CHARACTERIZATION OF HSP20 EXPRESSION IN RAT MYOMETRIUM DURING PREGNANCY

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Characterization of HSP20 Expression in Rat Myometrium During Pregnancy

by

© Brandon Edgar Hezekiah Cross

A thesis submitted to the School of Graduate Studies

in partial fulfilment of the requirements for the degree of

Master of Science

Division of BioMedical Sciences

Memorial University of Newfoundland

January 2009

St. John's Newfoundland & Labrador

Abstract

The underlying mechanisms regulating uterine contractions during labour are still poorly understood. Heat shock protein 20 (HSP20) is a stress protein present at high levels in vascular smooth muscle and is implicated in cyclic nucleotide dependent smooth muscle relaxation, yet before this study was undertaken HSP20 expression and regulation in uterine smooth muscle, or myometrium, were completely unknown. Since HSP20 has been implicated in smooth muscle relaxation, I hypothesized that HSP20 would be highly expressed in the rat myometrium during early and mid pregnancy, but its expression would be downregulated during the late stages of pregnancy, as the muscle becomes activated and the onset of labour approaches. Northern blot analysis demonstrated that HSP20 mRNA detection was significantly decreased from day (d) 22 - d23 of gestation compared to non-pregnant (NP) samples and from d22 - one day post-partum (PP) compared with d6 ($p<0.05$). Immunoblot analysis showed that detection of HSP20 was significantly decreased at d23 compared to d12 and d15 ($p<0.05$). HSP20 detection also significantly decreased at PP compared to d15 ($p<0.05$). Immunofluorescence analysis demonstrated that after d15, plasma membrane-associated localization of HSP20 decreased markedly in both circular and longitudinal muscle layers. My results demonstrate that HSP20 mRNA and protein are highly expressed during early and mid- pregnancy and then expression markedly decreases during late pregnancy and labour.

During pregnancy, the myometrium exhibits a very noticeable change in size and phenotype which is believed to be modulated by both mechanical and hormonal influences

originating, in part, from within the fetal genome. Since decreased HSP20 expression near term correlates with decreased levels of circulating progesterone, I also studied the effects of progesterone delayed labour and early progesterone withdrawal, using the receptor antagonist RU486, on HSP20 mRNA and protein detection. HSP20 was detectable near cell membranes at much higher levels in the longitudinal muscle layer of progesterone-treated pregnant rats (delayed labour) at all gestational timepoints examined compared with controls. Early progesterone withdrawal had no effect on HSP20 mRNA or protein detection in the myometrium.

I also examined the role of uterine stretch in regulation of HSP20 expression during late pregnancy and labour. Using unilaterally pregnant rats, I investigated the changes in HSP20 mRNA and protein detection in the myometrium. Gravidity did not affect the detection of HSP20 mRNA or protein at d19, but did cause a significant decrease in HSP20 mRNA ($p < 0.10$) and protein ($p < 0.05$) detection in the gravid horn at labour as compared to the non-gravid horn. Immunofluorescence analysis did not demonstrate any differences in HSP20 detection *in situ* at d19, but verified the decrease in HSP20 detection in the gravid horn at labour, with a decrease in staining near myocyte membranes. These findings suggest that expression of HSP20 in rat myometrium is not likely dependent on circulating progesterone levels, but may be negatively affected by stretch of the myometrium at term labour.

Acknowledgements

Many people have been a part of my graduate education, as friends, teachers and colleagues. Dr. Daniel MacPhee, first and foremost, has been all of these. The best supervisor I could have asked for, he is actively involved in the work of all of his students, and clearly always has their best interests in mind. Dr. MacPhee, you always gave the kind of supervision that most other supervisors didn't have time for.

I would also like to extend my thanks to all of the friends that I have made since I began working in Dr. MacPhee's lab. To Bryan White, for teaching me most of the procedures that were critical to my research. Also, for sharing his samples to use and for giving me a hand whenever I needed it; but, most of all, for always being able to find a way to distract me from what I should have really been doing in the lab. To Pia Elustondo, whose wonderful personality brightened the lab every day. To Trina Kirby, without whom I would have never learned of the joys of insulin and the sugar-free chocolate bar. Finally, to the other friends that I have made in our lab, Joy Williams, Luke Pike and Heather O'Dea. It was a pleasure to meet and get to know all of you.

Finally, I would like to thank my supervisory committee, Drs. Ken Kao and Karen Mearow, for their suggestions and help and especially to Dr. Mearow, for her critical reading of my manuscript and thesis.

This research was supported by an operating grant from NSERC (National Sciences and Engineering Research Council) and a stipend from the Office of Research and Graduate Studies - Memorial University School of Medicine.

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Abbreviations

ANOVA	-	analysis of variance
cAMP	-	cyclic adenosine monophosphate
CAP	-	contraction-associated proteins
cDNA	-	complementary deoxyribonucleic acid
cGMP	-	cyclic guanosine monophosphate
Cx-43	-	connexin-43
ddH_2O	-	double distilled water
DEPC	-	diethyl pyrocarbonate
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetraacetic acid
FITC	-	fluorescein isothiocyanate
HPA	-	hypothalamic-pituitary-adrenal
HRP	-	horseradish peroxidase
HSP	-	heat shock protein
IgG	-	immunoglobulin
MLCK	-	myosin light chain kinase
MOPS	-	3-(N-morpholino) propane sulfonic acid
mRNA	-	messenger ribonucleic acid
OTR	-	oxytocin receptor
PBS	-	phosphate buffered saline
P_4	-	progesterone
PGE_2	-	prostaglandin E_2
$PGF_{2\alpha}$	-	prostaglandin $F_{2\alpha}$
PGI_2	-	prostacyclin
pHSP20	-	phosphorylated HSP20
PKA	-	cAMP-dependent protein kinase
PKG	-	cGMP-dependent protein kinase
PR-A/B/C	-	progesterone receptor A/B/C
RIPA	-	radioimmunoprecipitation assay
RNA	-	ribonucleic acid
rRNA	-	ribosomal ribonucleic acid
RU486	-	mifepristone
SDS	-	sodium dodecyl sulfate
sHSP	-	small heat shock protein
SSC	-	sodium chloride / sodium citrate
SSPE	-	sodium chloride-sodium phosphate ethylenediaminetetraacetic acid
TBST	-	tris-buffered saline tween
Tris	-	tris hydroxymethyl aminomethane

Chapter One

Introduction

1.1 Pre-Term Birth

In humans, pre-term birth is defined as birth occurring before week 37 of the gestational period and occurs when there is asynchrony between the labor process and maturation of the fetus (Challis *et al.*, 2000; Challis *et al.*, 2002). Premature labor occurs in 5 - 10 percent of all pregnancies in North America, with pre-term birth accounting for 7.6 percent of all births in Canada in 2001 (Challis *et al.*, 2002; Loudon *et al.*, 2003). This problem is a major public health concern as prematurity is associated with approximately 75 percent of neonatal deaths and 70 percent of cases of neonatal morbidity. To put this into perspective, infants born prematurely are 40 times more likely to die than those born at term (Challis, 2001; Lyndon, 2006). Those infants who do survive pre-term birth still live with an increased risk of cerebral palsy, neurological and pulmonary disorders. In addition, babies born prematurely are at significantly higher risk of childhood and adolescent learning disabilities (Lye *et al.*, 2001).

Pre-term birth can occur due to a variety of causes. Approximately 30 percent of pre-term births occur as the result of an underlying infection, whereas nearly 50 percent of cases are of an idiopathic, or unknown, nature (Challis *et al.*, 2002; Gibb & Challis, 2002). Pre-term labour is a major medical problem in both developed and developing countries and its prevention is a major aim of modern obstetrics. For decades research in this field has been focussed on the development of drugs which could inhibit the contractile activity of the

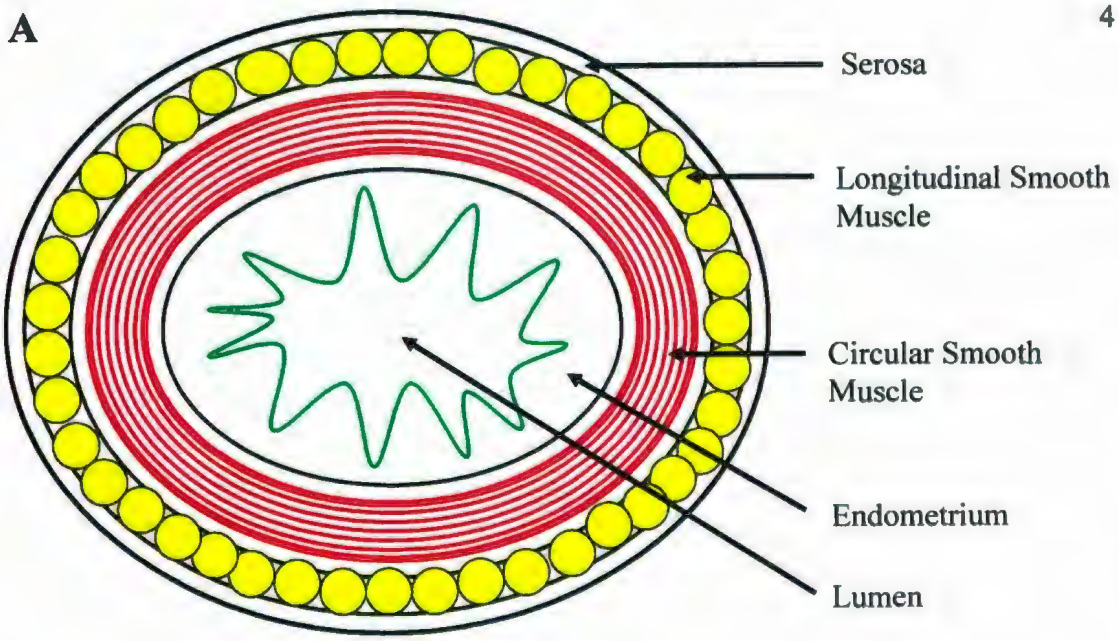
uterine smooth muscle (myometrium). However, this research has not yet led to a reduction in the incidence of pre-term birth (Challis *et al.*, 2002; Lye *et al.*, 2001). To date, there are no effective diagnostic indicators or treatments for pre-term birth. The major hurdle which needs to be overcome in developing effective tools for pre-term labour is the acquiring of a complete understanding of the normal physiology of human parturition. Thus, any future research into pre-term labour needs to first determine the underlying biochemistry involved in the normal labour process before any effective treatments can be created (Challis *et al.*, 2001; Slattery & Morrison, 2002).

1.2 Uterine Smooth Muscle (Myometrium)

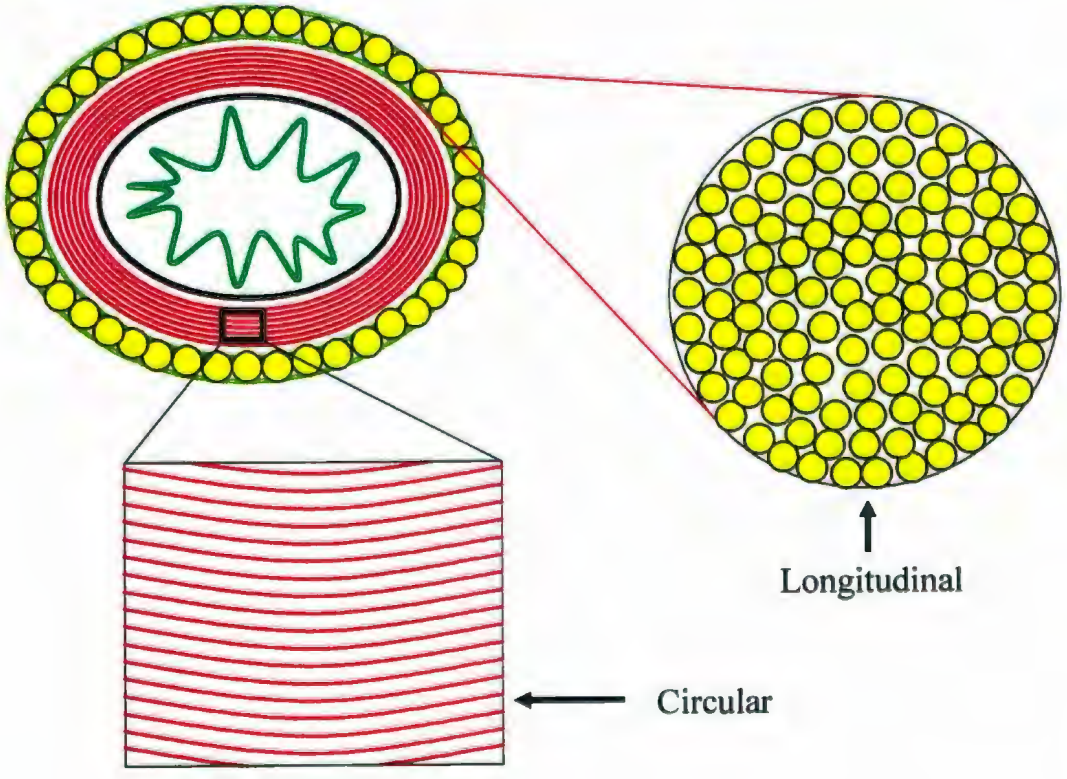
The rat uterus is essentially composed of four layers: the serosa, the outer longitudinal muscle layer, inner circular muscle layer and the endometrium (Fig 1.1). The two muscle layers can be easily differentiated when the uterus is observed under a microscope. The longitudinal muscle layer is composed of numerous muscle bundles which align along the long axis of the uterus, while the circumferentially oriented circular muscle layer surrounds the base of the endometrium. The two layers are separated by the vascular plexus, an extensive network of blood vessels that provide the muscle layers with nourishment (Shynlova *et al.*, 2005). Both muscle layers are composed of smooth muscle interspersed with marked amounts of connective tissue. Internally, individual smooth muscle cells contain two fibrillar domains: (i) a contractile domain consisting of actin "thin" filaments, actin-associated proteins and myosin "thick" filaments; and (ii) a cytoskeletal domain, consisting of non-muscle actin and intermediate filament proteins, which gives the cell its structural

Figure 1.1. (A) Diagrammatic representation of a cross section of the rat uterine horn (B) The two muscle layers can be easily differentiated when a uterine tissue section is observed under a microscope. The longitudinal muscle layer is composed of numerous muscle bundles which align along the long axis of the uterus, while the circumferentially oriented circular muscle layer surrounds the base of the endometrium.

A


Serosa
Longitudinal Smooth Muscle
Circular Smooth Muscle
Endometrium
Lumen

B


Longitudinal
Circular

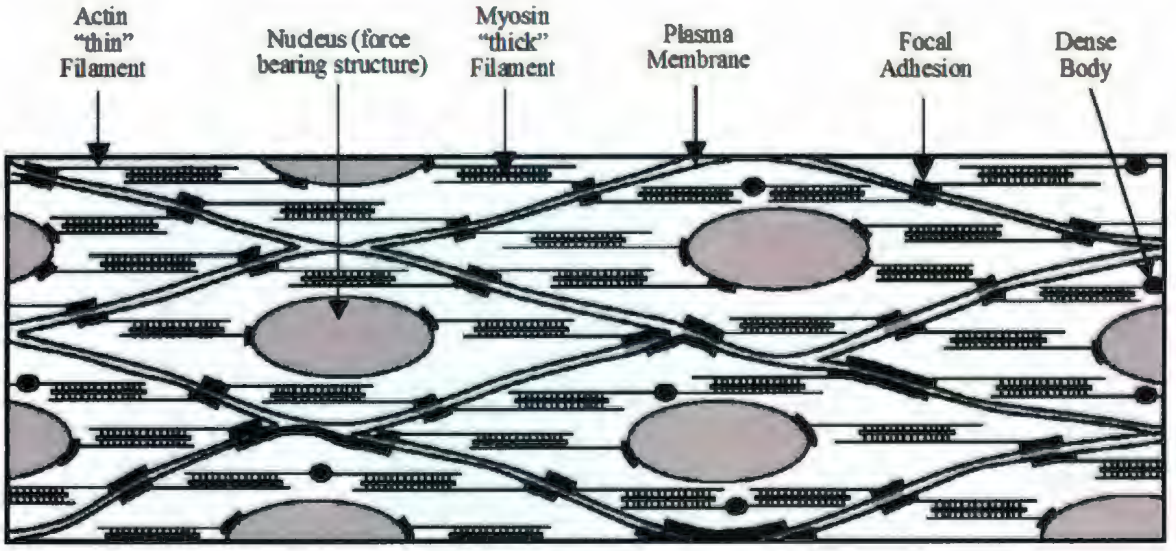
integrity (Tessier *et al.*, 2003).

Force generation in smooth muscle is thought to be a result of cyclic interaction between myosin crossbridges and actin filaments, as it is in striated muscle (Ali *et al.*, 2005). In order for uniform force transmission to be achieved along the length of a bundle of smooth muscle, the same amount of force has to be transmitted across each section of the smooth muscle bundle. These forces are actively generated and carried by the contractile filaments and passively borne by the nuclei. Mechanical connections among the cells are provided by intermediate junctions that contain two opposed dense plaques from adjacent cells (Kuo & Seow, 2004). This arrangement of smooth muscle cells allows an aggregate of cells to form a functional unit, or syncytium, in which force can be generated and transmitted uniformly from cell to cell (Fig 1.2; Kuo & Seow, 2004).

1.3 Myometrial Activation

During pregnancy, physiological growth of the uterus occurs by two mechanisms: an increase in cell number (hyperplasia) followed by an increase in cell size (hypertrophy) (Shynlova *et al.*, 2006). Throughout the majority of pregnancy, the myometrium remains relatively quiescent. Contractions that do occur are poorly synchronized and weak. At term, the myometrium becomes highly excitable and generates the high amplitude, high frequency contractions characteristic of labour (Gibb *et al.*, 2006). Uterine contractility during pregnancy and labour can be divided into four distinct phases. *Phase 0* corresponds to pregnancy, a time of relative uterine quiescence. Throughout the majority of pregnancy, the uterus is unresponsive to uterotonins and contractions are poorly coordinated. The

Figure 1.2. Schematic representation of contractile filament architecture in a bundle of smooth muscle cells. Contraction in smooth muscle involves cyclic interaction of myosin and actin. Mechanical connections provided by dense plaques (or focal adhesions) on opposing cells allow transfer of the force from one cell to another and, thus, the formation of a functional syncytium. Adapted from Kuo and Seow, 2004.


Actin
"thin"
Filament
Nucleus (force
bearing structure)
Myosin
"thick"
Filament
Plasma
Membrane
Focal
Adhesion
Dense
Body

myometrium is acted on by one or more of a variety of paracrine or endocrine inhibitors, which include progesterone, prostacyclin (PGI_2), relaxin, and nitric oxide. Each of these inhibitors act via different pathways, but result in the same endpoint: an increase in intracellular levels of cAMP and/or cGMP (Dayes, 2008; Grammatopoulos & Hillhouse, 1999; Negishi *et al.*, 1995; Riemer *et al.*, 2008). These nucleotides, respectively, activate cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), which can promote phosphodiesterase activity and myosin light chain kinase (MLCK) dephosphorylation, leading to its inactivation. Phosphorylation of the myosin light chain is critical for contraction of the uterine myocyte (Moore & López Bernal, 2001). Activation of PKA also stimulates the uptake of cytoplasmic calcium into intracellular storage sites. Relaxation of uterine smooth muscle is also promoted by processes that maintain actin in its globular form, therefore preventing formation of the actin fibrils required for contraction (Challis *et al.*, 2000; Gibb & Challis, 2002; Smith, 2007).

