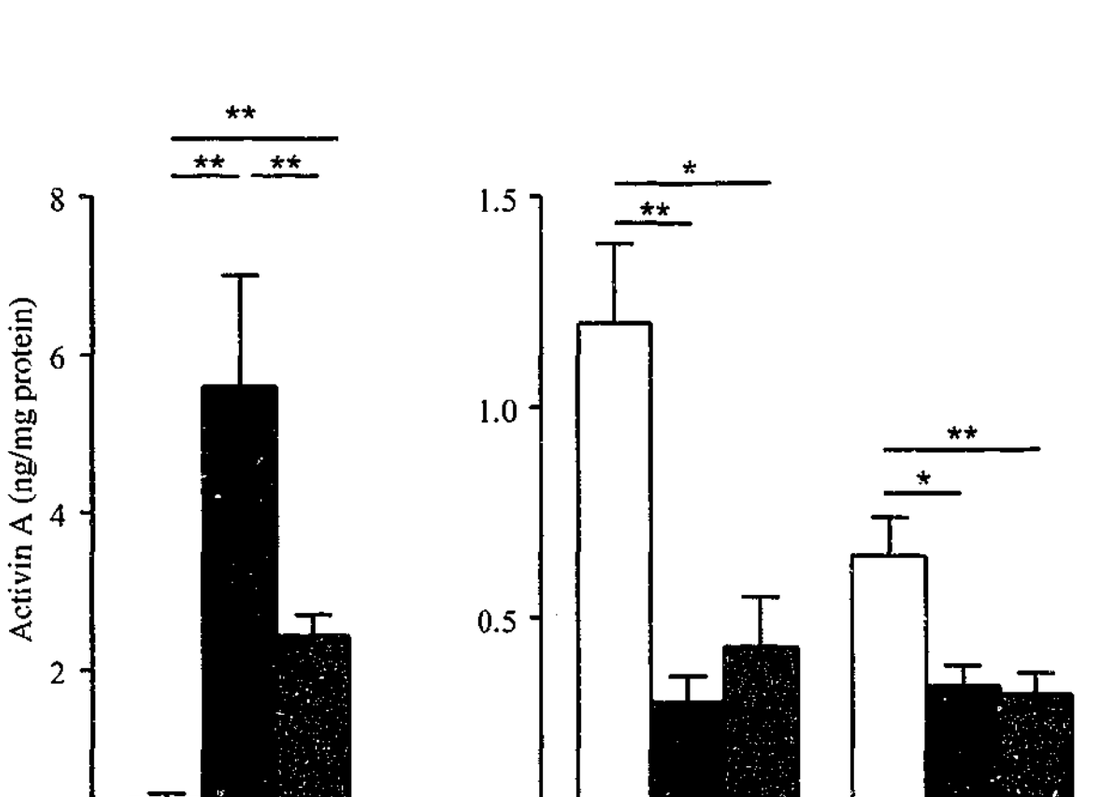


Figure 9.2 Mean ± SEM total activin A concentrations in gestational tissue from healthy term controls (T C) □ , pre-term pre-eclamptic (PT PE) ■ , and term pre-eclamptic women (T PE) ■ .

with no significant difference between PT PE and T PE amnion. Activin A concentrations in choriodecidua were lower in T PE compared to controls (P = 0.009) with no significant differences between the PT PE and the T PE groups.

In 18 pre-eclamptic pregnancies, paired samples of maternal serum and tissue samples were analysed (PT PE, N = 8; T PE, N = 10) and a significant positive correlation was detected between serum and placental activin A content (r = 0.84, P = 0.01, figure 9.3).

