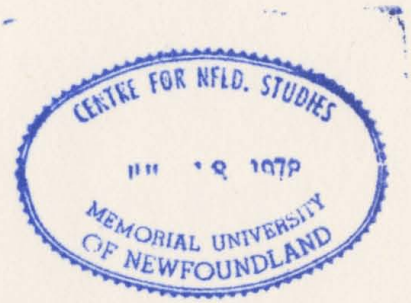
THE EXPRESSION AND REGULATION OF HSPB1 (Hsp27) IN THE RAT MYOMETRIUM DURING PREGNANCY AND LABOUR

BRYAN GLOVER WHITE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The Expression and Regulation of HSPB1 (Hsp27) in the Rat Myometrium During Pregnancy and Labour

By

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Abstract

One avenue available to combat the significant problem of pre-term labour is through a better understanding of regulation of uterine smooth muscle function and contraction. Although there is minimal research into the role of HSPB1 (Hsp27) in uterine smooth muscle, it has been proposed to play an important role in cytoskeletal arrangement and contraction in colonic smooth muscle. It was hypothesized that HspB1 would be highly expressed in the rat myometrium during pregnancy and regulated by the endocrinological environment as well as uterine stretch. Utilizing northern blot, immunoblot, and immunofluorescence analysis, the expression and regulation of HSPB1 mRNA and HSPB1 protein in rat uterine smooth muscle (myometrium) was assessed. Through gestation, HSPB1 mRNA increased to a maximum at day 19 and decreased afterwards, while total HSPB1 and HSPB1 phosphorylated on serine-15 (pSer15 HSPB1) increased from d19 to a maximum at labour, falling thereafter. Labour also signalled a switch from peri-nuclear /membrane-associated HSPB1 localization in myometrial cells earlier in pregnancy to cytoplasmic localization *in situ*. To analyze regulation of HSPB1 by ovarian steroids, experimental models including administration of progesterone or progesterone receptor antagonist (RU486) to pregnant rats were utilized as well as 17β-estradiol administration to non-pregnant rats. Progesterone increased HSPB1 expression, prevented the late-pregnancy increase in HSPB1, and forestalled the normal labour-associated localization shift *in situ*, while RU486 had the opposite effect. 17β-estradiol stimulated a very rapid (<1hr) increase in pSer15 HSPB1 expression, with an increase in

total HSPB1 after 12-24hrs. To analyse the role of myometrial stretch on HSPB1 expression, unilaterally pregnant and non-pregnant ovariectomized rat models were utilized. Regardless of the endocrine environment, myometrial stretch facilitated an increase in both HSPB1 and pSer15 HSPB1 expression. However, total HSPB1 only increased when the hormone environment was intact, which also directed its sub-cellular localization *in situ*. In total these data validate our hypothesis and specifically show that hormonal and stretch-facilitated signalling pathways, perhaps in concert, regulate HSPB1 gene expression. These data also support an ongoing hypothesis in the lab that prior to labour HSPB1 may facilitate proper structural organization of the actin cytoskeleton and incorporation into focal adhesions on the cell membrane while at labour HSPB1 may facilitate association of myosin with actin in the cytoplasm, to allow for the high amplitude contractions necessary for parturition.

Acknowledgements

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List of Abbreviations

Δ - *HSPB1* construct with the WDPF domain removed.

14-3-3 protein - Family of conserved eukaryote reglatory molecules.

18S - 18S component of the small eukaryotic ribosomal subunit.

24mer - Oligomer of 24 individual units.

3β-HSD - 3β-hydroxysteroid Dehydrogenase.

17β-HSD - 17β-hydroxysteroid Dehydrogenase.

^{32}P - Radioactive isotope of phosphorus.

^{32}P-dCTP - ^{32}P-radiolabelled Cytidine Triphosphate.

3T3 - Fibroblast cell line.

AA - Constitutively non-phosphorylated *HSPB1*

ACTH - Adrenocorticotropin Hormone.

ADP - Adenosine Diphosphate.

Akt - Protein Kinase B.

ANOVA - Analysis of Variance.

AP-1 - Activator Protein 1 (AP-1) transcription factor.

Ask1 - Apoptosis Signal-regulating Kinase 1.

ATP - Adenosine Triphosphate.

ATPase - Enzyme that catalyzes ATP hydrolysis to produce a free phosphate ion.

Bad - Bcl-2-associated death promoter.

BCL - B cell lymphoma gene.

Bcl-2 - B cell lymphoma 2.

BrdU - Bromodeoxyuridine.

Ca^{2+} - Calcium ion.

$[Ca^{2+}]_I$ - Intracellular concentrations of free calcium ion.

CaM - Calmodulin.

CaMK - Ca^{2+}/calmodulin-dependent kinase.

cAMP - Cyclic Adenosine Monophosphate.

CAP - Contraction Associated Protein.

cDNA - Cyclic DNA.

cGMP - Cyclic Guanosine Monophosphate.

CHiP - Chromatin Immunoprecipitation.

ClpA - Regulatory component of HSPH.

ClpB - Caseinolytic peptidase B protein homologue.

ClpP - Protease component of HSPH.

ClpX - Regulatory component of HSPH.

Con - Pre-immune serum control.

COX-1 - cyclooxygenase-1.

COX-2 - cyclooxygenase-2.

CRE - cAMP Responsive Element.

CREB - cAMP Responsive Element Binding protein.

CRH - Corticotropin Releasing Hormone.

CT - Polyclonal HSPB1 antisera raised against its c-terminus.

cv - Cardiovascular Hsp (HSPB7).

cx-26 - 26kDa Connexin.

cx-43 - 43kDa Connexin.

d1-d23 - Day 1-Day 23 of parturition.

DAG - Diacylglycerol.

DAXX - Death-associated protein 6.

ddH_2O - Double distilled H_2O.

DEPC - Diethylpyrocarbonate.

DHEA - Dehydroepiandrosterone.

DHEA-S - Dehydroepiandrosterone-sulfate.

DHFR - Dihydrofolate Reductase.

DNA - Deoxyribonucleic Acid.

DNAJ - 40kDa heat shock protein.

DP - Progesterone receptor type DP.

E - Empty vector.

E_1 - Estrone.

E_2 - Estradiol.

E_3 - Estriol.

ECM - Extra-cellular Matrix.

EDTA - Ethylenediaminetetraacetic acid.

EE - Constitutively phosphorylated *HSPB1*

ELISA - Enzyme-linked immunosorbent assay.

EP - Progesterone receptor type EP.

ER - Estrogen Receptor.

ERα - Estrogen Receptor Alpha.

ERβ - Estrogen Receptor Beta.

ERE - Estrogen Responsive Element.

ERK - Extracellular Signal-Regulated Kinase.

ERK2 - Extracellular Signal-Regulated Kinase 2.

EtOH - Ethanol.

F-actin - Filamentous actin.

FAK - Focal Adhesion Kinase.

FITC - Fluorescein isothiocyanate.

FP - Progesterone receptor type FP.

G-actin - Globular actin.

$G_{\alpha q/11}$ - G-Protein coupled receptor αq/11.

GST - Glutathione S-transferase.

HeLa - An immortalized cervical cancer cell line isolated from patient Henrietta Lacks.

Hic-5 - Transforming growth factor beta-1-induced transcript 1 protein (Paxillin Homologue).

HPA - Hypothalamus-Pituitary Axis.

HPLC - High-performance Liquid Chromatography.

HRP - Horseradish Peroxidase.

HSE - Heat Shock Element.

HSF - Heat Shock Factor .

HSF1-4 - Heat Shock Factor type 1-4.

HSP - Heat Shock Protein.

HSP16.3 - 16.3kDa Bacterial Heat Shock Potein.

HSP16.5 - 16.5kDa Bacterial Heat Shock Potein.

HSP16.9 - 16.9kDa Heat Shock Protein of *M. jannaschii.*

HSP26 - 26kDa Heat Shock Protein of *Saccharomyces cerevisiae.*

HSP27 - 27kDa Heat Shock Protein (HSPB1).

HSP70 - 70kDa Heat Shock Protein (HSPA).

HSP90 - 90kDa Heat Shock Protein (HSPC).

HSPA - Heat Shock Protein A (70kDa Heat Shock Protein).

HSPB1 - Heat Shock Protein B1 (27kDa small Heat Shock Protein).

HSPB2 - Heat Shock Protein B2 (Myotonic Dystrophy Protein Kinase-Binding Protein).

HSPB3 - Heat Shock Protein B3 (27kDa-like small Heat Shock Protein).

HSPB4 - Heat Shock Protein B4 (αA-Crystallin).

HSPB5 - Heat Shock Protein B5 (αB-Crystallin).

HSPB6 - Heat Shock Protein B6 (20kDa small Heat Shock Protein).

HSPB7 - Heat Shock Protein B7 (Cardiovascular Heat Shock Protein).

HSPB8 - Heat Shock Protein B8 (22kDa small Heat Shock Protein).

HSPB9 - Heat Shock Protein B9 (Testis Specific Heat Shock Protein).

HSPB10 - Heat Shock Protein B10 (Sperm Outer Dense Fiber Protein).

HSPB11 - Heat Shock Protein B11 (16.2kDa small Heat Shock Protein).

HSPC - Heat Shock Protein C (90kDa Heat Shock Protein).

HSPD - Heat Shock Protein D (60kDa Heat Shock Protein).

HSPE - Heat Shock Protein E (Chaperonin 10).

HSPH - Heat Shock Protein H (100kDa Heat Shock Protein).

hTERT-HM - A human myometrial cell line.

I-1 - Phosphorylate Inhibitor-1.

IgG - Immunoglobulin G.

IP - Progesterone receptor type IP.

IP_3 - Inositol Triphosphate.

IUD - Intra-uterine Device.

L-type - Long-lasting Type.

M11 - A human myometrial cell line.

MAPK - Mitogen Activated Protein Kinase.

MAPKAP K-2 - Mitogen Activated Protein Kinase Activating Protein K-2.

MAPKAP K-3 - Mitogen Activated Protein Kinase Activating Protein K-3.

MAPKAP K-5 - Mitogen Activated Protein Kinase Activating Protein K-5.

MCF-7 - A breast cancer cell line.

Mg^{2+} - Magnesium ion.

MgATP - ATP with magnesium bound to activate it.

MK2 - MAPK-activated Protein Kinase-2.

MKBP - Myotonic Dystrophy Protein Kinase Binding Protein (HSPB2).

MLC - Myosin Light Chain.

MLC_{17} - Myosin 17kDa Essential Light Chain.

MLC_{20} - Myosin 20kDa Regulatory Light Chain.

MLCK - Myosin Light Chain Kinase.

MLCP - Myosin Light Chain Phosphatase.

MOI - Multiplicity Of Infection.

MOPS - 3-(N-morpholino)propanesulfonic acid.

mPR - Membrane Progesterone Receptor.

mRNA - Messenger Ribonucleic Acid.

MYPT - 130kDa regulatory myosin-binding subunit of MLCP.

Na_2VO_3 - Sodium orthovanadate.

NAC - Nascent Chain-associated Complex.

NaCl - Sodium chloride.

NAD^+ - Nicotinamide Adenine Dinucleotide.

NP - Non-Pregnant.

nPR - Nuclear Progesterone Receptor.

NSERC - National Science and Engineering Research Council.

OM-1 - Ovine myometrium cell line.

OTR - Oxytocin Receptor.

OVX - Ovariectomized rat model.

P-HSPB1 - Phosphorylated HSPB1.

p38 - p38 Mitogen activated protein kinase.

p38 MAPK - p38 Mitogen Activated Protein Kinase.

P_4 - Progesterone.

p450arom - Aromatase.

$p450_{C17}$ - Steroid 17-alpha-monooxygenase.

p450scc - Cytochrome P450 family 11 subfamily A polypeptide 1.

$p70^{RSK}$ - Ribosomal protein S6 kinase II.

PBS - Phosphate Buffered Saline.

PC12 - Neuronal tumour cell line.

PCNA - Proliferating Cell Nuclear Antigen.

PFD - Prefoldin.

PG - Prostaglandins.

PGD_2 - Prostaglandin D_2.

PGDH - Prostaglandin Dehydrogenase.

PGDS - PGD_2 Synthase,

PGE_2 - Prostaglandin E_2.

PGES - PGE_2 Synthase.

$PGF_{2\alpha}$ - Prostaglandin $F_{2\alpha}$.

PGH_2 - Prostaglandin H_2.

PGHS - PGH_2 Synthase.

PGHS-2 - PGH_2 Synthase 2.

PGI_2 - Prostaglandin I_2.

pH - A measure of the acidity or basicity of a solution.

PHM1 - A Primary Human Myometrial cell line.

Pi - Inorganic phosphate.

PIP_2 - Phosphatidylinositol 4,5-bisphosphate.

PIP_3 - Phosphatidylinositol 3,4,5-triphosphate.

PKA - cAMP-dependent Protein Kinase.

PKB - Protein Kinase B.

PKC - Protein Kinase C.

PKCα - Alpha isoform of Protein Kinase C.

PKG - cGMP-dependent Protein Kinase.

PLA_2 - Phospholipase A_2.

PLB - Phospholamban.

PLC - Phospholipase C.

PP - 1 day Post Parturition.

PPs - Protein Phosphatases.

PP1 - Protein Phosphatase 1.

PP2A - Protein Phosphatase 2A.

PP2B - Protein Phosphatase 2B.

PR - Progesterone Receptor.

PR-A - Progesterone Receptor A.

PR-B - Progesterone Receptor B.

PR-C - Progesterone Receptor C.

Pregnenolone-S - Pregnenolone Sulfate.

PTHrP - Parathyroid Hormone Related Peptide.

RepA - A DNA replication initiator protein.

RhoA - Ras homolog gene family, member A.

RIPA - Radioimmunoprecipitation assay buffer (Protein extraction buffer).

RNA - Ribonucleic Acid.

ROCK-II - Rho-associated protein kinase II.

rRNA - Ribosomal RNA.

RU486 - A progesterone receptor antagonist known as Mifepristone.

S15 - Polyclonal antisera raised against serine-15 phosphorylated HSPB1.

pSer HSPB1 - HSPB1 phosphorylated on serine 15.

SDS - Sodium dodecyl sulfate.

Ser - Serine.

Ser-15 - Serine residue located at amino acid 15 of HSPB1.

Ser-78 - Serine residue located at amino acid 78 of HSPB1.

Ser-82 - Serine residue located at amino acid 82 of HSPB1.

Ser-86 - Serine residue located at amino acid 86 of HSPB1.

SHM - Syrian hamster uterine smooth muscle cell line.

sHSP - Small Heat Shock Protein.

siRNA - Small Interfering Ribonucleic Acid.

SP1 - Specificity Protein 1human transcription factor.

SR - Sarcoplasmic Reticulum.

Src - Proto-oncogene tyrosine-protein kinase.

SRQL - Polyclonal HSPB1 antisera raised against amino acids 73-83 of the human HSPB1 sequence including amino acids 78-81 comprised of serine(S)-arginine(R)-glutamine(Q)-leucine(L).

SSC - Saline Sodium Citrate.

SSPE - Saline Sodium Phosphate EDTA.

T-HSPB1 - Total HSPB1.

T-type - Transient Type.

TATA - A cis-regulatory element found in the promoter region of eukaryotic genes.

Thr - Threonine.

TP - Progesterone receptor type TP.

Tris-CL - Tris(hydroxymethyl)amino methane.

WDPF - A structural motif of small heat shock proteins comprised of Tryptophan (W), Aspartic Acid (D), Proline (P), and Phenylalanine (F).

WT *HSPB1* - Wild-type *HSPB1*.

WT - Wild-type.

Chapter 1

Literature Review

1.1 Pregnancy

Pregnancy and parturition (the act of giving birth) are processes that are fundamental to the continuation of human life. They are highly regulated sequences of events that promote the growth of a foetus to the point where it is ready to be expelled from the uterine environment and able to survive on its own. Regulatory inputs arise from the mother, foetus, and the placenta. With the proper signals, a foetus will be ready for survival when born, but if pregnancy is disturbed for some reason, the foetus may be born prematurely.

Pre-term birth is described as a human birth that occurs prior to 37 weeks gestation as a result of an underlying asynchrony between fetal maturation and the labour process (Challis *et al.*, 2000, 2002). This remains a significant problem in many countries. For example, approximately 5-10% of all pregnancies in North America happen pre-term (Challis, 2001; Lye & Olson, 1996). Canadian statistics from 1996 showed that approximately 7.1% of all pregnancies occurred pre-term (Public Health Agency of Canada, 1999). Although this number is relatively stable, a continual and gradual increase has been observed over the past 30 years. For example, 6.3% of live births in 1981 occurred pre-term, with this number rising to 6.8% by 1991, and subsequently 7.1% by 1996 (Joseph *et al.*, 1998; Public Health Agency of Canada, 1999). To further emphasize this health problem, preterm birth accounted for 75-85% of

neonatal deaths, making it the leading cause of neonatal morbidity and mortality, as babies born premature have 40% higher mortality than those born at term (Challis, 2001; Gibb & Challis, 2002; Lye & Olson, 1996; Lyndon, 2006; Public Health Agency of Canada, 1999). If we then consider the infants born premature that do survive, these individuals have a higher risk of having neurological and pulmonary disorders, including cerebral palsy, blindness and deafness (Lye *et al.*, 2001). These individuals also have an increased risk for problems later in life such as learning disabilities and behavioural problems (Marlow *et al.*, 2007; Sullivan & McGrath, 2003).

There exist many causes of pre-term birth, with approximately 30% due to an underlying infection (Challis *et al.*, 2002; Gibb & Challis, 2002), and 50% due to unknown causes (idiopathic). Interestingly, in cases of pregnancy problems such as preeclampsia, preterm birth often has to be surgically initiated. Other risk factors for pre-term birth include stress, smoking, drug usage, low body weight, and even previous repeated abortions (Goffinet, 2005; Reedy, 2007). Much of the research that has been undertaken to combat pre-term labour has targeted on drug development to inhibit myometrial contractility. Even though greater understanding of uterine contraction has been achieved, this has not translated into a decreasing of pre-term birth occurrence (Challis *et al.*, 2002; Lye *et al.*, 2001). In order for a better control of, and increased prevention of, preterm labour to occur, future research must focus on a greater understanding of the underlying mechanisms of uterine contraction (Slattery & Morrison, 2002). This research could lead to a better recognition of, and therapies for, premature

delivery.

1.2 Uterine Structure (Myometrium)

In many ways, the uterus during pregnancy is unlike any other organ. Although it is only used for a brief period of time to house the foetus, it is required to function properly to facilitate continuation of the species. It must quickly change its responsiveness to a wide variety of biochemical signals, that originate from various sources. This transition can range from hours to months, with the signals coming from both the local environment and the overall systemic endocrine environment.

The extent to which the uterus is able to adapt and change, is evident in the physical characteristics of this organ. The human pre-pregnancy uterus is around 40-70g in weight with an internal volume of 10mL, whereas the human uterus at labour averages 1100-1200g and can hold upwards of 5L (Monga & Sanborn, 2003). In addition, the size of human myometrial cells themselves have been found to increase up to 10 times in length and 3 times in width during pregnancy (Monga & Sanborn, 2003). These changes describe not only an alteration in the overall size due to fetal growth-induced uterine distention, but also an increase in mass due to growth of the uterus. As described later (See section 1.5: Phases of Myometrial Activation), these changes in mass and size correspond to distinct uterine phases of pregnancy (Shynlova *et al.*, 2009). As stated, regulation of these changes/phases is due to a multitude of sources, however the main influences are the circulating hormonal milieu and uterine mechanical stretch, and will

comprise the main focus of this thesis.

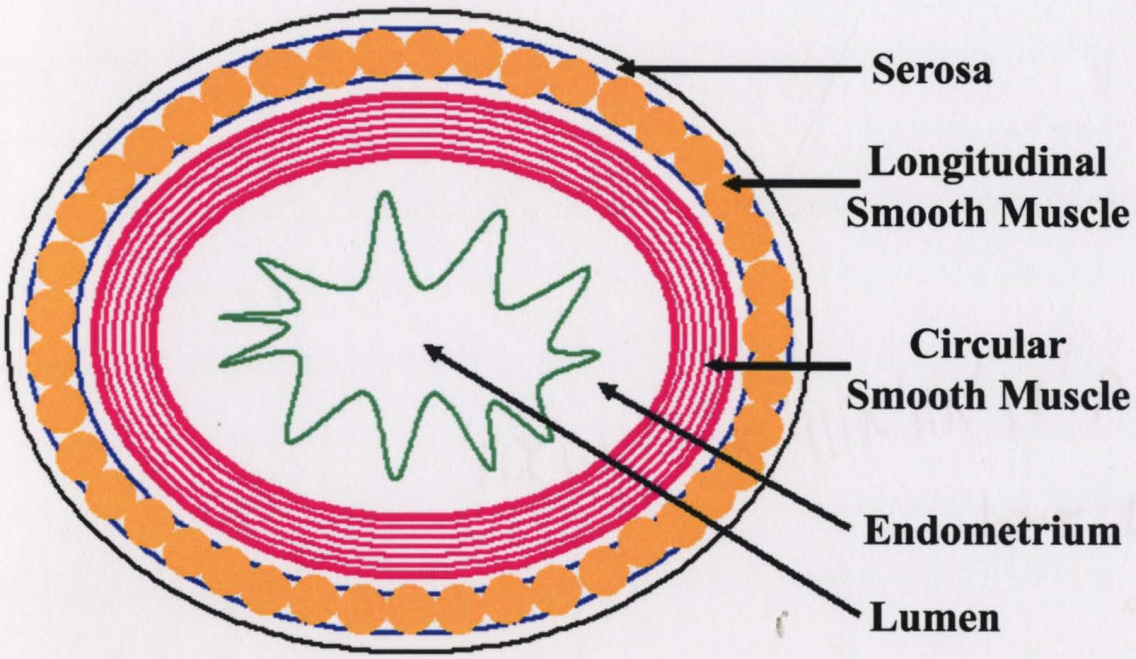
The majority of mammals, including rats used as a model in this thesis, have a uterus that is bicornuate (V-shaped) with an ovary at the distal end of each horn, connected in the centre to the cervical opening. It is comprised of four distinct layers (Figure 1-1); (from outside to inside) serosa, outer longitudinal muscle, inner circular muscle, and the endometrium (Shynlova *et al.*, 2005). The two myometrial layers are separated by a vascular plexus which provides the blood supply, and are easily distinguishable by microscopy in cross section by the direction of the muscle fibres. The longitudinal layer is comprised of muscle bundles that run the length of the uterus, while the circular layer bundles run in a circumferential manner. This separation of layers is further emphasized in the differing lineages of the two layers. The longitudinal layer is derived from the mesenchyme of the subperimetrial zone of the urogenital ridge, while the circular layer originates from the middle layer of mesenchyme in the urogenital ridge, near the Mullerian duct, and develops first (Brody and Cunha, 1989; Williams *et al.*, 2010). As such, the two layers have different physiological characteristics and respond to differing stimuli for much of pregnancy, yet late in pregnancy stimulation of the two layers become more coordinated through a development of the gap-junction and cytoskeletal components (Tomiyasu, 1988; Tuross *et al.*, 1987)

As a caveat, this layered organization of uterine smooth muscle is not observed in humans (Young, 2007). Human uteri are composed of an intermixing of the various myometrial cell types, that cannot be easily distinguished. It is debated whether human

Figure 1-1

Graphical representation of the various layers of the rat uterus.

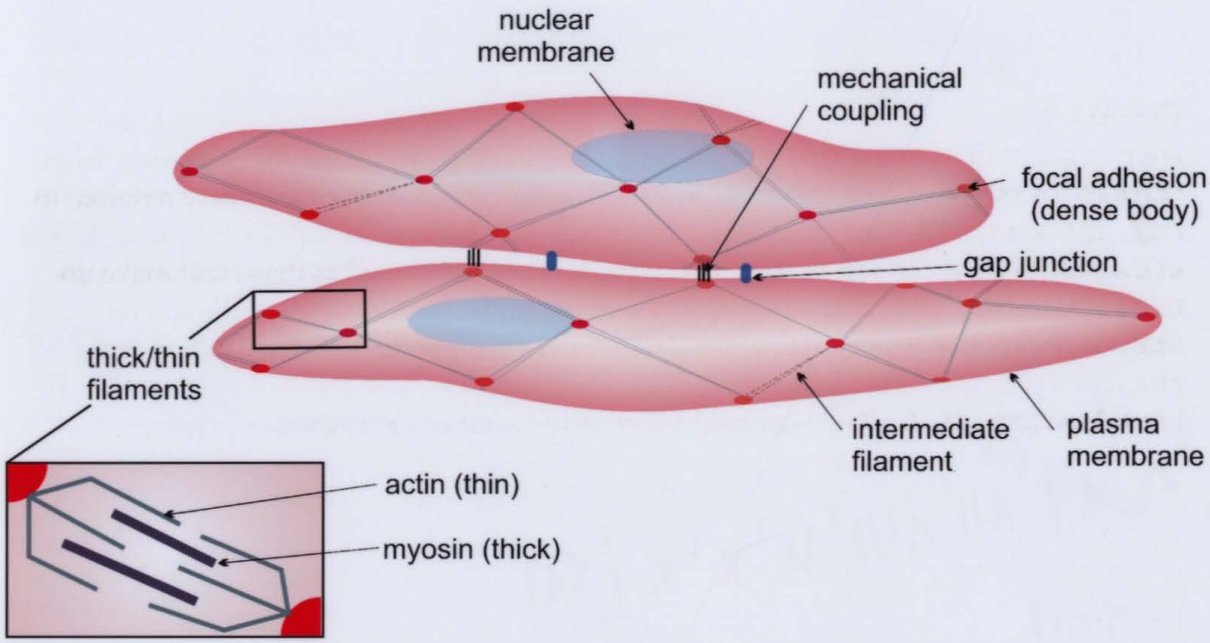
Rat Uterine Horn Organization



uteri thus have the same distinct muscle layer signalling that other mammals have, or whether the signalling is unitary.

Muscle cells, including smooth muscle, have important intramolecular structures, as well as intermolecular. Myometrial cells, not unlike all mammalian cells, have a cytoskeleton of interconnected microfilaments, intermediate filaments, and microtubules, composed of such proteins as actin, vimentin, and tubulin (Figure 1-2; Dahl *et al.*, 2008; Wade, 2007). In smooth muscle, these filaments are connected together through structures termed dense bodies which are dispersed throughout the cytoplasm and nuclear membrane (Small & Gimona, 1998). Connection to the external environment is facilitated through cellular membrane attachment points termed focal adhesions or dense plaques (MacPhee & Lye, 2000). These focal adhesions are composed of a large complex of structural and signalling molecules, such as vinculin, paxilin, talin, and focal adhesion kinase (FAK), ultimately ending with a connection to the extracellular matrix (ECM) through integrin molecules. Through this organization, not only can force be transmitted through neighbouring cells, but outside:inside signalling cascades can be produced from stretch signals originating within the ECM. Acting through integrins in the membrane, stretch can signal molecules within the focal adhesion and thus into the cell itself. It has been suggested that force applied to a uterine smooth muscle cell can be transmitted along the muscle bundle in a similar way to that in other types of smooth muscle. Forces applied to one cell can pass through the cytoskeleton and the nucleus, and onto an adjacent cell through focal adhesions in each cell that are located opposite one another

Figure 1-2
Architecture of the myometrial smooth muscle cell. The cytoskeleton of the myometrium undergoes a substantial remodelling process that equips it with the machinery required to respond to contractile stimuli at the time of parturition onset. This involves rearrangement of the proteins that constitute the thin and thick filaments as well as those that make up the intermediate filaments. Electrical communication between adjacent cells can be achieved through gap junctions whilst mechanical coupling through focal adhesions provides a means for mechanotransduction.
From MacIntyre *et al.*, 2007. Reproduced by permission of the author.


nuclear membrane
mechanical coupling
focal adhesion (dense body)
gap junction
thick/thin filaments
intermediate filament
plasma membrane
actin (thin)
myosin (thick)

(Figure 1-3). In this way the cells in a bundle act as one to generate and transmit force, forming a functional syncytium (Kuo & Seow, 2004).