Near term there is a switch from *phase 0* to *phase 1* of uterine contractility, known as *activation*. This switch is the result of the effects of uterotropins, the removal of inhibitory factors, and the generation of mechanical forces due to uterine stretch. Mechanical stretch and/or uterotropic priming lead to upregulation of a cassette of genes required for coordinated contractions of the uterus. These genes encode the contraction-associated proteins (CAP), which include the oxytocin receptor, prostaglandin receptors, connexin-43 (Cx-43; a key component of gap junctions), and ion channels. The result of activation is a switch from a muscle which is quiescent during the majority of pregnancy to one which is

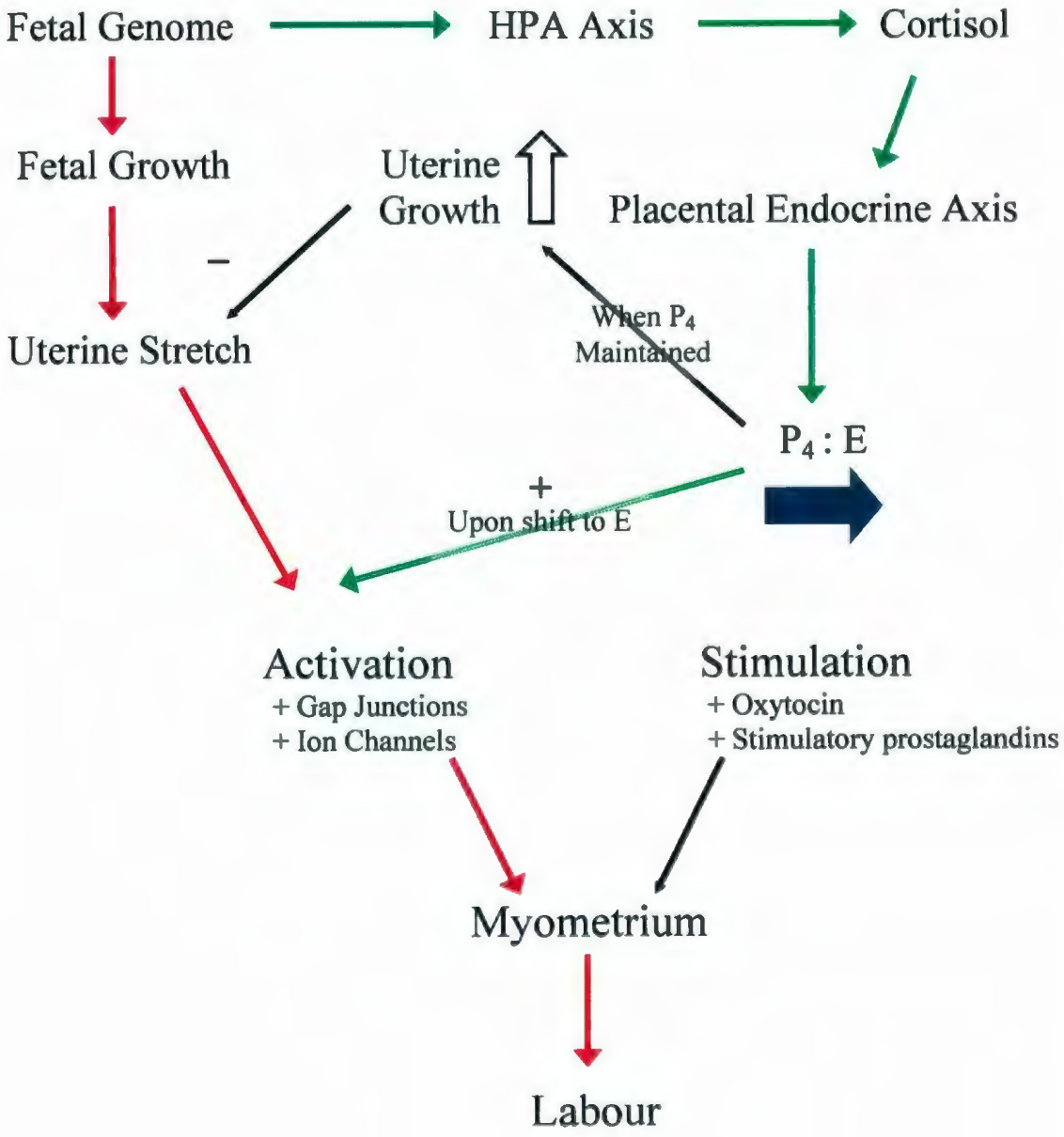
spontaneously active, excitable and highly responsive to uterotonic stimuli (Challis, 2001; Gibb *et al.*, 2006). *Phase 2* corresponds to the forceful contractions of labour upon *stimulation* by uterotonins, including prostaglandins and oxytocin. Finally, *Phase 3* is characterized by the postpartum involution of the uterus and expulsion of the placenta, which has been mainly attributed to the effects of oxytocin (Gibb *et al.*, 2006; Gibb & Challis, 2002).

Although the molecular mechanisms underlying the progression from a quiescent myometrium to one activated for labour are not well understood, it is known that activation of myometrial function is driven through the fetal genome and effected through two separate but interdependent pathways (Fig 1.3). Activation involves an endocrine cascade, which includes the fetal hypothalamic-pituitary-adrenal (HPA) axis, as well as mechanical signals imparted by uterine stretch due to growth of the fetus (Challis, 2001; Gibb & Challis, 2002). Recent studies have examined the role of these two pathways in regulating expression of the CAP genes. These studies have found that CAP genes can be regulated by mechanical signals, hormonal signals, or a combination of both. For example, it has been found that Cx-43 gene expression is regulated by both the endocrine and mechanical pathways, whereas expression of the $PGF_{2\alpha}$ receptor depends only upon hormonal control (Gibb *et al.*, 2006; Ou *et al.*, 1997; Ou *et al.*, 2000; Petrocelli & Lye, 1993).

1.4 The Endocrine Pathway and the HPA Axis

During the late stages of pregnancy, maturation of the fetal HPA axis occurs. As term approaches, the placenta secretes increasing concentrations of corticotropin-releasing

Figure 1.3. The dual pathway by which the fetal genome contributes to the onset of labour via mechanical and endocrine signals. The fetal hypothalamic-pituitary-adrenal (HPA) axis leads to initiation of endocrine cascades that cause a shift (blue arrow), in most animals, in the progesterone:estrogen ratio which favours estrogen just prior to labour. The mechanical stretch of the uterine muscle due to fetal growth leads to stretch related increases in contraction associated proteins, which include the gap junction protein connexin-43 and ion channels. Adapted from Lye *et al*, 2001.


Fetal Genome
HPA Axis
Cortisol
Fetal Growth
Uterine Growth
Placental Endocrine Axis
–
When P_4 Maintained
Uterine Stretch
P_4 : E
+
Upon shift to E
Activation
+ Gap Junctions
+ Ion Channels
Stimulation
+ Oxytocin
+ Stimulatory prostaglandins
Myometrium
Labour

hormone, leading to a boost in the production of corticotropin by the fetal pituitary. This stimulates the release of cortisol, as well as heightened steroidogenesis, from the fetal adrenal glands (Smith, 2007). The heightened levels of cortisol lead to an increase in the synthesis of prostaglandins (PG) such as PGE_2, which has been identified as a key mediator of uterine contractility (Challis, 2001; Whittle *et al.*, 2001). Increased levels of PGE_2 sustain HPA axis activation and also direct preferential estrogen synthesis at the expense of progesterone in the placenta (Loudon *et al.*, 2003; Whittle *et al.*, 2001). This increase in estrogen production occurs in humans as well as many animal species; however, in most animals there is also a marked decrease in the placental and/or ovarian output of progesterone before birth (Gibb *et al.*, 2006; Gibb & Challis, 2002). A high progesterone:estrogen ratio and suppression of CAP gene expression are required for uterine growth. In most animals, the increase in estrogen production at term coupled with a decrease in progesterone leads to a decline in progesterone influence on the myometrium and an increase in CAP gene expression (Gibb & Challis, 2002). However, a characteristic of human pregnancy is that, unlike other mammals, the level of circulating progesterone does not fall with the onset of labour.

Until recently, it was not understood how labour could proceed in the presence of such high levels of progesterone. It has been found that, late in pregnancy, the myometrium responds differently to progesterone due to alterations in the progesterone response machinery. These findings have demonstrated that three different isoforms of the progesterone receptor (PR-A, PR-B & PR-C) exist. Progesterone receptor B mediates the actions of progesterone, while the other two receptors function as dominant repressors of the

function of PR-B (Giangrande *et al.*, 2000; Wei *et al.*, 1996). Studies have provided evidence that the relative activities of the different isoforms of the progesterone receptor change as the uterus approaches labour, with PR-A increasing late in pregnancy. This change in isoform levels is believed to contribute to the functional withdrawal of progesterone in human myometrium (Mesiano *et al.*, 2002). This withdrawal also occurs as a result of the alteration of other cellular response mechanisms, including changes in the expression of co-activators and co-repressors of progesterone (Condon *et al.*, 2003).

1.5 The Mechanical Stretch Pathway

Although the endocrine pathway is important, complete activation of the myometrium would not occur with the effects of this pathway alone. Mechanical signals imparted by uterine stretch (as a result of fetal growth during pregnancy) are also required for myometrial activation. Kasai *et al.* demonstrated that in vitro stretch of the rat uterus caused transient smooth muscle contractions and Ca^{2+} influx (Kasai *et al.*, 1995). The increase in myometrial stress can likely explain why pregnancies involving multiple fetuses (ie. twins, triplets, etc.) have a higher incidence of pre-term birth (Challis, 2001; Gardner *et al.*, 1995; Lye *et al.*, 2001).

Growth of the uterus can be divided into three phases. The first phase of growth occurs during the first trimester, results in uterine hyperplasia, and is regulated by endocrine factors. During the second phase, which occurs during the second and third trimesters, growth of the uterus occurs in parallel with growth of the fetus, giving the fetus enough space for its own growth. Uterine growth ceases toward the end of pregnancy (the third phase) and

the resulting increase in tension of the uterine wall signals the onset of parturition (Gibb *et al.*, 2006; Smith, 2007).

Early in pregnancy, stretch stimulates the production of prostacyclin, an inhibitory prostaglandin, which lessens the uterotonic actions which occur with an increase in myometrial stress (Lye *et al.*, 2001). Progesterone is required to stimulate the stretch-induced hypertrophy which occurs during early pregnancy, preventing activation of the uterus due to mechanical stretch. Upon maturation of the fetal HPA axis near term, the decreased effect of progesterone on the myometrium leads to discordance between fetal growth and uterine size. The increased tension, combined with the effects of estrogen on the myometrium, leads to an increase in gap junction formation as well as increased expression of the oxytocin receptor and Cx-43 (the CAP genes), allowing labour to occur (Gibb *et al.*, 2006; Gibb & Challis, 2002; Lye *et al.*, 2001). As mentioned previously, stretch is required to fully activate expression of a number of the CAP genes. In unilaterally pregnant rats labour resulted in no increase in CAP gene expression in non-gravid horns when compared to gravid horns, despite both horns being in the same endocrine environment (Ou *et al.*, 1998).

1.6 Small Heat Shock Proteins (sHSPs)

The small heat shock proteins (sHSPs) are a family of proteins that range in mass from 12 to 43 kDa and exhibit chaperone activity. They participate in the folding of proteins in both normal conditions and extreme conditions such as heat shock, oxidation and exposure to toxins such as anti-cancer drugs (Gusev *et al.*, 2002; Gusev *et al.*, 2005). sHSPs are involved in the renaturation of partially denatured proteins and the complete elimination of

fully denatured proteins. These proteins also possess various other common properties. All members of this family of proteins contain a conserved α-crystallin domain, consisting of 80 - 100 amino acid residues, which spans two putative actin-binding domains. The majority of these proteins also undergo phosphorylation by protein kinases, which is thought to affect their function within the cell. Members of this family also tend to form high molecular mass oligomers with other small heat shock proteins, which range in size from 150 to 800 kDa (Gusev *et al.*, 2005). Both the conserved α-crystallin domain and phosphorylation of sHSPs have been shown to play important roles in the formation of these oligomers (Gusev *et al.*, 2002).

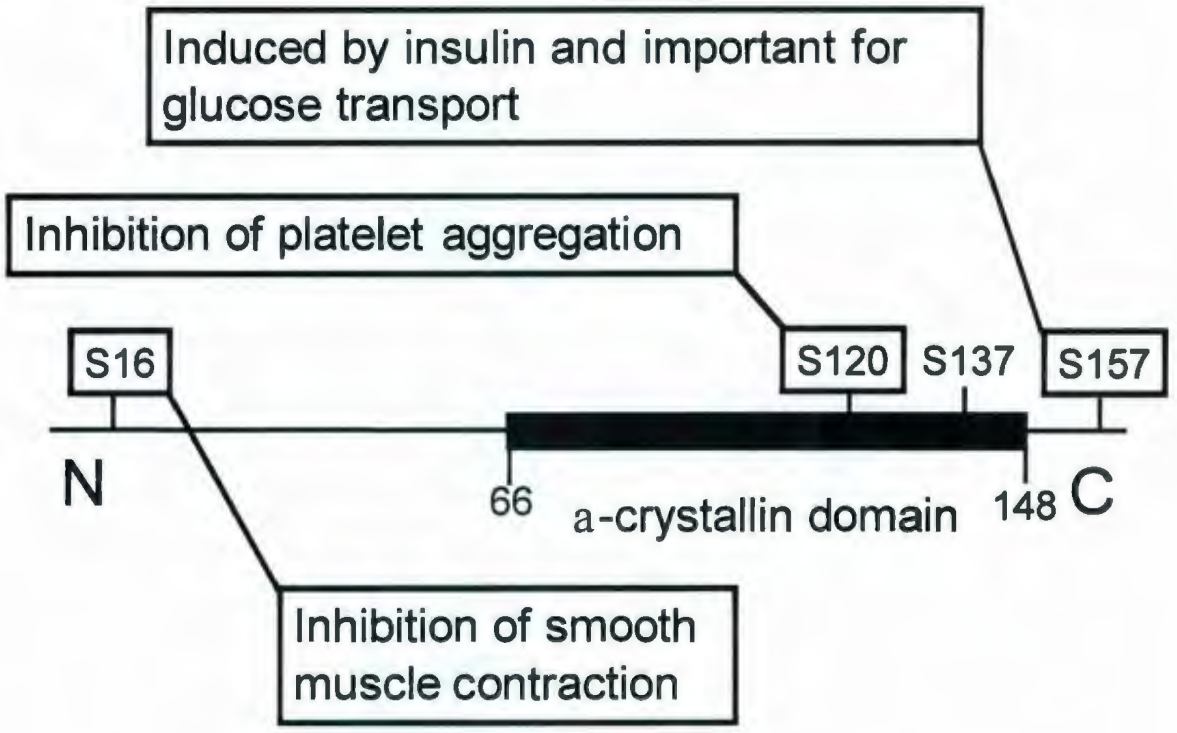
sHSPs are found in bacteria, archaea and eukaryotes, with the abundance of each of the sHSPs varying according to the cell type and/or organism studied. The mammalian family of sHSPs is comprised of ten known members, namely HSP27 (HSPB1), myotonic dystrophy protein kinase binding protein (MKBP, HSPB2), HSPB3, αA-crystallin (HSPB4), αB-crystallin (HSPB5), HSP20 (HSPB6), cvHSP (HSPB7), HSP22 (HSPB8), HSPB9, and sperm outer dense fiber protein (ODFP, HSPB10) (Golenhofen *et al.*, 2004). Levels of sHSPs in specific tissues range from nonexistent to about one percent in the case of certain forms of muscle cells and many sHSPs are present at particularly high levels during muscle formation, starting from the earliest stages of myocyte differentiation (Davidson *et al*, 2002; Haslbeck & Buchner, 2002). Some sHSPs, such as αB-crystallin and HSP27, are expressed in a variety of tissues, while others are expressed in a tissue-specific manner (Bukach *et al.*, 2004).

1.7 Heat Shock Protein 20 (HSP20; HSPB6)

The sHSP of importance for this thesis is HSP20, a 20 kDa protein composed of approximately 160 amino acid residues. This protein is expressed in practically all tissues, but reaches a maximal level of 1.3% of total proteins in skeletal and smooth muscle (Bukach *et al.*, 2004). HSP20 was first described as a by-product of the purification of two other sHSPs, αB-crystallin and HSP27, and, like other proteins in this family, participates in the formation of large aggregates of sHSPs in cells (Kato *et al.*, 1994). The structure of HSP20 is very similar to that of both αB-crystallin and HSP27, proteins known to affect actin filament dynamics (Rembold *et al.*, 2000).

HSP20 contains four serine residues whose phosphorylation impart various functions to this protein (Fig 1.4). HSP20 can be phosphorylated on serine 16 by both cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) and increases in HSP20 phosphorylation are associated with smooth muscle relaxation (Beall *et al.*, 1999; Rembold *et al.*, 2000). HSP20 is unique among members of the sHSP family in that it contains a PKA/PKG consensus sequence surrounding serine 16. Thus, it is believed to be the only sHSP which is involved in activation of the cyclic nucleotide dependent pathway of smooth muscle relaxation (Beall *et al.*, 1999; Kato *et al.*, 1994; Woodrum *et al.*, 1999). Further demonstrating the importance of phosphorylated HSP20 (pHSP20) in smooth muscle relaxation are experiments which have shown that introducing phosphopeptide analogues of HSP20 into bovine carotid artery smooth muscle inhibits agonist induced muscle contractions (Beall *et al.*, 1999).

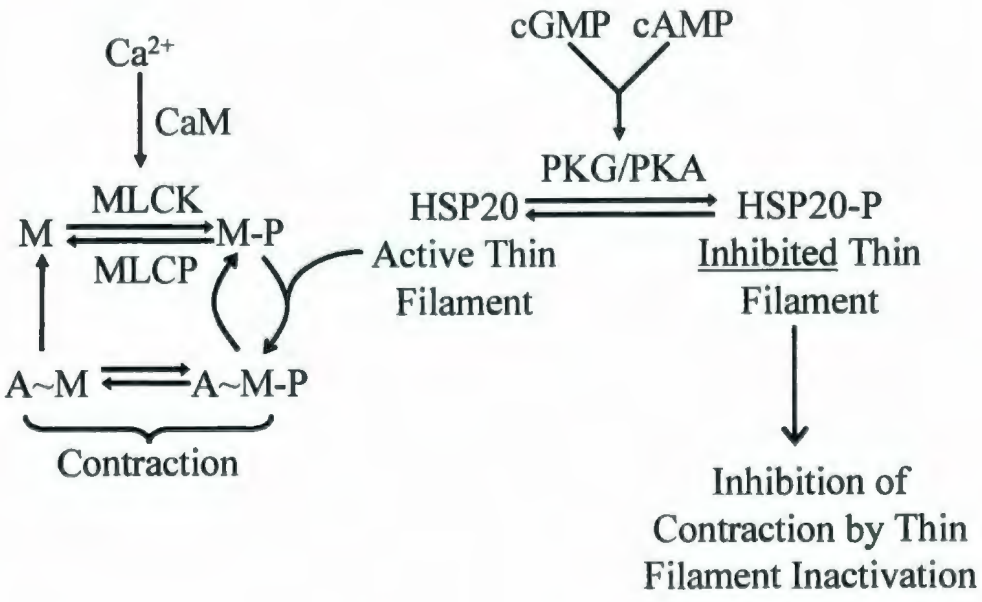
Figure 1.4. Scheme of the structure of HSP20 demonstrating location of the α-crystallin domain and serine phosphorylation sites. Phosphorylation on Ser157 by phosphatidylinositol 3-kinase is induced by insulin and is important for glucose transportation within the cell. Phosphorylation on Ser120 occurs during the inhibition of platelet aggregation. Phosphorylation of Ser16 by PKA/PKG is induced by smooth muscle antagonists and is important for the inhibition of smooth muscle contraction; boxed numbers = serine residues


Induced by insulin and important for glucose transport
Inhibition of platelet aggregation
S16
S120
S137
S157
N
66
a-crystallin domain
148
C
Inhibition of smooth muscle contraction

The role of HSP20 in smooth muscle relaxation appears to be centred upon its association with actin within the cell. Phosphorylation of HSP20 increases its affinity for the actin thin filament, as well as the actin associated protein α-actinin, which is abundant within focal adhesions and is known to form the dynamic cross-links between actin filaments (Rembold *et al.*, 2000; Tessier *et al.*, 2003). HSP20 contains an amino acid region (residues 110-121) which bears a high degree of sequence homology with the actin binding region of cardiac and skeletal troponin I, termed the troponin I inhibitory region. This actin binding region is also conserved in other sHSPs such as αB-crystallin and HSP27 and altering this region of HSP20 reduces its ability to bind to the actin thin filament (Rembold *et al.*, 2000). This knowledge has led to a proposed mechanism of action, whereby pHSP20 binds to and somehow alters the conformation of actin thin filaments, inhibiting the formation of phosphorylated cross bridges (Fig 1.5). In essence, binding of pHSP20 to actin "turns off" thin filaments so that they are unable to interact with phosphorylated myosin, and contraction cannot occur (Rembold *et al.*, 2001).