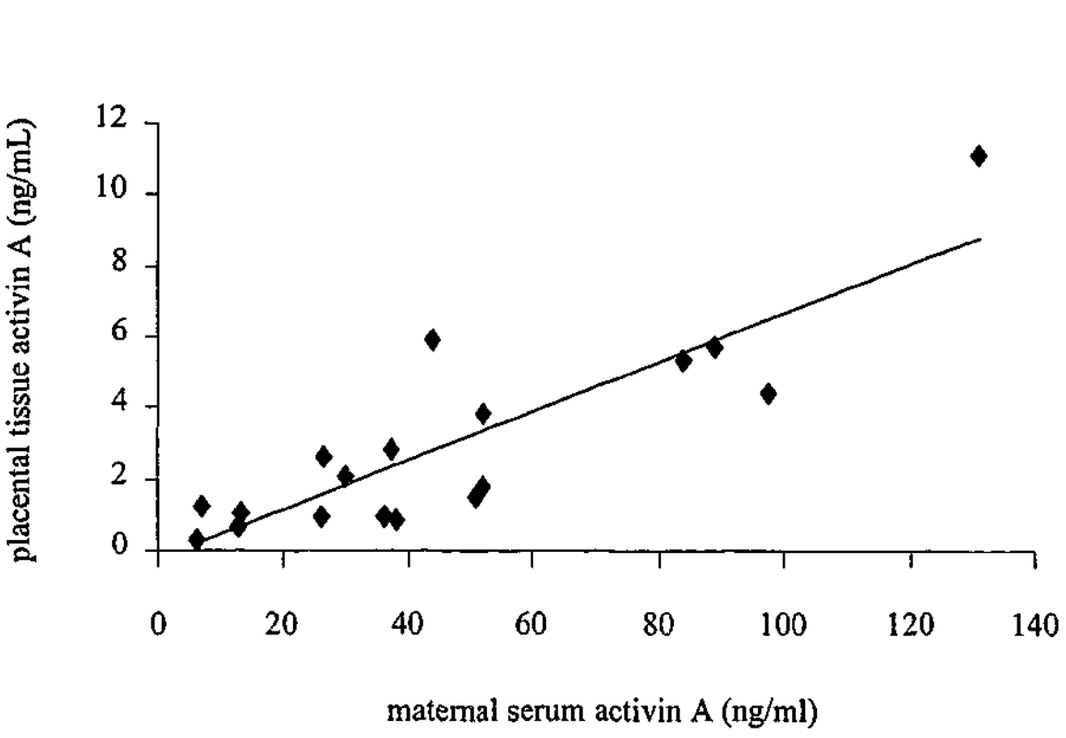


Figure 9.3 Correlation between matched activin A levels in pre-eclamptic maternal serum and placental tissue samples. r = 0.84, P = 0.01.

9.4. DISCUSSION

Several recent studies have shown that the levels of maternal serum activin A are significantly elevated in women with severe pre-eclampsia, whereas levels of follistatin in the same compartment remain unchanged (Muttukrishna et al, 1997, 2000a, Silver et al, 1999, D'Antona et al, 2000). The placenta is generally acknowledged as the major source of these proteins in pregnancy (de Kretser et al, 1994, Wallace and Healy, 1996). The results of this study support and extend earlier work by confirming significantly increased maternal serum activin A levels in pre-eclampsia. In both preterm and term pre-eclampsia, activin A protein content was significantly elevated placental samples compared to normal pregnancy. In addition, this is the first study to demonstrate that the serum levels are highly correlated with

the placental tissue content of activin A suggesting that increased levels of activin A observed in the maternal circulation of pre-eclamptic women are due to increased placental production.

This study was undertaken because, apart from increased placental production, several other mechanisms may contribute to the increased maternal serum concentrations of activin A during pre-eclampsia. Impaired renal function which results in reduced clearance has been proposed as the underlying cause of the elevated levels of activin A observed in serum of pre-eclamptic women. However, maternal serum levels are elevated well before the onset of renal involvement (Muttukrishna et al, 2000a) precluding a renal involvement as the cause.