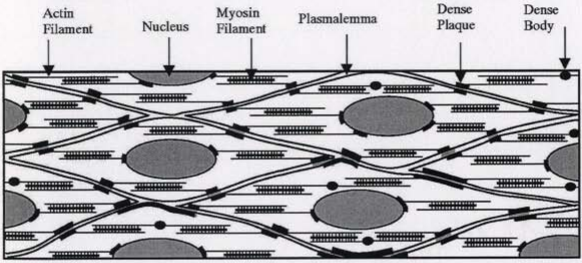
1.3 Endocrine Control

The major hormones that play a role in uterine smooth muscle regulation include progesterone, estrogens, oxytocin, and the prostaglandin hormone family. As a general overview, these hormones are concerned with maintenance of the uterine growth environment, uterine musculature development, and stimulation of contraction, respectively. Each of these will be addressed individually and then tied together in an overall model of hormonal control of pregnancy.

1.3.1 Progesterone.

Progesterone, a C-21 steroid hormone, is intimately involved in the regulation of pregnancy. It is primarily produced by the ovaries (corpus luteum), the adrenal glands, and the placenta (Hadley, 2000). As with all steroid hormones, synthesis of progesterone originates with cholesterol. Cholesterol from the maternal circulation is processed in the placenta by cytochrome p450scc, and after three hydrolysis reactions produces pregnenolone (Tuckey, 2005). Pregnenolone forms the basis of virtually all further steroid hormone production. Following pregnenolone production, the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme facilitates the conversion to progesterone through two steps involving oxidization and tautomerization. Both pregnenolone and progesterone are

Figure 1-3
Schematic representation of a functional syncytium within a smooth muscle bundle. From Kuo & Seow, 2004. Reproduced by permission of the author.


Actin Filament
Nucleus
Myosin Filament
Plasmalemma
Dense Plaque
Dense Body

subsequently utilized as precursors for synthesis of other steroid hormones through conversion to either cortisol or androstenedione (Figure 1-4; Gibb *et al.*, 2006).

Circulating progesterone levels naturally fluctuate throughout the menstrual cycle, but during pregnancy however, expression is much more stable (Jones, 1997). Initially, progesterone is produced in humans by the corpus luteum to prevent menstruation and aids in implantation and initiation of pregnancy (Schindler, 2004; Pepe & Albrecht, 1995). Following implantation and subsequent placental growth, progesterone production from the corpus luteum is replaced by that of the placenta (Tuckey, 2005). Serum levels at the beginning of pregnancy are similar to the highest menstrual cycle levels (~20ng/mL), but begin increasing quickly as pregnancy progresses (Yen, 1991). In humans, circulating levels increase throughout pregnancy, reaching a maximum of up to 200ng/mL at labour (Porterfield & White, 2007). Levels drop back to basal levels (~2ng/mL) shortly after birth, with expulsion of the placenta (Yen, 1991).

In the rat model, progesterone is produced solely by the corpus luteum, but has the same function as placental progesterone (Sugimoto *et al.*, 1997). In the majority of mammals, excluding humans, circulating progesterone levels remain high, maintaining quiescence into late pregnancy, at which time it decreases to basal levels (~5ng/mL) by parturition (Pepe & Rothchild, 1974). More specifically, in the rat, circulating progesterone levels begin increasing within three days of pregnancy, rising to a maximum of 130ng/ml between days 15-19 (Figure 1-5; Rosenblatt *et al.*, 1979). Subsequently, levels drop by half within 2 days (d21), and to undetectable levels by labour (d23; Pepe &

Figure 1-4
Pathway of steroid hormone synthesis in human females during pregnancy.
3β-HSD = 3ß-hydroxysteroid dehydrogenase, 17β-HSD = 17ß-hydroxysteroid dehydrogenases, DHEA = Dehydroepiandrosterone, DHEA-S = Dehydroepiandrosterone sulfate, Fetal = fetal adrenal gland, Maternal = maternal circulation, p450arom = Aromatase, p450c17 = steroid 17-alpha-monooxygenase, p450scc = Cytochrome P450 family 11 subfamily A polypeptide 1, Placental = within the placenta, Pregnenolone-S = Pregnenolone sulfate.
NOTE - all items in italics are enzymes involved in steroid biosynthesis.

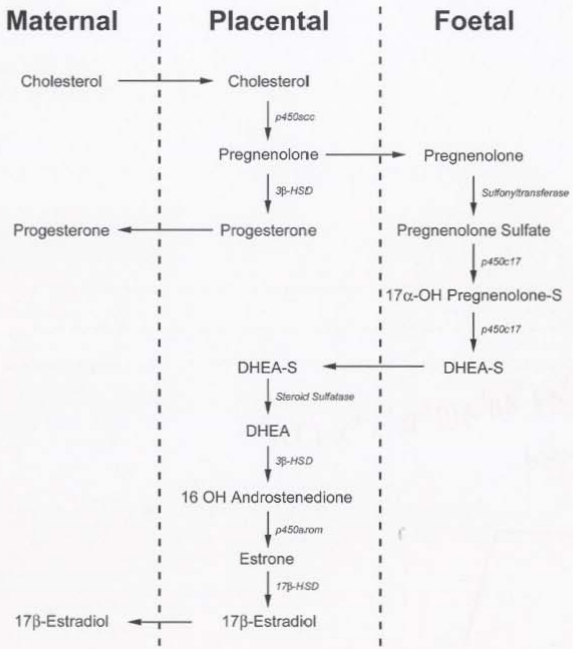
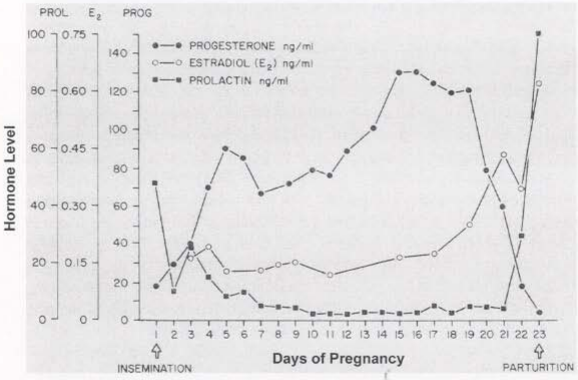

Maternal
Placental
Foetal
Cholesterol
Cholesterol
p450scc
Pregnenolone
Pregnenolone
3β-HSD
Sulfonyltransferase
Progesterone
Progesterone
Pregnenolone Sulfate
p450c17
17α-OH Pregnenolone-S
p450c17
DHEA-S
DHEA-S
Steroid Sulfatase
DHEA
3β-HSD
16 OH Androstenedione
p450arom
Estrone
17β-HSD
17β-Estradiol
17β-Estradiol

Figure 1-5
Plasma hormone concentrations during pregnancy in the rat.
From Rosenblatt *et al.* (1979). Reproduced by permission of Academic Press

Circulating Levels of Progesterone, 17β-Estradiol, and Prolactin During Pregnancy in the Rat



Rothchild, 1974). In rats, this decrease in progesterone is due to the degradation of the corpus luteum, while in sheep it is due in part to increasing conversion of progesterone into 17β-Estradiol by the enzyme $p450_{C17}$ (Challis & Lye, 1984; Whittle *et al.*, 2001).

1.3.2 Estrogen.

Estrogen, as is evident from usage of the term in naming the menstrual cycle in animals (Estrus cycle), is the quintessential pregnancy hormone, directing everything from fetal development, to coordination of muscle contractions. In humans, three major forms of estrogen exist; estrone (E1), estradiol (E2), and estriol (E3) (Hadley, 2000). 17β-estradiol is the most abundant, and is generally referred to as estrogen. These estrogens are primarily produced in the corpus luteum of the ovaries throughout the menstrual cycle, and the placenta during pregnancy. As previously mentioned, the production of estrogen begins with conversion of cholesterol to pregnenolone in the placenta or corpus luteum and fetal adrenal gland. At this stage estrogen production moves between various locations within the feto-placental unit (Gibb *et al.*, 2006; Hill *et al.*, 2010). Pregnenolone is circulated to the fetal adrenal gland where it is sulfonated via a sulfonyltransferase (Hill et al., 2010). The sulfonated pregnenolone is then hydroxylated twice by 17α-hydroxylase ($p450_{C17}$) to produce dehydroepiandrosterone-sulfate (DHEA-S). Fetal adrenal production of DHEA-S is essential in human pregnancy since the human placenta lacks the $p450_{C17}$ enzyme (Smith *et al.*, 1998). DHEA-S then circulates back to the placenta where it is converted to dehydroepiandrosterone (DHEA) by a steroid sulfatase. DHEA is further

converted into androstenedione through the actions of 3β-HSD. Androstenedione is another divergent point in steroid production, as it is further converted into either an estrogen or testosterone. Through an aromatase reaction (p450arom), it is converted into estrone, and then further converted by 17β-hydroxysteroid dehydrogenase (17ß-HSD) into 17β-estradiol (Figure 1-4). This reaction can also proceed through testosterone as an alternative starting constituent.

As with progesterone, estrogen levels fluctuate during the menstrual cycle, and it has a very different expression pattern during pregnancy. In humans, estrogen levels start increasing shortly after the initial progesterone surge from the corpus luteum, and increases steadily up till labour (Johnson, 2007). In all other mammals, estrogen levels remain relatively low throughout the majority of pregnancy, spiking shortly prior to labour, partially in response to increased cortisol production (Challis & Lye, 1994, Lye *et al.*, 1993). In the rat model, circulating levels of 17β-estradiol throughout most of pregnancy remain around ~15ng/ml, with a surge beginning around day 19 and increasing up to ~60ng/ml at day 23, *i.e.* labour (Figure 1-5; Rosenblatt *et al.*, 1979).

1.3.3 Prostaglandins.

Prostaglandins (PG), are lipid derived hormones that are found in virtually all nucleated cells. They are proposed to be the initiators and mediators of labour, in both normal gestation and preterm. Prostaglandins exist in four forms, including PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2 and are produced in almost every nucleated cell (Olson, 2003). During pregnancy, enzymes that produce prostaglandins are highly expressed in the placenta and

fetal membranes (Freed *et al.*, 1997; Slater *et al.*, 1999). The highest levels however, are found in the amnion and the amniotic fluid (Gibb & Sun, 1996; Johansen *et al.*, 2000; Salmon & Amy, 1973). In contrast to the steroid hormones, PG production begins with lipids. Initially, diacylglycerol (DAG) or a phospholipid are converted to arachidonic acid through the actions of either phospholipase C (PLC) or phospholipase A_2 (PLA_2), respectively. In the amnion, this primarily progresses through the actions of cytosolic PLA_2, whose activity increases throughout pregnancy (Skannal *et al.*, 1997). Once arachidonic acid is produced, it can then follow two different metabolic pathways. Through the actions of lipooxygenase, arachidonic acid is converted to hydroperoxyeicosatetraenoic acid thus leading to the production of leukotrienes. PG synthesis however, proceeds through the conversion of arachidonic acid to PGH_2 via the PGH_2 synthase (PGHS) enzyme; alternatively referred to as cyclooxygenase-1 or -2 (COX-1 or COX-2; Challis *et al.*, 1999, 2002; Smith *et al.*, 2000). A third form of PGHS, COX-3, is a splice variant of COX-1, but has not been demonstrated to be a functional protein. Although these three different potential PGHS enzymes exist in humans, PGHS-2 is the primary pregnancy related form. PGH_2 is then further processed by a series of synthases and an isomerase in order to produce the various active forms of prostaglandin; PGD synthase produces PGD_2, prostacyclin synthase produces PGI_2, and PGE synthase produces PGE_2. An isomerase further converts PGE_2 into $PGF_{2\alpha}$. PGHS levels vary by placental layer throughout pregnancy, but overall there is an increase at term of PGHS-2 in all layers (Alfaidy *et al.*, 2003; Cheung *et al.*, 1990; Hirst *et al.*, 1995; Slater *et al.*, 1998). Due to factors such as normal biological variation, precise site of collection,

thickness of sample, and labouring versus non-labouring phenotype, investigation of human myometrial expression of PGHS at labour has ellicited varying conclusions (Moore *et al.*, 1999; Sparey *et al.*, 1999; Zuo *et al.*, 1994). Giannoulias and colleagues (2002) have shown that PGHS levels in the myometrium do not change with labour, while Erkinheimo and colleagues (2000) have shown a 15-fold increase in PGHS mRNA levels in myometrial specimens for labouring versus non-labouring women.. Results from rodent models on the other hand, have been consistent with the other placental layers, showing an increase during labour (Tsuboi *et al.*, 2000)

Perhaps more important to overall PG levels, is their rate of degradation. Metabolism of prostaglandins is mediated by a type 1 NAD^+ (Nicotinamide adenine dinucleotide) dependant PG dehydrogenase (PGDH), and similarly varies by placental layer (Braithwaite & Jarabak, 1975; Cheung *et al.*, 1992; Matsuo *et al.*, 1997). PGDH is primarily found in the chorionic layer, and is thought to act as a biological barrier to impede PGs produced in the amnion from reaching the myometrium. Levels of PGDH remain high in the chorion throughout most of pregnancy, but have been found to be significantly reduced at term or preterm labour (Sangha *et al.*, 1994; Van Meir *et al.*, 1997). In addition, protein levels and activity of PGDH in the myometrium has been found to decrease with labour (Giannoulias *et al.*, 2002). In this way it is thought that the decrease in PGDH production at labour allows PGs to stimulate the myometrium.

Overall, PG levels (PGE_2 & $PGF_{2\alpha}$) within the placenta and myometrium progressively increase during late pregnancy with a significant spike during labour (Challis *et al.*, 2002; Salmon & Amy, 1973; Sellers *et al.*, 1981). This increase in

expression is due to the regulation of both PG synthesis as well as PG breakdown. So to facilitate this overall increase, levels of PGHS-2 increase in labour, while levels of PGDH decrease (Challis *et al.*, 1999). However, differential regulation of PG in the myometrium through changes in synthesis and degradation also occurs locally within discrete regions of the uterus. Durn and colleagues (2010) found that while levels of $PGF_{2\alpha}$ remain high in the myometrium before and during labour, levels of PGE_2 are significantly increased at labour specifically in the lower uterine myometrium. This allows for contraction of the upper uterine myometrium, while facilitating relaxation and cervical ripening of the lower segment. In addition to changes in regulation of prostaglandin synthesis and degredation, regulation PG signalling is also prevalent in the myometrium through changes in PG receptor expression (Astle et al., 2005; Grigsby *et al.*, 2006; Leonhardt *et al.*, 2003; See section 1.5.3: Phase 2 - Stimulation).

1.3.4 Oxytocin and corticotropin releasing hormone (CRH).

The last major hormones involved with pregnancy are oxytocin and CRH. These two molecules are both polypeptide hormones and neuropeptides (Zingg & Lefebvre, 1988; Florio *et al.*, 2003). They are synthesized by the paraventricular nucleus of the hypothalamus and subsequently are transported to the pituitary. Once in the posterior pituitary, oxytocin is released into the circulatory system, while CRH mediates the production of other steroid hormones. Both of these molecules are also produced by the placenta during gestation and are involved in the regulation of myometrial contraction (Lefebvre *et al.*, 1992, Petraglia *et al.*, 1996). During pregnancy, placental production of

oxytocin is approximately fivefold higher than pituitary production, making this the main reproductive source (Blanks & Thornton, 2003).

Oxytocin, whether produced in the brain or placenta, is found in both the maternal and fetal circulatory systems (Gibb *et al.*, 2006; Thornton *et al.*, 1992). Levels of oxytocin increase exponentially during labour, and accompany contractions in most species (Petraglia *et al.*, 1996). Mitchell and colleagues (2005) have shown that levels of oxytocin mRNA in the uterus of rats increase at least 20-fold just prior to labour.

CRH is produced at two different locations, neuronal and placental, and although these proteins have identical structure and function they are regulated independently (McLean & Smith, 2001). CRH is thought to be directly involved with regulation of the timing of labour (McLean *et al.*, 1995; McLean & Smith, 2001). Circulating levels of CRH are dramatically increased during late pregnancy and labour, but unlike oxytocin, CRH levels are increased prior to the increase in contractility (Petraglia *et al.*, 1996). This has implicated CRH as a possible marker of impending preterm birth, as women who deliver preterm have been shown to have higher levels of circulating CRH (McLean *et al.*, 1995). CRH is in an active form while circulating freely. It can however bind to a placenta-produced CRH binding protein, that upon complexing with it, inactivates it.

1.4 Mechanotransduction

The endocrine environment is not the only factor modulating myometrial function. The physical state of the muscle also plays an important role. Mechanical distension and/or stress that is applied to muscle cells themselves facilitates important functional

changes (Ingber, 2008; Katz *et al.*, 2000; Vogel, 2006). This signalling from mechanical forces is termed mechanotransduction, and is an important regulator of the function of all muscle types. Prior research has demonstrated mechanotransduction signalling specific to the myometrium during pregnancy (Burghardt *et al.*, 2009; Galbraith *et al.*, 2002; Li *et al.*, 2007; Ou *et al.*, 1997; Shynlova *et al.*, 2004; Wu *et al.*, 2008).

As the foetus grows within the uterine environment, the myometrium is progressively and increasingly stretched, with growth of the muscle cells unable to keep up with the physical demands for space. This stretch increases the expression of a variety of molecules including signalling molecules such as inositol tri-phosphate and c-fos, as well as structural molecules such as type IV collagen and laminin-γ2 (Sadoshima & Izumo, 1993; Shynlova *et al.*, 2004, 2009; Tanaka *et al.*, 1994). As well, stretch induces an increase in the expression of contraction associated proteins (CAPs) such as connexin-43, the oxytocin receptor, prostaglandin $F_{2\alpha}$ and E_2, and their associated receptors (EP_4 type and $F_{2\alpha}$ receptor) (Lye *et al.*, 1993, 2001; Manabe *et al.*, 1983; Martineau *et al.*, 2004; Ou *et al.*, 1997, 1998; Wu *et al.*, 1999). Stretch also increases phosphorylation and activation of mitogen activated protein kinases (MAPKs) such as extracellular signal-regulated protein kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK (Oldenhof *et al.*, 2002).

This is further emphasized by observing the non-pregnant, and unilaterally pregnant rat models. Investigators have shown that stretch of non-pregnant rat uteri has produced increases in total protein expression, and that pregnant animals with one empty uterine horn (unstretched by pups) and one gravid horn have significantly decreased

protein expression in the empty horn (Ou *et al.*, 1997, 1998). Utilizing this model, Shynlova and colleagues (2009) have shown that stretch of the uterus, due to the presence of foetuses, led to a significant increase in basement membrane proteins collagen IV & laminin-γ2. Further research using this model has shown that a lack of uterine stretch due to lack of foetuses significantly decreases expression of connexin-43 and the oxytocin receptor, and that if this stretch is recapitulated through surgical insertion of stretching device (plastic tube) expressions levels return to their normal fetal-stretched levels (Ou *et al.*, 1997, 1998). In addition to increasing protein expression, stretch has also been shown to play a role in activation and post translational modification of molecules, often through phosphorylation. Stretch induced activation of protein kinase C (PKC), mitogen activated protein kinases (MAPKs), phospholipase C & D, myosin light chain (MLC), and COX-2 has been shown in cell types such as arterial and myometrial smooth muscle, cardiac muscle, and kidney cells (Barany *et al.*, 1985; Martineau *et al.*, 2004; Oldenhof, *et al.* 2002; Sadoshima & Izumo, 1993; Sooranna *et al.*, 2004). Oldenhof and colleagues (2002) have shown that *in vitro* static stretch of rat uterine smooth muscle cells using a flexible bottomed culture plate, and *in vivo* stretch due to the presence of rat foetuses, facilitate a very quick phosphorylation (activation) of various MAPKs. Stretch-induced mechanotransduction also increases the expression of focal adhesion proteins such as focal adhesion kinase (FAK), paxillin, caldesmon, and extracellular signal-regulated kinases (ERK). In addition to changes in the functional state of these proteins through changes in their phosphorylation, stretch can also regulate their specific subcellular localization (Li *et al.*, 2007; Ou *et al.*, 1997; Shynlova *et al.*, 2009). Myometrial stretch in

the rat model has been shown by Shynlova and colleagues (2009) to promote changes in the organization of extracellular basement membrane proteins into regular structures surrounding the plasma membrane. In addition, focal adhesion proteins such as paxillin and vinculin showed a translocation from the cytoplasm to the cell periphery, often into focal adhesion structures.

The importance of mechanotransduction driven signalling in the myometrium can also be observed through investigation of the statistics surrounding multiple-foetus pregnancies (ie. twins, triplets, etc...). In these pregnancies, it is accepted that there is an increase in stretch of the uterus due to the combined increase in size of the growing foetuses, as compared to singleton pregnancies, yet there has been very little research comparing myometrium from twin or single-birth pregnancies (Turton *et al.*, 2009). It has been shown that multiple-foetus pregnancy correlates with varying fetal outcomes (Garite *et al.*, 2004a, 2004b; Shinwell, 2005). In the United States in 2006, the incidence of pre-term birth was six times higher with multiple pregnancies, versus that of singletons (61% vs 11%) Not only has it been shown that the incidence of preterm birth increases with multiple pregnancies, but as stated previously, these births also have a much higher incidence of neonatal morbidity and mortality.

These demonstrations of cellular signalling through force-dependent expression and/or phosphorylation are naturally influenced by, and interact with, the underlying endocrine environment (Giannone & Sheetz, 2006; MacPhee & Lye, 2000, Vogel, 2006). This has been demonstrated for many CAPs and focal adhesion proteins (Lye *et al.*, 2001; Shynlova *et al.*, 2009). It has been shown that progesterone plays a regulatory role in the

dynamics of stretch signalling. Utilizing a non-pregnant model of uterine stretch through surgical interventions (surgical insertion of a plastic tube into one uterine horn), Ou and colleagues found that the significant stretch-induced increases of connexin-43 could be counteracted and returned to a basal level with exogenous injections of progesterone. Progesterone, whether endogenous or exogenous, decreases stretch induced expression of many proteins such as OTR and cx-43, likely through stimulation of uterine growth that then counteracts the distension. (Lye *et al.*, 2001; Mesiano & Welsh, 2007; Ou *et al.*, 1997, 1998; Shynlova *et al.*, 2009). Thus all of these signals work together to influence the development and regulation of the uterine musculature.

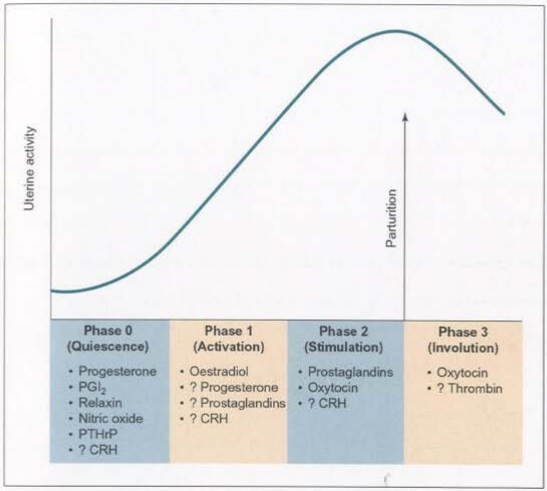
1.5 Phases of Myometrial Activation

There are distinct phases that the myometrium goes through during pregnancy and labour (Figure 1-6). These phases are characterized by changes in both structure and function of the myometrium (Shynlova *et al.*, 2006, 2009).

1.5.1 Phase 0 - Quiescence.

The majority of pregnancy is represented by the quiescent phase of myometrial activation (Gibb *et al.*, 2006). This stage is characterized by a lack of marked contractility of the myometrium, with contractions of the muscle being weak and poorly synchronized due to the unresponsiveness of the myometrium to uterotonins (substances that modulate myometrial tone and contractility). These "contractures" occur throughout pregnancy, and correspond to Braxton-Hicks contractions in women (Harding *et al.*, 1982; Lye, 1994).

Figure 1-6
Phases of uterine activity. A listing of the various agents involved during quiescence (phase 0), activation (phase 1), stimulation (phase 2) and involution (phase 3) of the uterus during pregnancy are represented.
PGI_2 = prostacyclin, PTHrP = parathyroid hormone related peptide, CRH = corticotrophin releasing hormone.
From Challis *et al.*, 2002.


Uterine activity
Parturition
Phase 0 (Quiescence)
• Progesterone
• PGI_2
• Relaxin
• Nitric oxide
• PTHrP
• ? CRH
Phase 1 (Activation)
• Oestradiol
• ? Progesterone
• ? Prostaglandins
• ? CRH
Phase 2 (Stimulation)
• Prostaglandins
• Oxytocin
• ? CRH
Phase 3 (Involution)
• Oxytocin
• ? Thrombin

Through paracrine and endocrine regulatory pathways, the myometrium remains in this silenced state. Regulating molecules include progesterone, PGI_2, relaxin, and nitric oxide. Although acting through different pathways, these molecules facilitate the inhibition of Ca^{2+} release within myometrial cells (Challis, 2001). The pathways stimulate an increase in the levels of cAMP and cGMP, that activate cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) within uterine muscle cells (Dayes, 2008; Negishi *et al.*, 1995; Reimer *et al.*, 2008). Activation of PKA and PKG leads to the activation of phosphodiesterase which dephosphorylates and inactivates myosin light chain kinase (MLCK). Since activated (phosphorylated) MLCK is essential for smooth muscle contraction, the myometrium remains quiescent (Moore & López Bernal, 2001). In addition, regulatory elements affect contractility by modulation of actin filament dynamics. Cofilin (also called actin depolymerization factor) promotes the breakdown of F-actin into its subunits (G-actin) by disrupting the attachment of actin subunits though steric alteration of the tertiary structure of F-actin (Pfaendtner, 2010; Scoville *et al., 2009)*. Also, HSPB1 has been shown to cap F-actin, preventing elongation, and to bind G-actin thus preventing it's incorporation into actin chains (See section 1.8.2.3.1: Actin regulation). These cofactors promote the overall maintenance of G-actin, thereby preventing the contractile machinery from being constructed (Challis, 2000; Smith *et al.*, 2007).

PKA also plays another role in the maintenance of myometrial quiescence. Sarcoplasmic reticulum (SR) calcium ATPases that actively remove calcium from the cytoplasm into SR stores, are inhibited by sarcoplasmic reticulum (SR) membrane protein

phospholamban (PLB) (Kawashima *et al.*, 2009). PKA acts to phosphorylate Ser-16 and Thr-17 of PLB, which alleviates this inhibition and allows efficient calcium uptake (Wegener, 1989). Alternatively, protein phosphatases (PPs) such as PP1 decrease calcium uptake by dephosphorylating PLB. PKA also acts to phosphorylate inhibitor-1 (I-1), which is an inhibitor of PP1, on Thr-35 to activate it and aid in calcium uptake into the SR (Endo *et al.*, 1996). In this way, PKA facilitates the removal of Ca^{2+} within myometrial cells by promoting uptake into intracellular stores.