HSP20 has been shown in extracts from heart, diaphragm, and soleus muscle cells to associate in macromolecular aggregates with HSP27. HSP20 and HSP27 may regulate smooth muscle contraction and relaxation through interdependent interactions, whereby HSP20 may modulate the function of HSP27, and vice versa (Brophy *et al.*, 1999; Fuchs *et al.*, 2000; Tessier *et al.*, 2003). In addition, previous research in our lab has demonstrated that HSP27 is present in rat myometrium and that its expression is upregulated during late pregnancy and labour, implicating HSP27 as a candidate contraction-associated protein

Figure 1.5. Proposed mechanism for inhibition of smooth muscle contraction by HSP20 phosphorylation. Ca^{2+} binds to calmodulin (CaM), activating myosin light chain kinase (MLCK) which phosphorylates myosin (M→M-P), allowing attachment to the actin (A) thin filament allowing contraction to occur. Adenylyl cyclase or guanylyl cyclase are activated via hormone receptors, producing cAMP or cGMP, respectively. cAMP and cGMP activate PKA and PKG, respectively. These kinases phosphorylate HSP20 leading to inactivation of the actin thin filament, preventing its binding to myosin, and thus inhibition of contraction. Adapted from Rembold *et al.*, 2001.


Ca2+
CaM
MLCK
M
M-P
MLCP
A~M
A~M-P
Contraction
Active Thin Filament
cGMP cAMP
PKG/PKA
HSP20
HSP20-P
Inhibited Thin Filament
Inhibition of Contraction by Thin Filament Inactivation

(White *et al.*, 2005).

1.8 Objectives and Hypothesis

The main goal of this thesis was to characterize the expression of HSP20 in rat myometrium and to relate its expression to the stages of pregnancy and labour. To accomplish this goal I set out two main objectives:

Objective 1: To determine the spatial and temporal expression of HSP20 in rat myometrium during pregnancy and labour.

Objective 2: To determine whether the endocrine environment and mechanical distension of the uterus affect the expression of HSP20 in rat myometrium.

Due to the apparent importance of HSP20 in relaxation of smooth muscle, coupled with its interaction with the potential contraction-associated protein HSP27, I hypothesized that HSP20 would be expressed in the rat myometrium during early to mid pregnancy; however, its expression should be downregulated during the late stages of pregnancy, as the muscle becomes activated and the onset of labour approaches

Co-Authorship Statement

Chapter Two has recently been published in Reproduction (Cross *et al*, 2007). BE Cross performed the majority of the experiments, with the exception of some protein extraction and SDS polyacrylamide gel electrophoresis, which were performed by HM O'Dea. The manuscript was written primarily by BE Cross and was corrected and edited by DJ MacPhee.

Chapter Two

Expression of Small Heat Shock-related Protein 20 (HSP20) in Rat Myometrium is Markedly Decreased during Late Pregnancy and Labour.

Cross, BE, O'Dea HM, MacPhee, DJ.

Published in Reproduction: April 2007

133: 807-817

2.1 Abstract

The underlying mechanisms regulating uterine contractions during labour are still poorly understood. Heat shock protein 20 (HSP20) is known to be present at high levels in smooth muscle and implicated in muscle relaxation, but HSP20 expression in the myometrium is completely undetermined. Since HSP20 has been implicated in smooth muscle relaxation, we hypothesized that HSP20 would be highly expressed in the rat myometrium during early and mid pregnancy when the myometrium is relatively quiescent. Northern blot analysis demonstrated that HSP20 mRNA detection was significantly decreased from day (d) 22 - d23 of gestation compared to non-pregnant (NP) samples and from d22 - one day post-partum (PP) compared with d6 ($p<0.05$). Immunoblot analysis showed that detection of HSP20 was significantly decreased at d23 compared to d12 and d15 ($p<0.05$). HSP20 detection also significantly decreased at PP compared to d15 ($p<0.05$). Immunofluorescence analysis demonstrated that after d15, plasma membrane-associated localization of HSP20 decreased markedly in both circular and longitudinal muscle layers. In addition, HSP20 was detectable near cell membranes at much higher levels in the longitudinal muscle layer of progesterone-treated pregnant rats (delayed labour) at all gestational timepoints examined compared with controls. Our results demonstrate that HSP20 mRNA and protein are highly expressed during early and mid- pregnancy and then expression markedly decreases during late pregnancy and labour. The observed patterns of HSP20 expression are consistent with a potential role for HSP20 in facilitating myometrium quiescence during early and mid- pregnancy.

2.2 Introduction

The underlying mechanisms regulating uterine contractions during pregnancy and labour are still poorly understood. It is, however, clear that the initiation of parturition is controlled by the integration of endocrine and mechanical (uterine stretch) signals which originate within the fetal genome (Lye *et al*, 2001). The combination of these signalling pathways leads to phenotypic changes in the myometrium, resulting in myometrial activation. Endocrine signalling requires the activation of a cascade which involves the fetal hypothalamic-pituitary-adrenal axis. This endocrine cascade yields, in most species, an increase in circulating estrogen levels and a decrease in circulating progesterone levels in the maternal plasma (Challis *et al*, 2002). Although important, complete activation would not occur with the influence of the endocrine pathway alone. Mechanical signals imparted by uterine stretch (as a result of fetal growth during pregnancy) are also required for myometrial activation (Ou *et al*, 1997).

The result of activation is a switch from a muscle which is quiescent during the majority of pregnancy to one which is spontaneously active, excitable and highly responsive to uterotonic stimuli (Challis *et al*, 2002). This activation leads to an increase in the expression of a cassette of genes known collectively as contraction-associated proteins, which include ion channels, gap junction proteins and the oxytocin receptor (Challis *et al*, 2002)

The small heat shock proteins (sHSPs) are a family of proteins which range in mass from 12 to 43 kDa and exhibit chaperone activity, participating in the folding of proteins in

both normal conditions and extreme conditions such as heat shock, oxidation and exposure to toxins such as anti-cancer drugs (Gusev *et al*, 2002; Gusev *et al*, 2005). These proteins also possess other common properties, including the presence of an 80-residue conserved α-crystallin domain which spans two putative actin binding domains (Gusev *et al*, 2005).

HSP20 is expressed in practically all tissues, but reaches a maximal level of 1.3% of total proteins in skeletal and smooth muscle (Bukach *et al*, 2003). This 20 kDa protein can be phosphorylated by both cAMP- and cGMP-dependent protein kinase (PKA/PKG) and increases in phosphorylation are associated with smooth muscle relaxation (Beall *et al*, 1999). Beall *et al* (1999) have shown that the introduction of phosphopeptide analogs of HSP20 into bovine carotid artery smooth muscle inhibited agonist-induced muscle contractions. Upon phosphorylation, HSP20 has been shown to associate with actin (Rembold *et al*, 2000) and the actin-binding protein α-actinin (Tessier *et al*, 2003), which is located at focal adhesions in smooth muscle cells. Amino acid residues 110-121 of HSP20 bear sequence homology with the actin binding region of both cardiac and skeletal troponin I and this region is necessary for binding of HSP20 to actin filaments (Rembold *et al*, 2000). This binding is believed to inhibit cross-bridge cycling (myosin-actin interactions), thus leading to relaxation of smooth muscle (Rembold *et al*, 2001). HSP20 has been shown in both skeletal and smooth muscle to exist in macromolecular aggregates with HSP27, another small heat shock protein that we have identified as a candidate contraction-associated protein in the myometrium (White *et al*, 2005). This interaction is thought to be a functional one, with HSP20 regulating the function of HSP27 (Brophy *et al*, 1999).

The apparent importance of HSP20 in smooth muscle contraction, coupled with its interaction with the potential contraction-associated protein HSP27 led us to the hypothesis that HSP20 would be expressed in the rat myometrium during early and mid pregnancy, but that its expression would be downregulated during the late stages of pregnancy, as the muscle becomes activated and the onset of labour approaches.

2.3 Materials and Methods

2.3.1 Animals

Sprague Dawley rats were obtained from the Mount Scio Vivarium (Memorial University of Newfoundland, St John's, NL, Canada). Animals were held and cared for under standard environmental conditions (12h light, 12h darkness) 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, MO, USA) and water was available *ad libitum*. All experiments were approved by the institutional animal care committee under animal care protocols 02-02-DM to 06-02-DM. Virgin female rats (~220 g each) were mated with stud males, and observation of vaginal plugs the following morning was designated day 1 of pregnancy. The time of delivery under these standard conditions was day 23 of gestation.

2.3.2 Experimental Design

2.3.2.1 Normal pregnancy and term labour

Individual animals were each placed in an euthanasia chamber and exposed to an increasing concentration of carbon dioxide gas resulting in death within 5-10 minutes. Tissues were collected at the following timepoints: non-pregnant (NP), gestation days (d) 6, 12, 15, 17, 19, 21, 22, 23 (labour) and 1 day postpartum (PP). Labour samples were taken during active labour and only after the rat had delivered two to three pups.

2.3.2.2 Progesterone-delayed labour

The onset of labour is coupled with a withdrawal of the inhibitory effects of progesterone on the myometrium following a decline in plasma levels of this steroid. To determine whether progesterone withdrawal might modulate HSP20 expression, pregnant rats were given either a daily injection of progesterone (4 mg s.c. in 0.2 ml of corn oil) to maintain elevated plasma levels of this steroid, or vehicle alone (0.2 ml of corn oil, s.c.) beginning on d20 of gestation. Individual animals were each placed in an euthanasia chamber and exposed to an increasing concentration of carbon dioxide gas resulting in death within 5-10 minutes. Samples were collected from vehicle control animals on d21, d22, and d23 (during delivery) while samples were collected from progesterone-treated rats on d21, d22, d23 and d24; however, on d23 and d24, the rats were not in labour.

2.3.3 Tissue Collection

For Northern blot and immunoblot analysis, uterine horns were removed, opened longitudinally and fetuses and placentae discarded. Uterine tissue was placed in ice cold phosphate-buffered saline (PBS; pH 7.4) and endometrial tissue was removed by gentle scraping with a scalpel blade. All myometrium samples were flash-frozen in liquid nitrogen and stored at −80 °C. For immunocytochemistry, rat uterine tissue samples were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at room temperature with shaking. Tissues were paraffin embedded, sectioned and mounted on microscope slides by the Histology Unit of Memorial University of Newfoundland School of Medicine. Sections of rat uterine tissue

contained both longitudinal and circular smooth muscle layers and sections were processed under identical conditions and at the same time in each experiment.

2.3.4 Northern Blot Analysis

2.3.4.1 RNA isolation

Northern blot analysis for both normal pregnancy and delayed labour regimes was performed on four separate, independent sets of RNA samples (n = 4; i.e. four rats used per gestational time point). RNA was isolated from tissues using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) exactly according to the manufacturer's instructions. RNA quality and quantity ($A_{260/280}$) were determined using a Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, ON, Canada) and samples were stored at –70°C.

2.3.4.2 Electrophoretic separation and capillary transfer of RNA

RNA samples (10 µg per lane) were loaded on a 1% agarose gel containing 0.66 M formaldehyde and 1 × MOPS buffer (0.02 M MOPS pH = 7.0, 2 mM sodium acetate, 10 mM EDTA pH = 8.0) and electrophoretically separated at 80V in 1 × 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 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate) in DEPC-treated ddH_2O. RNA was cross-linked to nylon membrane with a UVC-508 ultraviolet cross-

linker (Ultra-Lum Inc., Paramount, CA, USA) and all blots were stored at −20°C until required.

2.3.4.3 Northern blot hybridization

Northern blot hybridization procedures have been described in detail elsewhere (White *et al.* 2005; Williams *et al.* 2005). Briefly, membranes were pre-hybridized in hybridization buffer then hybridized overnight at 42 °C in hybridization buffer containing a ^{32}P-labelled HSP20 cDNA probe. The 729 bp Bam HI/Xba I fragment of the rat HSP20 cDNA (Genbank Accession: NM_138887) was kindly provided, within the vector pRTL2, by Dr. Colleen Brophy and Dr. Charles Robb Flynn (Arizona State University, Tempe, AZ, USA). Radiolabelled cDNA probes were prepared with a Megaprime DNA Labelling Kit (Cat. No. RPN 1607; Amersham Biosciences, Little Chalfont, Buckinghamshire, England) according to manufacturers specifications. Following hybridization, blots were washed in 2 × SSC/0.1% SDS and exposed to X-ray film (Hyperfilm MP; Amersham Pharmacia Biotech), with the exception of the delayed labour experiments where blots were exposed to multisensitive phosphor imaging screens (Cyclone Storage Phosphor Screen; Perkin Elmer, Boston, MA, USA). Multiple exposures were produced for each Northern blot to ensure the results were within the linear range of the film or screens.

Following analysis of HSP20 gene expression, Northern blots were stripped by incubating membranes for 2 h in a solution of 1 M Tris-Cl, 1 mM EDTA, 0.1 × Denhardt's solution in ddH_2O. Membranes were washed at room temperature with 0.1 × SSPE and then

subsequently analyzed for expression of 18S rRNA using the same procedures described above. A 750 bp EcoRI fragment of the rabbit 18S ribosomal cDNA (Genbank Accession: X06778) was generously provided, within the vector pBluescript, by Dr I Skerjanc (University of Western Ontario, London, Ontario, Canada; Petropoulos and Skerjanc, 2002; Rogerson *et al.* 2002). 18S rRNA is constitutively expressed in rat myometrial cells and has been utilized as a loading control for analysis of myometrial gene expression (Oldenhof *et al.* 2002; Shynlova *et al.* 2004; White *et al.* 2005; Williams *et al.* 2005).

2.3.5 Immunoblot Analysis

Immunoblot analysis for both normal pregnancy and delayed labour experiments was performed on four separate, independent sets of protein samples (n = 4; i.e. four rats used per gestational time point) according to the method described by MacPhee and Lye (2000). Briefly, frozen rat myometrial samples were pulverized under liquid nitrogen and homogenized in RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, and 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 × *g* at 4°C for 15 min, and the supernatants were collected. Protein concentrations were determined by the Bradford assay (Bradford, 1976) using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and protein samples (100 μg/lane) were separated by polyacrylamide gel electrophoresis in 12% resolving gels according to the method described by Laemmli (1970). Gels were electroblotted to BioTrace NT

nitrocellulose membranes (Pall Life Sciences, Pensacola, FL, USA).

Membranes were rinsed in Tris-buffered saline-Tween-20 (TBST; 20 mM Tris base, 137 mM NaCl, and 0.1% Tween-20; pH 7.6) and blocked in 5% milk powder/TBST for 45 min. Unless otherwise stated, all incubations were performed at room temperature and with constant agitation. Rabbit polyclonal antisera raised against HSP20 (Cat. No. 07-490; Upstate Biotechnology, Lake Placid, NY, USA) or mouse monoclonal antisera raised against smooth muscle calponin (Cat. No. C2687; clone hCP; Sigma-Aldrich Canada, Oakville, Ontario, Canada) used at dilutions of 1:2000 and 1:50 000, respectively, were incubated with blots for 1 h and then blots were rinsed with TBST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) or HRP-conjugated goat anti-mouse IgG (H + L) (Cat. No. 31460 and 31430, respectively; Pierce, Rockford, IL, USA) were used as secondary antisera at dilutions of 1:10 000 and 1:150 000, respectively. Proteins were detected using the Pierce SuperSignal West Pico chemiluminescent substrate detection system (MJS Biolynx, Inc., Brockville, Ontario, Canada) and multiple exposures were produced to ensure the linearity of the film exposures.

Following detection of HSP20, all blots were stripped with Restore Western blot stripping solution (Pierce) according to the manufacturer's instructions. Analysis of calponin protein expression was subsequently performed. We have determined that calponin protein is constitutively expressed in non-pregnant and pregnant rat myometrial tissue under our protein extraction conditions (i.e. RIPA buffer extraction protocol) and, as a result, serves as a sufficient loading control for our analyses (White *et al.* 2005; Williams *et al.* 2005).

2.3.6 Immunocytochemistry

Two separate, independently collected sets of rat tissues (n = 2; i.e. 2 rats used per gestational time point) were utilized for immunocytochemistry experiments and the experiments were repeated three times. Following de-waxing and rehydration, tissue sections were permeabilized with 0.125% trypsin in PBS for 10 min at room temperature and then rinsed with PBS. Unless otherwise noted, all incubations were performed at room temperature and with constant agitation. Tissue sections were blocked with 5% normal goat serum/1% horse serum in PBS for 30 min. Sections were then incubated overnight with constant agitation at 4°C in rabbit anti-HSP20 (Cat. No. H1830-48D; US Biological, Swampscott, MA, USA) at a dilution of 1:200 in blocking solution or rabbit IgG (Cat. No. 011-000-003; Jackson ImmunoResearch Labs, Inc., West Grove, PA, USA) at the same final concentration to serve as a negative control. Tissue sections were washed in PBS and then incubated in fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG (Cat. No. F7512; Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:200 in blocking solution for 30 min. For nuclear staining, TO-PRO-3 iodide (Cat. No. T3605; Molecular Probes, Eugene, OR, USA) was added at a dilution of 1:250 to the secondary antibody solution. Sections were washed with cold PBS containing 0.02% Tween-20, followed by mounting in Vectashield (Vector Laboratories Inc., Burlington, Ontario, Canada). Prepared slides were observed and images were collected with an Olympus Fluoview laser scanning confocal microscope (Olympus Optical Company Ltd, Melville, NY, USA). For each immunocytochemistry experiment (ie normal pregnancy and term labour, progesterone-delayed labour) image collection parameters (PMT, Gain, and Black level settings) were the same within each

muscle layer.