While levels of activin A were elevated in placental tissue in pre-eclampsia, interestingly, in amnion, levels were significantly lower than in healthy pregnancy suggesting that the production of activin A in these tissues is differentially regulated. A similar finding for inhibin A and activin A was reported in placenta and fetal membranes in cases of Down's syndrome (Wallace et al, 1997, Wallace et al, 1999). It is nevertheless surprising to find reduced levels of activin A in the amnion of pre-eclampsia because levels of TNFα are elevated in the amniotic fluid of these women (Wang and Walsh, 1996) and TNFα and IL-1β increase activin A synthesis in placental, amnion and choriodecidual explant cultures (Keelan et al, 1998, 2000a). Perhaps this finding argues against increased TNFα as the mechansism behind the increased levels of activin A. In addition, Petraglia et al (1997b) and Keelan et al (1999a) did not detect significant increases in the levels of activin A in amnion and chorion from pregnancies complicated by *in utero* infection arguing against an inflammatory mechansim underlying the increased levels of activin A in pre-

eclampsia and the decreased concentrations detected in the amnion. Further, activin A inhibits TNFα production in cultures of term placental explants (Keelan et al, 2000b). In the context of pre-eclampsia, both TNFα and IL-1β are increased in placentae (Conrad and Benyo, 1997). Consistent with this as a possible mechanism underlying increased activin A concentrations, levels of maternal serum activin A are elevated prior disease manifestation (Muttukrishna et al, 2000a) and it would be important to explore whether this was also evident for TNFα and IL-1β.

The balance between the vasoconstrictor thromboxane (TX) and the vasodilator prostacyclin (PGI_2) is altered in pre-eclampsia (Walsh, 1985, Walsh and Wang, 1995, Blackburn and Loper, 1992) in favour of excess TX levels and reduced PGI_2 levels. This imbalance is thought to underlie the reduction in uteroplacental and placental blood flow that leads to tissue hypoxaemia and damage to the placenta. As has been discussed in chapters 5 and 8, decreased oxygenation may result in increased production of activin A in gestational tissues. Activin A was shown to induce TX synthetic activity in murine erythroleukemia cells (Yamashita et al, 1991) and in an murine macrophage cell line (Nusing et al, 1995) and it is possible that activin A could also stimulate TX in gestational tissues affecting inflammatory processes in haematopoietic systems. However, experiments investigating the relationship between activin A and these factors in normal pregnancy and pre-eclampsia are still outstanding.

Recently, our group has shown that activin receptors are localised in the endothelium of placental blood vessels in both normal and pre-eclamptic tissues with altered levels of receptor expression in women affected by this disease (Manuelpillai et al, 2001). It

is possible that in pre-eclamptic placentae, which are characterised by systemic endothelial damage, activin A is secreted as a result of localised utero-placental ischaemia and may be involved in the regulation of blood flow in the placenta and surrounding tissues. Indeed, previous studies have shown that activin A and follistatin can directly modulate vascular endothelial cell activity. McCarthy and Bicknell (1993) have demonstrated that activin A inhibits capillary endothelial cell growth *in vitro*, whereas follistatin can oppose these effects and induce angiogenesis (Kozian et al, 1997). There is further evidence showing that activin A can potentiate the proliferation and differentiation of erythroid progenitor cells *in vitro* (Broxmeyer et al, 1988, Yu et al, 1989, Shiozaki et al, 1998) and also induce an increase in the levels of red blood cells and haemoglobin under the influence of oestrogen (Schwall et al, 1989). These observations suggest that activin A may be released in situations which require actions to counteract the depressed levels of oxygen encountered in cases such as pre-eclampsia or IUGR. These actions may be processed via auto- and/or paracrine alterations at the receptor levels within the endothelium of placental vessels (Manuelpillai et al, 2001). However, it is unclear if activin A is released as a secondary factor produced as a result of reduced blood flow or released as a direct compensatory mechanism aimed at improving the disease state.

Clearly, the preliminary data presented in this chapter point to potentially important roles for activin A in normal and abnormal pregnancy, roles that merit addressing in future studies.

