THE ROLE OF INTEGRINS IN UTERINE SMOOTH MUSCLE CONTRACTION DURING PREGNANCY AND LABOUR

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The Role of Integrins in Uterine Smooth Muscle Contraction during Pregnancy and Labour

By

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Abstract

Preterm labour is the leading cause of perinatal morbidity and mortality in North America and its prevention is essential to improve pregnancy outcomes. Unfortunately, the basic biochemical and molecular mechanisms of myometrial function during pregnancy and labour remain poorly understood making it difficult to anticipate and prevent preterm labour. Focal adhesions are important to properly anchor growing cells to their ECM and it is becoming appreciated that they are reorganized in myometrial cells during pregnancy and labour to facilitate the significant uterine growth seen during this time. Integrins are an important component of focal adhesions. In this thesis, I initially investigated the gestation profile of ITGA5, ITGA1, ITGA3 and ITGB1 in rat myometrium during pregnancy and labour. The gene expression of all 4 integrins was elevated during late pregnancy and the apparent accumulation of all 4 integrin proteins at myocyte membranes may contribute to the cell-ECM interactions required for the development of a mechanical syncytium and the coordinated contractions of labour. Delaying labour, through the administration of progesterone, maintained ITGA5 gene and protein expression. Inducing preterm labour, by administering RU486, a progesterone receptor antagonist, resulted in a slight increase in only ITGA5 expression and its accumulation at myocyte membranes. In a unilateral pregnant rat model, ITGA5 gene and protein expression was increased with gravidity.

In further experiments, we examined the effect of ITGA5 silencing on fibronectin deposition and secretion by uterine smooth muscle cells, which is important to aid in the development of a fibronectin matrix. We found that silencing *Itga5* gene expression

resulted in a decrease in ITGA5 protein expression and decreased fibronectin deposition and secretion in human myometrial cells. Our results support the idea that ITGA5B1 plays an integral part in FN fibrillogenesis in uterine myometrial cells.

We suggest that these series of experiments support the concept that a mechanical syncytium may develop in the myometrium to facilitate the coordinated contractions of labour. In addition, ITGA5B1 may be required for fibronectin matrix assembly in the myometrium and initiate the process of cellular cohesion, which is dependent on correct cytoskeletal filament organization, focal adhesion formation, cell-cell and cell-ECM interactions and these processes may be components of myometrial activation aiding in the development of a mechanical syncytium.

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List of Abbreviations

ABP	actin binding protein
ACTH	adrenocorticotrophic hormone
ADMIDAS	adjacent to metal-ion-dependent adhesion site
ANOVA	analysis of variance
AP	activator protein
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AVP	arginine vasopressin
BM	basement membrane
$[Ca^{2+}]_i$	intracellular calcium
Ca^{2+}	calcium
CaM	calmodulin
CaMKII	Calcium-calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CAP	contration associated protein
CBG	corticosteroid-binding globulin
CBP	cAMP-responsive element binding protein
cDNA	complementary DNA
cGMP	cyclic guanine monophosphate
Cl^-	cloride
CLIP	corticotrophin-like intermediate lobe peptide
CNN	calponin protein
cPLA2	cytosolic phospholipase A2
CR	calreticulin
CRE	cAMP response element
CRH	corticotrophin releasing hormone
CRH-BP	CRH-binding protein
CSQ	calsequestrin
Ct	cycle threshold
Cx26	connexin26
Cx43	connexin43
DEPC	diethylpyrocarbonate
DHEAS	dehydroepiandrosterone sulfate
DNA	deoxyribonucleic acid
E2	estrogen
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	estrogen receptor
Erk	extracellular signal regulated kinase
EtOH	ethanol
FAK	focal adhesion kinase

FERM	four-point-one, ezrin, radixin, moesin
Fn	fibronectin mRNA
FN	fibronectin protein
FP	prostaglandin F2α receptor
GEF	guanine exchange factor
GPI	glycosylphosphatidylinositol
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GSK	glycogen synthase kinase
GTPase	guanine triphospatase
h	hours
H^+	hydrogen
HPA	hypothalamic-pituitary-adrenal
HRP	horseradish peroxidase
HSD	hydroxysteroid dehydrogenase
IAP	integrin associated protein
IL	interleukin
ILK	integrin-linked kinase
IP	PGI receptor
IP3	inositol 1,4,5-triphosphate
IP3R	IP3 receptor
Itga1	integrin α1 mRNA
ITGA1	integrin α1 protein
Itga3	integrin α3 mRNA
ITGA3	integrin α3 protein
Itga5	integrin α5 mRNA
ITGA5	integrin α5 protein
Itgb1	integrin β1 mRNA
ITGB1	integrin β1 protein
ITGA5B1	integrin α5β1
JNK	jun N-terminal kinase
K^+	potassium
K_{ATP}	ATP-sensitive potassium channel
K_{ca}	calcium-activated potassium channel
K_{ir}	inward rectifier potassium channel
K_v	voltage-gated potassium channel
LC20	20kDa myosin light chain
LDL	low density lipoprotein
LDV	leucine-aspartic acid-valine
MAPK	mitogen activated protein kinase
MEKK	mitogen-activated protein/Erk kinase
Mg^{2+}	magnesium
MIDAS	metal-ion-dependent adhesion site
min	minutes

MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MSH	melanocyte stimulating hormone
Na^+	sodium
NCoR	nuclear receptor corepressor
NFκB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
NP	nonpregnant
OT	oxytocin
OTR	oxytocin receptor
P4	progesterone
PAK	p21 associated kinase
PBS	phosphate buffered saline
PBT	phosphate buffered saline with tween 20
PC	prohormone convertase
PCR	polymerase chain reaction
PGDH	prostaglandin H dehyrodgenase
PGE2	prostaglandin E2
PGES	prostaglandin E synthase
PGF2α	prostaglandin F2α
PGHS	prostaglandin H synthase
PI3K	phosphatidylinositol 3 kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
POMC	proopiomelanocortin
PP	postpartum
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PTD	phosphotyrosine binding domain
PVN	paraventricular nucleus
PXN	paxillin
RGD	arginine-glycine-asparatic acid
Rn18S	18S RNA mRNA
RNA	ribonucleic acid
ROD	relative optical density
ROK	rho-associated kinase
RT	reverse transcriptase
RT-PCR	real time polymerase chain reaction

RyR	ryanodine receptor
SDS	sodium dodecyl sulfate
SE	standard error
SEM	standard error of means
SERCA	sarcoplasmic/endoplasmic reticulum calcium-ATPase
SH	Shc homology
SMC	smooth muscle cell
SMRT	silencing media for retinoid and thyroid hormone receptors
SP-1	specific protein 1
sPLA2	secretory phospholipase A2
SR	sarcoplasmic reticulum
SRC	steroid hormone receptor co-activator
SSC	sodium chloride sodium citrate
SSPE	sodium chloride-sodium phosphate-EDTA
TBS	tris buffered saline
TBST	tris buffered saline with tween 20
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor β
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor
uPAR	urokinase plasminogen activator receptor
VEGF	vascular endothelial growth factor
vWFA	von Willebrand factor type A
ZBF	zinc buffered fixative

CHAPTER 1

INTRODUCTION

1.1 Preterm Birth

Parturition is the process by which the fetus is expelled from the uterus and results from a complex interaction between maternal, placental and fetal factors. It requires that the uterus shift from a state of relative quiescence during pregnancy to one of coordinated contractility and the cervix must dilate to allow for the passage of the fetus. In addition, maturation of the fetal organs necessary for extrauterine life and maternal changes required for lactation must have occurred.

Preterm birth results from asynchrony between the labour process and fetal maturation and occurs before 37 weeks of gestation [Challis et al., 2002]. In North America, preterm birth complicates 7-12% of all births and is the leading cause of perinatal morbidity and mortality, accounting for 75-85% of perinatal deaths in Canada [Martin et al., 2006; Public Health Agency of Canada, 1999]. Preterm infants have an increased risk of cerebral palsy, blindness and deafness, as well as neurological and pulmonary disorders [Lye et al., 2001]. In addition, preterm infants have a higher incidence for later educational underachievement, due to learning disabilities and behavioral problems [Marlow et al., 2007; Sullivan and McGrath, 2003].

Approximately 50% of preterm births are of unknown cause, 30% are associated with an underlying infectious process, and 20% are due to elective preterm labour for the benefit of either the fetus or mother [Tucker et al., 1991; Challis et al., 2002; Gibb &

Challis, 2002]. Preterm labour can be induced in pregnancies where there are considerable complications such as preeclampsia or fetal distress. Premature rupture of the membranes can also lead to preterm labour with or without infection. Primary prevention consists of promoting health and avoiding or reducing risk factors. Factors that may have an association with preterm labour include very low body mass index, low gestational weight gain and repeated abortions, while risk factors such as smoking, drug use and stress are more easily controlled [Goffinet, 2005; Reedy, 2007]. However, many preterm births occur in women who have no identifiable risk factors. Despite research that has made advances in understanding the factors involved in the regulation of term and preterm labour, the rates of preterm labour have remained relatively unchanged since the mid-1980's [Challis et al., 2002; Gibb and Challis, 2002]. There are many drugs available to suppress uterine contractions (i.e. tocolytics), however, most are not universally effective and may have serious side effects for either the mother or the fetus.

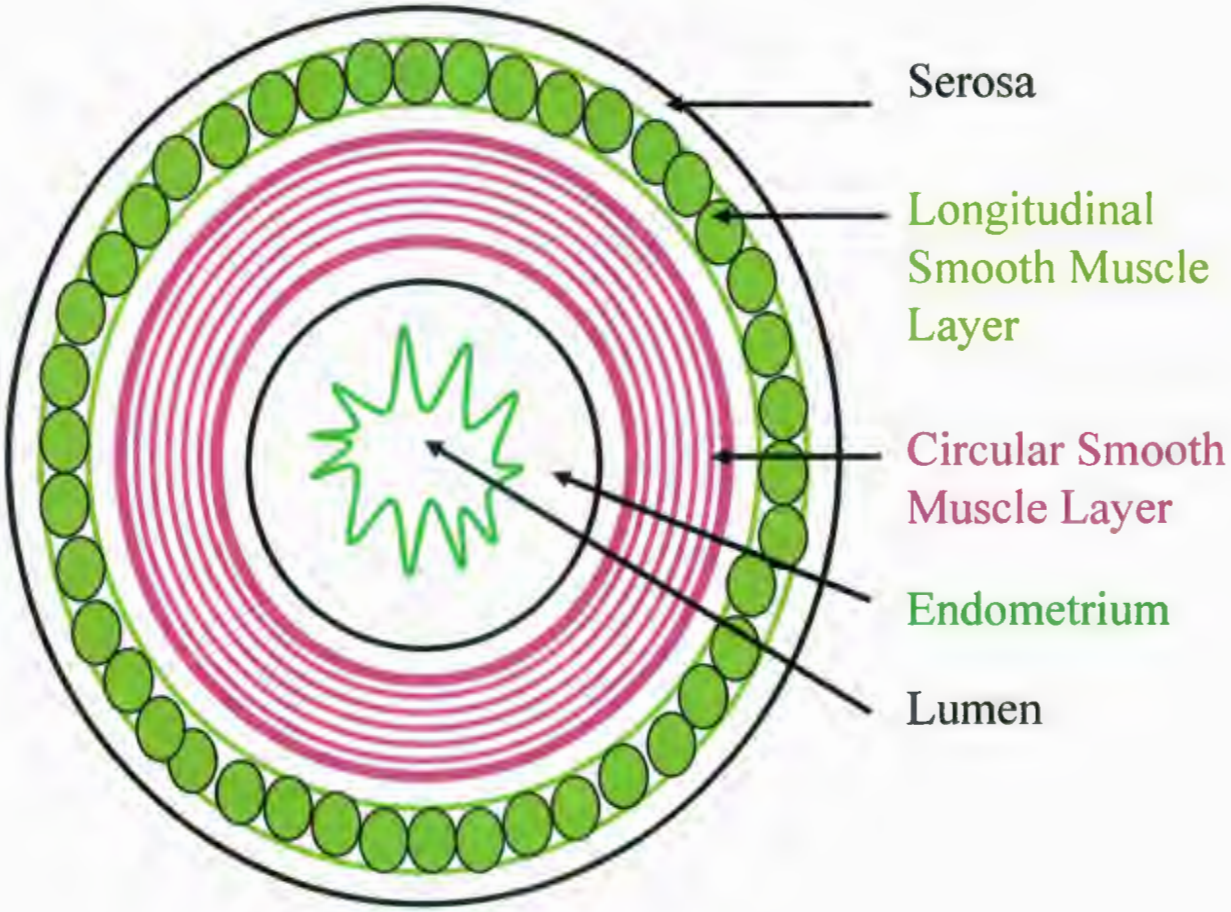
The prevention of preterm birth is vital to improve pregnancy outcomes; however the basic biochemical and molecular mechanisms of myometrial function during pregnancy and labour still remain poorly understood. It is hoped that studies to advance the understanding of the mechanisms involved in labour both at term and preterm will provide information that can be used to better predict those patients in preterm labour who will deliver. Further research to aid in the complete understanding of human parturition is required before effective diagnostic indicators or treatments for preterm labour can be created and utilized in clinical practice.

1.2 Uterine Smooth Muscle (Myometrium)

The majority of our knowledge of the physiology of parturition has been gained by research in only a few species, specifically the sheep, pig, rat, rabbit, some non-human primates and the human [reviewed by Young, 2001]. Sheep may be a preferred species to work with, in part due to the similarities of parturition to human parturition. These similarities include typically having 1 or 2 lambs with birth weights similar to human babies and the ewe placenta produces the majority of progesterone after it develops as in humans. However, there are several limitations to working with sheep, a significant one being the high cost of purchase and maintenance and the large space that they require. Therefore, rats are commonly used because of cheaper costs, easier surgical manipulation, and the breadth of knowledge now available on rat physiology during pregnancy.

As typical with animals with a bicornuate uterus, the rat uterus consists of 4 distinct layers; the serosa, outer longitudinal smooth muscle layer, vascular plexus, inner circular smooth muscle layer and the endometrium [Figure 1.1; Shynlova et al., 2005]. The two muscle layers have different origins and temporal patterns of development: the circular muscle layer develops first from the middle layer of mesenchyme in the urogenital ridge, near the Mullerian duct, and the longitudinal layer originates from mesenchyme of the subperimetrial zone of the ridge (Brody and Cunha, 1989). Thus, the two layers have different pharmacological and physiological characteristics during pregnancy [Kawarabayashi and Osa, 1976; Chow and Marshall, 1981; Tomiyasu et al., 1988; Mlynarczyk *et al.*, 2003]. For example, throughout gestation the magnitude and sensitivity of the contracile response to oxytocin is always lower in the circular layer;

Figure 1.1 Cartoon of a cross section of one horn of the rat uterus. The lumen of the uterus is surrounded by the endometrium, myometrium and the serosa. The myometrium is made up of 2 distinct layers, the longitudinal smooth muscle layer, which runs the length of the horn and circular smooth muscle layer, which sounds the circumference of the horn. Between the two muscle layers is a thin vascular plexus.


Serosa
Longitudinal Smooth Muscle Layer
Circular Smooth Muscle Layer
Endometrium
Lumen

however, at term the sensitivity of both layers is similar [Tuross et al., 1987]. In addition, studies have shown a co-ordination between the two smooth muscle layers near term that was not observed during early to mid gestation [Osa and Katase, 1975; Tomiyasu et al., 1988]. Specifically, these authors demonstrated that electrical stimulation of one muscle layer of the rat uterus near term generated action potentials in the other muscle layer. The human myometrium is made up of smooth muscle, though it does not have defined layers as in the rat. The endometrium lines the uterine lumen and a loose tissue, called the perimetrium, surrounds the myometrium (similar to the serosa in the rat).

1.3 Mechanisms of Myometrial Contraction

1.3.1 Structure of the Contractile Apparatus

Individual smooth muscle cells contain both a contractile and a cytoskeletal domain. The contractile domain consists of actin "thin" filaments, actin-associated proteins and myosin "thick" filaments, while the cytoskeletal domain consists of non-muscle actin and intermediate filaments that assist in maintaining cell integrity [Tessier *et al.*, 2003]. The myosin filament is a large polypeptide protein, composed of two heavy chains, each having two light chains. Each heavy chain dimer contains a slightly elongated globular head at the N-terminus. The myosin head is connected to a long α-helical coiled tail that aggregates to form the rod-like backbone of the thick filament. The myosin head contains the functional motor domain and an actin binding site, and produces the adenosine triphosphatase (ATPase) activity. The actin filament is made up of globular actin molecules that polymerizes into long, thin filaments (F-actin). A number of proteins are bound to actin such as tropomyosin, caldesmon and calponin

[Horowitz *et al.*, 1996]. Other proteins, such as filamin (also called actin binding protein (ABP)) [Janmey *et al.*, 1990], the calponin-like protein SM22 [Lees-Miller *et al.*, 1987], myosin light chain kinase [Lin *et al.*, 1997] and heat shock protein 27 [Larsen *et al.*, 1997], may also be tightly bound to actin. Actin filaments are more abundant than myosin filaments, are densely packed and run in parallel to the longitudinal axis of the cell. In vertebrates, there are 6 different isoforms of actin and smooth muscle cells typically contain a mixture of muscle (α and γ) and cytoplasmic (β and γ) isoforms [North *et al.*, 1994]. While all three isoforms are expressed in myometrial cells, α-actin was found to be highly expressed throughout gestation and γ-actin demonstrated an increased expression and altered localization towards term [Shynlova *et al.*, 2005].

In electron micrographs, bundles of actin can be seen penetrating the inner surface of dense plaques of the smooth muscle plasma membrane [Ashton *et al.*, 1975; Small, 1985]. Dense plaques mediate the transmission of force between the contractile apparatus and either the extracellular matrix (ECM) or adjacent cells. Dense plaques are thought to be analogous to focal adhesions of cultured cells [Burridge and Chrzanowska, 1996; Gerthoffer and Gunst, 2001]. Electron dense areas can also be seen within the cell cytoplasm that are referred to as dense bodies. Actin filaments appear to be associated with these dense bodies, as does the actin cross linking protein, α-actinin [Geiger *et al.*, 1981; Fay *et al.*, 1983; Small, 1985].

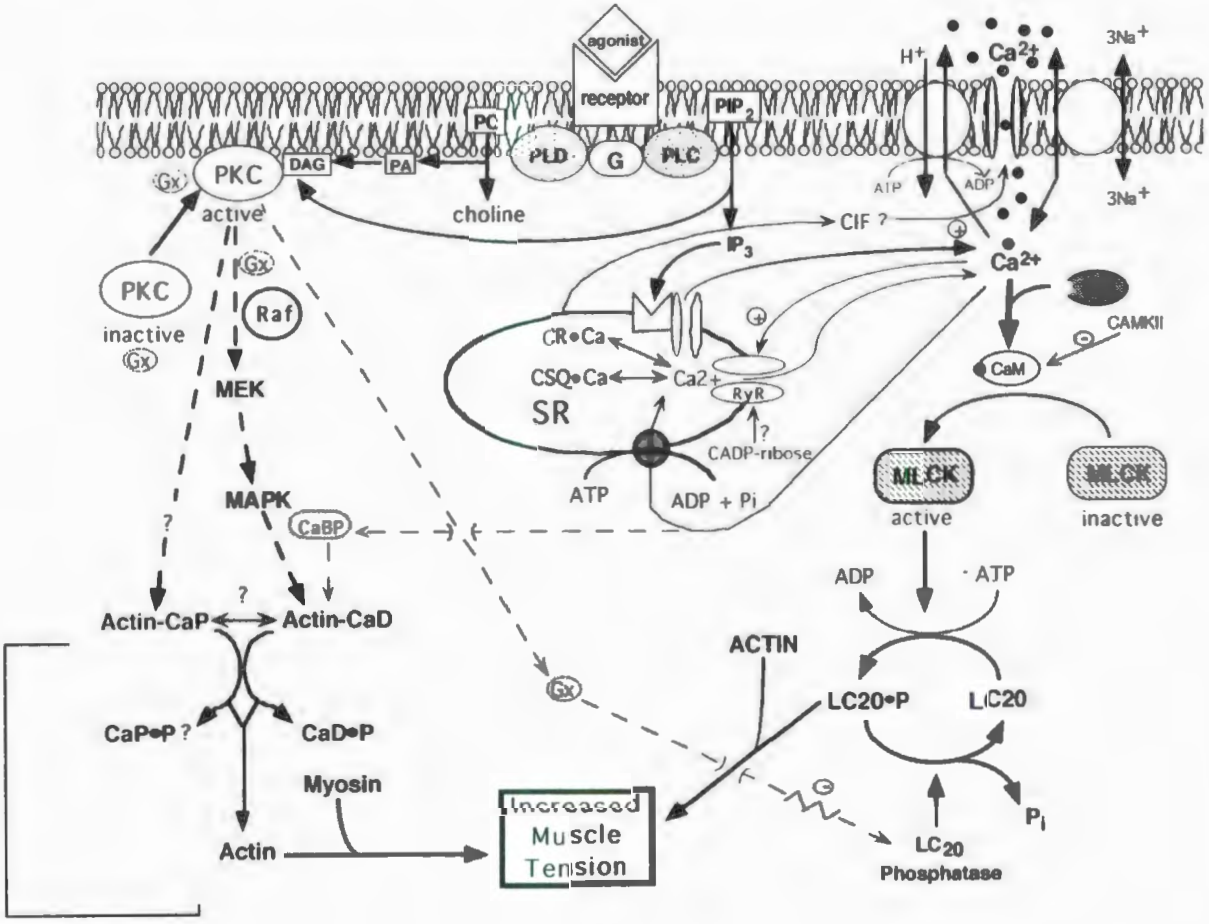
Contraction of the myometrium at term or preterm requires conformational changes in actin and myosin (cross-bridge cycling), allowing them to slide over each other, which results in a shortening of the myocyte and conversion of the chemical

energy from ATP into mechanical energy (i.e. contraction). Interestingly, smooth muscle is capable of generating more force per cross-sectional area than striated muscle, even though smooth muscle cells contain about one-fifth the myosin content of striated muscle [Murphy *et al.*, 1974]. Myosin filaments are longer in smooth muscle resulting in a greater number of cross-bridges and thus a greater number of contractile units which would result in a greater unitary force [Ashton *et al.*, 1975; Warshaw *et al.*, 1987].

Conventional models of force transmission in smooth muscle have shown the contractile filaments obliquely oriented relative to the longitudinal axis of the cell, however, this model is based on isolated cells which, when contracted, typically shorten in a corkscrew-like manner [Fisher and Bagby, 1977; Warshaw et al., 1987]. This model is currently under scrutiny because isolated cells have lost their connections to other cells and the ECM and it is doubtful whether the corkscrew like shortening would occur in an intact muscle bundle. Kuo and Seow (2004) have recently suggested that transmission of the force generated in smooth muscle cells is a result of the cells in a tissue bundle working as a mechanical syncytium. They found that the orientation of the contractile filaments lie parallel to the longitudinal axis of the cell and the axis of force transmission in the muscle bundle, allowing for uniform force transmission to be achieved.

A major factor in contractility of any smooth muscle is the level of intracellular free calcium ($[Ca^{2+}]_i$) and an increase in it results in the activation of calmodulin (CaM)-dependent myosin light chain kinase (MLCK) (Figure 1.2). Calmodulin binds four Ca^{2+} and Ca^{2+}-calmodulin interacts with MLCK to induce a conformational change in MLCK which results in phosphorylation of the myosin light chains. MLCK is inactivated by

Figure 1.2 Signal transduction mechanisms in vascular smooth muscle. G, heterotrimeric GTP-binding protein; PLC, phospholipase C; PIP2, phosphatidylinositol 4, 5-bisphosphate; PC, phosphatidylcholine; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; SR, sarcoplasmic reticulum; MLCK, myosin light chain kinase; PKC, protein kinase C; CaD, caldesmon; CaP, calponin; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; Gx, small GTP-binding protein; CaBP, calcium-binding protein; CR, calreticulum; CSQ, calsequestrin; PA, phosphatidic acid; CaM, calmodulin; RyR, ryanodine receptor; LC20, 20-kDa myosin light chain; CaMKII, Ca2+/calmodulin protein kinase II. Dashed lines indicate pathways that may require kinases or cofactors not yet defined. *Copyright 1996, Physiological Reviews.* Reprinted with permission to include copyright material (Appendix 1) from Horowitz *et al.*, Physiological Reviews. 1996; 76(4): 867-1003.


agonist
receptor
PC
PIP2
PLD
G
PLC
DAG
PA
choline
PKC
active
Gx
PKC
inactive
Raf
MEK
MAPK
CaBP
?
Actin-CaP
Actin-CaD
CaP•P ?
CaD•P
Myosin
Actin
Increased
Muscle
Tension
H+
Ca2+
3Na+
ATP
ADP
CIF ?
IP3
Ca2+
CAMKII
CaM
CR•Ca
CSQ•Ca
Ca2+
RyR
SR
CADP-ribose
ATP
ADP + Pi
MLCK
active
MLCK
inactive
ADP
ATP
ACTIN
LC20•P
LC20
Pi
LC20
Phosphatase

phosphorylation via cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and protein kinase C (PKC). The affinity of MLCK for Ca^{2+}-calmodulin is also reduced by phosphorylation of MLCK, predominately by Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII), but also by Rac/Cdc42-associated kinase (p21-associated kinase (PAK)) [Word, 1995; Sanders *et al.*, 1999]. Myosin light chain phosphatase (MLCP) dephosphorylates myosin and is regulated positively by cGMP and negatively by PKC and rhoA via rho-associated kinase (ROK) [Savineau and Marthan, 1997].

The inhibitory functions of the regulatory proteins caldesmon and calponin, associated with the thin filaments, are removed as a result of phosphorylation of these proteins [Adam *et al.*, 1989; Winder and Walsh, 1990]. Caldesmon is phosphorylated by mitogen-activated protein kinase (MAPK) and PAK, while calponin is phosphorylated by PKC and CaMKII [Savineau and Marthan, 1997; Foster *et al.*, 2000].

1.3.2 Regulation of Intracellular Free Calcium Concentrations

Intracellular free Ca^{2+} is a central second messenger in smooth muscle contraction and its regulation is essential [Wray *et al.*, 2001]. During contraction, smooth muscle $[Ca^{2+}]_i$ increases either as a consequence of an influx of Ca^{2+} from the extracellular space or a release of Ca^{2+} from intracellular stores [Horowitz *et al.*, 1996]. Influx across the plasma membrane occurs via ion channels or exchangers, while release from intracellular stores proceeds via inositol 1,4,5-trisphosphate (IP_3)-regulated channels or by Ca^{2+}-induced Ca^{2+} release via ryanodine receptors-regulated channels [Tribe, 2001]. During relaxation there is a decrease in $[Ca^{2+}]_i$ via the membrane Ca^{2+}-ATPase or the Na^+/Ca^{2+} exchange pump or via the Ca^{2+}-ATPase on the sarcoplasmic reticulum (SR) membrane.

Calcium influx into the cell via ion channels can be either Ca^{2+} dependent or independent, as well as voltage dependent or independent. The only voltage-dependent Ca^{2+} channels that have been found in smooth muscle cells are the transient (T)- and long lasting (L)-type channels [McDonald *et al.*, 1994]. L-type channels are activated (change from the closed to open state) by membrane depolarization, while deactivation is caused by repolarization and hyperpolarization. Intracellular free calcium reversibly inhibits L-type channel-mediated Ca^{2+} influx and phorbol esters can enhance this influx in some smooth muscle types [Ohya *et al.*, 1988; Vivaudou *et al.*, 1988]. Studies have suggested that the number of voltage-dependent L-type channels increase during pregnancy. Mironneau *et al.* (1993) found an increase in Ca^{2+} channel density per cell with gestation up to day 18 in the rat. T-type channels are found in relatively high density in spontaneously active smooth muscle types and they are activated at very low negative membrane potential, thus it is thought that they are associated with the generation of action potentials. It has been reported that T-type currents in pregnant human myometrial cells exhibit an increased sensitivity to magnesium than L-type currents in the same cells [Young *et al.*, 1993].

The plasma membrane contains both Na^+/Ca^{2+} exchange pumps and Ca^{2+}-ATPases. Movement of Ca^{2+} via the Na^+/Ca^{2+} exchange pump is driven by the gradient for Na^+, with 3 Na^+ exchanged for 1 Ca^{2+} [reviewed in Blaustein and Lederer, 1999]. The pump has been shown to be important for both Ca^{2+} influx and efflux, representing contraction and relaxation, respectively. The Ca^{2+}-ATPase is a Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase exchanging Ca^{2+} for H^+. Ca^{2+}-ATPase activity is regulated by

calmodulin as well as phosphorylation and requires the hydrolysis of ATP to extrude Ca^{2+} against its' gradient into the extracellular space. PKA-mediated phosphorylation has been reported to activate the pump, however this phosphorylation is inhibited by calmodulin [Strehler, 1991].

The SR has two types of channels, IP_3 and ryanodine receptors (IP_3R and RyR, respectively), as well as a Ca^{2+}-ATPase pump. IP_3 receptors are located in the membrane of the SR and isoforms IP_3R type I, II, and III have been detected in myometrial smooth muscle [Morgan *et al.*, 1996]. IP_3 binding is required to open the receptors and is modulated by Ca^{2+}. Lower Ca^{2+} concentrations within the cell cytoplasm promote channel opening and higher Ca^{2+} concentrations inhibits channel function [Thrower *et al.*, 2001]. Upon G protein-coupled receptor stimulation on the plasma membrane, phospholipase C hydrolyzes phosphatidyl inositol to IP_3 and diacylglycerol. IP_3 binds to its receptor on the SR membrane, releasing SR Ca^{2+} into the cytoplasm. RyRs can also release Ca^{2+} into the cytoplasm, however they do so by the binding of Ca^{2+} to the cytoplasmic side of the channel. RyRs exhibit a similar sensitivity to Ca^{2+} as IP_3 does; maximal activation at low Ca^{2+} concentrations and channel closure at higher Ca^{2+} concentrations [Bezprozvanny *et al.*, 1991]. Ca^{2+} uptake into the SR from the cytoplasm occurs via Ca^{2+}-ATPase pumps (Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)), which drive Ca^{2+} against its gradient through the hydrolysis of ATP. This Ca^{2+}-ATPase is also stimulated by Ca^{2+} and Mg^{2+} dependent pathways.

Relaxation of smooth muscle is mediated by hyperpolarization of the plasma membrane, reducing the entry of Ca^{2+} and by removal of Ca^{2+} from the cytoplasm

[Reviewed in Buxton, 2004]. It is well established that K^+ channels aid in hyperpolarizing the cell. Five major types of K^+ channels have been identified as modulators of smooth muscle membrane potential; voltage-gated (K_v), large conductance Ca^{2+}-activated (K_{ca}), small conductance Ca^{2+}-activated, inward rectifier (Kir) and ATP-sensitive (K_{ATP}) K^+ channels. A number of K^+ channel types have been described in the myometrium and more than one type has been detected in individual myometrial cells. Calcium-activated K^+ channels (K_{ca}), also called maxi-K channels, are large voltage-dependent channels. Blockage of K_{ca} channels depolarizes human myometrial cells and is associated with an increase in $[Ca^{2+}]_i$ [Anwer *et al.*, 1993]. K_{ca} channels are activated by β-adrenergic agents and other uterine relaxants, most of which elevate cAMP [Tritthart *et al.*, 1991; Anwer *et al.*, 1992]. K_{ATP} channels are thought to be voltage-dependent and inhibited by cytoplasmic ATP and thus activated with ATP depletion during hypoxia or metabolic inhibition. These channels have been implicated in myometrial function but their concentration during pregnancy and labour have not been measured directly.

Stretch activated ion channels, the open time of which is increased upon stretch or deformation due to pressure or shear stress, are still poorly understood, but are thought to gate Cl^- or Na^+. These stretch channels are thought to be activated upon depolarization, resulting in activation of voltage-dependent calcium channels, increases in $[Ca^{2+}]_i$ and contraction [Welsh *et al.*, 2000; Slish *et al.*, 2002]. In colonic and pulmonary artery smooth muscle, the mechanism mediating the activation of these stretch-activated Cl^-

channels is suggested to involve PKC, as PKC inhibitors increase and PKC activators reduce current through these channels [Dick *et al.*, 1998; Zhong *et al.*, 2002].

1.3.3 Coordination of Contractile Activity

Coordination of contractions among cells is achieved by coupling responses of individual cells to their neighboring cells. This coupling is accomplished chemically and/or electrically. In myometrial cells, electrical coupling is achieved where low resistance bridges physically connect individual cells and enable the transfer of electrical signals carried by ions or second messengers. Gap junctions are thought to provide these low resistance bridges between neighboring cells [Gilula *et al.*, 1972; Loewenstein, 1981]. Gap junctions are channels composed of proteins called connexins, which connect the interiors of two cells and allow molecules up to 1000Da to pass through them. Connexins are arranged into hexameric hemichannels, which become aligned between cells to form an interconnecting pore [Risek *et al.*, 1990]. Hundreds of pores are arranged to form a gap junction plaque.

Studies have found that during most of pregnancy, gap junctions are either undetectable or present in very low numbers [Garfield, 1985; Garfield, 1988]. With the onset of labour, there is an increase in the number and size of the gap junctions, associated with an increase in electrical conductivity and the development of spontaneous, well coordinated contractions [Miller *et al.*, 1989]. Within the myometrium, the major gap junction-forming connexin protein has been identified as connexin-43 (Cx-43). The expression of Cx-43 is barely detectable until late pregnancy and is at its highest level during delivery in the rat [Lye *et al.*, 1993]. In contrast,

connexin-26 (Cx-26) expression in the rat increased midgestation, was at its highest between days 19 and 21 and decreased before labour [Orsino *et al.*, 1996]. In the human myometrium, Cx-43 mRNA expression was significantly elevated towards term and a further significant increase was observed with labour [Chow and Lye, 1994]. Regulation of gap junction formation is complex. Adminstration of progesterone to pregnant rats abolished the increase in Cx-43 mRNA at term, while functional progesterone withdrawal, via a progesterone receptor antagonist RU486, significantly elevated Cx-43 mRNA expression during mid-pregnancy, when Cx-43 transcripts are normally low [Petrocelli and Lye, 1993]. In contrast, progesterone maintained Cx-26 mRNA expression when it normally would have decreased and RU486 treatment prevented the normal increased in mRNA expression seen midgestation in the rat [Orinso *et al.*, 1996]. In addition, administration of estradiol to nonpregnant rats significantly increased Cx-43 mRNA [Petrocelli and Lye, 1993]. Mechanical stretch has also been reported to increase Cx-43 mRNA expression in the rat myometrium but it failed to increase Cx-26 mRNA expression [Ou *et al.*, 1997]. Interestingly, the expression of Cx-43 mRNA was not increased in the myometrium of women with multiple fetuses compared to singleton pregnancies [Lyall *et al.*, 2002].

1.4 Phases of Parturition

1.4.1 Pregnancy: Phase 0 of Parturition

During pregnancy the uterus undergoes significant morphological changes, including increases in size, weight and capacity. These changes are caused by 2 mechanisms: an increase in cell number (hyperplasia) and an increase in cell size

(hypertrophy). Hyperplasia predominately occurs in the first half of pregnancy, while hypertrophy predominately occurs in the second half in the rat [Shynlova et al., 2006]. In the human nonpregnant state, the uterus weighs 40 to 70 g and only has a capacity of 10 ml; while at term, it weighs 1100 to 1200 g and has a capacity of 5 L. Cell size also increases in length from 50 to 500 μm and in width from 5 to 15 μm in the human uterus [Monga and Sanborn, 2004]. Hypertrophy and hyperplasia are associated with an increase in cell protein synthesis, extracellular matrix protein synthesis, an increase in cell organelles and transitions in contractile protein content and organization [Gabella, 1990].

Pregnancy is a period of relative uterine quiescence, where low-frequency, low-amplitude contractions occur that are associated with episodes of increased electromyographic activity lasting 2 to 5 minutes [Gibb *et al.*, 2006]. These contractions are commonly referred to as Braxton Hicks contractions in women and are poorly coordinated thus making the uterus unresponsive to uterotonins [Lye, 1997; Harding *et al.*, 1982]. Uterine quiescence is due to several effectors which act in either a paracrine or endocrine fashion to inhibit contractions and it is believed that withdrawal of one or more of these effectors may lead to delivery [reviewed by Buston, 2004; Bernal, 2007]. They include progesterone (P4), prostacyclin and nitric oxide (NO) which all act via different pathways but lead to the same end result – inhibition of intracellular calcium release and phosphorylation of MLCK, which is required for shortening of the myofilaments [Challis, 2001; Gibb and Challis, 2002; Gibb *et al*, 2006].

Progesterone's role in pregnancy maintenance was first demonstrated when the removal of the corpus luteum of gravid rabbits terminated pregnancy [Corner and Allen, 1929]. In a variety of mammals (eg, mice, rats and rabbits), the corpus luteum is the principal source of progesterone and a drop in circulating maternal progesterone levels (due to the regression of the corpus luteum) precedes the initiation of labour. This drop is due to a regression of the corpus luteum mediated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Prostaglandins are a group of hormone-like substances that are produced in various mammalian tissues, mediate a wide range of physiological functions, and are particularly important for late pregnancy and parturition. When the $PGF_{2\alpha}$ receptor (FP) is knocked out in mice, the corpus luteum remains intact and pups are not delivered at term [Sugimoto *et al.*, 1997]. In human pregnancy, the corpus luteum is the main source of progesterone up to the 7^{th} week of gestation; at which point, the luteo-placental shift occurs where the developing placenta becomes the principal site of progesterone synthesis [Zakar and Hertelendy, 2007].

1.4.2 Activation: Phase 1 of Parturition

The switch from phase 0 to phase 1 of pregnancy is termed *myometrial activation* and results from the expression of a cassette of proteins termed contraction-associated proteins (CAPs) as well as the removal of the inhibitory effectors that are present during uterine quiescence. CAPs include ion channels, agonist receptors (e.g., oxytocin (OT) receptor (OTR) and prostaglandin (PG) receptors for PGE_2 and $PGF_{2\alpha}$), and gap junctions. The increase in CAPs allow the muscle to generate coordinated high-

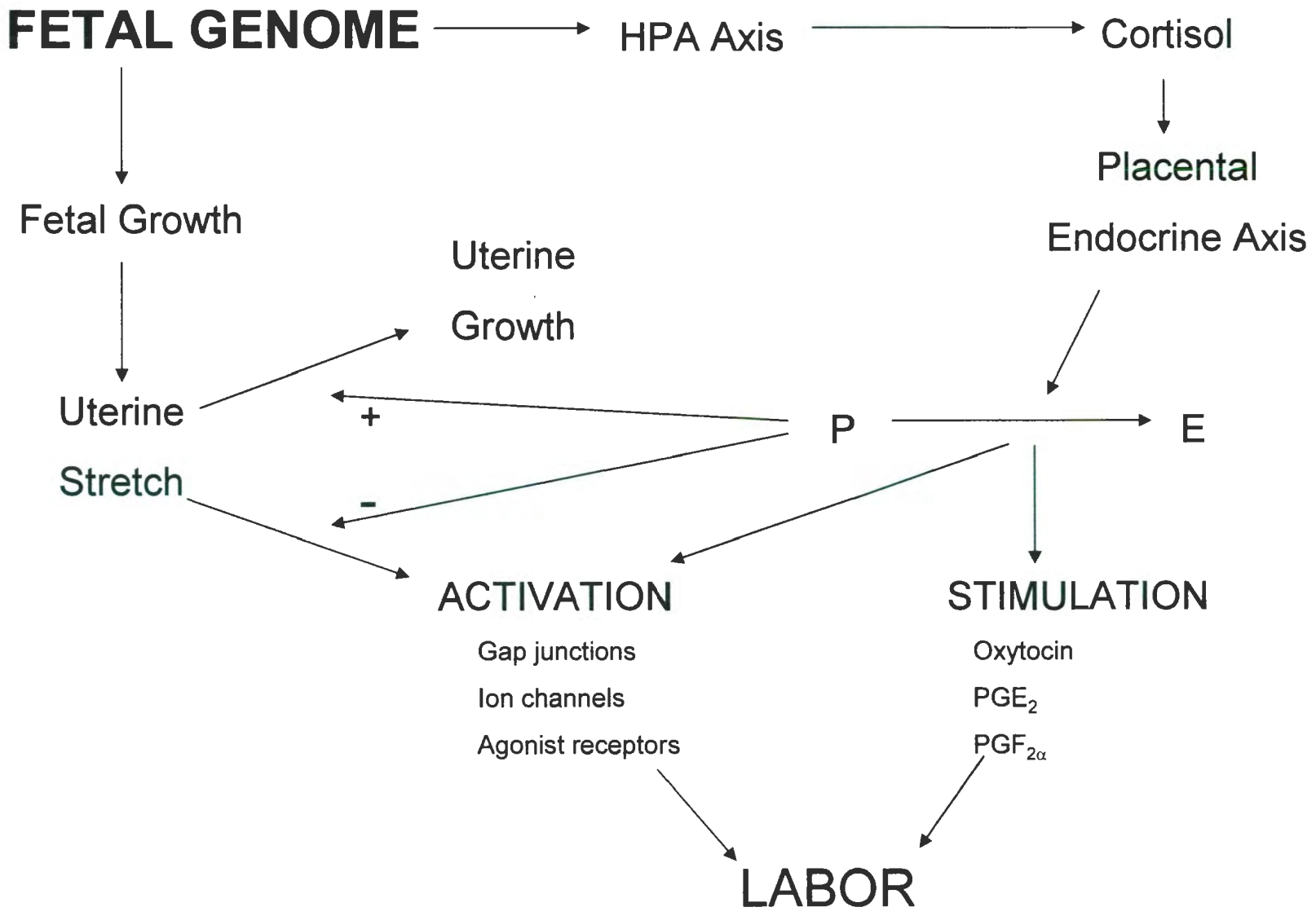
frequency, high amplitude contractions and enable it to respond to stimulation from uterotonins, such as OT, PGE_2, $PGF_{2\alpha}$.

Since the fetus must remain in utero to develop properly, it makes sense that the onset of labour is dictated by the fetus, specifically the fetal genome. This idea was first suggested after pregnant ewes ingested a plant called *Vibrio californicum* resulting in a prolonged gestation due to pituitary and adrenal abnormalities in the fetuses [Liggins, 1969a; Liggins *et al.*, 1973; Thorburn and Challis, 1979]. This observation lead investigators to examine the importance of the fetus in the timing of labour. Kitts *et al.* (1984; 1985) implanted sheep embryos from long gestion breeds into show gestation ewes and from short gestation breeds into long gestation ewes. It was observed that the length of gestation was appropriate for the fetus rather than the ewe.

The fetal genome exerts its effects through 2 different pathways, an endocrine/paracrine pathway and a fetal growth pathway (Figure 1.3). These pathways are not independent of each other, as changes in progesterone and estrogen modulate uterine growth and thus the ability of uterine stretch to exert effects on the expression of genes associated with activation [Reviewed in Gibb *et al.*, 2000]. Progesterone supports the stretch induced hypertrophy of the uterus during midgestation in combination with an increase in fetal size. As progesterone levels fall at term, there is an increase in uterine tension due to a decrease in uterine growth relative to fetal growth, thus causing an increase in CAP gene expression, resulting in myometrial activation [Lye *et al.*, 1998].

1.4.2.1 The Fetal Hypothalamic-Pituitary-Adrenal (HPA) Axis

Figure 1.3 The onset of labour is dictated by the fetal genome proceeding through either a fetal growth pathway with increases in uterine stretch or fetal endocrine pathway involving activation of the fetal HPA axis. These two arms are not independent because changes in progesterone and estrogen modulate the ability of uterine stretch to increase expressions of genes associated with myometrial activation. *Copyright 2000, The Endocrine Society.* Reprinted with copyright permission (Appendix 1) from Challis *et al.*, Endocrine Reviews 2000; 21: 514-550.


FETAL GENOME
HPA Axis
Cortisol
Placental
Endocrine Axis
Fetal Growth
Uterine
Growth
Uterine
Stretch
+
-
P
E
ACTIVATION
Gap junctions
Ion channels
Agonist receptors
STIMULATION
Oxytocin
PGE_2
$PGF_{2\alpha}$
LABOR

The importance of the fetal HPA axis in parturition was established more than 30 years ago [Reviewed in Liggins and Thorburn, 1994]. In vivo studies have found that fetal hypophysectomy, adrenalectomy, disruption of the pituitary stalk, and lesions of the paraventricular nucleus (PVN) resulted in prolonged gestational length [Gluckman *et al.*, 1991; McDonald *et al.*, 1992]. It was also found that infusion of adrenocorticotrophic hormone (ACTH) or the synthetic glucocorticoid dexamethasone in utero resulted in premature parturition [Thorburn *et al.*, 1991; McLaren *et al.*, 1996].

In response to an acute stimulus, such as hypoxia, psychological stress, or systemic hypotension, the fetal hypothalamus releases corticotrophin releasing hormone (CRH). CRH mRNA is localized to the parvocellular region of the PVN of the fetal hypothalamus [Palkovits *et al.*, 1983; Matthews *et al.*, 1991; Myers *et al.*, 1993]. CRH has been detected as early as day 60 of gestation and was found to increase, peaking at term and subsequently declining postpartum in sheep [Brieu *et al.*, 1989; Watabe *et al.*, 1991; Matthews and Challis, 1995]. The secretion of CRH from the PVN of the hypothalamus drives the anterior pituitary to synthesize proopiomelanocortin (POMC) and secrete ACTH [Gibb *et al.*, 2006]. In sheep, levels of POMC mRNA increased with gestation [Matthews *et al.*, 1994]. POMC is cleaved by prohormone convertase 1 (PC1) producing ACTH, ACTH is then cleaved by prohormone convertase 2 (PC2) to form corticotrophin-like intermediate lobe peptide (CLIP) and α-melanocyte-stimulating hormone (α-MSH). It was found that PC-1 mRNA and not PC-2 mRNA increased with gestational age in the anterior pituitary, although it did not change further at labour

[Holloway *et al.*, 2000]. This suggests that the ACTH produced is not increasingly cleaved to form CLIP and α-MSH.

Early in gestation, adrenal tissue responds to ACTH stimulation with increased cortisol output, which is decreased mid-gestation and increases again near term [Matthews and Challis, 1996; Wintour *et al.*, 1975]. Fetal cortisol acts on the sheep placenta to trigger the subsequent endocrine changes; progesterone output falls and estrogen concentrations increase. It was believed that the increase in fetal cortisol caused an increase in $P450_{C17}$ hydroxylase activity in the placenta [Ma *et al.*, 1999]. C21 steroids (pregnenolone, progesterone) reaching the placenta could then be metabolized to C19 steroids via $P450_{C17}$ hydroxylase and these C19 steroids could be converted to estrogen because the placenta expresses P450 aromatase. This model of increased $P450_{C17}$ hydroxylase activity is now thought to be in error [Challis et al., 2000]. A progressive increase of estrogens in maternal plasma and urine throughout the latter part of gestation has been observed, well before the terminal increase in placental $P450_{C17}$ hydroxylase activity [Dwyer and Robertson, 1980; Challis, 1971; Challis and Patrick, 1981]. In addition, placental output of PGE_2 and $PGF_{2\alpha}$ is not confined to the immediate 24-48 hours before spontaneous parturition, as it once was thought; PG production increases progressively through the last 20-25 days of gestation in the sheep. Prostaglandin H synthase (PGHS) enzymes are involved in the synthesis of PGs from their precursors and 2 isoforms exist, PGHS-2 and PGHS-1. It has been shown that expression and activity of PGHS-2 increases during the last 20 days of gestation before the last surge of estrogens [Boshier *et al.*, 1991; Rice *et al.*, 1995; Wimsatt and

Nathanielsz, 1995; Gibb *et al.*, 1996]. A study by Gyomorey *et al.* (2000), using placental tissues from sheep obtained at different intervals at term (before labour, and during early labour as well as active labour), demonstrated that the increased expression of PGHS-2 preceded the induction of the $P_{450}C_{17}$ hydroxylase enzyme. In contrast, no change in expression of the PGHS-1 enzyme has been found [Rice *et al.*, 1995; Gibb *et al.*, 1996].

It is well established that expression of PGE2 increases progressively over the last 15-20 of gestation in the fetal sheep circulation, matching closely to that of cortisol [Lye *et al.*, 1998]. Since PGE2 has been shown to stimulate $P450_{C17}$ hydroxylase expression in other tissues (ovine and bovine adrenal tissue) and cortisol stimulates PGHS-2 expression in human amnion cell cultures and chorion trophoblast cells, it has been proposed that fetal cortisol is responsible for increasing PGHS-2 expression and PGE2 acts to increase the expression of $P450_{C17}$ and is involved in the maturation of the HPA axis [Challis *et al.*, 2002]. Increased expression of $P450_{C17}$ results in an increase in estrogen production, which acts primarily on the myometrium to increase CAP expression and increase PGHS-2, resulting in increased PGF2α in the myometrium.

1.4.2.2 Role of Mechanical Stimuli

The ability of the myometrium to generate contractions is dependent upon both endocrine regulation and mechanical stretch of the uterine smooth muscle due to fetal growth. There are three phases of uterine growth during gestation: an initial phase of hyperplasia, controlled by endocrine factors and occurs during the first trimester; a second phase, where uterine growth mimics fetal growth during the second and third

trimesters; and a final phase where the uterus grows at a slower rate than the fetus, resulting in increased uterine wall stretch and tension [Lye *et al.*, 1998].

The resulting tension contributes to the triggering of increases in the expression of CAP genes, such as Cx-43 [Lye *et al.*, 1993; Chow and Lye, 1994], as well as the oxytocin receptor (OTR) [Ou *et al.*, 1998]. Mechanical stretch of the nonpregnant [Csapo *et al.*, 1965; Cullen and Harkness, 1968; Douglas *et al.*, 1988] and unilaterally pregnant [Goldspink and Douglas, 1988] rat uterus has been shown to promote growth and remodeling. Studies have found that expression of Cx-43 and OTR were increased in the gravid horn when compared to the non-gravid horn in unilaterally pregnant rats [Ou *et al.*, 1997; Ou *et al.*, 1998]. In addition, ECM proteins, such as fibronectin, elastin, laminin B2 and collagen IV, are positively regulated by stretch [Shynlova *et al.*, 2004].