2.3.7 Data Analysis

Densitometric analysis of Northern blots and immunoblots was performed with the aid of Scion Image software (Scion Image Corporation, Frederick, MD, USA). Densitometric measurements of HSP20 mRNA were normalized to those of 18S ribosomal RNA while measurements of HSP20 protein on immunoblots were normalized to those of calponin. Statistical analysis was performed with GraphPad Instat version 3.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and data graphed using GraphPad Prism version 4.0 (GraphPad Software). Data from Northern blot and immunoblot analyses of HSP20 expression during normal gestation were subjected to a one-way analysis of variance (ANOVA) and a Tukey-Kramer multiple comparisons test. Data from analysis of HSP20 expression during progesterone-delayed labour were subjected to a two-way ANOVA and a Bonferroni post-test. Values were considered significantly different if $p < 0.05$.

2.4 Results

2.4.1 Normal Pregnancy and Labour

2.4.1.1 Expression of HSP20 mRNA and Protein

To characterize the levels of HSP20 mRNA within myometrial samples, Northern blots of myometrial total RNA from NP, d6, d12, d15, d17, d19, d21, d22, d23 and PP were analysed with radiolabelled probes generated from a rat HSP20-specific cDNA (Fig. 2.1A). Our experiments demonstrated that HSP20 mRNA detection significantly decreased during late gestation (one-way ANOVA, $p < 0.05$; $n = 4$). Specifically, HSP20 was highly detectable in NP and d6 samples, then detection appeared to decrease by d12. HSP20 mRNA detection was significantly decreased at d22 and d23 when compared to NP samples and between d22 and PP, inclusive, when compared with d6 samples (Tukey-Kramer post test, $p < 0.05$; Fig. 2.1A,B).

Immunoblot analysis using HSP20-specific antisera demonstrated that HSP20 detection decreased significantly during late gestation (one-way ANOVA, $p < 0.05$; $n = 4$). Protein detection levels remained high until d15 of gestation and then began to decrease thereafter. Detection levels at d23 were significantly lower than those at d12 and d15, while post-partum levels were significantly decreased compared to d15 samples (Tukey-Kramer post test, $p < 0.05$; Fig. 2.2A,B).

2.4.1.2 Immunocytochemical Detection of HSP20

Detection of HSP20 in the longitudinal muscle layer demonstrated that HSP20 was primarily localized at or near cell membranes from NP and throughout gestation (Figs.

Figure 2.1. Northern blot analysis of HSP20 mRNA detection in rat myometrium during pregnancy, labour and 1 day post-partum. (A) Representative Northern blots of HSP20 mRNA detection and 18S rRNA detection. Analysis was performed using an HSP20-specific rat cDNA and an 18S-specific rabbit cDNA as templates for radiolabelled probe production. Ten micrograms of total RNA were utilized per lane. (B) Densitometric analysis illustrating the decrease in HSP20 mRNA detection during pregnancy and labour. Detection levels on d22, d23 were significantly decreased ($*p < 0.05$) when compared with NP and values on d22 to PP were significantly decreased ($**p < 0.05$) when compared with d6. Values are from four independent experiments ($n = 4$) ± SEM. Days 6, 12, 15, 17, 19, 21, 22, and 23 represent gestational timepoints. NP, nonpregnant; PP, 1 day postpartum.

A


NP d6 d12 d15 d17 d19 d21 d22 d23 PP
HSP20
18S

B

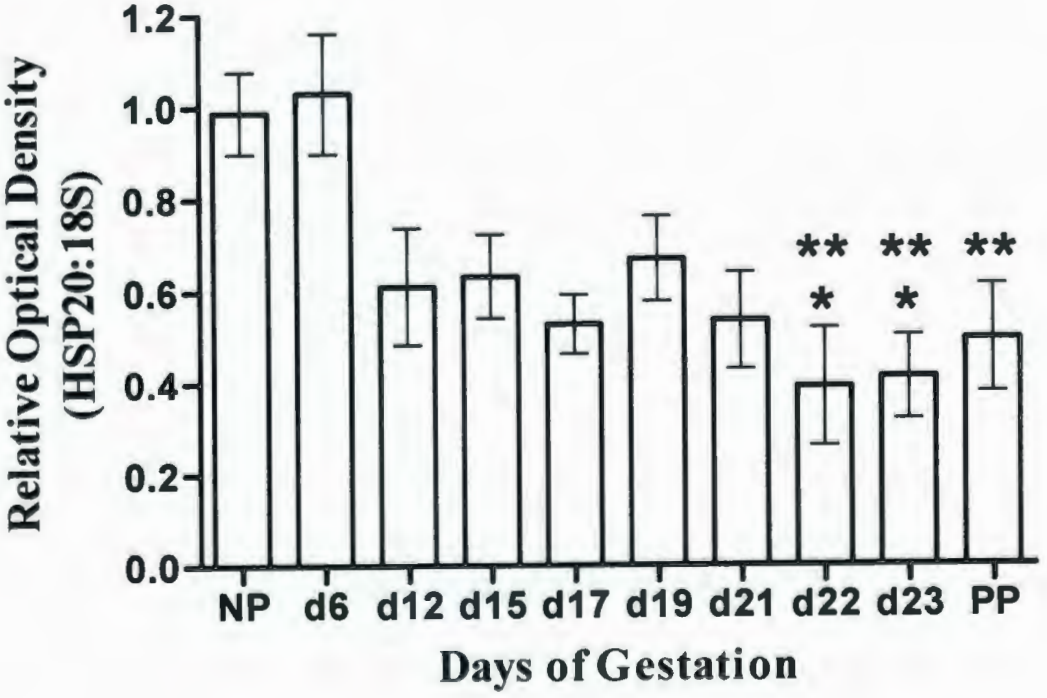
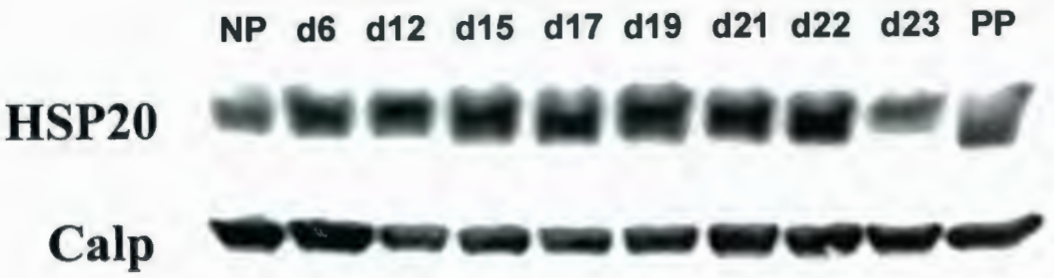
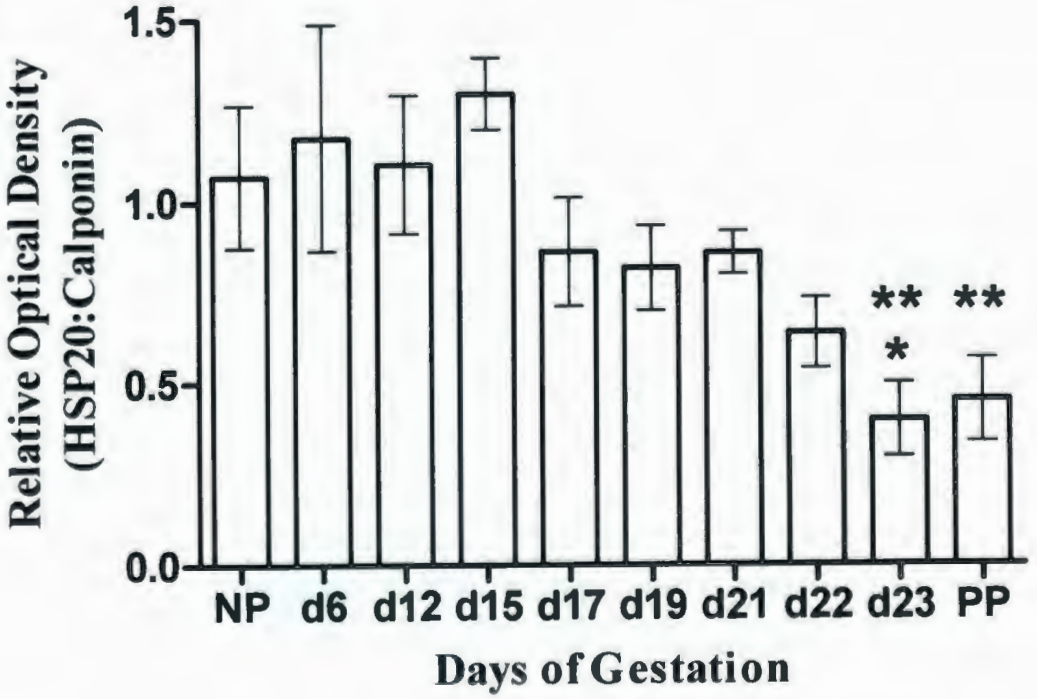

Relative Optical Density
(HSP20:18S)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
** ** **
* *
NP d6 d12 d15 d17 d19 d21 d22 d23 PP
Days of Gestation

Figure 2.2. Immunoblot analysis of HSP20 protein detection in rat myometrium during pregnancy, labour and 1 day post-partum. (A) Representative immunoblots of HSP20 protein and calponin detection. (B) Densitometric analysis illustrating the decrease in HSP20 protein detection during pregnancy and labour. Values on d23 were significantly decreased (*$p<0.05$) when compared with d12 and values on d23, PP were significantly decreased (**$p<0.05$) when compared with d15. Values are from four independent experiments ($n = 4$) ± SEM. Days 6, 12, 15, 17, 19, 21, 22, and 23 represent gestational timepoints. NP, nonpregnant; PP, 1 day postpartum.

A


NP d6 d12 d15 d17 d19 d21 d22 d23 PP
HSP20
Calp

B


Relative Optical Density
(HSP20:Calponin)
1.5
1.0
0.5
0.0
** **
*
NP d6 d12 d15 d17 d19 d21 d22 d23 PP
Days of Gestation

2.3,2.4). The level of detection gradually decreased throughout gestation, reaching its lowest detection level in the PP samples; however, detection was lower on d12 than at the adjacent d6 and d15 time points. A similar pattern of down regulation of HSP20 detection was seen in the circular muscle layer (Figs. 2.5, 2.6). Although HSP20 appeared to be localized at or near cell membranes, there was also evidence of HSP20 detection in the cytoplasm.

2.4.2 Progesterone-induced Delayed Labour

2.4.2.1 Expression of HSP20 mRNA and Protein

Throughout the majority of pregnancy, circulating levels of progesterone in the rat are high, reaching peak levels between d15 and d19 then declining thereafter including at labour (Lye *et al*, 1993). The finding that HSP20 expression also decreased significantly during late gestation and labour suggested that the decline in progesterone levels and its subsequent effects on the myometrium might be responsible, at least in part, for our results. Animals treated with progesterone beginning on d20 of gestation did not enter labour on d23. Our experiments demonstrated that, at each time point, there were no significant differences in HSP20 mRNA detection levels between the progesterone-treated and vehicle-treated animals (two-way ANOVA, $p > 0.05$; $n = 3$; Fig. 2.7). Immunoblot analysis also demonstrated that there were no significant differences in HSP20 protein detection levels (two-way ANOVA, $p > 0.05$; $n = 4$; Fig. 2.8).

2.4.2.2 Immunocytochemical Detection of HSP20

Figure 2.3. Immunocytochemical analysis of HSP20 protein detection in the longitudinal smooth muscle layer of rat myometrium between NP and d12 of pregnancy using polyclonal rabbit anti-HSP20 antisera. These images demonstrate the observed localization of HSP20 (green) at or near the cell membrane. TO-PRO-3 iodide was used for nuclear staining (red). Cont = control, rabbit IgG. Scale bar = 50 μm.

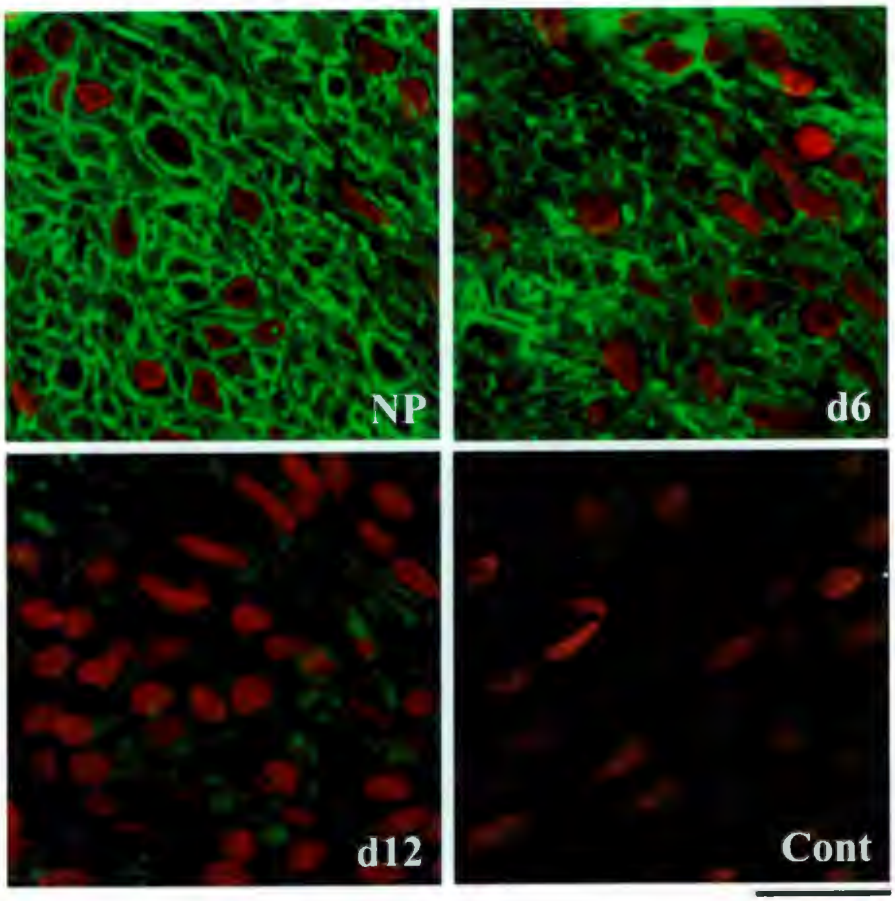

NP
d6
d12
Cont

Figure 2.4. Immunocytochemical analysis of HSP20 protein detection in the longitudinal smooth muscle layer of rat myometrium between d15 of pregnancy and PP using polyclonal rabbit anti-HSP20 antisera (green). TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μm.

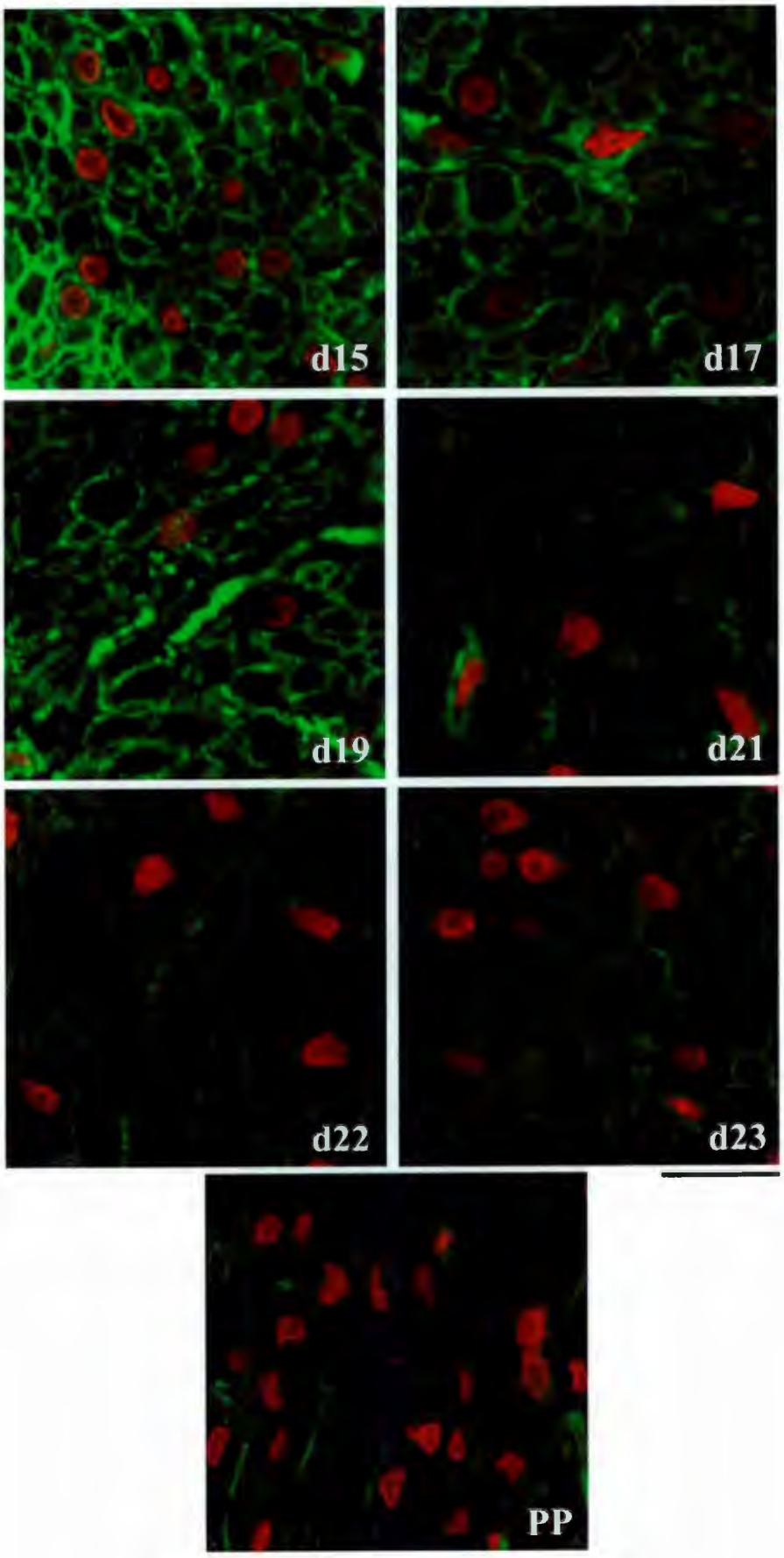

d15
d17
d19
d21
d22
d23
PP

Figure 2.5. Immunocytochemical analysis of HSP20 protein detection in the circular smooth muscle layer of rat myometrium between NP and d12 of pregnancy using polyclonal rabbit anti-HSP20 antisera (green). TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μm.