Progesterone is required to maintain pregnancy in virtually all mammals, and has a consistent functional role; to maintain a quiescent myometrium and prevent abortion (Csapo *et al.*, 1972; Graham & Clarke, 1997; Haluska *et al.*, 1997; Zakar & Hertelendy, 2007). This is supported by investigations utilizing the progesterone receptor antagonist RU486 or administration of exogenous progesterone (Baulieu, 1991; Petrocelli & Lye, 1993). Initial research using RU486 in the rat model showed that administration of the drug to pregnant rats would not only stimulate preterm birth, but would also increase the contractile activity of the myometrium, with animals going into labour within a short time after RU486 administration (Arkaravichien & Kendle, 1992; Chabbert-Buffet *et al.*, 2005; Garfield *et al.*, 1987; Gemzell-Danielsson & Marions, 2004). Fang and colleagues (1997) found that RU486-blockage of the P_4 receptor produced the labour-associated increase in oxytocin receptor (OTR) and prostaglandin levels well before normally observed. In contrast, administration of progesterone to pregnant rats starting several days prior to labour delays the onset of labour for at least 24 hours (Hendrix *et al.*, 1995; Ou *et al.*, 1998). Petrocelli and Lye (1993) established that administration of 68ng/ml of

progesterone was sufficient to prevent the normal labour-associated fall in circulating plasma progesterone. Progesterone signalling is facilitated though both genomic and non-genomic actions mediated by progesterone receptors A & B on the nucleus (nPR) and cell membrane (mPR; Mesiano & Welsh, 2007). The genomic signalling pathways function through progesterone activation of nPRs that then affect gene regulation of target proteins; such as contraction associated proteins (CAPs) during pregnancy. Once progesterone binds to a receptor, it then translocates to the nucleus, where it can bind to progesterone-response elements in gene-specific promoters and stimulate or suppress gene transcription. Ou and colleagues (2000) found that injection of 4mg of P_4 per animal, to pregnant rats starting three days prior to parturition, inhibited the normal labour-associated increase in $PGF_{2\alpha}$ receptor expression. Unlike genomic signalling, nongenomic progesterone signalling is very rapid and occurs through progesterone affecting intracellular signal transduction pathways (Perusquía, 2001). The nongenomic effects may be due to association of the mPRs to other membrane receptors (e.g. G-protein coupled receptor) or other signaling molecules that stimulate activation of Src/MAPK cascade and/or by an interaction with neurotransmitter and peptide hormone receptors such as OTR (Thomas *et al.*, 2007; Karteris *et al.*, 2006).

Through these combined signalling mechanisms, progesterone has the effect of both decreasing expression/preventing increased expression of some proteins like fibronectin, laminin, connexin-43 (cx-43), and oxytocin receptor (OTR), while preventing the fall/increasing expression of others like collagen III, integrin α5 and activated FAK (Lye *et al.*, 1993; MacPhee & Lye, 2000; Ou *et al.*, 1997; Ou *et al.*, 1998; Shynlova *et al.*,

2004, 2009; Williams *et al.*, 2005). For example, Williams and colleagues (2005) found that progesterone prevented the labour-associated decrease of Integrin α5 mRNA and protein expression, while Shynlova and colleagues (2004) found that progesterone administration prevented the late-pregnancy drop in collagen III mRNA, as well as causing a significant decrease in both fibronectin and laminin mRNA expression. In contrast, progesterone also facilitates changes in cellular trafficking of proteins. For example, Hendrix and colleagues (1995) found that progesterone decreases the translocation of cx-43 proteins to the membrane in order to decrease formation of gap junctions.

In addition to signalling through the PR's, progesterone also maintains uterine quiescence through interfering with the late-pregnancy signalling normally associated with increased levels of circulating 17β-estradiol, and with uterine stretch. As previously mentioned, progesterone has been shown to counteract the changes in expression of many proteins in late pregnancy (e.g. CAPs such as OTR), but it also mediates the overall reduction of 17β-estradiol signaling (Lye *et al.*, 2001; Mesiano & Welsh, 2007; Ou *et al.*, 1997; Ou *et al.*, 1998; Shynlova *et al.*, 2009). Early experiments by Pinto and colleagues (1967) found that the myometrial stimulatory action of intravenous administration of 17β-estradiol (200mg in 1hr) could be counteracted by administration of progesterone. Since the inhibitory action of P_4 occurred very quickly (within 10 minutes), this was evidence that progesterone could inhibit estrogenic signalling through a nongenomic pathway of action (Bishop & Stormshak, 2008). This response is facilitated by a reduction of 17β-estradiol signaling through steric hindrance of the 17β-estradiol receptor by progesterone,

thus physically blocking the estrogen from binding. There is also an increasing body of research suggesting that progesterone may also act genomically on estrogen signalling by decreasing ERα expression (Katzenellenbogen, 2000; Leavitt *et al.*, 1987; Mesiano *et al.*, 2002; Mesiano & Welsh, 2007; Zakar & Hertelendy, 2007; Zelinski *et al.*, 1980). Through the combined action of these multiple mechanisms, progesterone works to maintain a quiescent uterine environment.

During the first part of phase 0, myometrial cells are characterized by a period of rapid proliferation (hyperplasia), thus increasing the total number of myometrial cells (Shynlova *et al.*, 2009). Early experiments by Goldspink and Douglas (1988) showed that rat myometrial protein and DNA synthesis were increased during early-mid rat gestation, and that protein degradation was significantly decreased, illustrating a phase of rapid cell division and growth. In addition, the presence of the foetus (whether through hormonal or mechanical control) is a necessary component to these changes as assessed through use of a unilaterally pregnant rat model (Goldspink & Douglas, 1988). In rats, it has been shown through the use of BrdU staining that the amount of cellular proliferation and myometrial growth is high early in gestation and decreases as gestation progresses (Shynlova *et al.*, 2006). As well, much of this growth takes place specifically in the longitudinal muscle layer as evidenced by a very significant level of cellular growth as compared to circular muscle (Shynlova *et al.*, 2006). In addition to overall cellular growth, expression of many proteins such as FAK, Cx-43, and integrins, that act to anchor cells, are low until late gestation (MacPhee *et al.*, 2000; Orsino *et al.*, 1996; Williams *et al.*, 2005). Williams and colleagues (2005) have shown that the levels of integrin alpha 5 subunit in the

myometrium increases as gestation progresses in the rat, while MacPhee and colleagues (2000) have shown that FAK remains unphosphorylated and hence unactivated early in gestation. These results show that focal adhesion formation is minimal in myometrium as compared to late pregnancy, allowing cells to divide and grow more freely. As well, Lye and colleagues (1993) have shown that connexin-43, which is an integral part of gap junctions that connect neighbouring cells, is low early in pregnancy (See section 1.6.4: Mechanical Syncytium).

1.5.2 Phase I - Activation.

Late in pregnancy, a large shift in both regulation of uterine contractility and protein expression profiles occur as the myometrium enters the activation stage of contractility. Overall, there are three factors that stimulate this change; a decrease in progesterone signalling, an increase in circulating estrogen levels, and an increased physical stretch of the uterus (Gibb *et al.*, 2006). This is characterized by increasing uterine hypertrophy (increase in cell size; Shynlova *et al.*, 2009). Due to these changing signals, the cells of the myometrium synthesize a multitude of proteins that are necessary for the remodelling of the cellular architecture to facilitate the contractile response. These proteins fall under the classification of contraction associated proteins (CAPs). This term classically defines a set of specific ion channels (e.g. calcium channels), gap junctions (e.g. connexin-43), and agonist receptors (e.g. oxytocin receptor), but is often extended to include putative CAPs such as actin/myosin regulators (e.g. MLCK; Lye *et al.*, 2001). Garfield and colleagues (1988) have shown that for the majority of pregnancy,

myometrial cells have a high electrical resistance and poor coordination of contractions due to the lack of gap junctions (MacKenzie *et al.*, 1985). It can thus be observed that each of the classical CAPs act to enable the uterine smooth muscle to produce the high-amplitude contractions needed for labour by increasing calcium transport, signal coordination, and contractile agonist response (Sakai *et al.*, 1992). Investigations performed by members of the lab of Dr. Stephen Lye have shown that connexin-43 (cx-43) RNA and protein expression in the myometrium increases on day 21 of rat pregnancy to a maximum at labour, and that progesterone, estrogen, and mechanical stretch, affect it's regulation (Lye *et al.*, 1993; Mitchell *et al.*, 2003; Orsino *et al.*, 1996; Ou *et al.*, 1997; Piersanti & Lye, 1995; See section 1.6.4: Mechanical syncytium). Expression of oxytocin receptor (OTR) has been shown to parallel that of cx-43 (Mitchell *et al.*, 2003; Ou *et al.*, 1998). OTR RNA and protein expression in the myometrium of rats increases on day 21 of gestation up to a maximum at labour, and then returns to basal levels 1 day post parturition. In addition, experiments involving exogenous progesterone administration to either pregnant or ovariectomized rats show that progesterone prevents the late pregnant rise in OTR expression. However, once the effects of progesterone decrease, stretch of the myometrium is a potent stimulator of OTR expression, as illustrated through utilization of the unilaterally pregnant model (Ou *et al.*, 1998).

Shynlova and colleagues (2006), have proposed a model of hypoxia stimulated transition to activated myometrium. This is based on their own multiple publications detailing myometrial regulation, as well as prior characterizations of rat pregnancy (Reynolds, 1949, 1950; Shynlova *et al.*, 2004, 2005, 2006, 2009, 2010a). The growing rat

foetus remains spherical until mid gestation when maximum spherical radius is reached, after which the foetus elongates rather than growing in diameter (Reynolds, 1949). At this point, growth of the foetus causes strain on the surrounding uterine muscle, causing a pinching of the surrounding blood vessels. The consequent reduction in circulation promotes an ischaemic environment and hypoxia within the surrounding tissues that potentially stimulates the progression into phase 1 of pregnancy (Reynolds, 1950). Adding upon previous evidence for this proposed mechanism (Shynlova *et al.*, 2004, 2006), Shynlova and colleagues (2010a) utilized a unilaterally pregnant rat model to further characterize this process. Utilization of two hypoxia markers illustrated that hypoxia within the pregnant rat myometrium significantly increased at ~d14 of gestation and that it was predominantly localized to the circular muscle layer. Through western blot and immunohistochemical analysis, they also presented that expression and localization of activated caspase parallelled the pattern of myometrial hypoxia, occurring at the transition into phase 1 of pregnancy. In addition, caspase activation was almost exclusively observed in the gravid (i.e. stretched) uterine horn. Increasing the levels of stretch in the non-gravid horn through surgical intervention increased caspase activation almost to the level of that observed in the gravid horn. Since they didn't observe this recapitulation at other times during pregnancy, they have concluded that this stretch-induced hypoxia signalling was dependent on the underlying endocrine environment, and the associated caspase cascade possibly promotes transition into phase 1 of pregnancy.

Shynlova and colleagues (2006) found that early in pregnancy myometrial tissue has a high level of hyperplasia (cell growth) as evidenced through high levels of BrdU

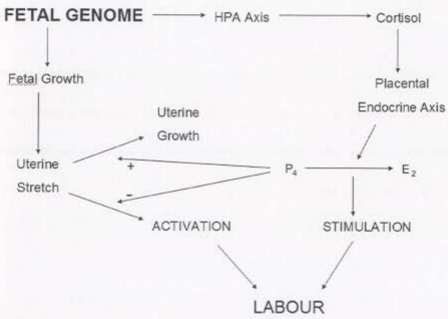
incorporation into cells, high levels of PCNA, and a low protein:DNA ratio. However between days 12 and 15 of rat gestation, they discovered an activation of apoptosis with an increase in activation of initiator caspase 9 and an increase in downstream caspases 3, 6, and 7, as well as a concomitant increase in bcl-2 levels. Following this caspase activation, the myometrial tissues investigated exhibited a hypertrophic phenotype as illustrated through an increased protein:DNA ratio and decreased proliferation, facilitating development of a myometrium that will be highly responsive and excitable, and spontaneously active (Challis *et al.*, 2001; Gibb *et al.*, 2006; Shynlova *et al.*, 2006).

This hypertrophy-induced caspase cascade is one example of how the foetus regulates the progression of pregnancy. Early experiments by Liggins (1969) and Thorburn (1979) illustrated this crucial role in the regulation of pregnancy and parturition. They found that ingestion of the plant *Veratrum Californicum* by pregnant ewes caused a prolongation of gestation, through blocking the development of the fetal HPA axis. It has subsequently been shown that activation of the myometrium is established through actions of the fetal genome in stimulating CAP expression, and follow two distinct pathways; mechanical and endocrine (Challis, 2000).

1.5.2.1 Fetal genome.

The endocrine regulation of myometrial activation is through activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis (Figure 1-7), by two mechanisms (Liggins & Thorburn, 1994). Physiological stresses, like the ischaemia-induced hypoxia previously discussed, cause the fetal hypothalamus to begin releasing CRH, thus

Figure 1-7
Graphical pathway of how the fetal genome dictates the onset of labour. This proceed through either a mechanical or endocrine pathway. Mechanical signalling is through increases in uterine stretch due to increases in fetal size, while the fetal endocrine pathway is through the activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis. Both of these signalling pathways are interdependent on each other, due to cross signalling. Changes in steroid hormone levels modulate the ability of uterine stretch to facilitate gene expression.
Based on figure in Challis & Lye, 2002.


FETAL GENOME
HPA Axis
Cortisol
Fetal Growth
Placental
Endocrine Axis
Uterine
Growth
Uterine
Stretch
+
-
P_4
E_2
ACTIVATION
STIMULATION
LABOUR

stimulating adrenocorticotropin hormone (ACTH) production by the pituitary (McMillen *et al.*, 2004). During this time the placenta begins to produce increasing amounts of CRH locally that also stimulate the fetal HPA axis (Gibb *et al.*, 2006). Throughout most of pregnancy, the placenta also produces CRH-binding protein which acts to sequester circulating CRH thus reducing its activity. However, at term concentrations of CRH-binding protein decrease, while CRH production increases, facilitating an overall increase in circulating CRH activity. This increase in circulating CRH mediates an increase in ACTH production that stimulates the fetal adrenal tissue to produce increasing amounts of cortisol to be secreted into the fetal and maternal circulations, as well as stimulating an increase in fetoplacental estrogen production (Gibb *et al.*, 2006).

Cortisol induces an increased expression of placental CRH which mediates many changes. CRH, through the CRH receptors (Type 1 and 2), facilitates an increase in protein kinase A, which in turn increases activation of cAMP responsive element binding protein (CREB). CREB can then bind to CRE elements in gene promoters, thus directing gene expression. By this mechanism, CRH has been shown to increase production of prostaglandins from the placenta through an upregulation of PGHS-2 production due to stimulation of gene expression, and by downregulation of PGDH activity through stimulation of cAMP production; a negative regulator of PGDH (Challis *et al.*, 2000; Jones & Challis, 1990).

Subsequently, PGs and cortisol have been shown to increase the production of estrogen and decrease the production of progesterone (Challis, 1971; Challis & Patrick, 1981; Flint *et al.*, 1975; Jenkin & Thorburn, 1985). In many mammals this occurs through

cortisol causing an increase in the conversion of progesterone to estrogen, primarily through the actions of placental $p450_{C17}$ enzyme but also through fetal adrenal derived sources (Challis, 2000; Challis *et al.*, 2002; Whittle *et al.*, 2001). In addition, PGs facilitate the breakdown of the corpus luteum in the ovaries (Driancourt *et al.*, 2000). Since this is the primary source of progesterone production in many mammals (e.g. rat placenta doesn't produce progesterone), this results in a decrease in the circulating levels of progesterone. This "switch" from progesterone to estrogen is one of the key regulatory events for the activation of the myometrium and progression of labour (Challis & Lye, 1984; Lye, 1994; Whittle *et al.*, 2001).

The role of 17β-estradiol in pregnancy is to promote uterine plasticity, and to change the uterus from a quiescent state into an activated form to prepare it for labour (Gibb *et al.*, 2006; Shynlova et al., 2006). As the switch from progesterone to estrogen proceeds, progesterone suppression of CAPs decline while 17β-estradiol activation of both CAPs expression and stimulation of prostaglandin production increases (Lye, 1994; Lye *et al.*, 1993; Mitchell *et al.*, 2003; Orsino *et al.*, 1996; Ou *et al.*, 1997; Piersanti & Lye, 1995). Like progesterone, estrogen acts through associations with it's specific steroid hormone receptors to, in general, exert genomic effects (Greene *et al.*, 1984; Levin, 2005). There are two specific estrogen receptors (ERα & ERβ) that when not bound to ligand, are complexed with heat shock proteins (Pratt *et al.*, 2004) to maintain proper receptor folding and to maintain functional state (Bohen & Yamamoto, 1994; Greene *et al.*, 1984; Mosselman *et al.*, 1996; Wolffe *et al.*, 1984). It has been shown recently that even the small heat shock proteins such as HSPB1 play a role in ER

regulation (Miller *et al.*, 2005). Protein-binding assays, such as immunoprecipitation, utilizing HeLa cell extracts show a specific interaction between HSPB1 and ERβ. In addition, through HeLa cells either overexpressing (stable transfection) or underexpressing (siRNA knockdown) *HSPB1*, HSPB1 appears to modulate receptor availability for signalling. When HSPB1 levels were increased estrogen responsive element (ERE) signalling was decreased by 20%, while decreasing HSPB1 (siRNA) increased ERE signalling by ~45%. Upon binding to estrogen, the ERs release from the HSP's, translocate to the nucleus, dimerize, and then act upon EREs, along with co-modulator proteins, to initiate transcription (Brosens *et al.*, 2004). This nuclear interaction can be through full EREs, or half ERE sites in conjunction with other recognition sites like the SP1 site in *HSPB1* (Kita *et al.*, 2009; Porter *et al.*, 1996; Safe & Kim, 2008). The actions of estrogen increase with the concomitant spike in circulating levels during late pregnancy, resulting in increased CAP expression.

With mechanical and estrogenic signalling promoting complimentary increases in gene expression, the myometrium begins to develop its contractility. Through a coordination of CAP expression, focal adhesion formation, cytoskeletal remodelling, and an increase in PG formation and PG/oxytocin receptor production, the changes that occur during this process are critical for a healthy pregnancy. Without proper myometrial regulation, sustained, powerful contractions would never develop enough to expel the growing foetus, or would lead to improper signalling and preterm birth.

1.5.2.2 Human myometrial activation.

The human situation however, differs from most mammals, as there is no observable drop in circulating progesterone levels during myometrial activation. Normal circulating estrogen level increases are observed in late pregnancy, but progesterone levels continue to increase until placental expulsion following labour (Dawood, 1976). It seems that in humans, there are two mechanisms that promote myometrial activation (Zakar & Hertelendy, 2007). First, the overall ratio of circulating progesterone to 17β-estradiol plays an important role with promoting labour and/or preterm birth (Chaim & Mazor, 1998). Additionally, researchers have observed a functional progesterone withdrawal in the myometrium during pregnancy, due to a switch in the type of progesterone receptor available (Allport VC *et al.*, 2001; Mesiano, 2004, Merlino *et al.*, 2007). There are three different human forms of the progesterone receptor (PR-A, B, C), each being regulated differently and having different functions. PR-B has been shown to facilitate the activation of progesterone responsive genes, while PR-A and PR-C function to repress the actions of PR-B through competitive ligand binding (Giangrande *et al.*, 2000; Wei *et al.*, 1996). As such, research has suggested that it is this change in the progesterone receptor ratio that is important in facilitating the functional withdrawal of progesterone signalling (Zakar & Hertelendy, 2007). It has also been shown that specific localization of the various PR isoforms is important in myometrial regulation. Levels of PR-B reportedly increased in the upper fundal area, but not lower segments, of the human uterus, while PR-A levels were constant in both locations (Condon *et al.*, 2006; Messiano, 2004). Besides specific localization of PR isoforms, it has also been shown

that the isoforms can be differentially regulated by various stimuli such as different prostaglandins. $PGF_{2\alpha}$ was shown to selectively increase PR-A, and thus increase the PR-A/PR-B ratio (Madsen *et al.*, 2004).

In addition to PR modulation, functional progesterone withdrawal is facilitated through changes in the expression and actions of the co-regulators (co-activators and co-repressors) of the progesterone receptors (Zakar & Hertelendy, 2007). Like PR, these co-regulators have been shown to vary by location within the uterus as well. During labour, expression of cAMP-response element-binding protein (CREB)-binding protein and steroid receptor coactivators 2 and 3 was significantly decreased in the fundal area of the human uterus. Levels of histone H3 acetylation, which promotes chromatin remodelling and increased access to PR coactivator gene promoters, were decreased at term leading to a functional progesterone withdrawal. In addition, prevention of deacetylation with histone deacetylase inhibitor caused a delay of labour due to a sustaining of progesterone signalling (Condon *et al.*, 2003).

1.5.3 Phase 2 - Stimulation.

Where phase 1 of pregnancy was characterized by weak, uncoordinated muscle contractions, phase 2 is characterized by strong, coordinated contractions that lead to parturition (Gibb & Challis, 2002; Shynlova *et al.*, 2009). These contractions are driven by action potentials that facilitate coordinated contraction due to proper preparation of the myometrial cells themselves (cytoskeleton, gap junction, ion channels, etc...; see section 1.6.4: Mechanical Syncytium). Uterotonins, such as oxytocin, PGs, and CRH, act to

stimulate these contractions. As we have seen, the upregulation of estrogenic and mechanical signaling act to increase not only expression of these molecules, but also stimulate an increase in the expression of their corresponding receptors (OTR, PG receptor; Gibb & Challis, 2002; Shynlova *et al.*, 2009).

Prostaglandins have a critical role in facilitating a progression into labour. This is achieved through an induction of myometrial contractility/motility, as well as through a regulation of membrane components such as Ca^{2+} transporters (Bennett *et al.*, 1987; Challis *et al.*, 2002; Keirse, 1993; Porterfield & White, 2007). Additionally, PGs have been implicated in the maintenance of placental blood flow, the stimulation of cervical ripening, and the activation of the fetal HPA axis (Challis *et al.*, 2000; Challis, 2001; Thorburn, 1992). Due to this, PGE_1 (Misoprostol) and PGE_2 are administered as a labour stimulating agent.

The actions of PGs are mediated through binding to specific cell surface seven-transmembrane G-protein-coupled receptors (Hertelendy & Zakar, 2004). There are 5 types of PG receptor that are named due to ligand type; DP, EP, FP, IP, & TP. Additionally, within each receptor type are multiple subtypes that have different effects and locations. For example, the EP receptor type has 4 subtypes (EP_{1-4}), that have opposing functions. EP_2 and EP_4 facilitate relaxation of the myometrium, while EP_1 and EP_3 facilitate contraction through increasing Ca^{2+} entry into cells and through a decrease in cAMP production. Here exists a simple mechanism for the regulation of prostaglandin function between different tissues, or within the same tissue over time. For example, in the myometrium EP_2 exhibits an increase of expression in the middle of the gestational

period, with a significant decrease towards labour, while FP receptor expression is low for most of gestation, increasing late in pregnancy and labour (Brody-Eppley & Myatt, 1998; Leonhardt *et al.*, 2003). There is also specific regulation of subcellular localization during pregnancy, with EP_1 and EP_4 moving to nucleus at labour (Al-Matubsi *et al.*, 2001; Grigsby, *et al.*, 2006). In humans, myometrial PG receptor expression has been shown to differ throughout the uterus (Astle *et al.*, 2005). Seminal work done by the Wiqvist laboratory utilized human uterine samples from caesarean sections performed prior to and during active labour to investigate contractility differences due to prostaglandins (Wiqvist *et al.*, 1985). For these experiments, they removed small strips of myometrium from the fundal (upper) and isthmus (lower) regions of the uterus and mounted them into a superfusion chamber to measure contractility changes upon addition of prostaglandins. It was shown that prior to spontaneous labour, PGE_2 had a biphasic dose-dependent response (stimulatory then inhibitory) in both areas, while $PGF_{2\alpha}$ had no effect on the fundal tissue but caused contraction of the isthmus tissue. Tissue from myometrium in spontaneous labour however had a completely different response. In this tissue both PGE_2 and $PGF_{2\alpha}$ had a stimulatory effect on fundal myometrium, while in the isthmus myometrium $PGF_{2\alpha}$ had no effect and PGE_2 actually inhibited contraction. Research performed by Astle and colleagues (2005) also showed that PG receptor expression varied by uterine regions. They found that overall, expression of EP2 was significantly higher in the lower isthmus region, while EP3 was significantly higher in the upper fundal region (Astle *et al.*, 2005). Therefore the combination of production of different prostaglandins and prostaglandin receptors, may allow for specific regulation of the contractility of the

upper fundal and the lower/cervical region to facilitate expulsion of the foetus; namely, contraction of the fundus and relaxation of the isthmus (Myatt & Lye, 2004).

Like PGs, oxytocin is involved with both myometrial contraction during labour, but also with stimulation of lactation (Soloff *et al.*, 1979). It is thought to stimulate coordinated, strong contractions, partially in response to stretch of the cervix (Russell *et al.*, 2003). However, oxytocin does not initiate labour, as levels do not rise until after labour begins. In addition, oxytocin knockout mice prepared through homologous recombination delivered pups normally, but were unable to lactate in order to feed them, insinuating that there must exist redundancy in the stimulating action of oxytocin (Young *et al.*, 1996). Unfortunately, these authors focused on neural-derived oxytocin production rather than placental, therefore during reproduction these animals may not be complete knockouts. That said, oxytocin injections have been used to induce parturition in the clinical setting, producing the same response as a normal labour. The mechanism of action of oxytocin involves binding to the oxytocin receptor; a seven transmembrane G-protein coupled ($G_{\alpha q/11}$) receptor that associates with, and signals through, phospholipase C to effect production of IP3 and hence calcium release (Burbach *et al.*, 2006). Oxytocin-receptor signalling can also release other contraction stimulating molecules such as prostaglandins, in the myometrium; likely through MAPK signalling (Molnar *et al.*, 1999; Soloff *et al.*, 2000; Ticconi *et al.*, 1998). Additionally, there exists an estrogen responsive element in the oxytocin gene, and it has been shown that exogenous administrations of estrogen, taken orally as a contraceptive, stimulate significant increases in circulating oxytocin levels by 12 hours after ingestion (Amico *et al.*, 1981). Alternatively, various

uterine stimuli have been shown to change oxytocin receptor (OTR) expression (Ou *et al.*, 1998). Uterine stretch through utilization of a unilaterally pregnant model has been demonstrated to cause an increase in OTR levels within the myometrium, however injections of progesterone prevents this increase, illustrating further the importance of the steroid hormone switch.

The role of CRH is also thought to be one of a stimulator of contraction, due to it causing an increased sensitivity of the myometrium to prostaglandins and oxytocin (McLean *et al.*, 1995; McLean & Smith, 2001). CRH has been suggested to be a clock that regulates the timing of labour. It has been shown that high levels of maternal plasma CRH correlate with pre-term labour, while low levels of CRH correlate with pregnancies that go past the due date (Korebrits *et al.*, 1998). CRH causes an increase in the production of estrogen and cortisol which propagates a self-amplifying positive feedback loop as discussed previously (See section 1.5.2.1: Fetal Genome). Additionally, increases in CRH during parturition stimulate PG production through an upregulation of PGHS-2 and a downregulation of PGDH (See section 1.5.2.1: Fetal Genome).

Through a concerted action of the multiple uterotonins, the uterus achieves the high amplitude, high frequency contractions necessary for successful parturition.

1.5.4 Phase 3 - Involution.

Phase 3 of pregnancy involves the expulsion of the placenta and the subsequent involution of the uterus. This process is primarily controlled by specific effects of oxytocin (Gibb *et al.*, 2006). The role of oxytocin in this phase is essentially a

continuation of its parturition function. Oxytocin stimulated contractions of the uterus after birth act to both detach and expel the placenta, as well as to aid in the recoil and involution of the vastly stretched myometrium. Like labour, this is driven through oxytocin stimulation of calcium release, as well as prostaglandin synthesis (Makino *et al.*, 2007). Prostaglandin $F_{2\alpha}$ has been shown to be associated with, and stimulate, uterine involution (Lindell *et al.*, 1982, Lindell & Kindahl, 1983). The process of uterine involution requires a great increase in tissue reorganization, and is accomplished though induction of matrix metalloproteinases, degradation of the extracellular matrix, and an increase in apoptosis. Due to an increase in myometrial insulin-like growth factor-1 (IGF-1) and IGF binding protein-5 gene expression post-parturition, it is hypothesized that these proteins may stimulate the involution-associated increase in apoptosis similar to that observed in mammary tissue (Shynlova *et al.*, 2009).