CHAPTER 10

GENERAL DISCUSSION

The aim of the work presented in this thesis was to investigate the roles of activin A during late pregnancy and parturition. Evidence that activin A may be physiologically important during pregnancy and delivery has come from small, cross-sectional and longitudinal studies measuring activin A in the maternal circulation (Muttukrishna et al, 1995, Woodruff et al, 1997, Fowler et al, 1998, O'Connor et al, 1999) and from *in vitro* experiments which demonstrated that activin A can regulate uterogenic factors such as prostaglandins, OT, CRH and ACTH, all of which in turn can stimulate contractions of the myometrium (Sawchenko et al, 1988, Plotsky et al, 1991, Petraglia et al, 1993a, Keelan et al, 2000b).

The results of the detailed studies debated in this thesis clearly demonstrate that levels of activin A in maternal serum, presumably reflecting placental output, rise during gestation with the major increase in late pregnancy until about three weeks prior to delivery and remain stable thereafter until they decline following delivery. The function and role of activin A during pregnancy and in particular during late pregnancy when levels surge, remains poorly understood. The studies in which activin βA-subunit was localised in first and second trimester and at term have shown that activin A staining is present in the syncytiotrophoblast throughout pregnancy and that the fetal membranes and the fetus appear to contribute to the activin A measured in maternal serum and amniotic fluid. Since the levels of activin A in the maternal circulation do not undergo changes with labour onset or labour progression in normal, healthy women it appears unlikely that activin A is a direct regulator of parturition. This is further supported by the studies into activin βA-subunit and activin receptor

protein in gestational tissues. It was shown that the expression of activin βA-subunit and activin receptor protein in gestational tissues before and after spontaneous labour do not change with labour onset. Furthermore, the mode of delivery did not influence the levels of activin A observed in the maternal peripheral circulation. No differences in the levels of activin A were recorded between women undergoing a spontaneous vaginal delivery, an instrumental delivery or an elective Caesarean section. It was further shown that in the human, in contrast to the rat, activin A does not have binding sites in the myometrial smooth muscle at term, either before or after onset of uterine contractions. These data suggest that if activin A does stimulate myometrial contractions, the pathways must involve other, secondary factors, possibly prostaglandins. Taken together, these studies provide considerable evidence against activin A as a direct modulator of human parturition.

As described above, the levels of maternal serum activin A were similar in all women regardless of mode of delivery with the exception of women undergoing an emergency Caesarean section. Circumstances requiring an emergency Caesarean section are commonly associated with extended periods of ineffective contractions during first stage of labour and failure to progress from first stage of labour into second stage. Such prolonged labour can, in some cases, impact on the well-being of the fetus, resulting in progressive fetal hypoxia and, in extreme cases acidaemia. Since the promoter region of the activin gene contains an hypoxia inducible response element (David Phillips, personal communication) it was proposed that fetal hypoxia, may stimulate the release activin A from either the fetus or the placenta, possibly with downstream stimulation of the fetal adrenal gland and subsequent release of cortisol

(Spencer et al, 1992, Mesiano et al, 1997). The resulting increased output of activin A by the fetus may thus be the underlying cause of the increased maternal serum levels observed in women requiring an emergency Caesarean delivery.

The association between activin A and placental oxygen supply may also explain the profile of maternal serum activin A observed during healthy pregnancies. It is well established that in late pregnancy, fetal venous pH declines, with concurrent increasing partial pressure of carbon dioxide (PCO_2) and decreasing partial pressure of oxygen (PO_2) (Gregg & Wiener, 1993). The decrease of oxygen available to the fetus at term may act as a trigger to stimulate biochemical pathways that eventually lead to the delivery of the fetus. These events may also play a part in premature onset of labour. We know that preterm infants are more likely than term infants to become significantly hypoxaemic during labour and the elevated levels of maternal serum and amniotic fluid activin A in association with preterm delivery observed by Petraglia et al (1997b) may reflect an hypoxaemic response of the fetus and the gestational tissues.

Our findings into umbilical arterial levels of activin A at delivery have provided further support for this hypothesis. Umbilical artery levels of activin A were elevated at delivery after an emergency Caesarean section (delay during first stage) or instrumental delivery (delay during second stage) compared to gestation-matched healthy fetuses delivered normally or by elective Caesarean section. Larger sample sizes will be required to establish if the difference in levels of activin A observed in the umbilical artery is real and cases of severe fetal distress at delivery will have to be

investigated to assess the potential link between fetal well-being during labour and maternal serum activin A levels.