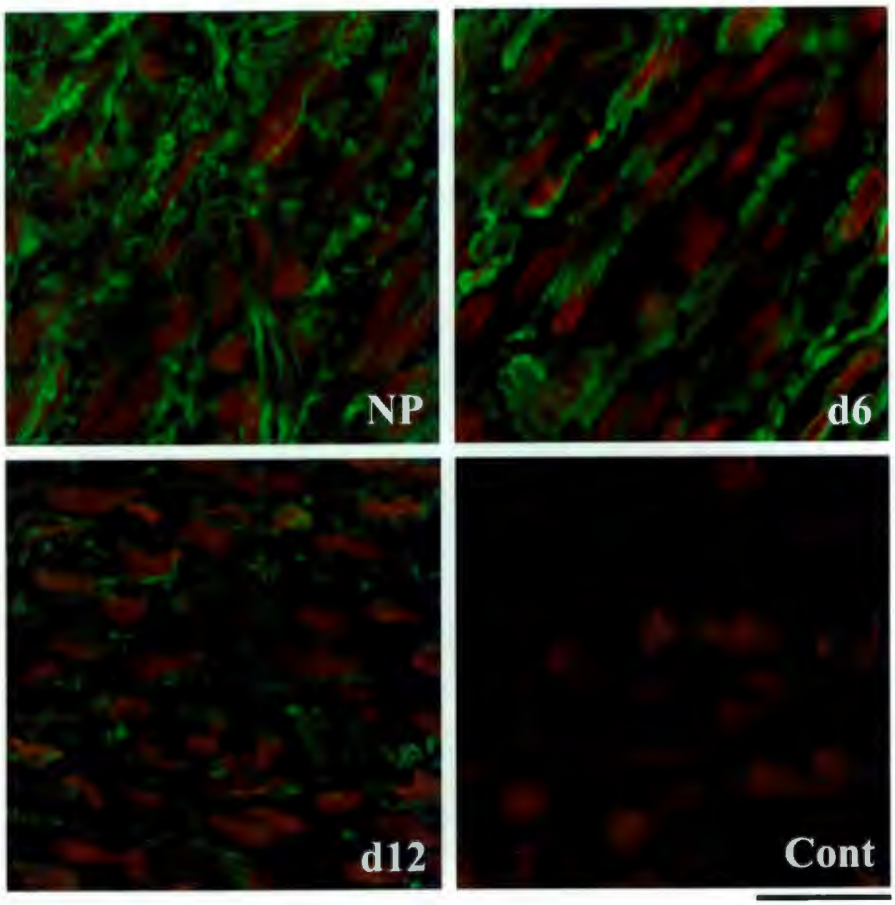

NP
d6
d12
Cont

Figure 2.6. Immunocytochemical analysis of HSP20 protein detection in the circular smooth muscle layer of rat myometrium between d15 of pregnancy and PP using polyclonal rabbit anti-HSP20 antisera (green). TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μm.

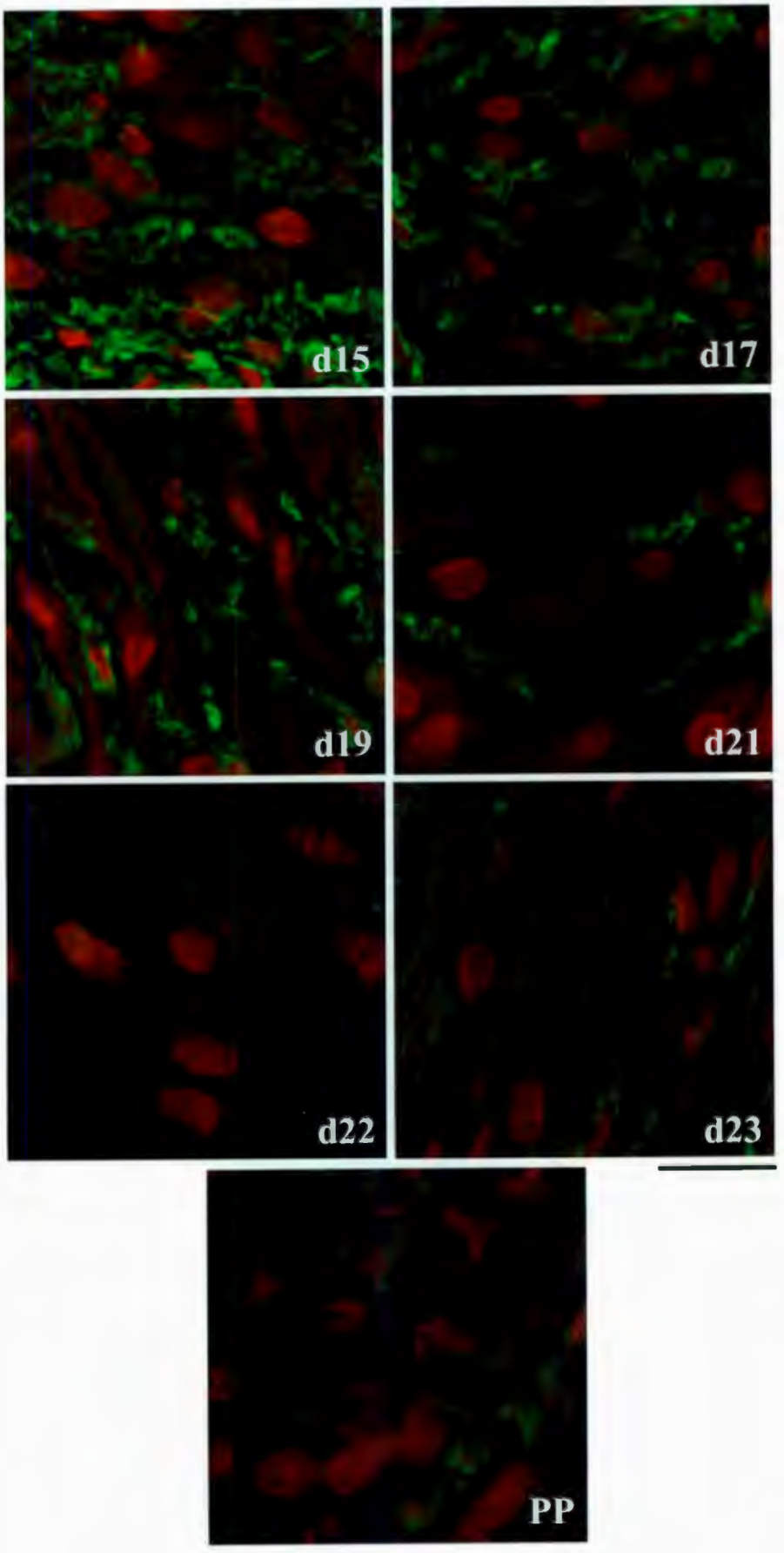
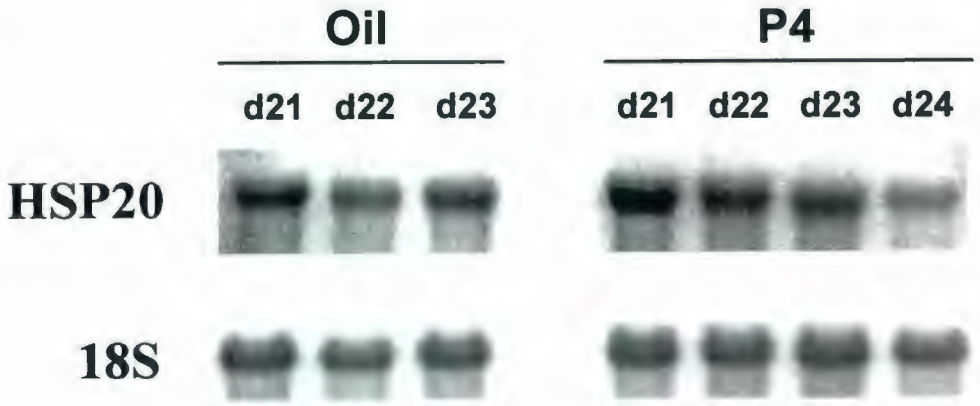

d15
d17
d19
d21
d22
d23
PP

Figure 2.7. Northern blot analysis of HSP20 mRNA detection in a delayed-labour model following administration of progesterone or corn oil (vehicle control) to pregnant rats. (A) Representative Northern blots of HSP20 mRNA detection and 18S rRNA detection. (B) Densitometric analysis illustrating no significant change in the level of detection between treatment groups. Values are from three independent experiments (n = 3) ± SEM. P4, progesterone. Designations 21-Oil, 22-Oil, 23-Oil, 21-P4, 22-P4, 23-P4, 24-P4 represent gestational time points in the two treatment groups.

A


Oil
P4
d21 d22 d23
d21 d22 d23 d24
HSP20
18S

B

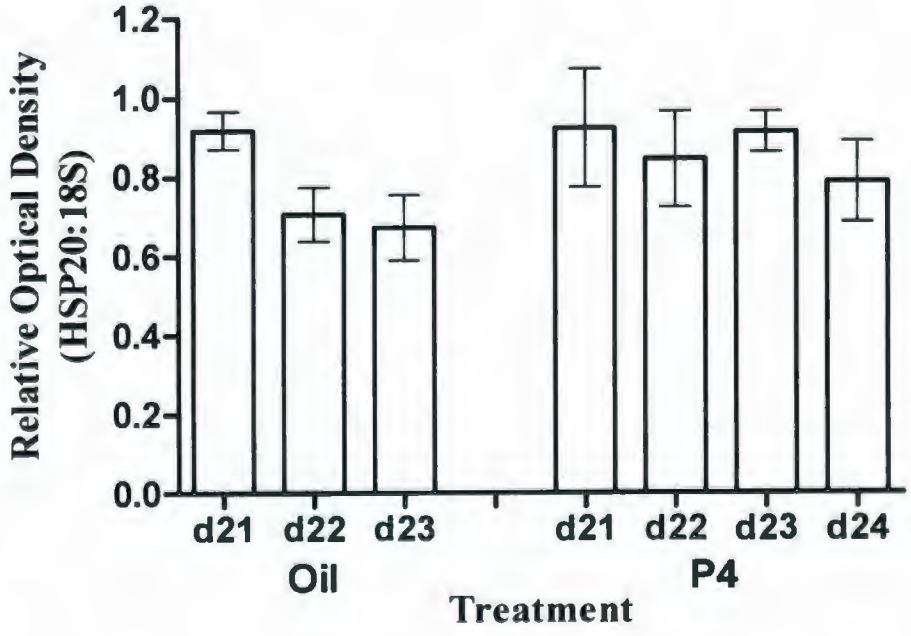
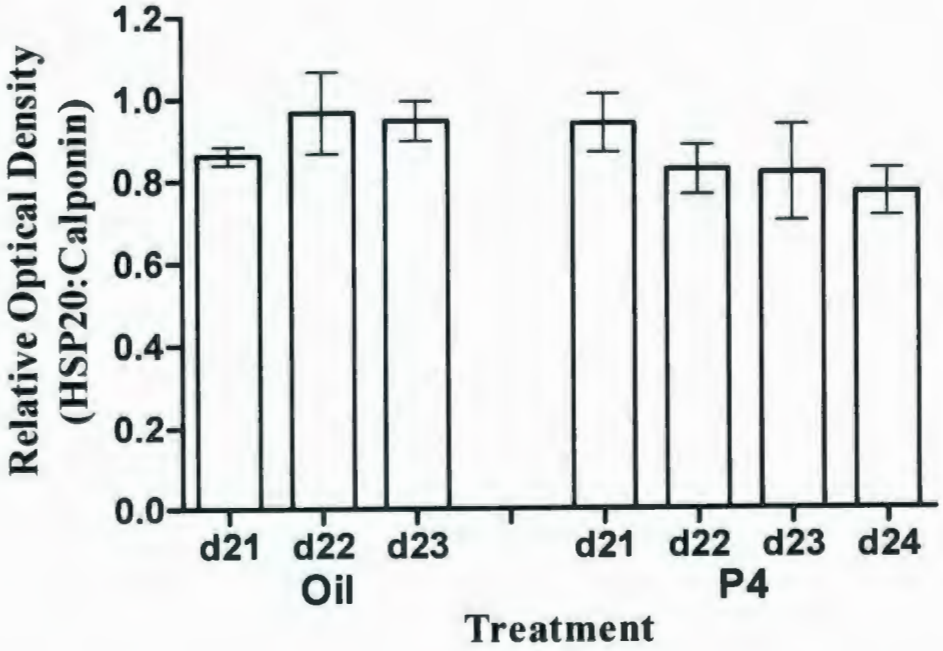

Relative Optical Density
(HSP20:18S)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
d21 d22 d23
Oil
d21 d22 d23 d24
P4
Treatment

Figure 2.8. Immunoblot analysis of HSP20 protein detection in a delayed-labour model following administration of progesterone or corn oil (vehicle control) to pregnant rats. (A) Representative immunoblots of HSP20 and calponin detection. (B) Densitometric analysis illustrating no significant change in the level of detection between treatment groups. Values are from four independent experiments (n = 4) ± SEM. P4, progesterone. Designations 21-Oil, 22-Oil, 23-Oil, 21-P4, 22-P4, 23-P4, 24-P4 represent gestational time points in the two treatment groups.

A


Oil
P4
d21
d22
d23
d21
d22
d23
d24
HSP20
Calp

B


Relative Optical Density
(HSP20:Calponin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
d21
d22
d23
Oil
d21
d22
d23
d24
P4
Treatment

There was a striking difference in detection levels between progesterone-treated and vehicle control tissue samples in the longitudinal muscle layer (Fig. 2.9). HSP20 detection in the progesterone-treated samples was markedly higher than that of the vehicle controls at all examined gestational timepoints. In both cases, HSP20 was also localized at or near cell membranes. In the circular muscle layer, no marked differences in HSP20 detection levels or spatial localization were observed between the progesterone-treated and vehicle control samples (Fig. 2.10).

Figure 2.9. Immunocytochemical analysis of HSP20 protein (green) detection in the longitudinal smooth muscle layer of rat myometrium in a delayed-labour model following administration of progesterone (4 mg in 0.2 mL of corn oil, s.c.) or oil (vehicle control; 0.2 mL corn oil, s.c.). These images illustrate the observed maintenance of HSP20 detection in the progesterone treatment samples. P = progesterone; O = oil; Cont = control, rabbit IgG. Days 21-O, 22-O, 23-O, 21-P, 22-P, 23-P, 24-P represent gestational time points. TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μm.

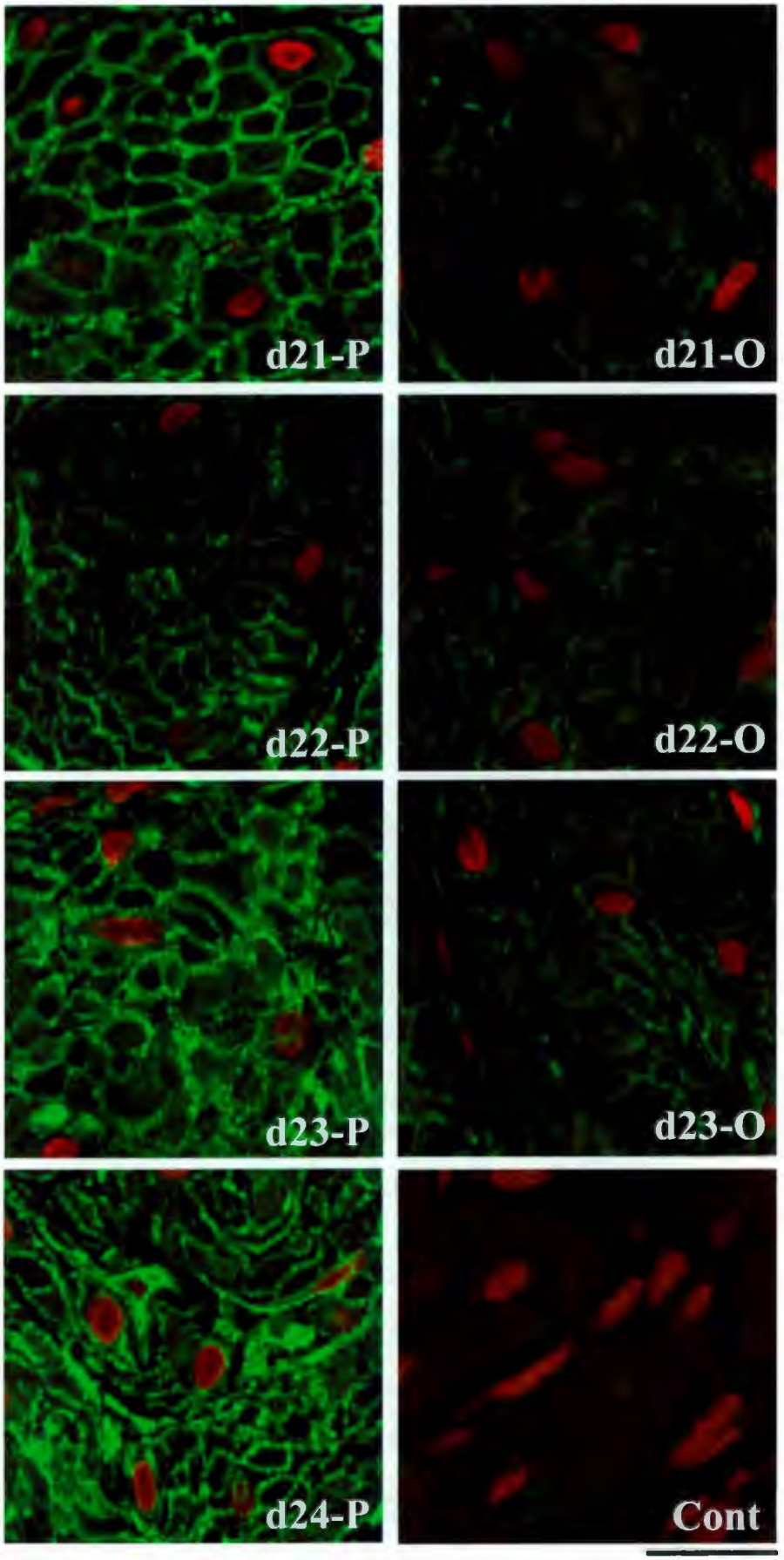
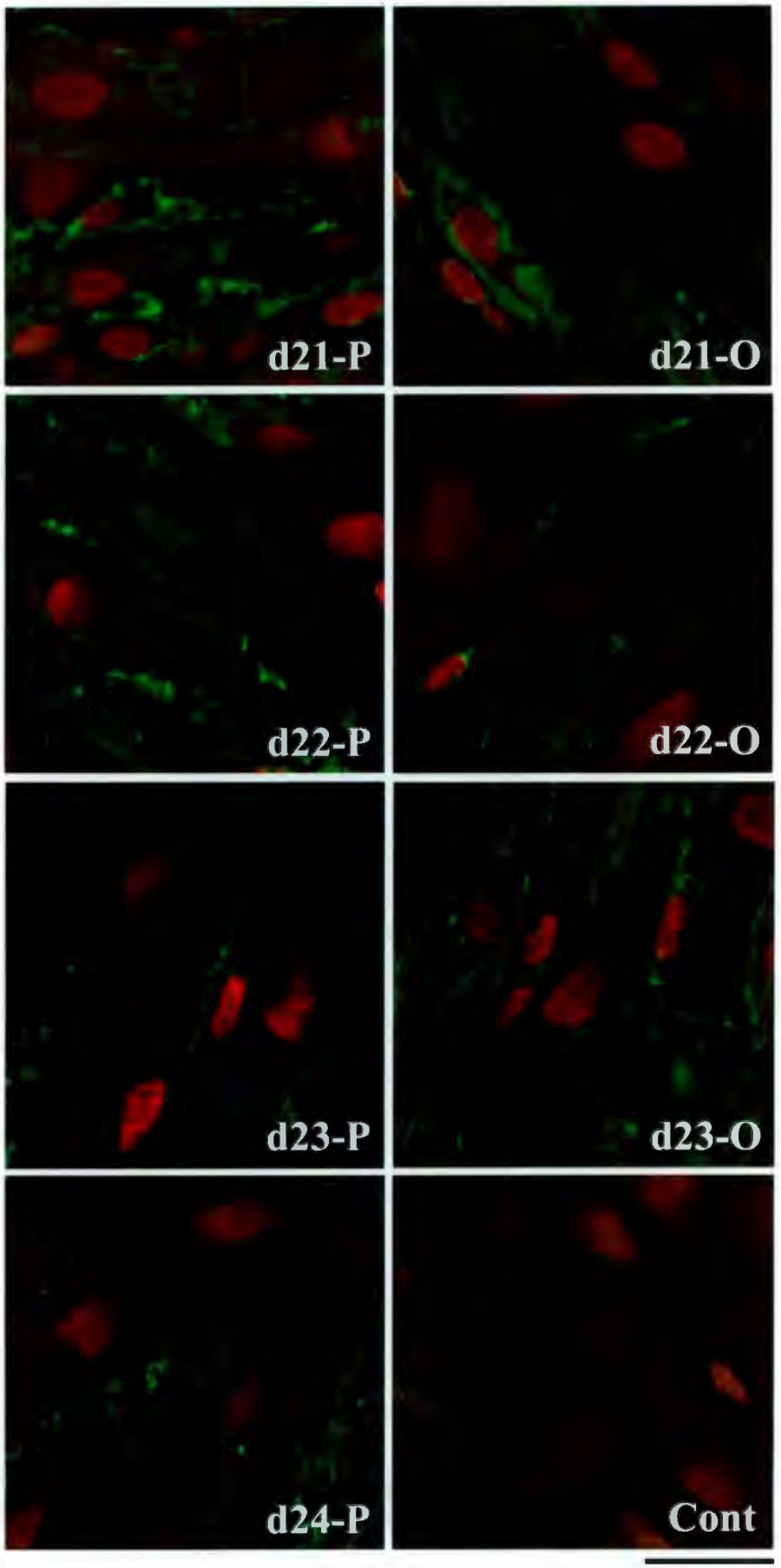

d21-P
d21-O
d22-P
d22-O
d23-P
d23-O
d24-P
Cont

Figure 2.10. Immunocytochemical analysis of HSP20 protein (green) detection in the circular smooth muscle layer of rat myometrium in a delayed-labour model following administration of progesterone (4 mg in 0.2 mL of corn oil, s.c.) or oil (vehicle control; 0.2 mL corn oil, s.c.). P = progesterone; O = oil; Cont = control, rabbit IgG. Days 21-O, 22-O, 23-O, 21-P, 22-P, 23-P, 24-P represent gestational time points. TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μm.


d21-P
d21-O
d22-P
d22-O
d23-P
d23-O
d24-P
Cont

2.5 Discussion

We recently reported that HSP27 was highly expressed in the rat myometrium during pregnancy and postulated that it could be a contraction-associated protein (White *et al*, 2005). Until now the expression of no other sHSP had been reported in the rat myometrium during pregnancy. HSP20 is highly expressed in smooth muscle (Bukach *et al*, 2003) , may have a role in smooth muscle relaxation (Beall *et al*, 1999), and can exist in macromolecular aggregates with HSP27 (Brophy *et al*, 1999). Therefore, we hypothesized that HSP20 would be expressed in the rat myometrium during early and mid pregnancy, but that its expression would be downregulated during the late stages of pregnancy, as the muscle became activated and the onset of labour approached.