The incidence of preterm birth is high in pregnancies complicated by increased uterine size due to multi-fetal pregnancies or polyhydramnios. The perinatal mortality rate of twins is 4-11 times higher than that of singletons and the morbidity of twins is higher due to a higher preterm birth rate. A study by Gardner *et al* (1995) for The March of Dimes found that 54% of twins were born premature compared with 9.6% singleton pregnancies. It would make sense that the growth of the fetus would have some influence on the timing of birth and explain the higher incidence of preterm birth in multifetal pregnancies. However, studies have not demonstrated any differences in the expression of Cx-43 in tissues obtained from twin and singleton pregnancies [Gibb *et al.*, 2006]. Thus, it is believed that the expression of CAPs and the ability of the uterus to respond to

uterotonins is a result of a complex interplay of interactions between the mechanical and endocrine pathways [Challis and Lye, 1994; Challis *et al.*, 2000].

It is thought that increased uterine volume produces a membrane depolarization through a stretch-induced alteration in ion channel activity, modulating the propagation of electrical and mechanical activity in uterine smooth muscle. While much is known about the signal transduction pathways that modulate stretch responses, little is understood about how cells sense and integrate mechanical signals to produce cellular responses such as contraction, growth and remodeling.

1.4.2.3 Steriods

The major steroid hormones produced during pregnancy are estrogens and progesterone; progesterone acts to inhibit contractions, while estrogens act to increase contractions in most species [Challis and Patrick, 1981; Jenkin and Thorburn, 1985]. In experimental animals, parturition is preceded by an increase in plasma estrogen levels and a decrease in plasma progesterone levels [Virgo and Bellward, 1974; Challis, 1971]. The sites of production of these steroids vary in different animals. In mice and rats, progesterone is produced by the corpus luteum of the ovary and estrogen is produced only at the end of gestation by the placenta when there is a decrease in progesterone production due to luteolysis; while in the sheep and human, both progesterone and estrogen are produced by the placenta [Gibb *et al.*, 2006; Zakar and Hertelendy, 2007]. In the human, maternal LDL cholesterol is the principal precursor of placental progesterone. Estriol synthesis depends mostly on androgen precursors from the fetal adrenal and liver, and estrone and estradiol synthesis depends on precursors (such as

dehydroepiandrosterone sulfate (DHEAS)) from both the fetal and maternal adrenal glands [Tulchinsky *et al.*, 1972].

In the mid-1950's, Csapo first hypothesized that if progesterone was essential for pregnancy maintenance, then progesterone withdrawl must be required for labour to occur [Csapo, 1956]. This theory is supported by the observation that the use of mifepristone (RU486) induces labour in pregnant rats within 24 hours [Fang *et al.*, 1997; Shynlova *et al.*, 2007]. RU486 is also an emergency contraceptive pill sometimes used by women to prevent implantation or to terminate a pregnancy [Sitruk-Ware *et al.*, 1990]. In both cases, RU486 acts as a competitive progesterone receptor antagonist, preventing progesterone from acting, causing an increased rate of spontaneous labour, increased ripening of the cervix and increased sensitivity to oxytocin compared with placebo-treated controls [Frydman *et al*, 1992; Lelaidier *et al.*, 1994; Chwalisz *et al.*, 1991].

In humans and higher primates, the theory of progesterone withdrawal is more complicated because circulating levels of maternal plasma progesterone remain elevated during labour [Boroditsky *et al.*, 1978; Tulchinsky *et al.*, 1972; Walsh *et al.*, 1984]. The concept of functional progesterone withdrawal has been proposed, mediated through changes in progesterone receptor (PR) expression or the existence of a progesterone receptor suppressor [reviewed in Zakar and Hertelendy, 2007]. It has been suggested that functional progesterone withdrawal is fundus-specific, leading investigators to speculate that elevated circulating progesterone levels may be necessary to relax the lower uterine segment and these high levels are blocked in the fundus to promote contraction at labour

[Sparey *et al.*, 1999]. This theory is supported by the observation of decreased expression of Cx-43 in the lower segment compared to the fundus [Sparey *et al.*, 1999].

PRs are members of the nuclear receptor superfamily and function as transcription factors when bound to progesterone. The occupied PRs undergo activation, dimerize and translocate to the nuclei, where they bind to specific sequences in the promoter regions of progesterone-responsive genes. Three subtypes of PRs have been identified: PR-A, PR-B and PR-C. PR-B functions predominately as an activator of progesterone responsive genes, while PR-A acts as a modulator or repressor of PR-B function. The responsiveness of target tissues to progesterone is dependent not only on the level, but also on the ratio of PR isoforms. Both isoforms are expressed in the human myometrium and there are increasing levels of PR-A and PR-B, as well as the PR-A/PR-B ratio in labouring human myometrium [Pieber *et al.*, 2001; Mesiano *et al.*, 2002]. Pieber *et al* (2001) showed that exogenous PR-A administration dose-dependently repressed PR-B transactivation and suggested that increasing the PR-A/PR-B ratio decreases progesterone responsiveness in myometrial cells.

It has been suggested that only PR-A and PR-B are of significance in the myometrium [Mesiano, 2004], however Condon *et al* (2006) recently examined PR isoforms in term pregnant myometrium and found evidence of a role for PR-C. They found increases in PR-B levels in fundal myometrium but not in the lower segment in women in labour; and PR-A levels were low and unchanging in both segments before or during labour. In addition, they found marked increases in levels of PR-C in fundal myometrium of women in labour.

There is evidence that suggests that PR isoform levels are regulated differentially by various agonists and regulatory pathways in uterine cells. Madsen *et al* (2004) reported that PGF2α induced PR-A mRNA but not PR-B mRNA expression, and PGE2 increased PR-A/PR-B ratio in an immortalized pregnant human myometrial cell line. The authors also found that phorbol ester treatment increased PR-A mRNA and the PR-A/PR-B ratio, suggesting a role for PKC in the upregulation of PR-A in pregnant myometrium [Madsen *et al.*, 2004].

There are co-regulatory proteins that interact with PRs to enhance or repress their transcriptional activities. The two families of co-activators are the steroid hormone receptor co-activators (SRC)-1, 2 and 3 and p300/cAMP-responsive element binding protein (CBP). PR-B has a higher affinity for SRC-1 and SRC-2 than PR-A [Giandrande *et al.*, 2000] and SRC-2 and -3 levels were found to decrease in the fundal human myometrium at labour [Condon *et al.*, 2003]. The levels of CBP were reported to decrease in the fundal region of the myometrium of labouring women while levels of p300 remained unchanged [Condon *et al.*, 2003; Long *et al.*, 2005]. Corepressor proteins, such as protein silencing media for retinoid and thyroid hormone receptors (SMRT) or nuclear receptor corepressor (NCoR), bind and inhibit PRs functions. The involvement of these corepressors has been documented in cell culture models (a human hepatocellular liver carcinoma cell line and a cervical cancer cell line) [Giandrande *et al.*, 2000; Wagner *et al.*, 1998].

Placental production of estrogens increase progressively during pregnancy and the uterine actions of estrogen are generally antagonistic to progesterone. In humans,

maternal estrogen levels are low in the first trimester, increase markedly at midgestation and then remain at an elevated level until labour while in other species circulating levels of estrogen only dramatically increase at labour [Ticconi *et al.*, 2006]. Estrogens have been found to increase the synthesis of CAPs, including OTR and Cx-43, as well as PG production [Challis *et al.*, 2000; Hatthachote and Gillespie, 1999]. Estrogens can regulate the relative expression of NO synthase (NOS) isoforms in pregnant myometrium and thus estrogens are thought to play a role in priming and preparing the myometrium for labour. In pregnant sheep, administration of estradiol results in premature delivery [Liggins *et al.*, 1972; Cahill *et al.*, 1976], however, estrogens are not clinically effective in inducing labour in women. Normal pregnancy and labour can occur in women with very low levels of circulating estrogens due to deficiencies of placental sulphatase or aromatase.

The actions of estrogens are mediated through estrogen receptor (ER) and the human ER exists as two subtypes, ESR1 and ESR2. An increase in the expression of ESR1 has been observed in labouring human myometrium and a correlation was observed between the PR-A/PR-B ratio and ESR1 expression in nonlabouring term myometrium [Mesiano *et al.*, 2002]. For most of pregnancy, the myometrium is refractory to estrogenic actions, even though the level of estrogens is high [Walsh *et al.*, 1984] and Mesiano *et al.* (2002) suggested that this insensitivity is due to the suppression of ESR1 expression by progesterone. In the pregnant rhesus monkey, RU486 not only induces parturition, it also increased myometrial ER expression [Haluska *et al.*, 1990]. Mesiano *et al.* (2002) demonstrated a positive correlation between the levels of ESR1 and

the expression of PGHS-2 and OTR in nonlabouring term myometrium. This association indicates that estrogen responsiveness is related to ESR1 levels, because while PGHS-2 is not known to be up-regulated by estrogen, OTR expression is known to be up-regulated by estrogen in the pregnant human myometrium [Young *et al.*, 1998; Quinones-Jenab *et al.*, 1997].

1.4.3 Stimulation: Phase 2 of Parturition

This phase of parturition is characterized by the strong, episodic uterine contractions of labour. The onset of labour is associated with a change in the contractile activity of the myometrium; the frequency and amplitude, as well as duration, of contractions begin to increase [Lye and Freitag, 1990]. Uterine contractions are dependent on action potentials generated and propagated by the muscle cells. Action potentials are influenced by the number of cells enlisted, the synchronicity of their electrical activity and the types of currents activated in these cells. The frequency and duration of action potentials determine the frequency and duration of the uterine contractions, while the amplitude of a contraction is dependent on the propagation of action potentials and consequently on the number of muscle cells recruited during a contraction [Garfield *et al.*, 1988]. During this phase, the uterus is now able to optimally respond to various uterotonins, such as OT and PGs, which stimulate the strong myometrial contractions.

1.4.3.1 Role of Oxytocin

Oxytocin (OT) is a potent uterotonic nonapeptide that is synthesized in the hypothalamus and it is then released into circulation from the posterior pituitary [Zingg

and Lefebvre, 1988]. Oxytocin is also synthesized by the decidua, amnion, chorion and to a lesser extent the placental trophoblast [Lefebvre *et al.*, 1992]. In addition to its ability to stimulate the myometrium in late gestation and labour, OT also stimulates milk release from the mammary glands during lactation [Petraglia *et al.*, 1996; Fuchs, 1985]. Oxytocin is thought to contribute to labour, as synthetic OT is clinically used to induce contractions in the myometrium and the labour pattern induced is indistinguishable from normal spontaneous labour. However, the role of OT in parturition does not appear to be essential because OT null mice deliver normally [Young *et al.*, 1996; Nishimori *et al.*, 1996] and oxytocin receptor (OTR) antagonists are ineffective in preventing preterm labour [Honnebier *et al.*, 1989].

Oxytocin protein is found in the plasma of both mother and fetus during late gestation but concentrations do not rise before labour [Leake *et al.*, 1981; Thornton *et al.*, 1992]. In the human, plasma concentrations increase during the process of labour and a high amplitude surge of OT protein is released into the maternal blood at labour [Petraglia *et al.*, 1996; Fuchs *et al.*, 1982]. Secretion of OT during labour is thought to be pulsatile and in species where singleton birth is the norm, such as sheep, cow and rhesus monkey, maximal OT secretion is observed at expulsion of the fetus [Glatz *et al.*, 1981; Landgraf *et al.*, 1983; Hirst *et al.*, 1993]. In the rabbit, OT protein peaks at the delivery of the first fetus [Fuchs and Dawood, 1980], while in the pig, a pulse of OT is detectable at the explusion of each fetus and in the rat, baseline plasma OT is elevated throughout labour with larger pulses coinciding with each pup expulsion [Higuchi *et al.*, 1986]. In addition, OT mRNA expression increased in the human and rat uterus and fetal

membranes at the time of parturition [Lefebvre *et al.*, 1992; Lefebvre *et al.*, 1993; Chibbar *et al.*, 1993].

Oxytocin usually exerts its action on the myometrium by binding to receptors on uterine smooth muscle cells. In addition, OT can modulate the local release of substances with potential effects on the contractile state of the myometrium in intrauterine tissues. Oxytocin can stimulate the release of several arachidonate cyclooxygenase and lipoxygenase products, including PGs and leukotrienes [Ticconi *et al.*, 1998].

The sensitivity of the myometrium to OT can also be increased before and during labour by increasing the numbers of OTRs, thus changes in circulating OT levels would not be necessary for the peptide to have a physiological role in labour [Riemer *et al.*, 1986; El Alj *et al.*, 1990; Fuchs *et al.*, 1982]. In all species studied, including humans, an increase in OTR protein at term and preterm labour is observed [Petraglia *et al.*, 1996; Blanks and Thornton, 2003; Soloff *et al.*, 1979; Riemer *et al.*, 1986; Wathes *et al.*, 1996]. The concentration of OTRs and the expression of OTR mRNA was found to be decreased in human myometrium during oxytocin-induced labour or prolonged labour in which oxytocin was used [Phaneuf *et al.*, 2000], suggesting that the OTR is sensitive to receptor loss during human labour.

The OT gene contains an oestrogen response element and high concentrations of plasma OT have correlated with high concentrations of oestradiol in humans and rats [Richard and Zingg, 1990; Amico *et al.*, 1981; Yamaguchi *et al.*, 1979]. In addition, oestrogen treatment can increase plasma OT concentrations in women [Amico *et al.*, 1981]. Studies investigating a role for progesterone regulation of OT have been

conflicting. In rats, RU486 caused a reduction in uterine OT mRNA near term [Fang *et al.*, 1997]. Progesterone has also appeared to 'block' the oestrogen-induced increase in OT mRNA [Chibbar *et al.*, 1995]. Uterine stretch has also been found to induce the expression of the OTR. Ou *et al.* (1998) found that progesterone attenuated the increase of OTR mRNA at term and the level of OTR mRNA was highly expressed in the gravid horns as well as the nongravid stretched horns, compared to the nongravid nonstretched horns in unilaterally pregnant rats.

Oxytocin also stimulates PG production in human fetal membranes and decidua and it has been shown to increase PGHS-2 expression in the myometrium through G-protein-coupled MAPK [Molnar *et al.*, 1999]. In explant culture of human chorio-decidua, OT markedly increased the production of $PGF_{2\alpha}$ and PGE_2 in contrast to the amnion where PGE_2 is the primary PG product [Fuchs *et al.*, 1981; Wilson *et al.*, 1988]. In the rabbit amnion, OT increasees cytosolic phosholipase A2 (PLA_2), PGHS-1 and PGHS-2 activity leading to PGE_2 formation [Soloff *et al.*, 2000].

1.4.3.2 Role of Prostaglandins

PGs are thought to play a central role in the initiation and progression of labour at both normal term and preterm in most species. Specifically, PGs have been found to induce myometrial contractility as well as play a role in regulating changes in ECM composition associated with cervical ripening at term [Ritchie *et al.*, 1984; Bennett *et al.*, 1987; Rath *et al.*, 1993; Keirse, 1993]. In addition, PGs are thought to be involved in fetal adaptation to the labour process, upregulation of the fetal HPA axis, membrane rupture and maintenance of uterine and placental blood flow [Thorburn, 1992; Challis *et*

al., 2000; So, 1993; Sastry *et al.*, 1997; Challis, 2001]. The best evidence for the role of PGs in parturition is the increased PG output before the onset of myometrial contractions [Romero *et al.*, 1996; Brown *et al.*, 1998a]. Clinically, PGs are used to induce uterine contractions and drugs that block PG synthesis are known to delay labour [Sawdy *et al.*, 1997, 2003; Mitchell and Olson, 2004].

1.4.3.2.1 PG Synthesis

Biologically active eicosanoids (PGs, leukotrienes, lipoxin and other 20-carbon fatty acids) are formed from arachidonic acid, which is a constituent of membrane phospholipids. The initial step in PG synthesis is the hydrolysis and liberation of arachidonic acid through the direct catalytic action of PLA_2 or indirectly by the action of phospholipase C (PLC). PLA_2 has 3 different isoforms which differ in their specificity for arachidonic acid: an intracellular form, a cytosolic form ($cPLA_2$) and a secretory form ($sPLA_2$). The $cPLA_2$ isoform is expressed in the placenta, amnion, chorion and myometrium, with the highest levels found in the amnion and chorion [Freed *et al.*, 1997; Johansen *et al.*, 2000]. In the human amnion, $cPLA_2$ is the main enzyme involved in arachidonic acid release and its' activity increases with gestation and then decreases during term labour and preterm labour [Skannal *et al.*, 1997].

Free arachidonic acid can now be metabolized through different lipoxygenase pathways to form leukotrienes and hydroxyeicosatetraenoic acids, and through the PGHS pathway to form PGs. Studies have suggested that metabolism is directed towards lipoxygenase products during human pregnancy, but a progressive switch to PGHS products occurs at term [Rose *et al.*, 1990]. Arachidonic acid is now processed through

several steps to form the intermediate PGH_2 [Smith and DeWitt, 1996; Smith *et al.*, 2000]. This is catalyzed by PGHS, also known as cyclo-oxygenase (COX). There are three isoforms of PGHS (PGHS-1,-2 and -3) and while isoforms PGHS-1 and -2 have been widely studied in relation to parturition, the PGHS-2 isoform appears to be particularly important in parturition. Interestingly, PGHS-2 null mice were found to be infertile while PGHS-1 null mice were able to produce live offspring [Dinchuk *et al.*, 1995; Langenbach *et al.*, 1995]. PGHS-2 gene expression can be stimulated by various growth factors and cytokines, while PGHS-1 appears to be a housekeeping gene. At this time, there is no evidence that PGHS-3 synthesizes PGs for the process of labour. The next step of PG synthesis is the conversion of PGH_2 to one of the biologically active PGs (D_2, E_2, $F_{2\alpha}$, or I_2) through the activity of specific isomerases and synthases [Olson, 2003].

In human pregnancy, the PG synthesizing and metabolizing enzymes are compartmentalized between the amnion, chorion, decidua and myometrium. The human amnion is a major site of PG synthesis (predominately PGE_2) [Lundin-Schiller and Mitchell, 1990; Olson *et al.*, 1991; Gibb and Sun, 1996]. There is an increase in PG synthesis and levels of PGHS-2 but not PGHS-1 at term and preterm labour in the amnion [Hirst *et al.*, 1995; Lopez-Bernal *et al.*, 1989; Teixeira *et al.*, 1993]. Output of PGE_2 increases at term and preterm labour within the amnion [Teixeira *et al.*, 1994; Hirst *et al.*, 1995; Fuentes *et al.*, 1996; Gibb and Sun 1996] and no changes in PGE synthase (PGES) were observed with term or preterm labour [Alfaidy *et al.*, 2003; Meadows *et al.*, 2003].

Interposed between the amnion and the decidua is the chorion, where there is an increase in PGHS-2 mRNA expression with the onset of labour [Slater *et al.*, 1995, 1998]. There are also high concentrations of PG dehydrogenase (PGDH) in the trophoblast cells of the chorion [Sangha *et al.*, 1994]. This high expression of PGDH is thought to be a metabolic barrier preventing the passage of PGs generated in the amnion or chorion from reaching the underlying decidua or myometrium. The presence of a barrier suggests that the PGs that are stimulating myometrial activity are likely derived from the decidua or the myometrium. However, in some instances of preterm labour, the patients were found to have a relative deficiency of PGDH in the chorion and it has been suggested that this deficiency might allow PGs generated in the amnion or chorion to reach the underlying myometrium and decidua and provoke uterine contractions to initiate premature delivery [Sangha *et al.*, 1994].

The decidua, a well-vascularized maternal tissue lying next to the myometrium, consisting of a mixture of decidualized stromal cells, bone marrow-derived macrophages and other cell types, contains a low concentration of both PGHS-1 and -2 and shows minimal PGDH staining [Casey and MacDonald, 1988; Cheung *et al.*, 1990; Teixeira *et al.*, 1994; Hirst *et al.*, 1995]. In the decidua, little overall change in PGHS-1 or PGHS-2 occurs with labour and the low PG production that is observed may be a result from constitutive expression of PGHS-1 rather than PGHS-2 [Gibb and Sun, 1996; Alfaidy *et al.*, 2003].

It is unclear whether there are changes in PGHS activity in the human myometrium at the time of labour. Studies on PG synthesis in the myometrium during

gestation are contradictory mainly because of the difficulty in obtaining tissue for experimentation and it is thought that enzyme expression varies according to the region of the myometrium sampled and/or with the proximity of the woman to labour [Zuo *et al.*, 1994; Erkinheimo *et al.*, 2000; Sparey *et al.*, 1999; Moore *et al.*, 1999]. In rats, both PGHS-1 and PGHS-2 were reported to increase with the onset of labour [Tsuboi *et al.*, 2000]. In human myometrium, increases, decreases and no changes in PGHS-2 expression at labour have been reported [Zuo *et al.*, 1994; Erkinheimo *et al.*, 2000; Sparey *et al.*, 1999; Moore *et al.*, 1999]. In the baboon, there is an increase in PGHS-2 during late gestation and during labour [Nathanielsz *et al.*, 2004] and the earliest increase was first observed in the lower uterine segment and the cervix [Wu *et al.*, 2000].

1.4.3.2.2 PG Metabolism

The levels of PGs depend on their rates of metabolism, as well as their rates of synthesis. The major pathway in the metabolism of PGE_2 and $PGF_{2\alpha}$ involves the action of a type 1 NAD^+-dependent PGDH [Matsuo *et al.*, 1997]. PGDH is localized to the trophoblast cells of the chorion and thus is thought to act as a barrier to the passage of unmetabolized PGs generated in the amnion or chorion from reaching the decidua or myometrium [Cheung *et al.*, 1990; 1992]. Activity, as well as mRNA and protein levels, of PGDH are lower at spontaneous term labour than at elective cesarean section and, in addition, its expression is reduced significantly in idiopathic preterm labour without infection and even further reduced in preterm labour with an underlying infection [Sangha *et al.*, 1994; van Meir *et al.*, 1997]. In this latter case, loss of PGDH is associated with a reduction in the numbers of chorionic trophoblast cells [van Meir *et al.*,

1997]. It is possible that during preterm labour, the PGDH 'metabolic barrier' may break down, allowing PGs generated elsewhere within the fetal membranes to reach the underlying myometrium and provoke premature delivery.

In idiopathic preterm labour, PGDH activity is specifically regulated in chorionic trophoblasts. Glucocorticoids (cortisol, beta-methasone and dexamethasone) inhibit PGDH gene expression and activity in chorion trophoblast cells in culture [Patel *et al.*, 1999, 2003; Gibb and Challis, 2002]. Synthetic progestins have been found to increase PGDH expression and inhibitors of progesterone action, such as RU486, or synthesis, such as trilostane, all decrease PGDH activity [Patel *et al.*, 1999]. Inhibition of PGDH by cortisol was found to be reversed by the addition of progesterone, which is thought to compete with and displace cortisol from the glucocorticoid receptors [Challis *et al.*, 1999]. Proinflammatory cytokines, such as IL-1β and TNFα, act to decrease PGDH expression in chorionic trophoblasts, which can attenuated by the anti-inflammatory cytokine IL-10 [Mitchell *et al.*, 2000; Brown *et al.*, 1998b; Challis *et al.*, 1999].

1.4.3.2.3 PG Receptors

PGs generate their effect through specific receptors, which have been classified based on responses to various agonists/antagonists into 5 main subtypes (IP, TP, DP, EP and FP), one for each of the major classes of PGs. PG receptors signal various G-proteins to stimulate formation of second messengers such as cAMP or IP3 [Myatt and Lye, 2004]. The main 4 receptor subtypes for PGE_2 are EP1, EP2, EP3 and EP4, while $PGF_{2\alpha}$ has one FP receptor. Of the four EP receptors, two lead to uterine contraction (EP1 and EP3) and two lead to uterine relaxation (EP2 and EP4). EP1 and EP3 receptors

increase intracellular Ca^{2+} mobilization and inhibiting intracellular cAMP generation. EP2 and EP4 receptors increase adenylate cyclase to relax smooth muscle. The presence of different receptor subtypes or of different isoforms, which give opposing actions for the same PG, indicates that differential expression of these isoforms may determine tissue response. Interestingly, it has been reported that FP null mice failed to deliever pups and EP2 null mice were infertile [Sugimoto *et al.*, 1997; Tilley *et al.*, 1999].

In pregnant rat myometrium, EP2 mRNA was highest mid-gestation and decreased towards labour, while FP mRNA was low mid-gestation and increased towards labour [Brody-Eppley and Myatt, 1998]. Localization of the FP receptor appears to change during late pregnancy and labour, translocating from the nucleus into the cytosol during the postpartum period [Al-Matubsi *et al.*, 2001]. In human myometrium from pregnant women, the expression of EP3 and FP receptors was found to be decreased, compared to myometrium from non-pregnant women [Matsumoto *et al.*, 1997]. In the lower segment of the myometrium, EP2 mRNA was decreased in term tissues compared with preterm tissues and FP mRNA expression was also decreased with advancing gestation [Brodt-Eppley and Myatt, 1999]. Lye *et al.* (1998) have found a decreased FP expression and increased EP4 expression in the lower segment of the myometrium with the onset of labour. Interestingly, both PGE_2 and $PGF_{2\alpha}$ were found to stimulate upper segment of the myometrium, while PGE_2 inhibited contractility and $PGF_{2\alpha}$ had no effect in lower segment tissues [Wikland *et al.*, 1984; Wiqvist *et al.*, 1985]. This is consistent with the idea that during labour, the lower segment relaxes to allow passage of the fetus, while the upper segment contracts to push the fetus out.

1.4.3.3 Role of CRH

The placenta produces CRH during human pregnancy and increasing levels of CRH mRNA are expressed in the placenta with advancing gestation, resulting in increased concentrations of CRH peptide in the placenta and an exponential increase in CRH concentration in maternal peripheral plasma [Petraglia *et al.*, 1996]. The biological activity of CRH in maternal plasma is attenuated by CRH-binding protein (CRH-BP), which is produced in the placenta and the liver [Challis *et al.*, 1995; Potter *et al.*, 1991]. CRH-BP inhibits CRH stimulation of ACTH release from pituitary cells and inhibits CRH effects on PG production in uterine tissues [Petraglia *et al.*, 1996]. CRH-BP concentrations decrease during the last 5-6 weeks of normal pregnancy and before preterm labour, which coincides with the increase in maternal CRH concentrations [Linton *et al.*, 1993; McLean *et al.*, 1995]. In the placenta, CRH is produced by syncytiotrophoblast and intermediate trophoblasts and is localized to these layers as well as mononuclear trophoblast cells in the chorion [Jones *et al.*, 1989; Riley *et al.*, 1991]. In vivo, within 24 hours of administering synthetic glucocorticoids to patients to promote fetal lung maturation, plasma CRH concentrations were elevated [Marinoni *et al.*, 1998; Korebrits *et al.*, 1998].

It has been suggested that CRH acts as a placental clock regulating the timing of parturition [Smith, 1999; McLean and Smith, 2001]. These authors suggest that increasing maternal plasma CRH concentrations may be used to predict women destined to enter preterm labour. Increased maternal plasma concentrations of CRH have been shown as early as 14-16 weeks of gestation in women who subsequently deliver preterm,

and lower concentrations of CRH in the plasma of women who delivered post term [McLean *et al.*, 1995]. Korebrits *et al.* (1998) found that maternal plasma CRH concentrations were higher in patients at weeks 28-32 of gestation with an initial diagnosis of threatened preterm labour, who delivered within 48 hours. However, the concentration of CRH was within the normal range in patients who had the same initial diagnosis who proceeded to delivery at term [Korebrits *et al.*, 1998].

1.5 Integrin Receptors

To support the dramatic growth of the uterus and the substantial ECM remodeling that is observed during late pregnancy, cell-ECM contacts must be reorganized to properly anchor growing cells to their ECM. Focal adhesion kinase (FAK) is a regulator of cell-ECM contact reorganization and is extensively expressed and highly active in rat myometrium during late pregnancy [MacPhee and Lye, 2000]. In smooth muscle, these contacts are termed dense plaques, which are analogous to focal adhesions described in cultured cells [Burridge and Chrzanowska-Wodnicka, 1996]. Focal adhesions are comprised of clusters of integrins that occur at sites where the cell makes contact with the extracellular matrix (ECM). Integrins are non-covalently linked, heterodimeric, type I transmembrane receptors composed of a α and a β subunit and the combinations of these subunits form various ECM receptors [Reddy and Mangale, 2003]. Mammalian genomes contain 18 α and 8 β subunit genes, and to date 24 different combinations have been identified, which are expressed in a tissue-dependent manner [Humphries *et al.*, 2006]. Integrin receptors exhibit overlap in their ligand binding specificities and a particular ECM molecule can bind to more than one integrin. For example, the αvβ3 receptor can

bind vitronectin, fibronectin, collagen, tenascin-C, thrombospondin and fibrinogen; and fibronectin can bind to at least 7 different integrin receptors (α5β1, α3β1, α4β1, α8β1, α9β1, αvβ3, and αvβ5) [Reddy and Mangale, 2003]. Integrins bind ligands on other cells or in the ECM, connect to the cytoskeleton inside the cell and regulate intracellular signaling pathways [Brakebusch *et al.*, 2002]. Integrins can signal through the cell in either direction; the extracellular binding activity of integrins is regulated from inside the cell (inside-out signaling), while the binding of the ECM elicits signals that are transmitted into the cell (outside-in signaling). Integrins have been implicated in signaling pathways that lead to wound healing, cell migration, cell growth, cell survival, and cell differentiation [Brakebusch *et al.*, 2002].

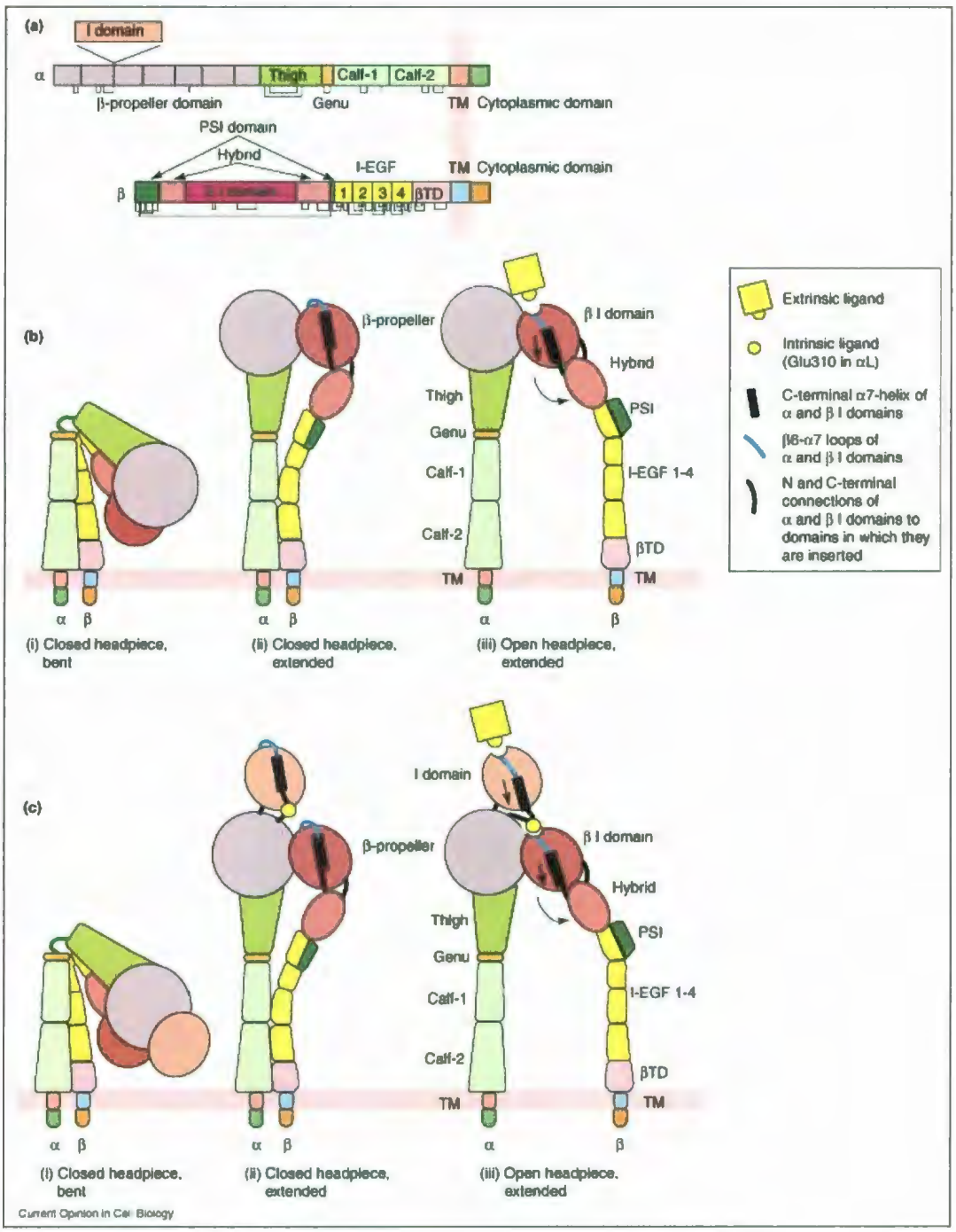
1.5.1 Molecular Structure

It is possible to cluster the majority of integrin-ligand combinations into four main groups, based on the structural interaction: those that bind to the Arginine-Glycine-Aspartic Acid (RGD) tripeptide, the Leucine-Aspartic Acid-Valine (LDV) tripeptide, the αI domain (also known as αA or von Willebrand Factor type A (vWFA)) and the non-αI domain integrins. The first group contain integrins that recognize ligands containing an RGD tripeptide active site, which binds at the interface between the α and β subunits [Ruoslahti, 1996]. Many ligands are shared by this group of integrins, however, the binding affinity varies, reflecting the preciseness of the fit of the ligand RGD conformation with the active site pockets [Humphries *et al.*, 2006]. LDV is an acidic motif that is structurally related to RGD and integrins that bind to the LDV motif are believed to bind similarly to RGD in these integrins [Ruoslahti, 1996]. Four α subunits

(α1, α2, α10 and α11) contain an αI domain and combine with β1 to form a laminin/collagen-binding subfamily. A glutamate within a collagenous GFOGER motif provides the key cation interacting residue [Emsley *et al.*, 2000]. The non-αI domain integrins are a subfamily of integrins that are highly selective for laminin. These integrins bind to different regions of the ligand than the αI domain-containing integrins [Humphries *et al.*, 2006].

The structure of the extracellular portion of α and β subunits has been investigated in both ligand-bound and unbound states and have revealed a compact, V-shape conformation, with each leg bent in the unbound state [Xiong *et al.*, 2001, 2002; Figure 1.4]. The α subunit consists of four domains: an N-terminal seven-bladed β-propeller, an Ig-like Thigh domain and two large β-sandwich domains, Calf-1 and Calf-2 [Arnaout *et al.*, 2007]. In addition, some α subunits contain an αI domain, which mediates divalent cation binding to extracellular ligands in αI-containing integrins [Michishita *et al.*, 1993]. The β subunit contains eight domains: an N-terminal cysteine-rich Plexin-Semaphorin-Integrin (PSI) domain, four epidermal growth factor (EGF)-like domains and a proximal novel tail domain (βTD) [Arnaout *et al.*, 2007]. The propeller domain from the α subunit and the βI domain from the β subunit assemble into a 'head' structure. The head sits on top of the α and β subunit 'legs' formed of the Thigh and Calf-1 domains from the α subunit and the PSI, Hybrid, the four EGF and βTD domains from the β subunit. In the closed conformation (unbound), the legs are bent at both 'knees', located between the Hybrid and Calf-1 domains in the α subunit and between EGF1 and EGF2 in the β subunit [Arnaout *et al.*, 2007]. This bent conformation is stabilized by multiple contacts

Figure 1.4 Integrin architecture and conformational changes associated with affinity regulation. (a) Organization of domains within the primary structures. (b,c) Conformational change of integrins lacking an I domain (b) or containing an α I domain (c). The domains are shown with the same color scheme as in (a). *Copyright 2006, Elsevier Science.* Reprinted with permission (Appendix 1) from Luo and Springer, Current Opinion in Cell Biology 2006; 18:579-586.


(a)
I domain
α
Thigh
Calf-1
Calf-2
β-propeller domain
Genu
TM
Cytoplasmic domain
PSI domain
Hybrid
I-EGF
TM
Cytoplasmic domain
β
1
2
3
4
βTD
(b)
β-propeller
β I domain
Hybrid
Thigh
PSI
Genu
I-EGF 1-4
Calf-1
Calf-2
βTD
TM
TM
α
β
(i) Closed headpiece, bent
(ii) Closed headpiece, extended
(iii) Open headpiece, extended
Extrinsic ligand
Intrinsic ligand (Glu310 in αL)
C-terminal α7-helix of α and β I domains
β6-α7 loops of α and β I domains
N and C-terminal connections of α and β I domains to domains in which they are inserted
(c)
I domain
β-propeller
β I domain
Hybrid
Thigh
PSI
Genu
I-EGF 1-4
Calf-1
Calf-2
βTD
TM
TM
α
β
(i) Closed headpiece, bent
(ii) Closed headpiece, extended
(iii) Open headpiece, extended
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between the upper (PSI-Hybrid-βI, EGF1) and lower (EGF2-4, βTD) leg domains of the β subunit [Arnaout *et al.*, 2007]. There are also several contacts between the Calf domain of the α subunit and the lower leg domains of the β subunit that aid in stabilizing the conformation [Luo and Springer, 2006].

1.5.2 Integrin Activation

Two models have been proposed to account for the conformational changes in integrins seen during activation. In the switchblade model, the bent conformation represents the low affinity state and priming and ligand binding induce a large conformational change where the integrin 'head' extends with a 'switchblade'-like motion [Takagi *et al.*, 2002; Kim *et al.*, 2003; Xiao *et al.*, 2004]. To provide the necessary space for the swing out of the head domain, this model proposes that the integrin should first fully extend its knees, converting the bent into a linear asymmetric conformation [Nishida *et al.*, 2006]. Full extension is also believed to relieve the steric hindrance of ligand binding by the plasma membrane expected in the bent conformation. The deadbolt model incorporates structural features in βA domain that are lacking in the αI domain: the absence of a metal ion in the conserved metal-ion-dependent adhesion site (MIDAS) in the unliganded state, presence of a site adjacent to the MIDAS (ADMIDAS), and proximity of the novel βTD to the βA and Hybrid domains [Arnaout *et al.*, 2007]. The deadbolt model proposes that activating changes in α1 helix and F/α7 loop in the β subunit is possible with only slight structure changes at the βTD interface with βA and possible Hybrid domains. The bound ligand provides the energy for the Hybrid swing out, which may be associated with various degrees of knee extension. Although Hybrid

swings and knee extensions are conformational changes in both models, they are considered necessary for activation in the switchblade model but a feature of only outside-in signaling in the deadbolt model [Arnaout *et al.*, 2007].

1.5.3 Integrin Signalling

The integrin β subunit cytoplasmic domain is required for integrin activation, whereas in most cases, the α subunit cytoplasmic domain plays a regulatory role [Ginsberg *et al.*, 2005]. The cytoplasmic domains of integrins are generally short and lack any enzymatic activity and therefore must associate with adaptor proteins to link to the actin cytoskeleton or for further signaling pathways [Giancotti and Ruoslahti, 1999]. Ligand binding to integrins leads to integrin clustering and recruitment of actin filaments and signaling proteins to the cytoplasmic domain [Miyamoto *et al.*, 1995]. While these signaling centres are still in the process of forming, they are called focal complexes and when they have matured into larger complexes, they are refered to as focal adhesions. Those focal adhesions that are formed through interactions with fibronectin mature into structures known as fibrillar adhesions [Geiger *et al.*, 2001].

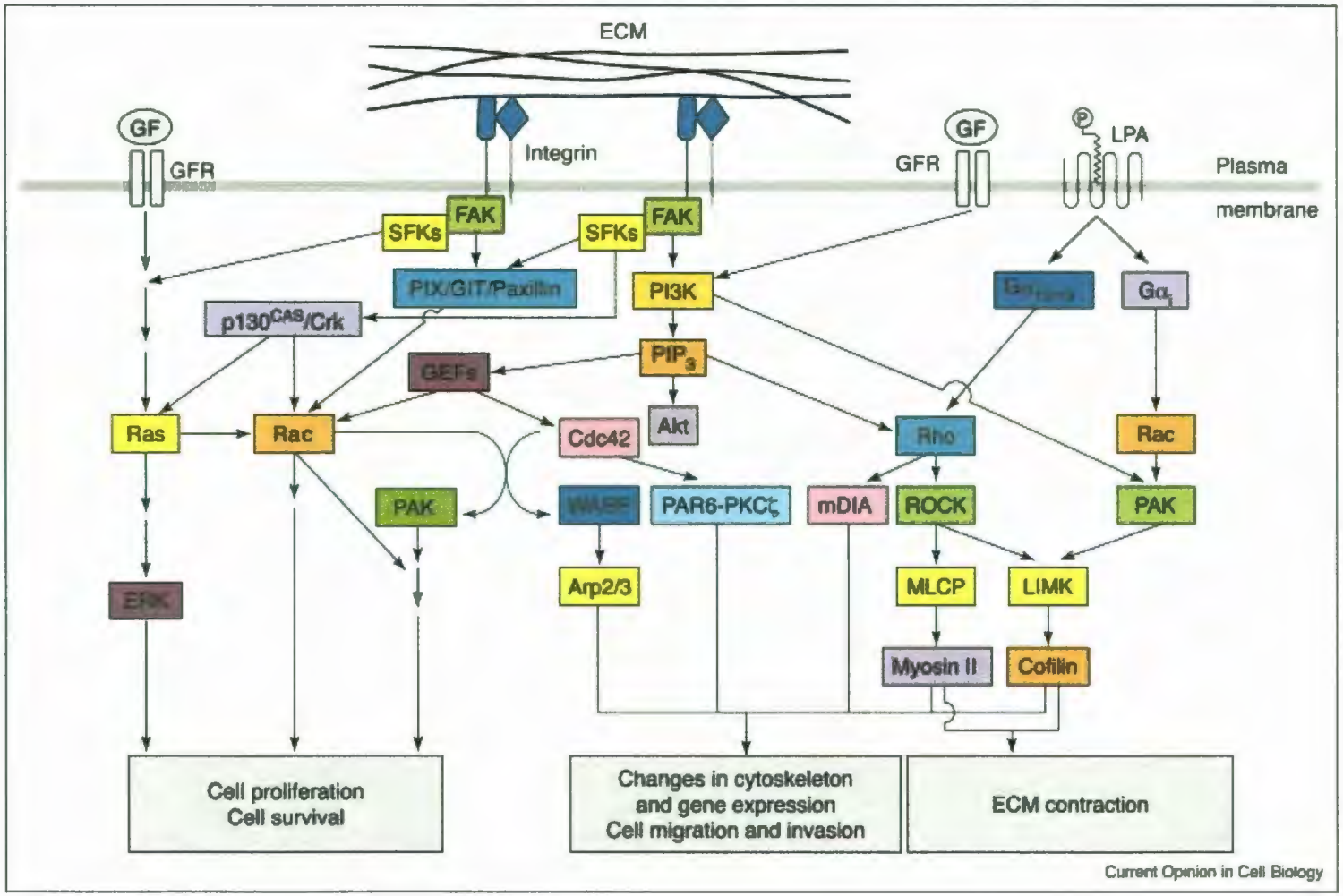
Inside-out signal transduction plays a pivotal role in diverse biological processes including embryonic development, hemostasis and angiogenesis [Bennett and Vilaire, 1979; Byzova *et al.*, 2000]. The binding of talin to the β subunit is believed to be the initial step in integrin activation during inside-out signaling. Talin binds to integrins via the band Four-point-one, Ezrin, Radixin, Moesin homology (FERM) domain located in its head domain [Calderwood *et al.*, 1999]. The membrane-proximal regions of α and β cytoplasmic tails are believed to interact through a salt bridge that keeps integrins in the

inactive state. The talin head domain is reported to disrupt the salt bridge formed [Vinogradova *et al.*, 2002], leading to tail separation and then integrin activation.

Outside-in signaling intermediates include enzymes (e.g. FAK/c-Src complex, Ras and Rho GTPases) and adaptor proteins (Cas/Crk, paxillin) that assemble within dynamic adhesion structures (Figure 1.5; Larsen *et al.*, 2006). Integrins associate with other receptors and cell membrane proteins (such as growth factor receptors and caveolae) and integrin signaling pathways often collabourate with those pathways activated by these plasma membrane receptors. Binding of ECM ligands to integrins initiates outside-in signaling by first inducing conformational changes which are transmited to the transmembrane and cytoplasmic domains and then causing integrin clustering [Ginsberg *et al.*, 2005]. Integrin clustering can result in association with the cytoskeleton and promotion of the assembly of actin filaments. The reorganization of actin filaments into larger stress fibers causes more integrin clustering, resulting in a positive feedback loop which enhances matrix binding and integrin organization. In migrating cells, the local reorganization of actin filaments initiated by integrins, promotes different types of membrane protrusions at the leading edge of the cell, while at the rear of the cell, integrins detach from the ECM, break the link to the cytoskeleton and are partially recycled to the front of the cell [Ballestrem *et al.*, 2001; Laukaitis *et al.*, 2001].

Several proteins with no intrinsic enzymatic activity and various protein tyrosine kinases bind directly or indirectly to integrins [reviewed in Liu *et al.*, 2000 and Schwartz, 2001]. Talin [Pfaff *et al.*, 1998], α-actinin [Otey *et al.*, 1990; Pavalko *et al.*, 1991], filamin [Pavalko *et al.*, 1989; Loo *et al.*, 1998], paxillin [Schaller *et al.*, 1995; Liu *et al.*,

Figure 1.5 Generalized schematic diagram of integrin signaling. Integrins signal through recruitment of FAK, recruitment and activation of SFKs, and activation of PI3K. Src phosphorylates $p130^{CAS}$ and recruits Crk to activate Rac. Rac is also activated by FAK via stimulation from PIX/GIT/paxillin complexes. FAK activates ERK signaling that, together with Rac downstream signaling, exerts a regulatory effect on cell proliferation and survival. Signaling downstream of PI3K affects activation of Akt and the small GTPases Rac, Cdc42, and Rho to induce changes in the cytoskeleton, cell contractility, cell migration, invasion and gene expression. Crosstalk between integrin and GFR signaling pathways ensures proper integration of integrin- and GFR-mediated signaling required for optimal cell function. LPA, acting through a seven-transmembrane G-protein-coupled receptor, signals through PAK and cofilin, and cooperates with the ROCK/MLCP/myosin II pathway to promote collagen matrix contraction. Abbreviations: guanine nucleotide-exchange factors, GEFs; growth factor, GF; LIM kinase, LIMK; mammalian diaphanous, mDIA; myosin light chain phosphatase, MLCP; phosphatidylinositol-3,4,5-trisphosphate, PIP_3; protein kinase C, PKC; Src-family kinases, SFKs; Wiskott-Aldrich syndrome protein, WASP. *Copyright 2006, Elsevier Science*. Reprinted with permission (Appendix 1) from Larsen *et al*. Current Opinion in Cell Biology 2006; 18:463-471.


ECM
GF
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Integrin
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LPA
Plasma membrane
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FAK
PIX/GIT/Paxillin
PI3K
p130CAS/Crk
PIP3
GEFs
Ras
Rac
Cdc42
Akt
Rho
Rac
PAK
WASP
PAR6-PKCζ
mDIA
ROCK
PAK
Erk
Arp2/3
MLCP
LIMK
Myosin II
Cofilin
Cell proliferation
Cell survival
Changes in cytoskeleton
and gene expression
Cell migration and invasion
ECM contraction
Current Opinion in Cell Biology

1999; Chen *et al.*, 2000] and integrin-linked kinase (ILK) [Hannigan *et al.*, 1996; Li *et al.*, 1999] are examples of adaptor proteins that can bind directly to the β subunit cytoplasmic domain. The proteins and protein tyrosine kinases found within focal adhesions allow for integrins to connect to the actin cytoskeleton and induce changes such as actin polymerization and cross linking of actin filaments [DeMali *et al.*, 2002].

Protein tyrosine kinases activated by integrin-mediated adhesion include focal adhesion kinase (FAK), Src-family kinases and Abl [Jockusch *et al.*, 1995; Renshaw *et al.*, 2000]. FAK is activated by most integrins and is only present in focal adhesions. Binding of FAK to integrins is thought to be direct to the β subunit, however since the 'integrin binding region' of FAK is not required for localization of FAK to focal adhesions and the 'FAK binding region' of β subunits is not required for FAK activation, it is possible that the in vivo interaction of FAK and integrin is indirect [Tahiliani *et al.*, 1997; Shen and Schaller, 1999]. FAK is known to bind strongly to talin and paxillin. Upon cell attachment, FAK autophosphorylates Tyr^{397} either directly by integrin activation or after phosphorylation of tyrosines 576 and 577 by Src, which enhances the catalytic activity of FAK [Schaller *et al.*, 1994]. Autophosphorylation of FAK creates a binding site for the Src homology 2 (SH2) domain of Src or Fyn, which also enhances the activity of FAK [Cobb *et al.*, 1994; Xing *et al.*, 1994]. Subsequently, Src can phosphorylate a number of focal adhesion components, including paxillin, tensin, and $p130^{CAS}$ (a docking protein that recruits adaptor proteins Crk and Nck) [Burridge and Chrzanowska-Wodnicka, 1996]. FAK also interacts with and activates PI3-K and this interaction can be either directly or through Src. Src phosphorylates FAK at Tyr^{925},

creating a binding site for Grb2 and Ras guanine exchange factor mSOS, which link FAK to signaling pathways that modify the cytoskeleton and activate MAPK cascades [Schlaepfer *et al.*, 1994]. Rho-GTPases can be activated by FAK through several mechanisms to ultimately modify the actin cytoskeleton. For example, FAK interacts with paxillin with the adapter GIT1 and with GEFs of the Cool/PIX family, which activate Rac1 and Cdc42 [Turner *et al.*, 1999; Zhao *et al.*, 2000].

Integrin transmembrane and extracellular domains have been found to associate with other membrane proteins that might serve as adaptors to promote signaling. One such protein is integrin-associated protein (IAP) and binding to integrin leads to the formation of a signaling complex that includes cholesterol [Green *et al.*, 1999]. IAP has been found to have a role in integrin-mediated roles such as the increase of intracellular calcium in endothelial cells during adhesion to fibronectin and integrin cross-talk [Schwartz *et al.*, 1993; Blystone *et al.*, 1994]. Integrins also associate with tetraspanin proteins, a family of small membrane proteins with four transmembrane domains. The cytoplasmic domains of these proteins can associate with signaling proteins such as PKC, and the downstream effects include cell migration and cytoskeletal modulation [Zhang *et al.*, 2001; Maecker *et al.*, 1997]. In addition, integrins associate with caveolin upon attachment of the cell to the ECM [Giancotti and Ruoslahti, 1999]. Caveolae are a subtype of cholesterol-enriched membrane microdomains that appear as an Ω-shaped invagination in the plasma membrane [Anderson, 1998]. Caveolin-1 is the major protein of caveolae and contributes to membrane and cholesterol homeostasis. Cell detachment from the ECM results in translocation of caveolin-1 from focal adhesions to caveolae

inducing internalization of caveolae [Echarri and Pozo, 2006]. The interaction of integrin and caveolin-1 is important because it is linked to phosphorylation of Shc by Src kinases, activation of Ras-Erk pathway, cell proliferation and survival [Schwartz, 2001]. Integrins also associate with the urokinase plasminogen activator receptor (uPAR), which is found in caveolae. uPAR is a GPI-linked protein that promotes ligand binding by bringing integrins into proximity of other caveolae components [Wei *et al.*, 1999].