Nevertheless, evidence for the findings in the human is provided by the chronically cannulated sheep model, where it has indeed been shown recently that fetal activin A levels rise dramatically during acute feto-placental hypoxia and return to normal levels within 30 minutes of return to normoxia (Jenkin et al, 2001). Importantly, the increases of activin A were followed closely by significant increases of feto-placental PGE_2, a vasodilator, and also later by increases of $PGF_{2\alpha}$, a potent uterotonic factor (Challis and Olson, 1994), raising the possiblity that in the sheep model, rising activin A levels act as a trigger to the release of PGE_2 and $PGF_{2\alpha}$ from placenta, the fetal membranes and decidua. The elevated concentrations of PGs in turn may regulate placental blood flow and also stimulate the smooth muscle of the uterus to contract thereby initiating labour.

It is important to note that in the studies reported here none of the infants delivered by an emergency Caesarean section or instrumental vaginal delivery showed evidence of overt compromise at delivery as indicated by Apgar scores and acid-base assessment of the umbilical arterial blood. At this stage, without further evidence of fetal distress, the association between elevated levels of activin A and possible hypoxic episodes during labour remain speculative. Nevertheless, this proposed hypothesis is consistent with earlier observations showing that in the adult, mRNA for activin βA-subunit is upregulated during hypoxic ischaemia in the rat brain (Lai et al, 1996) and in the infant rat, an hypoxic ischaemic insult results in the release of activin A from the brain (Wu et al, 1999). Activin A has also been shown to be an important

neuroprotectant *in vivo* (Tetter et al, 2000). Increased concentrations of activin A in the brain provoke the release of OT, CRH and ACTH (Sawchenko et al, 1988, Plotsky et al, 1991, Petraglia et al, 1997a, 1997d), all of which can act on the fetal "stress" response and on the myometrium to induce contractions and initiate parturition. An hypoxic event may thus evoke the stimuli necessary to "rescue" a stressed fetus from its environment by setting in motion the processes of parturition. The preliminary data demonstrating a correlation between umbilical artery activin A and umbilical artery pH support this hypothesis.

The studies into acute hypoxia during labour were extended to include investigations into fetuses that are chronically hypoxic. Such fetuses are commonly affected by severe intra-uterine growth restriction, believed to be caused, in the majority of cases, by placental insufficiency (Nicolaides et al, 1989). Depending on the specific causes, these fetuses can suffer increased morbidity and mortality and require intense fetal monitoring to optimise outcomes (Gregg and Wiener, 1993).

Assessment of the well-being of the small fetus can be difficult. Essentially, it is important to distinguish between the growth restricted, "stressed" fetus and the small-for-dates, but otherwise healthy, normal fetus. While this is sometimes possible at the time of presentation, it requires serial ultrasound examinations. The results of the studies reported in chapter 8 have shown that in maternal serum, levels of activin A are elevated three-fold in association with IUGR when compared to normally grown or healthy, small-for-dates, gestation matched fetuses. Importantly, the 95% confidence intervals between these two groups did not overlap and the positive

predictive value was 71% (at MoM of 2.5) making activin A a potential useful clinical marker of fetal well-being in late pregnancy.