It can be observed through the above explanation, that the regulation of pregnancy and parturition is a multifaceted and a complex process. It is regulated by interactions of both mechanical and endocrine signaling processes, and through properly regulating these systems the strong, coordinated contractions of labour that are characteristic of a successful pregnancy can occur at the proper time in fetal development.

1.6 Smooth Muscle Contraction

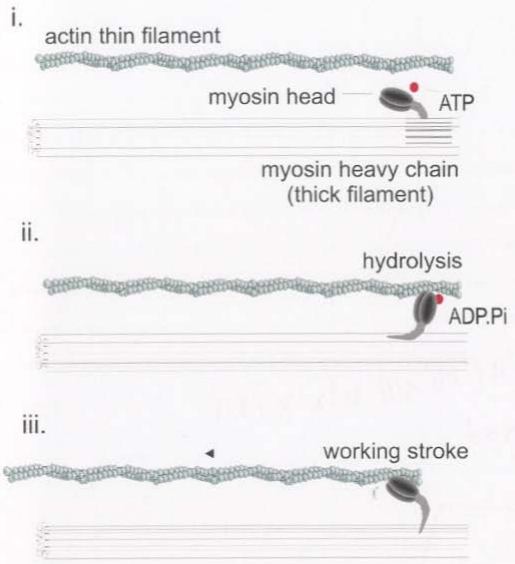
Near the end of term, the myometrial smooth muscle cells begin to change from quiescent cells into ones undergoing spontaneous contraction that have the ability to expel the developed foetus. This change requires a major transformation not only in the

signals being sent to the uterus, but also in the physical structure of the smooth muscle and how they respond to those signals (MacIntyre *et al.*, 2007). The cells gain not only the ability to contract, but to do so in coordination with the rest of the muscle. The structural components of smooth muscle cells are composed of thin filaments (actin), thick filaments (myosin), microtubules/intermediate filaments (e.g. cytokeratin and tubulin), and focal adhesion sites/dense plaques (Figure 1-2; Taggert & Morgan, 2007). These components are divided into two overall categories; one for cellular structural integrity that is composed of non-muscle actin and intermediate filaments, the other for smooth muscle contraction that is composed of actin and myosin (Tessier *et al.*, 2003). Although the contractile phenotype will be the primary focus of the subsequent section, the maintenance of cell shape by intermediate fibres is critical for a normal, non-contractile state.

1.6.1 Smooth muscle structure.

Smooth muscle thin filaments (6-8nm diameter) are a double stranded helical structure (Figure 1-8), comprised of multiple molecules of globular actin (G-actin) that have polymerized together into its filamentous form (F-actin) (Bitar, 2003; Somlyo *et al.*, 1983). Three isoforms of actin have been found in uterine smooth muscle; α, β, and γ-actin. α-actin is the most abundant isoform and expressed at consistent levels through pregnancy, while γ-actin is increased towards labour and has an altered localization pattern (North *et al.*, 1994; Shynlova *et al.*, 2005). Overall, γ-actin expression increased in the circular muscle layer after mid-gestation in only the gravid horn, but remained at a

Figure 1-8
Illustration of the molecular structure of fibrillar actin and myosin and the process of actomyosin cross-bridge cycling.
Actomyosin cross-bridge cycling:
(i) ATP binds to the myosin head in the thick filament where it is
(ii) hydrolysed to ADP and inorganic phosphate (Pi). Myosin heads can then interact with actin thin filaments forming a cross bridge
(iii) Actin-myosin interaction triggers the release of ADP and Pi from the myosin head resulting in a conformational change in the myosin cross bridge and a subsequent 'working stroke' or pulling of the actin filament. The myosin head then releases the actin thin filament and returns to its original confirmation ready to interact with another region of the actin filament.
Modified and used with permission of the author. Based on MacIntyre *et al.*, 2007.

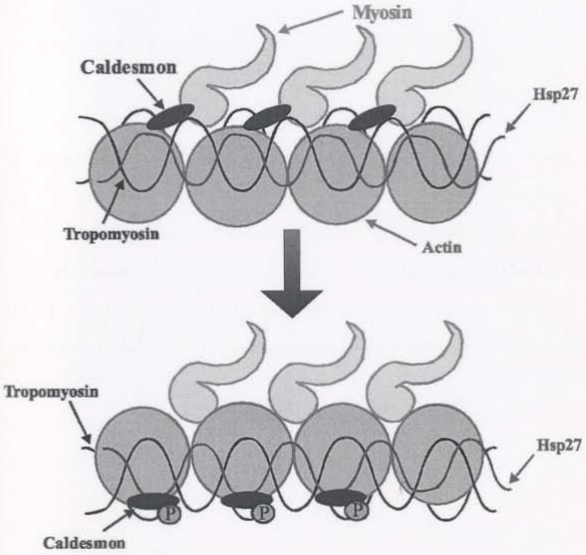

i.
actin thin filament
myosin head
ATP
myosin heavy chain
(thick filament)
ii.
hydrolysis
ADP.Pi
iii.
working stroke

similar expression in the longitudinal layer throughout pregnancy (Shynlova *et al.*, 2005). As well, it was shown that γ-actin localization changed from a cytoplasmic distribution in myometrial cells during early-mid gestation, to a membrane association during late pregnancy. It is suspected that a regulatory mechanism for contraction exists through altering the different isoforms expressed, since the overall actin level in the myometrium does not change over pregnancy (Word *et al.*, 1993). Due to the γ isoform translocating to the membrane of myometrial cells in late pregnancy/labour, it is proposed that it acts to connect the contractile machinery to the focal adhesions in the membrane and thus to the ECM components to allow for proper transmission of force (Shynlova *et al.*, 2005). The β isoform has been shown to be located in the centre of dense plaques and connecting intra and inter-cellular dense plaques, proposing a role of setting cellular connections to facilitate contraction of the other smooth muscle actin isoforms (North *et al.*, 1994).

Smooth muscle myosin thick filaments (15-18nm diameter) are composed of three distinct regions; a "tail", a "neck", and a "head" (Figure 1-8; Alberts *et al.*, 2002). These regions are composed of two heavy chains and two copies of each of two light myosin chains. Each of the heavy chains form a globular "head" domain as well as a long "tail", with the the two tails intertwining with each other to form one large myosin unit. The myosin light chains are comprised of a 17kDa myosin essential light chain (MLC_{17}) and a 20kDa regulatory molecule (MLC_{20}), and contains two domains; an actin binding domain and magnesium-adenosine triphosphate binding site (MgATP) (Guilford & Warshaw, 1998). These chains bind to the "neck" region (region between the head and tail) of each myosin heavy chain to regulate its activation, as the myosin heads cannot bind to actin

without phosphorylation, and hence activation, of the MLC_{20} domain (Webb, 2003). Actin and myosin filaments inter-twine with each other in a manner similar to a striated muscle sarcomere, and facilitate contraction through actomyosin crossbridge cycling (Ali, *et al.*, 2005). Through ATP hydrolysis, the head domains can be activated into a cocked position, bind actin, and contract upon release of ADP. The repeating attachment and release of actin facilitates a ratchet-like "stepping" motion along the thin filaments (Figure 1-8). Activation of the myosin head is dependent on phosphorylation of MLCs by myosin light chain kinase (MLCK), and as such can be deactivated through the actions of the myosin light chain phosphatase (MLCP; Savineau & Marthan, 1997). Actin-myosin interaction can also be regulated to facilitate contraction. The myosin binding site on actin is naturally occupied by a complex of proteins (Figure 1-9) including tropomyosin, caldesmon, HSPB1, and calponin, preventing myosin association (Somara and Bitar, 2006). When this complex of proteins is removed through mechanisms such as an acetylcholine-induced increase in intracellular calcium levels, myosin is able to bind actin and facilitate contraction. Additionally, caldesmon and calponin both inhibit actomyosin ATPase activity, thus impeding the ability of myosin from cycling ATP and contracting. Therefore, removal of caldesmon from the myosin binding site on actin removes this inhibition. As such, it can be observed how these proteins regulate not only actin-myosin interaction, but also myosin cross-bridge cycling. Through this contractile mechanism, smooth muscle can generate more contractile force than striated muscle per cross-sectional area, and with much fewer myosin molecules (Murphy *et al.*, 1974).

Figure 1-9
Association of phospho-caldesmon with HSPB1 results in dissociation of phospho-caldesmon from tropomyosin allowing tropomyosin to slide on actin, exposing myosin-binding sites for sustained contraction. During the relaxed state, caldesmon is bound to tropomyosin and actin. On stimulation, HSPB1 is phosphorylated. Phospho-HSPB1 helps translocation and phosphorylation of PKC. Phospho-PKC may associate with caldesmon and with tropomyosin resulting in PKC-dependent phosphorylation of caldesmon and tropomyosin. On phosphorylation of caldesmon, phospho-PKC dissociates from phospho-caldesmon. Simultaneous association of phospho-caldesmon and phospho-tropomyosin with phospho-HSPB1 results in dissociation of phospho-tropomyosin from phospho-caldesmon. This results in sliding of tropomyosin on actin and thus exposing the myosin-binding sites on actin. The net result is exposing the myosin-binding sites on actin for sustained smooth muscle contraction. From Somara & Bitar, 2006.

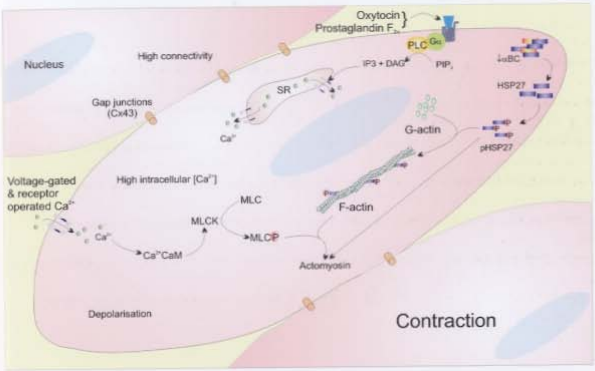

Myosin
Caldesmon
Hsp27
Tropomyosin
Actin
Tropomyosin
Hsp27
P
P
P
Caldesmon

1.6.2 Ca^{2+} regulation.

The contraction of uterine smooth muscle (Figure 1-10), like all muscle types, is primarily regulated by the available intracellular concentrations of the free calcium ion ($[Ca^{2+}]_i$; Monga & Sanborn, 2004; Wray *et al.*, 2001). Increase in $[Ca^{2+}]_i$ is regulated by two means; the amount of influx and the amount of removal (Horowitz *et al.*, 1996). Both of these mechanisms are important, and highly regulated.

Ca^{2+} influx into the cell is through two available stores; extracellular and intracellular (Horowitz *et al.*, 1996). The extracellular entry of Ca^{2+} is mainly facilitated by ion channels and exchange pumps, and can be regulated by either varying Ca^{2+} levels (Ca^{2+} dependent/independent) or alterations to voltage (voltage dependent/independent). The two types of voltage dependent channels that exist in smooth muscle are L-type (long-lasting) and T-type (transient) channels (McDonald *et al.*, 1994). L-type channels have been shown to be increased during pregnancy, and are activated through membrane depolarization. T-type channels are highly expressed in spontaneously active myometrium and are activated by negative membrane potentials due to action potentials. The presence of Ca^{2+} dependent channels has been well documented in the myometrium, and are triggered by release of Ca^{2+} from internal stores (Montell *et al.*, 2002; Young *et al.*, 2001; Yang *et al.*, 2002). Intracellular entry of Ca^{2+} originates with the Ca^{2+} stores present in the sarcoplasmic reticulum (SR; Noble *et al.*, 2009; Sanborn, 2001). Stimulatory agents outside of the cell, such as oxytocin, trigger signal cascades that ultimately open the SR stores into the cytoplasm (Monga & Sanborn, 2004). These molecules bind to G-protein coupled receptors in the membrane and activate phospholipase C (PLC). Active PLC can

Figure 1-10
Contraction in human myocytes. At labour, human myocytes develop high cell-cell connectivity through gap junctions formed primarily by connexin 43 (cx-43). Intracellular Ca^{2+} concentrations rise rapidly via influxes from outside the cell through voltage-gated and receptor operated channels. Contractile agonists such as oxytocin and prostaglandin $F_{2\alpha}$ activate phospholipase C (PLC) initiating the production of inositol triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidyl inositol-3,4,5-triphosphate (PIP_3). This leads to the release of Ca^{2+} from intracellular stores in the sarcoplasmic reticulum (SR). The increased intracellular Ca^{2+} concentration drives the formation of the Ca^{2+}-CaM complex and the activation of MLCK and subsequent phosphorylation of MLC. At the same time, αB-crystallin levels are decreased and HSPB1 phosphorylation increases promoting its relocation to the actin cytoskeleton. Here it stabilises f-actin, and aids in the removal of tropomyosin from it's myosin binding site, enabling it to interact with myosin and drive contraction.
Modified and used with permission of the author. Based on MacIntyre *et al.*, 2007.


Oxytocin
Prostaglandin F2α
PLC
Gαq
PIP2
IP3 + DAG
αBC
HSP27
pHSP27
G-actin
F-actin
Nucleus
High connectivity
Gap junctions
(Cx43)
SR
Ca2+
Voltage-gated
& receptor
operated Ca2+
High intracellular [Ca2+]
MLC
MLCK
MLCP
Ca2+CaM
Actomyosin
Depolarisation
Contraction

then convert phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is able to bind to its receptor in the SR membrane and release the stores into the cell. It is also suspected that stretch activated ion channels also alter Ca^{2+} levels, ultimately opening other voltage gated channels (Slish *et al.*, 2002).

Ca^{2+} removal from a myometrial cell, following contraction, is due first to repolarization of the membrane to stop any further entry, and subsequent removal of the Ca^{2+} present by membrane-bound ATP-dependent Ca^{2+} pumps (Kosterin *et al.*, 1994; Sanborn *et al.*, 2005). Some pumps, such as plasma membrane Ca^{2+}/Mg^{2+} ATPase, are inhibited by contractile stimulants like oxytocin, and stimulated by relaxants (Soloff & Sweet, 1982). Similarly, the SR stores are also refreshed through the actions of a Ca^{2+}-ATPase pumps located in the SR membrane (Wray & Burdyga, 2010). The plasma membrane also contains ion exchangers that bring in sodium to remove Ca^{2+}, but these have a much lower affinity for Ca^{2+} (Morishita *et al.*, 1995; Sanborn, 2000).

Regardless of how Ca^{2+} is increased within the myometrial cell, it ultimately affects contraction via interaction with the Ca^{2+} binding protein calmodulin (Figure 1-10; Gallagher *et al.*, 1997; Sanborn, 2001). Calmodulin binds to four Ca^{2+} that subsequently initiates a conformational change in the protein. Once Ca^{2+}-bound, it is able to interact with multiple other proteins and influence their regulation. MLCK has a natural auto-inhibitory domain that impedes phosphorylation of the protein itself and subsequent activation. The Ca^{2+}-calmodulin complex binds to MLCK to make the phosphorylation site available and aiding activation, thus leading to MLC activation. Additionally,

removal of caldesmon from the myosin binding groove on actin is reportedly facilitated by the Ca^{2+}-calmodulin complex, enabling actin-myosin interaction and contraction (Hemric *et al.*, 1993).

1.6.3 Regulation by phosphorylation.

Protein phosphorylation plays a critical role in the regulation of uterine smooth muscle contraction (Figure 1-10). Many of the proteins involved with facilitation of smooth muscle contraction rely on phosphorylation for activation, some dependent on Ca^{2+} while others are dependent on external stimuli such as 17β-estradiol or uterine stretch.

Most obvious is the activation of MLCK. MLCK is activated by phosphorylation due to Ca^{2+}-dependent protein kinase, and deactivated by protein phosphatase 1 and 2A (Monga & Sanborn, 2004, Nomura *et al.*, 1992). Additionally, the functional state of all of the proteins bound to the myosin binding site on actin are altered due to phosphorylation. Phosphorylation of tropomyosin, caldesmon, calponin and HSPB1 is necessary for making the actin binding site available to myosin (Somara & Bitar, 2006; Winder & Walsh, 1990). All of the regulatory molecules have been shown to be phosphorylated by protein kinase C (PKC), while caldesmon has also been shown to be phosphorylated by mitogen activated protein kinase (MAPK; Foster *et al.*, 2000; Savineau & Marthan, 1997; Somara & Bitar, 2006)

HSPB1 has been implicated as a potential regulator of the entire phosphorylation

event with the actin binding proteins. HSPB1 is primarily phosphorylated by mitogen activated protein kinase activating protein K-2 (MAPKAP K-2) through the p38 MAPK signalling cascade (Dorion & Landry, 2002; Kato *et al.*, 2002). It has been shown that when constitutively phosphorylated, HSPB1 aids in the phosphorylation and translocation of PKCα to actin where it can phosphorylate caldesmon, calponin, and tropomyosin (Patil *et al.*, 2004a; Savineau & Marthan, 1997; Somara & Bitar, 2006). In addition, it has also been proposed that phosphorylated HSPB1 facilitates an inhibition of myosin phosphatase activity by acting as a scaffold protein to increase association of myosin phosphatase, RhoA and ROCK-II. This complex promotes the phosphorylation and deactivation of myosin phosphatase, thus increasing contraction (Patil & Bitar, 2006).

1.6.4 Mechanical syncytium.

Through *in vitro* studies, it was believed that smooth muscle cells acted independently of each other, contracting in a spiral fashion (Warshaw *et al.*, 1987). With that type of organization, force would be unable to be effectively transmitted along the muscle bundle, thus making it very inefficient. In order for coordinated contractions to occur, muscle bundle signalling must be coordinated and contractile forces generated within the cell must be able to be transmitted through adjacent cells (Figure 1-3).

Coordination of muscle cell contraction is achieved through effective transmission of electrical and/or chemical signalling. In myometrial cells, this is facilitated through the formation of gap junctions; areas of connection between cells that form low resistance

bridges (Gilula *et al.*, 1972; Loewenstein, 1981; Lye *et al.*, 1993; MacKenzie *et al.*, 1985). Proteins called connexins are arranged into hexameric hemichannels in the plasma membrane and align themselves with connexins of neighbouring cells, forming a gap junction that allow molecules up to 1000kDa to move between the cells (Risek *et al.*, 1990). The amount of gap junction formations are variable, constantly being regulated. For example, it has been shown that gap junctions are present at a very low level in the myometrium for most of pregnancy, with a huge increase in their number and size just prior to labour (Garfield, 1985; Garfield, 1988; Miller *et al.*, 1989). The main connexin in the myometrium, connexin-43 (cx-43), follows this trend with levels peaking at labour in the rat model (Lye *et al.*, 1993). Many stimuli affect cx-43 expression (positively and negatively), including steroid hormones and mechanical stress (Orsino *et al.*, 1996; Ou *et al.*, 1997, Petrocelli & Lye, 1993). Petrocelli *et al.* (1993) found that administration of exogenous 17β-estradiol increased cx-43 gene expression, while progesterone decreased it, and that expression increased in very late pregnancy to a maximum at labour. Additionally, Ou *et al.* (1997) found that stretch of the myometrium through either presence of a foetus or inserted polyvinyl tubes increased expression of cx-43 gene expression, and that this affect was attenuated by presence of progesterone. This leads the to hypothesis of a possible regulation of muscle connectivity and contraction through regulation of gap junction components (Orsino *et al.*, 1996; Ou *et al.*, 1997).

Recently, investigators have proposed that the arrangement of cytoskeletal components of smooth muscle cells *in vivo* allow for force transmission equally along the

length of a muscle bundle (Kuo & Seow, 2004). For this to occur, cytoskeletal elements within an individual cell must be able to transmit the force from one side of the cell to the other effectively (Figure 1-3). This is achieved with an organization of structural elements between focal adhesions (dense plaques) in the plasma membrane, to the nucleus, and then out to the membrane again on the opposite side. Subsequently, for the force to continue being transmitted, there exists an alignment of focal adhesion sites between neighbouring cells. Forces generated by actin-myosin interaction in one cell can be transferred to those surrounding it. The authors found that cells contained cytoskeletal components that were organized to be parallel to the longitudinal axis of the cell and the muscle bundle. This organization within and between smooth muscle cells of a muscle bundle was refered to as a "functional syncytium", where cells of a muscle bundle act as one to transmit force equally along it (Figure 1-3). This organization of smooth muscle cells to achieve maximal force transmission has begun to be verified in mouse myometrial smooth muscle cells (Wu *et al.*, 2008). Electron microscopy of mouse myometrium revealed that dense plaques of neighbouring cells align on either side of a narrow extracellular cleft, and have an increase expression and activation of dense plaque-associated proteins at term as determined by immunoblot analysis.

1.7 Heat Shock Proteins (HSPs)

Early experiments by Tissières (1974) on salivary glands of Drosophila lead to the discovery of a family of proteins referred to as the Heat Shock Proteins (HSPs). As the

name suggests, they were initially found to be quickly increased in Drosophila larvae in response to mild heat stress. We now know that these proteins are present in most cells of the body, and are highly expressed with a sudden heat shock, or by exposure to any number of other cellular stressors such as oxidative stress, anoxia, exposure to certain metals, altered pH, cytotoxic chemicals, and mechanical stress (Beck *et al.*, 2000; Choi *et al.*, 1991; Hightower, 1991; Tanguay *et al.*, 1993). Due to this response, they are referred to now as "stress proteins". Additionally, some of the more newly characterized stress proteins such as HSPB6 and HSPB11 do not exhibit the classical heat shock response, but do have sequence and functional similarities (Kirbach & Golenhofen, 2010).

Upon exposure to stressors, heat shock proteins are primarily upregulated through stimulation of transcription by a family of proteins known as heat shock factors (HSF; Prahlad & Morimoto, 2009; Rabindran *et al.*, 1991). Although HSFs such as HSF1 are normally associated with HSPs, when cellular stressors cause proteins to misfold HSPs release HSFs and bind the misfolded proteins. Unbound HSFs associates into trimers, translocates to the nucleus, and binds to various heat shock elements (HSEs) in HSP gene promoters. Once bound, HSF can be phosphorylated, thus mediating initiation of transcription (Dokladny *et al.*, 2008; Lindquist *et al.*, 1986; see section 1.8.2.2.1: HSF).

1.7.1 HSP families.

There is much debate on the actual number of families, and members, that make up the heat shock protein groups. Following the guidelines by Kampinga (2009), there are

7 overall families of heat shock proteins that vary by size, structure and function (HSPA-H & DNAJ), with several questionable families omitted (Table 1-1). The actual number of different proteins in these families range from 1 (HSPD) to 50 (DNAJ), representing a wide category of biologically very important proteins. The fact that 50 proteins fall in the same family is evidence that HSPs are generally highly conserved, even between various organisms. For instance, the sequence homology between human and E-coli HSPA is approximately 50% (Hunt & Morimoto, 1985). Additionally, many of these proteins are found to be expressed ubiquitously in cells and often found to be critical for normal cell growth in unstressed cells, giving them a homeostatic role other than just being involved in thermotolerance or cellular stressors (Hendrick and Hartl, 1993, 1995). Another factor that is similar between these proteins is their reliance on, and binding to, ATP (Ellis, 2005). HSPA, C, D, and H all have ATP-binding sites and hydrolyse ATP, which is required for the normal functioning of these chaperones. Even though ATP binding is important for the regulation of HSPCs interaction with accessory proteins, the ATPase activity of the protein is low. The remaining HSPs (HSPB, HSPE, & DNAJ) all lack an ATP-binding site, however ATP does somehow affect the structure of the small heat shock proteins (sHSPs) and their interaction with protein substrates.

1.7.2 Chaperone function.

The HSPs are also known to be molecular chaperones, due to the common chaperone function in both stressed and unstressed cells, that is characteristic of this

Table 1-1: Heat Shock Protein Superfamilies

Family	Previous Names	# of Members	Function
HSPA	HSP70, BiP, Grp75, DnaK	13	Molecular chaperone, assist protein folding, transport and degradation, inhibitor of apoptosis, facilitates immune response
HSPB	small HSP's (12-35 kDa)	11	See Table 1-2
HSPC	HSP90, HtpG, TRAP1	5	Molecular chaperone, assist protein folding, transport, and degradation, glucocorticoid receptor (GR) activation and translocation, inhibitor of apoptosis
HSPD	HSP60, GroEL	1	Chaperonin protein, aids in protein folding and transport
HSPE	HSP10, GroES	1	Chaperonin protein, acts in concert with HSPD
HSPH	HSP100, Clp	4	Molecular chaperone, assist protein re-folding and degradation
DNAJ	HSP40	50	co-chaperone of HSPA, regulation of HSPA ATPase activity.

overall group of proteins. The term molecular chaperone, first used by Ellis in 1987, is best defined as a large and diverse group of proteins that share the property of assisting the non-covalent assembly/disassembly of other macromolecular structures but which are not permanent components of these structures when these are performing their normal biological functions (Ellis, 2005). Molecular chaperone support of protein assembly includes aiding with nascent polypeptide chain folding both while being translated and after being released from the ribosomal machinery, and aiding with the oligomeric formation of polypeptide chains. These proteins also aid with disassembly procedures through multiple mechanisms such as partial unfolding or denaturing of proteins to facilitate their normal interactions, and the degradation or re-solubilization of aggregated or denatured proteins. Examples of this include the maintenance of glucocorticoid receptor in a form receptive to steroid binding through the actions of HSPC, and of the ability of HSPH to re-dissolve insoluble protein aggregates facilitating their degradation (Parsell, 1994; Smith, 2000; Weibezahn, 2005). Cellular stressors, such as heat shock, cytotoxic chemicals, and anoxia, can cause proteins to begin to unfold or prevent proteins from achieving their correct structure. If this is left unchecked, it can lead to protein aggregation that can be potentially fatal to cells. Chaperones are very quickly produced in response to these stressors to interact with proteins that are partially denatured or partially aggregated, and aid in either promoting their re-folding or disposal of these proteins. This quick response is produced via three mechanisms, including altering mRNA synthesis, mRNA stability, and translation efficiency of the chaperone gene products. Molecular

chaperones also facilitate the transport of molecules throughout the cell, and across cellular membranes. There are various other functions of heat shock proteins, however since the focus of this thesis is on the small heat shock proteins, only these family members will be discussed. For an overview of the other heat shock protein families, please refer to the articles in parentheses (Ben-Zvi & Goloubinoff, 2001; Ellis, 2005; Henderson & Pockley, 2005; Horwich *et al.*, 1999; Horwich *et al.*, 2007; Morimoto *et al.*, 1990, 1994; Pratt *et al.*, 2004; Tsan & Gao, 2009).