1.5.4 Mechanotransduction

One outcome of the close relationship between integrins and the actin cytoskeleton is mechanotransduction. Mechanical forces due to physical stresses from outside the cell can be transmitted to the cytoskeleton through integrins to modify the cytoskeleton and influence signaling and cells can then generate forces that regulate their own cytoskeleton and are transmitted to the ECM to modulate its assembly. It is also thought that integrins transduce physical forces into chemical signals as well [Katsumi *et al.*, 2004]. Increased tension within adhesions can trigger increased integrin clustering and integrin-independent pathways, such as specific stretch-activated channels, appear to be involved [Katsumi *et al.*, 2005]. Integrins have been implicated in several forms of mechanotransduction, including cellular responses to stretch, elevated hydrostatic pressure, fluid shear stress and osmotic forces [Katsumi *et al.*, 2004]. Several studies have shown that increasing the force on an adhesion strengthens and enlarges the adhesion. Increasing tension on integrins leads to a fast response in the form of rapid recruitment of vinculin, zyxin and other focal adhesion components as well as a slow response, where tension triggers conformational activation of some of the cells

unoccupied integrins, inducing their binding to the ECM, and activation of FAK and c-src [Katsumi *et al.*, 2005, 2004].

Several studies have investigated the effect of tension on the expression of integrins. Mechanical tension applied to bladder smooth muscle cells resulted in increased synthesis of collagen types I and III and increased integrin β1, β3 and αv subunit expression [Coplen *et al.*, 2003; Upadhyay *et al.*, 2003]. During gestation, the mechanical signal pathway that contributes to myometrial activation and the initiation of labour dictates that uterine myocytes require a mechanism to sense mechanical signals to create biochemical responses to them. It has been suggested that focal adhesions (i.e. smooth muscle dense plaques) are these sites of mechanotransduction [MacPhee and Lye, 2000; Challis and Lye, 2004].

Recently, mechanotransduction was shown to be altered during pregnancy in mice due to the progressive increase in absolute force production at each optimal stretch [Wu *et al.*, 2008]. To uniformly transmit force across a smooth muscle bundle, the same amount of force has to be transmitted across each section of the muscle bundle. Kuo and Seow (2004) have suggested that the tissue bundle works as a mechanical syncytium during contraction of airway smooth muscle. This model requires correct cytoskeletal filament organization, focal adhesion formation, cell-cell and cell-ECM interactions. We have suggested that a similar model of a mechanical syncytium exists in the myometrium during late pregnancy that facilitates efficient force transduction of the contractions during labour [Williams *et al.*, 2005].

1.5.5 Focal and Cell Adhesion Molecules in the Myometrium

As previously stated, myometrial activation is a result of the increased expression of various CAPs, which includes a cell adhesion protein termed gap junctions [Challis and Lye, 1994]. The major protein forming myometrial gap junctions is Cx-43 and its mRNA expression in the rat is elevated near term and maximal during delivery [Lye *et al*, 1993]. In contrast, the expression of Cx-26 is highest during late pregnancy, but falls to low levels during labour [Orsino *et al.*, 1996]. Cx-43 expression at term is correlated with an increase in the estrogen/progesterone (E:P) ratio in maternal plasma [Lye *et al.*, 1993], while decreasing Cx-26 expression at term occurs concomitantly with progesterone withdrawal that occurs during the increase in the E:P ratio [Orsino *et al.*, 1996]. Stretch of the uterus during labour is required for full expression of Cx-43, however, stretch is not involved in the regulation of Cx-26 during pregnancy [Ou *et al.*, 1997]. It has been noted that Cx-43-deficient mice die shortly after birth due to heart defects and Cx-26 deficiency is embryonically lethal [Reaume *et al.*, 1995; Gabriel *et al.*, 1998]. However, Cx-43 is important for parturition as 1) ablation of Cx-43 in uterine smooth muscle of the mouse resulted in delayed parturition [Doring *et al*, 2006] and 2) a dominant loss-of-function Cx-43 mutant mouse exhibited impaired parturition [Tong *et al*, 2009].

Cadherins are membrane glycoproteins that mediate calcium-dependent cell adhesion in a homophilic manner [Potter *et al.*, 1999]. They appear essential for mammalian development as mice deficient in both E- and N-cadherin are embryonically lethal during early development [Larue *et al.*, 1994; Radice *et al.*, 1997]. However, the role for cadherins in the myometrium is unclear; in nonpregnant myometrium, weak

staining was found for E-cadherin and moderate staining for N-cadherin [Taylor *et al.*, 1996]. However, Tai *et al* (2003) failed to detect E-cadherin expression in nonpregnant myometrium. There have been no reports of cadherin expression in the pregnant myometrium.

The expression of several focal adhesion proteins has been found to be altered in the myometrium during pregnancy. Tyrosine phosphorylation of FAK and paxillin exhibited a significant increase during late pregnancy and a dramatic fall with the onset of labour in rat myometrium [MacPhee and Lye, 2000]. These authors also showed that progesterone blocked the onset of labour and prevented the fall of tyrosine phosphorylated FAK. Hic-5 is a paxillin homologue and is localized to focal adhesions [Shibanuma *et al.*, 1994; Fujita *et al.*, 1998]. Hic-5 has been found to be highly expressed in the rat myometrium during late pregnancy and labour and co-localizes with FAK *in situ* [Croke *et al.*, 2007].

Another important component of focal adhesions are the ECM molecules. Substantial remodeling of the ECM in the myometrium is observed during pregnancy. Nishinaka and Fukuda (1991) were the first to observe the deposition of type IV collagen, laminin and fibronectin around smooth muscle cells in rat myometrium during late pregnancy. Shynlova *et al* (2004) recently investigated the mRNA expression of several ECM molecules during pregnancy and labour in the rat myometrium. Expression of fibronectin, laminin β2 and collagen IV mRNA was low during early gestation but increased markedly to labour. Type I and III collagens peaked midgestation and decreased towards labour, while elastin remained elevated from midgestation onward.

Shynlova *et al* (2004) found that administration of progesterone caused dramatic reductions in the levels of fibronectin and laminin and prevented the fall in collagen III levels on day 23. Treatment with the progesterone antagonist RU486 resulted in a premature increase in collagen IV, fibronectin and laminin. The authors also showed that most of the changes in ECM gene expression occurred specifically in the gravid horn, revealing a role of mechanical stretch in regulation of ECM expression.

In addition, several cytokeletal proteins have been found to be altered in the myometrium during pregnancy. The small heat shock proteins are a family of proteins that exhibit chaperone activity and the expression of two of the family members has been shown to change during pregnancy in rat myometrium. The expression of Hsp27 and phosphorylated Hsp27 are highly upregulated during late pregnancy and labour in the rat myometrium [White *et al.*, 2005]. Interestingly, MacIntyre *et al.* (2008) recently showed that while total Hsp27 levels were unchanged, phosphorylation of Hsp27 was 3-fold higher at labour, compared to non-labouring human myometrial samples. Hsp27 has been found to regulate actin cytoskeleton dynamics and regulate contractile protein activation in addition to its chaperone role. Phosphorylation of Hsp27 is proposed to induce a conformational change in Hsp27 that aids in the direct binding of Hsp27 to actin filaments [Lambert *et al.*, 1999; Gerthoffer and Gunst, 2001; Mounier and Arrigo, 2002]. It has been shown that phosphorylation of Hsp27 promotes the association of actin and myosin and this association has been suggested to be facilitated by tropomyosin [Bitar, 2002].

In contrast to Hsp27, Hsp20 appears to have a role in relaxation of the myometrium. After phosphorylation of Hsp20, it has been shown to associate with actin and α-actinin, an actin-binding protein [Rembold *et al.*, 2000; Tessier *et al.*, 2003]. The actin binding sequence in Hsp20 shows sequence homology with the actin-binding sequence of troponin I in skeletal and cardiac muscle and it is believed that binding of Hsp20 to actin inhibits cross-bridge cycling, leading to relaxation of the myometrium [Rembold *et al.*, 2000, 2001]. Recently, it has been shown that Hsp20 expression is highly expressed during early and mid-pregnancy and expression markedly decreases during late pregnancy and labour [Cross *et al.*, 2007]. These results support the potential role of Hsp20 in facilitating uterine quiescence during pregnancy.

Actin is a major component of both the contractile and the cytoskeletal domains of SMCs and differentiated SMCs usually contain both muscle (α and γ) and cytoplasmic (β and γ) actin isoforms [North *et al.*, 1994]. The cytoskeleton has been suggested as a potential mechanosensor of the hypertrophied myometrial cell during pregnancy. It has also been suggested that myometrial growth during pregnancy is associated with alterations in the structure of the contracile apparatus. Shynlova *et al.* (2005) have demonstrated that both α-SM-actin and γ-actin were detected in the rat myometrium. Expression of α-SM-actin was high throughout pregnancy, while expression of γ-actin increased significantly mid to late pregnancy [Shynlova *et al.*, 2005].

1.6 Hypothesis and Objectives

While there are reports of integrins in nonpregnant human myometrium [Taylor *et al.*, 1996], there were no reports of integrin expression in the myometrium during

pregnancy and labour when I began my PhD studies. Thus, the main focus of my thesis was to investigate the gestation profile of several different integrin subunits. As it is very difficult to obtain myometrial samples from human patients during a broad period of gestation, pregnant rat myometrium was used for the experiments. Because the major ECM components that are altered during gestation are type IV collagen, laminin and fibronectin, I decided to focus my thesis on α1, α3, α5 and β1 integrin subunits. We hypothesized that integrin expression would be altered during gestation to accommodate the significant ECM remodeling. Progesterone and stretch are both known regulators of gene expression during pregnancy. Therefore, the effect of these regulators on integrin expression was examined. The most significant changes occurred with α5 integrin subunit expression and therefore, I decided to investigate the effect of silencing α5 integrin in cells in vivo on fibronectin expression and deposition.

Objective 1: To investigate the gestation profile of integrin subunits ITGA1, ITGA3, ITGA5 and ITGB1 in the rat myometrium. A) Use Northern Blot analyses to identify the mRNA expression of *Itga1, Itga3, Itga5* and *ItgB1* in myometrium from non-pregnant rats and from animals at days 6, 12, 15, 17, 19, 21, 22 of gestation, labour and 1 day post partum. B) Use Immunoblot analyses to identify the protein expression of ITGA1, ITGA3, ITGA5 and ITGB1 in myometrium from the same time points. C) Using Immunofluorescence, determine the cellular localization of ITGA1, ITGA3, ITGA5 and ITGB1 in the myometrium from the same timepoints. (Chapters 2 and 3).

Objective 2: To examine the effect of progesterone, stretch and the progesterone antagonist RU486 on *Itga5* and ITGA5 expression and localization. A)

Progesterone levels decline in the rat myometrium during late pregnancy and labour so to determine whether progesterone withdrawl might modulate ITGA5 expression, pregnant rats were injected with progesterone and ITGA5 expression examined by Immunoblot analysis and Immunofluorescence (Chapter 2). To further elucidate a role of progesterone in ITGA5 regulation, the effect of RU486 on ITGA5 expression was also evaluated in a similar manner (Chapter 4). B) To investigate the effect of stretch, unilaterally pregnant rats were used and ITGA5 expression examined (Chapter 4).

Objective 3: Investigate the effect of ITGA5 subunit silencing on fibronectin expression and deposition. Using a human myometrial cell line, M11 cells were transfected with siRNA to silence *Itga5* expression and then ITGA5 and FN expression were evaluated to determine if there was an effect on fibronectin expression and deposition (Chapter 5).

Co-authorship Statement

I, Joy Williams, am the first or co-first author for all manuscripts that are contained within this thesis (chapters 2-5). However, each of these chapers has been co-authored by several individuals. The contributions from the individuals have been invaluable in the completion of this work. The specific contribution of each author to each manuscript is described below. Chapters 2 and 4 are published manuscripts, while the manuscripts in chapters 3 and 5 are in preparation.

Chapter 2, "Expression of α5 integrin (*Itga5*) is elevated in the rat myometrium during late pregnancy and labour: implications for development of a mechanical syncytium". As the principle author, I participated in the experimental design and performed all experimental work and data analysis for the completion of this manuscript. Mr. Bryan White provided help in collection of samples, while Dr. MacPhee provided help with the experimental design and writing of the first to final drafts of this manuscript (Williams *et al.*, 2005).

Chapter 3, "Temporal and spatial expression of α1, α3 and β1 integrin subunits in rat myometrium during pregnancy and labour". As a co-first author, I participated in the experimental design and performed all work and data analysis for Northern Analysis, Immunoblot Analysis, and Immunofluorescence Analysis (figures 3.1, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, and 3.9). Dr. Oksana Shynlova, also co-first author, provided the experimental design and performed all work and data analysis for Real Time-Polymerase Chain Reaction Analysis (figure 3.2). Mr. Bryan White provided help in collection of samples,

while Dr. MacPhee provided help with correction and improvement of the first to final drafts of this manuscript.

Chapter 4, "Uterine stretch regulates temporal and spatial expression of fibronectin protein and its alpha 5 integrin receptor in myometrium of unilaterally pregnant rats". As a co-first author, I participated in the experimental design and performed all work and data analysis for Northern Analysis, Immunoblot Analysis, and Immunofluorescence Analysis of *Itga5* mRNA and ITGA5 protein (figures 4.1, 4.2, 4.3, 4.6, 4.7, 4.9 and 4.10). Dr. Oksana Shynlova, also co-first author, provided the experimental design and proformed all work and data analysis for Real Time-Polymerase Chain Reaction Analysis as well as Immunoblot and Immunofluorescence Analysis of FN protein (figures 4.4, 4.5 and 4.8). Mr. Bryan White provided help in collection of samples, as well as the isolation of RNA from RU486 samples. Ms. Haley Draper provided help to Dr. Shynlova. Dr. MacPhee provided help with correction and improvement of the first to final drafts of this manuscript (Shynlova *et al.*, 2007).

Chapter 5, "Silencing of α5 Integrin Decreases Fibronection Deposition and Secretion". As the principle author, I participated in the experimental design and performed all experimental work and data analysis for the completion of this manuscript. Dr. MacPhee participated in the experimental design, correction and improvement of this manuscript.

CHAPTER 2

Expression of α5 Integrin is Elevated in the Rat Myometrium during Late Pregnancy and Labour: Implications for Development of a Mechanical Syncytium

(Published as is in Biology of Reproduction, 2005, 72, 1114 – 1124)

2.1 Introduction

The underlying mechanisms controlling uterine contractions during labour are still poorly understood. Signals that initiate labour are known to reside in the fetal genome and involve both mechanical (uterine stretch) and endocrine pathways [Challis and Lye, 2004]. These signals ultimately lead to phenotypic changes in the myometrium, collectively termed myometrial *activation*, that are marked at the molecular level by the increased expression of a group of genes that encode contraction-associated proteins such as ion channels and gap junctions [Challis *et al.*, 2002]. The result at term is a muscle that is spontaneously active, excitable, and responsive to agonists of uterine contraction.

Prior to myometrial *activation*, the uterus undergoes dramatic growth during late pregnancy primarily due to myometrial hypertrophy under the influence of progesterone and estrogen [Monga and Sanborn, 2004]. Uterine distension by artificial means or as a result of growing fetuses has also been demonstrated to stimulate growth, particularly hypertrophy [Goldspink and Douglas, 1988; Douglas *et al.*, 1988]. To support hypertrophy, cell-ECM contacts must be reorganized to properly anchor growing cells to

their ECM, consequently a regulator of cell-ECM contact reorganization named focal adhesion kinase (FAK) is highly expressed and activated in rat myometrium during late pregnancy [MacPhee and Lye, 2000]. In smooth muscle, these cell-ECM contacts are termed dense plaques, which are analogous to the focal adhesions described in cultured cells in vitro (for simplicity, hereafter dense plaques will be referred to as focal adhesions) [Burridge and Chrzanowska-Wodnicka, 1996; Gerthoffer and Gunst, 2001].

Late pregnancy is also a period marked by substantial remodelling of the ECM itself [Monga and Sanborn, 2004]. Type IV collagen, laminin and fibronectin are deposited around smooth muscle cells in rat myometrium during late pregnancy [Gerthoffer and Gunst, 2001]. At the molecular level, both fibronectin mRNA (*Fn*) and fibronectin protein (FN) expression have been reported to increase in the myometrium prior to labour [Nishinaka and Fukuda, 1991; Shynlova *et al.*, 2004]. Of significance, the interaction of fibronectin with its major receptor, α5β1 integrin (ITGA5B1), is important for fibronectin matrix assembly and strong intercellular cohesion [Wierzbicka-Patynowski and Schwarzbauer, 2003; Robinson *et al.*, 2003; Robinson *et al.*, 2004]. Integrins receptors are integral components of focal adhesions. These ECM-binding, heterodimeric, transmembrane receptors are composed of α and β subunits and the composition of some of these heterodimeric receptors can be quite specific. For instance, α5 integrin (ITGA5) partners solely with β1 integrin (ITGB1) in cell membranes to form a fibronectin receptor [Reddy and Mangale, 2003]. Integrins can mediate tension transmission between the contractile apparatus of the cell and the ECM yet they lack enzymatic activity and therefore must associate with numerous adaptor proteins,

cytoplasmic kinases or transmembrane growth factor receptors to connect them to the actin cytoskeleton and/or trigger biochemical signalling pathways [Gerthoffer and Gunst, 2001; Wang *et al.*, 1993; Brakebusch and Fassler, 2003; Schlaepfer *et al.*, 1999].

As in non-muscle cells, there is evidence that integrins play a pivotal role in mediating functional adjustments in smooth muscle cells in response to changes in their external environment [Gerthoffer and Gunst, 2001]. Functional adjustments can include changes in contractility resulting from interaction of ECM proteins with appropriate integrin receptors. For example, ligand binding to ITGA5B1 can increase L-type Ca^{2+} current in arteriolar smooth muscle and alter contractility [Wu *et al.*, 2001]. In addition, in renal vascular smooth muscle cells, the binding of ligands containing Arg-Gly-Asp (RGD)-peptides to integrins stimulates the release of intracellular Ca^{2+} that can alter smooth muscle contractility [Chan *et al.*, 2001].

While the detection of integins in non-pregnant human myometrium and leiomyomas has been reported [Taylor *et al.*, 1996], there have been no reports of integrin expression in the myometrium during pregnancy and labour. Since integrins are components of focal adhesions and late pregnancy involves significant uterine growth, ECM and focal adhesion remodelling, we hypothesized that *Itga5* mRNA and ITGA5 protein expression would be induced in the rat myometrium during late pregnancy and labour.

2.2 Materials and Methods

2.2.1 Animals

Sprague-Dawley rats were obtained from the Mount Scio Vivarium (Memorial University of Newfoundland, St. John=s, Newfoundland, Canada). Animals were individually housed and cared for under standard environmental conditions (12 hour light and 12 hour dark) in the Animal Care Unit at the Health Sciences Centre, Memorial University of Newfoundland. Rats were fed LabDiet Prolab RMH 3000 (PMI Nutrition International, Brentwood, Missouri, USA) and water *ad libitum*. The institutional animal care committee approved all experiments under animal care protocols 02-02-DM - 02-05-DM. Virgin female rats (~220g each) were mated with stud males and observation of vaginal plugs the following morning was designated day 1 post coitum. The time of delivery under these standard conditions was day 23 of gestation.

2.2.2 Experimental Design

2.2.2.1 Normal Pregnancy and Term Labour

All animals were killed by carbon dioxide inhalation on the desired day of sampling and pregnancy (e.g. non-pregnant (NP), days 6, 12, 15, 17, 19, 21, 22, labour (L), and 1 day post partum (PP)). The estrous cycle stage was not determined prior to obtaining nonpregnant samples. Labour samples were taken during active labour and only after the rat had delivered two to three pups.

2.2.2.2 Progesterone-Delayed Labour

The onset of labour is coupled with a withdrawal of the inhibitory effects of progesterone on the myometrium following a fall in plasma levels of this steroid. To determine whether progesterone withdrawal might modulate *Itga5* expression, pregnant

rats were given either a daily injection of progesterone (4mg, sc, in 0.2 mL corn oil), to maintain elevated plasma levels of this steroid, or vehicle alone (corn oil, 0.2 mL sc) beginning on day 20 of gestation [Petrocelli and Lye, 1993]. Animals were killed by carbon dioxide inhalation on the desired day of sampling. Vehicle control animals were killed on days 21, 22, and labour (during delivery). Progesterone-treated rats were killed on the same gestation days and on day 24; however, on days 23 and 24 the rats were not in labour.

2.2.3 Tissue Collection

Uterine horns were removed, opened longitudinally, and fetuses and placentas discarded. The uterine horns were placed in ice-cold phosphate buffered saline. The endometrium was carefully removed by scraping the luminal surface of the uterus, and myometrial tissue was flash-frozen in liquid nitrogen and stored at -80°C.

2.2.4 Northern Blot Analysis

Northern blot analysis for the normal pregnancy regime was performed on four separate, independent sets of RNA samples (n=4, i.e. 4 rats used per gestational timepoints) while analysis for the delayed labour regime was performed on three separate, independent sets of RNA samples (n=3). RNA was isolated from tissues using TRIzol® Reagent (Invitrogen Corporation, Carlsbad, California, USA). Approximately 100mg of tissue was ground up using a mortar and pestle on dry ice. The tissue was added to 1ml of TRIzol® Reagent and homogenized using the Polytron for

approximately 30 seconds. The sample was then incubated at room temperature for 5 minutes, 200µl chloroform was added, and incubated at room temperature for 3 minutes. The sample was then centrifuged for 15 minutes at approximately 12,000 x g at 2°C. The aqueous phase of the sample was transferred to a new microcentrifuge tube, 500µl isopropanol was added and then the sample was incubated at room temperature for 10 minutes. The sample was then centrifuged for 10 minutes at 12,000 x g at 2°C. The supernatant was then aspirated and the sample was washed with 1ml 75% diethyl pyrocarbonate (DEPC)-treated Ethanol (EtOH). The sample was centrifuged for 5 minutes at 7,500 x g at 2°C. The supernatant was aspirated and the pellet was allowed to air dry for 10 minutes. The RNA was re-dissolved in 100µl DEPC-treated twice distilled water (ddH_2O) at 55°C for 10 minutes. RNA purity and quantity ($A_{260/280}$) were determined using a Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, Ontario, Canada) and samples stored at -70EC.

Ten micrograms of each RNA sample in sample buffer (50% formamide, 0.066 M formaldehyde, and 1X MOPS buffer in DEPC-treated ddH_2O) were loaded on a 1% agarose gel containing 0.66 M formaldehyde and 1X MOPS buffer (0.02 M MOPS pH=7.0, 2 mM sodium acetate, 10 mM ethylenediaminetetraacetic acid (EDTA) pH=8.0) and electrophoretically separated at 80V in 1X MOPS / 0.22 M formaldehyde running buffer. RNA was transferred overnight to a nylon membrane (Hybond-XL; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) by upward capillary action using 2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate) in DEPC-treated ddH_2O. RNA was crosslinked to nylon membrane with a UVC-508 ultraviolet crosslinker

(Ultra-Lum Inc., Paramount, California, USA). All blots were stored at -20EC until required.

pOTB7 vector containing the human *Itga5* cDNA was purchased from the American Type Culture Collection (Manassas,Virginia, USA, www.atcc.org ; #MGC-3697). Digestion of pOTB7 with the restriction endonucleases EcoRI and XhoI resulted in the production of 4 DNA fragments including a 2.4 kb *Itga5* cDNA fragment that was subsequently isolated with a Qiagen Gel Extraction Kit (Qiagen, Inc, Mississauga, Ontario, Canada), according to the manufacturer's instructions and used for the production of random primed, radiolabeled cDNA probes. The 2.4 kb fragment of the human *Itga5* cDNA (Genbank Accession #BC008786) was found to have 89 % identity with the rat *Itga5* cDNA (Genbank Accession #XM_235707).

Membranes were pre-hybridized in hybridization buffer consisting of 50% formamide, 5X sodium chloride-sodium phosphate EDTA (SSPE; 0.75 M sodium chloride, 0.05 M sodium phosphate, 0.005 M EDTA), 1% SDS, 5X Denhardt=s Solution, and 0.1 mg/mL Herring Sperm DNA for 1-2 hours at 42EC in a hybridization oven (Hybaid Instruments, Franklin, Massachusetts). Radiolabelled cDNA probes were prepared with a Megaprime DNA Labelling kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). Approximately 30ng of cDNA was added to primers and DEPC-treated ddH_2O and boiled at 95°C on the dry heating block for 5 minutes. Labelling buffer, ^{32}P-α-dCTP, and Klenow Enzyme were added to the DNA mixture and incubated in a hybridization oven for 30 minutes. The cDNA probe was cleaned by centrifuging it through a G50 sephadex column. The probe was boiled for 5 minutes and

then added to the membrane with hybridization buffer. Hybridization was performed overnight at 42EC. Blots were washed 1X 15 mins and 3X 5 mins at 65EC in 0.2X SSC and 0.2% SDS and exposed to x-ray film (Hyperfilm MP; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Multiple exposures were produced for each northern blot to ensure the results were within the linear range of the film.

Following analysis of *Itga5* mRNA expression, northern blots were analyzed for expression of 18S rRNA (*Rn18s)*, as described above, utilizing a rabbit *Rn18s* cDNA template generously provided by Dr. I. Skerjanc (University of Western Ontario, London, Ontario, Canada). *Rn18s* RNA is constitutively expressed in rat myometrial cells and has been utilized, in the past, as a loading control for analysis of myometrial gene expression [Shynolva *et al.*, 2004; Mitchell and Lye, 2002; Oldenhof *et al.*, 2002].

2.2.5 Immunoblot Analysis

Immunoblot analysis for both normal pregnancy and delayed labour regimes was performed on four separate, independent sets of protein samples (n=4, i.e. 4 rats used per gestational timepoint). Approximately 250mg of frozen rat myometrial tissue was pulverized under liquid nitrogen with a mortar and pestle and homogenized in RIPA lysis buffer [50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS] containing 100 μM Na_2VO_3 and COMPLETE, Mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Laval, Quebec, Canada). Samples were centrifuged at 15,000 x *g* at 4 ^{0}C for 15 mins and the supernatants collected. Protein concentrations were determined by the Bradford Assay

[Bradford, 1976] using Bio-Rad protein assay dye reagent (Bio-Rad Labouratories, Mississauga, Ontario, Canada). Protein samples (50 ug/lane) were separated under non-reducing conditions by polyacrylamide gel electrophoresis in 9% resolving gels according to the method of Laemmli [1970] and gels were electroblotted to Pierce 0.45 μm nitrocellulose membrane (MJS BioLynx, Inc., Brockville, Ontario, Canada).

Membranes were rinsed in Tris buffered saline (20mM Tris base, 137 mM NaCl, pH 7.6) with 0.1% Tween-20 (TBST) for 5 mins. Unless otherwise stated, all incubations were done at room temperature and with constant agitation. Blots were blocked in 5% BSA/TBST for 30 mins. Rabbit polycolonal antisera raised against ITGA5 (AB1928; Chemicon International, Temexula, CA) or mouse monoclonal antisera raised against smooth muscle calponin (CNN) (C2687; clone hCP; Sigma-Aldrich, Oakville, ON, Canada) used at dilutions of 1:125,000 and 1:100,000, respectively, were incubated with blots for 1 h. The ITGA5-specific antibody produced a single band and has previously been used and specificity was verifed [Proulx *et al.*, 2003; Garcia *et al.*, 2004]. Blots were rinsed once for 15 min in TBST, followed by two rinses for 5 min in TBST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (H+L) or HRP-conjugated goat anti-mouse IgG (H+L) were used at dilutions of 1:100,000 and 1:150,000, respectively, and incubated with blots for 1 hr. Blots were washed once for 15 min in TBST, then four times for 5 min each in TBST. Proteins were detected using the Pierce SuperSignal West Pico Chemiluminescent Substrate (MJS BioLynx, Inc., Brockville, Ontario, Canada) detection system and multiple exposures were generated to ensure the linearity of the film exposures.

Following immunoblot analysis of ITGA5 expression, analysis of CNN expression was subsequently performed. We have determined that CNN is constitutively expressed in non-pregnant and pregnant rat myometrial tissue under our protein extraction conditions.

2.2.6 Immunocytochemistry

Two separate, independently collected sets of rat tissues (n=2, i.e. 2 rats used per gestational timepoint) were utilized for immunocytochemistry experiments and experiments were repeated four times. Tissues were fixed overnight at room temperature with constant agitation in zinc-buffered fixative (ZBF; 100mM Tris, pH 7.4, 3mM calcium acetate, 27 mM zinc acetate, 37 mM zinc chloride) [Beckstead, 1994] and then rinsed in PBS overnight at room temperature with constant agitation. Tissues were paraffin embedded, sectioned, and mounted on microscope slides by the Histology Department of Memorial University of Newfoundland School of Medicine.

Slides were dried overnight at 37 ^{0}C. Sections were dewaxed in xylene (3 x 5 min), rehydrated in descending grades of ethanol and soaked in 1X PBS. Heat-induced epitope retrieval was accomplished using a solution of 0.01M SSC, pH 6.0. This solution was heated for approximately 3 mins (until boiling) using a microwave. Slides were immersed in the hot solution for 10 mins and then air dried for 5 mins. This was repeated an additional 3 times and then slides were rinsed in PBS. Sections were blocked in 5% normal goat serum/1% horse serum in PBS for 30 min at room temperature with constant agitation. Sections were then incubated for 1 hour at room temperature in Rabbit anti-

ITGA5 (AB1928; Chemicon Intenational) at diluations of 1:500 (4ug/ml) in blocking solution or rabbit IgG (011-000-003; Jackson Immunoresearch Labs Inc, West Grove, PA) at the same concentration to serve as a negative control. Tissue sections were washed in PBS 3 x 5 min and then incubated in fluorescein isothiocyanate-conjudated (FITC)-conjugated sheep anti-rabbit IgG (F7512; Sigma, St. Louis, MO) at a dilution of 1:250 in blocking solution for 30 min with constant agitation at room temperature. Sections were washed with cold PBS containing 0.02% Tween-20 (PBT) 3 x 5 min with constant agitation. Tissues were mounted in Vectashield (Vector Labouratories, Inc., Burlington, Ontario, Canada) before viewing with an Olympus Fluoview laser scanning confocal microscope (Olympus Optical Company Ltd., Melville, New York, USA).

2.2.7 Data Analysis

Densitometric analysis of northern blots and immunoblots were performed with the aid of Scion Image software (Scion Image Corporation, Frederick, Maryland, USA). Densitometric measurements of *Itga5* mRNA was normalized to those of *Rn18s* RNA while measurements of ITGA5 protein on immunoblots were normalized to those of CNN. Statistical analysis was performed with GraphPad Instat version 3.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com) and data graphed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com). Data from northern blot and immunoblot analysis of *Itga5* and ITGA5 expression, respectively, during normal gestation were subjected to a One-way Analysis of Variance and a Tukey-Kramer Multiple Comparisons test. Data from

northern blot analysis of *Itga5* mRNA expression during progesterone-delayed labour were subjected to a Two-way ANOVA and a Bonferroni post-test. Values were considered significantly different if $p < 0.05$.

2.3 Results

2.3.1 Normal Pregnancy and Labour

2.3.1.1 Expression of *Itga5* mRNA and ITGA5 protein

To characterize the levels of *Itga5* mRNA within myometrial samples, northern blots of myometrial total RNA from non-pregnant (NP), days 6, 12, 15, 17, 19, 21, 22, labour and PP were analyzed with radiolabelled probes generated from a human *Itga5*-specific cDNA (Fig. 2.1A). Our experiments demonstrated that *Itga5* mRNA expression significantly increased during late gestation (One-way ANOVA, $p<0.0001$; $n=4$). Specifically, *Itga5* mRNA expression was barely detectable from NP to day 12 and then began to increase by day 15 of gestation. *Itga5* mRNA expression then became significantly elevated (Tukey-Kramer post-test, $p<0.05$) between d17 – labour, inclusive, compared to NP, day 6, day 12 and PP timepoints (Fig. 2.1A, B).

Immunoblot analysis utilizing ITGA5-specific antisera demonstrated that ITGA5 was readily detectable and ITGA5 levels in myometrial tissue lysates increased gradually during gestation (One-way ANOVA, $p<0.05$; $n=4$). Specifically, ITGA5 protein levels during labour were significantly elevated over NP timepoints (Tukey-Kramer post-test, $p<0.05$; $n=4$; Fig. 2.2A, B).

Figure 2.1. Northern blot analysis of *Itga5* mRNA expression in rat myometrium during pregnancy, labour and 1 day postpartum. (A) Representative northern blots of *Itga5* mRNA expression and *Rn18s* RNA expression. Analysis was performed with an *Itga5*-specific human cDNA and an *Rn18s*-specific rabbit cDNA as templates for radiolabelled probe production. (B) Densitometric analysis illustrating the increase of *Itga5* mRNA expression during pregnancy and labour. Expression levels were significantly higher ($p<0.05$) on days 17-labour over NP, d6, d12 and 1dPP (*), while days 19 through 22 were significantly higher ($p<0.01$) than d15 (#). Values are from 4 independent experiments ($n=4$) ± SE. Days 6, 12, 15, 17, 19, 21, 22 and labour represent gestational timepoints. NP = non-pregnant, L = active labour (2-3 pups born), PP = 1 day postpartum. (Williams *et al.*, Biology of Reproduction, 2005, 72, 1114 – 1124)

A


NP d6 d12 d15 d17 d19 d21 d22 L PP
Itga5
Rn18s

B

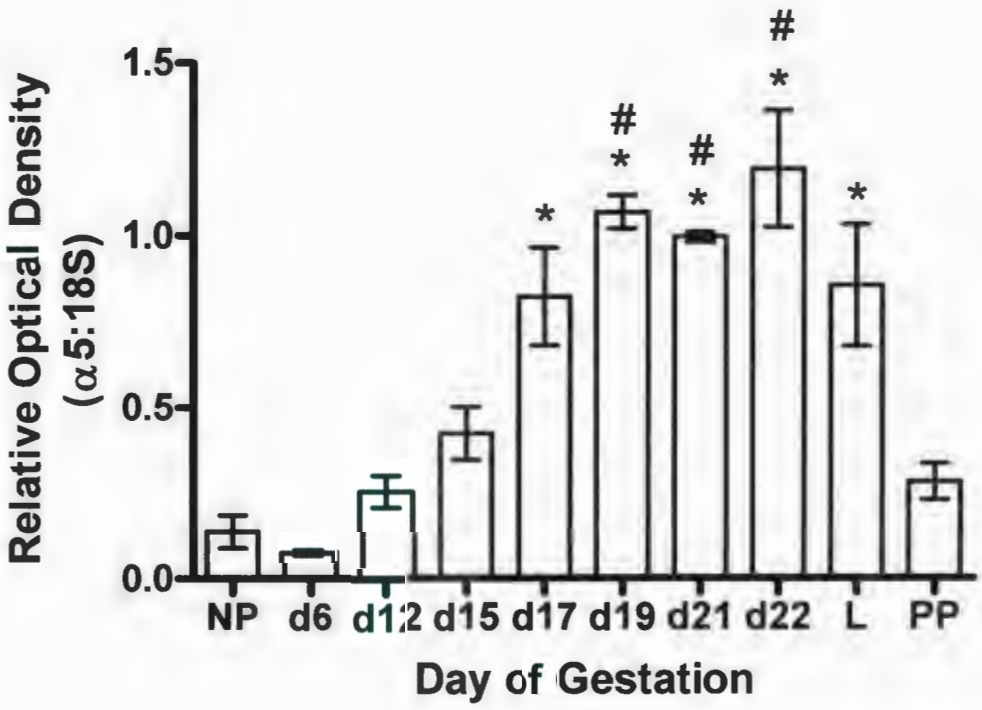
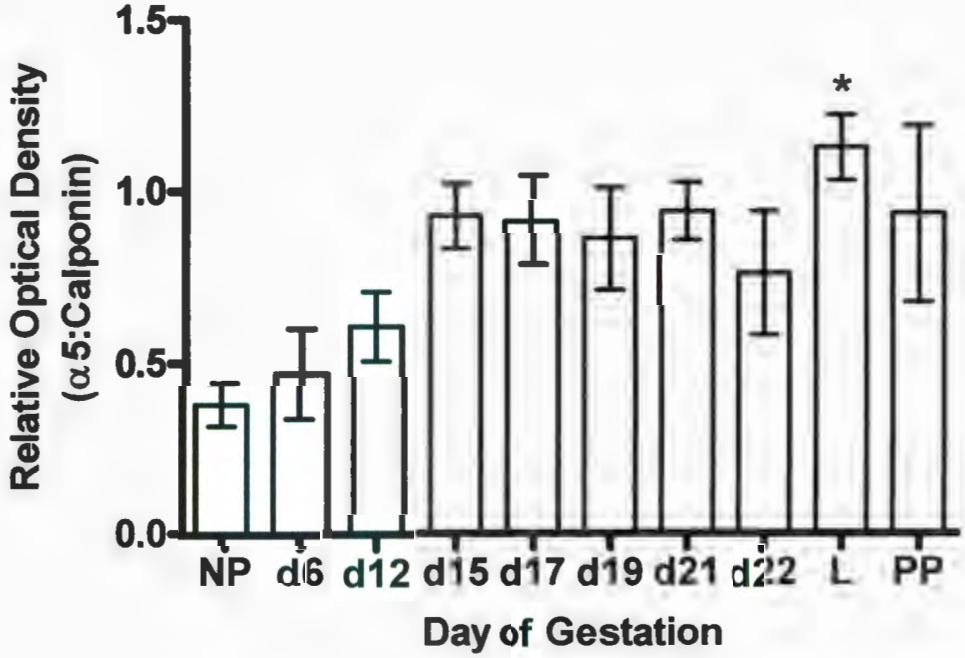

Relative Optical Density
(α5:18S)
1.5
1.0
0.5
0.0
NP d6 d12 d15 d17 d19 d21 d22 L PP
Day of Gestation

Figure 2.2. Immunoblot analysis of ITGA5 integrin protein expression in rat myometrium during pregnancy, labour and postpartum. (A) Representative immunoblots of ITGA5 protein and CNN expression. (B) Densitometric analysis illustrating the increasing linear trend in ITGA5 protein expression during pregnancy and labour. Expression levels were significantly higher ($p<0.05$) at labour over NP (*). Values are from 4 independent experiments (n=4) ± SE. Days 6, 12, 15, 17, 19, 21, 22 and labour represent gestational timepoints. NP = non-pregnant, L = active labour (2-3 pups born), PP = 1 day postpartum. (Williams *et al.*, Biology of Reproduction, 2005, 72, 1114 – 1124)

A


NP d6 d12 d15 d17 d19 d21 d22 L PP
ITGA5
Calponin

B


Relative Optical Density
(α5:Calponin)
1.5
1.0
0.5
0.0
*
NP d6 d12 d15 d17 d19 d21 d22 L PP
Day of Gestation

2.3.1.2 Immunocytochemical detection of ITGA5

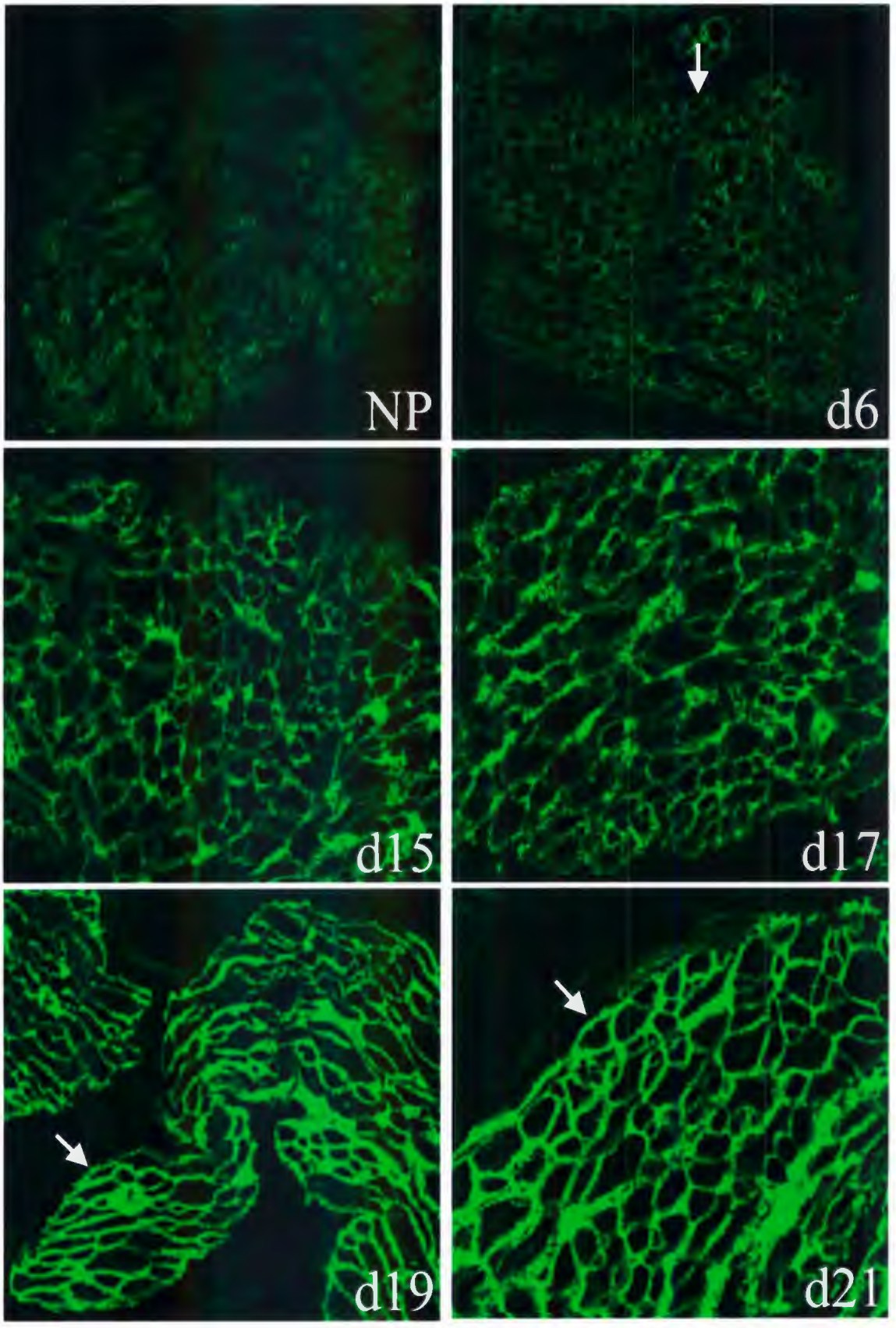
In the longitudinal muscle layer of the myometrium, ITGA5 was readily and exclusively localized to myometrial cell membranes from NP to PP (Fig. 2.3). A slight decrease in detection of ITGA5 expression was consistently observed PP. In the circular muscle layer of the myometrium ITGA5 was barely detectable on NP and day 6, then was primarily localized to cell membranes from day 15 to PP (Fig. 2.4). However, detection of ITGA5 on day 15 and day 17 was low with a more punctate staining pattern compared with the expression of ITGA5 at later gestational timepoints. As gestation progressed, detection of ITGA5 increased and a more continuous membrane-staining pattern was observed.

2.3.2 Progesterone-Induced Delayed Labour

2.3.2.1 Expression of *Itga5* mRNA and ITGA5 Protein

Throughout the majority of pregnancy, circulating levels of progesterone in the rat are high and then subsequently decline between day 19 and labour [Lye *et al.*, 1993]. Since *Itga5* mRNA expression became significantly elevated from day 17 to labour, this suggested that the effects of progesterone on the myometrium might influence *Itga5* mRNA expression during late pregnancy. Animals treated with progesterone beginning on day 20 of gestation, did not exhibit labour on day 23 and our experiments demonstrated that *Itga5* mRNA expression was sustained through to day 24, a full day after normal labour would have occurred and when *Itga5* expression would have dropped

Figure 2.3. Immunolocalization of ITGA5 protein in the longitudinal smooth muscle layer of rat myometrium during the nonpregnant state and throughout gestation. The first six panels show NP-Day 21 as labeled; the last four panels show Day 22-PP, and control, as labeled. Numbers represent gestational timepoints. L, Labour (2-3 pups born), Control, rabbit IgG . Arrows highlight membrane-specific staining. Scale bar = 10μm. (Williams *et al.*, Biology of Reproduction, 2005, 72, 1114 – 1124)


NP
d6
d15
d17
d19
d21

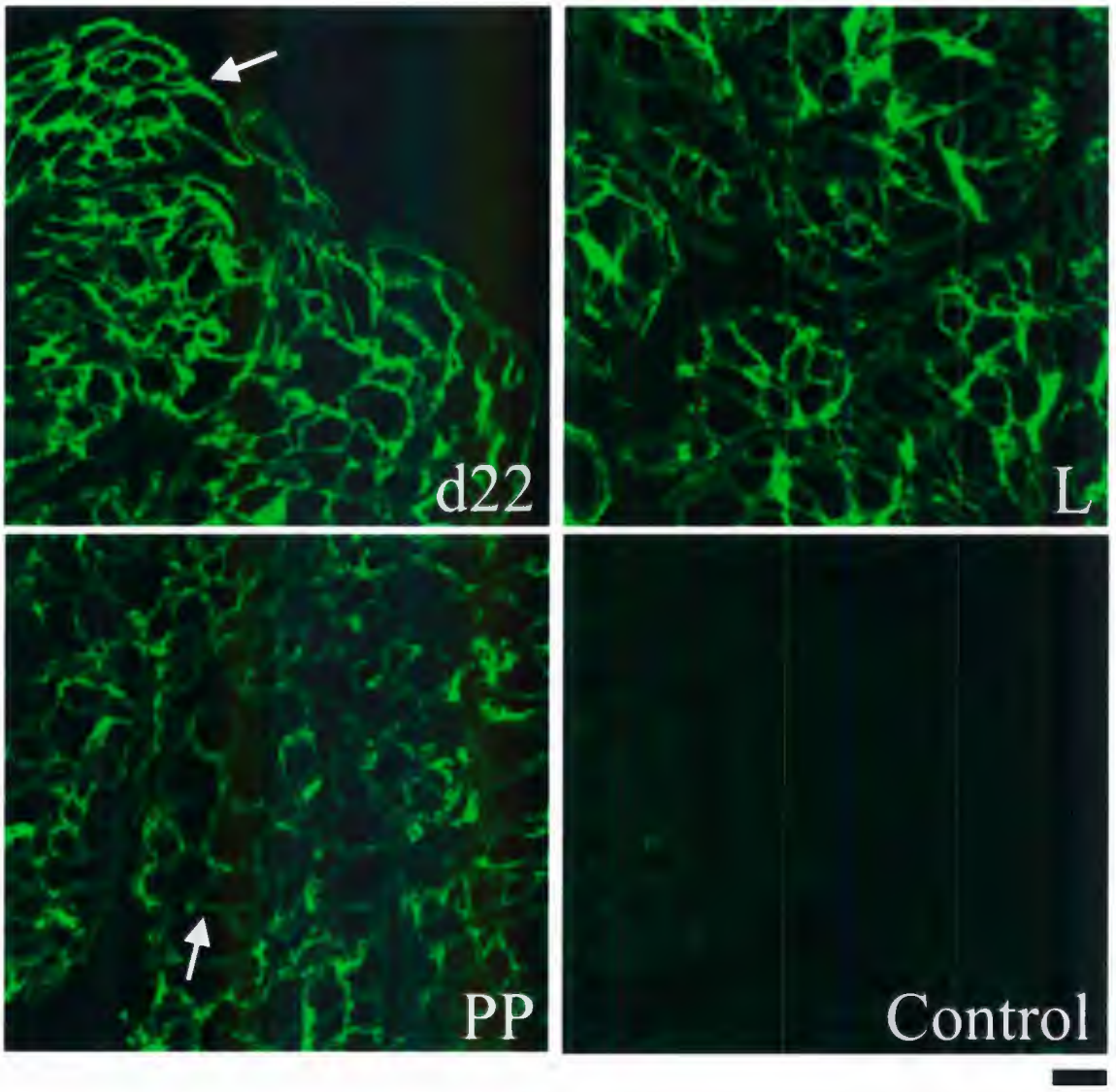
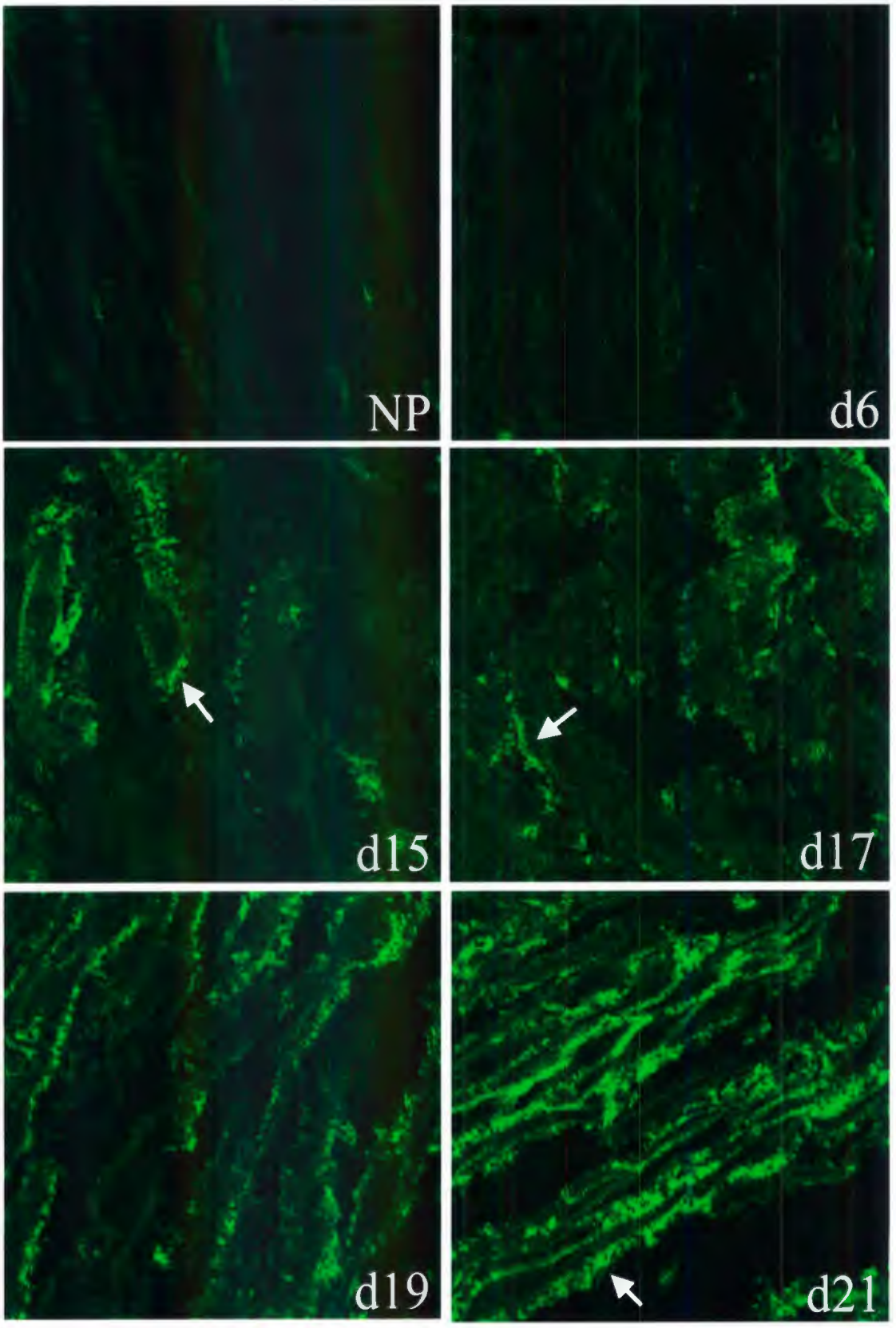
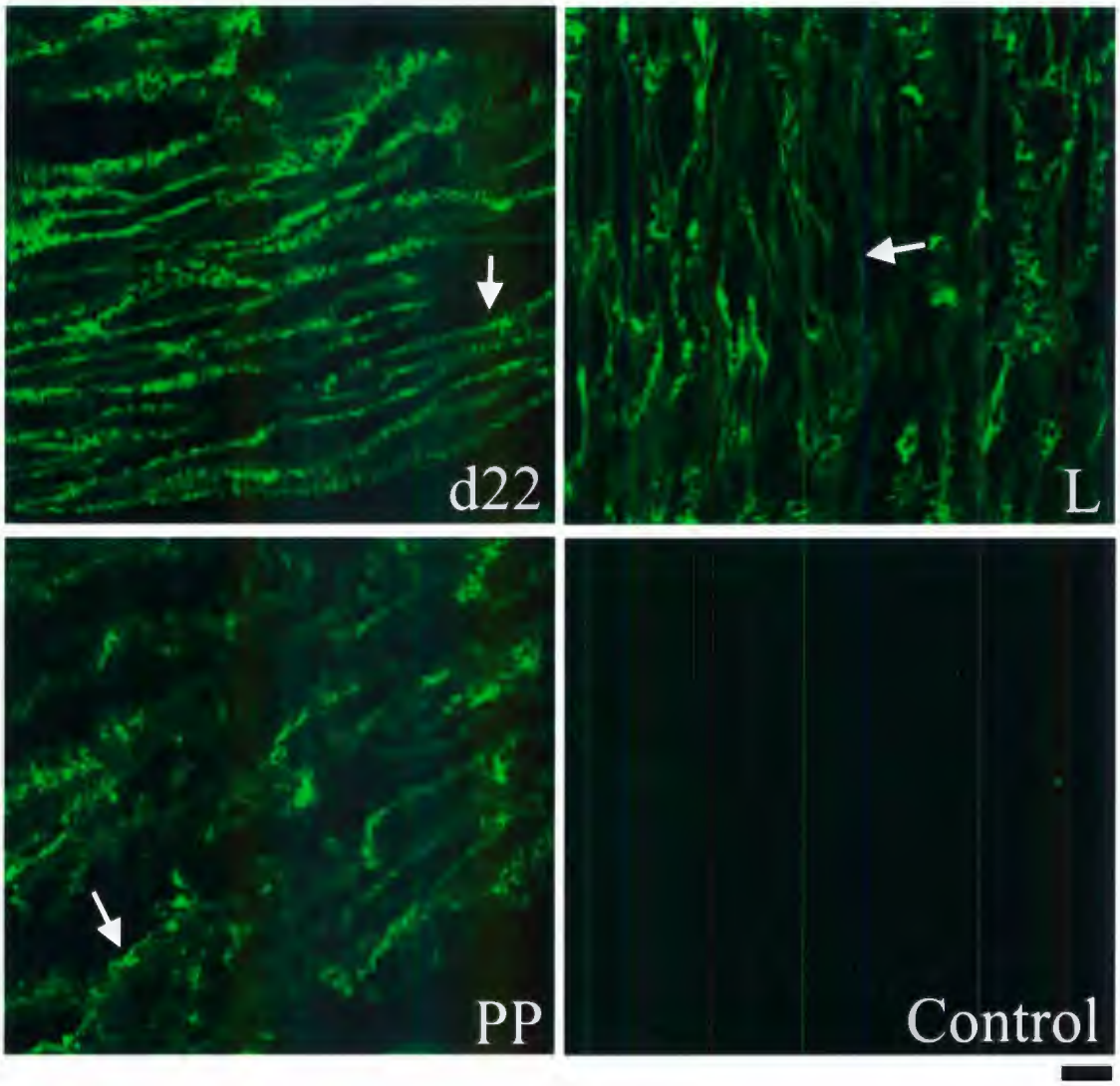

d22
L
PP
Control

Figure 2.4. Immunolocalization of ITGA5 protein in the circular smooth muscle layer of rat myometrium during the nonpregnant state and throughout gestation. The first six panels show NP-Day 21 as labeled; the last four panels show Day 22-PP and control, as labeled. Numbers represent gestational timepoints. L, Labour (2-3 pups born), Control, rabbit IgG. Arrows highlight membrane-specific staining. Scale bar = 10μm. (Williams *et al.*, Biology of Reproduction, 2005, 72, 1114 – 1124)


NP
d6
d15
d17
d19
d21


d22
L
PP
Control

significantly PP. Specifically, there was no significant decrease of *Itga5* mRNA levels in progesterone-treated animals on day 24 compared with vehicle-treated animals during labour (Two-way ANOVA, $p>0.05$; $n=3$; Fig. 2.5). Immunoblot analysis also demonstrated that ITGA5 levels in myometrial tissue lysates were maintained and similar in progesterone-treated animals on day 24 compared with vehicle-treated animals during labour (Two-way ANOVA, $p>0.05$; $n=4$; Fig. 2.6).