Another pathological condition of pregnancy associated with chronic feto-placental hypoxia is pre-eclampsia. Pre-eclampsia is characterised by trophoblast dysfunction thought to be due to abnormal invasion at the time of implantation and early placentation. This results in deficient maternal blood supply to the placenta rendering the placenta ischaemic. In previous studies it was shown that maternal serum levels of activin A and inhibin A are significantly elevated in women with severe pre-eclampsia (Muttukrishna et al, 1997, 2000a, Silver et al, 1999, D'Antona et al, 2000). It is not known why activin A and inhibin A are elevated in these women, but other hormones synthesised by the syncytiotrophoblast, such as CRH and hCG are also elevated (Ashour et al, 1997, Perkins et al, 1995). One underlying cause may be that activin A is released from the placenta as a result of the severe systemic inflammatory response associated with pre-eclampsia (Redman et al, 1999). Inflammatory cells, such as monocytes have been shown to produce activin A (Yu and Dolter, 1997) and Muttukrishna et al (2000b) were able to demonstrate that monocytes in the peripheral circulation and placental cytokines TNFα and ILβ induce the release of activin A in women with pre-eclampsia (Conrad and Benyo, 1997). The levels of activin A measured in the peripheral circulation may therefore be not only a product of the placenta but also of inflammatory cells. However, based upon the studies related in chapter 8, it was hypothesised that the elevated levels activin A observed in maternal serum of severely pre-eclamptic women, are primarily due to an ischaemic placental environment. Reduced levels of oxygen and the accompanying release of imflammatory cytokines probably stimulate increased production of activin A by the

syncytiotrophoblast. The strong correlation between maternal serum and placental concentrations of activin A in pre-eclampsia indicates, however, that inflammatory cells are not likely to be a major source of activin A in the maternal peripheral circulation.

Interestingly, as described in chapter 7, in contrast to first and second trimester, at term activin A receptors are expressed in vascular endothelium of placental blood vessels. So while the syncytiotrophoblast continues to release activin A during the entire duration of pregnancy, at term, rather than act in an autocrine fashion on syncytiotrophoblast cells, it appears likely that activin A may act in a paracrine fashion by targeting the placental vascular endothelial cells. This novel finding is supported by earlier work demonstrating direct effects of activin A on vascular endothelial cell activity in other vessels, such as adrenal capillary, pulmonary artery and aortic and umbilical vein endothelial cells (McCarthy and Bicknell, 1993, Kozian et al, 1997). *In vitro*, activin A was shown to inhibit the proliferation of vascular endothelial cells, while follistatin counteracts these actions and can stimulate angiogenesis. Although the functions of activin A in placental or myometrial vessels are not yet known, it may be possible that towards the end of pregnancy, activin A may modulate vascular endothelial cell proliferation and placental blood flow in the placenta and the uterus either directly or via other mediators such as PGs. Recent *in vitro* experiments have demonstrated that the release of activin A into the medium of cultured first trimester and term trophoblast cells is downregulated during hypoxia (Manuelpillai et al, 2000). However, these studies were carried out on trophoblast cells and since activin receptors appear to be present on the endothelium of placental vessels, future experiments will need to investigate the association between hypoxia

and activin A in cultured placental vascular endothelial cells since they appear to be the predominant cells targeted by activin A, at least at term. Comparisons to hypoxic responses between vascular endothelial cells from normal placentae and from placentae affected by severe IUGR or PE may provide further clarifications to the underlying mechanisms of activin A synthesis in these disease states.

Based on the results of the studies reported in this thesis it is suggested that activin A levels are increased by the placenta and the fetus when the uterine oxygen levels drop below threshold levels. Activin A may therefore act as a compensatory mechanisms to counteract a low oxygen environment, either by modulating blood flow to the tissues or, in extreme cases, by stimulating fetal and gestational tissue to release uterotonic factors which stimulate contractions of the myometrium and deliver the fetus from a hostile environment. The proposed pathways of events are shown in figure 10.1

The results of the work presented here suggest that activin A is not directly involved in the initiation of normal, uneventful parturition in the human. The functions of activin A, as well as follistatin during pregnancy remain unclear. However, our studies have provided considerable support to link the production of activin A by gestational tissues to acute and chronic intra-uterine hypoxic events