2.5.1 Normal pregnancy and labour

Our Northern blot analysis showed that the detection of HSP20 mRNA decreased significantly from d22 to PP while detection of HSP20 protein on immunoblots showed a significant decrease by labour. Thus, the observed decrease in HSP20 protein expression could be the result of decreased HSP20 mRNA available for subsequent translation. Together with our results from immunofluorescence experiments demonstrating a marked reduction in HSP20 immunostaining after d15, the observed patterns of HSP20 expression during pregnancy and labour potentially support a role for HSP20 in regulating myometrial quiescence during early and mid- pregnancy. Previous research in smooth muscle has demonstrated an association between HSP20 and actin which has been suggested to lead to actin depolymerization and inhibition of contraction (Beall *et al*, 1999; Tessier *et al*, 2004;

Dreiza *et al*, 2005). A decrease in HSP20 expression late in gestation could also lead to a decrease in the association of HSP20 with actin thereby relieving, at least in part, contractile inhibition of the myometrium. Significant rat uterine contractile activity only becomes evident within the last 24 hours prior to labour (Buhimschi and Garfield,1996; Buhimschi *et al,* 1998).

Immunocytochemistry experiments revealed that HSP20 was primarily localized at or near cell membranes. HSP20 has been shown to be associated with the actin-binding protein α-actinin, which is a known component of focal adhesions (or dense plaques) in smooth muscle cells (Tessier *et al*, 2003). Furthermore, the spatial immunolocalization of HSP20 correlates, at least in part, with Serine 15 (Ser15) phosphorylated HSP27 expression, as Ser15- phosphorylated HSP27 was also localized to cell membranes prior to labour (White *et al*, 2005). The significance of this finding is not yet clear but Fuchs *et al* (2000) has demonstrated that phosphorylated HSP27 can inhibit phosphorylation of HSP20 leading to reduction of vascular smooth muscle relaxation. In total, our findings are consistent with a report that HSP20 and HSP27 proteins may exist in aggregates in smooth muscle (Brophy *et al*, 1999) and our future work will examine the phosphorylation state of HSP20 in the myometrium during gestation and the interdependence of HSP20 and HSP27 in uterine smooth muscle function.

Physiological growth of the pregnant uterus occurs by two mechanisms: an increase in cell number (hyperplasia) followed by an increase in cell size (hypertrophy) (Shynlova *et al*, 2006). The transition between these two phases of growth is associated with a transient

activation of the caspase cascade, at ~d12-d14 of gestation, that initiates the differentiation of uterine smooth muscle but does not lead to the biochemical or morphological features of apoptosis (Shynlova *et al*, 2006). Interestingly, within relatively the same period we observed a transient but marked decrease in immunodetection of HSP20 in both muscle layers by d12 of gestation followed by an increase in detection by d15. We believe these results may be related to the initiation of uterine smooth muscle differentiation. The subsequent increase in HSP20 detection by d15 in both muscle layers could be a reflection of a protective mechanism against cell death since HSP20 has previously been shown to protect against apoptosis in cardiomyocytes (Fan *et al*, 2004).

2.5.2 Progesterone-Induced Delayed Labour

Circulating levels of progesterone in the rat peak at d19 of gestation declining thereafter including at labour (Lye *et al*, 1993); thus, the observed drop in HSP20 expression late in pregnancy could be related to the decline in circulating levels of this steroid. Evidence suggests that expression of sHSPs can be regulated by both progesterone and estrogen (Ciocca *et al*, 1993; Tabibzadeh *et al*, 1996; Bany and Schultz, 2001; Kato *et al*, 2002). Progesterone administration to pregnant rats did not result in significant induction of HSP20 mRNA and protein expression compared to vehicle controls as measured by Northern blot and immunoblot analysis, respectively. However, immunocytochemical analysis demonstrated a maintenance of HSP20 expression at or near cell membranes from d21-d24 in the longitudinal muscle layer of progesterone-treated rats compared to vehicle controls suggesting a regional sensitivity of HSP20 expression to progesterone. It has been reported

that the longitudinal and circular muscle layers exhibit different contractile and physiological characteristics (Osa & Katase, 1975; Kawarabayashi & Osa, 1976; Chow & Marshall, 1981; Mlynarczyk *et al*, 2003); however, it is important to note that we demonstrated similar HSP20 immunolocalization patterns in the two muscle layers across normal gestation and particularly during early to mid-pregnancy when circulating levels of progesterone are high (Lye *et al*, 1993). Thus, the reason(s) for increased HSP20 expression from d21-d24 in the longitudinal muscle layer during progesterone-induced delayed labour is unknown and requires future clarification. In total, our findings indicate that progesterone regulation of HSP20 expression, if any, may be quite complex. Future work will attempt to more specifically determine the role of both progesterone and estrogen in the regulation of HSP20 gene expression.

2.5.3 HSP20 Expression and Relevance to Initiation of Labour

A recent study by Girotti and Zingg (2003) using DNA microarrays of rat uterus has found that as the uterus progresses toward labour, the degree of gene suppression is greater than the degree of gene activation. This finding suggests that for the initiation of labour, the suppression of genes related to the control of quiescence may be equally as important as the up-regulation of the contraction-associated proteins. Our work has identified that HSP20 expression decreases as the uterus progresses toward labour. Although it is not yet possible to determine the specific functional role(s) of HSP20 in the rat myometrium during pregnancy and labour, our results, coupled with documented evidence of a role for HSP20 in smooth muscle relaxation, implicate a role for HSP20 in the regulation of myometrial

quiescence. Subsequent downregulation of HSP20 expression prior to and during labour may also be important for the initiation of parturition.

2.6 Acknowledgements

We would like to acknowledge the assistance of Judy Foote and Art Taylor for tissue processing and sectioning of rat myometrial tissue for our immunocytochemistry experiments. We also thank Dr. Karen Mearow for critically reading the manuscript. The research described in this manuscript was funded by the Natural Sciences and Engineering Research Council of Canada, Grant #250218-02 and aided by a New Opportunities Fund infrastructure grant from the Canada Foundation for Innovation (Project # 74119). BE Cross and HM O'Dea were partially funded by Summer Undergraduate Research Awards from the Faculty of Medicine (Memorial University of Newfoundland).

Chapter Three

Effect of Mechanical Distension and Progesterone on Expression of HSP20 in Rat Myometrium

3.1 Abstract

During pregnancy, the myometrium exhibits a very noticeable change in size and phenotype which is believed to be modulated by both mechanical and hormonal influences originating, in part, from within the fetal genome. HSP20 is known to be involved in relaxation of smooth muscle and may play a role in maintaining quiescence of the myometrium during early to mid pregnancy. I have previously found a dramatic decrease in the detection of HSP20 in pregnant rat myometrium near term. The objective of this study was to examine the role of uterine stretch in regulation of HSP20 expression during late pregnancy and labour. Using unilaterally pregnant rats, I investigated the changes in HSP20 mRNA and protein detection in myometrium from gravid and non-gravid horns by northern blot and immunoblot analysis, respectively. I also examined the temporal/spatial localization of HSP20 using immunofluorescence analysis. As decreased HSP20 expression near term correlates with decreased levels of circulating progesterone, I also studied the effects of early progesterone withdrawal on HSP20 mRNA and protein detection, using the receptor antagonist RU486. Gravidity did not affect the detection of HSP20 mRNA or protein at d19, but did cause a significant decrease in HSP20 mRNA ($p < 0.10$) and protein ($p < 0.05$) detection in the gravid horn at labour. Immunofluorescence analysis did not demonstrate any differences in HSP20 detection at d19, but verified the decrease in HSP20 detection in the gravid horn at labour, with a decrease in staining at or near myocyte membranes. Early progesterone withdrawal had no effect on HSP20 mRNA or protein detection in the myometrium. These findings, combined with previous results, suggest that expression of

HSP20 in rat myometrium is not likely dependent on circulating progesterone levels, but may be negatively affected by stretch of the myometrium at term labour.

3.2 Introduction

Pregnancy is a remarkable series of events during which the uterus undergoes a variety of changes. Most notable is the growth of the uterus which occurs to accommodate the growing fetus(es). During pregnancy, the myometrium exhibits a very noticeable change in size and phenotype which is believed to be modulated by both mechanical and hormonal influences originating from within the fetal genome (Lye *et al.*, 2001; Shynlova *et al.*, 2004). Hormonal signalling occurs through a cascade involving the fetal hypothalamic-pituitary-adrenal (HPA) axis and results, in most species, in an increase in circulating levels of estrogen towards term at the expense of circulating progesterone levels in the maternal plasma (Challis *et al.*, 2002). Mechanical signals are imparted upon the myometrium through increased fetal and placental growth and increasing amniotic fluid volume during pregnancy (Ou *et al.*, 1997). It has been shown that both of these signalling pathways play roles in altering the expression of genes believed to be important during pregnancy and labour.

Using a unilaterally pregnant rat model, Shynlova *et al.* demonstrated that mechanical distension of the uterus alone caused an increase in the detection of γ-actin mRNA (Shynlova *et al.*, 2005). This model was also used to study the expression of the gap junction protein connexin-43 (Cx-43). In this study uterine stretch, due either to the presence of pups or the insertion of an inert plastic tube to create stretch in the non-gravid horn, led to an increase in the expression of Cx-43 (Ou *et al.*, 1997). Thus, expression of some genes appears to be controlled by the effect of one pathway; however, in many cases, altered gene expression is due to the concerted effect of both hormonal and mechanical signals. Expression of the

oxytocin receptor (OTR) is controlled by the presence of progesterone as well as stretch of the uterus. Ou *et al.* showed that injecting rats with progesterone delayed labour and also blocked the increase in OTR mRNA expression that normally occurs at term, whereas removal of the effects of progesterone had the opposite effect on OTR mRNA expression. At labour, unilaterally pregnant rats showed an increase in OTR mRNA expression in both gravid horns and non-gravid stretched horns, but not in empty horns (Ou *et al.*, 1998). Previous research in our lab has shown that expression of HSP27, a member of the small heat shock protein family, is also affected by progesterone and mechanical distension of the myometrium, in a similar fashion to that of OTR expression (White & MacPhee, 2008).

HSP20, another member of the small heat shock protein family, is thought to play an important role in the cyclic-nucleotide dependent pathway of relaxation in smooth muscle cells. Upon phosphorylation by cAMP or cGMP-dependent protein kinase (PKA/PKG), HSP20 binds to the actin thin filament and prevents the formation of cross-bridges between actin and myosin, thus leading to relaxation of smooth muscle (Rembold *et al.*, 2000; Rembold *et al.*, 2001). I have previously shown that HSP20 expression peaks at day 15 of pregnancy, and decreases thereafter, reaching statistical significance at labour (Cross *et al.*, 2007). Thus, published findings and my experiments suggest that HSP20 may play an important role in maintaining myometrial quiescence during the early stages of pregnancy.

I have also previously utilized a delayed labour experimental regimen to analyse the effects of progesterone on HSP20 expression. Progesterone was able to maintain detection of HSP20 *in situ* at or near the cell membrane in the longitudinal muscle layer of the uterus

up to d24 (1 day after normal labour would occur); however, progesterone was unable to maintain detection in the circular muscle layer or affect overall expression of HSP20 in the myometrium as measured by immunoblot analysis, indicating that progesterone regulation of HSP20 expression, if any, may be quite complex (Cross *et al.*, 2007). In order to further delineate the role that progesterone may play in regulation of HSP20 expression, this study utilized a rat experimental model whereby pregnant rats were administered the progesterone receptor antagonist RU486 on day 18 of pregnancy, resulting in functional withdrawal of progesterone and delivery of fetuses on d19. Thus, these experiments will address the following question: will HSP20 expression decrease upon removal of the effects of progesterone?

During the same time frame that HSP20 expression decreases, the uterus also experiences considerable mechanical stress due to the growth of the developing fetus(es). As previously mentioned, mechanical signals can also regulate the expression of genes in the myometrium. It is therefore possible that mechanical force may negatively effect HSP20 expression. Therefore, the effect of uterine distension on HSP20 expression was also investigated using a unilaterally pregnant rat model.

3.3 Materials and Methods

3.3.1 Animals

Sprague Dawley rats were obtained from the Mount Scio Vivarium (Memorial University of Newfoundland, St John's, NL, Canada). Animals were held and cared for under standard environmental conditions (12h light, 12h darkness) 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, MO, USA) and water was available *ad libitum*. All experiments were approved by the institutional animal care committee under animal care protocols 02-02-DM to 06-02-DM. Virgin female rats (~220 g each) were mated with stud males, and observation of vaginal plugs the following morning was designated day 1 of pregnancy. The time of delivery under these standard conditions was day 23 of gestation.

3.3.2 Experimental Design

3.3.2.1 RU486-induced preterm labour

On Day 18 of gestation, two groups of rats were treated with either RU486 (10 mg/kg, s.c., in 0.5 ml corn oil containing 10% ethanol, Mifepristone; 17β-hydroxy-11β-[4-dimethylaminophenyl]-17-[1-propynyl]-estra-4,10-dien-3-one; Sigma-Aldrich, St. Louis, MO) or vehicle. Myometrial samples were collected from both groups of animals on day 20. RU486 treated rats were killed during active labour when one to three pups had been delivered (n = 4).

3.3.2.2 Unilaterally pregnant rats

Under general anaesthesia virgin female rats underwent tubal ligation through a flank incision to ensure that they subsequently became pregnant in only one horn. 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 19 (n = 3) and 23 (labour; n = 4). Labour samples were taken during active labour and only after the rat had delivered two to three pups .

3.3.3 Tissue Collection

For Northern blot and immunoblot analysis, uterine horns were removed, opened longitudinally and fetuses and placentae discarded. Uterine tissue was placed in ice cold phosphate-buffered saline (PBS; pH 7.4) and endometrial tissue was removed by gentle scraping with a scalpel blade. All myometrium samples were flash-frozen in liquid nitrogen and stored at −80 °C. For immunocytochemistry, rat uterine tissue samples were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at room temperature with shaking. Tissues were paraffin embedded, sectioned and mounted on microscope slides by the Histology Unit of Memorial University of Newfoundland School of Medicine. Sections of rat uterine tissue contained both longitudinal and circular smooth muscle layers and sections were processed under identical conditions and at the same time in each experiment.

3.3.4 Northern Blot Analysis

3.3.4.1 RNA isolation

RNA was isolated from tissues using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) exactly according to the manufacturer's instructions. RNA quality and quantity ($A_{260/280}$) were determined using a Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, ON, Canada) and samples were stored at –70°C.

3.3.4.2 Electrophoretic separation and capillary transfer of RNA

RNA samples (10 µg per lane) were loaded on a 1% agarose gel containing 0.66 M formaldehyde and 1 × MOPS buffer (0.02 M MOPS pH = 7.0, 2 mM sodium acetate, 10 mM EDTA pH = 8.0) and electrophoretically separated at 80V in 1 × 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 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate) in DEPC-treated ddH_2O. RNA was cross-linked to nylon membrane with a UVC-508 ultraviolet cross-linker (Ultra-Lum Inc., Paramount, CA, USA) and all blots were stored at −20°C until required.

3.3.4.3 Northern blot hybridization

Northern blot hybridization procedures have been described in detail elsewhere (White *et al.*, 2005; Williams *et al.*, 2005). Briefly, membranes were pre-hybridized in

hybridization buffer then hybridized overnight at 42°C in hybridization buffer containing a ^{32}P-labelled HSP20 cDNA probe. The 729 bp Bam HI/Xba I fragment of the rat HSP20 cDNA (Genbank Accession: NM_138887) was kindly provided, within the vector pRTL2, by Dr. Colleen Brophy and Dr. Charles Robb Flynn (Arizona State University, Tempe, AZ, USA). Radiolabelled cDNA probes were prepared with a Megaprime DNA Labelling Kit (Cat. No. RPN 1607; Amersham Biosciences, Little Chalfont, Buckinghamshire, England) according to manufacturers specifications. Following hybridization, blots were washed in 2 × SSC/0.1% SDS and exposed to multisensitive phosphor imaging screens (Cyclone Storage Phosphor Screen; Perkin Elmer, Boston, MA, USA). Multiple exposures were produced for each northern blot to ensure the results were within the linear range of the screens.

Following analysis of HSP20 gene expression, Northern blots were stripped by incubating membranes for 2 h in a solution of 1 M Tris-Cl, 1 mM EDTA, 0.1 × Denhardt's solution in ddH_2O. Membranes were washed at room temperature with 0.1 × SSPE and then subsequently analyzed for expression of 18S rRNA using the same procedures described above. A 750 bp EcoRI fragment of the rabbit 18S ribosomal cDNA (Genbank Accession: X06778) was generously provided, within the vector pBluescript, by Dr I Skerjanc (University of Western Ontario, London, Ontario, Canada; Petropoulos & Skerjanc, 2002; Rogerson *et al.* 2002). 18S rRNA is constitutively expressed in rat myometrial cells and has been utilized as a loading control for analysis of myometrial gene expression (Oldenhof *et al.*, 2002; Shynlova *et al.*, 2004; White *et al.*, 2005; Williams *et al.*, 2005).