2.3.2.2 Immunocytochemical Detection of ITGA5

ITGA5 in the longitudinal muscle layer was continuously localized around myometrial cell membranes and detection was maintained on day 24 (Fig. 2.7). In addition, ITGA5 in progesterone-treated animals consistently appeared to be accumulating at high levels in cell membranes on days 23 and 24, compared to vehicle controls, giving the appearance of thicker membranes. In the circular muscle layer, ITGA5 was also primarily localized to myometrial cell membranes and expression was sustained on day 24 (Fig. 2.8). Similar to the longitudinal muscle layer, ITGA5 in progesterone-treated animals consistently appeared to be accumulating at high levels in cell membranes on days 23 and 24, compared to vehicle controls, however the increased detection was primarily in the form of punctate staining.

2.4 Discussion

2.4.1 Normal Pregnancy and Labour

2.4.1.1 Comparison of *Itga5* and *Fn* Gene Expression

Figure 2.5. Northern blot analysis of *Itga5* mRNA expression in a delayed-labour model following administration of progesterone or corn oil (vehicle control) to pregnant rats. (A) Representative northern blots of *Itga5* mRNA expression and *Rn18s* rRNA expression. (B) Densitometric analysis illustrating the maintenance of *Itga5* mRNA expression at day 24 of gestation. Values are from 3 independent experiments (n=3) ± SE. P4, Progesterone; L, active labour (2-3 pups born). Designations 21-Oil, 22-Oil, L-Oil, 21-P4, 22-P4, 23-P4 and 24-P4 represent gestational time-points in the two treatment groups. (Williams *et al.*, Biology of Reproduction, 2005, 72, 1114 – 1124)

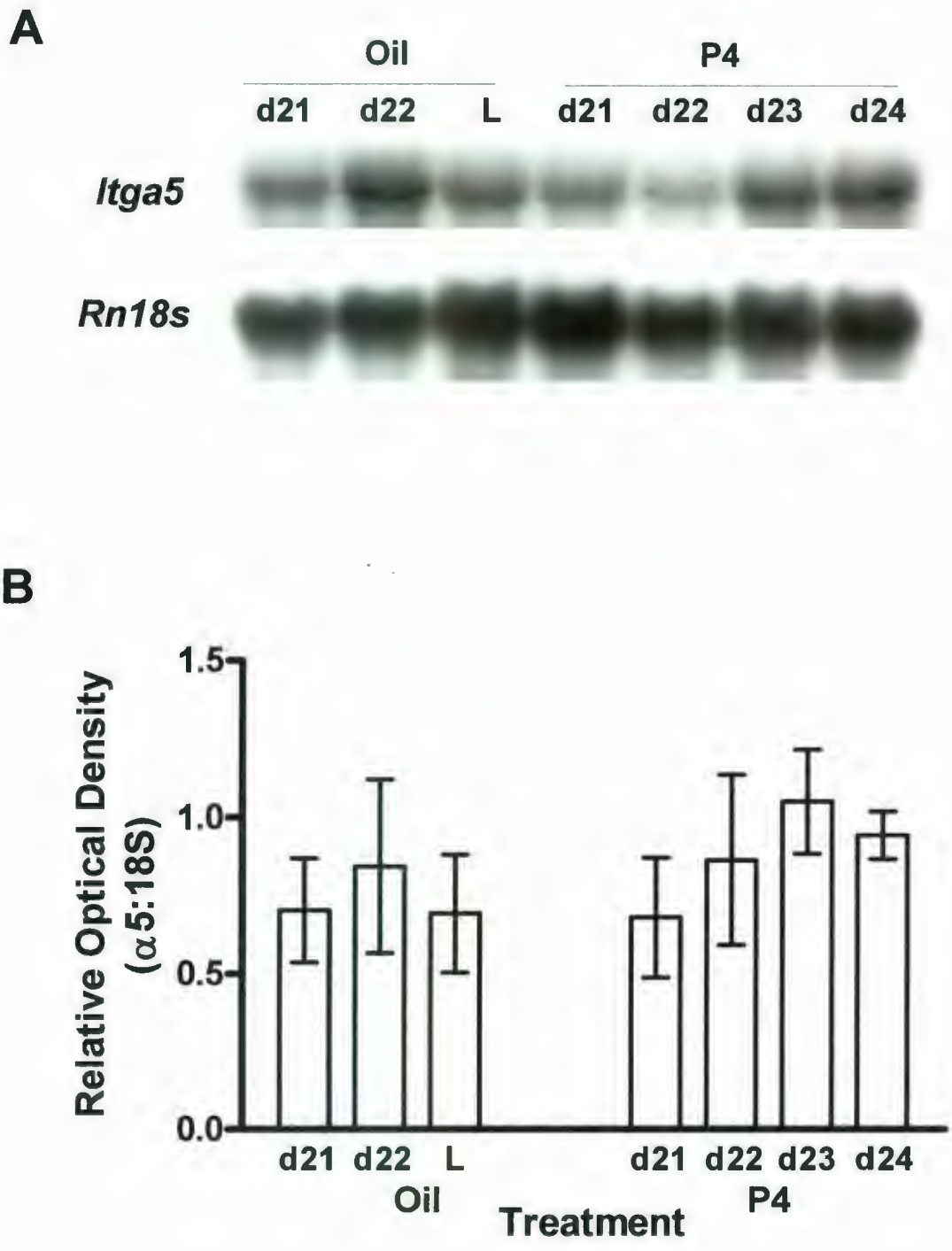
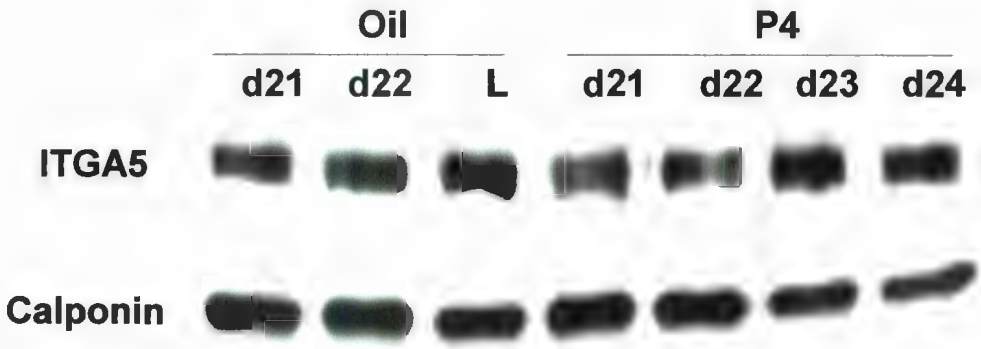

A
Oil
P4
d21
d22
L
d21
d22
d23
d24
Itga5
Rn18s
B
Relative Optical Density
(α5:18S)
1.5
1.0
0.5
0.0
d21
d22
L
d21
d22
d23
d24
Oil
P4
Treatment

Figure 2.6. Immunoblot analysis of ITGA5 protein expression in a delayed-labour model following administration of progesterone or corn oil (vehicle control) to pregnant rats. (A) Representative immunoblots of ITGA5 and CNN protein expression. (B) Densitometric analysis illustrating maintenance of ITGA5 protein expression on day 24 of gestation. Values are from 4 independent experiments (n=4) ± SE. P4, Progesterone; L, active labour (2-3 pups born). Designations 21-Oil, 22-Oil, L-Oil, 21-P4, 22-P4, 23-P4 and 24-P4 represent gestational time-points in the two treatment groups. (Williams *et al.*, Biology of Reproduction, 2005, 72, 1114 – 1124)

A


Oil
P4
d21
d22
L
d21
d22
d23
d24
ITGA5
Calponin

B

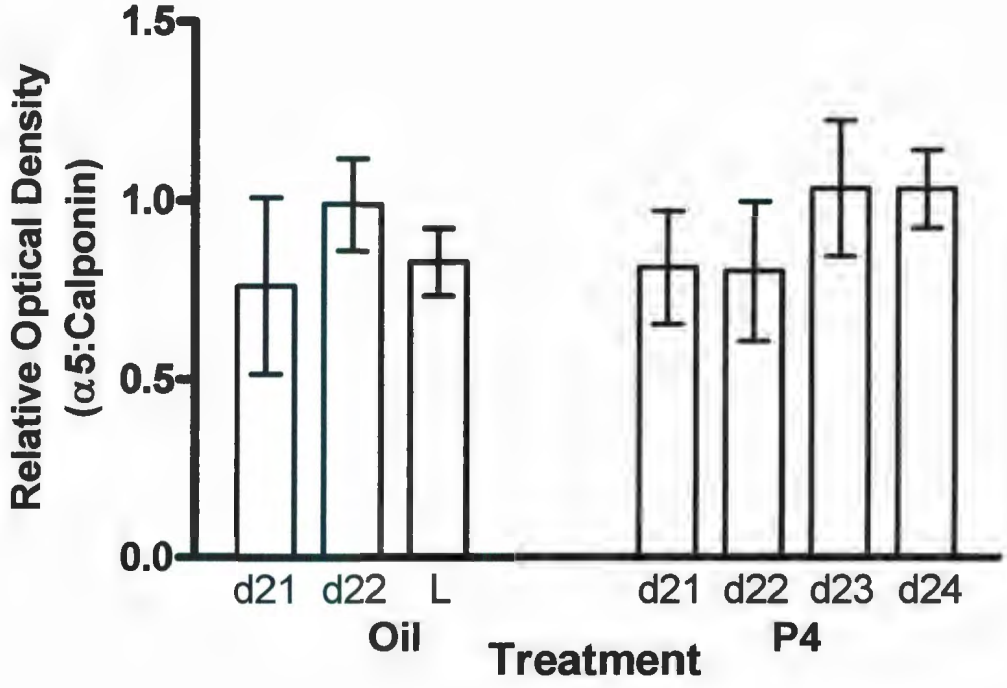

Relative Optical Density
(α5:Calponin)
1.5
1.0
0.5
0.0
d21
d22
L
d21
d22
d23
d24
Oil
P4
Treatment

Figure 2.7. Immunocytochemical analysis of ITGA5 protein expression in the longitudinal smooth muscle layer of rat myometrium in a delayed-labour model following administration of progesterone (4mg in 0.2ml corn oil sc) or oil (vehicle control; 0.2ml corn oil sc). P, Progesterone; O, Oil; L, active labour (2-3 pups born); Control, rabbit IgG. Days 21-O, 22-O, L-O, 21-P, 22-P, 23-P and 24-P represent gestational time-points. Arrows highlight localization of ITGA5 protein at cell membranes. Scale bar = 15μm. (Williams *et al.*, Biology of Reproduction, 2005, 72, 1114 – 1124)

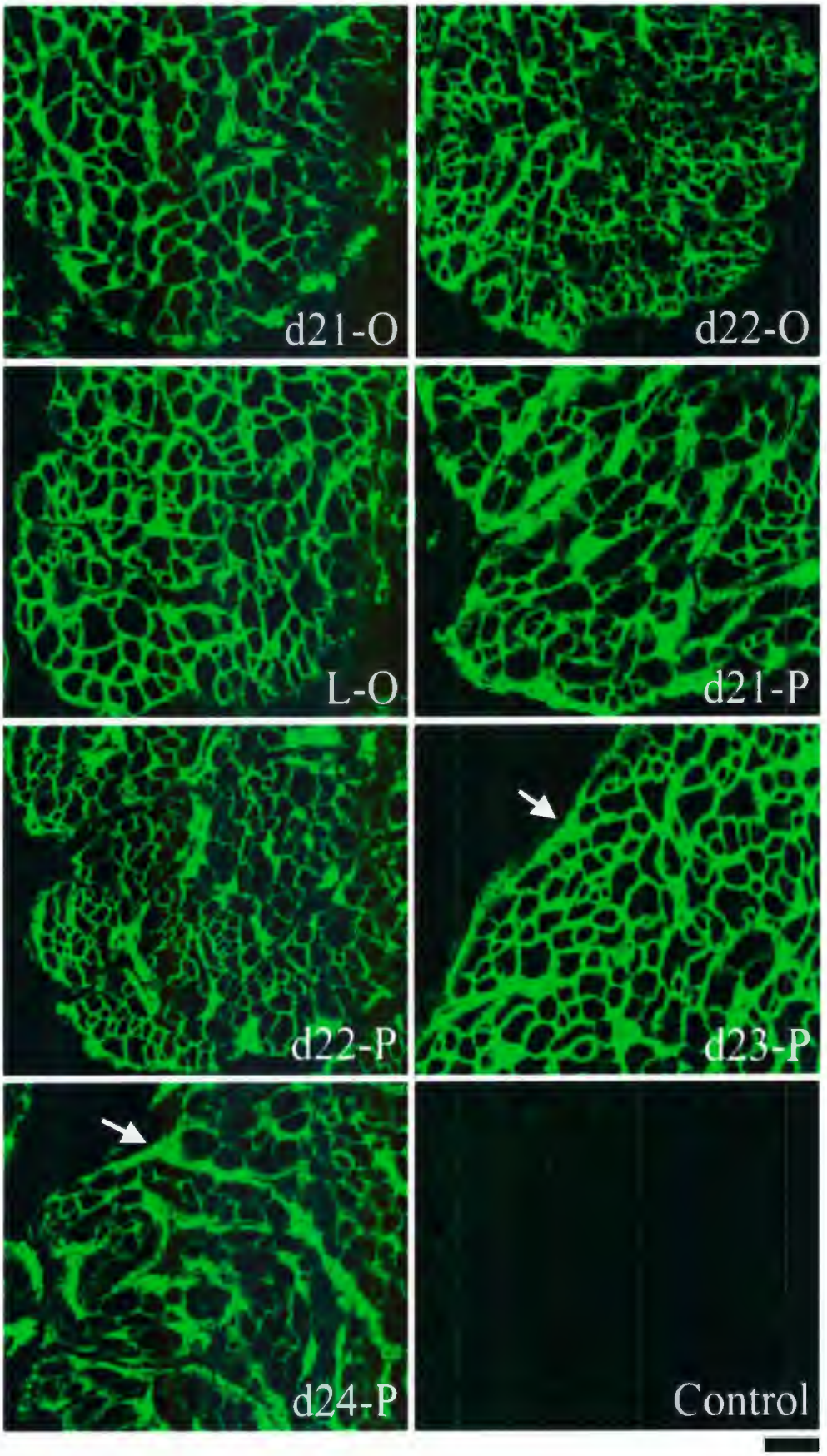
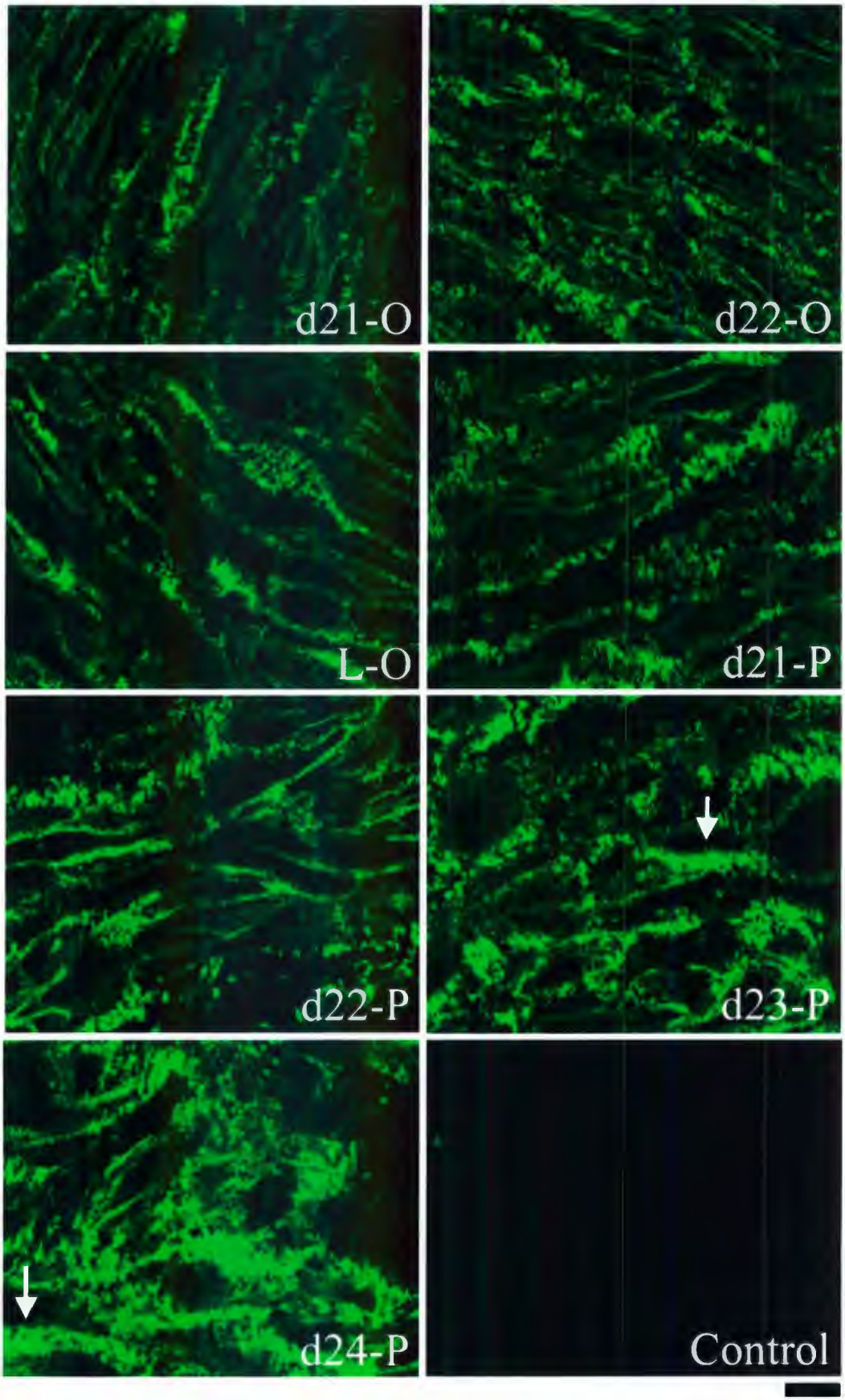

d21-O
d22-O
L-O
d21-P
d22-P
d23-P
d24-P
Control

Figure 2.8. Immunocytochemical analysis of ITGA5 protein expression in the circular smooth muscle layer of rat myometrium in a delayed-labour model following administration of progesterone (4mg in 0.2ml corn oil sc) or oil (vehicle control; 0.2ml corn oil sc). P, Progesterone; O, Oil; L, active labour (2-3 pups born); Control, rabbit IgG. Days 21-O, 22-O, L-O, 21-P, 22-P, 23-P and 24-P represent gestational time-points. Arrows highlight localization of ITGA5 protein at cell membranes. Scale bar = 15μm. (Williams *et al.*, Biology of Reproduction, 2005, 72, 1114 – 1124)


d21-O
d22-O
L-O
d21-P
d22-P
d23-P
d24-P
Control

Our northern blot analysis demonstrated that a significant increase in *Itga5* mRNA expression by day 17 of gestation preceded the reported increase in *Fn* mRNA expression just prior to labour [Shynlova *et al.*, 2004]. MacPhee and Lye [2000] previously reported that the tyrosine kinase PTK2 and a PTK2-binding adapter protein named paxillin (PXN) were highly activated and tyrosine phosphorylated in rat myometrium, respectively, by day 21 of gestation, indicative of a significant period of focal adhesion remodelling at this time to support stretch and hormonally-induced myometrial hypertrophy. Since integrins can be components of focal adhesions, induction of *Itga5* mRNA expression may be required for subsequent translation into ITGA5 protein to aid this focal adhesion turnover and reorganization prior to labour.

Our immunoblot analysis demonstrated that ITGA5 was readily detectable in myometrial tissue lysates during late pregnancy and labour reaching statistically significant levels at labour. Although immunocytochemical analysis demonstrated that ITGA5 in the longitudinal muscle layer was highly detected around individual myometrial cells (membrane localized) at all gestational timepoints examined, ITGA5 on NP and day 6 was almost undetectable in the circular muscle layer while detection on days 15 and 17 was low with a more punctate staining pattern compared to later timepoints. Therefore, detection of significantly more ITGA5 at labour versus NP by immunoblot analysis may reflect the fact that ITGA5 is primarily expressed in only longitudinal muscle in NP rat myometrium unlike the rat myometrium at labour. Furthermore, increased detection of ITGA5 by day 19 in the circular muscle layer would appear to be relatively coordinated with increased levels of *Itga5* mRNA by day 17 of

gestation. However, the lack of significant changes in protein expression in the longitudinal muscle layer and after day 19 in the circular muscle layer that would closely correlate with the significant elevation of *Itga5* mRNA expression prior to and during labour, suggests that *Itga5* expression may be translationally regulated. It is also possible that an increase in ITGA5 protein turnover during late pregnancy and labour, perhaps in concert with focal adhesion remodeling during late pregnancy, may be occuring. Nonetheless, the immunoblot and immunocytochemistry data do compare favourably with the reported increased immunofluorescent detection of fibronectin around individual hypertrophic smooth muscle cells by days 16 and 19 of rat pregnancy, in accordance with increased thickness of basement membranes [Nishinaka and Fukuda, 1991].

One day PP, ITGA5 continued to be readily detectable and localized around individual myometrial cells, in contrast to analysis of fibronectin localization 3 days post-partum in the rat which demonstrated that fibronectin became localized in discontinuous areas of myometrial cell membranes similar to localization in non-pregnant and early pregnant (day 7 and day 10) rat myometrium [Nishinaka and Fukuda, 1991]. We believe that these differences in immunolocalization patterns, compared to late pregnancy and labour, are simply due to the significant temporal differences in post-partum analysis of these proteins.

Because ITGA5 partners solely with ITGB1 in cell membranes to form a fibronectin receptor [Reddy and Mangale, 2003], and ITGB1 is detectable throughout gestation in myometrial lysates by immunoblot analysis (data not shown), our results as a whole suggest that ITGA5B1 – FN interaction is occurring during late pregnancy and

labour in the rat. This interaction is likely a significant event during late pregnancy and labour because ITGA5B1 is unique among the FN-binding integrins in that it is the only integrin that naturally assembles FN into a fibrillar matrix [Robinson *et al.*, 2003].

Post-translational regulation of integrin proteins has been suggested to be required for the function of the heterodimers. It has been shown that glycosylation of ITGA5B1 is required for heterdimer formation and proper integrin-matrix interactions [reviewed in Gu *et al.*, 2004; Bellis, 2004]. Phosphorylation has also been suggested as a post-translational modification regulating integrin function [reviewed in Leggate and Fassler, 2009]. It is possible that these modifications are required for integrin heterodimer function and matrix elabouration in vivo in the myometrium during pregnancy and labour; however, they have not been investigated.

Late pregnancy is a period of increasing uterine stretch due to growing fetuses and, as a result, stretch-induced myometrial hypertrophy [Goldspink and Douglas, 1988]. Therefore, existing ITGA5 proteins in myometrial cell membranes may act as the initial mechanical stress gauges during late pregnancy and be involved in signal transduction pathways that ultimately induce additional *Itga5* mRNA expression, cytoskeletal reorganization and *Fn* gene expression. This process is likely aided by the known association of integins with the actin cytoskeleton via adapter proteins [Horwitz *et al.*, 1986; Lui *et al.*, 2000], facilitating the sensing of mechanical forces both from the exterior and interior of the cell. Considerable evidence has accumulated that mechanical stress induces integrin gene expression and ECM synthesis/remodeling [Stewart *et al.*, 1995; Wernig *et al.*, 2003].

2.4.2 Progesterone-Induced Delayed Labour

In our study, we demonstrated that *Itga5* mRNA expression was maintained at high levels on day 24 during progesterone-induced delayed labour. Further work is clearly required to determine whether progesterone and/or any number of other biochemical signals are potential regulators of *Itga5* mRNA expression during pregnancy, labour or post-partum; however, it is possible that the prolongation of the gravid state of the uterus may be the mechanism underlying sustained *Itga5* mRNA expression. Additional stretch and stretch-induced hypertrophy, as a result of delayed labour, may maintain *Itga5* mRNA expression on day 24 since recent work by Shynlova et al [2004] has demonstrated that gravidity positively regulates *Fn* mRNA expression.

While our immunoblot analysis demonstrated the maintenance of ITGA5 levels in myometrial tissue lysates on day 24 during delayed labour, immunocytochemistry experiments showed ITGA5 was consistently detectable at higher levels in cell membranes of both myometrial layers on days 23 and 24 in progesterone-treated animals compared to vehicle controls. These results suggest an increased recruitment of ITGA5 to cell membranes on days 23 and 24 of delayed labour, likely to facilitate increased ITGA5B1-FN interaction during a period of extended gravidity, subsequent myometrial stretch and hypertrophy, and increased focal adhesion remodeling marked by sustained PTK2 activation during delayed labour [MacPhee and Lye, 2000]. The increased localization of ITGA5 in cell membranes was not reflected in increased total ITGA5 levels from our immunoblot analysis. We postulate that these results might be explained

by increased ITGA5 turnover during delayed labour. Newly translated ITGA5 might be quickly recruited to membranes to replace ITGA5 proteins that were degraded during focal adhesion remodeling and maintain relatively comparable total myometrial ITGA5 levels in the process.

2.4.3 The Role of α5 Integrin in Promoting Contractility

While there is evidence that integrins and their ligands can regulate smooth muscle contractility, largely through changes in intracellular Ca^{2+} concentration [Wu *et al.*, 2001; Chan *et al.*, 2001], our results demonstrate that considerable expression of *Itga5* mRNA and ITGA5 protein occurs many days before labour occurs, implying that ITGA5 may have a broader role during late pregnancy. We propose that ITGA5, partnered with ITGB1, is important during late pregnancy in promoting cellular cohesion. Robinson et al [2003] has elegantly demonstrated that ITGA5B1 confers stronger cohesivity to 3D tissue aggregates than that conferred by N-cadherin; a member of the Cadherin family that are traditionally regarded as a primary regulator of tissue cohesivity. This cohesivity appears to be dependent on FN matrix assembly [Robinson *et al.*, 2004], a process that requires activation by binding to ITGA5B1 and integrin clustering [Wierzbicka-Patynowski and Schwarzbauer, 2003].

Recently, Kuo and Seow [2004] used electron microscopic and functional evidence to report that airway smooth muscle cells in a tissue bundle work as a mechanical syncytium during contraction, dependent on correct cytoskeletal filament organization, focal adhesion formation, and cell-cell and cell-ECM interaction.

Therefore, in the rat myometrium during late pregnancy ECM remodelling, focal adhesion turnover, increased expression of *Itga5* and subsequent ITGA5B1 -FN interaction may facilitate proper smooth muscle cellular cohesion in myometrial tissue prior to labour. These processes may be components of myometrial activation by contributing to the development of a mechanical syncytium that will facilitate efficient force transduction of the sustained, coordinated and powerful contractions of labour.

CHAPTER 3

Temporal and Spatial Expression of ITGA1, ITGA3 and ITGB1 Subunits in Rat Myometrium During Pregnancy and Labour

(In Preparation)

3.1 Introduction

The onset of coordinated, high frequency and high amplitude uterine smooth muscle contractions (labour) is precisely controlled to ensure the timely delivery of a term fetus whose organ systems are sufficiently mature for extrauterine survival. Signals that contribute to the initiation of labour are known to reside, at least in part, in the fetal genome and originate within two pathways- an endocrine and a mechanical pathway [Challis and Lye, 2004; Gibb *et al.*, 2006]. The signals produced in these endocrine and mechanical pathways ultimately lead to a phenotypic switch or *activation* of the myometrium during late pregnancy - from a muscle that is quiescent to one which is spontaneously active, excitable, highly responsive to uterine agonists, and exhibits a high degree of cell-cell coupling [Challis and Lye, 2004]. This myometrial *activation* results from the increased expression of contraction associated proteins in this tissue such as ion channels, agonist receptors and gap junctions; however, myometrial growth and subsequent remodelling of the actin cytoskeleton and cell-ECM contacts during late pregnancy also appear to be key aspects of myometrial *activation* [MacPhee and Lye, 2000; Challis and Lye, 2004; Shynlova et al, 2005; Breuiller-Fouche and Germain, 2006].

For the mechanical pathway to exist, uterine myocytes require a mechanism to sense mechanical signals and create biochemical responses to them. It has been suggested that focal adhesions (i.e. smooth muscle dense plaques) are these sites of mechanotransduction [MacPhee and Lye, 2000; Challis and Lye, 2004]. Focal adhesions are comprised of clusters of integrins that occur at sites where the cell makes contact with the ECM. Integrins are heterodimeric, transmembrane receptors composed of a α and a β subunit and the combinations of these subunits form various ECM receptors [Reddy and Mangale, 2003]. Evidence exists that these focal adhesions reorganize during pregnancy to support myometrial hypertrophy by allowing cells to properly anchor to their ECM [MacPhee and Lye, 2000]. Integrins, and their ECM ligands, have also been previously implicated in initiating signaling events that alter the contractility of smooth muscle cells. For example, the binding of RGD peptides to integrins induced the release of intracellular Ca^{2+} from Ca^{2+} stores in renal vascular smooth muscle cells [Chan *et al.*, 2001]. Furthermore, stimulation of contraction in canine tracheal smooth muscle cells caused an increase in the association of ITGB1 with α-actinin, a known focal adhesion protein that binds to both integrins and actin filaments [Zhang and Gunst, 2006].

Individual myometrial smooth muscle cells are surrounded by an ECM that is composed of structural proteins, such as collagens, and adhesion molecules such as fibronectin and laminin [Gibb *et al.*, 2006]. Late pregnancy is associated with extensive remodeling of the ECM and the increased expression of mRNA and protein of major components of smooth muscle basement membrane, laminin and type IV collagen [Shynlova et al, 2004; Nishinaka & Fukuda, 1991]. In addition, increased deposition of

type IV collagen and laminin around smooth muscle cells has been demonstrated in the rat myometrium during late pregnancy [Nishinaka & Fukuda, 1991].

While both ITGA1B1 and ITGA3B1 have been reported to have affinity for both laminin and collagen [Wayner and Carter, 1987; Kramer and Marks, 1989; Forsberg *et al.*, 1990], it appears that ITGA1B1 is the major receptor for collagen and ITGA3B1 is the major receptor for laminin [Hynes, 2002; Wiesner et al, 2005]. There have been reports of integrin expression in nonpregnant human myometrium [Taylor *et al.*, 1996], and we have previously reported an increase in ITGA5 subunit detection during late pregnancy and labour in the rat myometrium and that uterine stretch regulates temporal and spatial expression of ITGA5 [Williams *et al.*, 2005, Shynlova *et al.*, 2007]. Importantly, there have been no reports of ITGA1, ITGA3 and ITGB1 expression in uterine smooth muscle during pregnancy and labour. With the importance of these integrins in binding collagen and laminin, the goal of this study was to investigate the expression of ITGA1, ITGA3 and ITGB1 in non-pregnant, pregnant, and post-partum rat myometrium.

3.2 Material and Methods

3.2.1 Animals

Sprague-Dawley rats were obtained from the Mount Scio Vivarium (Memorial University of Newfoundland, St. John=s, Newfoundland, Canada). Animals were individually housed and cared for under standard environmental conditions (12 hour light and 12 hour dark) in the Animal Care Unit at the Health Sciences Centre, Memorial

University of Newfoundland. Rats were fed LabDiet Prolab RMH 3000 (PMI Nutrition International, Brentwood, Missouri, USA) and water *ad libitum*. The institutional animal care committee approved all experiments under animal care protocols 02-02-DM - 02-05-DM. Virgin female rats (~220g each) were mated with stud males and observation of vaginal plugs the following morning was designated day 1 post coitum. The time of delivery under these standard conditions was day 23 of gestation.

3.2.2 Tissue Collection

All animals were killed by carbon dioxide inhalation on the desired day of sampling. The estrous cycle stage was not determined prior to obtaining nonpregnant samples. Labour samples were taken during active labour and only after the rat had delivered 2-3 pups. Uterine horns were removed, opened longitudinally, and fetuses and placentas discarded. The endometrium was carefully removed by scraping the luminal surface of the uterus, and myometrial tissue was flash-frozen in liquid nitrogen and stored at -70°C or fixed overnight in zinc buffered fixative (ZBF; 100mM Tris buffer pH 7.4, 3mM calcium acetate, 27mM zinc acetate, 37mM zinc chloride; Beckstead, 1994; Williams *et al.*, 2005) while shaking at room temperature. Tissues for immunofluorescence experiments were embedded in paraffin, sectioned and mounted on microscope slides by the Histology Unit of the Faculty of Medicine at Memorial University of Newfoundland.

3.2.3 Northern Blot Analysis

RNA isolation and Northern blot analysis was performed according to methods previously described in detail [Williams et al., 2005]. Four separate, independent sets of RNA samples were used (n=4, i.e. 4 rats used per gestational timepoints) for analysis.

The pBS-SK$^-$ vector containing the rat *Itga1* cDNA was generously provided by Dr. Louis Reichardt (University of California, San Francisco, San Francisco, California). Digestion of pBS-SK$^-$ with restriction endonuclease EcoRI resulted in the production of 2 fragments including a 4kb *Itga1* cDNA fragment. The pBS-SK$^+$ vector containing the mouse *Itga3* cDNA (CD7) was generously provided by Dr. Tsutomu Tsuji (Hoshi University School of Pharmaceutical Sciences, Tokyo, Japan). Digestion of pBS-SK$^+$ with restriction endonucleases SalI and BamHI resulted in the production of 3 fragments including a 3kb *Itga3* cDNA fragment. The mouse *Itga3* cDNA (Genbank accession number NM_013563) was found to have 93% identity with the rat *Itga3* cDNA (Genbank accession number XM_340884). The pBJ-1 vector containing the human *ItgB1* cDNA was generously provided by Dr. Lucia Languino (University of Massachusetts Medical School, Worcester, MA). Digestion of pBJ-1 with restriction endonuclease EcoRI resulted in the production of 2 fragments including a 3kb *ItgB1* fragment. The human *ItgB1* integrin cDNA (Genbank Accession # NM_002211) was found to have 86% identity with the rat *ItgB1* cDNA (Genbank Accession # NM_017022). Fragments were subsequently isolated with a Qiagen Gel Extraction Kit (Qiagen, Inc, Mississauga, Ontario, Canada), according to instructions provided by the manufacturer, and utilized for the production of random primed radiolabelled cDNA probes as previously described [Williams et al., 2005].

Northern blots were pre-hybridized in hybridization buffer for 1-2 hours at 55EC in a hybridization oven (Hybaid Instruments, Franklin, Massachusetts). Hybridization was performed overnight at 55EC. Blots were then washed once for 15 min and three times 5 min at 55EC in 2X SSC and 0.1% SDS and then exposed to Cyclone Storage Phosphor Screens (PerkinElmer, Boston, MA, USA).

Following analysis of *Itga1, Itga3 and Itgb1* mRNA expression, northern blots were analyzed for expression of 18S rRNA (*Rn18S*), utilizing a rabbit 18S ribosomal cDNA template generously provided by Dr. I. Skerjanc (University of Western Ontario, London, Ontario, Canada). *Rn18S* is constitutively expressed in rat myometrial cells and has been utilized, in the past, as a loading control for analysis of myometrial gene expression [Shynlova *et al.*, 2004; White *et al.*, 2005].

3.2.4 Real Time-Polymerase Chain Reaction (PCR) Analysis

Total RNA was extracted from the frozen tissues (4 different sets) using TRIZOL (Gibco BRL, Burlington, ON) according to manufacturer's instructions. RNA samples were column purified using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada), and treated with 2.5µl DNase I (2.73 Kunitz units/µl, Qiagen) to remove genomic DNA contamination. Reverse transcription (RT) and real-time PCR (RT-PCR) was performed to detect the mRNA expression of *Itga1, Itga3* and *Itgb1* subunit genes in rat myometrium. 2µg of total RNA was primed with random hexamers to synthesize single-strand cDNAs in a total reaction volume of 100µl using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as described earlier

[Shynlova et al, 2006]. 20 ng of cDNA from the previous step was subjected to RT- PCR using the following specific sets of primers designed using Primer Express software (Version 2.0.0, Applied Biosystems): ***Itga1*** (GenBank accession #NM_030994), 5'-TCTGCCAAACTCAGTCCACGA-3'(sense primer) and 5'-TGACGATCAGCAGGCTCTTTT-3' (antisense primer); ***Itga3*** (GenBank accession #NM_013565), 5'- TGACTACTTGCAGACCGGCAT-3' (sense primer) and 5'-CAGTCCTTCCGCTGAATCATG-3' (antisense primer); ***Itgb1*** (GenBank accession #NM_017022), 5'-CAGAAGGTGGCTTTGATGCAA-3'(sense primer) and 5'-AACCCAGCATCCGTGGAAA -3' (antisense primer);*Rn18S* (GenBank accession #X01117), 5'-GCGAAAGCATTTGCCAGAA-3' (sense primer) and 5'-GGCATCGTTTATGGTCGGAAC-3' (antisense primer). A total reaction volume of 25μl was utilized for these assays (Applied Biosystems). RT-PCR was performed in an optical 96-well plate with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), using the SYBR Green detection chemistry. The run protocol was as follows: initial denaturation stage at 95°C for 10min, 40 cycles of amplification at 95°C for 15sec and 60°C for 1min. After PCR, a dissociation curve was constructed by increasing temperature from 65°C to 95°C for detection of PCR product specificity. In addition, a no-template control (H_2O control) was analyzed for possible contamination in the master-mix. A cycle threshold (Ct) value was recorded for each sample. PCR reactions were set up in triplicates and the mean of the 3 Cts was calculated. A comparative Ct method (delta delta (ΔΔ) Ct method) was applied to the raw Ct values to find a relative gene expression across normal gestation. To obtain experimental results,

the expression of an individual gene at every gestational day was (1) normalized to *Rn18S* and (2) a fold change was calculated relative to the expression of the same gene in the corresponding NP sample using an arithmetic formula (see ABI User Bulletin #2). Validation experiments were performed to ensure the PCR efficiencies between the target genes and 18S were approximately equal.

3.2.5 Immunoblot Analysis

Protein extraction and immunoblot analysis were performed according to methods previously described in detail [Williams et al., 2005; White et al., 2005]. Four separate, independent sets of protein samples were used (n=4, i.e. 4 rats used per gestational timepoint) for analysis. Protein samples (50 ug/lane) were separated under reducing conditions by polyacrylamide gel electrophoresis in 9% resolving gels. Appropriate primary antisera (Table 3.1) were incubated with blots for 1 hour at room temperature with constant agitation and then blots were rinsed with Tris-buffered saline-Tween-20 (TBST; 20mM Tris base, 137 mM NaCl, pH 7.6) with 0.1% Tween-20). Immunoblots were incubated in horseradish peroxidase (HRP)-conjugated secondary antisera (Table 3.1) for 30 min at room temperature with constant agitation and then rinsed in TBST. Proteins were detected using the Pierce SuperSignal West Pico Chemiluminescent Substrate (MJS BioLynx, Inc., Brockville, Ontario, Canada) detection system and multiple exposures were generated to ensure the linearity of the film exposures.

Following immunoblot analysis of ITGA1, ITGA3 or ITGB1 integrin expression, analysis of calponin protein expression was subsequently performed. We have

Table 3.1. Antisera utilized for Immunoblot and Immunofluorescence Experiments

Antisera	Method	Dilution	Company	Catalogue #
Rabbit anti-α1	IB	1:2500	Chemicon International, Temecula, California, USA	AB1934
Rabbit anti-α1 (R-164)	IF	1:100	Santa Cruz Biotechnology Inc, Santa Cruz, California, USA	sc-10728
Rabbit anti-α3	IB	1:2500	Chemicon International	AB1920
Rabbit anti-α3	IF	1:100	Chemicon International	AB1920
Mouse anti-β1	IB	1:5000	BD Transduction Labouratories, Mississauga, Ontario, Canada	610467
Rabbit anti-β1	IF	1:100	Chemicon International	AB1952
Mouse anti-Calponin; Clone hCP	IB	1:100,000	Sigma-Aldrich, Oakville, Ontario, Canada	C2687
FITC-Sheep anti-Rabbit IgG	IF	1:200	Sigma, St. Louis, Missouri, USA	F7512
ChromPure Rabbit IgG	IF	N/A*	Jackson ImmunoResearch Labs Inc, West Grove, USA	011-000-003
HRP-Goat anti-Rabbit IgG (H+L)	IB	1:5000	Pierce, Rockford, IL, USA	31460
HRP-Goat anti-Mouse IgG (H+L)	IB	1:10,000, 1:150,000	Pierce	31430

IF, Immunofluorescence; IB, Immunoblot; FITC, Fluorescein isothiocyanate; HRP, Horseradish Peroxidase; *Dependent on concentration of primary antisera utilized.

previously demonstrated that calponin protein is constitutively expressed in non-pregnant (NP) and pregnant rat myometrial tissue under our protein extraction conditions [Williams et al., 2005; White et al., 2005].

3.2.6 Immunofluorescence Analysis

Immunofluorescence was performed according to methods previously described in detail [Williams et al., 2005; White et al., 2005]. Two separate, independently collected sets of rat tissues (n=2, i.e. 2 rats used per gestational timepoint) were utilized for immunocytochemistry experiments and experiments were repeated 2 times. For experimental purposes, two uterine tissue sections per slide for each gestational timepoint were used. All tissue sections used were 5 μm in thickness, contained both longitudinal and circular smooth muscle layers, and were processed under identical conditions (e.g. the same volumes for blocking and antisera solutions). Epitope retrieval was performed in two ways. To detect ITGA1, tissue sections were incubated in 0.125% trypsin for 15 minutes at room temperature under constant agitation. To detect ITGA3 and ITB1 proteins, tissue sections underwent heat-induced epitope retrieval using a solution of 0.01M SSC, pH 6.0. This solution was heated for approximately 3 mins (until boiling) using a microwave. Slides were immersed in the hot solution for 10 mins and then air dried for 5 mins. This was repeated an additional 7 times and then slides were rinsed in PBS. Sections were then incubated for 1 hour at room temperature in primary antisera (Table 3.1) or affinity-purified IgG of the appropriate species, at the same concentration as the primary antisera, to serve as a negative control. After three washes in PBS, the

sections were incubated with FITC-conjugated secondary antisera (Table 3.1). Sections were washed with cold PBS containing 0.02% Tween-20 (PBT) with constant agitation. Tissues were mounted in Vectashield (Vector Labouratories, Inc., Burlington, Ontario, Canada) before viewing with an Olympus Fluoview laser scanning confocal microscope (Olympus Optical Company Ltd., Melville, New York, USA).

3.2.7 Data Analysis

For RT-PCR, gestational profiles were subjected to a One-way Analysis of Variance (ANOVA) followed by pairwise multiple comparison procedures (Student-Newman-Keuls method) to determine differences between groups. Statistical analysis was carried out using SigmaStat version 3.01 (Jandel Corp., San Rafael, CA) with the level of significance for comparison set at $P<0.05$.

Densitometric analysis of Northern Blots and Immunoblots was performed with the aid of Scion Image Software (Scion Image Corporation, Frederick, Maryland, USA). Densitometric measurements of *Itga3* and *Itgb1* were normalized to those of *Rn18S*, while ITGA1, ITGA3 and ITGB1 proteins were normalized to those of CNN. Statistical analysis was performed with GraphPad Instat version 3.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com) and graphed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com). Data from Northern blot analysis of *Itga3* and *Itgb1* expression during gestation were subjected to a One-way Anova and a Tukey-Kramer Multiple Comparions test. Data from immunoblot analysis of ITGA1, ITGA3 and ITGB1 expression during gestation

were subjected to a One-way ANOVA and a Tukey-Kramer Multiple Comparisons test. Values were considered significant if $p < 0.05$.