1.8 Small Heat Shock Proteins

1.8.1 Family members.

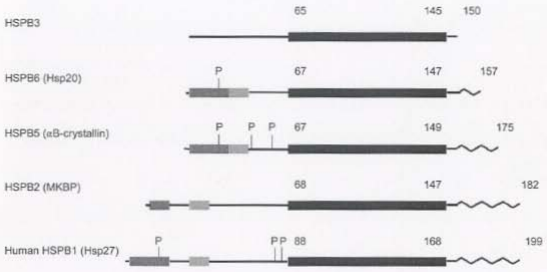
The small heat shock protein (sHSP) family is currently still expanding, with three new additions within the past 10 years. Presently, the mammalian sHSP family is comprised of eleven proteins with sizes ranging from 16-35kDa, although different investigators have different size classifications (Bellyei *et al.*, 2007; Kampinga *et al.*, 2009; Kappe *et al.*, 2003). As with the larger HSPs, the sHSPs were originally named by size or function, but a common standardized naming scheme is now being implemented (Table 1-2). These proteins are found in virtually all tissues of the human body, however some of the family members reside in specific tissues with extraordinary abundance (Up to 3% of total protein; Arrigo & Landry, 1994). For example, HSPB4 & 5 (the α-crystallins) are structural proteins of the lens within the eye and are found in copious amounts therein as originally investigated by Mörner in the 1800's (Mörner, 1894;

Table 1-2: Members of the Small Heat Shock Protein Superfamily

Gene Name	Previous Name	Function
HSPB1	HSP27, HSP25, HSP28	Molecular chaperone through protein stabilization, inhibition of apoptosis, regulation of actin filaments and smooth muscle contraction, cytoprotectant
HSPB2	MKBP (myotonic dystrophy protein kinase-binding protein)	Striated muscle maintenance
HSPB3	HSPL27	Proposed striated muscle maintenance
HSPB4	αA-crystallin	Molecular chaperone, structure protein of eye lens
HSPB5	αB-crystallin	Molecular chaperone, integrity of muscle cytoskeleton
HSPB6	HSP20	Molecular chaperone, muscle relaxation
HSPB7	cvHSP (cardiovascular HSP)	Striated muscle maintenance
HSPB8	HSP22	Molecular chaperone, regulation of cell proliferation and apoptosis, striated muscle regulation, possible cofactor of HSPB1
HSPB9	FLJ27437	Possible testis-specific cancer antigen
HSPB10	ODF1, ODFPG (sperm outer dense fiber protein)	Structural protein in sperm tail cytoskeleton
HSPB11	HSP16.2	Potential molecular chaperone and modulator of apoptosis

Bloemendal & Cate, 1959). Additionally, several of the sHSPs, including HSPB2, HSPB3 and HSPB7, are found in high levels within cardiac smooth muscle, offering stress protection to the heart (Fontaine *et al.*, 2005; Golenhofen *et al.*, 2004; Sugiyama *et al.*, 2000). In contrast to the other HSPs, it is generally accepted that the sHSP family members are not regulated by ATP (Jakob *et al.*, 1993; Van Montfort *et al.*, 2001). However, there are a few studies that show an interaction of ATP with α-crystallin (Biswas & Das, 2004; Muchowski & Clark, 1998). Additionally, molecular chaperone activity is common, it is not a function of every member of the small heat shock protein family (Arrigo, 2007). The singular defining characteristic of the small heat shock protein family is the presence of a conserved domain that is present in every family member (Gusev *et al.*, 2002; Haslbeck *et al.*, 2005; Kostenko & Moens, 2009). The α-crystallin domain is a stretch of 80-100 amino acid residues that are located in the C-terminal part of the protein (Figure 1-11). Homology of this region varies by family member, but ranges from 20-60% in mammals (Berengian *et al.*, 1999; de Jong *et al.*, 1998). This region contributes to the key observed characteristic of small HSPs; the formation of large oligomeric structures. However other regions, such as the variable N-terminal domain, have also been shown to play an important role (Chen *et al.*, 2010; Leroux *et al.*, 1997; Muchowski *et al.*, 1999; Wistow, 1985). The tertiary and quaternary structure of the sHSPs often determines the functional role that the molecule undertakes. Since these other regions are not observed in all sHSP family members, they will be discussed specifically with respect to HSPB1.

Figure 1-11
Scheme of the structure of several representatives of the family of small heat shock proteins. The dark shaded area marks the so-called WDPF-domain, the light shaded area marks conservative region in the N-terminal part of sHSP and the black area denotes conservative alpha-crystallin domain. P, the sites of phosphorylation; zigzag, flexible C-terminal region. Figures above the scheme denote number of amino acid residues. Gusev *et al.* 2002. Reproduced with permission of the author.


HSPB3
65
145
150
HSPB6 (Hsp20)
P
67
147
157
HSPB5 (αB-crystallin)
P
P
P
67
149
175
HSPB2 (MKBP)
68
147
182
Human HSPB1 (Hsp27)
P
P
P
88
168
199

1.8.2 HSPB1.

HSPB1, formerly HSP27, is one of the earliest identified and most investigated small heat shock protein, and has one of the largest heat shock responses. It is a 27kDa protein comprised of 205 amino acids, of which serine 15, 78, and 82 can be phosphorylated; serine 15 and 86 in rodents (reviewed by Kostenko & Moens, 2009). HSPB1 has been found in all species investigated and has a relatively high sequence homology among these species (Franck, 2004; Kostenko & Moens, 2009). Since it has been investigated so much over the years, much of the structure, function, and overall classification of the small HSPs comes from results found with HSPB1.

1.8.2.1 Structure.

The monomeric structure of HSPB1 is composed of 5 main domains (Figure 1-11); WDPF, α-crystallin, conserved N-terminal, variable, and extreme C-terminal domains (Gusev *et al.*, 2002; Thériault *et al.*, 2004; Salinthone *et al.*, 2008). As previously mentioned, the C-terminal domain is primarily composed of the highly conserved, and characteristic, α-crystallin domain located at residues 87-168. Through the use of fluorescence resonance energy transfer with various truncated forms of HSPB1, Bova and colleagues (2000) found that removing the majority of the N-terminal end and leaving primarily only the α-crystallin domain of HSPB1 caused the protein to exist in small multimeric groups of 3-4 subunits. However when only a small portion of the N-terminal region was excised, oligomeric formation was very common. This structural

control of unit interactions has also been shown through similar studies on αA-crystallin and bacterial sHSPs such as HSP16.3, and HSP16.5 (Koteiche *et al.*, 2002). Using site-directed spin labeling, Mchaourab *et al.* (1997) found that the α-crystallin domain of HSPB1 forms into β-strands that interact between oligomeric subunits. These β-sheet structures interact together to form stable dimers that further aggregate together to form tetramers (Mchaourab *et al.*, 1997). Upon tetramer formation, they will often form large oligomers of up to 6 tetrameric units, however with just α-crystallin input, they are unstable (Bova *et al.*, 2000). Due to the conserved nature of the α-crystallin domain, HSPB1 is able to form heterodimers not only with other sHSPs (HSPB5, B6, B8), but also with some of the large heat shock proteins (Brophy et al.,1999; Kato et al., 1994a; Pipkin et al., 2003). This illustrates the redundancy built into the overall HSP family and the molecular chaperone function.

Stability of the large oligomeric structures of HSPs is facilitated by the WDPF domain (Bova *et al.*, 2000; Theriault *et al.*, 2004). Located at the N-terminal end on residues 5-19, it is composed of a WDPF motif (residues 16-19, W - tryptophan, D - aspartic acid, P - proline, F - phenylalanine) that sometimes repeats, and a serine phosphorylation site at Ser-15. In addition to the WDPF motif and phosphorylation site, the entire domain has been found to be necessary for chaperone activity and thermotolerance (Thériault *et al.*, 2004). This functional role is a result of the effect on oligomeric formation and stability. The WDPF domain provides an extra bonding event between it and the α-crystallin domain during oligomerization to facilitate a stable

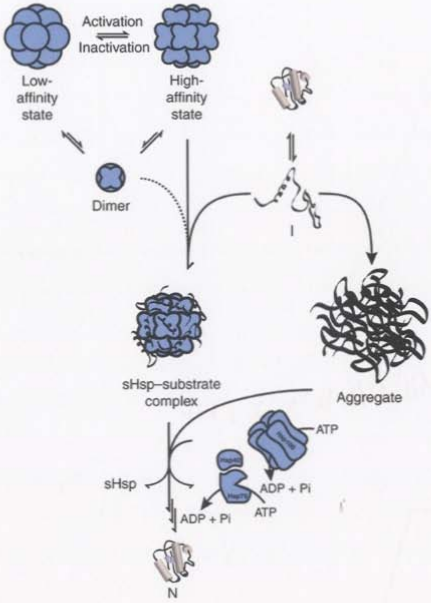
oligomeric structure (Bova *et al.*, 2000; Theriault *et al.*, 2004). Following the WDPF domain, there exists a short variable sequence containing a highly conserved sequence near the C-terminal end, with the primary structure PHSRLFDQAFGLPRL in human HSPB1 (NCBI, n.d.; Sugiyama *et al.*, 2000; Suzuki *et al.*, 1998). In humans, there are two additional phosphorylatable serines, located on sites Ser-78 and Ser-82 (Landry *et al.*, 1992). However, in the rat and mouse only one other phosphorylation site exists at Ser-86, with an absence of the corresponding Ser-78 residue (Lambert *et al.*, 1999). Since the three serine phosphorylation sites on human HSPB1 are located near these attachment domains, their phosphorylation causes a change in the interaction of these domains and thus impacts oligomeric structure. The outcome, is that HSPB1 dissociates from an oligomeric structure into smaller units when phosphorylated (Bitar, 2002; Gaestel, 2002; Gusev *et al.*, 2002; Kato *et al.*, 2002).

The final domain of HSPB1 is located after the α-crystallin domain, at the extreme C-terminal end. The domain is not well conserved across family members, and forms a flexible "tail" on the protein. Some experimental evidence indicates that this domain may not be essential for oligomeric formation, but potentially impacts secondary and tertiary structure (Lindner *et al.*, 2000). However, studies on other sHSPs have pointed to a critical role in oligomer stability (Kim *et al.*, 1998; van Montfort *et al.*, 2001). In addition, removal of this "tail" decreases the chaperone function with respect to some target proteins, leading to a potential role of the negatively charged C-terminal tail acting as the domain in HSPB1 that binds to misfolded proteins when HSPB1 undertakes a

chaperone function (Lindner *et al.*, 2000).

Despite this relative wealth of information, the overall structure is still relatively unknown, due to the current inability to form crystals of HSPB1 for x-ray crystallography. The crystal structure however, for HSP26 of *Saccharomyces cerevisiae* and HSP16.9 of *M. jannaschii* has been determined (Haslbeck *et al.*, 1999; Kim et al., 1998). Due to sequence similarities, this structural information has been used to estimate the oligomeric structure for mammalian HSPB1, and it is thought to be a hollow 600kDa ball held together by a number of interacting forces. The α-crystallin domain β-sheets initially are thought to promote dimerization of monomers, which further joins to form tetramers, and proceeding into an amalgamation of 6 tetramers to form the oligomer (Mchaourab *et al.*, 1997). The oligomeric structure is stabilized through an interaction of the WDPF domain with the exposed β-sheet structure on the α-crystallin domain (Bova *et al.*, 2000). The outside of this hollow ball is dotted with flexible C-terminal tails, that act as hydrophobic attachment points to attract unfolded or aggregated proteins (Haslbeck *et al.*, 2005; Lindner *et al.*, 2000). The proteins then bind to the protruding tails and coat the outside of the oligomer (Figure 1-12). It has been shown that up to 12 proteins can bind to one 24mer oligomer of HSPB1 (Lee *et al.*, 1997). Once bound to HSPB1, these proteins can be presented to other large HSPs that would then promote proper refolding (Ehrnsperger *et al.*, 1998). The importance of this tail domain has been shown in studies involving truncated HSPB1 proteins (Lindner *et al.*, 2000). Removal of the tail prevents HSPB1 from being able to interact with certain protein substrates such as α-lactalbumin. It has

Figure 1-12
Model for the chaperone function of HSPB1. Misfolded proteins are bound to the exterior of HSPB1 oligomers, thus preventing aggregation and degradation. These proteins are then presented to other molecular chaperones (large HSPs) to facilitate protein native-state refolding.
From Haslbeck *et al.*, 2005. Reproduced by permission of the author.


Activation
Inactivation
Low-affinity state
High-affinity state
Dimer
I
sHsp–substrate complex
Aggregate
ATP
sHsp
ADP + Pi
ATP
ADP + Pi
N

also been shown that smaller dimeric structures do have some chaperone activity, albeit greatly reduced; dropping from nearly 100% to as low as 10% (Rogalla *et al.*, 1999).

1.8.2.2 Regulation.

1.8.2.2.1 HSF.

The key regulator of *HSPB1* expression in response to cellular stressors such as heat shock or cytotoxic events, is a family of proteins called Heat Shock Factors (HSF) (Anckar & Sistonen, 2007; Prahlad & Morimoto, 2009; Wu *et al.*, 1990). In unstressed conditions, HSF's are in a transcriptionally inactive monomeric form, and are located within the nucleoplasm and cytosol as shown through immunofluorescence by Sarge *et al.*(1993). In this state, they are associated with a variety of proteins, including heat shock proteins such as HSPA and HSPC (Abravaya *et al.*, 1991; Mosser *et al.*, 1993; Zou *et al.*, 1998). Upon cellular stress such as heat shock, the cellular proteins begin to un-fold, mis-fold or aggregate. HSP's bind to these mis-folded proteins, thus freeing the bound HSF's. This allows the HSF's to bind each other to form an active trimeric structure that is often phosphorylated (Hensold, 1990; Sorger, 1990; Westwood *et al.*, 1991). In this active, multimeric, phosphorylated state, HSF relocates to the nucleus, and binds to the Heat Shock Element (HSE) which is composed of a repeat (minimum of three) of a pentameric sequence (nGAAn) located in the promoter sequence of heat shock proteins upstream of the transcriptional start point (Dokladny *et al.*, 2008; Sarge *et al.*, 1993; Wilkerson *et al.*, 2007). The actual mechanism of initiation of transcription is still unknown, however it is

proposed to be accomplished through the freeing of stalled polymerases thus allowing them to continue (Anckar *et al.*, 2007). This may be accomplished through interaction with the TATA Binding Protein thus releasing the polymerase, or through other factors bound to the trimeric HSF stimulating transcription at the nearby start site (Yuan *et al.*, 2000). Once the levels of misfolded proteins are decreased, HSF's can once again bind to HSP's leading to a decrease in the activated trimeric form. (Lindquist, 1986; Morimoto, 1998).

There exist four variants of the HSF family in vertebrates (HSF1-4). However, due to HSF3 being avian specific, humans have three members of the HSF family (HSF1, 2, &4; Pirkkala *et al.*, 2001). These three HSF's are regulated differently with differing effects on HSP production. HSF1, known solely as HSF for years, is the most researched factor and is the homologue of the HSF found in Drosophila and yeast (Sarge *et al.*, 1993). Seminal research by Sarge and colleagues investigated expression, localization, and regulation of mammalian HSF's using 3T3 (mouse fibroblast) and HeLa (human cervical cancer) cells. They initially showed that both HSF1 & HSF2 have a constitutive DNA binding activity, and that HSF1 was the dominant DNA binding factor. Sarge and colleagues also illustrated that heat shock differentially affects HSF1 post-translationally in mammalian cells through phosphorylation; there was a change in the observable size of the HSF1 protein on western blots when heat shocked, while no change was observed with HSF2. In addition to heat shock associated phosphorylation, oligomerization of HSF1 was also changed in heat shocked cells, and shown to be important for regulation

of HSE activation (Takemori, *et al.*, 2009). The most accepted explanation of HSF1 sub-cellular localization is that it moves from being distributed throughout the cell to a primarily nuclear localization upon heat shock (Sarge *et al.*, 1993). However, some researchers show that HSF1 is predominantly a nuclear protein, and that the cytoplasmic localization results are caused by specific extraction protocols (Mercier *et al.*, 1999).

Researchers have therefore determined that HSF1 is the traditional HSF that is activated by different forms of stress, and that it is the primary factor affecting HSPB1 production in response to heat shock. (Clos *et al.*, 1990; Ito *et al.*, 1996; Quivy *et al.*, 1996; Sarge *et al.*, 1993; Trinklein *et al.*, 2004; Zhong *et al.*, 1998; Zimarino *et al.*, 1987). Trinklein and colleagues (2004) found through microarray and ChIP experiments involving stressed and unstressed human erythroleukemic cells that HSF1 is the primary factor bound to the HSE of *HSPB1* in stressed cells, thus promoting its production. HSF2 however, is primarily stimulated by the developmental conditions associated with mitosis, as illustrated by Wilkerson and colleagues (2007). They found binding of HSFs to HSE in Jurkat cells (immortalized human T lymphocyte cells) undergoing mitosis was not mediated by the classical stress stimuli (Sarge *et al.*, 1993). In addition, blocking HSF2 in mitotic cells through siRNA application resulted in a decrease in HSPB1 production, thus illustrating that HSF2 binds the HSE of *HSPB1* and plays a role in it's developmental expression (Wilkerson *et al.*, 2007).

1.8.2.2.2 Hormone regulation.

Estrogen increases *HSPB1* gene transcription through a varying mechanism involving an SP1 binding site (Kita *et al.*, 2009; Porter *et al.*, 1996; Safe & Kim, 2008). It was initially discovered through research by Edwards and Adams on MCF-7 cells (an estrogen-responsive breast cancer cell line) that estrogen treatment elicited an increase in the expression of a 24 kDa protein that later proved to be HSPB1 (Adams *et al.*, 1980; Edwards *et al.*, 1980). Since then research has been done attempting to fully elucidate the regulatory mechanism that steroid hormones, such as estrogen, utilize in regulation of HSPB1, all primarily conducted in the MCF-7 cell line (Ciocca, 1983; Edwards *et al.*, 1981; Mendelsohn *et al.*, 1991). However, it was not until sixteen years later that the means of estrogen activation was fully understood through research on the promoter of *HSPB1* conducted by the Safe lab (Porter *et al.*, 1996). These researchers utilized competitive protein-binding experiments in conjunction with gel mobility shift assays, as well as cell transfection with mutant versus wild type estrogen responsive elements (ERE) and SP1 oligonucleutides, to understand the mechanism of *HSPB1* initiation. They found that the estrogen binding site on *HSPB1* was not a traditional complete sequence. Like the cathepsin D promoter, the *HSPB1* promoter had a half-palindromic 17β-estradiol response element separated by 10 nucleotides from a SP1 binding site. They illustrated that ER and SP1 bind in a complex to these binding sites in order to facilitate estrogen-induced *HSPB1* gene expression.

Estrogen stimulation of HSPB1 has been shown in a variety of cell types

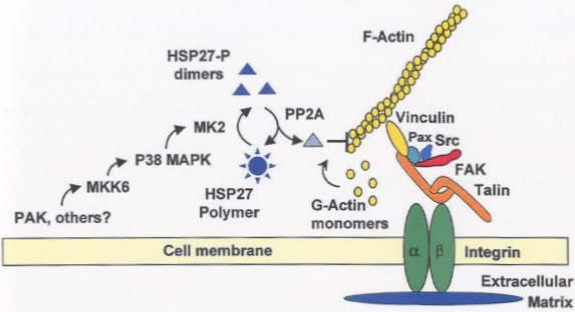
including breast cancer cells, small intestine smooth muscle, neuroblastoma cells, and bone osteoblast cells (Cooper *et al.*, 2000; Edwards *et al.*, 1981; Manthey *et al.*, 2006; Porter *et al.*, 1996; Shimomura *et al.*, 2006). In the past several years, additional means of regulation though possible direct interactions of HSPB1 with the nuclear estrogen receptors (ERα & ERβ) have been reported (Al-Madhoun *et al.*, 2007; Chen *et al.*, 2008; Miller *et al.*, 2005; Rayner *et al.*, 2008). Miller and colleagues (2005) utilized a yeast two hybrid assay to locate and subsequently sequence proteins that associate specifically with Erβ, which was further verified through immunoprecipitation and western blot analysis of HSPB1 and ERβ proteins. This association of HSPB1 with estrogen receptors and/or the hormone itself was observed to be dependent on the existence of a phosphorylated state (Al-Madhoun *et al.*, 2007). Immunoprecipitation of transfected, mutated, and truncated forms of HSPB1 showed that only the wild-type and constitutive phosphorylation mimic forms pull down ERβ, illustrating that phosphorylation is essential for interaction. Immunofluorescence experiments also supported the IP data, as it was observed that the phosphorylation-mimic HSPB1 exhibited a pattern of expression and response to 17β-estradiol treatments similar to WT, while the non-phosphorylation mimic had a different response. The localization of these mimics was also shown to differ with heat shock versus estrogen treatment, thus illustrating a differential stress response mediated though changes in phosphorylation state.

1.8.2.2.3 Phosphorylation.

Phosphorylation of HSPB1 is an event that is critical to both HSPB1 structure and function. As previously stated, human HSPB1 has three phosphorylation sites located on serines 15, 78, and 82, while the rat (our utilized animal model) only has two located at serine residues 15 and 86 (Kostenko & Moens, 2009). The rat serine 86 residue is analogous to the human serine 82, while the human serine 78 is not present in the rat protein sequence. Through phosphorylation studies starting with Benndorf and colleagues (1992), the primary phosphorylation pathway was discovered to be through the p38 mitogen activated protein kinase (MAPK) signalling pathway (Figure 1-13; Dorion & Landry, 2002; Kato *et al.*, 2002). This was established in part from the finding that after abolishing the phosphorylation activity of most other enzymes in Ehrlich ascites tumour cells, including cAMP-dependent protein kinase or protein kinase A (PKA), protein kinase C (PKC), cGMP-dependent protein kinase (PKG), and Ca^{2+}/calmodulin-dependent protein kinase (CaMK), the authors still could not fully inhibit HSPB1 phosphorylation (Benndorf *et al.*, 1992).

Cellular events trigger and activate (phosphorylate) MAPK, which directly phosphorylates mitogen activated protein kinase activating protein kinase-2 (MAPKAP K-2). Once phosphorylated and activated, MAPKAP K-2 directly phosphorylates HSPB1 (Stokoe *et al.*, 1992a). The importance of MAPKAP K-2 has been shown in various studies (Azuma *et al.*, 2001; Kayyali *et al.*, 2002; Pietersma *et al.*, 1997; Shi *et al.*, 2003; Vertii *et al.*, 2006). For example, Azuma and colleagues (2001) reported that inhibiting

Figure 1-13
Signal transduction pathway for regulating HSPB1 phosphorylation and interaction of HSPB1 with actin filaments. Signals to the p38 MAPK pathway are thought to culminate in phosphorylation of HSPB1 polymers by MK2, causing dissociation of the polymers to HSPB1 dimers or monomers. Phosphorylated HSPB1 is a substrate for type 2 phosphatases (PP2A), but the details of this pathway at the level of actin filaments in forming focal contacts are unknown. Actin capping activity of the nonphosphorylated HSPB1 dimer/monomer has been shown, and phosphorylation of HSPB1 is known to favor formation of F actin at various sites and to promote formation of focal contacts. From Gerthoffer & Gunst, 2001.


F-Actin
HSP27-P
dimers
PP2A
MK2
Vinculin
Pax
Src
P38 MAPK
FAK
MKK6
Talin
PAK, others?
HSP27
Polymer
G-Actin
monomers
Cell membrane
α
β
Integrin
Extracellular
Matrix

p38 activation with 1μM of SB-203580 abolished shear stress activation of MAPKAP K-2 and HSPB1 in endothelial cells, and was duplicated through transfection of dominant negative forms of p38-α into cells. Vertii and colleagues (2006) utilized cells derived from MAPKAP K-2 deficient mice to show that phosphorylation through the MAPKAP K-2 pathway is essential not only for stress-dependent HSPB1 phosphorylation, but also for the cellular stress response itself.

There are a plethora of stimuli that regulate phosphorylation of HSPB1 apart from MAPKAP K-2, and it has been shown *in vivo* and *in vitro* in smooth muscle cells that it is through multiple kinases. The list of enzymes that phosphorylate or are suspected to phosphorylate HSPB1 on the three serine residues include MAPKAP K-3, MAPKAP K-5, PKA, Akt/protein kinase B (PKB), PKC (specifically the δ isoform), Protein kinase D, PKG, Ribosomal protein S6 kinase II ($p70^{RSK}$), CaMK, and apoptosis signal-regulating kinase (Benndorf *et al.*, 1992; Butt *et al.*, 2001; Evans *et al.*, 2008; Gaestel *et al.*, 1991; S. Y. Huang *et al.*, 2006, 2007; Konishi *et al.*, 1997; Maizels *et al.*, 1998; McLaughlin *et al.*, 1996; Ni *et al.*, 1998; Peters *et al.*, 1999; Stetler *et al.*, 2008). Through the use of radio-labeled serine probes with stressed HeLa or MCF-7 cells, Landry and colleagues (1992) have shown that there are three phosphorylation sites within human HSPB1, with the major phosphorylation site *in vivo* being serine-82. Although HeLa cells treated with chemical (arsenite) and thermal stressors were primarily phosphorylated on serine-82, minor sites serine-78 and serine-15 were phosphorylated to a lesser extent as detected through western blot and HPLC analysis (Landry *et al.*, 1992).

Often, proteins with multiple phosphorylation sites are phosphorylated sequentially (Gardner & Montminy, 2005). It has been shown however, that phosphorylation of the three serine residues of HSPB1 does not follow a sequential order (Landry *et al.*, 1992). Therefore each of these sites may be phosphorylated independently and promote different functional roles. Lambert and colleagues, using chinese hamster *HSPB1* and creation of point mutation constructs that mimic the constitutively phosphorylated (serine 15 and/or serine 90 replaced with glutamate) or nonphosphorylatable (serine 15 and/or serine 90 replaced with alanine) forms of the protein, showed that phosphorylation of all serine residues led to formation of the monomeric structure (Benndorf *et al.*, 1994; Lambert *et al.*, 1999). However phosphorylation of just serine 90 (human serine 82) promoted formation of the dimeric form of the protein, while unphosphorylated led to a high molecular mass oligomeric structure (Kato *et al.*, 1994b, 2002). Since a large oligomeric state is related to is role as a molecular chaperone (Figure 1-12), phosphorylation of serine 82 (rat - serine 86, hamster - serine 90) may be the primary regulator of chaperone function, while phosphorylation of serine 15 may facilitate the activation of cytoskeletal functions due to promotion of a monomeric state (Jakob *et al.*, 1993).

HSPB1 phosphorylation has been shown to be reversible, and so it may also be regulated by dephosphorylation. A multitude of studies have been performed over the years to elucidate the phosphatases that act on HSPB1. The major phosphatase involved with HSPB1 seems to be protein phosphatase 2A (PP2A) (Berrou & Bryckaert, 2009;

Cairns *et al.*, 1994; Tar *et al.*, 2006). Cairns and colleagues (1994) initially discovered PP2A action through the use of a wide range of known phosphatase inhibitors to assess their effectiveness at decreasing HSPB1 phosphorylation. This has been verified by subsequent investigators who have shown that over expressing PP2A abolishes HSPB1 phosphorylation in response to stressors (Tar *et al.*, 2006). In addition, not only does PP2A abolish HSPB1 phosphorylation, but it has also been shown to dephosphorylate and inactivate MAPKAP K-2 (Stokoe *et al.*, 1992b). Therefore PP2A may act directly by dephosphorylating HSPB1 and indirectly by deactivating MAPKAP K-2, thus preventing it from phosphorylating HSPB1. It has also been shown that PP2B may have some ability to dephosphorylate HSPB1, although to a lesser amount, while no PP1-dependent HSPB1 dephosphorylation has been observed (Cairns *et al.*, 1994).

1.8.2.2.4 Mechanical regulation.

Stretch and mechanotransduction has been shown to upregulate expression and post-translational modification of a variety of proteins (See section 1.4: Mechanotransduction). Several studies have demonstrated an increase in HspA (Hsp70) expression in epithelial cells in response to stretch, yet very little is known about the regulation of HSPB1 by mechanical stress (Copland & Post, 2007; Luo *et al.*, 2007). There has also been a virtual absence of similar investigations in smooth muscle, but a couple of studies have been done using epithelial and skeletal muscle cells. In these experiments, increases in HSPB1 expression were promoted by stretch of epithelial cells

and heavy resistance training (electrical stimulation) of skeletal muscle (Luo *et al*, 2007; Murlasits *et al.*, 2005).

1.8.2.3 Functions.

In addition to the canonical molecular chaperone function, HSPB1 has several other important functions that will be discussed in the following sections. These range from stress survival to muscle contraction.