3.3.5 Immunoblot Analysis

Total protein was extracted from the frozen tissues using RIPA lysis buffer as was described earlier in detail (Cross *et al.*, 2007; Williams *et al.*, 2005). Protein samples (100 μg/lane) were separated by polyacrylamide gel electrophoresis in 12% resolving gels according to the method described by Laemmli (1970). Gels were electroblotted to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, FL, USA).

Membranes were rinsed in Tris-buffered saline-Tween-20 (TBST; 20 mM Tris base, 137 mM NaCl, and 0.1% Tween-20; pH 7.6) and blocked in 5% milk powder/TBST for 45 min. Unless otherwise stated, all incubations were performed at room temperature and with constant agitation. Rabbit polyclonal antisera raised against HSP20 (Cat. No. 07-490; Upstate Biotechnology, Lake Placid, NY, USA) or mouse monoclonal antisera raised against smooth muscle calponin (Cat. No. C2687; clone hCP; Sigma-Aldrich Canada, Oakville, Ontario, Canada) used at dilutions of 1:2000 and 1:50 000, respectively, were incubated with blots for 1 h and then blots were rinsed with TBST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) or HRP-conjugated goat anti-mouse IgG (H + L) (Cat. No. 31460 and 31430, respectively; Pierce, Rockford, IL, USA) were used as secondary antisera at dilutions of 1:10 000 and 1:150 000, respectively. Proteins were detected using the Pierce SuperSignal West Pico chemiluminescent substrate detection system (MJS Biolynx, Inc., Brockville, Ontario, Canada) and multiple exposures were produced to ensure the linearity of the film exposures.

Following detection of HSP20, all blots were stripped with Restore Western blot

stripping solution (Pierce) according to the manufacturer's instructions. Analysis of calponin protein expression was subsequently performed. We have determined that calponin protein is constitutively expressed in non-pregnant and pregnant rat myometrial tissue under our protein extraction conditions (i.e. RIPA buffer extraction protocol) and, as a result, serves as a sufficient loading control for our analyses (White *et al.*, 2005; Williams *et al.*, 2005).

3.3.6 Immunocytochemistry

Two separate, independently collected sets of rat tissues (n = 2) were utilized for immunocytochemistry experiments and the experiments were repeated twice with each tissue set. Following de-waxing and rehydration, tissue sections were permeabilized with 0.125% trypsin in PBS for 10 min at room temperature and then rinsed with PBS. Unless otherwise noted, all incubations were performed at room temperature and with constant agitation. Tissue sections were blocked with 5% normal goat serum/1% horse serum in PBS for 30 min. Sections were then incubated overnight with constant agitation at 4°C in rabbit anti-HSP20 (Cat. No. H1830-48D; US Biological, Swampscott, MA, USA) at a dilution of 1:200 in blocking solution or rabbit IgG (Cat. No. 011-000-003; Jackson ImmunoResearch Labs, Inc., West Grove, PA, USA) at the same final concentration to serve as a negative control. Tissue sections were washed in PBS and then incubated in fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG (Cat. No. F7512; Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:200 in blocking solution for 30 min. For nuclear staining, TO-PRO-3 iodide (Cat. No. T3605; 1mM stock solution, Molecular Probes, Eugene, OR, USA) was added at

a dilution of 1:250 to the secondary antibody solution. Sections were washed with cold PBS containing 0.02% Tween-20, followed by mounting in Vectashield (Vector Laboratories Inc., Burlington, Ontario, Canada). Prepared slides were observed and images were collected with an Olympus Fluoview laser scanning confocal microscope (Olympus Optical Company Ltd, Melville, NY, USA). For each immunocytochemistry experiment (ie RU486-induced preterm labour, unilaterally pregnant rats) image collection parameters (PMT, Gain, and Black level settings) were the same within each muscle layer.

3.3.7 Data Analysis

Densitometric analysis of northern blots and immunoblots was performed with the aid of Scion Image software (Scion Image Corporation, Frederick, MD, USA). Densitometric measurements of HSP20 mRNA were normalized to those of 18S ribosomal RNA while measurements of HSP20 protein on immunoblots were normalized to those of calponin. Statistical analysis was performed with GraphPad Instat version 3.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and data graphed using GraphPad Prism version 4.0 (GraphPad Software).

3.4 Results

3.4.1 RU486-Induced Preterm Labour

3.4.1.1 Expression of HSP20 mRNA and Protein

Treatment of pregnant animals at day 18 of gestation with RU486 induced preterm labour within 24 hours. Northern blot analysis of HSP20 mRNA expression in myometrial tissue collected from vehicle and RU486-treated rats indicated that progesterone withdrawal did not significantly effect HSP20 mRNA expression (Fig. 3.1). Immunoblot analysis showed a similar result, as HSP20 protein expression in myometrial lysates did not change upon RU486 administration or in vehicle controls (Fig. 3.2).

3.4.1.2 Immunocytochemical Detection of HSP20

In both the circular and longitudinal muscle layers of the myometrium, there was no discernible difference in detection levels of HSP20 between vehicle and RU486-treated samples (Fig. 3.3). Detection levels and localization patterns were similar to those observed at this same timepoint in the normal pregnancy model, as shown previously (Cross *et al*, 2007), with HSP20 detected, at a low level, at or near the cell membranes.

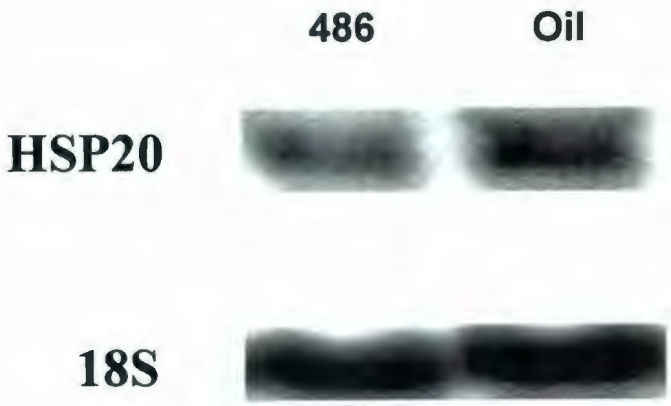
3.4.2 Unilaterally Pregnant Rat Model

3.4.2.1 Expression of HSP20 mRNA and Protein

To determine whether HSP20 mRNA expression was affected by mechanical stretch of the myometrium during late pregnancy, northern blot analysis was performed. Total RNA

Figure 3.1. Northern blot analysis of HSP20 mRNA detection in myometrium in a preterm labour model following administration of RU486 or corn oil (vehicle control) to pregnant rats. Treatment was administered on d18 and samples taken on d19. (A) Representative northern blots of HSP20 mRNA detection and 18S rRNA detection. Analysis was performed using an HSP20-specific rat cDNA and an 18S-specific rabbit cDNA as templates for radiolabelled probe production. Ten micrograms of total RNA were utilized per lane. (B) Densitometric analysis illustrating no significant change in the level of detection between treatment groups. Values are from four independent experiments (n = 4) ± SEM. T, RU486; O, vehicle control

A


486
Oil
HSP20
18S

B

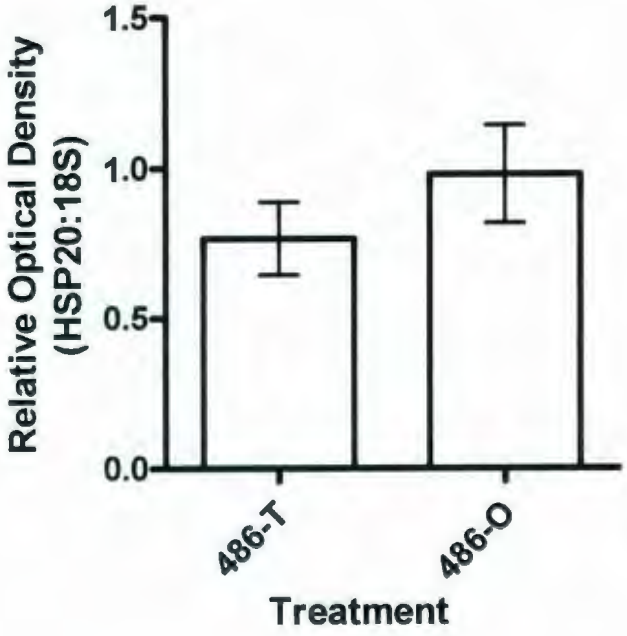

Relative Optical Density
(HSP20:18S)
1.5
1.0
0.5
0.0
486-T
486-O
Treatment

Figure 3.2. Immunoblot analysis of HSP20 protein detection in myometrium in a preterm labour model following administration of RU486 or corn oil (vehicle control) to pregnant rats. Treatment was administered on d18 and samples taken on d19. (A) Representative immunoblots of HSP20 and calponin detection. (B) Densitometric analysis illustrating no significant change in the level of detection between treatment groups. Values are from four independent experiments (n = 4) ± SEM. T, RU486; O, vehicle control

A


486
Oil
HSP20
Calp

B

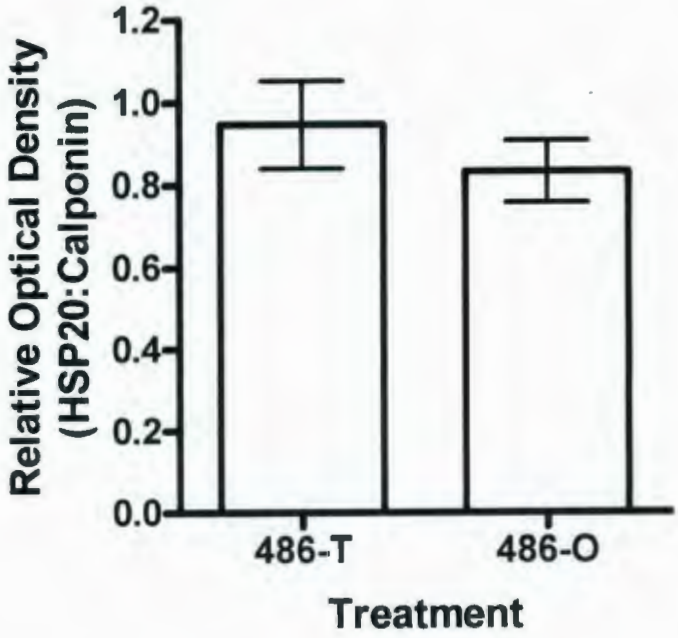
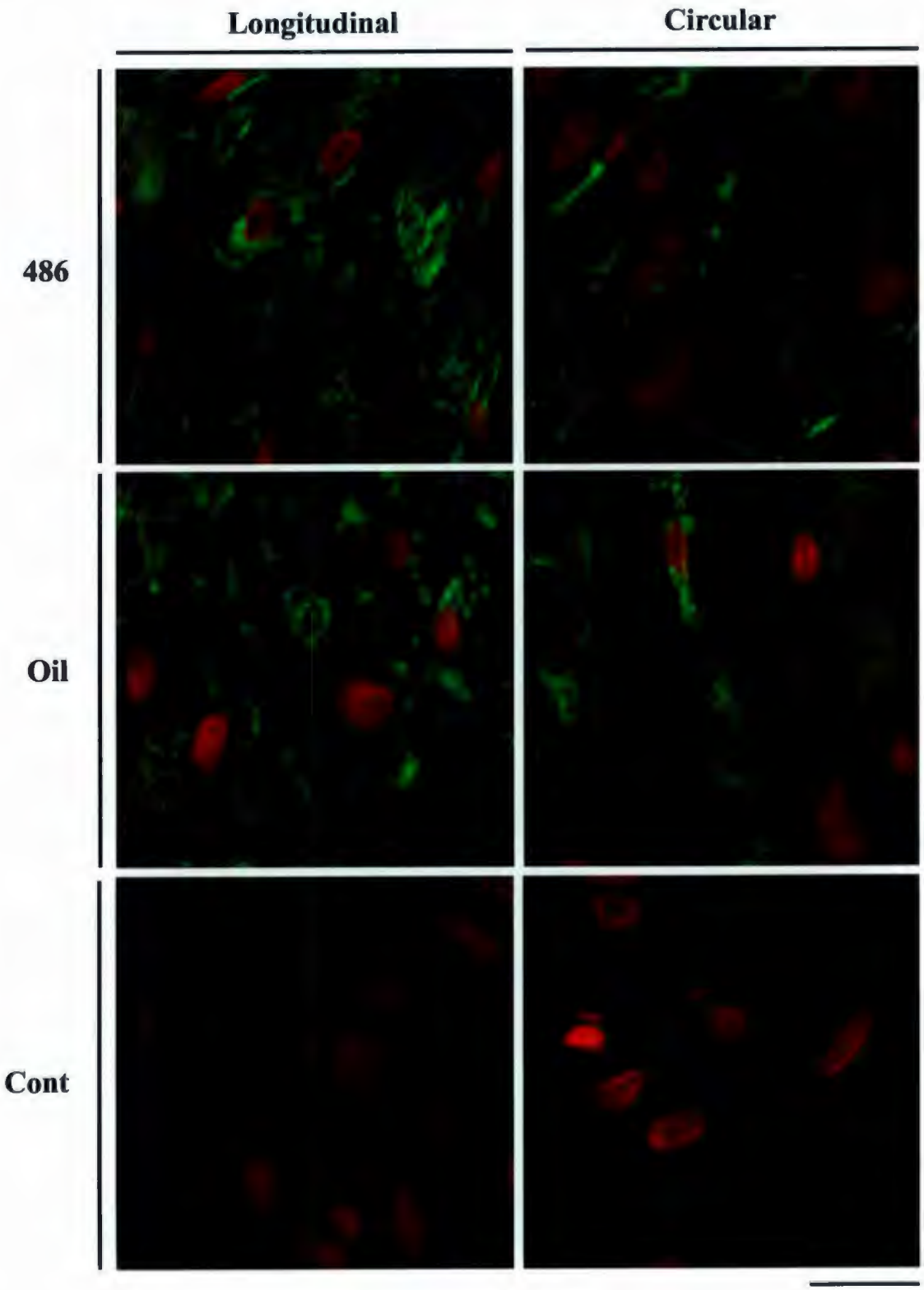

Relative Optical Density
(HSP20:Calponin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
486-T
486-O
Treatment

Figure 3.3. Immunocytochemical detection of HSP20 protein (green) in the longitudinal and circular smooth muscle layers of rat myometrium after treatment of pregnant rats with RU486 (486) or vehicle (oil). Treatment was administered on d18 and samples taken on d19. Cont = control, rabbit IgG. TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μm.


Longitudinal
Circular
486
Oil
Cont

was isolated from non-gravid and gravid horns of unilaterally pregnant rats. Expression of HSP20 mRNA appeared to be higher in the non-gravid horn at both d19 and d23 (labour). However, densitometric analysis revealed that HSP20 expression was only significantly greater in the non-gravid horn of d23 rats when compared to the gravid horn (unpaired t-test, $p < 0.10$; Fig. 3.4)

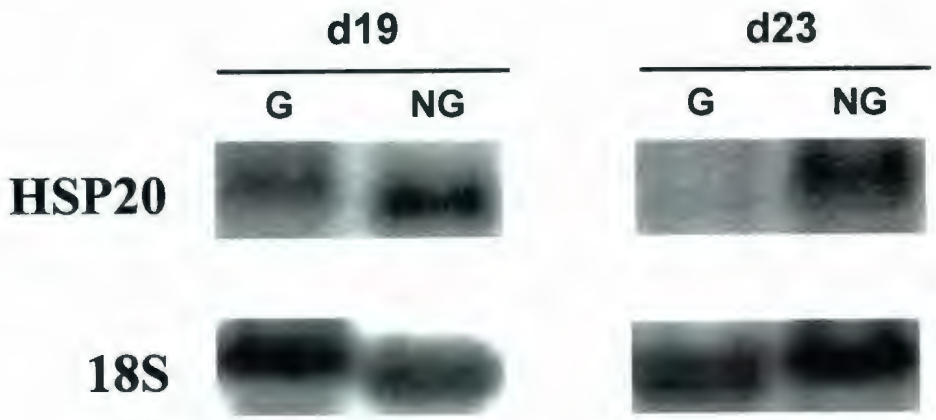
Immunoblot analysis showed that HSP20 protein expression mirrored HSP20 mRNA expression in the unilaterally pregnant rat myometrium. HSP20 protein expression was only significantly higher in the non-gravid horn at labour, when compared to the gravid horn ($p < 0.05$; Fig. 3.5).

3.4.2.2 Immunocytochemical Detection of HSP20

Detection of HSP20 *in situ* in the gravid and non-gravid horns reflected the observed expression of HSP20 protein shown through immunoblot analysis. In day 19 pregnant rat uterine samples, there was no observable difference in spatial localization of HSP20 and a small increase in detection levels in the non-gravid samples when compared to gravid uterine samples (Fig. 3.6). On the other hand, day 23 (labour) samples showed a much more noticeable increase in HSP20 detection within both myometrial layers of the non-gravid horn compared to gravid horns, as was observed with immunoblot analysis (Fig. 3.7).

Figure 3.4. Northern blot analysis of HSP20 mRNA detection in the myometrium of unilaterally pregnant rats during late gestation and labour. (A) Representative northern blots of HSP20 mRNA detection and 18S rRNA detection. (B) Densitometric analysis of HSP20 mRNA levels in non-gravid (NG) and gravid (G) horns illustrating a significant increase in HSP20 expression in the non-gravid horn at labour (* $p < 0.10$). Values for d19 and d23 are, respectively, from three ($n = 3$) and four ($n = 4$) independent experiments ± SEM.

A


d19
d23
G
NG
G
NG
HSP20
18S

B

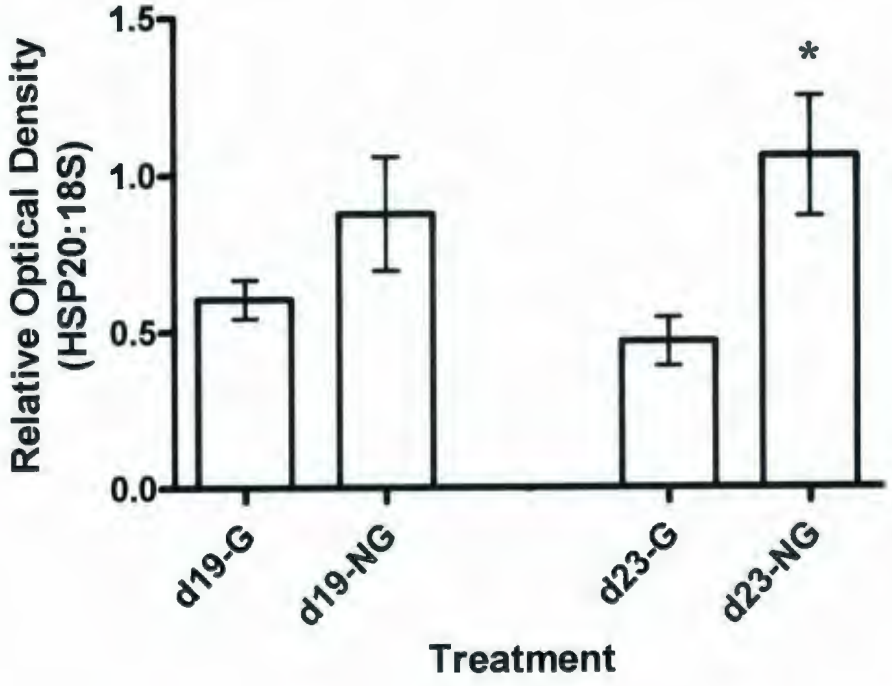
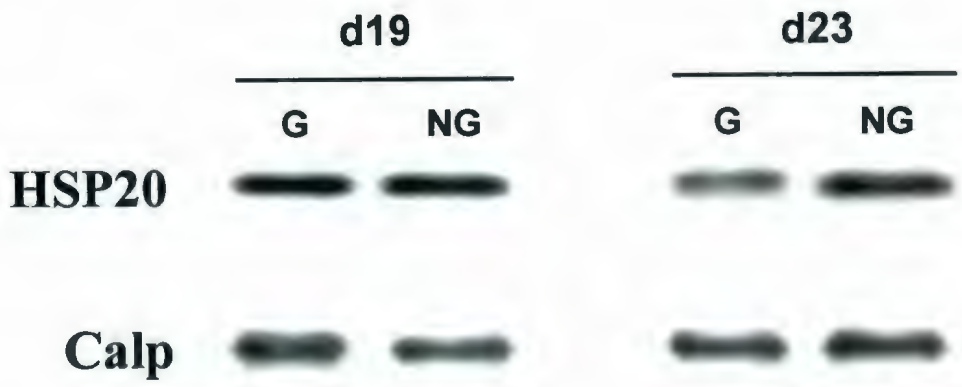

Relative Optical Density
(HSP20:18S)
1.5
1.0
0.5
0.0
*
d19-G
d19-NG
d23-G
d23-NG
Treatment

Figure 3.5. Immunoblot analysis of HSP20 protein detection in the myometrium of unilaterally pregnant rats during late gestation and labour. (A) Representative immunoblots of HSP20 and calponin detection. (B) Densitometric analysis of HSP20 protein levels in non-gravid (NG) and gravid (G) horns illustrating a significant increase in HSP20 expression in the non-gravid horn at labour (* $p < 0.05$). Values for d19 and d23 are from four ($n = 4$) independent experiments ± SEM.