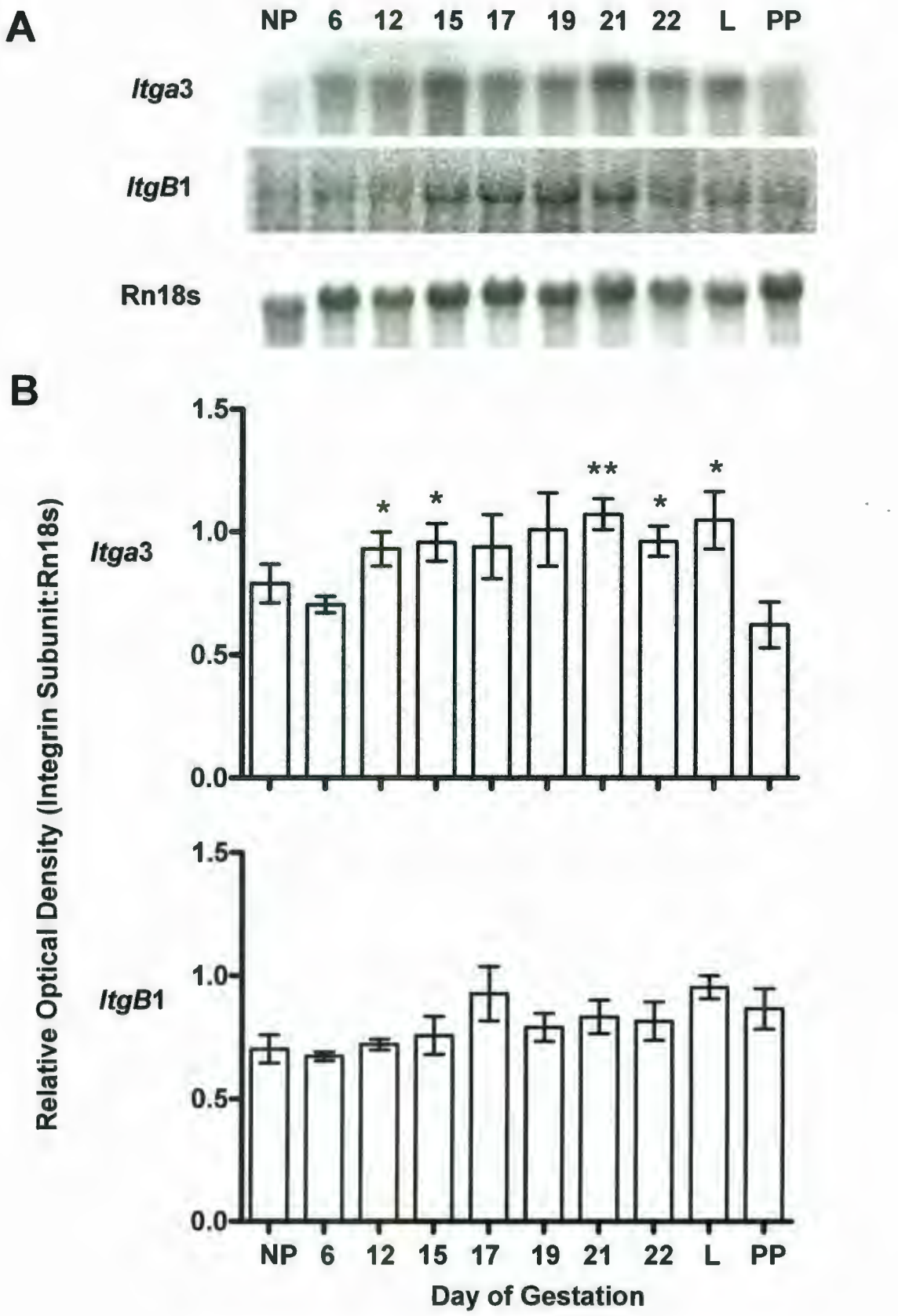
3.3 Results

3.3.1 Expression of *Itga1*, *Itga3* and *Itgb1* During Pregnancy and Spontaneous Term Labour

Northern blots of myometrial RNA isolated from non-pregnant (NP), d6, 12, 15, 17, 19, 21, 22, labour (L) and 1 day post-partum (PP) were analyzed with radiolabelled probes generated from either a rat *Itga1*-specific cDNA, a mouse *Itga3*-specific cDNA or a human *ItgB1*-specific cDNA (Figure 3.1A; *Itga1* not shown). Our experiments demonstrated that *Itga1* was not detectable under our experimental conditions, even when 3 different (2 rat and 1 human) *Itga1* cDNAs were utilized for generation of radiolabelled probes (data not shown). *Itga3* expression was found to gradually increase as gestation progressed (One-way ANOVA, $p < 0.05$, $n = 4$; Figure 3.1B). Specifically, expression levels were significantly higher on d12, d15, d22 and L compared to d6 and PP (Unpaired t-test, $p<0.05$; *), and expression on d21 was significantly higher than NP, d6 and PP (Unpaired t-test, $p<0.05$; **). While there appeared to be a slight increase in *ItgB1* expression as gestation progressed, the analysis of *ItgB1* over 4 independent sets of samples demonstrated that the increase was not statistically significant.

3.3.2 Comparative Characteristics of Integrin Gene Expression in Non-Pregnant, Pregnant and Post-Partum Rat Myometrium

Figure 3.1 Northern blot analysis of *Itga3* and *ItgB1* in rat myometrium during pregnancy, labour and 1 day postpartum. (A) Representative northern blots of *Itga3* and *Itgb1*expression and *Rn18S* expression. Analysis was performed with an *Itga3*-specific mouse cDNA, a *ItgB1*-specific human cDNA and an 18S-specific rabbit cDNA as templates for radiolabelled probe production. (B) Densitometric analysis of *Itga3* expression illustrated an increase of expression during pregnancy and labour. Expression levels were significantly higher ($p<0.05$) on d12, d15, d22 and L compared to d6 and PP (*) and expression at d21 was significantly higher than NP, d6 and PP (**). While there appeared to be a small gradual increase in *ItgB1* expression over gestation, densitometric analysis illustrated that there was no statistically significant changes in expression during pregnancy or with labour. Values are from 4 independent experiments (n=4) ± SE. Days 6, 12, 15, 17, 19, 21, 22 and labour represent gestational timepoints. NP, non-pregnant; L, active labour (2-3 pups born); PP, 1 day postpartum.


A
NP 6 12 15 17 19 21 22 L PP
Itga3
ItgB1
Rn18s
B
Relative Optical Density (Integrin Subunit:Rn18s)
Itga3
ItgB1
1.5
1.0
0.5
0.0
*
*
*
**
*
NP 6 12 15 17 19 21 22 L PP
Day of Gestation

A ΔΔCt method was used in our study to determine the gene expression for each integrin across gestation relative to the non-pregnant sample. This approach also allowed us to compare simultaneously the relative gestational changes of the three integrin genes expressed in rat myometrium in our study. *Itga1* expression was significantly increased from day (d) 6 to d23 compared to NP, 1 day post-partum (PP) and 4 days post-partum (4PP) timepoints ($p < 0.05$; Figure 3.2). In contrast, *Itga3* expression was significantly increased at d14, d21, and d22, compared to NP, d10, PP and 4PP ($p < 0.05$). *ItgB1* expression was significantly elevated throughout gestation, from d6 to d23 compared to NP and 4PP ($p < 0.05$; Figure 3.2), in a manner similar to *Itga1* expression. *Itga3* expression in non-pregnant (NP) rat myometrium was found to be the lowest among all integrins studied and therefore was chosen as a calibrator for an "all- integrins" relative gene expression study. Analysis of these data revealed *Itga1, Itga3* and *ItgB1* were characterized by very different mRNA abundances (Table 3.2). *ItgB1* expression was considerably higher than *Itga1* and *Itga3* throughout gestation. Of all the integrins we studied, the *ItgB1* demonstrated the most dramatic gestational changes and the highest expression levels in pregnant rat myometrium.

3.3.3 Detection of ITGA1, ITGA3 and ITGB1 Subunit Proteins during Pregnancy and Spontaneous Term Labour

3.3.3.1 Immunoblot Analysis

Immunoblot analysis demonstrated that ITGA1, ITGA3 and ITGB1 proteins were readily detectable in rat myometrium. Densitometric analysis revealed that there were no

Figure 3.2. The effect of gestational age on the mRNA expression of *Itga1* (A), *Itga3* (B) and *ItgB1* (C) integrin subunits in pregnant rat myometrium. Total RNAs were extracted from frozen myometrial tissues, single-strand cDNAs were synthesized as described under "Materials and Methods" and integrin mRNA levels were analyzed on the indicated days of gestation by Real-Time PCR. *Itga1* (A), *Itga3* (B) and *ItgB1* (C) integrin subunit mRNA levels (in relative fold changes) were normalized to 18S mRNAs. The bars represent mean ± SEM (n=4 at each time point). Days 6, 8, 10, 12, 14, 15, 17, 19, 21, 22 and labour represent gestational timepoints. NP, non-pregnant; L, active labour (2-3 pups born); PP, 1 day postpartum; 4PP, 4 days postpartum. Data labelled with different letters are significantly different from each other ($P < 0.05$).

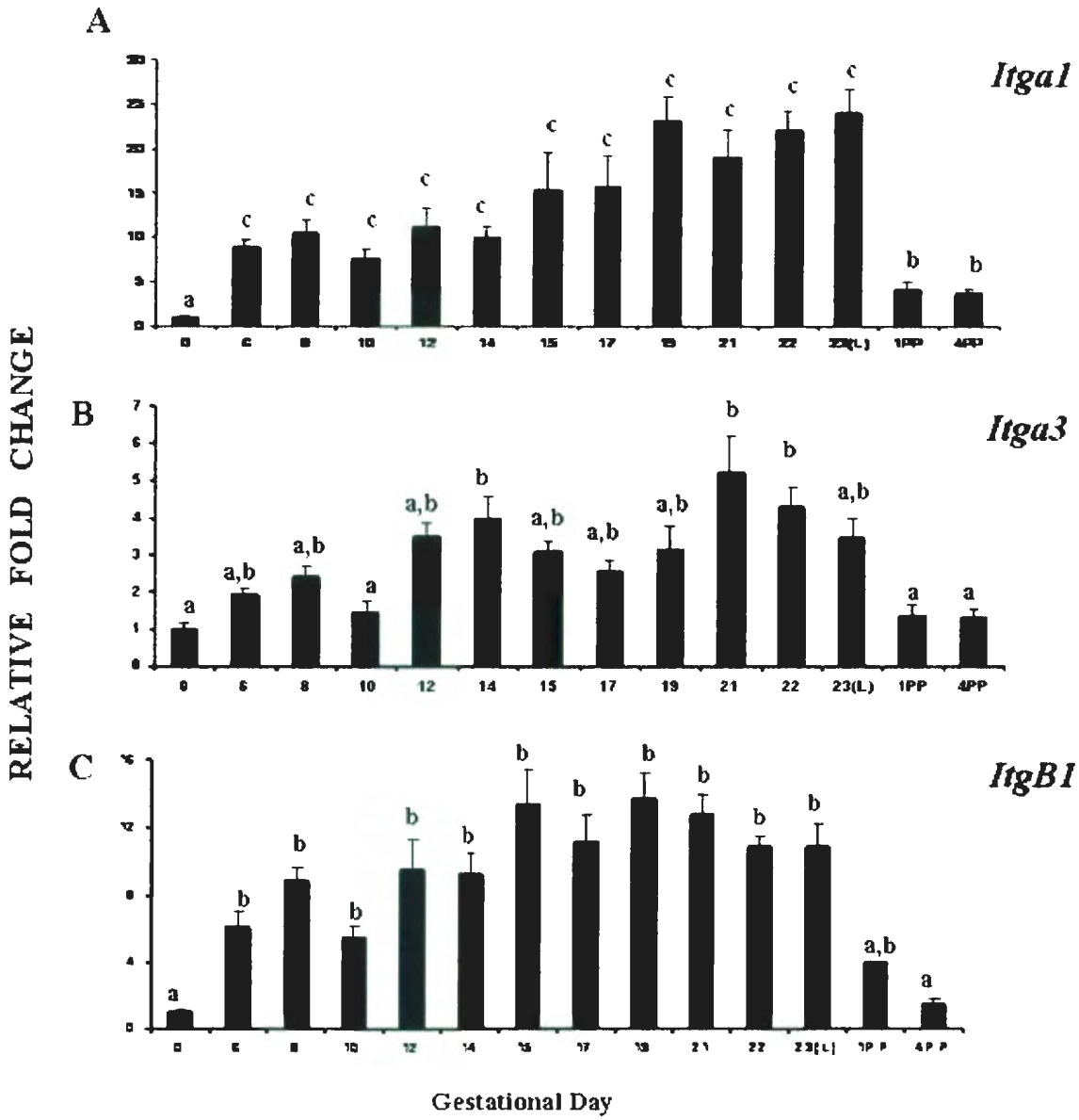

A
Itga1
B
Itga3
C
ItgB1
RELATIVE FOLD CHANGE
Gestational Day

Table 3.2. Comparative characteristics of integrin gene expression in non-pregnant, pregnant and post-partum rat myometrium. All integrin mRNA levels (recorded as a Ct values) were normalized to 18S mRNA and integrin mRNA levels are expressed in fold changes relative to integrin *Itga3* expression in NP rat myometrium. L = active labour (2-3 pups born).

Gestation	α3 integrin	α1 integrin	β1 integrin
0	1.0	15.6	100.7
6	1.9	87.2	408.6
8	2.4	101.6	596.2
10	1.5	73.5	360.4
12	3.5	108.2	632.2
14	4.0	97.8	614.8
15	3.1	149.5	896.9
17	2.6	153.7	748.0
19	3.1	226.1	916.3
21	5.2	186.2	852.0
22	4.3	214.9	725.1
23(L)	3.5	233.7	722.2
PP	1.4	40.2	264.3
4PP	1.3	36.3	98.3

temporally significant changes in total detection levels of ITGA1 during gestation (One-Way ANOVA, $p>0.05$, $n = 4$, Figure 3.3) while total detection levels of ITGA3 only decreased slightly from NP to 1 day post-partum (PP) (One-Way ANOVA, $p<0.05$, $n = 4$). Specifically, ITGA3 expression was only significantly decreased at d22 compared to NP (Tukey's Multiple Comparison Post-test, $p<0.05$, $n = 4$, Figure 3.3). In contrast, temporally there were no statistically significant changes in total ITGB1 detection levels during rat gestation (One-Way ANOVA, $p>0.05$, $n = 4$, Figure 3.3).

3.3.3.2 Immunofluorescence

In the longitudinal smooth muscle layer, ITGA1 was spatially localized to myometrial cell membranes. ITGA1 appeared to be accumulating at cell membranes on NP and by d6 of pregnancy but detection was particularly pronounced at myocyte membranes on d21 and d22, decreasing slightly at labour and more markedly at PP (Figure 3.4). In the circular smooth muscle, ITGA1 was detectable at a lower level compared to the longitudinal layer. Immunoreactive ITGA1 proteins were primarily detected in the cell cytoplasm from NP to d21 and detection increased consistently in myocyte membranes on d22. ITGA1 immunostaining remained evident in myocyte membranes at labour, but expression became more cytoplasmic at PP (Figure 3.5). During NP and d6 timepoints in the longitudinal smooth muscle layer, ITGA3 was detectable primarily in the cell cytoplasm (Figure 3.6). An increase in cytoplasmic detection was consistently observed on d15 until d19. On d21 of gestation, significant cytoplasmic detection of ITGA3 was abruptly lost and a low level of membrane

Figure 3.3. Immunoblot analysis of ITGA1, ITGA3 and ITGB1 expression in rat myometrium during pregnancy, labour and postpartum. (A) Representative immunoblots of ITGA1, ITGA3 and ITGB1 and CNN expression. (B) Densitometric analysis of ITGA1 revealed that there were no statistically significant changes over gestation. Densitometric analysis of ITGA3 illustrated a decrease in expression during late pregnancy and labour. Expression levels were significantly decreased ($p<0.05$) on d22 compared to NP (*). Densitometric analysis illustrated no significant changes in ITGB1 expression during gestation. Values are from 4 independent experiments ($n=4$) ± SE. Days 6, 12, 15, 17, 19, 21, 22 and labour represent gestational timepoints. NP, non-pregnant; L, active labour (2-3 pups born); PP, 1 day postpartum.

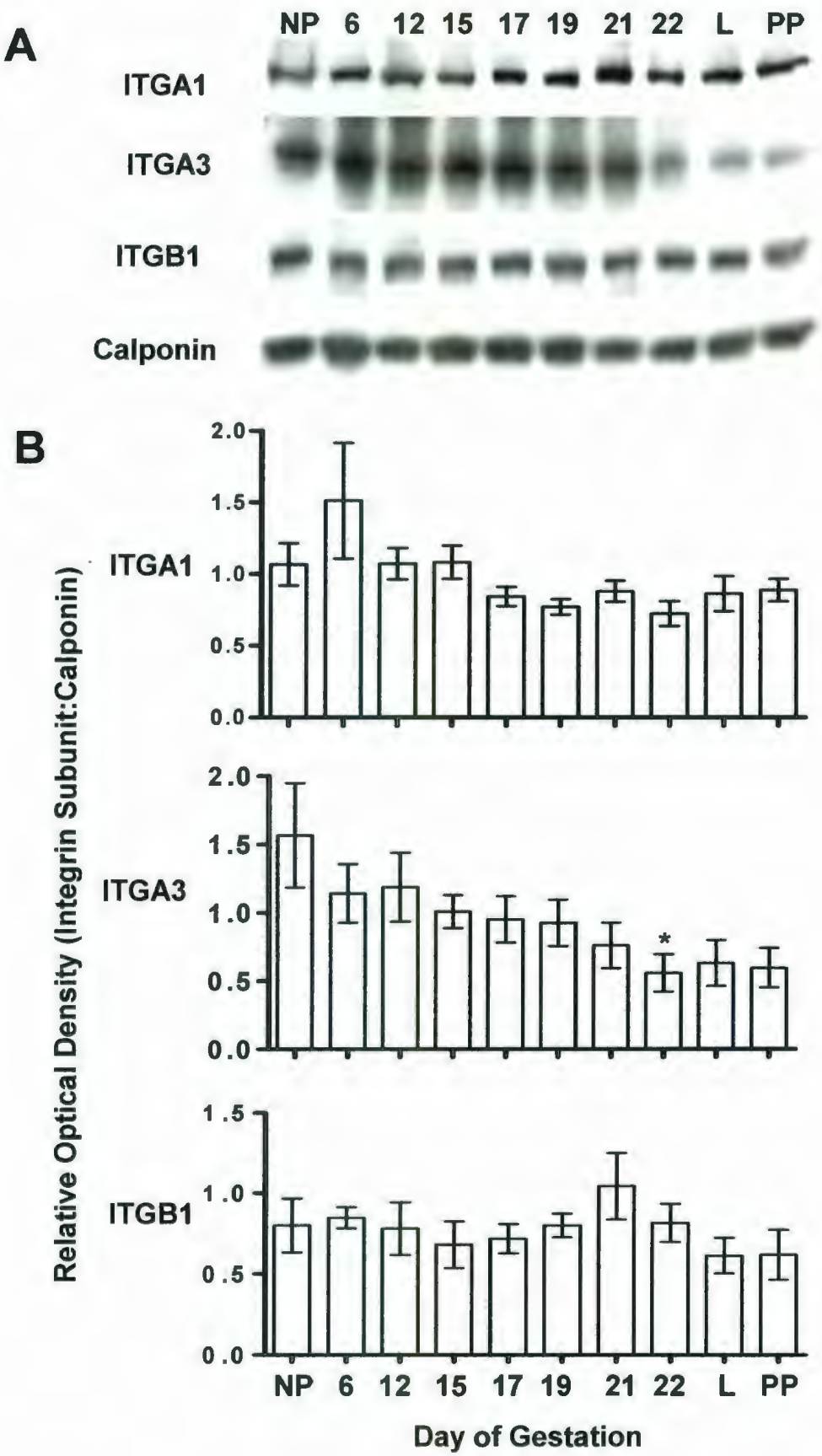

A
NP 6 12 15 17 19 21 22 L PP
ITGA1
ITGA3
ITGB1
Calponin
B
Relative Optical Density (Integrin Subunit:Calponin)
ITGA1
ITGA3
ITGB1
2.0
1.5
1.0
0.5
0.0
*
NP 6 12 15 17 19 21 22 L PP
Day of Gestation

Figure 3.4. Immunolocalization of ITGA1 in the longitudinal smooth muscle layer of rat myometrium between NP and 1 day postpartum. Numbers represent gestational timepoints. L, Labour (2-3 pups born); C, Control (Rabbit IgG). Arrows highlight membrane-specific staining. Scale bar = 10μm.

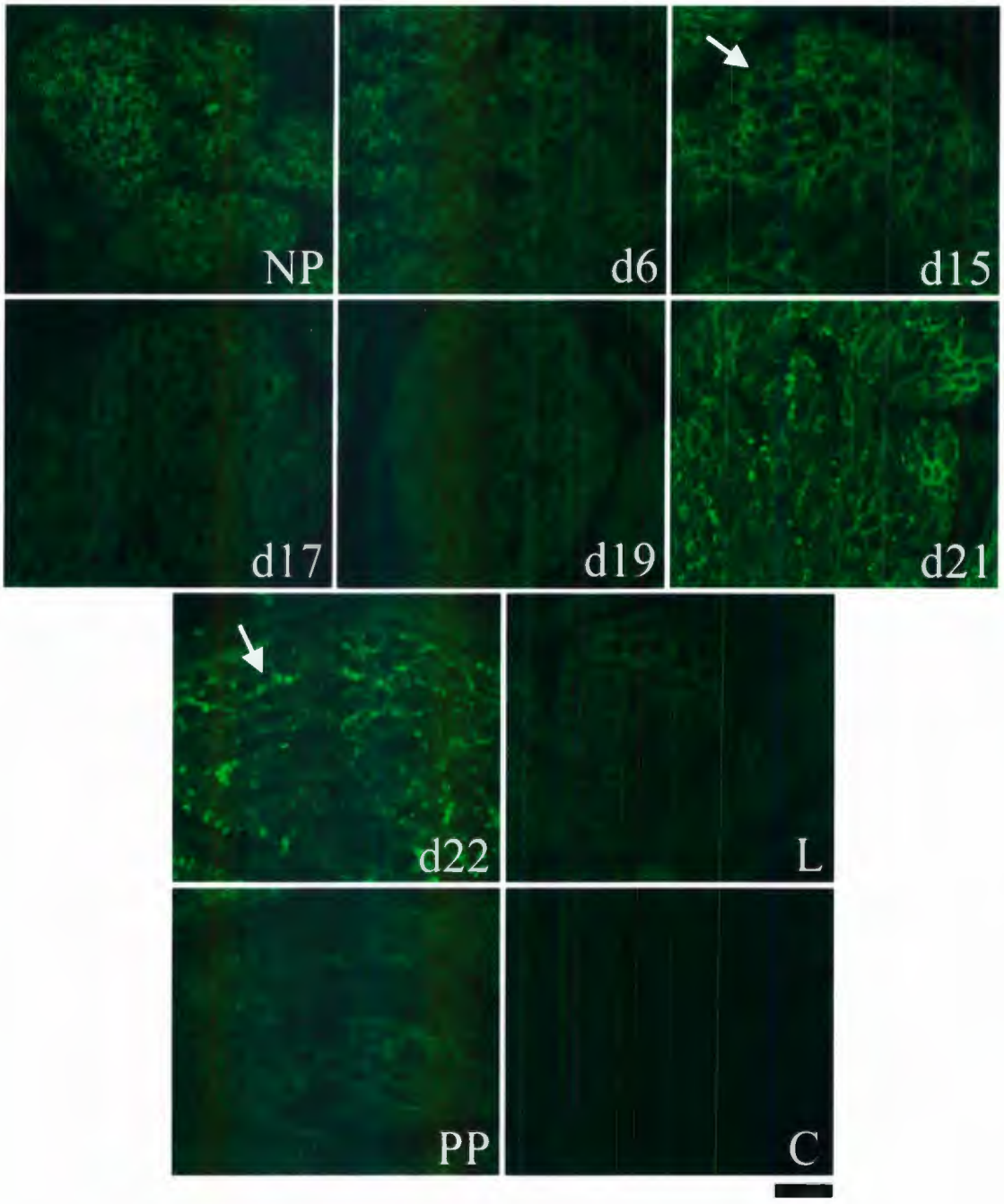

NP
d6
d15
d17
d19
d21
d22
L
PP
C

Figure 3.5. Immunolocalization of ITGA1 in the circular smooth muscle layer of rat myometrium between NP and 1 day postpartum. Numbers represent gestational timepoints. L, Labour (2-3 pups born); C, control (Rabbit IgG). Arrows highlight cytoplasmic and membrane-specific staining. Scale bar = 10μm.

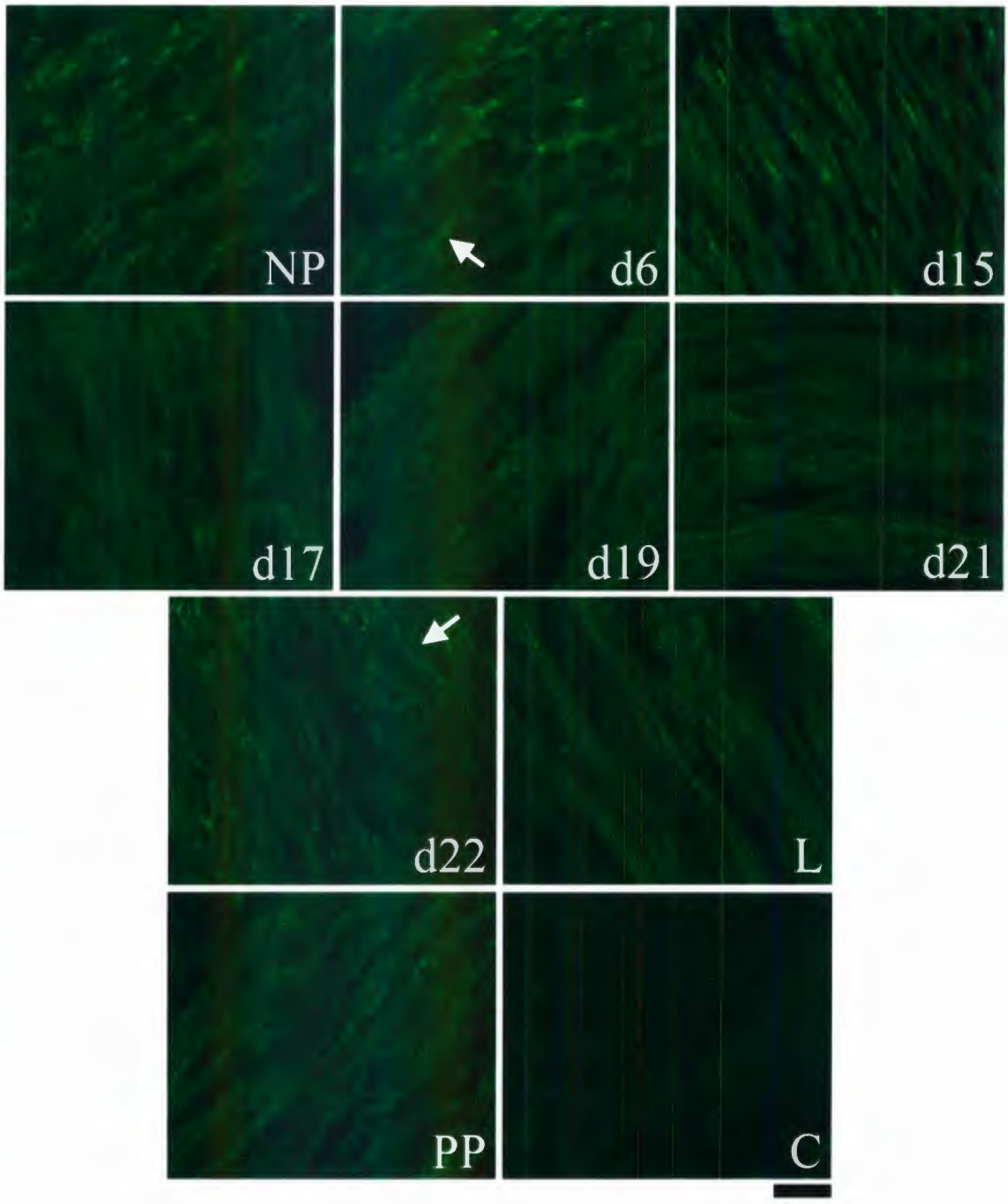
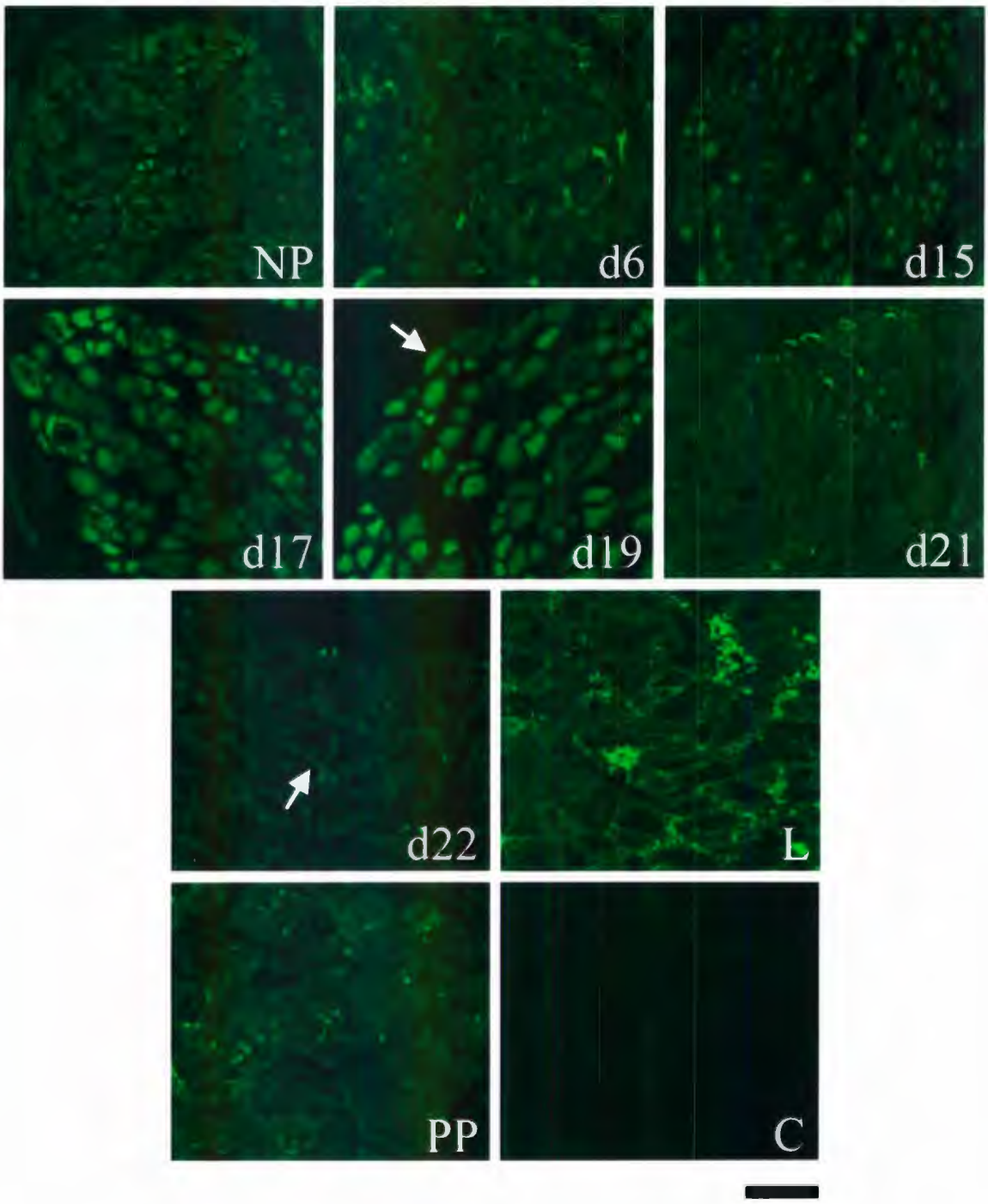

NP
d6
d15
d17
d19
d21
d22
L
PP
C

Figure 3.6. Immunolocalization of ITGA3 in the longitudinal smooth muscle layer of rat myometrium between NP and 1 day postpartum. Numbers represent gestational timepoints. L, Labour (2-3 pups born); C, Control (Rabbit IgG). Arrows highlight cytoplasmic and membrane-specific staining. Scale bar = 10μm.


NP
d6
d15
d17
d19
d21
d22
L
PP
C

localization was observed. The detection of ITGA3 in cell membranes consistently increased on d22, appeared to be greatest during labour, and was maintained until PP. In the circular smooth muscle layer, ITGA3 was detectable at a lower level compared to the longitudinal layer. ITGA3 was detected in the cytoplasm of NP rat myometrial cells and throughout gestation up to d21 (Figure 3.7). At d22 of gestation through to PP, ITGA3 was primarily detectable at myocyte cell membranes and at a very low level in the cytoplasm.

In the longitudinal smooth muscle layer, ITGB1was virtually undetectable on NP and d6, then was more detectable in the cytoplasm from d15 to d19 (Figure 3.8). On days 21 through PP, ITGB1 was localized to myocyte membranes and detection increased markedly from d21 to labour. In addition, a consistent decrease in ITGB1 membrane localization was observed at PP. Detection of ITGB1 in the circular smooth muscle layer was virtually undetectable from NP to d15 of gestation and then cytoplasmic detection was very low from d17 to d21 (Figure 3.9). From d22 to PP, ITGB1 was primarily detected in smooth muscle cell membranes. As seen with both ITGA1 and ITGA3, ITGB1 was also detected at a lower level in the circular layer compared to the longitudinal layer.

3.4 Discussion

It is beginning to be appreciated that smooth muscle tissue architecture, cell-ECM interactions and subsequent biochemical and molecular events associated with such interactions are key aspects of smooth muscle contractile activation and function [Meiss,

To evaluate the effect of *Itga5* siRNA on ITGA5 expression in M11 myometrial cells, cells were harvested 48 hours after transfection with NP-40 lysis buffer and a plastic cell scraper and then homogenized by pipetting the sample up and down several times. Samples were cleared by centrifugation and supernatants were retained for immunoblot analysis. For investigation of both ITGA5 expression in cell lysates and detection of secreted FN in conditioned media, protein concentrations were determined by the Bradford Assay [Bradford, 1976] using Bio-Rad protein assay dye reagent (Bio-Rad Labouratories, Mississauga, Ontario, Canada). Protein samples (10 μg/lane for cell lysates, 50 μg/lane for conditioned media and 10ul for matrix samples) were separated under non-reducing conditions by polyacrylamide gel electrophoresis in 9% resolving gels according to the method of Laemmli [Laemmli, 1970] and gels were electroblotted to Pierce 0.45 μm nitrocellulose membrane (MJS BioLynx, Inc., Brockville, Ontario, Canada).

Immunoblots were rinsed in Tris buffered saline (20mM Tris base, 137 mM NaCl, pH 7.6) with 0.1% Tween-20 (TBST) for 5 mins. Unless otherwise stated, all incubations were conducted at room temperature and with constant agitation. Blots were blocked in 5% BSA/TBST for 30 mins. Rabbit polyclonal antisera raised against ITGA5 (Cat # AB1928; Chemicon International, Temecula, California, USA) or fibronectin (FN; Cat # F3648; Sigma, Missouri, USA) used at dilutions 1:5,000 and 1:50,000, respectively, were incubated with blots for 1 hr. Blots were rinsed once for 15 min in TBST, followed by two rinses for 5 min in TBST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (H+L) was used at dilutions of 1:20,000 and 1:10,000, for

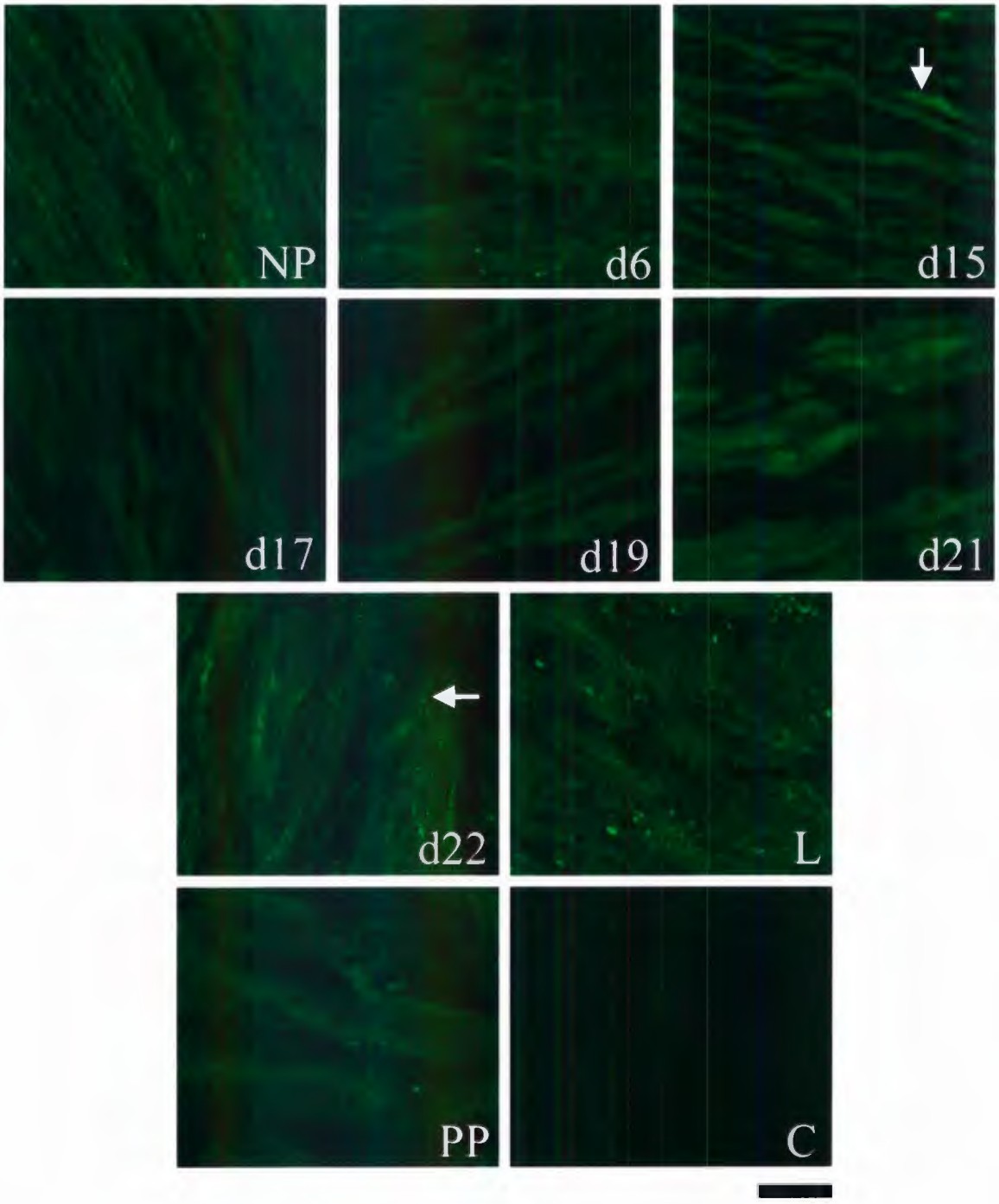

NP
d6
d15
d17
d19
d21
d22
L
PP
C

Figure 3.8. Immunolocalization of ITGB1 integrin protein in the longitudinal smooth muscle layer of rat myometrium between NP and 1 day postpartum. Numbers represent gestational timepoints. L, Labour (2-3 pups born); C, Control (Rabbit IgG). Arrows highlight cytoplasmic and membrane-specific staining. Scale bar = 10μm.

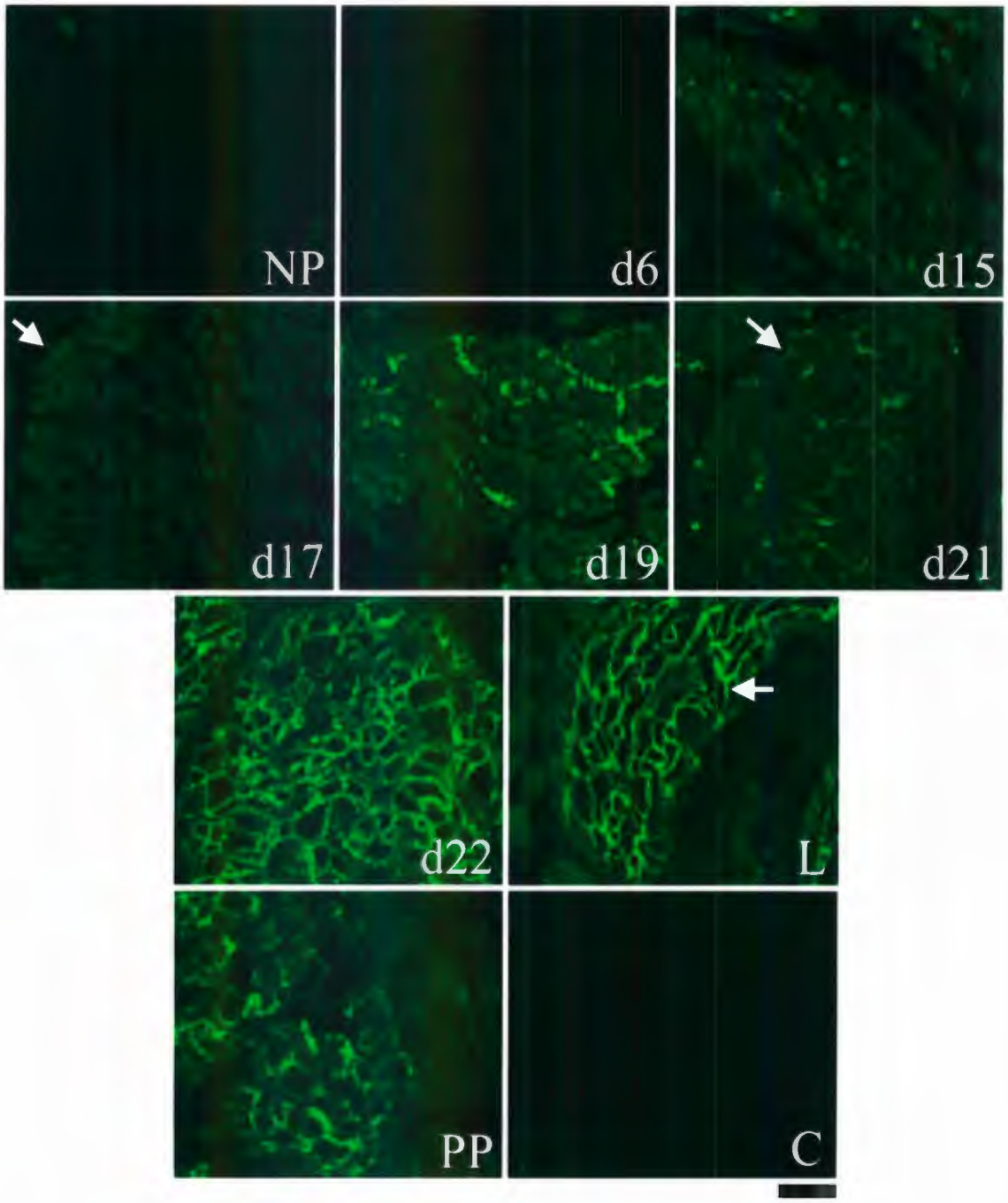
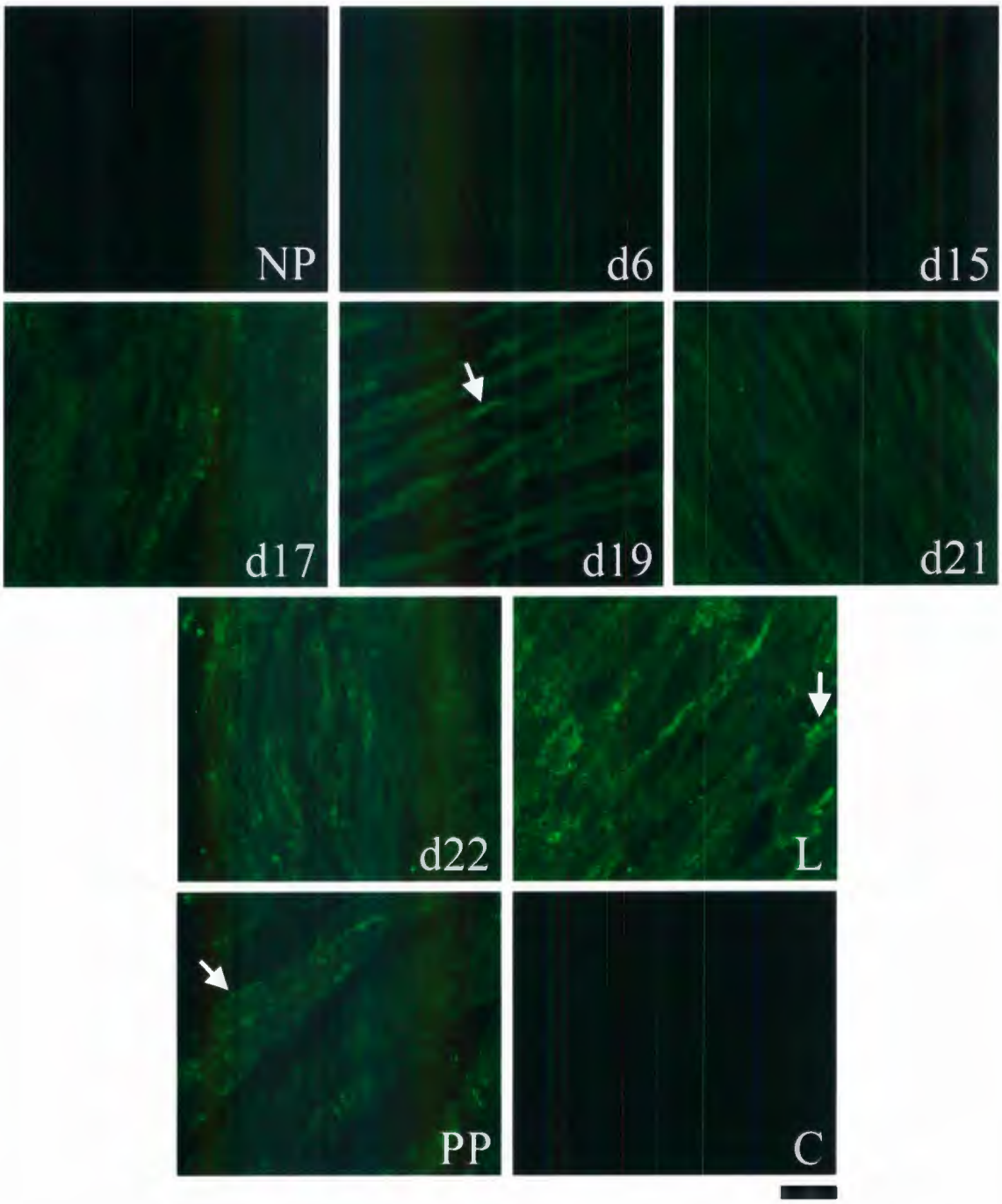

NP
d6
d15
d17
d19
d21
d22
L
PP
C

Figure 3.9. Immunolocalization of ITGB1 in the circular smooth muscle layer of rat myometrium between NP and 1 day postpartum. Numbers represent gestational timepoints. L, Labour (2-3 pups born); C, Control (Rabbit IgG). Arrows highlight cytoplasmic and membrane-specific staining. Scale bar = 10μm.


NP
d6
d15
d17
d19
d21
d22
L
PP
C

1997]. In uterine smooth muscle, the reorganization of focal adhesions during late pregnancy is thought to facilitate tissue remodeling during uterine distension-induced hypertrophic growth [MacPhee and Lye, 2000] and aid the proper anchoring of growing myometrial cells to their ECM. With the exception of *Itga5* [Williams et al, 2005], the expression of other integrins in the rat myometrium during pregnancy and labour has not been reported. With documented changes in collagen and laminin expression in rat myometrium during late pregnancy [Shynlova *et al*, 2005; Nishinaka & Fukuda, 1991], the goal of this study was to investigate the expression of the corresponding receptor subunits. Our hypothesis was that *Itga1, Itga3* and *ItgB1* would be expressed in the myometrium and that expression would be altered to accommodate ECM remodeling and focal adhesion turnover during late pregnancy.

Our initial investigation of mRNA expression using Northern Blot Analysis resulted in data that was variable at times. Quantification of Northern Blot results has its limitations. It is possible that the specific cDNAs used for radiolabelling may not have been optimal for random priming, inhibiting our ability to tease out specific changes in integrin mRNA expression. Despite the use of a number of different cDNA probes, Northern blot analysis of *Itga1, Itga3 and ItgB1* revealed that *Itga1* expression was undetectable, *Itga3* expression increased only slightly and no change in *ItgB1* was observed. As these results seemed questionable, Real-Time PCR was performed as it is more sensitive than Northern blot analysis. To offset the time required for learning and troubleshooting the use of a new real-time PCR machine in the lab, we collabourated with Dr. Stephen Lye (University of Toronto). The sequences they used for each integrin

subunit primer were entered into NCBI's Basic Local Alignment Search Tool (BLAST) and the resultant hits were only those of the appropriate integrin subunit.

Immunoblot analyses demonstrated that ITGA1, ITGA3, and ITGB1 integrin subunit proteins were readily detectable in rat myometrial cell lysates. However, the only integrin examined in this study that demonstrated significant changes in total detection levels was ITGA3 (d22 vs NP). ITGA1, ITGA3, and ITGB1 integrin subunits are ubiquitously expressed in tissues including endothelial cells and vascular smooth muscle cells (Asakura *et al.*, 1997; Martinez-Lemus *et al.*, 2003; Reddy and Mangale 2003). Shynlova *et al* (2005) has previously reported that myometrial blood vessels in the vascular plexus between the muscle layers increase in size throughout pregnancy. Thus, we cannot rule out the possibility of contributions of vascular integrin subunits to our immunoblot data as immunoblot analyses is not a sensitive quantitative technique, in contrast to real time PCR, and in this instance may not clearly distinguish myometrial smooth muscle-specific changes in integrin subunit expression in cell lysates that also contain such contributions from vascular integrin subunits. This was also postulated for immunoblot detection of γ-actin over gestation (Shynlova *et al*, 2005). Thus, we also employed immunofluorescence analyses and indeed detected temporally specific changes in the spatial localization of individual integrin subunits within the specific muscle layers of the myometrium during pregnancy and labour.