1.8.2.3.1 Regulation of the actin cytoskeleton.

HSPB1 regulates actin cytoskeletal dynamics through direct interaction as well as regulation of actin binding proteins (Liang & MacRae, 1997). This role of HSPB1 is critical for the normal functioning of smooth muscle contraction, as well as cell division, migration, and attachment (Bitar, 2002; Lee *et al.*, 2008; Piotrowicz *et al.*, 1998; Schneider *et al.*, 1998). It was first observed that HSPB1 and actin colocalize *in situ* in multiple muscle types, including cardiac, skeletal and smooth muscle (Benndorf *et al.*, 1994; Bitar, 2002; Ibitayo *et al.*, 1999; Lutsch *et al.*, 1997). Subsequently, it was shown that not only is there an association of HSPB1 and actin, but that HSPB1 regulates the formation of filamentous actin (Figure 1-13; An *et al.*, 2004; Bitar, 2002; Gerthoffer & Gunst, 2001; Gusev *et al.*, 2002; Miron *et al.*, 1991; Rogalla *et al.*, 1999). While investigating the actin-modulating properties of vinculin through chromatography, researchers discovered a variety of "contaminating" proteins bound to vinculin that were

possible mediators of the vinculin-actin interaction (Evans *et al.*, 1984; Wilkins & Lin, 1986). In 1988, Miron and colleagues purified one of these proteins (25kDa) and found that when it was added to solutions of G-actin it would reduce the amount of actin polymerization, as detected through measurements of low-shear viscosity. They later confirmed through sequence similarity and heat-shock response, that this protein was HSPB1 (Miron *et al.*, 1991). It was hypothesized that the method of action was through capping of the barbed end of an actin filament (Bendorf *et al.*, 1994). Through investigation of actin polymerization by spectroscopy and electron microscopy, Benndorf and colleagues (1994) illustrated that in an unphosphorylated, monomeric state, HSPB1 was able to inhibit the polymerization of actin filaments through binding to the leading edge of the filament. However, HSPB1 phosphorylation released this inhibition, allowing stimulation of F-actin formation to occur (Lavoie *et al.*, 1993b). Pichon (2004) provided additional evidence for this conclusion *in vivo* through investigation of smooth muscle cell migration. Through immunoflourescent localization of HSPB1 and actin within the lamellipodia of migrating smooth muscle cells, these authors showed that non-phosphorylated HSPB1 localized to the leading edge of lamellipodia, while phosphorylated HSPB1 was localized to the base of these structures. Since branching of actin is required for proper movement of lamellipodia, this finding reinforced the assertion that HSPB1 acts as an actin capping protein to help create these barbed ends and branching points. Pichon also proposed that in its phosphorylated form, HSPB1 could facilitate stabilization of F-actin away from the leading edge through association with

tropomyosin. In this way, signal transduction pathways that result in the activation of p38 MAPK (mechanotransduction through integrins) would be able to regulate actin cytoskeletal dynamics and thus smooth muscle contraction, through phosphorylation of HSPB1 (Figure 1-13; Gerthoffer & Gunst, 2001; Hedges *et al.*, 1999; Landry & Huot, 1995).

Recently however, studies have proposed that the method of HSPB1-induced regulation of actin polymerization is through interaction with the G-actin subunits as opposed to the actin filaments (During *et al.*, 2007). These authors performed *in vitro* actin nucleation and assembly assays to ascertain how HSPB1 affected actin assembly. They found that preincubation of HSPB1 with actin monomers, rather than with actin nuclei with open barbed ends, significantly decreased the rate of actin assembly. In addition, phosphorylation released actin monomers from HSPB1, thus allowing fibre formation. These conclusions lead them to postulate that it is through HSPB1 association with monomeric actin as opposed to capping of actin barbed-ends that facilitates regulation of actin polymerization, and helped to explain their *in vivo* results illustrating the shuttling of HSPB1 within migrating HeLa cells.

In addition to capping, HSPB1 also regulates actin cytoskeleton through filament stabilization and interactions with actin-binding proteins. This is illustrated in one study through experiments involving denaturation and/or aggregation of F-actin and HSPB1 (Pivovarova *et al.*, 2005). Addition of HSPB1 to solutions of F-actin prevented aggregation of this protein when heat-denatured. This fits with the established chaperone

role of HSPB1 in binding denatured proteins, preventing degradation and promoting refolding. Recently, through pull-down experiments with mouse fibroblast cells using GST-tagged actin-regulating proteins, it was shown that HSPB1 binds to the 14-3-3 protein and that this interaction is phosphorylation dependent (Vertii *et al.*, 2006). It was hypothesized that phosphorylated HSPB1 downregulates 14-3-3 expression. In muscle cells, cofilin in an active unphosphorylated form binds to G and F-actin and promotes depolymerization of the negative ("-") end of filaments, leading to actin remodelling (As reviewed by Gaestel, 2006). When cofilin is phosphorylated, 14-3-3 binds to it and acts to prevent cofilin from being dephosphorylated, thus promoting stress fiber formation. However, since phosphorylated HSPB1 binds to 14-3-3, it was hypothesized that this prevents 14-3-3 from stabilizing phosphorylated cofilin. Through this mechanism, and through interaction with actin and other regulating proteins, HSPB1 promotes actin stabilization.

1.8.2.3.2 Actin/myosin interaction (facilitation of contraction).

Bitar and colleagues have shown that HSPB1 plays an important role in actin-myosin interaction and thus contraction of colonic smooth muscle (Bitar, 2002). Using transfection of constitutively phosphorylated and constitutively unphosphorylated forms of *HSPB1* in extracted colonic smooth muscle cells, the authors showed through immunoprecipitation experiments that phosphorylated HSPB1 increased the association of actin and myosin while unphosphorylated decreased this interaction.

The common pathway of facilitation of actin-myosin interaction is through regulation of crossbridge cycling, through two different mechanisms. One mechanism is through HSPB1 interaction with RhoA protein (Patil *et al.*, 2004a; Patil & Bitar, 2006). In colonic smooth muscle cells, HSPB1 was shown to bind to RhoA and to ROCK-II proteins during acetylcholine-induced contraction (Patil *et al.*, 2004b). This in effect caused an upregulation of the associated signalling pathway through HSPB1 facilitation of the interaction of RhoA/ROCK-II with the 130-kDa regulatory myosin-binding subunit (MYPT; Patil & Bitar, 2006). The result of this was phosphorylation of MYPT, potentially through PKCα, leading to inhibition of myosin phosphatase activity and a sustained phosphorylation of myosin light chain (MLC; Patil & Bitar, 2006). This sustained activation led to an increase in actin/myosin interaction and contraction.

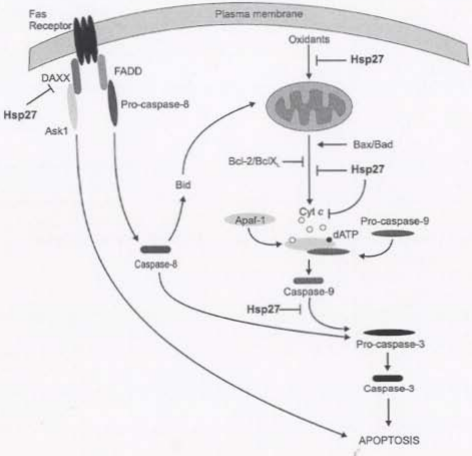
The second mechanism by which HSPB1 facilitates actin/myosin interaction is through regulation of the availability of the myosin binding site on actin (Bitar, 2002; Somara & Bitar, 2004, 2006). As previously described (See section 1.6.1: Smooth Muscle Structure and section 1.6.3: Regulation by Phosphorylation), when muscles are at rest the myosin binding groove on actin is occupied by tropomyosin, thus preventing actin-myosin interaction. With a rise in intracellular $[Ca^{2+}]_i$, tropomyosin is displaced, opening up the binding site (Reviewed by Marston & El-Mezgueldi, 2008). In skeletal and cardiac muscle, this displacement is facilitated by troponin (Reviewed by Gordon *et al.*, 2000). Troponin, a Ca^{2+} sensitive protein, binds to Ca^{2+} and displaces tropomyosin (Poole *et al.*, 2006). In smooth muscle however, troponin is absent from the contractile machinery,

being functionally replaced by caldesmon. Caldesmon, an ~87kDa Ca^{2+} sensitive protein, binds to actin and tropomyosin preventing myosin binding (Huber, 1997). It has been shown through immunoprecipitation of proteins from colonic smooth muscles cells transfected with mutant *HSPB1*, that HSPB1 plays a key role in the removal of caldesmon from, and hence the opening of, the myosin binding site of actin (Somara & Bitar, 2006). Phosphorylated HSPB1 first aids in the phosphorylation and translocation of PKCα to actin where it phosphorylates caldesmon and tropomyosin (Figure 1-9; Ibitayo *et al.*, 1999; Patil *et al.*, 2004a). Once phosphorylated, HSPB1, caldesmon, and tropomyosin associate together resulting in a release of caldesmon from tropomyosin and allowing tropomyosin to migrate away from the myosin binding site on actin (Bitar, 2002; Somara & Bitar, 2006;).

1.8.2.3.3 Other functions.

HSPB1 also plays a role in cell survival, thermoresistance, and oxidative/chemotactic stressors (Arrigo, 1998; Arrigo *et al.*, 2002; Concannon *et al.*, 2003; Fuqua *et al.*, 1994; Landry *et al.*, 1989; Lavoie *et al.*, 1993a). Many of these protective roles of HSPB1 are due to its aforementioned chaperone (Reviewed by Arrigo, 2007). Another cellular function that continues to emerge for HSPB1 is that of an inhibitor of apoptosis (Figure 1-14). This is achieved through multiple mechanisms. The Mearow laboratory has shown, through the use of the PC12 neuronal tumour cell-line, that HSPB1 reacts to various cell stressors with an increase in binding to Akt (PKB)

Figure 1-14
HSPB1 regulation of various apoptotic pathways. HSPB1 can bind to both cytochrome *c*, after its release from mitochondria, and pro-caspase-3 thus preventing apoptosome formation and events downstream of mitochondrial damage. Apart from the caspase-dependent apoptosis, HSPB1 is reported to block Daxx-mediated apoptosis. Daxx, a nuclear protein that translocates to the membrane during Fas-mediated apoptosis, binds at one end to the Fas receptor and at the other to Ask1, thus mediating a caspase-independent apoptosis. HSPB1 can prevent the translocation of Daxx to membrane and its interaction with Fas.
From Concannon *et al.*, 2003. Reproduced by permission of the publisher.


Fas
Receptor
Plasma membrane
Oxidants
Hsp27
DAXX
FADD
Hsp27
Pro-caspase-8
Ask1
Bax/Bad
Bcl-2/BclX
Hsp27
Bid
Apaf-1
Cyt c
dATP
Pro-caspase-9
Caspase-8
Caspase-9
Hsp27
Pro-caspase-3
Caspase-3
APOPTOSIS

(Dodge *et al.*, 2006; Mearow *et al.*, 2002). This binding is essential for Akt activation and proceeds through HSPB1 chaperone activity, resulting in a protection and stabilization of Akt (Konishi *et al.*, 1997; Rane *et al.*, 2003). Once activated, Akt proceeds to inhibit apoptosis through multiple mechanisms, such as interaction with the Bad/Bcl-2 pathway, inhibition of cytochrome-c release, and inhibition of apoptotic caspases (Cardone *et al.*, 1998; Duronio, 2008; Garrido *et al.*, 1999).

HSPB1 is also implicated in multiple direct interactions with members of the apoptotic cascade (Figure 1-14), such as DAXX, a mediator of Fas-induced caspase-independent apoptosis (Charette *et al.*, 2000; Concannon *et al.*, 2003). DAXX associates with both the Fas receptor in the membrane, and the Ask1 protein which induces apoptosis. Phosphorylated HSPB1 sequesters DAXX, preventing initiation of this cascade. If the apoptotic pathway is already started however, HSPB1 still plays an inhibitory role to prevent apoptosis. This inhibition is in part due to the regulation of the cytochrome c pathway by HSPB1 (Bruey *et al.*, 2000; Garrido *et al.*, 1999; Paul *et al.*, 2002). HSPB1 not only inhibits the release of cytochrome-c from the mitochondria, but also binds to cytochrome-c molecules that are released into the cytoplasm (Paul *et al.*, 2002). Through immunoprecipitation experiments on human leukaemic cell lines transfected with *HSPB1* constructs, Bruey and colleagues (2000) showed that immunoprecipitation of either HSPB1 or cytochrome c also pulled down the other, illustrating a direct interaction between HSPB1 and the cytochrome c molecule. Through these means, HSPB1 likely sequesters cytochrome c, preventing it from activating pro-

caspase-9 (an initiator caspase) to facilitate downstream apoptotic signalling. HSPB1 has also been shown to inhibit the downstream caspase cascade through sequestering procaspase-3 and preventing it's activation (Concannon *et al.*, 2001; Pandey *et al.*, 2000).

1.8.2.4 Knockout mouse.

One consistent problem with investigations into sHSPs, especially HSPB1, *in vivo*, is teasing out the exact physiological role for HSPB1. One tool that often aids in this investigation is the knockout mouse. It has long been believed that a *HSPB1* knockout would be lethal, however investigators recently developed a viable *HSPB1* knockout mouse (Huang *et al.*, 2007). This mouse was created by replacing ~60% of the coding sequence of *HSPB1* through fusing a lacZ gene in frame with the *HSPB1* start codon. No observable expression of HSPB1 was found in the uterus of adult heterozygous mice, although much expression was observed in skeletal and cardiac muscle. Of greater note was the observation that the homozygous *HSPB1* null offspring showed no apparent morphological or physiological differences from their heterozygous or wild-type littermates (Huang *et al.*, 2007). The *HSPB1* knockout was not lethal, they were born with the expected litter distribution, and were fertile. This is a very interesting experimental finding, as HSPB1 has been hypothesized to be critical for many normal cellular processes such as muscle contraction (Bitar, 2002; Somara & Bitar, 2006). A more detailed investigation by Huang and colleagues (2007) into the effect on muscle physiology, although still preliminary, showed no significant differences.

One explanation for the reported findings is based on the observed structural and functional similarities within the sHSPs, and between sHSPs and the larger HSP family. Not only do sHSPs form multi-member complexes, but are also able to replace certain member functions with other family members (Fontaine *et al.*, 2005; Sugiyama *et al.*, 2000; Sun *et al.*, 2004). For example, the Welsh and Benndorf labratories showed that HSPB8 can bind to, and form heterooligomers with, multiple sHSP family members (HSPB1, B2, B3, B5, B6, B7). In addition, both HSPB1 and HSPB8 exhibit chaperone activity with heat shock (Chowdary *et al.*, 2004; Arrigo et al., 1988). Therefore other sHSPs, such as HSPB8, may be able to compensate for the loss of HSPB1, especially with respect to chaperone function. Huang *et al.* (2007) have claimed however that they observed no changes in expression of other sHSPs in the homozygous mice as compared to the wild type littermates.

Another interesting finding from this mouse model was that thermotolerance was affected by the reduction of HSPB1 levels. Additionally, it was observed that HSPB1 and HSPA1 seemed to act synergistically in response to thermal stress. Huang and colleagues showed that HSPB1 and HSPA1 had similar expression patterns in response to heat stress, and that removal of either *HSPB1* or *HSPA1* (*HSPB1*-/- and *HSPA*-/- cells respectively) impaired cellular thermotolerance leading to increased apoptotic signalling (increased cleavage processing of caspase 3, caspase 9, and poly(ADP-ribose) polymerase). In addition, removal of both genes led to an even greater level of apoptosis, illustrating that effective thermotolerance requires the presence of both proteins in order

to function properly.

The only results presented in this article specific to the uterus was the report of no observable expression of HSPB1 in adult uterine tissue from knockout mice, no observable phenotypic differences between knockout mice and control littermates, and that knockout mice were fertile. The results presented tell very little about any of the proposed roles of HSPB1 in normal or pregnant rat uterine structure and function, requiring further investigation of the knockout mouse model with respect to uterine physiology.

1.9 Objectives

Although HSPB1 has had much investigation over the past 20 years, there is still a significant lack of research with respect to its presence and/or role in myometrial smooth muscle. The studies that have been done in the uterus with HSPB1 have generally been on epithelial tissue, or cervical tissues that include squamous cell carcinoma and cervical cancer cell lines such as HeLa cells (Fukushima *et al.*, 2011; Ono *et al.*, 2009; Shi *et al.*, 2011; Wataba *et al.*, 2001). For the present Ph.D. research project, we hypothesized that HSPB1 plays an important role in myometrial smooth muscle function, as has been demonstrated in other smooth muscle cell types. As with any initial investigation, I first undertook a profile of mRNA and protein expression changes that occur throughout pregnancy. I then proceeded with analysis and identification of the various factors that regulate HSPB1 expression in myometrial tissue. Techniques such as northern blotting,

immunoblotting, and immunofluorescence on various rat experimental models, as well as *in vitro* cell culture work, were used to investigate HSPB1 expression and regulation. In all immunoblot analyses, the 27kDa monomeric form of HSPB1 was investigated to assess overall changes regardless of oligomeric state.

Objective 1 - To investigate gestational changes in HSPB1 regulation in the rat myometrium.

The first goal of this project was to develop a complete gestational profile of HSPB1 expression throughout normal rat pregnancy. We hypothesized that HSPB1 expression would be highly induced during late pregnancy and labour in the rat. Steady state levels of *HSPB1* due to myometrial gene transcription was assessed over gestation by northern blotting. Myometrial samples were taken in the non-pregnant state, as well as on days 6, 12,15, 17, 19, 20, 21, 22, 23 (labour) of pregnancy, and 1 day post parturition (PP). Identification of HSPB1 protein expression and post-translationally modified protein was assessed by immunoblotting (western blotting) techniques. Cellular and sub-cellular localization of HSPB1 protein within the myometrium *in situ* was investigated with immunofluorescence procedures and the use of HSPB1-specific antisera. Through these experiments the spatial and temporal regulation of HSPB1 during pregnancy can be addressed.

Objective 2 - To investigate the hormonal regulation of HSPB1 in the myometrium.

Since steroid hormones including progesterone and estrogen play a major role in the regulation of both myometrium and pregnancy as a whole, we hypothesized that progesterone and 17β-estradiol regulate HSPB1 expression and subcellular localization. Regulation of HSPB1 expression by pregnancy-associated steroid hormones, the first of two signals originating from the fetal genome that initiates labour, was assessed through exogenous hormone administrations to non pregnant and pregnant rats. Since the majority of pregnancy related changes in HSPB1 expression occurred mid-late gestation, around the time the switch from progesterone to estrogen begins, this was a primary timepoint for our investigation. Circulating progesterone levels begin to drop after day 19 of rat gestation, so exogenous progesterone was administered to pregnant animals starting on day 20 and continuing until day 23. As this prevents labour from occurring at day 23, myometrial samples were taken daily between day 21 and day 24 of gestation. To assess the effect of a functional withdrawal of progesterone on HSPB1 expression, RU486 was administered to animals on day 18 of gestation, and samples were collected on day 19 when rats were undergoing pre-term labour due to withdrawal of the effects of progesterone. The regulatory effect of estrogen (17β-estradiol) on HSPB1 was investigated in a hormone-minimized non-pregnant, ovariectomized rat model. Exogenous estrogen was administered and samples were subsequently collected at various timepoints thereafter. Investigation of HSPB1 mRNA, protein, post-

translationally modified protein, and protein localization in all hormone investigation models, was performed using northern blotting, immunoblotting, and immunofluorescence techniques. The three animal models employed in this objective make significant progress towards understanding the hormonal regulation of HSPB1 in the myometrium during rat gestation.

Objective 3 - To investigate the regulation of HSPB1 expression by uterine distension.

The second signal for initiation of labour originating from the fetal genome is mechanical signalling. Mechanotransduction has been established as being important for signalling within the myometrium, and for initiation of contraction. Therefore we hypothesized that physical stress of the myometrium would increase HSPB1 expression within the uterine musculature. To study the impact that physical stretch of the uterus has on HSPB1 expression, we first conducted experiments utilizing a surgically prepared unilaterally pregnant rat model to investigate the effect of stretch in a normal pregnancy environment. These pregnancy investigations were performed at day 19 and day 23 of rat gestation. These points was chosen for three reasons. First, it is the point when *HSPB1* mRNA expression was the highest. Second, it is when HSPB1 protein levels have risen to a significant level and increase to a maximum at day 23, and third, there is significant physical stretch of the uterus at this point in pregnancy due to the growing foetuses (Monga & Sanborn, 2003). Following investigation into stretch in a pregnant state,

investigation of stretch in the myometrium of non-pregnant, ovariectomized (hormone minimized) rats was performed. This non-pregnant, ovariectomized model was used to help isolate any solely stretch-associated regulation from the combinatorial regulation of stretch and hormone environment associated with pregnancy. For all these models, investigation of HSPB1 mRNA, protein, post-translationally modified protein, and protein localization *in situ* was again performed using northern blotting, immunoblotting, and immunofluorescence techniques. Through these experimental models, this objective addresses the stretch regulation of HSPB1 in both a hormonally regulated pregnant uterine environment and a myometrium without pregnancy-related hormonal input.

Chapter 2

Small Heat Shock Protein 27 (Hsp27) Expression is Highly Induced in Rat Myometrium during Late Pregnancy and Labour.

(This article has been published in Reproduction (2005), 129, pp. 115-126, before the switch to the new nomenclature. Thus, Hsp27 has now been replaced with HSPB1 in the majority of this document.)

2.1 Introduction

The underlying mechanisms that induce or regulate uterine contractions during labour are still poorly understood; however, it is clear that the fetal genome contributes signals that facilitate the initiation of parturition (Challis *et al.*, 2000; Challis *et al.*, 2002). These signals ultimately lead to phenotypic changes in the myometrium, from a muscle which during pregnancy is quiescent to one which is spontaneously active, excitable, and responsive to uterine agonists and characteristically exhibits a high degree of cell-cell coupling. This myometrial *activation* results from the increased expression of a group of genes that encode contraction-associated proteins such as ion channels, agonist receptors, and gap junctions (Challis *et al.*, 2002).

Activation requires triggering of two separate, but interdependent signalling pathways in the foetus (Challis *et al.*, 2002). The first requires activation of an endocrine

cascade involving the fetal hypothalamic-pituitary-adrenal-placental axis. In most animals, the yield of this axis at term results in a decreased influence of progesterone on the myometrium. While important, this endocrine pathway is not sufficient for myometrial activation and work by Ou *et al* (1997) has demonstrated that mechanical distension (or stretch) within the myometrium (as a result of fetal growth during pregnancy) is also required. Ultimately, the endocrine and mechanical signalling pathways may potentially be integrated at focal adhesion sites (i.e. smooth muscle dense plaques) by integrin-mediated signal transduction via focal adhesion kinase (FAK) and downstream effectors (MacPhee and Lye, 2000).

The family of mammalian small heat shock proteins (sHSP) comprises seven members: HSPB4 and HSPB5 (αA and αB-crystallin), HSPB1 (Hsp27), HSPB6 (Hsp20), HSPB2 (Myotonic dystrophy protein kinase binding protein; MKBP), HSPB3 (Hsp27L) and HSPB7 (cardiovascular Hsp; cv Hsp) with molecular masses 15-30 kDa (Kato *et al.*, 2002). All members of this class share a structural domain named the α-crystallin domain in their carboxyl terminal halves that also span two putative actin binding domains (Quinlan, 2002). HSPB1, in particular, has been demonstrated to play an important role in actin polymerization, remodelling and even cross-bridge cycling in smooth muscle cells (Miron *et al.*, 1991; Lavoie *et al.*, 1993a, 1993b; Benndorf *et al.*, 1994; Ibitayo *et al.*, 1999; Gerthoffer and Gunst, 2001).

Phosphorylation is one of the most prominent modifications that occurs in sHSP and is considered to be very important for regulation of sHSP structure and function

(Gaestel, 2002). It is known to be one of the earliest events induced by a stress such as heat shock, oxidative stress, and stimuli such as cytokines and growth factors (Landry *et al.*, 1992). Two phosphorylation sites are known to exist for HSPB1 in rodents, Ser-15 and Ser-86 (homologous to Ser-82 in humans and Ser-90 in hamsters). The latter site has been demonstrated to be the major phosphorylation site for this protein and is necessary for the dissociation of large sHSP multimers, although at the cellular level it is not always sufficient (Lambert *et al.*, 1999; Gaestel, 2002; Kato *et al.*, 2002). In contrast, phosphorylation of HSPB1 on Ser-15 is proposed to produce a conformational change in HSPB1 that aids the direct binding of HSPB1 with actin filaments (Lambert *et al.*, 1999; Gerthoffer and Gunst, 2001; Mounier and Arrigo, 2002). Recent work by Bitar (2002) has demonstrated that agonist-induced phosphorylation of HSPB1 promotes the association of actin and myosin, likely through association with tropomyosin. Therefore, phosphorylation of HSPB1 could be a prerequisite for smooth muscle contraction (Gerthoffer and Gunst, 2001).

Small heat shock proteins have been detected in rat and mouse uterine tissue during implantation where they are thought to play a role in decidualization (Tabanelli *et al.*, 1992; Bany and Schultz, 2001). In addition, HSPB1 has been examined in human endometrial stromal cells during *in vivo* and *in vitro* decidualization (Tang *et al.*, 1993; Ciocca *et al.*, 1996; Shah *et al.*, 1998). Yet, there is a complete lack of information regarding the expression of sHSP in the myometrium during late pregnancy and labour. Since HSPB1 appears to be important in smooth muscle contraction, we hypothesized

that HSPB1 expression would be highly induced during late pregnancy and labour in the rat.

2.2 Materials and Methods

2.2.1 Animals.

Sprague Dawley rats were obtained from the Mount Scio Vivarium (Memorial University of Newfoundland, St. John's, Newfoundland and Labrador, Canada). Animals were held and cared for under standard environmental conditions (12-h light, 12-h dark) in the Animal Care Unit at the Health Sciences Centre, Memorial University of Newfoundland. Rats were fed LabDiet Prolab RMH 3000 (PMI Nutrition International, Brentwood, Missouri, USA) and water *ad libitum*. All experiments were approved by the institutional animal care committee under animal care protocol 02-02-DM. For our studies, virgin female rats (~220g each) were mated with stud males and the 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.

2.2.2 Tissue collection.

All animals were killed by carbon dioxide inhalation on the desired day of sampling and pregnancy (e.g. non-pregnant (NP), day (d) 6, 12, 15, 17, 19, 21, 22, 23 (labour), 1 day post-partum (PP)). Samples on d23 were taken during active labour and only after the rat had delivered 2-3 pups. Uterine horns were removed, opened

longitudinally, and foetuses and placentae discarded. Uterine tissue was placed in ice cold phosphate-buffered saline (PBS; pH 7.4) and endometrial tissue removed by gentle scraping with a scalpel blade. Liver and heart tissues were taken from non-pregnant rats for use as controls. All samples were flash-frozen in liquid nitrogen and stored at -80 C.

2.2.3 Northern blot analysis.

2.2.3.1 RNA isolation.

Northern blot analysis was performed on four separate, independent sets of RNA samples (n=4, ie. 4 rats used per gestational timepoint). RNA was isolated from tissues using TRIzol® Reagent (Invitrogen Corporation, Carlsbad, California, USA) exactly according to the manufacturer's instructions. RNA quality and quantity ($A_{260/280}$) were determined using an Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, Ontario, Canada) and samples stored at -70 C.