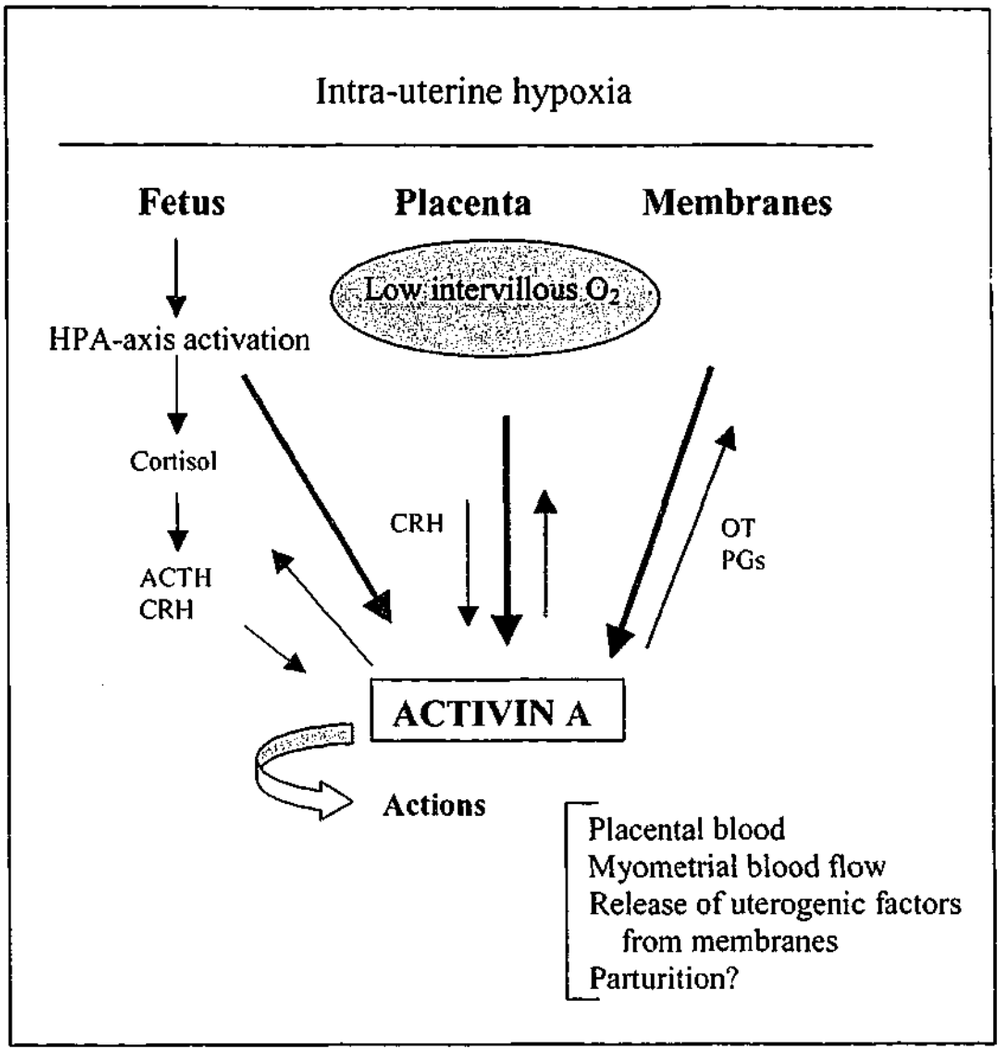


Figure 10.1. Proposed model for the pathways of activin A during intra-uterine hypoxia

We propose that activin A is secreted by the fetus and the placenta as a consequence of a low oxygen environment. Oxygen deprivation which can occur during pregnancy, as seen in cases of severe IUGR or pre-eclampsia, results in the release of stress-factors such as ACTH and CRH from the fetus and the gestational tissues which in turn stimulate activin A secretion from these compartments and the subsequent production of uterotonic factors, in particular the prostaglandins capable of initiating contractions which serve to expel the fetus from its hostile environment. Critical to this sequence of events appears to be the oxygen availability in the intervillous space. The possibility that activin A can counteract this scenario by targeting placental

vessels and modulating the intervillous blood flow by modulating the release of prostaglandins is suggested here but remains to be established in further experiments.

In conclusion, the results of our studies suggest that activin A may be useful as a non-invasive marker of fetal well-being during mid to late pregnancy and in the future may be able to assist in the detection and management of compromised fetuses.

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