Itga1 Subunit

Our RT-PCR analysis revealed that *Itga1* expression was significantly elevated by d6 of rat gestation and remained elevated until PP. Interestingly, immunofluorescence analysis demonstrated that ITGA1 was also detectable in situ in the myometrium throughout gestation, albeit at a much lower level in the circular muscle layer, but detection was particularly pronounced in myocyte membranes by ~d21-d22 in both muscle layers suggesting the existence of a post-translational mechanism(s) underlying delivery of ITGA1 to myocyte membranes during this time. The increased detection of ITGA1 integrin at myocyte membranes correlates with increased expression of type IV collagen, an ITGA1B1 ligand. The expression of type IV collagen mRNA and protein and subsequent deposition of type IV collagen around smooth muscle cells in the rat myometrium have been found to be upregulated during late pregnancy [Shynlova et al, 2004; Nishinaka & Fukuda, 1991]. Although our immunoblot analysis of total integrin subunit expression from myometrial tissue did not show a significant change in ITGA1 detection during these times, this analysis would not be sensitive enough to detect the myometrial layer-specific changes in ITGA1 detection documented by immunofluorescence analysis in longitudinal and circular muscle layers during late pregnancy.

Itga3 Subunit

RT-PCR analysis revealed a more complex pattern of *Itga3* expression than for either *Itga1* or *ItgB1*; however, the significant increase of mRNA expression at d14, d21, and d22 correlates well with observed changes in the detection and spatial

immunolocalization of ITGA3 in our immunofluorescence analysis. The increased detection of ITGA3 in the cytoplasm of myocytes in the longitudinal layer at ~d15-d19 was particularly notable, although ITGA3 was also detected in the cytoplasm in the circular layer at this time, albeit at a lower level than in the longitudinal layer. The accumulated pool of immunodetectable ITGA3 subunit observed in the cytoplasm prior to ~d21, particularly in the longintudinal layer, may have resulted from the observed increase in expression of *Itga3* by d14 of gestation and subsequent mRNA translation. The increased *Itga3* expression at d21 and d22 could then reflect an additional need for *Itga3* and ITGA3 translation during cell-ECM remodeling coincident with ITGA3 detection in myocyte membranes of both muscle layers. Membrane localization of ITGA3 during late pregnancy correlates well with the reported increased deposition of laminin, the ITGA3B1 ligand, around smooth muscle cells in the rat myometrium and the tissue remodeling that also occurs during this period of hypertrophic growth [Nishinaka & Fukuda, 1991].

ItgB1 Subunit

Similar to *Itga1* expression during gestation, *ItgB1* was significantly elevated by d6 and remained elevated until 4PP. In addition, we found marked changes in ITGB1 immunolocalization during gestation. Our experiments showed a considerable increase in cytoplasmic ITGB1 detection during pregnancy in both the longitudinal and circular muscle layers by d15 - d17, and d19, respectively. This may be a reflection of increased translation of the *ItgB1* during this time window. Similar to ITGA3 immunolocalization,

ITGB1 also became highly localized to myocyte membranes in the longitudinal and circular muscle layers on d21 and d22, respectively. ITGB1 at NP and d6 is virtually undetectable in situ in both the longitudinal and circular smooth muscle layers; however, Immunoblot analysis revealed that ITGB1 was readily detectable in myometial cell lysates at both NP and d6. This may reflect a contribution to ITGB1 proteins in lysates from additional components of the uterus, such as endothelial cells and vascular smooth muscle cells in blood vessels within the vascular plexus.

The pattern of ITGB1 immunolocalization in situ may indicate that the availability of the protein at uterine myocyte membranes may be a critical temporal event in the function of heterodimeric integrin receptors within the myometrium. For example, we previously detected ITGA5 in uterine myocytes in situ from NP to 1 day PP [Williams et al, 2005] and it is possible that ITGB1 expression could be the key regulating factor in fibronectin receptor (ITGA5B1) formation and function at the plasma membrane of uterine myocytes during late pregnancy when fibronectin is deposited around smooth muscle cells in rat myometrium at this time [Nishinaka and Fukuda, 1991]. Because the ITGB1 can also heterodimerize with ITGA1 and ITGA3 subunits to form functional collagen and laminin receptors, respectively [Hynes, 2002; Wiesner et al, 2005], it may also have similar regulatory roles with these integrin subunits and thus facilitate focal adhesion formation. Our comparative analyses of integrin gene expression in the non-pregnant and pregnant rat myometrium also supports such a role for ITGB1 as *Itgb1* expression was found to be dramatically higher than all other integrins examined.

Thus, a high level of *ItgB1* would be available, if necessary, for translation and incorporation of ITGB1 into a variety of integrin heterodimers.

3.4.1 Myometrial Layer-Specific Differences in Protein Detection

Immunofluorescence analyses revealed that all integrins appeared more readily detectable in the longitudinal muscle layer than the circular muscle layer. It is important to point out that the two muscle layers are reported to have different embryonic origins and exhibit different contractile and physiological characteristics [Osa and Katase, 1975; Kawarabayashi and Osa, 1976; Chow and Marshall, 1981; Mlynarczyk *et al.*, 2003]. Thus, these distinguishing characteristics may underlie the observed detection differences in the two muscle layers and also may, at least in part, contribute to the detection differences observed between immunoblot and immunofluorescence analyses since the former examines detection of a specific protein(s) from a total protein lysate prepared from bulk myometrium samples, and the latter allows examination of protein detection in the muscle bundles themselves. Interestingly, ITGA5 was also found to have higher detection levels in the longitudinal muscle layer than the circular muscle layer [Williams *et al.*, 2005].

3.4.2 Implications for Myometrial Function during Late Pregnancy and Labour

All three integrin subunits examined by immunofluorescence became detectable in myocyte membranes as labour approached. Late pregnancy is a period of increasing uterine stretch due to growing fetuses and, as a result, stretch-induced myometrial

hypertrophy [Goldspink and Douglas, 1988]. Considerable evidence has accumulated that mechanical stress induces integrin gene expression and ECM synthesis/remodeling [Wozniak et al, 2004]. Recently, we showed that *Itga5* was temporally and spatially regulated by uterine stretch in unilaterally pregnant rats [Shynlova *et al.*, 2007]. Thus, membrane localization of ITGA1, ITGA3 and ITGB1 during late pregnancy may also be induced by uterine distension. Subsequent integrin-ECM interaction may then lead to signal transduction to downstream effector molecules resulting in alterations in cytoskeletal organization and gene expression. This process would be aided by the known association of integins with the actin cytoskeleton via adapter proteins [Wozniak et al, 2004], facilitating the sensing of mechanical forces both from the exterior and interior of the cell.

We have previously suggested that a mechanical syncytium may develop in the myometrium to facilitate the coordinated contractions of labour [Williams *et al.*, 2005]. The highly specific expression of ITGA1, ITGA3 and ITGB1 in myocyte membranes during late pregnancy and labour noted in this study and the demonstrated upregulation of ITGA5, along with corresponding ECM molecules, could aid the cell-ECM interactions necessary to promote the development of such a syncytium.

CHAPTER 4

Uterine Stretch Regulates Temporal and Spatial Expression of Fibronectin Protein and its Alpha 5 Integrin Receptor in Myometrium of Unilaterally Pregnant Rats

(A Portion of this Chapter Published in Biology of Reproduction, 2007, 77, 880 – 888)

4.1 Introduction

The uterus undergoes dramatic physiological adaptations during the course of pregnancy to accommodate the developing fetus, placenta and amniotic fluid. The onset of labour requires triggering of the endocrine (the fetal hypothalamic-pituitary-adrenal-placental axis) and mechanical (stretch resulting from fetal growth during pregnancy) signalling pathways within the myometrium to activate the synchronized and time-coordinated labour contractions of the uterine smooth muscle. Both signals also play a determinant role in the growth of the uterus during late pregnancy that precedes myometrial activation [Challis and Lye, 2004]. Mechanical stretch has previously been shown to promote rapid and extensive uterine hypertrophy and remodelling in non-pregnant [Cullen and Harkenss, 1968], unilaterally pregnant [Goldspink and Douglas, 1988] and postpartum [Csapo *et al.*,1965] animals. Our previous data has suggested that the hypertrophic changes in pregnant myometrium are associated with significantly increased expression of ECM proteins in the second part of gestation [Shynlova *et al.*, 2004]. Specifically, we reported a decrease in expression of fibrillar collagens and a

coordinated temporal increase in expression of components of the basement membrane (type IV collagen, laminin) and fibronectin (FN) near term [Shynlova *et al.*, 2004].

These changes in ECM content reflect newly reorganized uterine tissue architecture, allowing hypertrophied uterine myocytes to properly anchor to the surrounding ECM. MacPhee and Lye (2000) discovered earlier that the expression and activity of the major regulator of cell-ECM contacts, focal adhesion kinase (FAK or PTK2), was up-regulated in rat myometrium at late gestation. It is well known that activation of PTK2 causes the formation of complex specialized structures termed focal adhesions, associated with the plasma membrane of SMCs. Integrin receptors are essential components of focal adhesions as they form clusters located at sites where SMCs make contact with the different ECM components [Hanks and Polte, 1997]. These ubiquitous large transmembrane receptors can bind to a number of ligands, primarily ECM molecules, establishing dynamic associations between ECM and the actin cytoskeleton. Integrins are involved in many cellular processes such as cellular adhesion and migration, regulation of the cellular phenotype in many cell types and stimulation of numerous signal transduction pathways [Wozniak *et al.*, 2004]. The interaction of ECM ligands with their corresponding integrins also plays an important role in embryo development, morphogenesis and tumorigenesis (reviewed in [Qin *et al.*, 2003]). Each integrin is a heterodimer that contains α and β subunits. The α/β pairings specify the ligand-binding selectivity of the integrin heterodimers. For example, integrin α5 subunit (ITGA5) partners exclusively with the β1 integrin (ITGB1). The repertoire of integrins expressed on particular cell types is unique and can vary temporally depending on many

factors. Cells often display multiple integrins capable of interacting with a particular ECM. Some integrins, such as α5/β1 (ITGA5B1), the "classic" fibronectin receptor, bind to a single ECM protein [Robinson *et al.*, 2004; Woodward *et al.*, 2001; Goldsmith *et al.*, 1999]. FN is a multifunctional, adhesive glycoprotein that is secreted by cells as a soluble dimer and that can be assembled on the cell surface into mesh-like fibrils [Hynes, 1990]. Interaction of FN with its receptor and the formation of fibrillar FN matrix is important for strong intercellular cohesion to allow for proper cell behavior [Robinson *et al.*, 2004; Wierzbicka-Patynowhki and Schwarzbauer, 2003].

We have previously reported an increase in *Fn* gene expression in the gravid horn of unilaterally pregnant rats subjected to a passive biological stretch by growing fetuses [Shynlova *et al.*, 2004]. Moreover, we demonstrated earlier that *Itga5* gene and ITGA5 protein expression were up-regulated in rat myometrium specifically at the second half of gestation when mechanical stretch of the uterine wall, imposed by the growing fetus, was apparent [Williams *et al.*, 2005]. Therefore, we decided to use the same animal model to examine the importance of mechanical stimulus on the expression of ITGA5 subunit in pregnant rat uterine tissue. In the present study we applied Real-Time PCR technique, northern blotting, immunoblotting in combination with immunofluorescence analysis to investigate the effect of gravidity on *Itga5* expression and ITGA5 subunit expression and localization as well as spatial and temporal distribution of FN. Our second aim was to investigate whether progesterone (P4) regulates ITGA5 expression in the myometrium. We have previously found that *Fn* gene expression was induced following early P4 withdrawal [Shynlova *et al.*, 2004]. As P4 plays a major role in myometrial ECM

remodelling, influencing the expression of the *Fn* gene, we analyzed the effects of blocking P4 receptor function on the expression of *Itga5* mRNA and ITGA5 localization in the rat myometrium during RU486-induced preterm labour.

4.2 Materials and Methods

4.2.1 Animals

Wistar rats (Charles River Co., St. Constance, Canada) or Sprague-Dawley rats (Memorial University of Newfoundland, St. Johns, Newfoundland, Canada) were housed individually under standard environmental conditions (12 hr light, 12 hr dark cycle) and fed Purina Rat Chow (Ralston Purina, St. Louis, MO) or LabDiet Prolab RMH 3000 (PMI Nutrition International, Brentwood, Missouri, USA) and water *ad libitum.* Female virgin rats were mated with male rats. Day one of gestation was designated as the day a vaginal plug was observed. The Samuel Lunenfeld Research Institute Animal Care Committee and Memorial University of Newfoundland Animal Care Committee approved all animal experiments. The Sprague-Dawley outbred strain was developed from the Wistar outbred strain and the main difference between the two strains is that Sprague-Dawley rats are less active and calmer, allowing for easier handling.

4.2.2 Experimental Design

4.2.2.1 Unilaterally Pregnant Rats

Under general anesthesia virgin female rats underwent tubal ligation through a flank incision to ensure that they subsequently became pregnant in only one horn [Ou *et*

al., 1998]. Animals were allowed to recover from surgery for at least 7 days before mating. Pregnant rat myometrial samples from non-gravid (empty) and gravid horns were collected on gestational days 15, 19 and 23 (Labour). Labour samples were taken during active labour and only after the rat had delivered 1-3 pups (n=3-4).

4.2.2.2 RU486-Induced Preterm Labour

On day 19 of gestation two groups of rats were treated with either RU486 (10mg/kg, sc, 10 am, in 0.5 ml corn oil containing 10% EtOH, Mifepristone;17ß-hydroxy-11ß-[4-dimethylaminophenyl]-17-[1-propynyl]-estra-4,10-dien-3-one; Biomol International, Plymouth Meeting, PA) or vehicle. Myometrial samples were collected from both groups of animals on day 20. RU486-treated rats were killed during active labour when they had delivered 1-3 pups (n=4).

4.2.3 Tissue Collection

Animals were killed by carbon dioxide inhalation. For RNA and protein extraction the uterine horns were placed into ice-cold PBS, bisected longitudinally and dissected away from both pups and placentae. The endometrium was carefully removed from the myometrial tissue by mechanical scraping on ice which we have previously shown removes the entire luminal epithelium and the majority of the uterine stroma [Piersanti and Lye, 1995]. The myometrial tissue was flash-frozen in liquid nitrogen and stored at –70°C. For immunohistochemical studies the intact uterine horns were placed in ice-cold PBS buffer, cut into 3-10-mm segments using a scalpel blade and fixed immediately in 4% paraformaldehyde solution at 4°C for 48 hours or overnight in zinc

buffered fixative (ZBF; 100mM Tris buffer pH 7.4, 3mM calcium acetate, 27mM zinc acetate, 37mM zinc chloride; [Williams *et al.*, 2005; Beckstead, 1994]) while shaking at room temperature. These segments were further cross-sectioned or sectioned longitudinally. For each day of gestation, tissue was collected from 4 different animals and processed for different experimental purposes.

4.2.4 Real Time Polymerase Chain Reaction (PCR) Analysis

Total RNA was extracted from the frozen tissues using TRIZOL (Gibco BRL, Burlington, ON) according to manufacturer's instructions. RNA samples were column purified using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada), and treated with 2.5µl DNase I (2.73Kunitz unit/µl, Qiagen) to remove genomic DNA contamination. Reverse transcription (RT) and real-time PCR (RT-PCR) was performed to detect the mRNA expression of *Itga5* gene in rat myometrium. 2µg of total RNA was primed with random hexamers to synthesize single-strand cDNAs in a total reaction volume of 100µl using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The thermal cycling parameters of RT were modified according to the Applied Biosystems manual. Hexamer incubation at 25°C for 10 min and RT at 42°C for 30 min was followed by reverse transcriptase inactivation at 95°C for 5 min. 20 ng of cDNA from the previous step was subjected to Real-Time PCR using specific sets of primers (see the legends to Fig.1) in a total reaction volume of 20µl. RT-PCR was performed in an optical 96-well plate with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), using the SYBR Green detection chemistry. The run protocol was

as follows: initial denaturation stage at 98°C for 2 min, 40 cycles of amplification at 95°C for 15sec and 60°C for 1min. After PCR, a dissociation curve was constructed by increasing temperature from 65°C to 95°C for detection of PCR product specificity. In addition, a no-template control (H_2O control) was analyzed for possible contamination in the master-mix. A Ct value was recorded for each sample. PCR reactions were set up in triplicates and the mean of the 3 Cts was calculated. A ΔΔCt method was applied to the raw Ct values to find a relative gene expression. To obtain experimental results, the expression of *Itga5* gene at specific gestational day was normalized to ribosomal *18S* mRNA. For unilaterally pregnant animals' *Itga5* gene expression was calculated as fold change relative to day 15 gravid horn mRNA level and for RU486-treated animals – as fold change relative to the vehicle sample using an arithmetic formula (see ABI User Bulletin #2). Validation experiments were performed to ensure the PCR efficiencies between the target genes and *18S* were approximately equal.

4.2.5 Northern Blot Analysis

RNA isolation and northern blot analysis of mRNA was performed according to methods previously described in detail [Williams *et al.*, 2005]. pOTB7 vector containing the human *Itga5* cDNA was purchased from the American Type Culture Collection (Manassas, Virginia, USA, www.atcc.org ; #MGC-3697). Isolation of a human *Itga5* cDNA fragment for probe production has been described previously [Williams *et al.*, 2005]. Probed membranes were exposed to x-ray film (Hyperfilm MP; Amersham Biosciences) with an intensifying screen at –70° C and analyzed by densitometry.

Multiple exposures were produced for each northern blot to ensure the results were within the linear range of the film. Following analysis of *Itga5* mRNA expression, northern blots were stripped and reprobed with similarly labeled 18S (*Rn18S*) probe (a rabbit *Rn18S* ribosomal cDNA template generously provided by Dr. I. Skerjanc, University of Western Ontario, London, Ontario, Canada). *Rn18S* RNA is constitutively expressed in rat myometrial cells and has been utilized as a loading control for analysis of myometrial gene expression [Shynlova *et al.*, 2004].

4.2.6 Immunoblot Analysis

Total protein was extracted from the frozen tissues using RIPA lysis buffer as was described earlier in detail [Williams *et al.*, 2005]. Protein samples (50 μg /lane) were separated under non-reducing conditions by polyacrylamide gel electrophoresis in 9% (for ITGA5) or 6% (for FN) resolving gels. Proteins were electroblotted to nitrocellulose (ITGA5) or polyvinylidene difluoride (FN) membranes (Millipore, Bedford, MA). Protein expression levels of ITGA5 were measured using primary polyclonal rabbit antibody (1:125000, Chemicon, Temecula, California, USA, catalog #AB1928). FN protein expression was measured using primary mouse monoclonal antibody (1:500, Santa Cruz, USA, catalog #sc-8422). Appropriate primary antisera were incubated with blots for 1 hour at room temperature under constant agitation and then blots were rinsed with Tris-buffered saline-Tween-20 (TBST; 20mM Tris base, 137 mM NaCl, pH 7.6 with 0.1% Tween-20). Immunoblots were incubated in horseradish peroxidase (HRP)-conjugated secondary antisera (1:100000, Pierce, Rockford, IL, USA or 1:50, EnVision,

Dako) for 30-60 min at room temperature with constant agitation and then rinsed in TBST. Proteins were detected using the Pierce SuperSignal West Pico Chemiluminescent Substrate detection system (MJS BioLynx, Inc., Brockville, Ontario, Canada) and multiple exposures were generated to ensure the linearity of the film exposures. Membranes were stripped and reprobed with anti-calponin (1:3000, clone hCP, Sigma-Aldrich, Oakville, Ontario, Canada, catalog # C2687) or anti-ERK antibodies (1:1000, Santa-Cruz Biotechnology, Santa Cruz, CA, catalog #sc-93) using the probing conditions described above for primary antibodies to control the loading variations. We have previously demonstrated that calponin protein (also known as CNN1) and ERK are constitutively expressed in non-pregnant and pregnant rat myometrial tissue under our protein extraction conditions [Williams *et al.*, 2005; Oldenhof *et al.*, 2002].

4.2.7 Immunocytochemistry

Immunofluorescence was performed according to methods previously described in detail [Williams *et al.*, 2005]. Two separate, independently collected sets of rat uterine tissues (n = 2, i.e. 2 rats used per timepoint) were utilized for immunocytochemistry experiments and experiments were repeated 2 times. For experimental purposes, two uterine tissue sections per slide for each gestational timepoint were utilized. All tissue sections used were 5 μm in thickness, contained both longitudinal and circular smooth muscle layers, and were processed under identical conditions (e.g. the same volumes for blocking and antisera solutions). Heat-induced epitope retrieval was accomplished using

a solution of 0.01M SSC, pH 6.0. One tissue section on each slide was then incubated for 1 hour at room temperature in primary rabbit anti-ITGA5 antisera (1:500, Chemicon, Temecula, California, USA) or mouse anti-FN (1:100, NeoMarkers, Fremont, CA, USA, clone FBN11, catalog #MS-1351-P1). At the same time, the remaining tissue section on each slide was incubated with affinity-purified IgG of the appropriate species, at the same concentration as the primary antisera, to serve as a negative control. After three washes in PBS, all of the tissue sections were incubated with FITC-conjugated secondary antisera (1:250, Sigma, St.Louis, USA, catalog # F7512). Sections were washed with cold PBS containing 0.02% Tween-20 (PBT) with constant agitation. Tissues were mounted in Vectashield (Vector Labouratories, Inc., Burlington, Ontario, Canada) before viewing with an Olympus Fluoview laser scanning confocal microscope (Olympus Optical Company Ltd., Melville, New York, USA) or Leica DMRXE microscope (Leica Microsystems, Richmond Hill, ON, Canada). With both instruments, image acquisition settings in each experiment were precisely maintained to allow examination of specific detection differences across the gestational profiles analyzed.

4.2.8 Data Analysis

Densitometric analysis of northern blots and immunoblots were performed with the aid of Scion Image software (Scion Image Corporation, Frederick, Maryland, USA). Densitometric measurements of *Itga5* mRNA were normalized to those of *Rn18S* RNA while measurements of ITGA5 protein on immunoblots were normalized to those of CNN1. Statistical analysis of northern and immunoblots was performed with GraphPad

Prism version 4.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com) and Real Time PCR – with SigmaStat version 2.01 (Jandel Corp., San Rafael, CA). Data from northern blot, Real-Time PCR and immunoblot analysis of *Itga5* and ITGA5 expression in the unilaterally pregnant model were subjected to a Two-way ANOVA followed by pairwise multiple comparison procedures (Student-Newman-Keuls method) to determine differences between groups. RU486 results were compared to vehicle using a t-test. Values were considered significantly different if $P < 0.05$.

4.3 Results

4.3.1 Gravidity Regulates FN and ITGA5 Receptor Expression in Unilaterally Pregnant Rat Myometrium

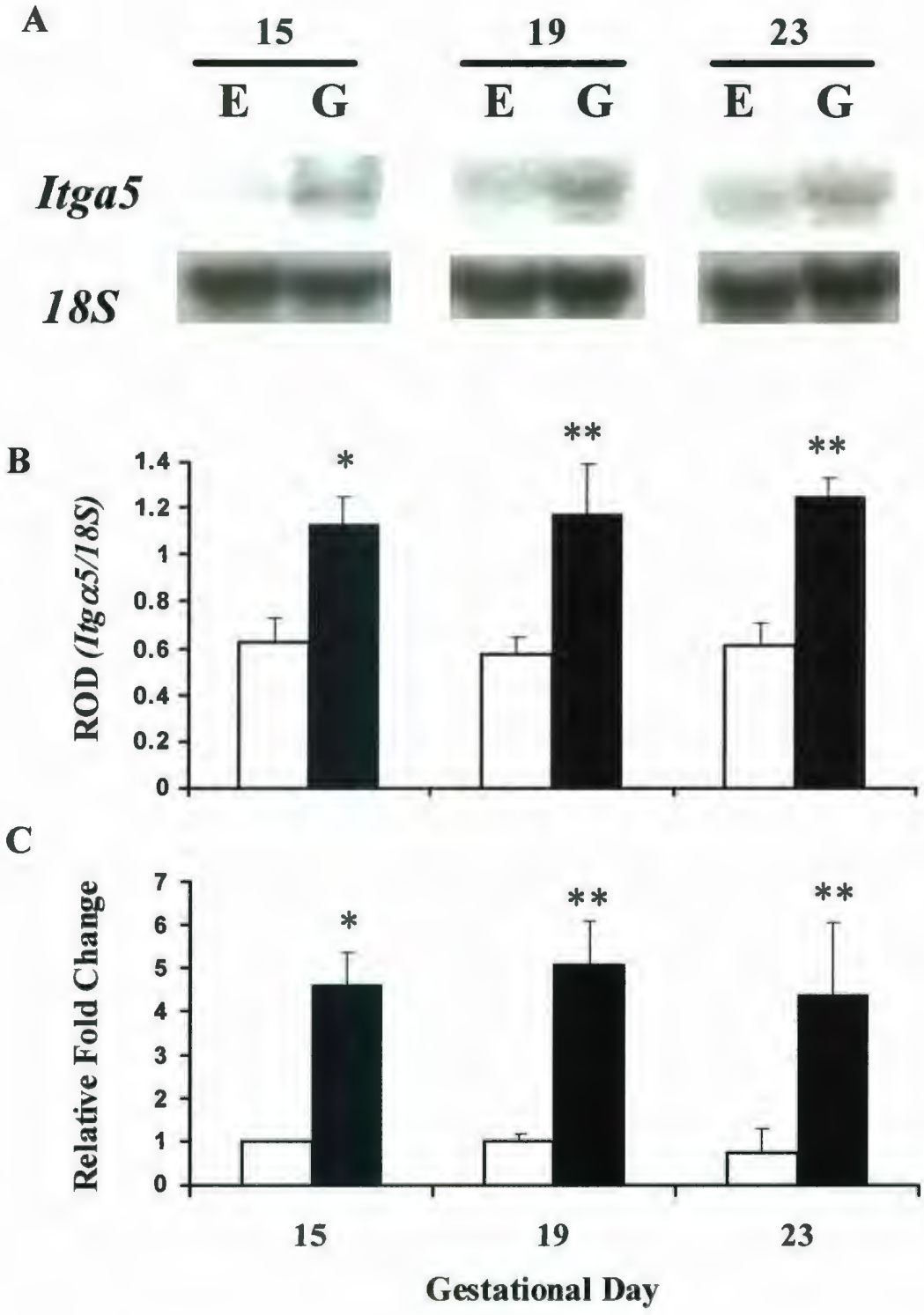
4.3.1.1 Expression of *Itga5* mRNA: Northern Blot Analysis

To determine whether *Itga5* gene expression was affected by mechanical stretch of the myometrium during late pregnancy, northern blot analysis was performed. We used total RNA isolated from empty and gravid uterine horns of unilaterally pregnant Sprague-Dawley rats. As shown on Figure 1A the expression of *Itga5* gene was elevated in the gravid horn compared to the empty one at day 15, day 19 and day 23 (labour). Densitometric analysis revealed that this increase was statistically significant (Figure 4.1A, B, $P<0.0001$).

4.3.1.2 Expression of *Itga5* mRNA: Real-Time PCR Analysis

The levels of *Itga5* mRNA were also analyzed in uterine tissue from gravid and

Figure 4.1. Expression of *Itga5* mRNA in the myometrium of unilaterally pregnant rats during late gestation. A. Representative northern blots show the expression of *Itga5* mRNA in gravid (G) and empty (E) horns of unilaterally pregnant rats. B. Densitometric analysis of *Itga5* mRNA levels in non-gravid (white bars) and gravid (black bars) horns (in relative units) normalized versus *Rn18S* RNA. C. *Itga5* mRNA levels were analyzed on the indicated days of gestation by Real-Time PCR. Specific forward and reverse primers were designed using Primer Express software, version 2.0.0 (Applied Biosystems), as follows: *Itga5* mRNA, 5'-CCTTCCTTCATTGGCATGGA-3' (sense primer) and 5'-TCTGCATCCTGTCAGCAATCC-3' (antisense primer; GenBank accession #: NM_012893); *Rn18S*, 5'-GCGAAAGCATTTGCCAGAA-3' (sense primer) and 5-'GGCATCGTTTATGGTCGGAAC-3' (antisense primer; GenBank accession #: X01117 K01593). *Itga5* gene expression levels in non-gravid (white bars) and gravid (black bars) (in relative fold changes) were normalized to *Rn18S* RNAs. The bars represent mean ± SEM (n=3 at each time point). A significant difference between gravid and non-gravid horn of the same day is indicated by * ($P < 0.05$) or ** ($P<0.01$). (Shynlova *et al.*, Biology of Reproduction, 2007, 77, 880 – 888)


A
15
19
23
E G
E G
E G
Itga5
18S
B
ROD (Itgα5/18S)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
C
Relative Fold Change
7
6
5
4
3
2
1
0
*
**
**
15
19
23
Gestational Day

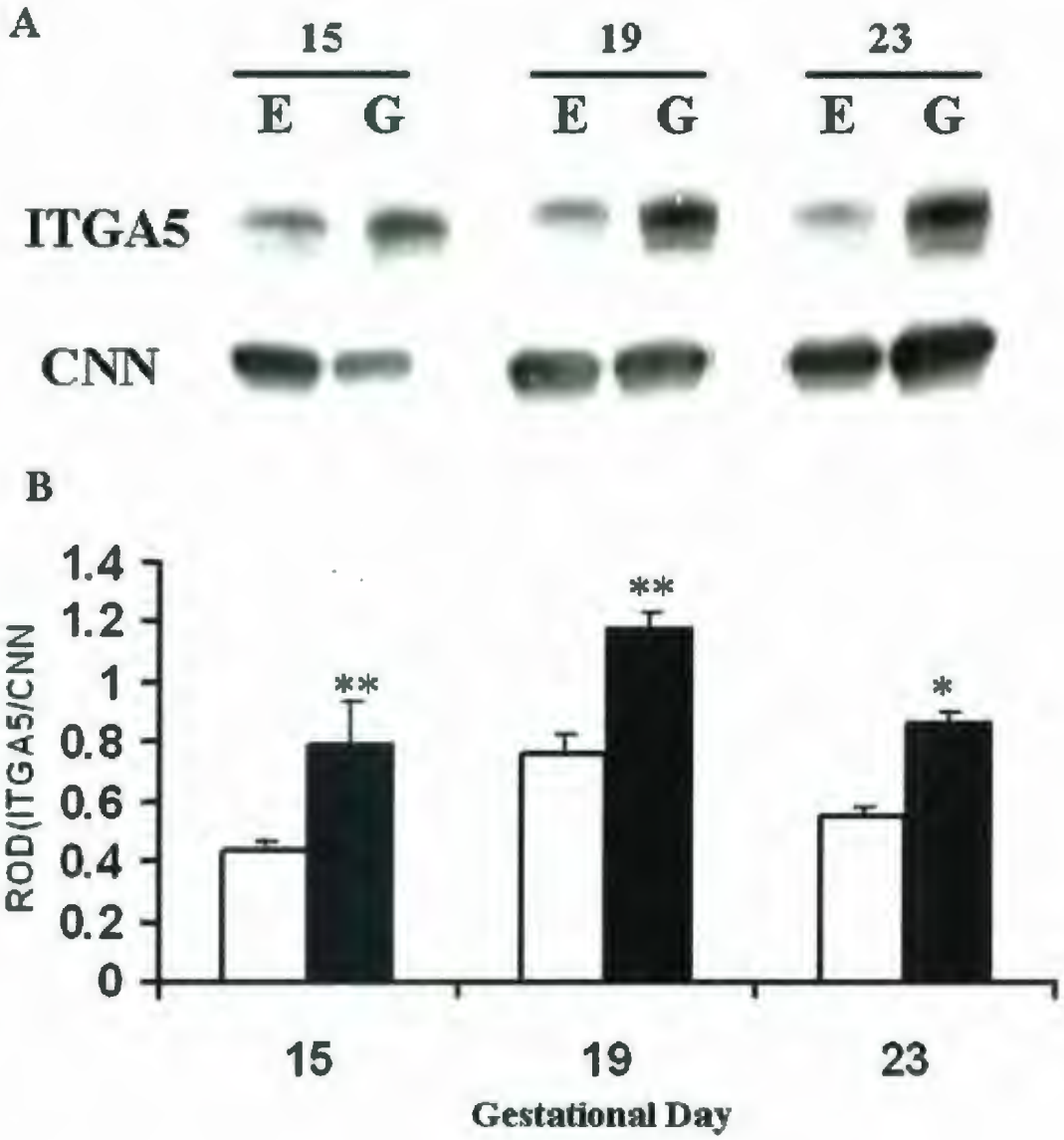
empty horns of unilaterally pregnant Wistar rats by Real Time PCR analysis. Similar to normal pregnant animals [Williams *et al.*, 2005] the mRNA levels for *Itga5* in the gravid horn of unilaterally pregnant rats remained high and relatively constant at late gestation (day 15-day 19) and during labour (day 23). In contrast, on all three gestational days *Itga5* transcript levels were significantly lower in the empty horn myometria compared to the gravid one (day 23, 4.4-fold increase in gravid *vs* empty horn, $P<0.001$) (Figure 4.1C).

4.3.1.3 Detection of ITGA5 Protein by Immunoblot and Immunofluorescence Analyses

Immunoblot analysis was performed using specific anti-ITGA5 antibodies to determine whether protein expression of this integrin subunit in empty and gravid horns of unilaterally pregnant rat myometrium at late gestation reflected its gene expression. ITGA5 protein levels were significantly higher in gravid uterine horn on gestational day 15, day 19 and day 23 (labour) compared to the empty one ($P<0.0001$, Figure 4.2A,B). Thus the expression of ITGA5 in rat myometrium was dependent on gravidity.

We also studied the spatial and temporal distribution of ITGA5 protein in the gravid and empty uterine horns at late gestation. ITGA5 *in situ* was detected in both (longitudinal and circular) myometrial layers. Strong immunoreactivity of ITGA5 was observed in the longitudinal muscle layer of all myometrial samples examined compared to the circular muscle layer. We also consistently observed a small increase in ITGA5 detection at day 15 and day 19 in the gravid horns compared to the empty horns. In the

Figure 4.2. Immunoblot analysis of myometrial ITGA5 protein expression in gravid (G) and empty (E) horns of unilaterally pregnant rats. (A) Representative immunoblots of ITGA5 and CNN protein expression. (B) Denstiometric analysis illustrating the increase in ITGA5 protein expression in the gravid horn compared to the empty one. ITGA5 protein expression levels were normalized to CNN. Bar graphs showing the mean ± SEM ROD (n=4 at each time point). A significant difference from the empty horn of the same gestational day is indicated by * ($P < 0.05$) or ** ($P<0.01$). (Shynlova *et al.*, Biology of Reproduction, 2007, 77, 880 – 888)


A
15
19
23
E G E G E G
ITGA5
CNN
B
ROD(ITGA5/CNN
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
*
15
19
23
Gestational Day

circular muscle layer of the empty horns, ITGA5 immunostaining was virtually undetectable while ITGA5 was readily observed in the gravid horns (Figure 4.3). However, we also observed a temporal alteration of ITGA5 detection in the circular muscle layer of gravid horns. ITGA5 detection in gravid horns was noticeably up-regulated in the circular muscle layer in late pregnant (day 19) and labouring myometrium (day 23) (Figure 4.3). These data potentially indicate differential regulation of ITGA5 expression between two muscle layers that could result in enhancement of contractile activity in the circular muscle of the gravid horn during late pregnancy. Spatial distribution of ITGA5 was similar in both muscle layers and was not affected by gravidity. In particular, we found that ITGA5 was predominantly localized to the myometrial cell membranes in the form of punctate staining (Figure 4.3).

4.3.1.4 Detection of FN Protein by Immunoblot and Immunofluorescence Analyses

We reported earlier that myometrial mRNA levels of *Fn* were dramatically up-regulated starting from day 15 and were significantly higher in the gravid horn as compared to the empty one of unilaterally pregnant rats suggesting that mechanical stretch may contribute to this change [Shynlova *et al.*, 2004]. Using antibodies that specifically recognize only fibrillar fibronectin, we found that this ECM protein was also up-regulated at mid-pregnancy exclusively in the gravid uterine horn (Figure 4.4). Interestingly, it coincided with the beginning of hypertrophic transformation of myometrial SMCs during the "synthetic" phase of myometrial differentiation.

To investigate spatial and temporal distribution of FN protein in the myometria

Figure 4.3. Temporal alterations of ITGA5 immunolocalization in gravid horn myometrium from unilaterally pregnant rats during late gestation. Uterine tissue was collected from empty **(E)** and gravid **(G)** horns of unilaterally pregnant rats on gestational days (d) 15, 19, and 23. Tissues were labeled with anti-ITGA5 antibody and fluorescence microscopy images of cross-sections were collected. Intense immunoreactivity of ITGA5 was detected in longitudinal (L) SMCs throughout late gestation in both myometrial layers. Relatively weak immunostaining was present in circular (C) myometrial layer of empty horn and of d15 from gravid horn myometria. Detection of ITGA5 in circular myometrial layer from a gravid horn was significantly elevated prior to labour (d19-d23, arrows). The lack of staining after incubation of myometrial tissue with non-specific Rabbit IgGs is shown in Figure 7 (control). Magnification is 400x; scale bar = 25μm. (Shynlova *et al.*, Biology of Reproduction, 2007, 77, 880 – 888)

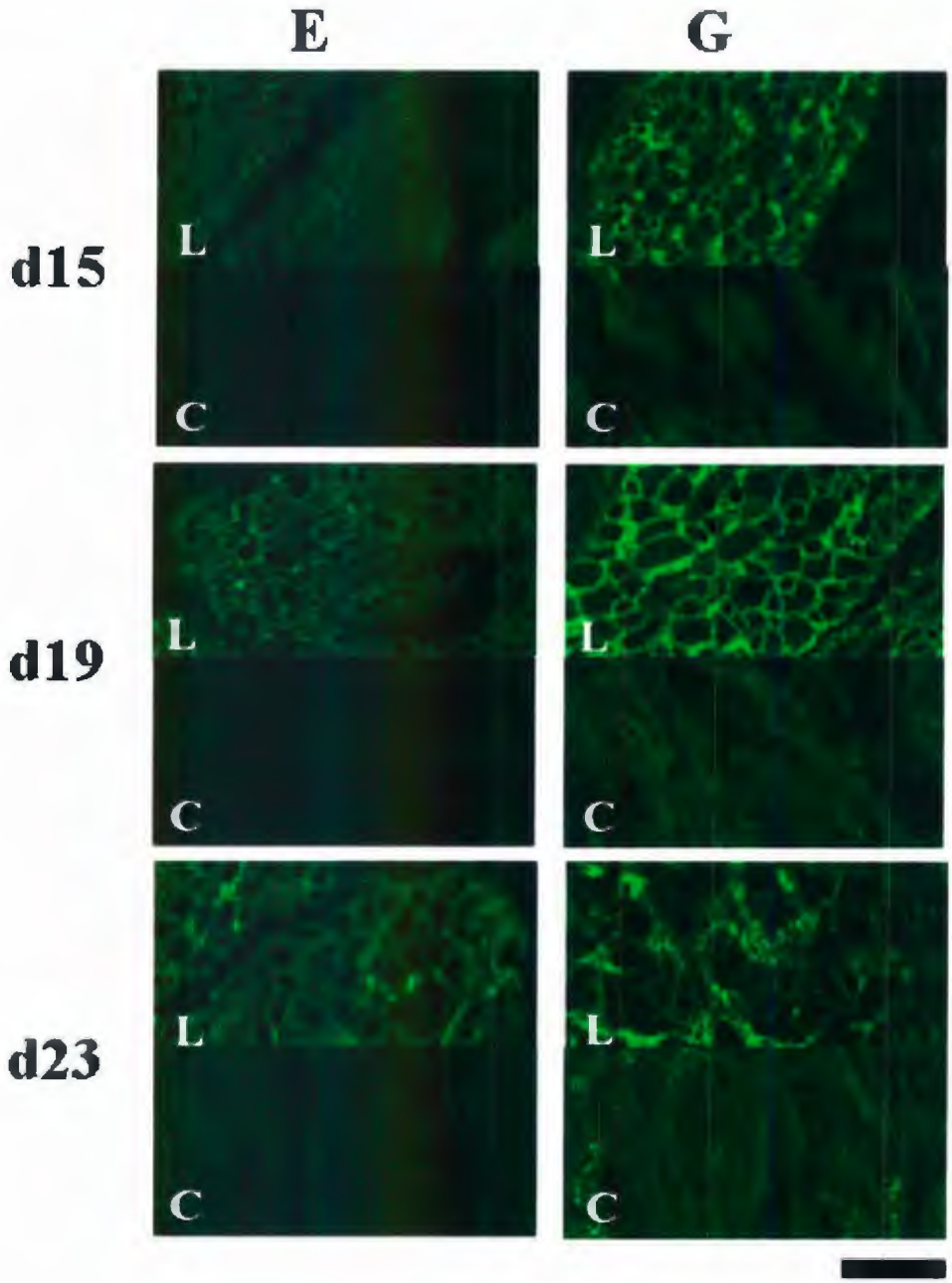
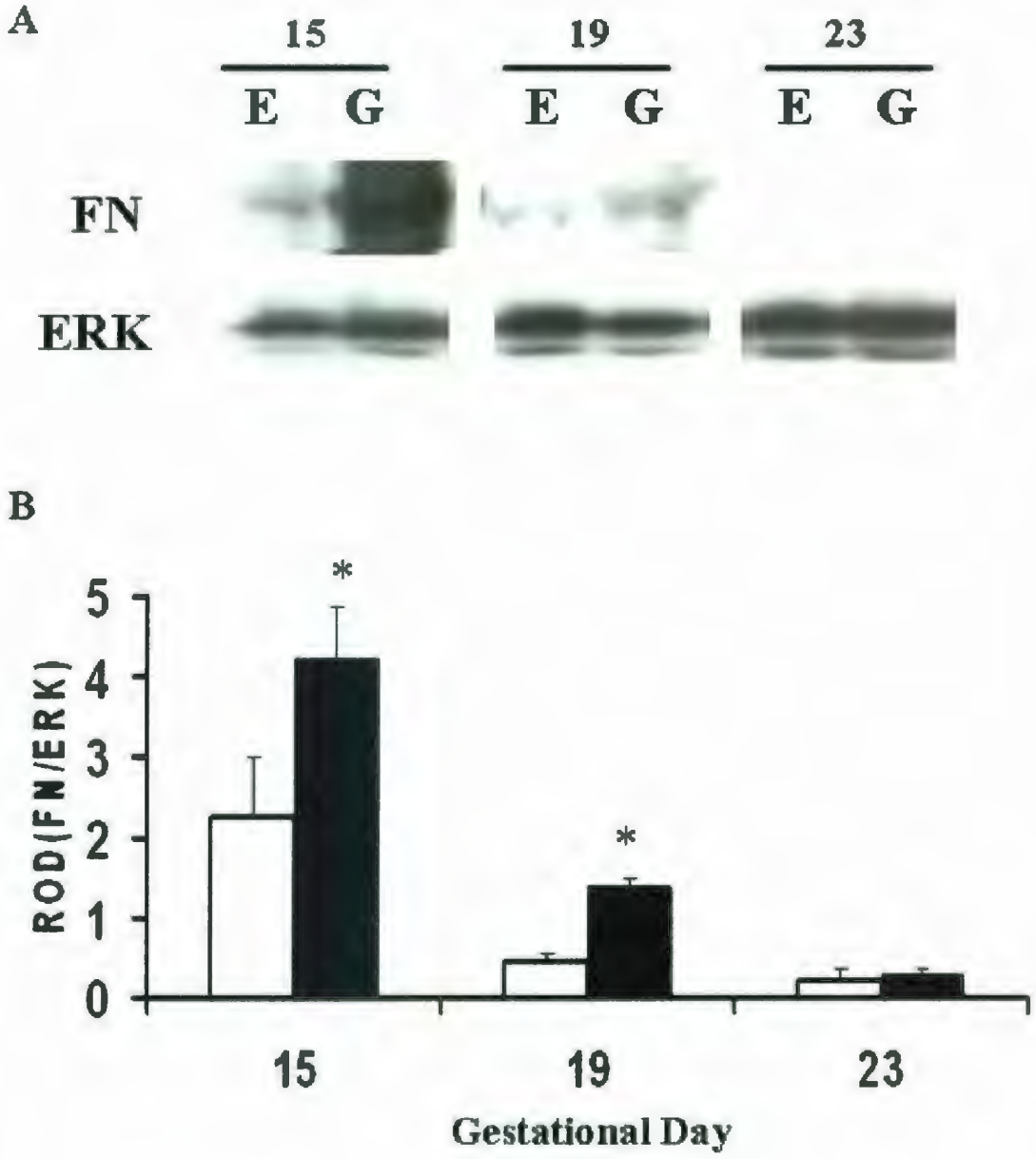

E
G
d15
d19
d23
L
C
L
C
L
C
L
C
L
C
L
C

Figure 4.4. Immunoblot analysis of FN protein expression in rat myometrium in gravid (G) and empty (E) horns of unilaterally pregnant rats. (A) Representative immunoblots of FN and ERK1/2 protein expression. (B) Denstiometric analysis illustrating the increase in ITGA5 protein expression in the gravid horn compared to the empty one. FN protein expression levels were normalized to ERK1/2. Bar graphs showing the mean ± SEM ROD (n=3 at each time point). A significant difference from the empty horn of the same gestational day is indicated by * ($P < 0.05$). (Shynlova *et al.*, Biology of Reproduction, 2007, 77, 880 – 888)


A
15
19
23
E G
E G
E G
FN
ERK
B
ROD(FN/ERK)
0
1
2
3
4
5
*
*
15
19
23
Gestational Day

from gravid and empty uterine horns of unilaterally pregnant rats at gestational days 15, 19, and 23 we applied immunofluorescence analysis (Figure 4.5). In the empty horn, FN immunostaining in the longitudinal muscle layer localized mostly around SM bundles, particularly at day 15 and day 19, however in the circular muscle layer staining was also found around individual SMCs. In the gravid horn, immunoreactive FN was found in both muscle layers surrounding the plasma membrane of each individual SMC, in connective tissue surrounding SMC bundles and in blood vessels. FN protein immunostaining at a higher magnification illustrated that FN was localized around individual SMCs exhibiting a continuous and regular, bead-like pattern of staining associated directly with the cell surface of hypertrophic myocytes (Figure 4.6). The immunofluorescence results would suggest that FN protein expression was not downregulated during late pregnancy and labour, in contrast to our immunoblot analysis. This difference may reflect the limitation of the two techniques utilized; immunoblot analysis was used to investigate total protein detection in a pooled lystate of both myometrial layers and the vascular plexus and our immunofluorescence analyses focused on spatial detection of the protein only within the muscle bundles.