2.2.3.2 Electrophoretic separation and capillary transfer of RNA.

RNA samples were prepared by precipitating RNA with two volumes of 100% ethanol / 2% potassium acetate and resuspending pellets in sample buffer (50% formamide, 0.066 M formaldehyde, and 1X MOPS buffer in DEPC-treated ddH_2O). Samples were incubated for thirty minutes at 65 C and quick cooled on ice for ten minutes. RNA samples (10 µg per lane) were loaded on a 1% agarose gel containing 0.66 M formaldehyde and 1X MOPS buffer (0.02 M MOPS pH=7.0, 2 mM sodium acetate, 10 mM EDTA pH=8.0) and electrophoretically separated at 80V in 1X MOPS / 0.22 M formaldehyde running buffer. RNA was transferred overnight to a nylon membrane

(Hybond-XL; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) by upward capillary action using 2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate) in DEPC-treated ddH_2O. RNA was crosslinked to nylon membrane with an UVC-508 ultraviolet crosslinker (Ultra-Lum Inc., Paramount, California, USA) and all blots were stored at -20 C until required.

2.2.3.3 Northern blot hybridization.

Membranes were pre-hybridized in hybridization buffer consisting of 50% formamide, 5X sodium chloride-sodium phosphate EDTA (SSPE; 0.75 M sodium chloride, 0.05 M sodium phosphate, 0.005 M EDTA), 1% SDS, 5X Denhardt's Solution, and 0.1 mg/mL Herring Sperm DNA for 1-2 hours at 42 C in a hybridization oven (Hybaid Instruments, Franklin, Massachusetts). Hamster *HSPB1* cDNA (Genbank Accession #X51747) was a kind gift from Dr. J. Landry (Laval University, Quebec, Canada). Radiolabelled cDNA probes were prepared with a Megaprime DNA Labelling kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) exactly according to manufacturers specifications. Hybridization was performed overnight at 42 C, then blots were washed 5X for 5 minutes at 42 C in 2X SSC/0.1% SDS and exposed to x-ray film (Hyperfilm MP; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Multiple exposures were produced for each northern blot to ensure the results were within the linear range of the film.

Following analysis of *HSPB1* gene expression, northern blots were stripped by

incubating membranes for two hours at 75 C in a solution of 1 M Tris-Cl, 1 mM EDTA, 0.1X Denhardts solution in ddH_2O. Membranes were washed at room temperature with 0.1X SSPE and then subsequently analysed for expression of *18S* rRNA using the same procedures described above and a rabbit *18S* ribosomal cDNA template generously provided by Dr. I. Skerjanc (University of Western Ontario, London, Ontario, Canada). *18S* rRNA is constitutively expressed in rat myometrial cells and has been utilized, in the past, as a loading control for analysis of myometrial gene expression (Mitchell and Lye, 2002; Oldenhof *et al.*, 2002, Shynlova *et al.*, 2004).

2.2.4 Immunoblot analysis.

Immunoblot analysis was performed on three separate, independent sets of protein samples (n=3, ie. 3 rats used per gestational timepoint) according to MacPhee and Lye (2000). Briefly, frozen rat myometrial samples were pulverized under liquid nitrogen and homogenized in RIPA lysis buffer (50mM Tris-HCl (pH7.5), 150mM NaCl, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS) containing 100 uM Na_2VO_3 and COMPLETE™, Mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Laval, Quebec, Canada). 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 (50 ug/lane) were separated by polyacrylamide gel electrophoresis in 9% resolving gels according to the method of Laemmli (1970). Gels were electroblotted to Pierce 0.45 µm nitrocellulose

membranes (MJS BioLynx, Inc., Brockville, Ontario, Canada).

Proteins were detected using the Pierce SuperSignal® West Pico chemiluminescent substrate detection system (MJS BioLynx, Inc., Brockville, Ontario, Canada) according to the manufacturer's instructions. Rabbit polyclonal antisera (S15) raised against serine-15 (Ser-15) phosphorylated HSPB1, rabbit polyclonal antisera (CT) raised against the c-terminus of HSPB1, or mouse monoclonal anti-smooth muscle calponin (clone hCP; Sigma-Aldrich Canada, Oakville, Ontario, Canada) were used as primary antisera at 1:2,000, 1:2000 or 1:50,000 dilutions, respectively. HRP-conjugated goat anti-rabbit IgG (H + L) (Pierce, Rockford, Illinois, USA) or HRP-conjugated goat anti-mouse IgG (H+L) (Pierce) were used as secondary antisera at 1:25,000 and 1:150,000 dilutions, respectively. Multiple exposures were generated to ensure the linearity of the film exposures.

Following detection of Ser-15 phosphorylated HSPB1, 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, followed by stripping and analysis of total HSPB1 protein expression. We have determined that calponin protein is constitutively expressed in non-pregnant and pregnant rat myometrial tissue under our protein extraction conditions (ie RIPA buffer extraction protocol) and, as a result, serves as a sufficient loading control for our analyses.

2.2.5 Immunocytochemistry.

Two separate, independently collected sets of rat tissues (n=2, ie 2 rats used per gestational timepoint) were utilized for immunocytochemistry experiments and experiments were repeated three times. 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.

Following de-waxing and rehydration, tissue sections were permeabilized with 0.125% Trypsin in PBS for 10 minutes at room temperature. Tissue sections were blocked with 5% normal goat serum / 1% horse serum and then incubated with rabbit anti-HSPB1 primary antisera (SRQL or S15) at dilutions of 1:200. To serve as negative controls for HSPB1 detection, separate tissue sections from the same gestational timepoints were incubated with pre-immune sera at the same concentration as the HSPB1-specific antisera. Tissue sections were incubated with FITC-conjugated sheep anti-rabbit IgG (Sigma-Aldrich Canada) at dilutions of 1:200 and then washed twice with cold 0.02% Tween-20 in PBS, followed by mounting in Vectashield® (Vector Laboratories Inc., Burlington, Ontario, Canada). Prepared slides were observed and images collected with an Olympus Fluoview laser scanning confocal microscope (Olympus Optical Company Ltd., Melville, New York, USA).

2.2.6 HSPB1 antisera.

Three HSPB1-specific antisera were employed for our experiments. A novel rabbit polyclonal antisera, designated SRQL for our experiments, was obtained from Affinity Bioreagents (Golden, Colorado, USA) and utilized exclusively for immunofluorescent detection of total pools of HSPB1. It was raised against a human HSPB1 sequence encompassing amino acids 73-83 (Genbank Accession # AB020027; YSRALSRQLSS). The targeted human sequence is 82 % identical with the rat sequence (Genbank Accession #M86389; FSRALNRQLSS) corresponding to amino acids 77-87. A rabbit polyclonal phosphorylation state-specific antisera, termed S15 for our experiments, was obtained from Affinity Bioreagents (Cat # PA1-018) and utilized for immunofluorescent detection and immunoblot analysis of Ser-15 phosphorylated HSPB1. It was raised against a phosphorylated peptide encompassing amino acids 10-20 (Genbank Accession # AB020027; LLRGP(pS)WDPFR) of the human sequence. The targeted human sequence is 82% identical with the corresponding rat sequence (Genbank Accession #M86389; LLRSP(pS)WEPFR). Lastly, a rabbit polyclonal antisera, designated CT, was obtained from Upstate Biotechnology (Lake Placid, NY, USA; Cat # 06-517) and utilized for immunoblot analysis of total pools of HSPB1 (Lavoie *et al.*, 1995; Lambert *et al.*, 1999). It was raised against a C-terminal sequence of hamster HSPB1 encompassing amino acids 204-213 (Genbank Accession # X51747; AGKSEQSGAK) and is specific for rodent HSPB1. The targeted hamster sequence is 70% identical to the corresponding rat sequence of amino acids 196-205 (Genbank

Accession #M86389; GPESEQSGAK).

2.2.7 Data analysis.

Densitometric analysis was performed with the aid of Scion Image software (Scion Image Corporation, Frederick, Maryland, USA). Densitometric measurements of *HSPB1* mRNA were normalized to those of *18S* ribosomal RNA while measurements of HSPB1 protein on immunoblots were normalized to those of calponin. Statistical analysis was performed with GraphPad Instat® version 3.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com) and data graphed using GraphPad Prism® version 4.0 (GraphPad Software). Data was subjected to a One-way Analysis of Variance and a Tukey-Kramer Multiple Comparisons test. Values were considered significantly different if $p < 0.05$.

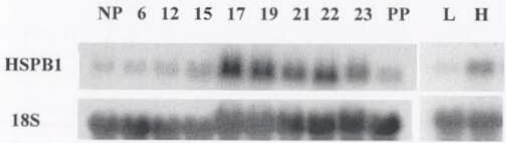
2.3 Results

2.3.1 Analysis of HSPB1 expression.

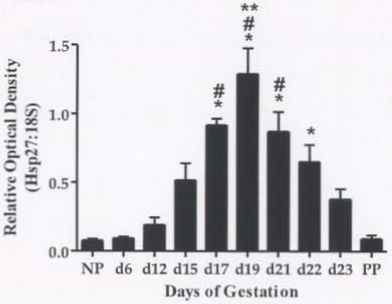
To characterize *HSPB1* mRNA expression within myometrial samples, northern blots of rat myometrial total RNA from non-pregnant (NP), d6, 12, 15, 17, 19, 21, 22, 23 (labour) of pregnancy, PP, heart and liver controls were analysed with radiolabelled probes generated from a hamster *HSPB1*-specific cDNA (Figure 2-1A). In agreement with a previous report (Lu and Das, 1993), *HSPB1* mRNA was detected in heart tissue, but very low expression was detected in liver tissue under our assay conditions. Our

Figure 2-1
Northern blot analysis of HSPB1 mRNA expression in rat myometrium during pregnancy, labour and post-partum. (**A**) A representative northern blot analysis of HSPB1 mRNA and *18S* rRNA expression. Northern blot analysis was performed with an HSPB1-specific hamster cDNA and an *18S*-specific rabbit cDNA as templates for radiolabelled probe production. 10μg of total RNA were utilized per lane. (**B**) Densitometric analysis illustrating the increase in HSPB1 mRNA expression during late pregnancy. Values on d17-d22 were significantly elevated ($p < 0.05$) over NP, d6 and PP (*) and values on d17-d21 were also significantly elevated ($p < 0.05$) over d12 and d23 (#). Expression on d19 was significantly elevated over d15 and d22 (**). Day 6, 12, 15, 17, 19, 21, 22, and 23 represent gestational timepoints. Values are from four independent experiments (n=4) ± SE. NP = non-pregnant, PP = 1 day post parturition, L, H = liver and heart tissue controls, respectively, Hsp27 = HSPB1.

A

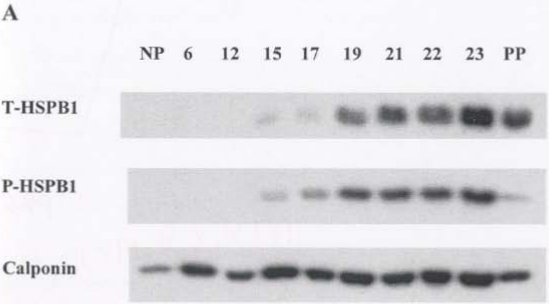

NP 6 12 15 17 19 21 22 23 PP L H
HSPB1
18S

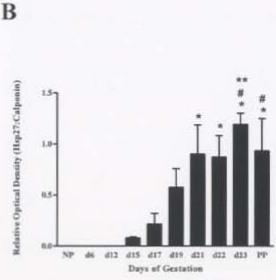
B

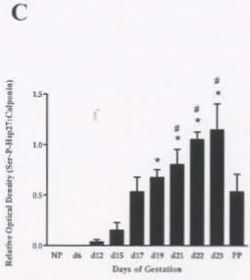

Relative Optical Density (Hsp27:18S)
1.5
1.0
0.5
0.0
NP d6 d12 d15 d17 d19 d21 d22 d23 PP
Days of Gestation

experiments demonstrated that *HSPB1* mRNA expression significantly increased during late pregnancy (One-way ANOVA, $p < 0.0001$; n=4). Specifically, *HSPB1* mRNA expression was barely detectable from NP - d12 and then began to increase by d15 of pregnancy. *HSPB1* mRNA expression then became significantly elevated (Tukey-Kramer post test, $p<0.05$) between d17 - d22, inclusive, compared to NP, d6, d12, d23 and PP timepoints (Figure 2-1A, 2-1B). Immunoblot analysis utilizing the HSPB1 CT antisera demonstrated that detection of total HSPB1 increased significantly during late pregnancy, labour and PP (One-way ANOVA, $p< 0.0001$; n=3). Specifically, HSPB1 protein was virtually undetectable from NP - d12 and then began to accumulate by d15. Detection of HSPB1 between d21 and PP, inclusive, then significantly increased over NP, d6 and d12 timepoints (Tukey-Kramer post test, $p<0.05$; n=3; Figure 2-2A, 2-2B). In addition, detection of HSPB1 on d23 and PP was also significantly elevated over d15 ($p<0.05$; n=3). Immunoblot analysis of Ser-15 phosphorylated HSPB1, utilizing the S15 antiserum, demonstrated that significantly increased detection of phosphorylated HSPB1 during late pregnancy and labour (One-way ANOVA, $p<0.0001$; n=3) paralleled the increased detection of total HSPB1. In particular, phosphorylated HSPB1 became detectable by d15 and between d19-d23, inclusive, detection of phosphorylated HSPB1 significantly increased (Tukey-Kramer post test, $p<0.05$; n=3) over NP, d6, d12, and d15 timepoints (Figure 2-2A, 2-2C). By PP, and in contrast to total HSPB1, detection of phosphorylated HSPB1 was markedly decreased.

Figure 2-2
Immunoblot analysis of serine 15 (S15) phosphorylated HSPB1 and total HSPB1 during pregnancy, parturition and post-partum.
(**A**) Representative immunoblots from analysis of HSPB1 and calponin expression during gestation.
(**B**) Densitometric analysis illustrating the dramatic increase in detection of total HSPB1 during pregnancy, labour and post-partum. Values on d21-PP were statistically significant ($p<0.05$) over NP, d6, and d12 (*) timepoints while values on d23-PP were statistically significant ($p<0.05$) over d15 (#) and expression on d23 was significantly elevated over d17 (**).
(**C**) Densitometric analysis illustrating the dramatic increase in detection of Ser-15 phosphorylated HSPB1 during pregnancy and labour. Values on d19-d23 were statistically significant ($p<0.05$) over NP, d6, and d12 (*) timepoints while values on d21-23 were statistically significant ($p<0.05$) over d15 (#). Day 6, 12, 15, 17, 19, 21, 22, and 23 represent gestational timepoints. Values are from three independent experiments (n=3) ± SE. NP = non-pregnant, PP = 1 day post parturition, T-HSPB1 represents Total HSPB1 and P-HSPB1 represents Phosphorylated HSPB1, Hsp27 = HSPB1.


A
NP 6 12 15 17 19 21 22 23 PP
T-HSPB1
P-HSPB1
Calponin


B
Relative Optical Density (Hsp27/Calponin)
1.5
1.0
0.5
0.0
NP d6 d12 d15 d17 d19 d21 d22 d23 PP
Days of Gestation


C
Relative Optical Density (Ser-P-Hsp27/Calponin)
1.5
1.0
0.5
0.0
NP d6 d12 d15 d17 d19 d21 d22 d25 PP
Days of Gestation

2.3.2 Immunocytochemical detection of HSPB1.

2.3.2.1 Localization in the circular muscle layer.

HSPB1 was virtually undetectable on d15 and d17 of gestation by immunocytochemical analysis with SRQL antisera. In contrast, HSPB1 was readily detectable on days 19-22, followed by a decrease in detection on day 23 and PP that approached background levels observed in pre-immune controls (Figure 2-3). Spatially, HSPB1 was localized to the cytoplasm in peri-nuclear regions on d19 - d22, likely representing newly synthesized HSPB1, but also became localized to myocyte membranes on d22.

Phosphorylated HSPB1 was also undetectable on d15 and d17 of gestation but became more readily detectable thereafter. On d19 - d22, phosphorylated HSPB1 was localized in peri-nuclear and membrane regions; however, in contrast to results with the SRQL antisera phosphorylated HSPB1 was also detected on d23 and PP. During these periods, phosphorylated HSPB1 was primarily restricted to the cytoplasm of myocytes (Figure 2-4) although not consistently to any specific sub-cellular domain (eg. peri-nuclear).

2.3.2.2 Localization in the longitudinal muscle layer.

Detection of HSPB1 in the longitudinal muscle layer with SRQL antisera demonstrated that HSPB1 was primarily localized to membrane regions from d15 and increasingly detected by d22, followed by decreased membrane detection on d23 and undetectable HSPB1 expression at PP that was similar to pre-immune controls (Figure 2-5). Utilizing S15 antisera, phosphorylated HSPB1 was also detected on d15 but readily

Figure 2-3

Immunocytochemical analysis of total HSPB1 protein expression in circular muscle layers of pregnant rat myometrium between d15 of pregnancy and PP using polyclonal anti-human HSPB1 antisera (SRQL). Arrows indicate peri-nuclear localization. Con = pre-immune serum control. Scale bar = 50μm.

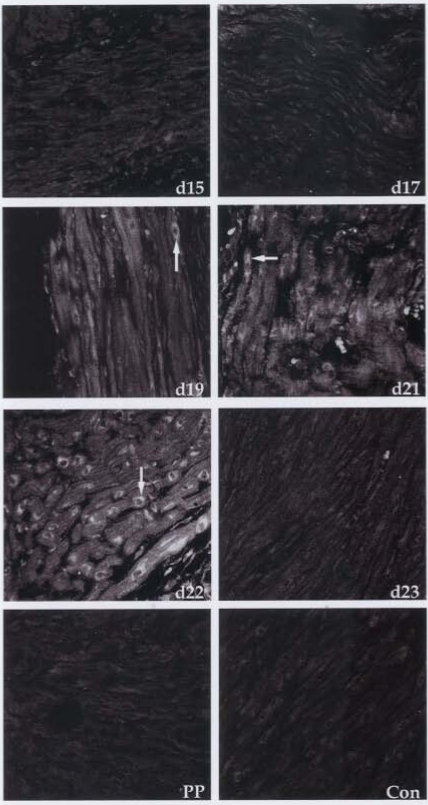

d15
d17
d19
d21
d22
d23
PP
Con

Figure 2-4
Immunocytochemical analysis of Ser-15 phosphorylated HSPB1 protein expression in circular muscle layers of pregnant rat myometrium between d15 of pregnancy and PP using phosphorylation-state specific polyclonal anti-human HSPB1 antisera (S15). Con = pre-immune serum control. Arrows on d21 indicate peri-nuclear localization while arrows on d22 demonstrate membrane localization. Scale bar = 50μm.

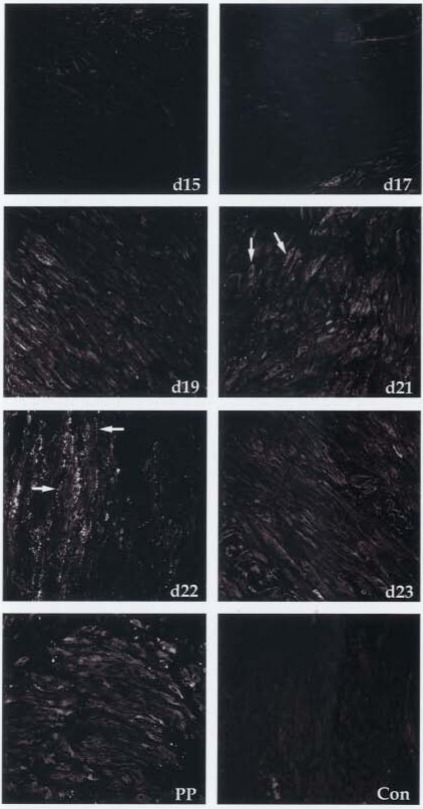
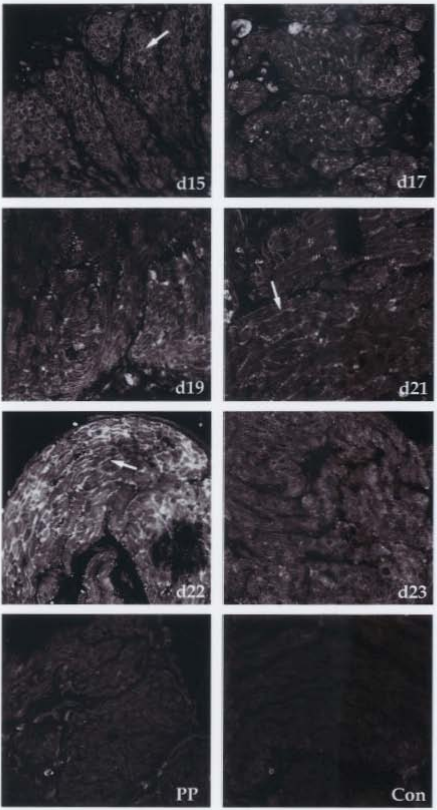

d15
d17
d19
d21
d22
d23
PP
Con

Figure 2-5
Immunocytochemical analysis of total HSPB1 protein expression in longitudinal muscle layers of pregnant rat myometrium between d15 of pregnancy and PP using polyclonal anti-human HSPB1 antisera (SRQL). Arrows indicate membrane localization. Con = pre-immune serum control. Scale bar = 50μm.


d15
d17
d19
d21
d22
d23
PP
Con

detectable on d17 - d22, primarily localized to myocyte membranes but also detectable at a low level in the cytoplasm of myocytes (Figure 2-6). In contrast to results with SRQL antisera, however, phosphorylated HSPB1 was readily detected on d23 and PP. In addition, phosphorylated HSPB1 was detected in the cytoplasm of myocytes during these two latter timepoints.

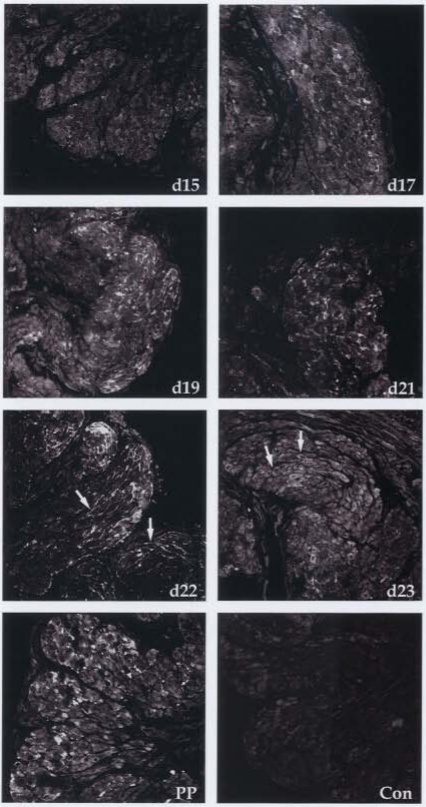
In addition to the temporal and spatial localization differences observed for total and Ser-15 phosphorylated HSPB1 expression during gestation, our immunocytochemistry experiments also demonstrated that HSPB1 was differentially expressed between the two muscle layers of the myometrium during gestation. Both total HSPB1 (measured with the SRQL antisera) and Ser-15 phosphorylated HSPB1 were much more highly detectable in the longitudinal muscle layer than in the circular muscle layer.

2.4 Discussion

2.4.1 HSPB1 expression during pregnancy.

Our northern blot analysis demonstrated that detection of *HSPB1* mRNA began to increase by d15 of pregnancy, increased significantly from d17-d22, and then dropped significantly thereafter. This pattern of expression may be due to gestation-specific changes in *HSPB1* mRNA synthesis. Putative AP-1-binding sites exist within the promoter region of sHSP genes (Iwaki *et al.*, 1990; Srinivasan and Bhat, 1994) and it is possible that AP-1 transcription factors, which are expressed in the myometrium during

Figure 2-6
Immunocytochemical analysis of Ser-15 phosphorylated HSPB1 protein expression in longitudinal muscle layers of pregnant rat myometrium between d15 of pregnancy and PP using phosphorylation-state specific polyclonal anti-human HSPB1 antisera (S15). Con = pre-immune serum control. Arrows on d22 indicate membrane localization while arrows on d23 demonstrate cytoplasmic localization. Scale bar = 50μm.


d15
d17
d19
d21
d22
d23
PP
Con

late pregnancy and labour (Piersant and Lye, 1995; Mitchell and Lye, 2002; Shynlova *et al.*, 2002), might at least be partially responsible for regulating *HSPB1* mRNA expression during gestation. We also cannot rule out the possibility that changes in *HSPB1* mRNA stability, in addition to mRNA synthesis, could be a contributing factor to the observed pattern of expression. Lastly, the steroid hormone environment during pregnancy may also regulate *HSPB1* mRNA expression. In most animal species, including the rat, the circulating levels of progesterone begin to drop significantly prior to labour and the circulating levels of estrogen sharply increase (Challis *et al.*, 2000). However, both progesterone and estrogen are thought to induce *HSPB1* expression (Tabibzadeh *et al.*, 1996; Kato *et al.*, 2002), therefore, the role these steroid hormones play in regulating *HSPB1* expression in the rat myometrium is unclear.

Detection of total HSPB1 protein, by immunoblot analysis, also began to increase by d15 and was significantly elevated during late pregnancy, labour, and PP. Despite the fact that HSPB1 was virtually undetectable from NP to d12 timepoints, it is still possible that low basal expression of HSPB1 exists because it may be beyond the detection level of our immunoblot analysis under the conditions utilized for our assay. Since the increased detection of phosphorylated HSPB1 towards labour paralleled the increase in detection of total HSPB1, our results suggest that at least a basal activity of an HSPB1 kinase is required for this post-translational modification. The markedly decreased detection of phosphorylated HSPB1 PP, compared to total HSPB1, suggests that this result was likely due to dephosphorylation of HSPB1 by a phosphatase.

Immunocytochemistry experiments revealed that Ser-15 phosphorylated HSPB1 was primarily localized to myocyte membranes during late pregnancy. Phosphorylated HSPB1 might be associated with focal adhesions during this period since HSPB1 has been reported to bind to the focal adhesion protein Hic-5, a member of the paxillin protein family (Turner, 2000). Focal adhesions are necessary for transmission of tension from the extracellular matrix to the internal contractile apparatus and may be remodeled during late pregnancy to support myometrial hypertrophy (MacPhee and Lye, 2000; Gerthoffer and Gunst, 2001). The phosphorylation of HSPB1 on Ser-15 is proposed to produce a conformational change in HSPB1 that aids the direct binding of HSPB1 with actin filaments (Lambert *et al*., 1999; Gerthoffer and Gunst, 2001; Mounier and Arrigo, 2002) and Benndorf *et al* (1994) have demonstrated HSPB1 phosphorylation can relieve HSPB1- inhibition of actin polymerization. Therefore, in the myometrium during late pregnancy, membrane-localized phosphorylated HSPB1 may regulate actin cytoskeleton dynamics at focal adhesion sites to support the hypertrophy-induced reorganization of these structures during late pregnancy.

Ser-15 phosphorylated HSPB1 was also readily detected in cytoplasmic regions of myocytes during labour. Phosphorylation of HSPB1 also appears to be an essential step for smooth muscle contraction by promoting actin-myosin interaction (Gerthoffer and Gunst, 2001; Bitar, 2002). In colonic smooth muscle cells, HSPB1 co-immunoprecipitates with actin, tropomyosin, and caldesmon (Ibitayo *et al*., 1999; Bitar, 2002). Colonic smooth muscle cells that were transfected with constitutively

phosphorylated HSPB1 mutants, and stimulated with contractile agonists, also exhibited an increased binding of actin with myosin (Bitar, 2002). Therefore, during labour phosphorylated HSPB1 in cytoplasmic regions might be associated with the contractile machinery mediating contraction.

Despite published evidence of a role for HSPB1 in both actin polymerization and smooth muscle contraction, we cannot rule out a role for phosphorylated HSPB1 as a chaperone. Phosphorylated HSPB1 was detected in the cytoplasm of myometrial cells during late pregnancy, labour, and PP. Actin cytoskeleton dynamics and protection require phosphorylated HSPB1 (Liang and MacRae, 1997; Mounier and Arrigo, 2002) and as a chaperone, phosphorylated HSPB1 may coat actin filaments to prevent access to actin-severing proteins and help maintain their organization in myometrial cells during gestation.