A


d19
d23
G
NG
G
NG
HSP20
Calp

B

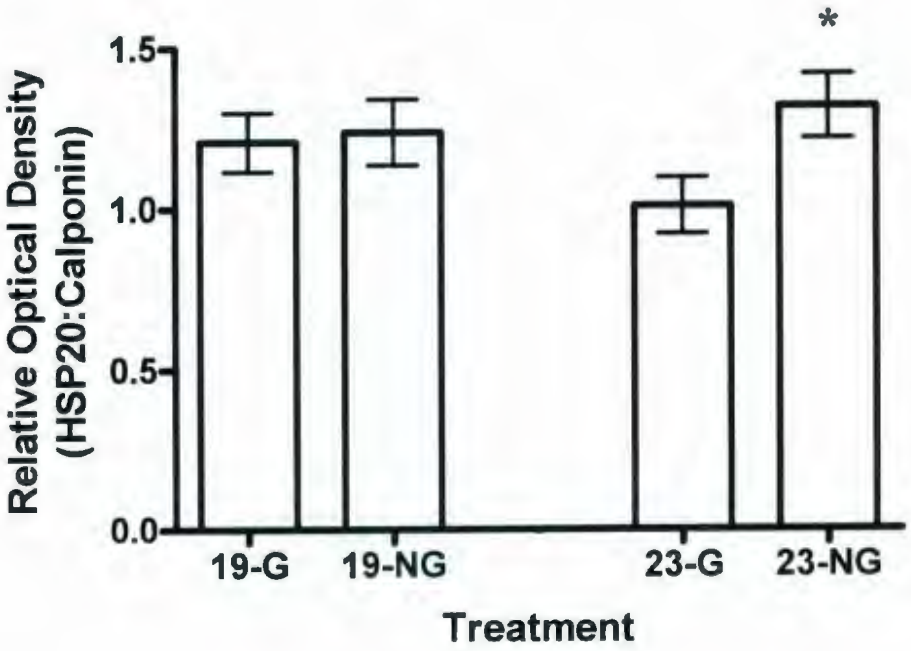

*
1.5
1.0
0.5
0.0
Relative Optical Density
(HSP20:Calponin)
19-G
19-NG
23-G
23-NG
Treatment

Figure 3.6. Immunocytochemical detection of HSP20 protein (green) in the longitudinal and circular smooth muscle layers of the gravid (G) and non-gravid (NG) horns of unilaterally pregnant rats at d19 of gestation. Cont = control, rabbit IgG. TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μm.

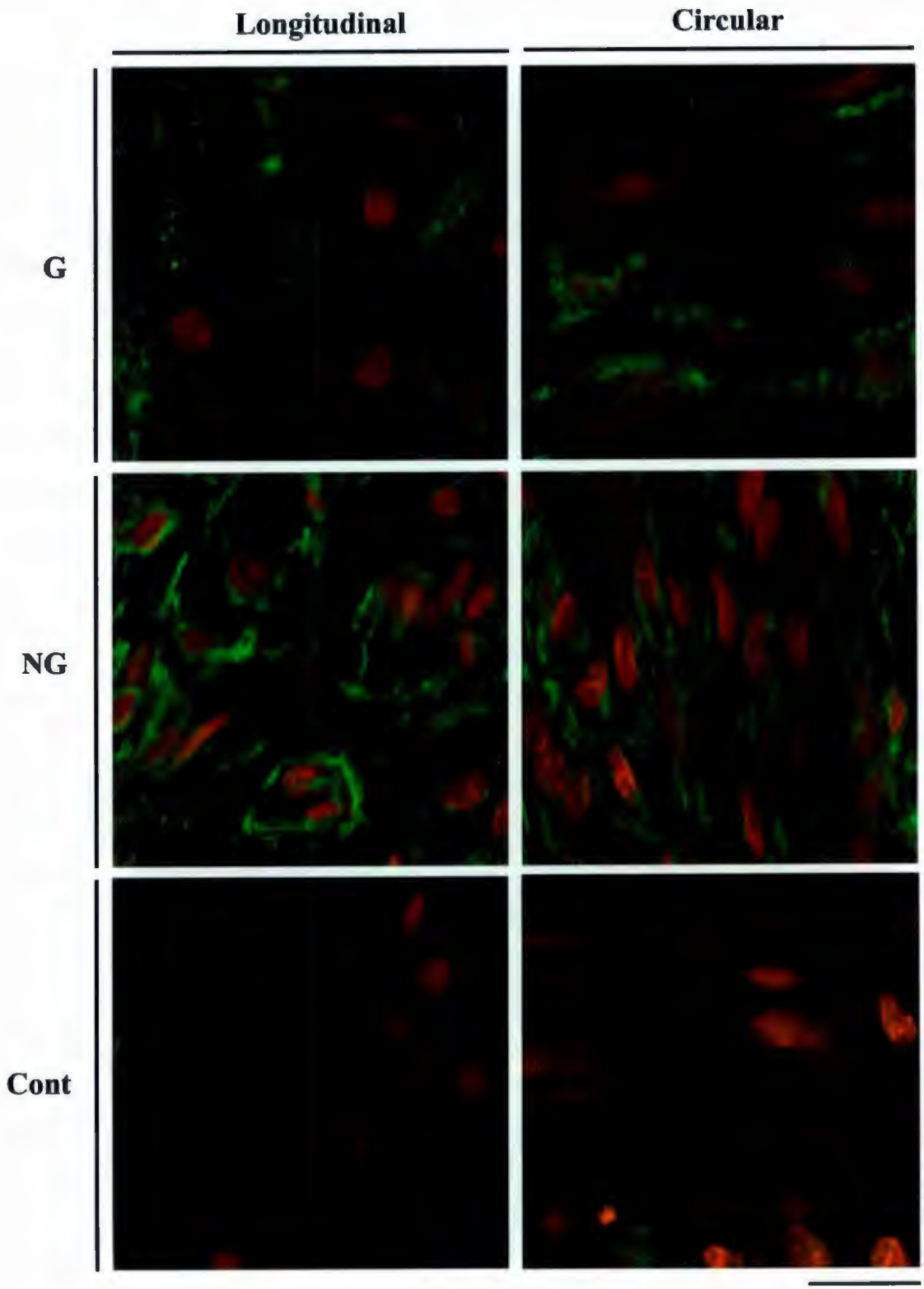
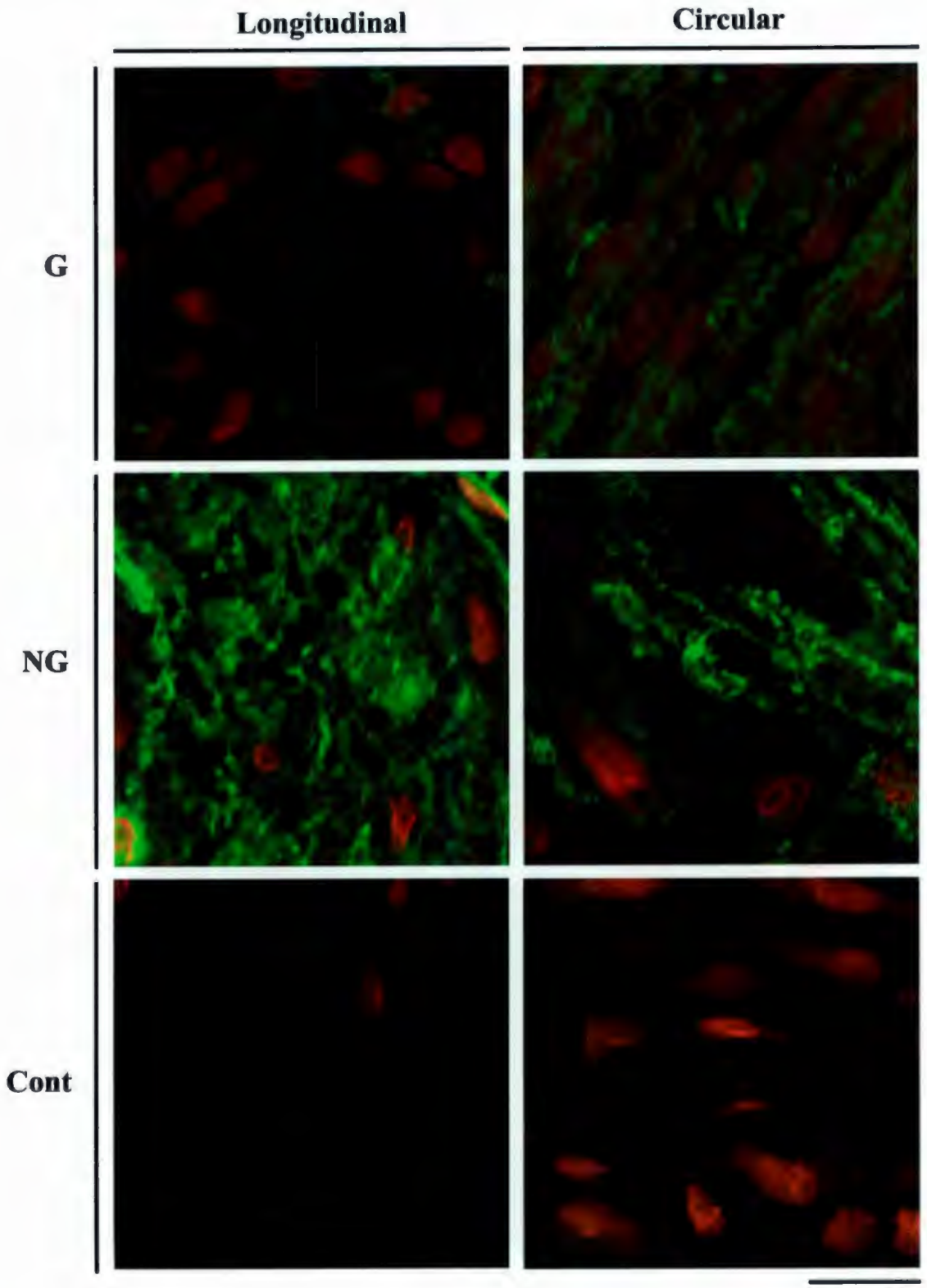

Longitudinal
Circular
G
NG
Cont

Figure 3.7. Immunocytochemical detection of HSP20 protein (green) in the longitudinal and circular smooth muscle layers of the gravid (G) and non-gravid (NG) horns of unilaterally pregnant rats at labour. Cont = control, rabbit IgG. TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μm.


Longitudinal
Circular
G
NG
Cont

3.5 Discussion

3.5.1 RU486-Induced Preterm Labour

Circulating levels of progesterone in rat maternal serum peak between day 15-19 and decline dramatically thereafter, until labour occurs (Lye *et al.*, 1993). I have previously shown, using a progesterone-delayed labour model, that administration of exogenous progesterone was able to maintain detection of HSP20 at or near myocyte membranes in the longitudinal smooth muscle layer, but not the circular muscle layer. This suggested that progesterone may play a role in HSP20 expression in rat myometrium (Cross *et al.*, 2007). Injection of pregnant rats with progesterone does not lead to a change in expression of another member of the heat shock protein family, HSP27, until d24 (1 day after normal labour) when expression significantly increases. Whether this change is due to the effects of progesterone or the increased stress at day 24 is unclear. Its expression decreases when the effects of progesterone are removed with administration of RU486 (White *et al*, in preparation).

In order to further determine the role progesterone plays in HSP20 expression in rat myometrium, I used an inhibitor of progesterone, RU486, to functionally withdraw the effects of progesterone on HSP20 expression beginning on d18 of gestation. This treatment also induces preterm labour at day 19. We found that inducing preterm labour did not lead to a significant decrease in the levels of HSP20 mRNA or protein, nor did it cause a change in the spatial localization of this protein within myometrial cells.

These findings suggest that progesterone withdrawal at day 19 of pregnancy does not

have a significant effect on the expression of relaxation-associated HSP20. However, it is possible that antagonizing the functional effects of progesterone at this stage in pregnancy may be too late to have an effect on HSP20 expression. My previous study has shown that, by day 19, HSP20 gene and protein expression has decreased markedly and may be beyond the point that a further decrease in progesterone would have any noticeable effect on its expression (Cross *et al.*, 2007).

It is also possible that the myometrial layer-specific down-regulation of HSP20 expression initially observed following administration of progesterone (Cross *et al.*, 2007) may be solely due to the effect of increased mechanical distension as fetal growth continues during progesterone-delayed labour. The circular muscle layer is the closest muscle layer to the endometrium and uterine lumen, and thus would be the first layer of myometrium to be exposed to the stretch caused by the growing fetus(es).

3.5.2 Unilateral Pregnancy

To examine the possible role of mechanical stretch on HSP20 expression in the myometrium, I utilized a unilaterally pregnant rat model during late pregnancy (d19) and labour. My results showed that gravidity did not significantly affect expression of HSP20 at d19 of pregnancy, but did lead to a significant decrease in HSP20 expression at labour. Rapid growth of the fetus occurs during late gestation, and this rapid growth leads to a second period of mechanical stretch, which ends at parturition (Shynlova *et al.*, 2006). The lack of an apparent effect at d19 suggests that a temporal relationship may exist whereby HSP20 expression is only robustly sensitive to mechanical distention at labour, when the effects of

stretch are maximal. Further evidence supporting this was observed with immunofluorescence analysis of *in situ* HSP20 expression at d19, where HSP20 detection did show a slight decrease in gravid versus non-gravid horns, in contrast to northern blot and immunoblot analysis. These results suggest that mechanical distention does decrease expression of HSP20, but this is not a finely controlled process. In total, however, we cannot exclude the possibility that the observed negative effect on HSP20 expression is solely dependant on distension. It is possible that the presence of feto-placental units in the gravid horn might contribute endocrinological signals, local or systemic, that could contribute to the observed effects.

Our lab has also shown that expression of the contraction-associated HSP27 is increased in the gravid versus non-gravid horn, which is in direct contrast to the expression of HSP20 (White & MacPhee, 2008); however, this result is not surprising, as HSP20 is associated with the relaxation of smooth muscle in other organs (Beall *et al.*, 1999; Brophy *et al.*, 1999; Bukach *et al.*, 2004) and HSP27 is associated with smooth muscle contraction (Bitar, 2002; Somara & Bitar, 2004). These findings together suggest a potential role for mechanical stretch in the regulation of HSP20 expression.

Chapter Four

Summary

The underlying biochemistry of parturition is not well understood. Studies such as this thesis can be used to help determine the roles that various proteins play in the regulation of uterine physiology in general and its contractile state specifically. HSP20, is known to play an important role in the relaxation of smooth muscle (Beall *et al.*, 1999; Bukach *et al.*, 2004), yet before this study was undertaken HSP20 expression and regulation in uterine smooth muscle were completely unknown.

My first set of experiments were performed to obtain a gestational profile for the expression of HSP20 mRNA and protein in rat myometrium throughout normal pregnancy and labour. It was found that HSP20 was highly expressed during the early stages of pregnancy, peaking at day 15, and that its expression significantly decreased towards labour, leading to the early suggestion that HSP20 played a role in facilitating myometrial quiescence during the early stages of pregnancy. The spatial localization of HSP20 within the layers of the myometrium at or near cell membranes coincided with its demonstrated association with the actin-binding protein α-actinin, which is a known component of focal adhesions in smooth muscle cells (Tessier *et al.*, 2003). This pattern of localization was similar to that of Ser15-phosphorylated HSP27, which was also found to be localized at or near myocyte membranes (White *et al.*, 2005) and is consistent with a report which demonstrated that HSP20 and HSP27 may exist in aggregates in smooth muscle cells (Brophy *et al.*, 1999). These findings suggest a possible interdependence in the roles of

HSP20 and HSP27 and their effects on the function of the myometrium.

As HSP20 expression decreased significantly at labour, these findings also suggested that HSP20 expression may be dependent on the circulating levels of progesterone within the rat; therefore, I conducted a series of experiments to determine whether progesterone plays a role in HSP20 expression. I found that total HSP20 mRNA and protein expression did not change when labour was delayed using progesterone, but that its detection *in situ* in the longitudinal myometrial layer was slightly increased upon progesterone administration and markedly decreased in the circular muscle layer.

To further examine the role of progesterone in HSP20 expression, I also utilized a RU486-induced preterm labour model to inhibit the functional effects of progesterone at day 19 and found that this did not appear to have any effect on HSP20 expression. It is, however, possible that antagonizing progesterone at day 19 of pregnancy may be too late to have an effect on HSP20 expression since, at this point, HSP20 gene and protein expression have decreased markedly and may be beyond the point that a further decrease in progesterone would have any noticeable effect on its expression. Therefore, future experiments could be conducted whereby functional withdrawal of progesterone is induced at day 15, the peak of HSP20 expression, to see if progesterone affects the expression of HSP20 earlier in gestation.

My next set of experiments examined the effects of mechanical distension (stretch) on the expression of HSP20 during late gestation. I found that removing the effects of the growing fetuses in one horn of the rat uterus led to a significant increase in HSP20 expression at labour in the non-gravid horn compared to the gravid horn. These findings

suggest that the trigger point for a significant decrease in HSP20 expression due to stretch is at or near labour, when uterine stretch is maximal. Future experiments may be conducted to determine the effects of stretch on HSP20 earlier in gestation; specifically at day 15 when HSP20 expression reaches its peak. Furthermore, it would be important to conduct experiments where laminaria tents are placed in non-gravid horns for 24 hours during pregnancy. These tents expand producing a dynamic stretch and would allow analysis of whether or not the dynamic stretch in the non-gravid horn could recapitulate the effects observed in the gravid horn.

This is the first study to show that HSP20 is highly expressed in rat myometrium during early and mid-pregnancy. Due to the pattern of expression of HSP20, it may have a role in maintaining uterine quiescence - a role that has been attributed to HSP20 in other smooth muscle-containing organs. This study has also demonstrated a potentially novel role for mechanical stretch in negatively regulating HSP20 expression.

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