4.3.2 Effect of RU486-Induced Preterm Labour on ITGA5 Expression

4.3.2.1 Expression of Itga5 mRNA

Circulating levels of P4 in rat maternal serum peak between day 15-day 19 and then dramatically decrease until day 23, the day of delivery [Pepe and Rothchild, 1974]. We have previously demonstrated a potential role for P4 in regulating *Itga5* and ITGA5

Figure 4.5. FN immunolocalization in gravid and empty horn myometria from unilaterally pregnant rats during late gestation. Uterine tissue was collected from empty **(E)** and gravid **(G)** horns of unilaterally pregnant rats on gestational days (d) 15, 19, and 23 Tissues were labelled with anti-FN antibody and fluorescent microscopy images of cross-sections were collected. FN localized mainly within the area surrounding SMC bundles and around individual SMCs in both, longitudinal (L) and circular (C) muscle layers of the gravid uterine horn. The lack of staining after incubation of myometrial tissue with affinity-purified IgG at the same concentration as the primary antisera, is shown in Control. Magnification is 200x; scale bar =50 μm. (Shynlova *et al.*, Biology of Reproduction, 2007, 77, 880 – 888)

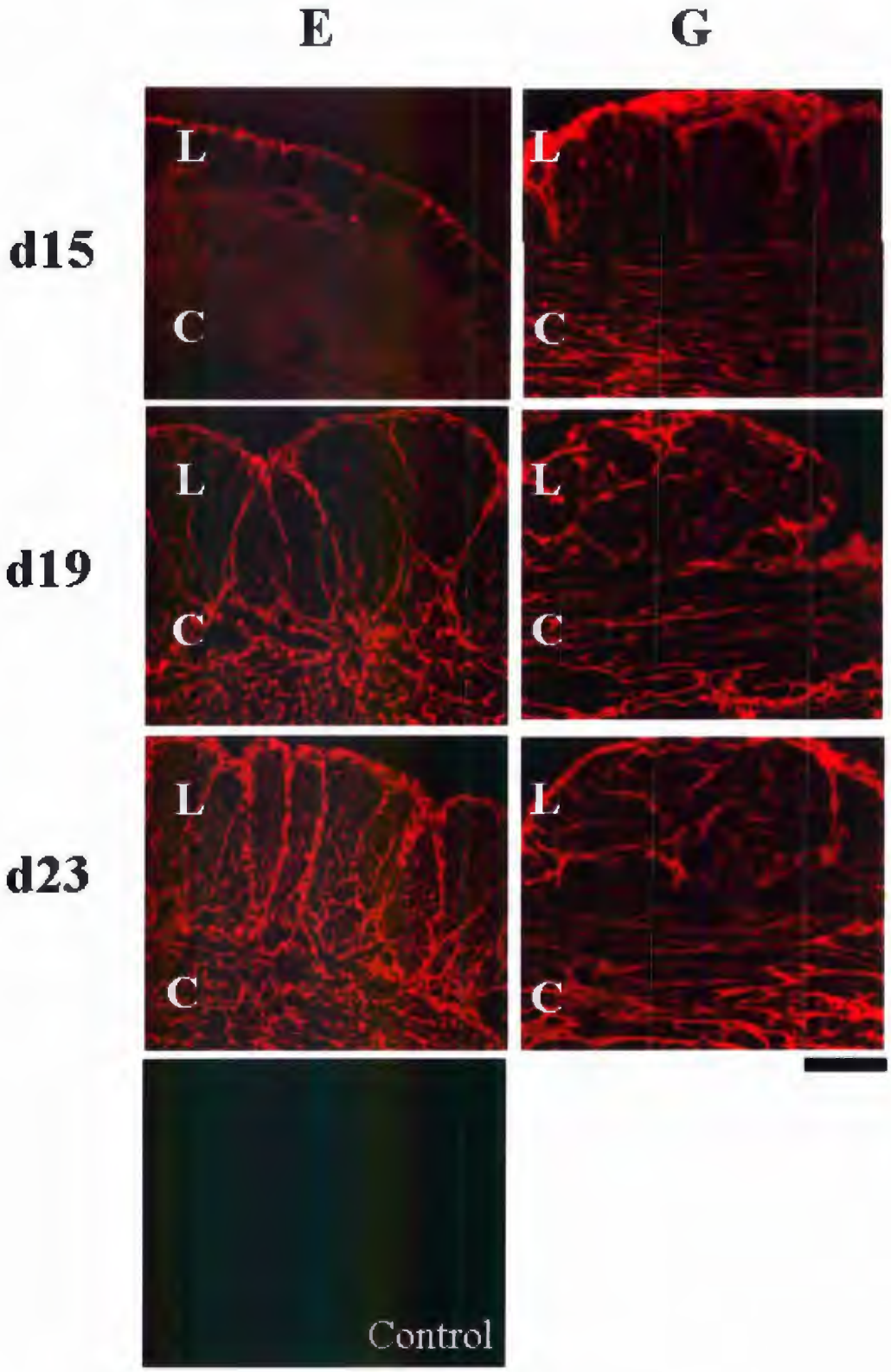
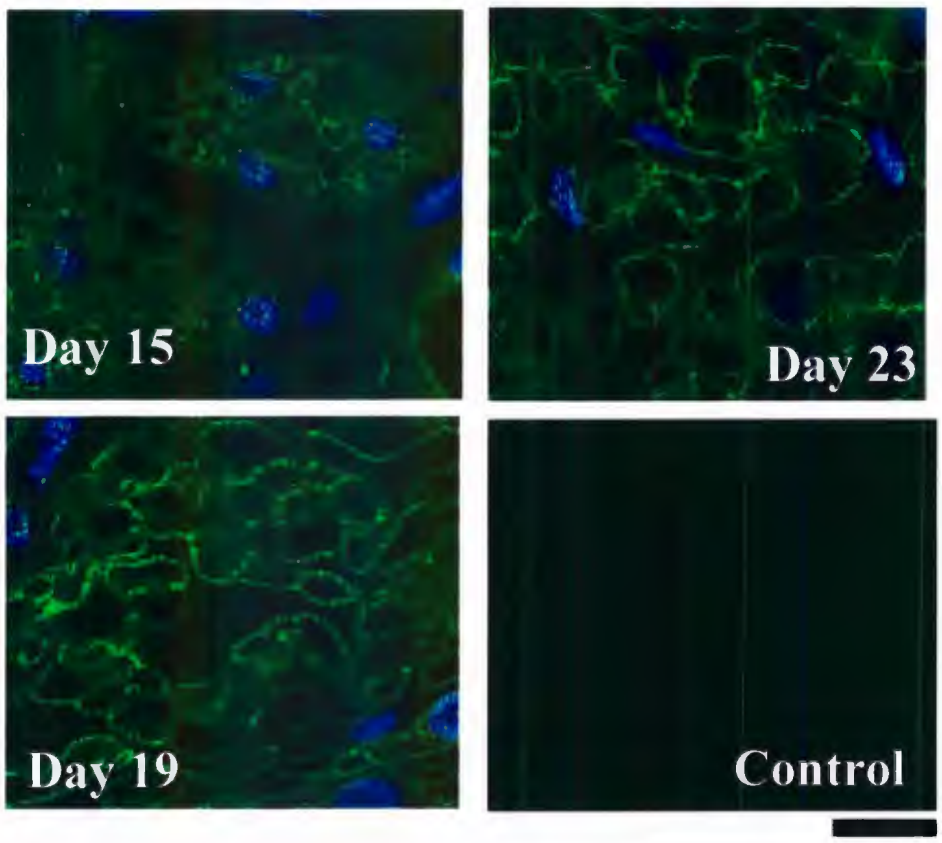

E
G
d15
d19
d23
L
C
L
C
L
C
L
C
L
C
L
C
Control

Figure 4.6. The organization of FN in the gravid horn myometrium. Shown are the cross-sections of myometrial cells from the longitudinal muscle layer of the gravid uterine horn. A bead-like FN staining localized around individual SMCs on gestational days 15, 19, 23. The lack of staining after incubation of myometrial tissue with affinity-purified IgG at the same concentration as the primary antisera, is shown in Control. DAPI nuclear counter stain (blue) is observed in 3 of the images. Magnification = 630x; scale bar 15μm. (Shynlova *et al.*, Biology of Reproduction, 2007, 77, 880 – 888)


Day 15
Day 23
Day 19
Control

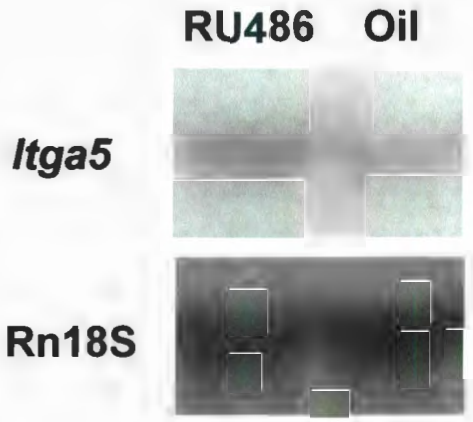
expression using a P4-prolonged labour model [Williams *et al.*, 2005]. Specifically, treatment of pregnant rats from day 20 with daily injections of P4 resulted in failure to initiate labour on day 23 and maintained *Itga5* and ITGA5 expression at day 24. To further examine the relationship between P4 plasma levels and *Itga5*/ ITGA5 expression, pregnant rats were treated with the P4 receptor antagonist, RU486. Treatment of pregnant animals at day 19 of gestation with RU486 induced preterm labour within 24 hours on day 20. Northern blot analysis of *Itga5* mRNA from vehicle and RU486-treated rats suggested that there was an increase in mRNA expression with induced preterm labour (Figure 4.7A), however this increase was not significant (t-test, $p = 0.1402$, $n = 4$; Figure 4.7B). Real-Time PCR analysis of myometrial tissue collected from vehicle and RU486-treated rats indicated that *Itga5* mRNA levels were significantly up-regulated after P4 blockade ($P<0.05$, Figure 4.8). In contrast to the significantly elevated expression of *Itga5* gene after administration of RU486, ITGA5 protein levels were only slightly increased and did not reach statistical significance (Figure 4.9).

4.3.2.2 Immunocytochemical Detection of ITGA5

In the longitudinal muscle layer, detection of ITGA5 in RU486-induced labour samples, appeared to be more intense compared to the vehicle controls (Fig. 4.10). Spatially, immunoreactive ITGA5 accumulated in regions closely associated with plasma membranes of hypertrophied uterine SMCs. Importantly, in the circular muscle layer ITGA5 also appeared to accumulate at cell membranes following RU486-induced labour compared to the vehicle control, mimicking changes that were observed towards normal

Figure 4.7. Northern blot analysis of *Itga5* expression in rat myometrium after treatment of d18 pregnant rats with RU486 or vehicle control. (A) Representative northern blots of *Itga5* mRNA expression and *Rn18s* RNA expression. Analysis was performed with an *Itga5*-specific human cDNA and an 18S-specific rabbit cDNA as templates for radiolabelled probe production. (B) Densitometric analysis illustrating that, while there was an increase in *Itga5* mRNA expression with administration of RU486, the increase was not significant. Values are from 4 independent experiments (n=4) ± SE. RU486, active labour (2-3 pups born); oil, vehicle control.

A


RU486
Oil
Itga5
Rn18S

B

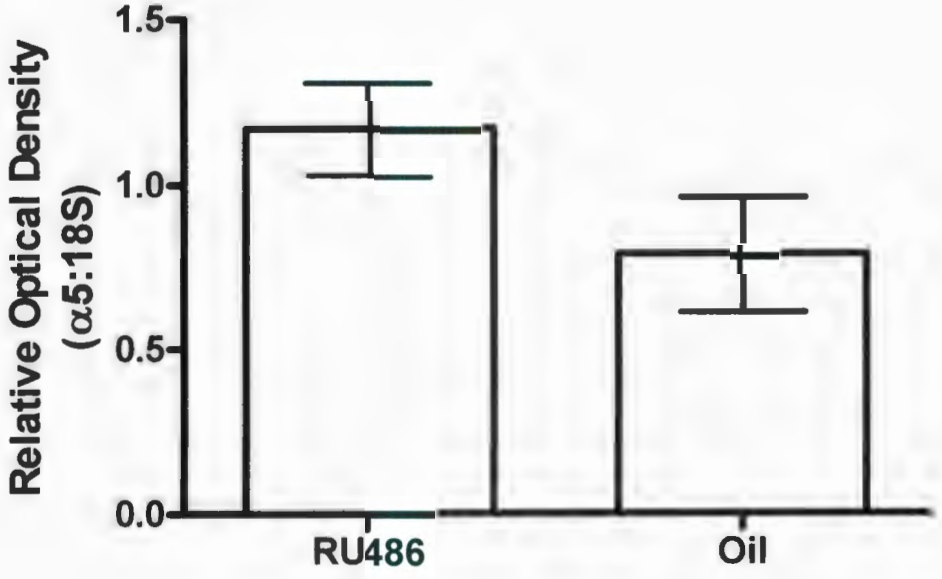

Relative Optical Density
(α5:18S)
1.5
1.0
0.5
0.0
RU486
Oil

Figure 4.8. Expression of *Itga5* mRNA in rat myometrium during RU486-induced preterm labour. Real-time PCR analysis of mRNA levels for *Itga5* expression normalized versus *Rn18S* RNA and expressed in fold change relative to the vehicle sample. See legend to Figure 1 for primers sequences. Shown are vehicle (white bar) and RU486-treated (black bar) samples. Values represent mean ± SD (n=4 at each time point). A significant difference is indicated by * ($P < 0.05$). (Shynlova *et al.*, Biology of Reproduction, 2007, 77, 880 – 888)

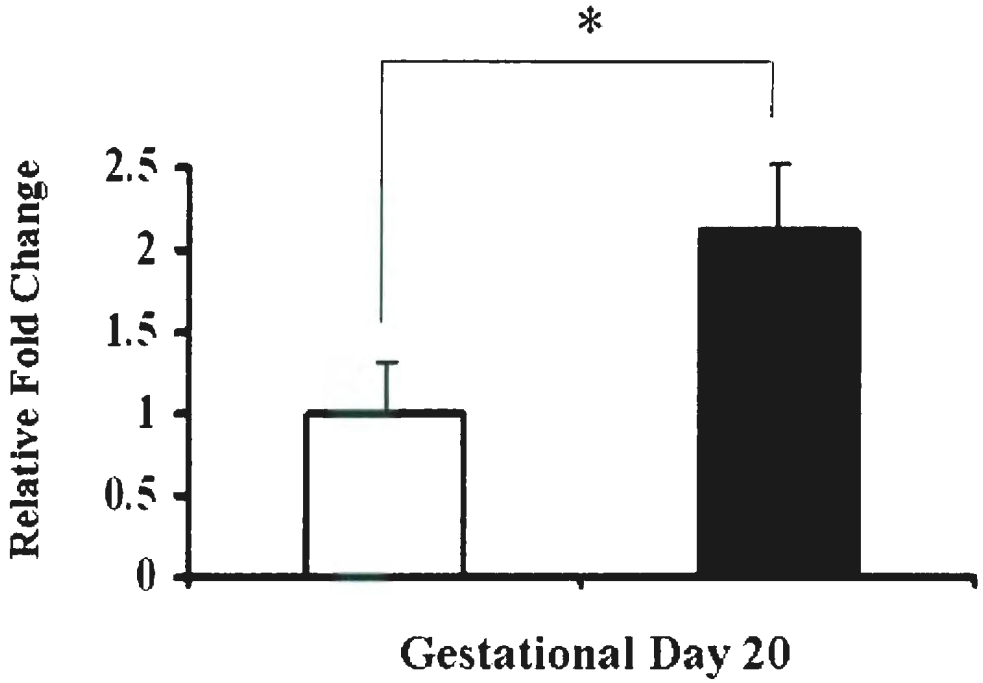
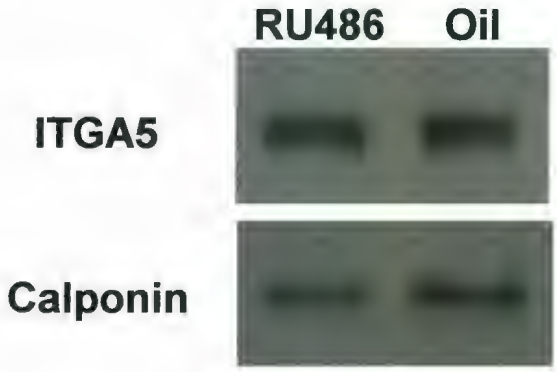

*
Relative Fold Change
2.5
2
1.5
1
0.5
0
Gestational Day 20

Figure 4.9. Immunoblot analysis of ITGA5 expression in rat myometrium after treatment of d18 pregnant rats with RU486 or vehicle control. (A) Representative immunoblots of ITGA5 and CNN protein expression. (B) Densitometric analysis illustrating that, while there was an increase in ITGA5 protein expression with administration of RU486, the increase was not significant. Values are from 4 independent experiments (n=4) ± SE. RU486, active labour (2-3 pups born); oil, vehicle control.

A


RU486
Oil
ITGA5
Calponin

B

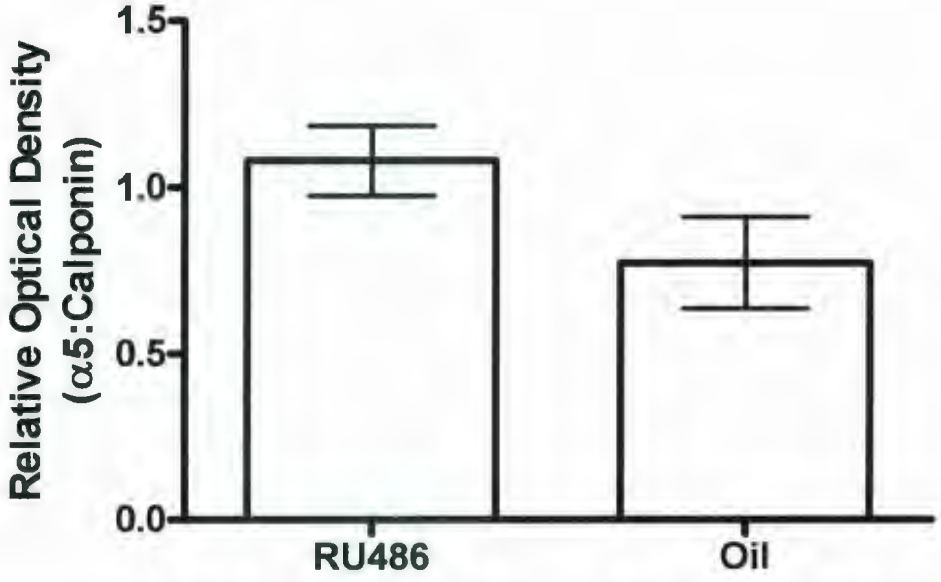
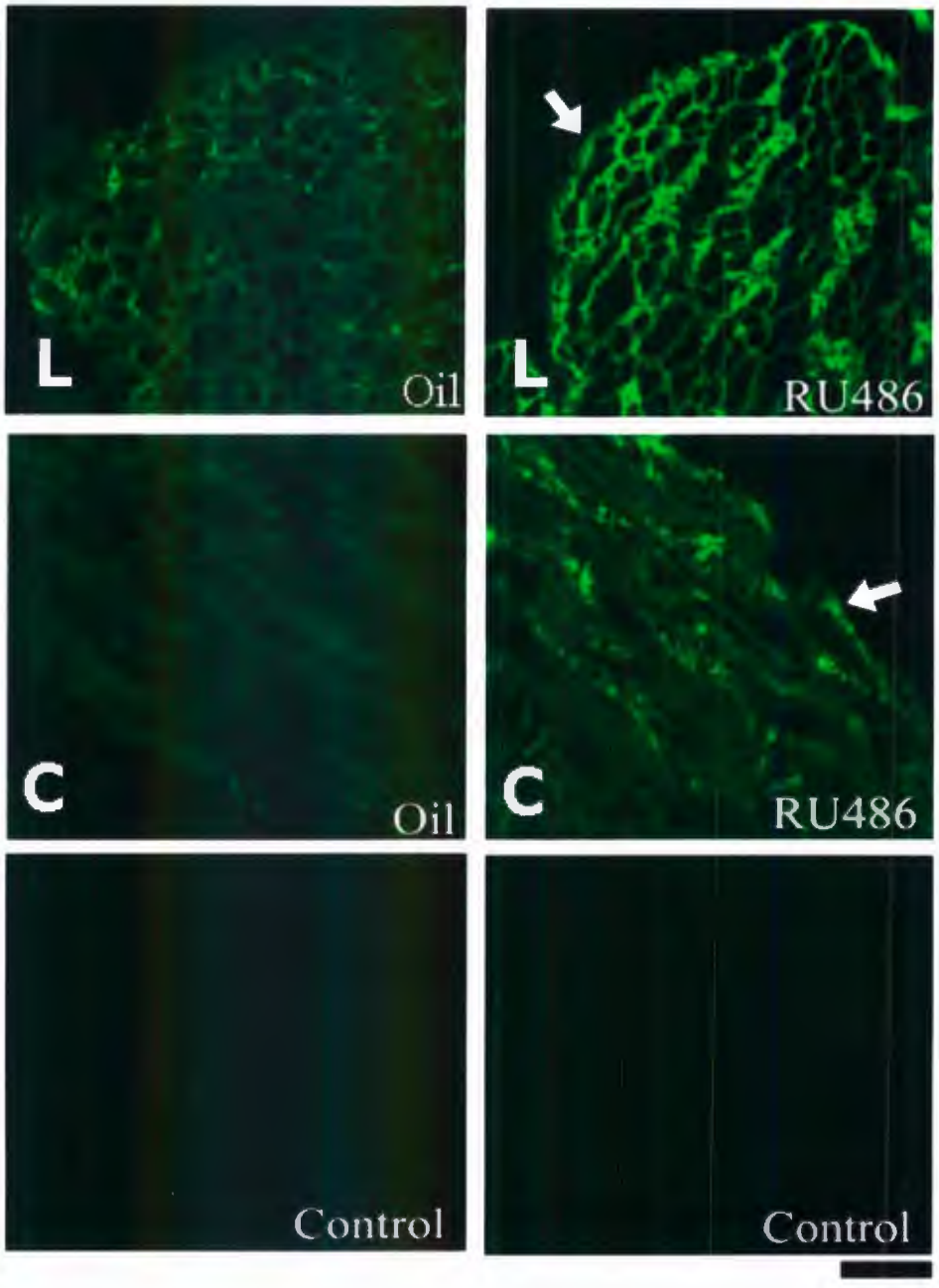

Relative Optical Density
(α5:Calponin)
1.5
1.0
0.5
0.0
RU486
Oil

Figure 4.10. Immunolocalization of ITGA5 protein in the longitudinal (L) and circular (C) smooth muscle layer of rat myometrium after treatment of pregnant rats with RU486 or vehicle (oil). Myometrial samples were collected during RU486 –induced preterm labour (2-3 pups born) and non-labouring day 20 pregnant vehicle-treated control rats. Negative control - rabbit IgG diluted to the same working dilution as primary anti-ITGA5 antibody. Arrows highlight membrane-specific staining. Magnification is 400x , scale bar = 25μm. (Shynlova *et al.*, Biology of Reproduction, 2007, 77, 880 – 888)


L
Oil
L
RU486
C
Oil
C
RU486
Control
Control

term labour (Figure 4.3;[Williams *et al.*, 2005]). Moreover, the staining was more punctate compared to the longitudinal smooth muscle layer.

4.4 Discussion

In the present study we have used the unilaterally pregnant rat model to investigate the role of biological mechanical stretch in the myometrial expression of FN and its unique FN-binding integrin partner ITGA5 during late pregnancy and labour. Our data primarily demonstrate that (1) a marked synchronous up-regulation of FN and ITGA5 occurs specifically in the gravid uterine horn of unilaterally pregnant rats, (2) the increase in ITGA5 detection during late pregnancy mostly occurs in the circular muscle layer of the gravid horn, which is subject to mechanical stimulation.

Previous publications have led us to conclude that the myometrium undergoes gradual changes in phenotype during pregnancy characterized by an early proliferative phase, an intermediate "synthetic" phase of cellular hypertrophy and matrix elabouration, and the final contractile/labour phase [Shynlova *et al.*, 2006; Shynlova *et al.*, 2007a]. Myometrial hypertrophy occurs specifically in the second half of gestation (starting from gestational day 15 in rats) when fetal growth is maximal. Several groups in the past have suggested that in addition to endocrine factors, the enlarging fetus might induce uterine growth by placing the uterine wall under tension [Goldspink and Douglas, 1988; Douglas *et al.*, 1988]. Using a unilaterally pregnant rat model, Douglas and Goldspink (1988) showed that cellular hypertrophy was dramatically increased in the gravid horn (subjected to stretch by growing fetus), while remaining unchanged in the empty horn.

Using the same model, we report that the gestational changes in *Fn* and *Itga5* gene and protein expression described in this study occurred specifically in the gravid horn of unilaterally pregnant rats beginning around mid-pregnancy, when myometrial SMCs acquired a synthetic phenotype. These changes in FN and ITGA5 protein expression coincided with the onset of hypertrophic changes in gravid rat myometrium. Studies on the heart suggest that multiple factors are responsible for the hypertrophic response and that integrins are an important component of this process with ITGB1 being directly linked to the hypertrophic cellular transformation of neonatal ventricular myocytes [Ross, 2002]. Moreover, ITGA1 and ITGB1 are up-regulated in the adult rat myocardium during aortic constriction mediated cardiac hypertrophy [Terracio *et al.*, 1991; Babbitt *et al.*, 2002; Keller *et al.*, 2001]. During the initial phases of pressure overload, expression of FN and ITGA5B1 in cardiomyocytes increase in parallel [Goldsmith *et al.*, 1999]. Thus we propose that a coordinated induction of FN and ITGA5 in pregnant rat myometrium is an important factor in the development of myometrial hypertrophy in rats. Of note, both ITGA1 and ITGB1 subunits are also increasingly detected at myometrial cell membranes in the second half of gestation (Williams, SJ, Shynlova, O, Lye, SJ, MacPhee, DJ, in preparation).

FN is a large multifunctional glycoprotein with wide tissue distribution and is essential for normal development and tissue repair [Hynes, 1990; Ross, 2002; Keller *et al.*, 2001; Schwarzbauer, 1991]. FN plays an important role in stabilizing ECM material by directly attaching cells to various substrates such as collagen fibers and proteoglycans [Pulkkinen *et al.*, 1984]. Integrin–FN interactions allow unfolding of the soluble protein

and its assembly into a detergent-insoluble fibrillar matrix that can modulate cell morphology, growth, and tissue architecture [Schwarzbauer and Sechler, 1999; Wierzbicka-Patynowski and Schwarzbauer, 2003]. In vascular tissue FN also appears to be involved in connecting the SMCs to each other, as suggested by the presence of FN-positive layers bridging the gaps between adjacent cells. Using antibodies that specifically recognize only fibrillar FN, in the present study we found that this ECM protein was up-regulated in a gravid horn at mid-pregnancy during the "synthetic" phase of myometrial differentiation. Likewise, it has been reported that the fibrillar form of FN was associated with the surface of dedifferentiated ("synthetic") vascular SMC. *In vitro* freshly isolated vascular SMCs adhere to a substrate of FN and in a few days of serum-free culture were converted from a contractile to a synthetic phenotype [Hedin *et al.*, 1989; Thyberg, 1996]. These experiments suggest that a substrate of FN may be sufficient to promote the shift in phenotype of vascular SMCs and that other exogenous macromolecules are not required in these processes. FN exerts its effect by interaction with integrin receptors, and this not only produces a physical linkage to the cells but also generates signals that adjust their behaviour.

Ligand-induced clustering of integrin receptors results in the formation of focal adhesions [Wozniak *et al.*, 2004]. These structures mediate tension transmission between cytoskeleton and ECM and also contain various adapter proteins and kinases that can promote cytoskeletal interaction. The resulting connection to cytoskeletal components ultimately allows focal adhesions to be mechanosensors while association with cytoplasmic kinases such as FAK also facilitates triggering of biochemical signalling

pathways or mechanotransduction [Schlaepfer *et al.*, 1999; Gerthoffer and Gunst, 2001]. Mechanical force applied to integrins results in increased clustering, perhaps as a result of increased actin and myosin recruitment and cytoskeletal assembly [Upadhyay *et al.*, 2003; Schoenwaelder and Burridge, 1999]. Similar to their function in pressure-induced cardiac hypertrophy [Ross, 2002] or stretched bladder SMCs [Upadhyay *et al.*, 2003], myometrial integrins are also components of mechanosensors that can sense and respond to increased stretch of the uterine walls imposed by the growing fetus(es). At early pregnancy, the uterus grows noticeably in longitudinal direction, followed by mostly circumferential growth from mid pregnancy onwards to accommodate the increasing fetal growth. It would thus be expected that tension on the uterine wall at late pregnancy is predominant in the circular SM layer. Indeed, in this study we observed a temporal change in ITGA5 protein detection in the circular muscle layers of the gravid horns compared to the longitudinal muscle layer. Such data provide additional support for a role of mechanical signals in the expression of ITGA5. The two muscle layers exhibit quite different phenotypes during pregnancy. For example, our previous studies have shown that the circular layer of the myometrium is more responsive to mechanical stretch than longitudinal [Doualla-Bell *et al.*, 1995; Shynlova *et al.*, 2005; Shynlova *et al.*, 2007b]. Such data imply specific roles for these myometrial layers during gestation. The circular muscle would provide the primary contractile response while the role of the longitudinal layer would be to shorten the uterus upon expulsion of each fetus (reviewed in [Shynlova *et al.*, 2005]). We propose that stretch-induced expression of ITGA5 in the circular myometrial layer stimulates FN-ITGA5 interaction and facilitates adhesion of

hypertrophied SMCs to ECM at late gestation contributing to myometrial growth and remodelling. We hypothesize further, that just prior to labour binding of FN to its receptor activates integrin signalling and assembly of FN fibrillar matrix around individual SMCs in circular muscle, which would contribute significantly to tissue cohesion [Wierzbicka-Patynowski and Schwarzbauer, 2003; Robinson *et al.*, 2003] and supports the specific role of this muscle layer during labour contractions. This mechanism would give rise to increased integrin signalling through FAK [Roovers and Assoian, 2003]. Mechanical stretch induces tyrosine phosphorylation of FAK, which is a critical event to stretch-induced MAP kinase activation in cardiomycytes [Aikawa *et al.*, 2002] and fibroblasts [Wang *et al.*, 2001]. Importantly, we discovered earlier that *in vivo* and *in vitro* static mechanical stretch of myometrial SMCs activated extracellular signal-regulated kinase (ERK) and p38 MAP kinase, allowing myometrial SMCs to sense mechanical stimuli and convert them to changes in gene expression [Oldenhof *et al.*, 2002].

We have previously documented that myometrial hypertrophic growth is dependent upon P4 and mechanical stretch. A fall in the P4 levels and an increase in E2 in rat maternal plasma at late gestation are believed to be essential to the activation of the myometrium at labour. Our present *in vivo* finding show that blockade of P4 signaling following administration of RU486 increases *Itga5* expression in the myometrium, but does not significantly increase ITGA5 levels, although immunoreactive ITGA5 in the circular and longitudinal layers accumulated in regions associated with myocyte plasma membranes compared to controls. If progesterone withdrawal alone positively regulated

Itga5 gene expression then a significant increase in *Itga5* mRNA and/or ITGA5 protein expression in gravid horns on day 23, compared to expression in gravid horns on d15 and d19, would have been expected since circulating progesterone levels would have declined by d23. This pattern of expression was not observed and as a result, it would appear that significant regulation of *Itga5* gene expression by progesterone is unlikely. The accumulation of immunoreactive ITGA5 at myocyte plasma membranes following RU486-induced labour may be a reflection of a need for ITGA5 at these sites during the labour process itself. These data suggest that increased *Itga5* expression levels in the myometrium during late pregnancy and labour [Williams *et al.*, 2005] are more likely due to increased stretch on the myometrium than changes in P4 levels. We do not believe that P4 serves as a negative regulator of ITGA5 as *Itga5* expression begins to increase long before circulating P4 levels begin to decrease. We have also reported earlier that blockade of P4 receptors by RU486 causes a significant up-regulation of *Fn* and basement membrane (BM) components collagen IV and laminin β2 [Shynlova *et al.*, 2004]. Thus, as a result of all our findings we speculate that the decrease in P4 and the subsequent increase in E2, in addition to a mechanical stimulation of myometrium might be responsible for an increase in the expression of FN-ITGA5 complexes. Additionally we can not rule out the possibility that at late gestation and during labour stimulation of FN-ITGA5 interaction could be triggered by other factor (s) derived from maternal decidua, extra embryonic membranes or fetus itself.

In summary, we suggest that increased expression of FN and ITGA5 may represent an important event in the preparation of the myometrium for the development

of optimal contractions during labour. At first, FN and ITGA5 promote rapid growth (hypertrophy) of pregnant myometrium. This "synthetic" phase of myometrial differentiation is associated with a formation of fibrillar FN matrix around individual SMCs, and is characterized by high FAK P-Tyr activity and increased focal adhesion turnover [MacPhee *et al.*, 2001]. During contractile phase of myometrial differentiation, a decrease in FAK activity stabilises SMC-ECM interactions, forming stable focal adhesions connecting FN matrix and actin cytoskeleton through clusters of ITGA5 molecules. The reinforcement of ligand-integrin interaction contributes significantly to anchorage of hypertrophied SMCs to the uterine ECM and facilitates proper smooth muscle intercellular cohesion before labour. During labour, the focal contacts, that were formed during late pregnancy, serve as major points of force transduction guaranteeing that myometrium works as a mechanical syncytium through each contraction.

CHAPTER 5

Silencing of *Itga5* Integrin Decreases Fibronection Deposition and Secretion.

(In Preparation)

5.1 Introduction

The uterus is a remarkably resilient system that that undergoes a rapid growth and differientiation during pregnancy and then resets itself after parturition. In the nonpregnant state, the human uterus weighs approximately 50 g and then increases to a weight of approximately 1200 g at term [Johansson, 1984]. This growth occurs by 2 mechanisms: an increase in cell number (hyperplasia), which is high during early gestation and decreases dramatically later, and an increase in cell size (hypertrophy), which is low at the beginning of gestation and increases with gestational age [Shynlova *et al.*, 2006]. Hypertrophy and hyperplasia are associated with an increase in cell protein synthesis, ECM protein synthesis, increases in number of cell organelles and transitions in contractile protein content and organization [Gabella, 1990]. This growth during pregnancy has been characterized in the rat uterus and several distinct phases were observed: 1) an early proliferative phase, 2) a transition period, 3) an intermediate phase of cellular hypertrophy and matrix elabouration, and 4) a contractile phase [Shynlova *et al.*, 2006]. Shynlova *et al* (2006) found an increase in protein levels of the cleaved form of caspases 3, 6, 7 and 9 at mid gestation and this triggered the differentiation of the uterine smooth muscle to a synthetic phenotype (intermediate phase) in the rat.

Integrins have been shown to initiate signaling events that alter the contractility of the cell, and in addition, stimulation of contraction has shown to increase association of integrins with focal adhesion proteins [Chan *et al.*, 2001; Zhang and Gunst, 2006]. We hypothesize that the presence of a proper ECM is very important for the proper differentiation of the myometrium into a contractile phenotype and ITGA5 may be a key molecule in driving the syntheis of a key matrix molecule.

We have previous suggested that the myometrium exists as a mechanical syncytium during gestation to facilitate efficient force transduction of the powerful contractions of labour [Williams *et al*, 2005]. We believe that ECM remodeling, focal adhesion turnover, increased expression of *Itga5* and subsequent ITGA5B1-FN interaction are important components of the development of a mechanical syncytium. Specifically, we proposed that ITGA5B1 may be important in promoting cellular cohesion during late pregnancy and it appears that this cohesivity is dependent on FN matrix assembly [Robinson *et al.*, 2004]. FN is secreted by cells as a soluble dimer and is assembled into an insoluble matrix at the cell membrane. Secreted FN exists as an inactive, compact dimer that is distributed diffusely over the cell surface and must be activated to undergo fibril assembly. FN matrix assembly is activated by the binding of soluble FN to ITGA5B1, resulting in integrin clustering and recruitment of signaling and cytoskeletal proteins into focal adhesions [Chen and Singer, 1982; Zamir *et al.*, 1999]. Kinase cascades stimulate a number of distinct intracellular responses, including reorganization of the actin cytoskeleton and changes in gene expression. Alterations in the cytoskeleton result in tension being produced, which expands and unfolds the FN

dimers, exposing sites for FN-FN interaction, and thus initiating FN fibril formation [Zhong *et al.*, 1998; Pankov *et al.*, 2000; Baneyx *et al.*, 2002]. It has been shown that disruption of the actin cytoskeleton results in loss of matrix assembly sites and decreased FN deposition [Christopher *et al.*, 1997; Wu *et al.*, 1995].

Previous studies have shown that antibodies to ITGA5B1 or to ITGB1 inhibit their function as well as inhibit FN assembly in vitro in fetal human lung fibroblast cells [Roman *et al.*, 1989; Akiyama *et al.*, 1989] and increasing the expression of ITGA5 by transfection has shown to increase FN deposition in the ECM in Chinese Hamster Ovary (CHO) cells [Giancotti and Ruoslahti, 1990]. Since the role of ITGA5 in FN secretion and deposition in myometrial cells has never been elucidated, the goal of this research was to investigate the effect of silencing *Itga5* gene expression, via *Itga5* specific siRNA, on these processes in the M11 human myometrial cell line.

5.2 Materials and Methods

5.2.1 Cell Culture

Myometrial M11 cells were a gift from John A. Copland, PhD (Mayo Clinic College of Medicine, Jacksonville, FL). These cells were derived from dispersed primary human myometrial cells by repeated passage without the use of any immortalizing or transforming agent. This cell line was chosen due to its ability to maintain a smooth muscle phenotype when cultured *in vitro,* unlike many available rat myometrium derived cell lines. M11 cells were maintained in DMEM high glucose (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Cansera International), and 100 U penicillin/100 μg

streptomycin (Invitrogen, Carlsbad, CA). Cells were cultured at 37°C in a humidified tissue culture incubator containing 5% CO_2 in air.

5.2.2 siRNA

To silence *Itga5* expression, Dharmacon ON-TARGET*plus SMART*pool siRNA reagent (Thermo Fisher Scientific, Lafayette. CO; Dharmacon Catalog # L-008003-00) targeting human *Itga5* (Accession # NM_002205) was used. The SMARTpool was chosen because the selection of siRNA duplexes is only as good as the target sequence used for the design. Errors in the sequence record as well as the presence of SNPs (single nucleotide polymorphisms) may interfere with the function of an individual siRNA. The use of a SMARTpool minimizes this risk by providing four highly functional duplexes that target different regions of the target gene. The siRNA was resuspended in Dharmacon 1X siRNA buffer, which was diluted from 5X siRNA buffer obtained from Dharmacon, to obtain a working concentration of 20 pmol/ul.

A positive control, siGLO RISC-free control siRNA (Thermo Fisher Scientific, Lafayette, CO; Dharmacon Catalog # D-001600-01), was co-transfected with *Itga5* siRNA to confirm the transfection efficiency and also used at times as a non-targetting control. This siGLO is a stable, fluorescent, non-targeting control siRNA with RISC-Free modification that enables co-transfection with functional siRNA without interfering with target gene silencing. After optimization experiments to test 100 nM, 80 nM, 50 nM and 20 nM, 20 nM *Itga5* siRNA and 20 nM siGLO concentrations effectively silenced

Itga5 gene expression while maintaining appropriate smooth muscle morphology and cell viability.

5.2.3 Transfection

Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, one day before transfection, M11 cells were plated in 75 cm^2 flasks in 10 ml of media without antibiotics so that they were approximately 80% confluent at the time of transfection for siRNA experiments. The next day, oligomer-Lipofectamine 2000 complexes were prepared as follows: 240 pmol (20 nM) of *Itga5* siRNA and 240 pmol (20 nM) of siGLO were diluted in serum-free, antibiotic-free media and gently mixed. Lipofectamine 2000 (30 µl) was diluted in serum-free, antibiotic-free media and gently mixed. These solutions were incubated for 5 minutes at room temperature. The diluted oligmer and the diluted Lipofectamine 2000 were then combined and incubated for 20 minutes at room temperature. The oligmer-Lipofectamine 2000 complexes were added to the flasks containing cells and serum-free media. Cells were incubated with complexes at 37°C in incubator for 24 hours. Cells were observed using a Leica DM-IRE2 inverted microscope (Leica Microsystems) equipped for epifluorescence illumination and attached to a Retiga EXi CCD camera (QImaging). Openlab Image Analysis software (Version 3.5.1; Improvision, Inc.) was used for image capture and analysis.

5.2.4 Collection of Secreted Fibronectin (Conditioned Media)

Cells were seeded ($2.5x10^5$/well) in 6 well plates 24 hours after siRNA transfection. Approximately 12 hours after seeding in 6 well plates, cells were washed in serum-free media for 2 hours and then incubated for an additional 24 hours in serum-free media containing 0.1% BSA. At the end of the 24 hours incubation, the media was collected in 15ml centrifuge tube and then centrifuged at 1000xg for 5 mins. The media was concentrated 10-fold using a Centricon centrifugal filter (10,000 molecular-weight cutoff; Ultracel YM-10, Millipore) by centrifugation for 30 mins at 5,000 x g according to the manufacturers' instructions.

5.2.5 Collection of Deposited Fibronectin Matrix

Cells were seeded ($2.5x10^5$/well) in 6 well plates 24 hours after siRNA transfection. After conditioned media was collected, the deposited matrix was collected. On ice, cells were washed once with PBS and then three times with 10mM Tris pH 8.0, 0.5% deoxycholate, 75 ug/ml phenylmethylsulphonyl fluoride (PMSF) to remove all but the deposited cell matrix. The matrix was then washed twice with 2mM Tris pH 8.0 and then once with PBS. Laemmli sample buffer (100ul, 2X) with 10% β-mercaptoethanol was added and each well was scraped thoroughly with a scraper. The plates were then tilted so that the solution pooled on one side of the well and was let to stand for 10 mins. Samples were then collected into microfuge tubes and boiled for 3 mins.

5.2.6 Immunoblot Analysis

ITGA5 and FN, respectively, and incubated with blots for 1 hr. Blots were washed once for 15 min in TBST, then four times for 5 min each in TBST. Proteins were detected using the Pierce SuperSignal West Pico Chemiluminescent Substrate (MJS BioLynx, Inc., Brockville, Ontario, Canada) detection system and multiple exposures were generated to ensure the linearity of the film exposures.

5.2.7 Data Analysis

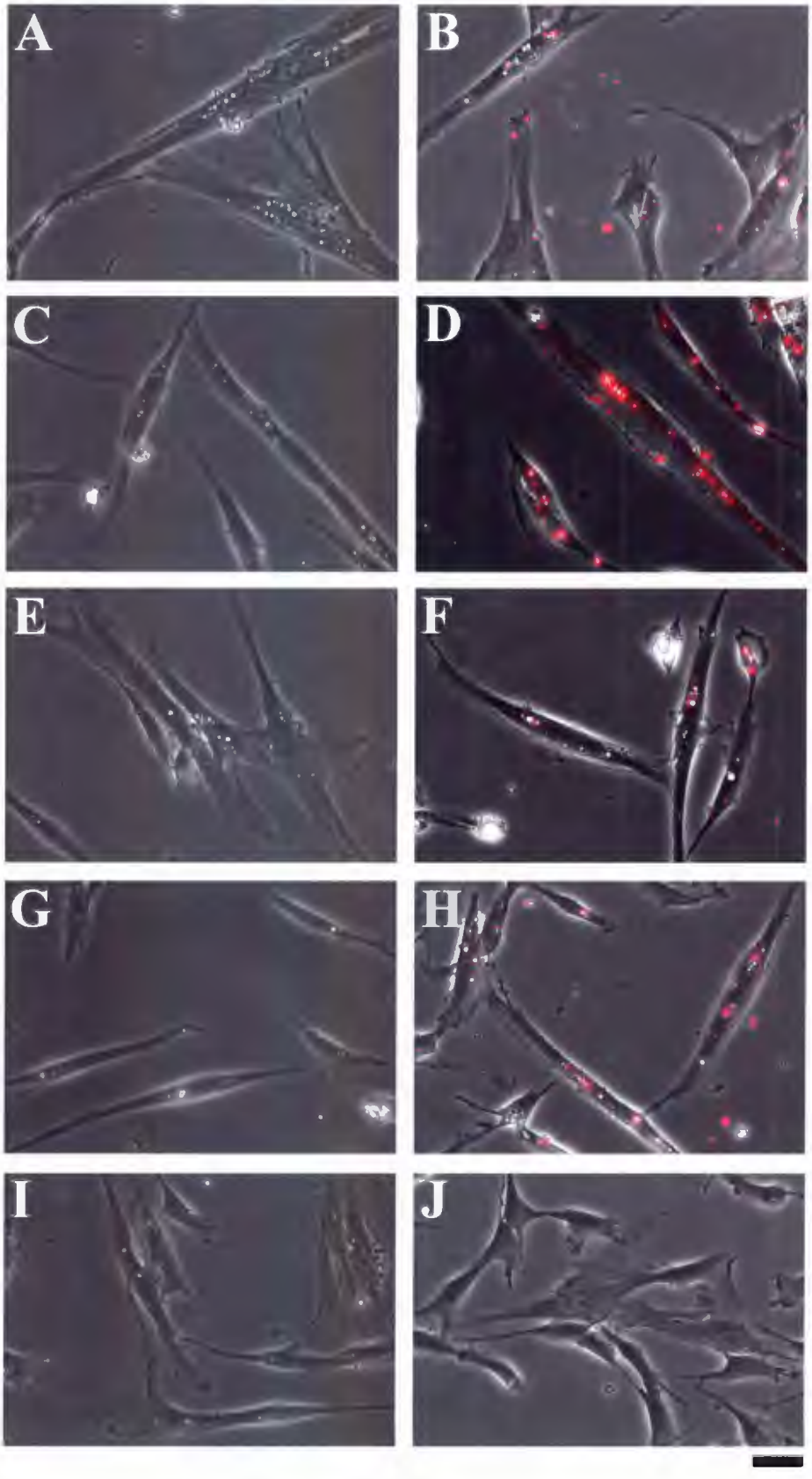
Densitometric analysis of immunoblots were performed with the aid of Scion Image software (Scion Image Corporation, Frederick, Maryland, USA). Statistical analysis was performed with GraphPad Instat version 3.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com) and data graphed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com). Data from Immunoblot analysis of ITGA5 and FN were subjected to a One-way Analysis of Variance and a Tukey-Kramer Multiple Comparisons test. Values were considered significantly different if $p < 0.05$.

5.3 Results

5.3.1 Effect of *Itga5* siRNA on ITGA5 Expression

Initial optimization experiments separately titrated *Itga5* siRNA and siGLO concentrations for use in transfections: 100 nM, 80 nM, 50 nM and 20 nM (Figures 5.1, 5.2, 5.3 and 5.4). Based on transfection efficiency and cell viability, both 50 nM and 20 nM concentrations were deemed appropriate with significant loss of ITGA5 and minimal

Figure 5.1 Photomicrographs of M11 cells 24 hours after transfection with various concentrations of *Itga5* siRNA or siGLO. Representative images of M11 cells transfected with: (A) 100 nM *Itga5* siRNA, (B) 100 nM siGLO, (C) 80 nM *Itga5* siRNA, (D) 80 nM siGLO, (E) 50 nM *Itga5* siRNA, (F) 50 nM siGLO, (G) 20 nM *Itga5* siRNA, (H) 20 nM siGLO, (I) Lipofectamine only and (J) Media only. Bar = 100 μm.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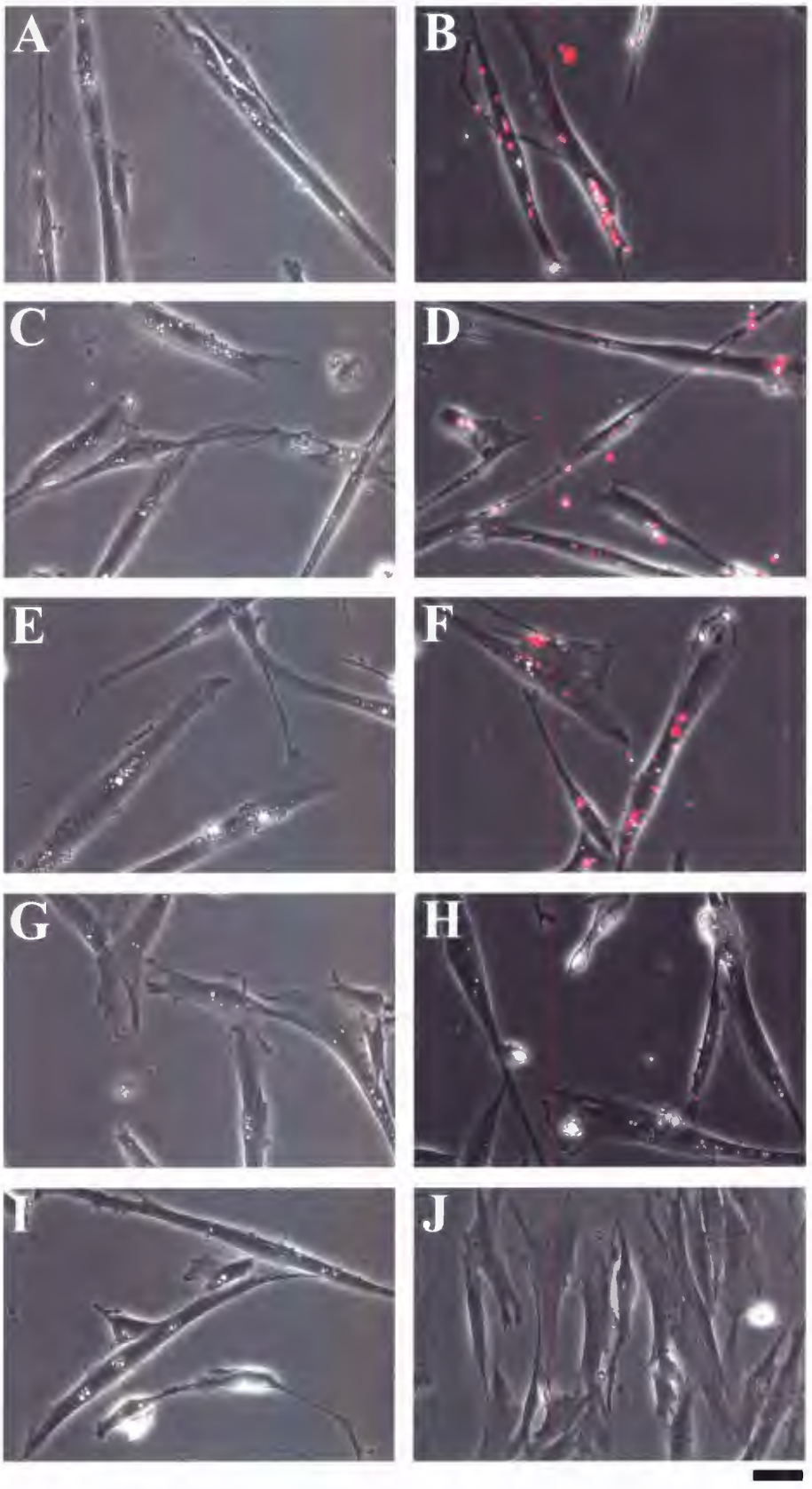
D
E
F
G
H
I
J

Figure 5.2 Photomicrographs of M11 cells 48 hours after transfection with various concentrations of *Itga5* siRNA or siGLO. Representative images of M11 cells transfected with: (A) 100 nM *Itga5* siRNA, (B) 100 nM siGLO, (C) 80 nM *Itga5* siRNA, (D) 80 nM siGLO, (E) 50 nM *Itga5* siRNA, (F) 50 nM siGLO, (G) 20 nM *Itga5* siRNA, (H) 20 nM siGLO, (I) Lipofectamine only and (J) Media only. Bar = 100 μm.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G
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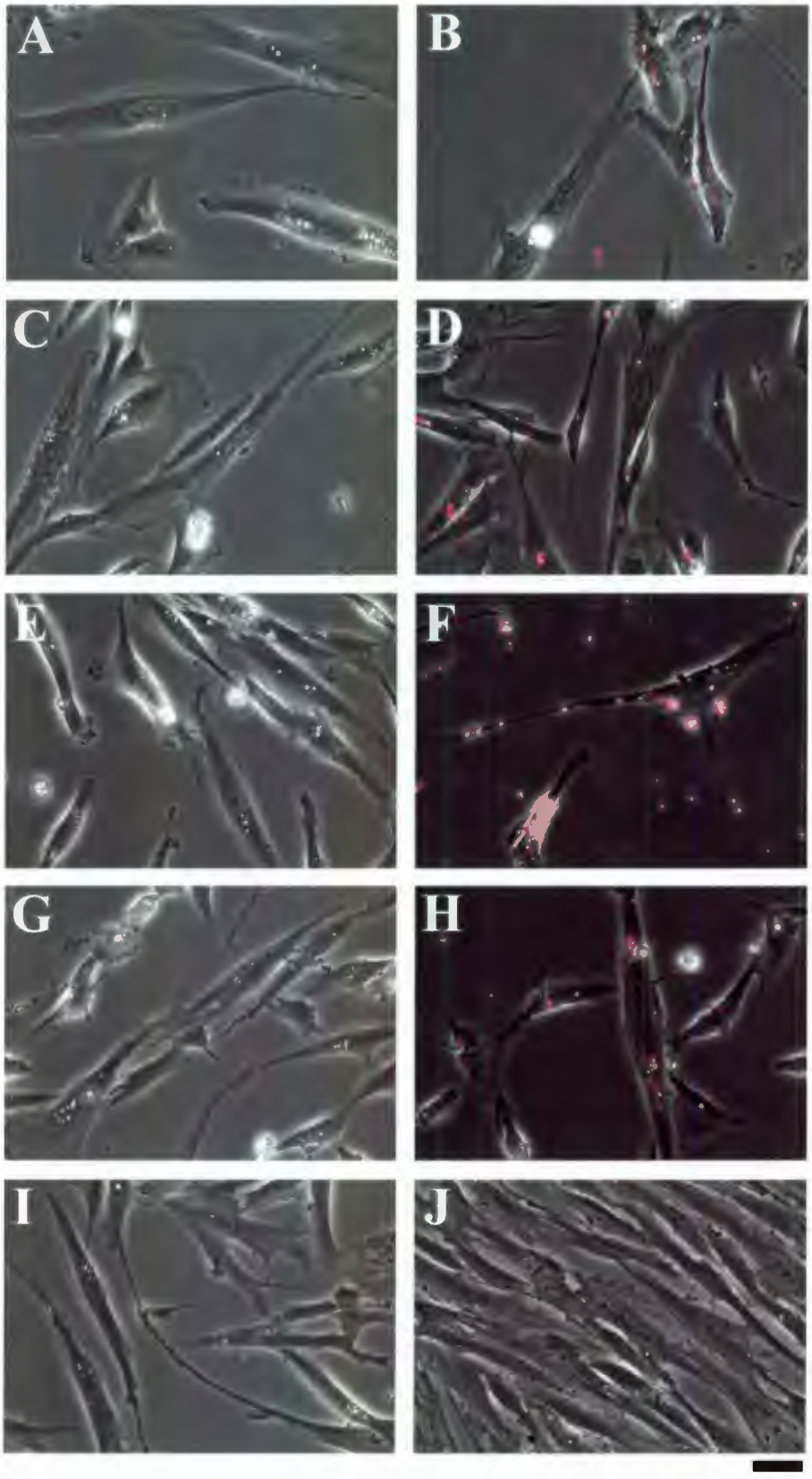
I
J

Figure 5.3 Photomicrographs of M11 cells 72 hours after transfection with various concentrations of *Itga5* siRNA or siGLO. Representative images of M11 cells transfected with: (A) 100 nM *Itga5* siRNA, (B) 100 nM siGLO, (C) 80 nM *Itga5* siRNA, (D) 80 nM siGLO, (E) 50 nM *Itga5* siRNA, (F) 50 nM siGLO, (G) 20 nM *Itga5* siRNA, (H) 20 nM siGLO, (I) Lipofectamine only and (J) Media only. Bar = 100 μm.


A
B
C
D
E
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G
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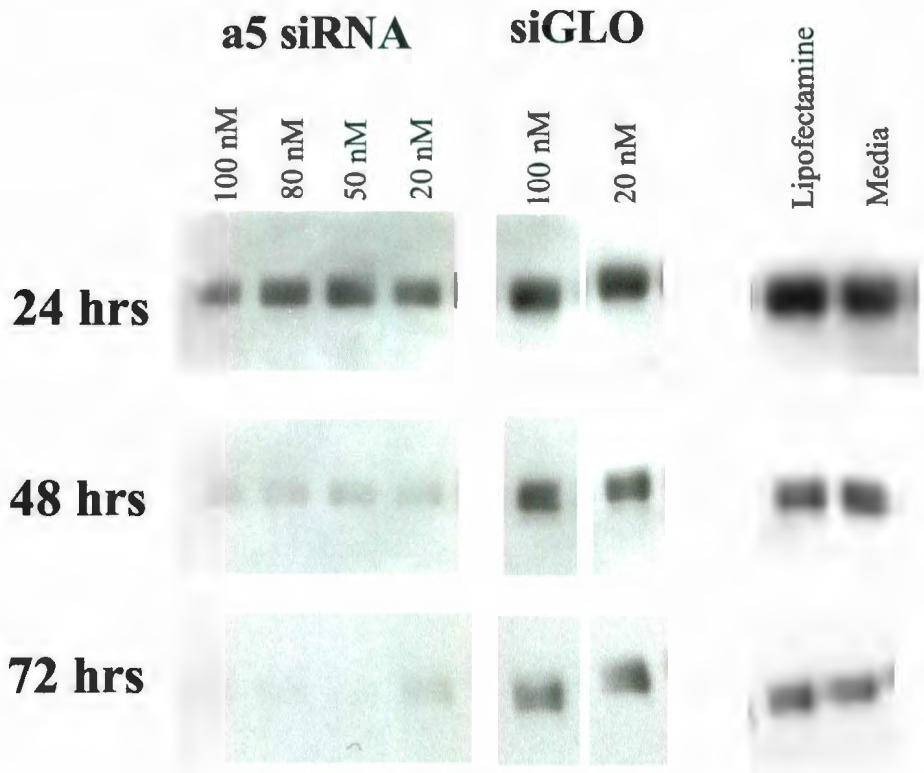
I
J

Figure 5.4 Immunoblot analysis of ITGA5 expression after transfection of M11 cells with various concentrations of *Itga5* siRNA or siGLO at 24, 48 or 72 hours after transfection.


a5 siRNA
siGLO
100 nM
80 nM
50 nM
20 nM
100 nM
20 nM
Lipofectamine
Media
24 hrs
48 hrs
72 hrs

cell loss. It appears that a decrease in ITGA5 is seen in lipofectamine and media only controls at 48 and 72 hrs compared to 24 hrs (Figure 5.4). This may be due to the exposure of cells to lipofectamine for an extended length of time resulting in cell death and the overgrowth of cells resulting in cell death in media only samples. Then co-transfection optimization experiments were performed on the different possible combinations of 50 nM and 20 nM siRNAs (50 nM *Itga5* siRNA + 50 nM siGLO; 50 nM *Itga5* siRNA + 20 nM siGLO; 20 nM *Itga5* siRNA + 50 nM siGLO; 20 nM *Itga5* siRNA + 20 nM siGLO) and it was deemed that 20 nM *Itga5* siRNA + 20 nM siGLO produced efficient silencing of *Itga5* while maintaining cell viability and would thus be used in future experiments (Figures 5.5 and 5.6). These results indicated that lipofectamine might have some effect on ITGA5 expression so a separate experiment was performed to determine whether lipofectamine could alter the expression of ITGA5. Lipofectamine did not appear to have a significant effect on ITGA5 expression (Figure 5.7).

After the optimization experiments were completed, we silenced *Itga5* gene expression in M11 myometrial cells in 8 independent experiments to determine the role of ITGA5 in FN matrix assembly. Immunoblot analysis using ITGA5-specific antisera demonstrated that ITGA5 was readily detectable in myometrial cell lysates and demonstrated that ITGA5 was readily detectable in myometrial cell lysates and expression was significantly decreased with *Itga5* siRNA transfection (one-way ANOVA, $P < 0.0001$; $n = 8$; Figure 5.8). Specifically, ITGA5 protein levels after *Itga5* siRNA transfection were significantly decreased over lipofectamine and media only controls (Tukey-Kramer post-test, $P < 0.001$; $n = 8$). Figure 5.9 shows Photomicrographs

Figure 5.5 Photomicrographs of M11 cells following co-transfection with different combinations of *Itga5* siRNA and siGLO at 24, 48 and 72 hours after transfection. Lipofectamine and media only images serve as control. Bar = 100 μm.

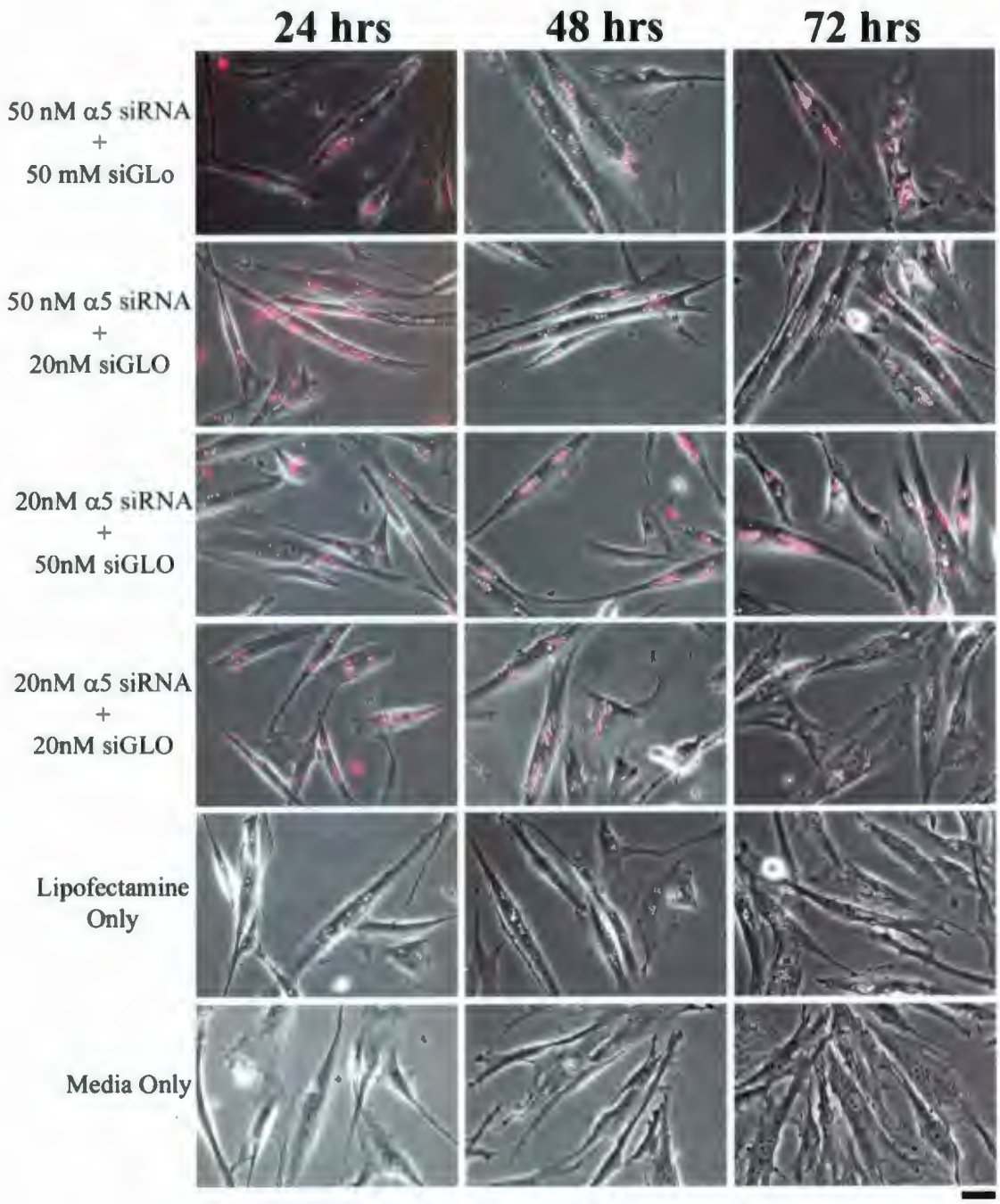

24 hrs
48 hrs
72 hrs
50 nM α5 siRNA + 50 mM siGLo
50 nM α5 siRNA + 20nM siGLO
20nM α5 siRNA + 50nM siGLO
20nM α5 siRNA + 20nM siGLO
Lipofectamine Only
Media Only

Figure 5.6 Knockdown of ITGA5 expression after co-transfection of *Itga5* siRNA and siGLO. Representative immunoblots of ITGA5 and CNN expression at (A) 24 hours after co-transfection, (B) 48 hours after co-transfection, (C) 72 hours after co-transfection. Lane 1 = 50nM *Itga5* siRNA + 50nM siGLO; Lane 2 = 50nM *Itga5* siRNA + 20nM siGLO; Lane 3 = 20nM *Itga5* siRNA + 50nM siGLO; Lane 4 = 20nM *Itga5* siRNA + 20nM siGLO; Lane 5 = Lipofectamine only; and Lane 6 = Media only.

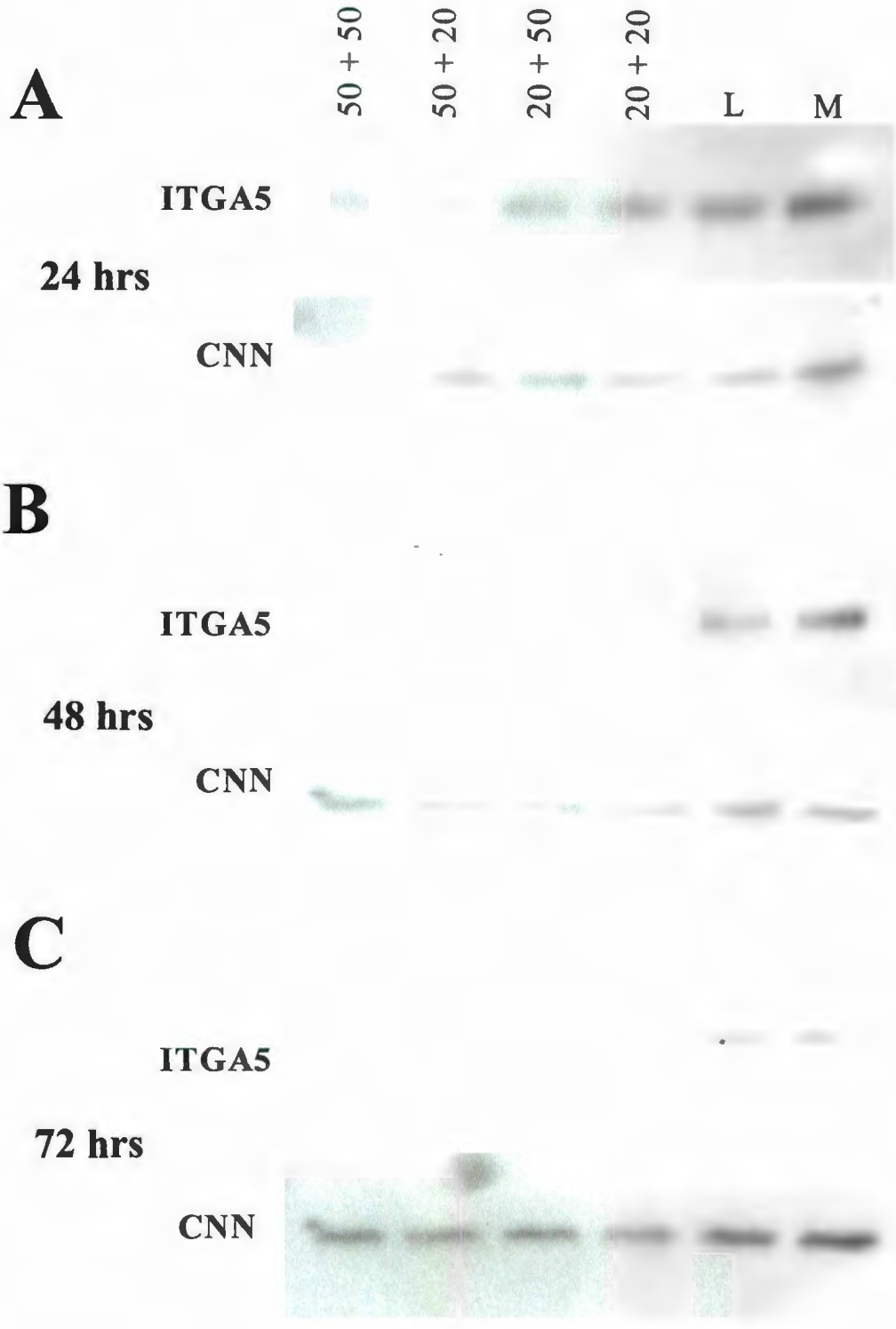

A
50 + 50
50 + 20
20 + 50
20 + 20
L
M
ITGA5
24 hrs
CNN
B
ITGA5
48 hrs
CNN
C
ITGA5
72 hrs
CNN

Figure 5.7 Immunoblot analysis of ITGA5 expression in Lipofectamine only and Media only samples. (A) Representative blots of ITGA5 and CNN expression at 24, 48 and 72 hours after transfection. (B) Densitometric analysis indicating no significant change in ITGA5 expression with the use of lipofectamine. L = lipofecatmine; M = Media.

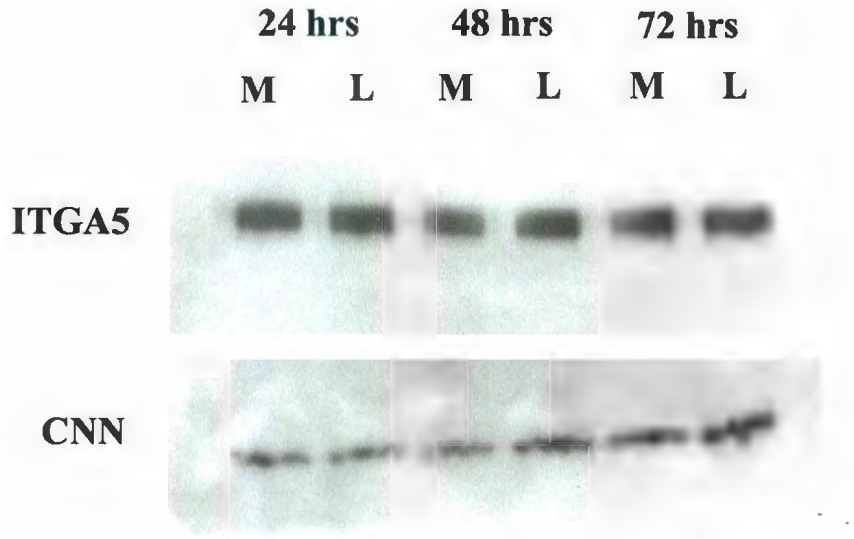
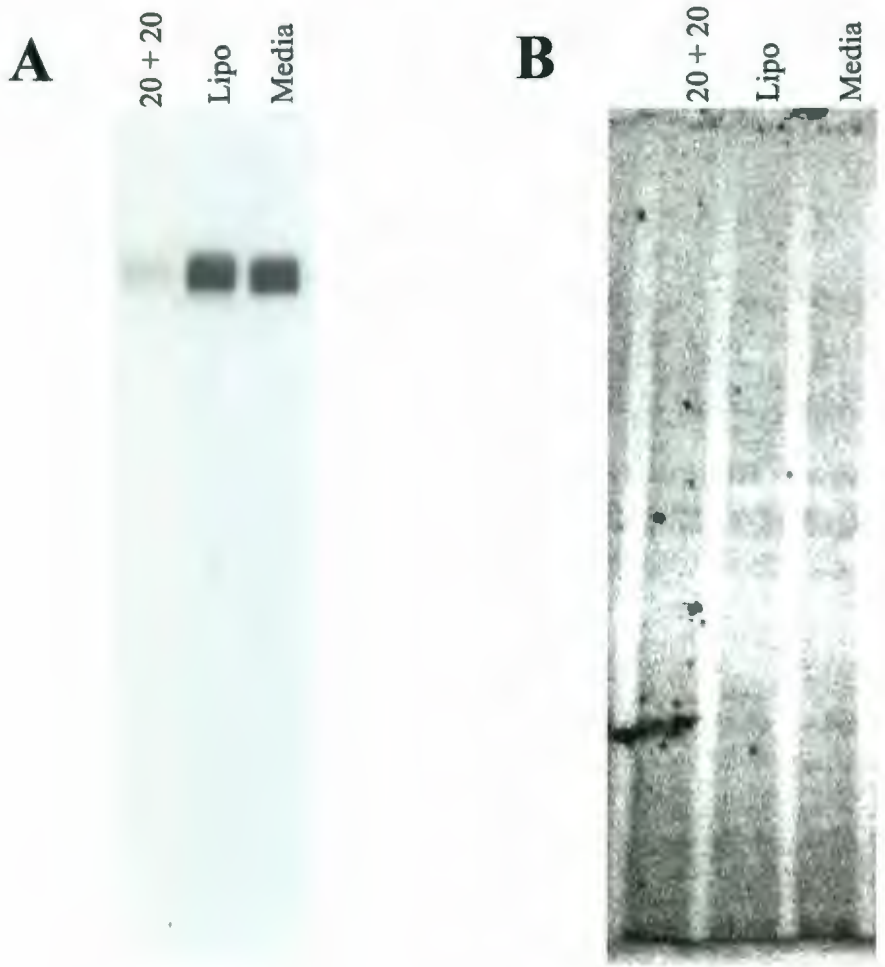

24 hrs
48 hrs
72 hrs
M
L
M
L
M
L
ITGA5
CNN

Figure 5.8. Knockdown of ITGA5 protein expression in M11 cells after transfection with *Itga5* siRNA. (A) Representative immunoblot of ITGA5 protein. Lane 20 + 20 represents 20 nM α5 siRNA + 20 nM siGLO transfection. (B) Gel picture of the same samples in A to illustrate equal loading. Lane 20 + 20 represents 20 nM α5 siRNA + 20 nM siGLO transfection. (C) Densitometric Analysis illustrating a significant decrease in ITGA5 with *Itga5* siRNA treatment ($P < 0.001$). Values are from 8 independent experiments ($n = 8$) ± SEM.


A
20 + 20
Lipo
Media
B
20 + 20
Lipo
Media

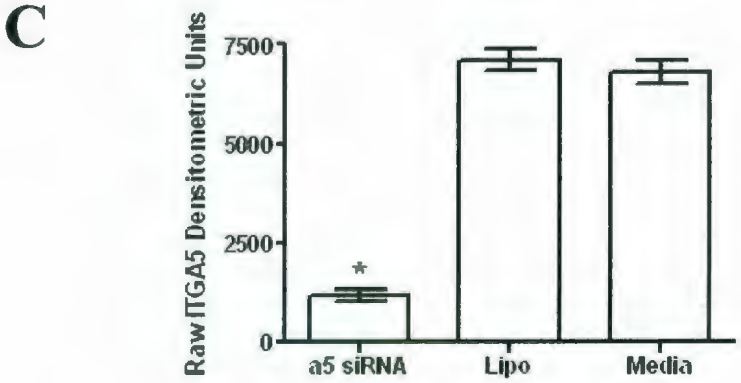
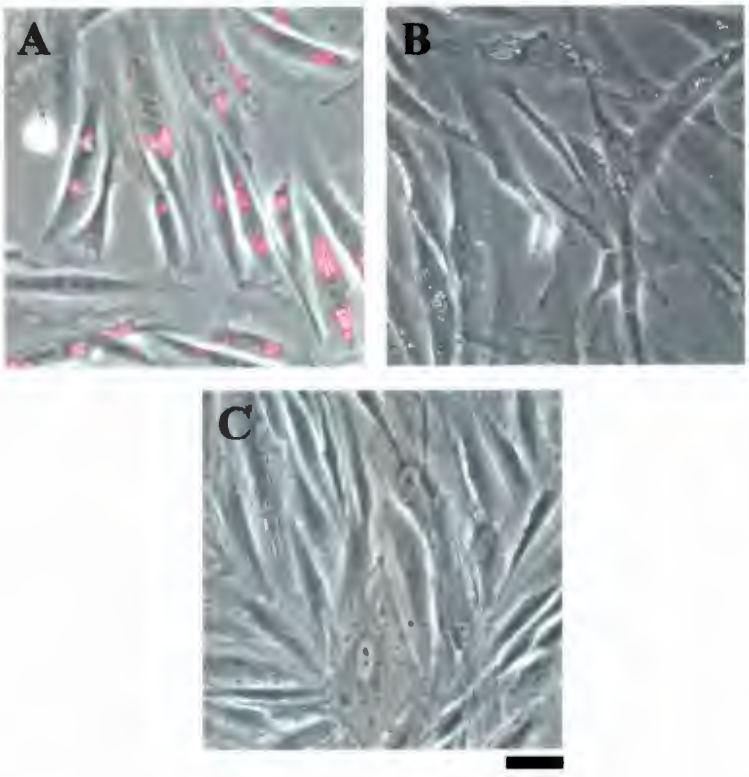

C
Raw ITGA5 Densitometric Units
7500
5000
2500
0
*
a5 siRNA
Lipo
Media

Figure 5.9. Photomicrographs of M11 cells following co-transfection with *Itga5* siRNA and siGLO. (A) Representative image of 20nM *Itga5* siRNA + 20nM siGLO. Fluorescence observed represents siGLO. (B) Representative image of lipofectamine only control. (C) Representative image of media only control. Bar = 100 μm.


A
B
C

of M11 cells after co-transfection of 20 nM *Itga5* siRNA and 20 nM siGLO with lipofectamine and media controls.

5.3.2 Effect of *Itga5* siRNA Knockdown of ITGA5 on FN Secretion and Deposition

Immunoblot analysis using FN-specific antisera revealed that FN protein matrix deposition was significantly decreased with *Itga5* silencing (one-way ANOVA, $P < 0.05$; $n = 8$; Figure 5.10). Specifically, FN protein matrix deposition was significantly decreased over lipofectamine (Tukey-Kramer post-test, $P < 0.001$) and media only (Tukey-Kramer post-test, $P < 0.05$) controls. A picture of the gel is not shown as a loading control because the staining was weak and while it could be seen by the eye, it was not possible to capture the image. Immunoblot analysis using FN-specific antisera also revealed that FN secretion into the media was significantly decreased with *Itga5* silencing (one-way ANOVA, $P < 0.05$; $n = 8$; Figure 5.11). A Tukey-Krame post-test ($n = 8$) revealed that FN secretion was significantly decreased compared to lipofectamine ($P < 0.05$) and media only ($P < 0.01$) controls.

5.4 Discussion

In the present study, we used a non-transformed human myometrial cell line to investigate the effect of *Itga5* gene silencing on FN matrix secretion and deposition. While matrix secretion and deposition is a complex process *in vivo* and typically involves several different cell types, it has been shown that individual cell types, including smooth muscle cells, secrete FN *in vitro* [Robinson *et al.*, 2003; 2004; Kim *et al.*, 2005]. Our

Figure 5.10. *Itga5* gene silencing significantly decreases FN protein in the deposited matrix of M11 cells. (A) Representative immunoblot of FN protein in the deposited matrix of M11 cells following transfection with *Itga5* siRNA. (B) Densitometric analysis illustrating a significant decrease in FN matrix deposition with *Itga5* siRNA treatment ($P < 0.001$). Values are from 8 independent experiments ($n = 8$) ± SEM.

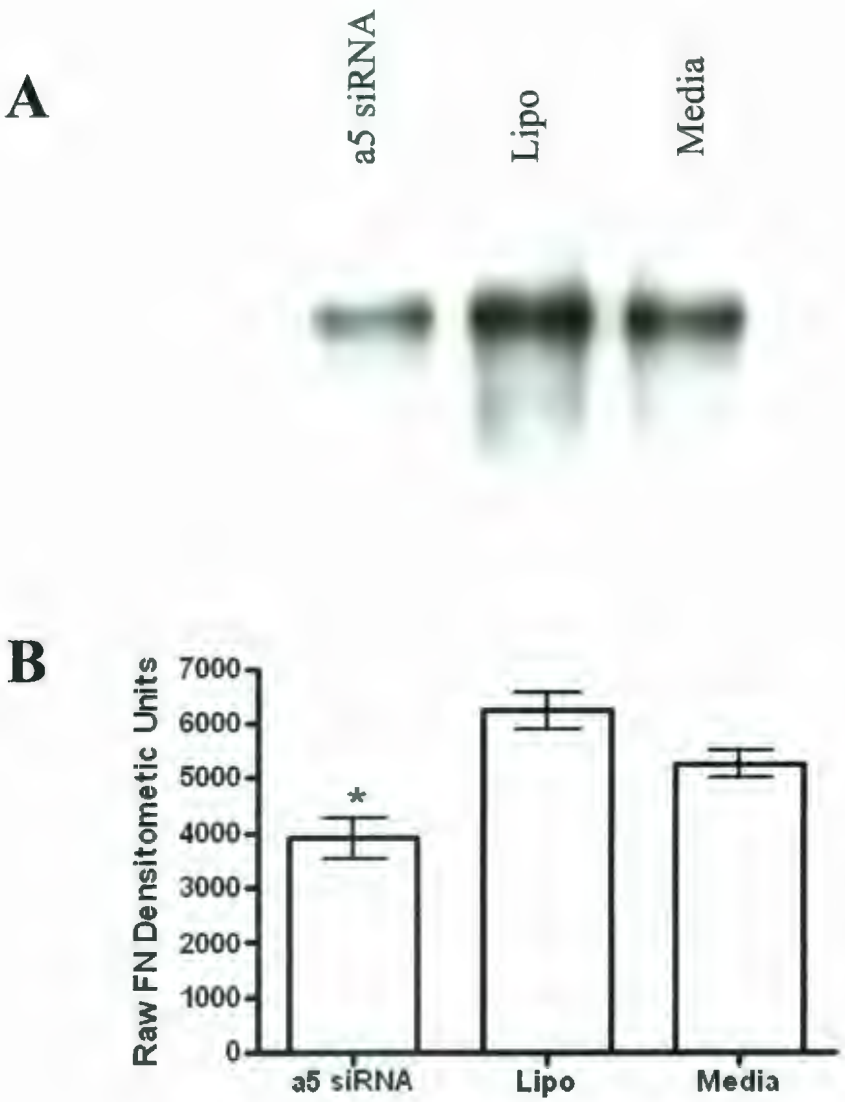

A
a5 siRNA
Lipo
Media
B
Raw FN Densitometic Units
7000
6000
5000
4000
3000
2000
1000
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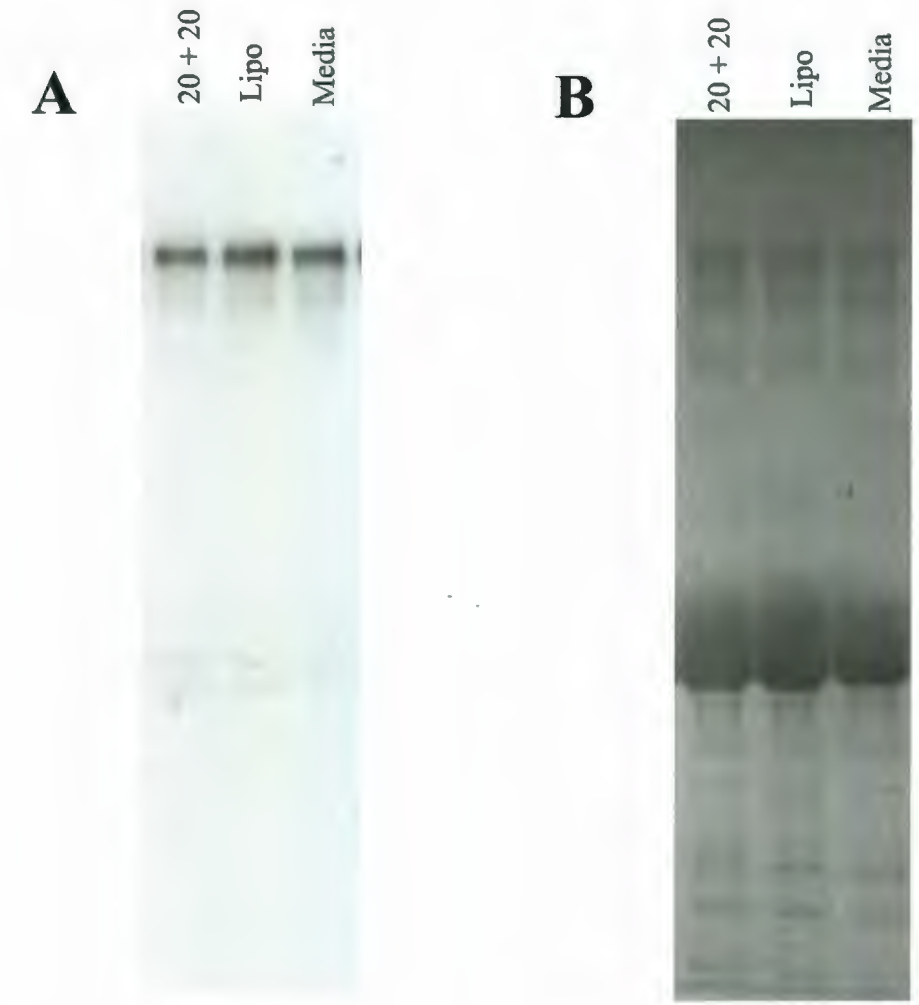
Figure 5.11. ***Itga5*** **gene silencing significantly decreases FN protein secreted into the media from M11 cells.** (A) Representative immunoblot of FN protein in the secreted media from M11 cells following transfection with *Itga5* siRNA. (B) Gel picture of the same samples in A to illustrate equal loading. (C) Densitometric analysis illustrating a significant decrease in FN protein secretion with *Itga5* siRNA treatment ($P < 0.05$). Values are from 8 independent experiments ($n = 8$) ± SEM.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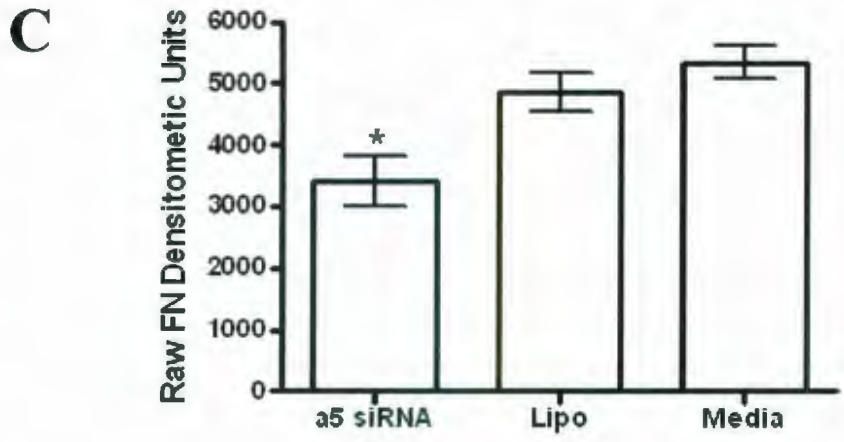
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data demonstrate that silencing *Itga5* gene expression in uterine myometrial cells resulted in significant inhibition of ITGA5 protein expression and subsequent inhibition of FN protein secretion and matrix deposition. It is possible that *Itga5* gene silencing may have other functional outcomes (such as a reduced adherence to the dish), but these were not investigated.

Our previous studies have led us to suggest that ITGA5B1 is a very important integrin receptor involved in signaling during late pregnancy and labour [Williams *et al.*, 2005; Shynlova *et al.*, 2007c]. We have demonstrated that *Itga5* gene and ITGA5 protein expression were up-regulated in the rat myometrium during the second half of gestation and a marked upregulation of *Itga5* gene and ITGA5 protein expression occurs specifically in the gravid uterine horn of unilaterally pregnant rats. It is now appreciated that the enlarging fetus, in addition to endocrine factors, induces uterine growth by placing the uterine wall under tension. These changes in ITGA5 coincide with an increase in *Fn* gene and FN protein expression during late gestation and in the gravid horn of unilaterally pregnant rats [Shynlova *et al.*, 2004; Shynlova *et al.*, 2007c]. As a result, we have proposed that the co-ordinated induction of ITGA5 and FN in the pregnant rat myometrium is an important factor in the development of myometrial hypertrophy in rats [Shynlova *et al.*, 2007c].

FN is a large ubiquitous and abundant glycoprotein that requires binding to integrins, most commonly ITGA5B1, to activate the process of fibril assembly. Recently, FN matrix assembly has been shown to contribute to cellular cohesion [Robinson *et al.*, 2004]. The authors found that inhibition of FN matrix assembly blocks the intercellular

associations required for tissue compaction and cellular cohesion. Compaction and cohesion have been found to be important processes in embryogenesis and wound remodeling [Flemming *et al.*, 2001; Khan *et al.*, 2003; Berry *et al.*, 1998] and increased FN expression and deposition during these processes have been observed [Downie and Newman, 1995; Clark, 1990]. FN also appears to be involved in connecting vascular smooth muscle cells together and studies suggest that the presence of FN may be sufficient to promote a shift in phenotype of vascular smooth muscle cells from contractile to synthetic [Hedin *et al.*, 1989; Thyberg, 1996]. In rat uterine smooth muscle cells, it has been suggested that gradual changes in their phenotype occur during pregnancy, with an early proliferative phase, followed by a synthetic phase of hypertrophy and matrix development, and a final contractile phase [Shynlova *et al.*, 2006; 2007a]. Both FN and ITGA5 expression begin to increase midpregnancy around the time the myometrial cells develop a synthetic phenotype.

We wanted to investigate the role of ITGA5 in FN matrix assembly in human myometrial cells. While FN can bind to at least 7 different integrin receptors (ITGA5B1, ITGA3B1, ITGA4B1, ITGA8B1, ITGA9B1, ITGAVB3, and ITGAVB5), all of which bind to the integrin binding motif Arg-Gly-Asp (RGD) found in FN, ITGA5B1 is the primary receptor responsible for FN fibrillogenesis [Reddy and Mangale, 2003]. Previous studies have investigated the role of inhibiting ITGA5B1 integrins by using blocking antibodies in fibroblast cells and found that inhibiting ITGA5B1 function resulted in inhibition of FN assembly [Roman *et al.*, 1989; Akiyama *et al.*, 1989]. We hypothesized that blocking *Itga5* gene expression would decrease expression of ITGA5

protein expression and result in an inhibition of FN secretion and matrix deposition. Interestingly, other integrins have been found to compensate for the absence of ITGA5B1 and in FN matrix assembly - ITGAV was found to replace ITGA5B1 in its absence [Wennerberg *et al.*, 1996; Yang and Hynes, 1996; Wu *et al.*, 1996]. ITGAV has been found in other smooth muscle types: vascular smooth muscle [Belmadani *et al.*, 2008; Lee *et al.*, 2006], bladder smooth muscle [Zhou *et al.*, 2005] and intestinal smooth muscle [Kuemmerle, 2006]. This might explain, at least in part, that while FN protein secretion and matrix deposition were significantly decreased, it was still detectable after *Itga5* gene silencing even though ITGA5 protein expression was almost completely abolished. It is possible that other integrins, such as ITGAV, were used to compensate for the loss of ITGA5 and, if so, it would appear that these other integrins were not as capable of aiding FN fibrillogenesis, since they were unable to maintain FN secretion and matrix deposition at normal levels in the presence of *Itga5* gene silencing.

Our results support the idea that ITGA5B1 plays an integral part in FN fibrillogenesis in uterine myometrial cells. It has been suggested that the role of ITGA5B1 in FN matrix assembly is probably not just limited to binding to soluble FN, leading to signaling events initiating fibrillogenesis. Wu *et al.* (1993) suggested that ITGA5B1 performed roles during FN matrix assembly, in addition to binding to soluble FN because Zhang *et al.* (1993) have shown that overexpression of ITGAVB1 promoted adhesion to FN, but couldn't substitute for ITGA5 in FN matrix assembly. Any additional roles that ITGA5B1 may play during FN matrix assembly still require investigation. ITGA5B1 is thought to function in the earliest detectible stages of matrix

assembly and once a FN matrix is established, FN binding to ITGA5B1 is not required [McDonald *et al.*, 1987; Roman *et al.*, 1989]. Interestingly, Wu *et al.* (1993) showed that the cytoplasmic domain of ITGA5B1 is not required for FN matrix assembly and the truncation of the ITGA5 cytoplasmic domain may enhance matrix assembly. The authors suggested that ITGA5 cytoplasmic domain could serve some regulatory function either by interaction with intracellular molecules or by modification such as phosphorylation.

We have previously suggested that a mechanical syncytium may develop in the myometrium to facilitate the coordinated contractions of labour [Williams *et al.*, 2005]. Based on our results, ITGA5B1 is likely required for FN matrix assembly in the myometrium in vivo beginning midpregnancy thus initiating the process of cellular cohesion in the uterine smooth muscle. This cellular cohesion is thus dependent on correct cytoskeletal filament organization, focal adhesion formation, cell-cell and cell-ECM interactions and these processes may be components of myometrial activation facilitating in the development of a mechanical syncytium to efficiently transduce labour contractions.

CHAPTER 6

SUMMARY

6.1 Summary of Major Findings

This thesis project has led to the following major findings:

1) *Itga5* and ITGA5 expression is highly elevated during late pregnancy and labour in the rat myometrium and immunocytochemistry experiments revealed that while ITGA5 is primarily localized to myometrial cell membranes during this time, myometrial layer-specific detection differences were observed. The longitudinal smooth muscle layer showed a more continuous ITGA5-specific staining pattern while the circular smooth muscle layer showed a more punctate staining pattern with reduced immunofluorescent detection compared to the longitudinal layer. Because integrins are important components of focal adhesions, induction of *Itga5* expression may be required for subsequent translation into ITGA5 protein to aid focal adhesion turnover and reorganization before labour.

2) Maintence of pregnancy with the administration of progesterone maintain *Itga5* expression as well as ITGA5 expression and localization. Blockade of progesterone signaling following administration of RU486 resulted in a significant increase in *Itga5* expression but an insignificant increase in total levels of ITGA5 in myometrial lysates, even though immunoreactive ITGA5 in situ accumulated in regions associated with myocyte membranes in both the longitudinal and circular smooth muscle layers

compared to controls. Thus, *Itga5* expression appears to be regulated, at least in part, by progesterone.

3) *Itga5* and ITGA5 expression were significantly elevated by uterine distension (stretch) as demonstrated in a unilateral pregnant rat model. An accumulation of ITGA5 at myocyte membranes was seen in both muscle layers of the gravid horn compared to the nongravid horn. Immunodetection of ITGA5 was observed to be stronger in the longitudinal smooth muscle layer compared to the circular smooth muscle layer at all timepoints studied; however there was more of a change in the detection of ITGA5 during late pregnancy in the circular layer of the gravid horn. Thus, increased ITGA5 expression reinforces the ligand-integrin interaction, allowing for properly anchored hypertrophied SMCs to the ECM, and facilitates proper smooth muscle intercellular cohesion before labour.

4) *Itga1, Itga3* and *Itgb1* expression were significantly elevated during late pregnancy however, there were no significant changes observed in ITGA1, ITGA3 or ITGB1 expression in total myometrial lysates. More specifically, in situ, immunoreactive ITGA1 accumulated in regions associated with myocyte membranes in the longitudinal smooth muscle layer over gestation. In the circular smooth muscle, immunoreactive ITGA1 proteins were primarily detected in the cell cytoplasm during early gestation and detection increased consistently in myocyte membranes on d22. In contrast, immunoreactive ITGA3 and ITGB1 was observed in the cytoplasm of myometrial smooth muscle cells during early gestation and then in myocyte membranes during late pregnancy in both smooth muscle layers. The highly specific expression of ITGA1,

ITGA3 and ITGB1 in myocyte membranes during late pregnancy and labour, along with corresponding ECM molecules, could aid the cell-ECM interactions necessary to promote the development of a mechanical syncytium.

5) Silencing of *Itga5* gene expression with *Itga5*-specific siRNA resulted in a significant decrease in ITGA5 expression in a human myometrial cell line. This decrease in receptor protein expression subsequently resulted in a significant decrease in both FN secretion and matrix deposition indicating a specific role for ITGA5 in FN-containing basement membrane organization.

6.2 Relevance to the Field of Parturition

The basic biochemical and molecular mechanisms underlying myometrial function during pregnancy and labour remain poorly understood making it difficult to anticipate and prevent preterm labour. Research to aid in the complete understanding of human parturition is required to create effective diagnostic indicators or treatments for preterm labour. Most drugs that are currently used for tocolysis are not universally effective and have a possibility for serious side effects for either the mother or the fetus. Research around the world is beginning to create an account of the signaling events that occur during late pregnancy and labour. The research conducted for this thesis has helped to lay the groundwork for increased understanding myometrial activation and contraction and may lead to the development of better therapeutic strategies to control the onset of labour.

Prior to this research within this thesis, the detection of integrin in nonpregnant human myometrium had been reported [Taylor *et al.*, 1996], but there were no studies published that had investigated the expression of integrins in the myometrium during pregnancy and labour. Williams *et al* (2005) was the first to show a temporal and spatial change in an integrin subunit in the myometrium. Taylor *et al* reported strong staining for ITGB1, and moderate staining for ITGA1, ITGA3, ITGA5, ITGA6, ITGAV and ITGB3 in the uterine smooth muscle. We hypothesized that integrin expression would be altered to accommodate the remodeling of the ECM that occurs in the myometrium during late pregnancy and labour.

We have suggested that the uterus exists as a mechanical syncytium during late pregnancy and labour to facilitate efficient force transduction of contractions. This stems from work by Kuo and Seow (2004), where they hypothesized that airway smooth muscle cells in a tissue bundle work as a mechanical syncytium. Earlier models of contractile filament organization in smooth muscle cells had been based on experiments on isolated cells, where a corkscrew-like shortening was observed. However, it is unlikely that individual cells will undergo corkscrew-like shortening without the whole tissue undergoing the same motion. Kuo and Seow (2004) suggested that to achieve uniform force transmission along the length of a muscle, the same amount of force has to be transmitted across each cross section, but not the same between individual cells of that cross section. This difference is accounted for by the observation that the contracile filaments lie parallel to the longitudinal axis of the cell bundle and correct focal adhesion

formation and cell-cell and cell-ECM interactions are required to efficiently transduce force.

These cell-cell and cell-ECM interactions occur at dense plaques (focal adhesions), where integrins are an integral component. Focal adhesions have been suggested as the site of mechanotransduction during myometrial activation [MacPhee and Lye, 2000; Challis and Lye, 2004] and integrins have been shown to be mechanosensors and transducers in a variety of cell and tissue behaviours [reviewed in Schwartz and Simone, 2008]. Previous work showed that focal adhesion signaling was abruptly terminated with the onset of labour [MacPhee and Lye, 2000]. Specifically, tyrosine phosphorylation of FAK and paxillin dramatically increased during late pregnancy and then decreased on day 23 of gestation (labour day) and treatment of rats with progesterone prevented the fall in tyrosine phosphorylation and also blocked the onset of labour. Studies show that remodeling of the actin cytoskeleton plays a key role in the regulation of smooth muscle contraction and FAK has been implicated to be involved in signaling pathways regulating cytoskeletal reorganization in other cell types. The goal of the research program in the labouratory is to identify which integrins and focal adhesion proteins are the major players during myometrial activation and contraction during late pregnancy and labour.

Based on work presented in this thesis and the work of Kuo and Seow (2004) and Robinson and colleagues (2003, 2004), we propose that the myometrium exists as a mechanical syncytium during late pregnancy and labour to facilitate force transduction of contractions and that ITGA5B1 may be an important component by promoting cellular

cohesion through signaling that leads to FN secretion and FN matrix deposition. In addition, the movement of ITGA1, ITGA3 and ITGB1 proteins to myocyte membranes during mid to late pregnancy also contributes to the syncytium model. We have suggested that the availability of the integrin proteins at uterine myocyte membranes may be a critical temporal event in the function of heterodimeric integrin receptors within the myometrium. Thus, the movement of ITGA1, ITGA3 and ITGB1 to myocyte membranes, along with corresponding ECM molecules, could aid the cell-ECM interactions necessary to promote the development of a mechanical syncytium.

6.3 Future Directions

Many questions still remain as to the role of integrins and integrin signaling during myometrial activation and contraction. Thus, more experiments and lines of work are required to further advance the knowledge base in this area.

1) We have determined that significant regulation of *Itga5* gene expression by progesterone is unlikely. An increase in 17-estradiol (E_2) in rat maternal plasma is observed during late gestation and E_2 has been shown to regulate the expression of CAPs in the pregnant and non pregnant rat myometrium. Specifically, the steroid has been found to increase the level of transcripts of Cx-43 and progesterone was found to both block and reverse this action of estrogen [Petrocelli and Lye, 1993]. Therefore, with increased immunodetection of ITGA5 and *Itga5* expression at late pregnancy and labour, it is possible that E_2 contributes in a minor way to the regulation of *Itga5* expression. To

determine if this steroid plays a role, experiments on both nonpregnant and pregnant rats need to be carried out. The effect of E_2 administration on *Itga5* and ITGA5 expression in the nonpregnant myometrium of ovariectomized rats can be established by injecting the rats with a single injection of E_2 and taking myometrial samples at various timepoints after injection for immunofluorescence, immunoblot and RT-PCR analyses. In tandem, whether E_2 has an effect on *Itga5* expression during pregnancy can be determined by treating rats with E_2 on day 15 of gestation and collecting myometrial samples at various timepoints after injection of E_2 for immunofluorescence, immunoblot and northern blot anayses (or RT-PCR). It is also possible to determine the effect of E_2 administration on *Itga5* gene expression by investigating the expression of *Itga5* and ITGA5 throughout the estrous cycle of nonpregnant myometrium in rats.

2) Focal adhesion signaling during pregnancy and labour is believed to be important in the transmission of force between the contractile apparatus of the cell and the ECM. We have suggested that there is significant focal adhesion turnover to facilitate the process of force transduction. Several focal adhesion proteins have been found to have increased expression and/or phosphorylation during late pregnancy and labour. Specifically, FAK was found to be highly expressed and activated during late pregnancy and paxillin was found to be highly tyrosine phosphorylated during this time [MacPhee and Lye, 2000]. Recently, our lab found that Hic-5, a focal adhesion protein, was highly expressed in the rat myometrium during late pregnancy and labour and co-localized with FAK in situ [Croke *et al.*, 2007]. Because there is significant focal adhesion turnover during late

pregnancy and labour, there is most likely a change in the binding partners to the ITGA5 receptor. To determine whether there is a change in binding partners, co-immunoprecipitation experiments need to be carried out. If an antibody for ITGA5 immunoprecipitations in rat tissue becomes available, it would be interesting to see whether there is a change in the binding partners of ITGA5 during late pregnancy and labour compared to nonpregnant and early pregnancy timepoints.

3) We have identified that *Itga5* silencing decreases FN deposition and secretion. It would be interesting to determine whether this silencing of *Itga5* has an effect on contraction. Devost and Zingg (2007) developed an in vitro system to measure myometrial cell contraction in response to OT. Using collagen type I from rat tail, myometrial cells were plated on collagen lattices and left overnight in the absence or presence of different concentrations of OT. Collagen contraction was expressed as percentage contraction, which was taken as the percentage of collagen lattice size reduction in comparison to the area size of the well. This system can be easily used to determine whether *Itga5* silencing has an effect on myometrial cell contraction. Myometrial cells only have to be transfected with *Itga5* siRNA prior to being plated on the collagen lattices. If silencing *Itga5* expression does have a negative effect on contraction, then there would be no change in the size of the lattice. If a change in the size of the lattice is still observed after *Itga5* silencing, then it's possible that either the ITGA5 receptor subunit plays little role during contraction or that the cells make up for

the lack of ITGA5 by using other subunits. It is known that FN can bind to several different integrin receptor subunit combinations in the event that ITGA5 is absent.

4) We have determined that *Itga1* and *Itga3* expression is significantly elevated in the rat myometrium during late pregnancy. While we found no change in ITGA1 or ITGA3 expression in total myometrial lysates, we did observe an accumulation of immunoreactive ITGA1 and ITGA3 in myocyte membranes in situ. In addition, Nishinaka and Fukuda (1991) observed an increased deposition of type IV collagen and laminin around smooth muscle cells in the rat myometrium during late pregnancy. Little data currently exists in the literature examining the contribution of other integrins to collagen and laminin deposition and secretion. In vascular SMCs, blocking of ITGB3 prevented the increase in collage I deposition that was observed in response to high glucose in hypertensive mice [Belmadani *et al.*, 2008]. We propose an experiment similar to the one in which we silenced *Itga5* expression, only in this case, *Itga1* and *Itga3* expression would be silenced and the effect on collagen and laminin secretion and deposition would be examined in the M11 human myometrial cell line.

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Figure 3.7. Immunolocalization of ITGA3 in the circular smooth muscle layer of rat myometrium between NP and 1 day postpartum. Numbers represent gestational timepoints. L, Labour (2-3 pups born); C, Control (Rabbit IgG). Arrows highlight cytoplasmic and membrane-specific staining. Scale bar = 10μm.