Our immunocytochemistry experiments showing that Ser-15 phosphorylated HSPB1 was detectable by S15 antisera in both circular and longitudinal muscle layers during labour and PP, was in stark contrast to HSPB1 detected with the SRQL antisera. In addition, immunoblot analysis of total HSPB1 with CT antisera also demonstrated detection of HSPB1 at labour and PP compared to the immunocytochemistry results with SQRL antisera. The reason(s) for the lack of detection of HSPB1 at labour and PP by SQRL antisera is unknown; however, we cannot discount the possibility that SRQL antisera specificity is sensitive to conformational changes in HSPB1 structure. In addition to phosphorylation for example, sHSP can undergo deamidation, acylation,

oxidation and glycation (Gaestel, 2002). Therefore, specific post-translational modifications during labour and PP might cause a conformational change in the HSPB1 molecule that could prevent the SQRL antisera from efficiently recognizing its epitope *in situ*.

Our data also indicates that HSPB1 is detectable at much higher levels in the longitudinal muscle layer than in the circular muscle layer during late pregnancy. The functional consequences of this expression pattern are unknown, but the two muscle layers do have different embryological origins and exhibit different contractile and physiological characteristics (Osa and Katase, 1975; Kawarabayashi and Osa, 1976; Chow and Marshall, 1981; Mlynarczyk *et al.*, 2003). Therefore, it is possible that HSPB1 has muscle layer-specific roles during pregnancy and labour.

2.4.2 HSPB1 and myometrial contractility.

Temporal-specific differences in uterine contractility have been reported during pregnancy in humans, sheep, rats and mice (Harding *et al.*, 1982; Bengtsson *et al.*, 1984; Buhimschi and Garfield, 1996; Buhimschi *et al.*, 1997; 1998; Hennan and Diamond, 1998; Mackler *et al.*, 1999). These reports have consistently provided evidence that uterine activity significantly increases just prior to labour. Specifically in the rat, Buhimschi and Garfield (1996) and Buhimschi *et al* (1998) used abdominal and myometrial surface electromyographic analysis as a means to assess uterine contractions. Only within the last 24 hours before active labour occurred did significant contractile

activity become evident. Reports of increased myometrial contractility just prior to labour coincide well with documented activation of the myometrium.

Myometrial activation is thought to be necessary for the myometrium to properly respond to contractile stimuli and is marked by the increase in expression of contraction-associated proteins (Challis *et al.*, 2000; Challis *et al.*, 2002). Interestingly, two families of heat shock proteins, Hsp70 and Hsp90, are already considered contraction-associated proteins in the myometrium of the ewe (Wu *et al.*, 1996). Although we cannot yet determine the specific role(s) of HSPB1 in the rat myometrium during pregnancy, labour and PP, our results demonstrating that HSPB1 expression is highly upregulated during late pregnancy and labour, and documented evidence of roles for HSPB1 in smooth muscle contraction and actin polymerization, suggest HSPB1 is a potential contraction-associated protein.

Chapter 3

Progesterone and 17β-Estradiol Differentially Regulate the Expression and Phosphorylation of the Small Heat Shock Protein HSPB1 in the Rat Myometrium.

3.1 Introduction

Critical to the understanding of myometrial contraction and potential treatment of associated problems, is a need for a greater understanding of the normal physiological processes occurring during pregnancy; how coordinated contractions develop and the mechanisms that control them. What we do know is that as pregnancy proceeds into labour, there is a very complex "communication" between the mother and foetus. This complex communication specifically controls the initiation of coordinated smooth muscle contractions that have a high frequency and amplitude, i.e. labour (Taggart & Morgan, 2007). It has been established that the communication signals originate within the fetal genome and follow two distinct, yet highly connected pathways; an endocrine and a mechanical pathway (Challis *et al.*, 2000; Vidaeff & Ramin, 2008). The endocrine pathway follows the fetal hypothalamic-pituitary-adrenal-placental axis leading to alteration of levels of pregnancy-related steroid hormones (Challis *et al.*, 2000). The importance of this very complex endocrine cascade has been known for over 100 years, yet the exact mechanisms of action are still being investigated (Zakar & Hertelendy, 2007). The mechanical pathway involves mechanotransduction within the myometrium due to physical stretch (Burghardt *et al.*, 2009; Galbraith *et al.*, 2002)

The two main pregnancy-related steroid hormones, progesterone and 17β-estradiol play critical roles in regulating myometrial contraction and initiation of labour. Both signals are necessary for a successful pregnancy, but in many ways have opposing functions. Progesterone, the major hormone for most of pregnancy, prevents labour through a maintenance of uterine quiescence as well as priming the uterus for the development of contractility (Csapo *et al.*, 1972; Haluska *et al.*, 1997). First described by Csapo in the 1950's, it is proposed that progesterone "blocks" the activation of the uterus through suppressing the propagation of electrical activity (Csapo *et al.*, 1955). A sudden withdrawal of the actions of progesterone is necessary for parturition in all mammals studied so far (Messiano *et al.*, 2002). The alternative steroid hormone of pregnancy, 17β-estradiol, has a role in the stimulation of labour. 17β-estradiol promotes contraction through facilitating an increase in the production of the family of proteins refered to as "contraction associated proteins" (CAPs) such as connexin-43 and through stimulation of prostaglandin production (Lye, 1994; Lye *et al.*, 1993). These proteins "activate" the myometrium, increasing contractility and responsiveness to labour-associated signals. In most mammals this switch from progesterone to 17β-estradiol is achieved through a decrease in circulating progesterone levels and an increase in circulating 17β-estradiol. Rosenblatt (1979) showed this switch began occurring around day 19 of rat gestation. Serum progesterone levels decreased in half between day 19 and day 21, decreasing to basal levels by labour, while serum levels of 17β-estradiol began increasing at day 17 leading to a 4 fold total increase at labour.

The regulation of these two hormones during pregnancy in humans differs from that observed in other mammals in that there is no labour-related change in circulating hormone levels (Zakar & Hertelendy, 2007). Circulating progesterone and 17β-estradiol levels remain fairly high and consistent throughout pregnancy and labour, leading to the development of the concept of a "functional" progesterone withdrawal (Casey & McDonald, 1997; Mendelson & Condon, 2005). This was shown to be likely achieved through a switching in the type of progesterone receptor available, as well as through the actions of co-regulator (co-activators and co-repressors) molecules (Allport *et al.*, 2001; McKenna *et al.*, 1999; Merlino *et al.*, 2007; Mesiano, 2004)

The mammalian small heat shock protein family is comprised of eleven proteins thus far (Bellyei *et al.*, 2007; Kappe *et al.*, 2003). The family includes: HSPB1 (HSP27), HSPB2 (MKBP - myotonic dystrophy protein kinase-binding protein), HSPB3, HSPB4 (αA-crystallin), HSPB5 (αB-crystallin), HSPB6 (HSP20), HSPB7 (cvHSP - cardiovascular heat shock protein), HSPB8 (HSP22), HSPB9, HSPB10 (ODFP - sperm outer dense fiber protein), and HspB11 (Hsp16.2) (Kampinga *et al.*, 2009). The HSPB# nomenclature has been introduced to replace the original format of naming by size or function (i.e. HSP27 is a 27KDa protein). All of these members share a common structural domain (e.g. α-crystallin domain), and many also share a common molecular chaperone function (see Arrigo & Müller, 2002; Arrigo, 2007). HSPB1 has another important role concerning the regulation of the actin cytoskeleton and actin-myosin interaction (Gerthoffer & Gunst, 2001). These differing roles are ultimately determined

by the phosphorylation state of the protein, which often determines quaternary structure (Kato *et al.*, 1994b; Kostenko & Moens, 2009). Unphosphorylated HSPB1 forms large oligomeric structures and functions as a molecular chaperone, while the phosphorylated form dissociates into smaller subunits (e.g. monomers and dimers) and functions to promote actin-myosin interaction (Benndorf *et al.*, 1994; Gerthoffer & Gunst, 2001; Ibitayo *et al.*, 1999; Lavoie *et al.*, 1993a, 1993b; Miron *et al.*, 1991). When in a phosphorylated form, HSPB1 is involved in actin polymerization, remodeling, and crossbridge cycling, and has been shown to be essential for the contraction of colonic smooth muscle (Benndorf *et al.*, 1994; Bitar, 2002; Gerthoffer & Gunst, 2001; Ibitayo *et al.*, 1999; Lavoie *et al.*, 1993a,1993b; Miron *et al.*, 1991). Another investigation into colonic smooth muscle functioning has proposed that caldesmon, a protein essential for smooth muscle contraction, may be regulated by HSPB1 (Somara & Bitar, 2006).

In terms of hormonal regulation of sHSPs, 17β-estradiol has been shown to increase the expression of HSPB1 in vascular smooth muscle and MCF-7 breast ductile carcinoma cells, and HSPB1 has been shown in various cell types to interact directly with the nuclear estrogen receptors (ERα & ERβ) and possibly even estrogen itself (Al-Madhoun *et al.*, 2007; Chen *et al.*, 2008; Edwards *et al.*, 1981; Rayner *et al.*, 2008). The 17β-estradiol responsiveness has been hypothesized to be due to a SP1 and half-palindromic 17β-estradiol response element (ERE) separated by 10 nucleotides in the *HSPB1* promoter and through interaction with the 17β-estradiol receptor (Porter *et al.*, 1996).

We have previously reported that HSPB1 expression is highly upregulated in late pregnancy and labour in the rat myometrium (White *et al.*, 2005). It was also seen immunohistochemically that there was a high prevalence of HSPB1 having a membrane-associated localization in both the circular and longitudinal muscle layers of the myometrium during late pregnancy. Our previous observations, in concert with published reports of a role for HSPB1 in colonic smooth muscle contraction (Bitar, 2002) suggested HSPB1 was a potential contraction-associated protein. It was also noted that alterations in HSPB1 expression appeared to be associated with changes in circulating steroid hormone levels during late rat pregnancy. Specifically, mRNA expression appeared associated with the increase and decrease in circulating progesterone levels, while protein expression and phosphorylation were associated with the increase in levels of circulating 17β-estradiol. In this study, we hypothesized that progesterone and 17β-Estradiol may regulate HSPB1 expression and subcellular localization. We tested this hypothesis through the use of northern blotting, immunoblotting, and immunocytochemistry techniques within three experimental rat models; a progesterone-delayed- labour model, an RU486-induced preterm labour model, and a non-pregnant 17β-estradiol-administration model.

3.2 Materials and Methods

3.2.1 Animals.

Sprague Dawley rats were obtained from the Mount Scio Vivarium (Memorial University of Newfoundland, St. John's, Newfoundland and Labrador, Canada). Animals

were held and cared for under standard environmental conditions (12-h light, 12-h dark) in the Animal Care Unit at the Health Sciences Centre, Memorial University of Newfoundland. Rats were fed LabDiet Prolab RMH 3000 (PMI Nutrition International, Brentwood, Missouri, USA) and water *ad libitum*. All experiments were approved by the institutional animal care committee under animal care protocol 03-02-DM - 08-02-DM. For our pregnancy studies, virgin female rats (~220g each) were mated with stud males and the 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. Day 23 samples were collected during active labour, as evidenced by the birth of one or two pups.

3.2.2 Experimental design.

3.2.2.1 Progesterone-delayed labour model.

To investigate the role that progesterone played in regulating levels of HSPB1 in the rat uterus, daily exogenous sub-cutaneous injections of either progesterone (4mg progesterone in 0.2ml corn oil/10%EtOH; P-3972, Sigma-Aldrich, St. Louis, MO, USA; n=3) or corn oil/10% EtOH (n=3) vehicle were administered to pregnant animals starting on day 20 of gestation. Administration of exogenous progesterone prevents the pregnant rat from going into labour on day 23 (Hendrix *et al.*, 1995, Ou *et al.*, 1998). Myometrial samples were collected at days 21, 22, and labour from vehicle-control animals, and at days 21, 22, and 23 (delayed labour) from progesterone-administered rats.

3.2.2.2 RU486-induced labour model.

To investigate the effect of functional progesterone withdrawal on HSPB1 expression, a competitive receptor antagonist, mifepristone (RU-486), was utilized. RU-486 (2.5mg in 0.5mL corn oil/10% ethanol; M-8046, Sigma-Aldrich, St. Louis, MO, USA) was administered sub-cutaneously at day 18 of gestation to facilitate collection of pre-term labour samples during active labour ~24 hours post injection (n=4). Corn oil/10% EtOH vehicle was also administered to control rats on day 18 with subsequent sample collection as normal on day 19 (n=4).

3.2.2.3 17β-Estradiol administration model.

To investigate the role of 17β-estradiol on HSPB1 expression, an ovariectomized rat model (Ou et al., 1997) was utilized, thus avoiding a contribution from endogenous ovarian 17β-estradiol. Virgin female rats (~280g) were anaesthetised by intramuscular injections of a 50:50 mix of ketamine and xylazine (100mg/kg ketamine, 20mg/kg xylazine; Ketaset®, Wyeth Animal Health, Guelph, ON, Canada; Rompun®, Bayer Inc., Toronto, ON, Canada) and ovariectomized through bilateral flank incisions (dorsal surface) approximately 1cm distal of the spine. All animals were monitored post-operatively until they revived. They were then allowed to recover at least 5 days before being given subcutaneous injections of either 17β-Estradiol (0.5μg in corn oil, E-8875, Sigma-Aldrich, St. Louis, MO, USA) or vehicle control (0.2ml corn oil with 10% ethanol). Following injection, animals were euthanised and samples were collected at 1h, 3h, 6h, 12h, and 24h post injection (n=4). Samples were also collected from vehicle-

treated baseline animals (0hr; n=4).

3.2.3 Tissue collection.

All animals were euthanized by carbon dioxide inhalation prior to sample collection. Upon removal of uterine horns, samples were taken for immunocytochemistry and prepared by fixation in 4% paraformaldehyde in 1X PBS overnight with rotation, followed by washing in 1X PBS for 24 hours. Tissue samples were then subjected to a graded ethanol series and xylene penetration prior to embedment in paraffin wax. For northern and western blot assays, uterine horns were opened longitudinally, the foetuses and placentae were discarded, and uterine tissue was placed in ice cold phosphate-buffered saline (PBS; pH 7.4). Gentle scraping with a scalpel was employed to remove any endometrial tissue, prior to flash freezing in liquid nitrogen and storage at -80°C (White *et al.*, 2005).

3.2.4 Northern blot analysis.

Northern blot analysis was performed on four independent sets of RNA samples for each experimental model (ie. 4 rats used per gestational time point). RNA extractions were completed using TRIzol® Reagent according to the manufacturers directions (Invitrogen Corporation, Carlsbad, California, USA). RNA quality and quantity ($A_{260/280}$) were determined using an Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, Ontario, Canada) and samples were stored at -70°C. The northern blot

preparation and hybridization procedures (ie. electrophoresis/membrane transfer and labelling) were completed as previously described in detail (White *et al.*, 2005). Membranes (Hybond-XL nylon membrane; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) were stored at -20 C until required for experiments.

Membranes were first pre-hybridized at 42 C for 1-2 hours. Next the blots were hybridized with radiolabelled probes (^{32}P), created using a Megaprime DNA Labelling kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) following the provided instructions with hamster *HSPB1* cDNA (Genbank Accession #X51747) provided as a kind gift from Dr. J. Landry (Laval University, Quebec, Canada). After blot washes, x-ray film exposures (Hyperfilm MP; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) of varying intensity were prepared to ensure the linearity of the film response. For normalization of northern blot data, all blots were stripped and re-probed for ribosomal *18S* RNA. Rabbit *18S* ribosomal cDNA (Genbank Accession #X06778) was generously provided by Dr. I. Skerjanc (University of Ottawa, Ontario, Canada). *18S* rRNA is constitutively expressed in rat myometrial cells and has been utilized, in the past, as a loading control for analysis of myometrial gene expression (Mitchell and Lye, 2002; Oldenhof *et al.*, 2002, Shynlova *et al.*, 2004).

3.2.5 Immunoblot analysis.

Immunoblot analysis was performed for each experimental model of study, as previously described in detail (White *et al.*, 2005). Protein from flash-frozen samples

were extracted by homogenization of myometrial tissue in RIPA lysis buffer containing COMPLETE™, Mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Laval, Quebec, Canada) and phosphatase inhibitors (PhosSTOP, Roche Diagnostics, Indianapolis, IN, USA). The Bradford Assay (Bradford, 1976) was utilized to determine protein concentrations and samples (50 or 100μg/lane) were electrophoretically separated using 10 or 12% SDS-polyacrylamide gels (Laemmli, 1970). After proteins were transferred to Pierce 0.45μm nitrocellulose membranes (Cat #PI-88018, Fisher Scientific, Ottawa, ON, Canada), immunoblot analysis was performed.

Following 1 hr incubations of blots at room temperature with appropriate primary antisera (Table 3-1), secondary antisera were also incubated with blots for 1 hr at room temperature. Detection of protein-antibody complexes was conducted with the Pierce SuperSignal® West Pico chemiluminescent substrate detection system (Cat #PI-34080, Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer's instructions. Multiple exposures were generated to ensure the linearity of the film response.

Detection of phosphorylated proteins was always the initial immunoblot analysis performed. Following this, stripping of the blots was performed using Restore™ western blot stripping solution (PI-21059, Fisher Scientific, Ottawa, ON, Canada Pierce) according to manufacturer's instructions, and then additional antibodies were used to re-probe the blots for specific proteins. In experimental models, calponin expression was used as a loading control, with the exception of the 17β-Estradiol administration study in

Table 3-1: Antisera Used for Immunobloting and Immunofluorescence

Antisera	Method	Dilution	Company	Catalogue #
Rabbit anti-pHSPB1 (S15)	IB	1:1000	Affinity BioReagents, Golden, CO, USA	PA1-016
Rabbit anti-HSPB1 (CT)	IB	1:1500	Millipore, Temecula, CA, USA	06-517
Mouse anti-calponin; Clone hCP	IB	1:100,000	Sigma-Aldrich Canada, Oakville, Ontario, Canada	C2687
Rabbit anti-ERK2	IB	1:2,000	Santa Cruz Biotechnology, Santa Cruz, CA, USA	sc-154
HRP-Goat anti-Rabbit IgG (H+L)	IB	1:10,000	Promega Corporation, Madison, WI, USA	W4011
HRP-Goat anti-Mouse IgG (H+L)	IB	1:150,000	Promega, Corporation, Madison, WI, USA	W4021
Rabbit anti-pHSPB1 (Ser 15)	IF	1:200	Affinity BioReagents, Golden, CO, USA	PA1-018
Rabbit anti-HSPB1 (CT)	IF	1:200	Millipore, Temecula, CA, USA	06-517
FITC-Sheep anti-Rabbit IgG	IF	1:200	Sigma-Aldrich, St. Louis, MO, USA	F7512

IF: immunofluorescence, IB: immunoblot, FITC: Fluorescein isothiocyanate, HRP: horseradish peroxidase

which ERK2 expression was used (Oldenhof *et al.*, 2002; Shynlova *et al.*, 2009; White *et al.*, 2005).

3.2.6 Immunocytochemistry.

Immunocytochemical analysis was performed on two independent sets of samples for each experimental design, and repeated at least twice. Fixed samples were paraffin embedded, sectioned, and tissue sections placed on silane-coated microscope slides by the Histology Unit of Memorial University of Newfoundland School of Medicine.

For immunocytochemistry, tissue sections were dewaxed in xylene then re-hydrated in a graded ethanol series followed by washing in PBS. Epitope retrieval of re-hydrated tissue sections was conducted by incubating samples in 0.125% Trypsin (in 1XPBS) for 15 minutes at room temperature. Blocking of tissue sections was performed with a solution of 5% goat, 1% horse, and 1% fetal bovine serum in 1XPBS for 1 hour at room temperature. Appropriate primary antiserum (Table 3-1) in blocking solution was incubated on tissue sections for 1 hour at room temperature with mild agitation on a shaker. Following PBS washes, appropriate secondary antiserum was incubated on tissue sections for 1 hour at room temperature. Sections were washed with cold 0.02% Tween-20 in PBS before being mounted with coverslips in Vectashield® (Vector Laboratories Inc., Burlington, Ontario, Canada). Prepared slides were observed and images collected with an Olympus Fluoview laser scanning confocal microscope (Olympus Optical Company Ltd., Melville, New York, USA).

3.2.7 Data analysis.

Densitometric analysis was performed with the aid of Scion Image software (Scion Image Corporation, Frederick, Maryland, USA). Densitometric measurements of *HSPB1* mRNA were normalized to those of *18S* ribosomal RNA while measurements of HSPB1 protein on immunoblots were normalized to those of calponin or total ERK2. Statistical analysis was performed with GraphPad Instat® version 3.1 (GraphPad Software, San Diego, California, USA, www.graphpad.com) and data graphed using GraphPad Prism® version 5.0 (GraphPad Software). Progesterone and 17β-estradiol data was subjected to a Two-way Analysis of Variance (ANOVA) with student t-tests. RU486 data was analysed using t-tests. Values were considered significantly different if $p < 0.05$.

3.3 Results

3.3.1 The effect of progesterone on HSPB1 expression in the pregnant rat myometrium.

3.3.1.1 Expression of HSPB1 mRNA.

Northern blot analysis was performed to assess whether or not *HSPB1* mRNA expression was induced by progesterone administration during late pregnancy. Myometrial samples from pregnant rats given exogenous progesterone beginning on d21 of pregnancy were used for RNA extraction and analyses. Overall, administration of progesterone had a significant effect on *HSPB1* mRNA expression (2-way ANOVA, $p<0.05$). In the vehicle controls, an expected significant decrease in *HSPB1* mRNA

expression was observed between day 21 and day 23 ($p<0.05$) as has been previously reported (White et al 2005). In contrast, progesterone administration significantly elevated *HSPB1* mRNA expression on day 23 (delayed labour) compared to day 23 in the vehicle control (active labour; Figure 3-1A; $p<0.05$).

3.3.1.2 Expression of HSPB1 protein.

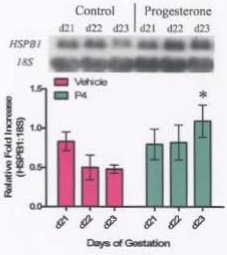
Immunoblot analysis was performed with total myometrial protein extracts to investigate any potential control of HSPB1 protein expression by progesterone. Utilizing an HSPB1 CT-specific antisera it was observed that the normal late pregnancy increase in HSPB1 expression (White et al, 2005) was observed in vehicle controls but that there was a cessation of significance with progesterone administration as indicated through a significant difference ($p<0.05$) between day 21 and day 23 vehicle, and between vehicle and progesterone samples at day 23 of rat gestation (Figure 3-1C). In addition, S15-phosphorylated HSPB1, analysed utilizing a pSer15-HSPB1-specific antisera, has a similar significant response ($p<0.05$) upon administration of progesterone (Figure 3-1B; White et al, 2005).

3.3.1.3 Immunofluorescence analysis.

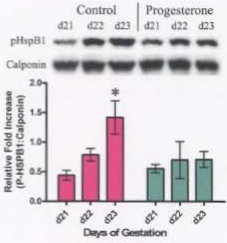
Immunofluorescence analysis was used to investigate any marked changes in cellular localization of HSPB1, due to progesterone administration, in the longitudinal and circular muscle layers of the myometrium. Detection of both total HSPB1 and pSer15 HSPB1 in longitudinal and circular muscle layers became much more readily

Figure 3-1
Progesterone administration prevents changes of HSPB1 normally observed in the myometrium during late pregnancy. Progesterone increases the levels of *HSPB1* mRNA, and prevents the late pregnancy increase in HSPB1 protein expression and phosphorylation. Representative A) Northern blots for *HSPB1* and ribosomal *18S* RNA, as well as protein immunoblots for B) pSer12-HSPB1, C) total HSPB1, and associated loading controls are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different (*) from their time-matched counterparts when $p<0.05$.

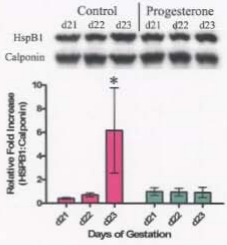
A


Control
Progesterone
d21 d22 d23 d21 d22 d23
HSPB1
18S
Vehicle
P4
Relative Fold Increase (HSPB1:18S)
1.5
1.0
0.5
0.0
*
d21 d22 d23 d21 d22 d23
Days of Gestation

B


Control
Progesterone
d21 d22 d23 d21 d22 d23
pHspB1
Calponin
Relative Fold Increase (P-HSPB1:Calponin)
2.0
1.5
1.0
0.5
0.0
*
d21 d22 d23 d21 d22 d23
Days of Gestation

C


Control
Progesterone
d21 d22 d23 d21 d22 d23
HspB1
Calponin
Relative Fold Increase (HSPB1:Calponin)
10
8
6
4
2
0
*
d21 d22 d23 d21 d22 d23
Days of Gestation

detectable in vehicle controls or upon progesterone administration as day 23 approached (Figures 3-2 – 3-5). However, in progesterone-treated rats total and pSer15 HSPB1 detection decreased in both muscle layers, compared to vehicle controls, throughout the time points examined (Figures 3-2 – 3-5; right panels).

The sub-cellular localization of both total and pSer15 HSPB1 in both muscle cell layers displayed a shift in vehicle controls; from a myometrial cell membrane-associated localization during late pregnancy to a more cytoplasmic localization in myometrial cells beginning at day 22 and markedly occurring at parturition (Figures 3-2 – 3-5; left panels). In progesterone-treated rats, HSPB1 and pSer15 HSPB1 were detectable in membrane-associated, punctate patterns in both muscle layers, but no marked shift of the proteins to the cytoplasmic regions of myometrial cells was observed on day 22 or day 23 (delayed labour) in either muscle layer (Figures 3 -2 – 3-5; right panels). Lastly, regardless of treatment, both total and phosphorylated HSPB1 detection in the longitudinal muscle (Figures 3-2, 3-3) layer was consistently higher than that in the circular muscle layer (Figures 3-4, 3-5).

3.3.2 The effect of progesterone withdrawal on HSPB1 expression.

3.3.2.1 Expression of HSPB1 mRNA.

Since progesterone administration during pregnancy indicated a potential regulatory role for this hormone on *HSPB1* mRNA expression, we investigated the effect of progesterone withdrawal on *HSPB1*. In the rat model, circulating progesterone levels

Figure 3-2
Immunofluorescence detection revealed a maintenance of membrane localization of pSer HSPB1 in the longitudinal muscle layer with progesterone administration. A pSer HSPB1-specific rabbit polyclonal antiserum was used to detect the phosphorylated form of HSPB1 in the longitudinal muscle layer of vehicle control (Oil) or progesterone treated pregnant rats. The oil control samples illustrated the the normal, labour associated translocation to the cytoplasm between day 21 (d21) and day 23 (d23) of rat gestation, while the progesterone-treated samples showed a maintenance of membrane localization, albeit with an overall lower expression. Pre = pre-immune serum control of day 23 vehicle control. Scale bar = 50μm.

Figure 3-2
Immunofluorescence detection revealed a maintenance of membrane localization of pSer HSPB1 in the longitudinal muscle layer with progesterone administration. A pSer HSPB1-specific rabbit polyclonal antiserum was used to detect the phosphorylated form of HSPB1 in the longitudinal muscle layer of vehicle control (Oil) or progesterone treated pregnant rats. The oil control samples illustrated the the normal, labour associated translocation to the cytoplasm between day 21 (d21) and day 23 (d23) of rat gestation, while the progesterone-treated samples showed a maintenance of membrane localization, albeit with an overall lower expression. Pre = pre-immune serum control of day 23 vehicle control. Scale bar = 50μm.

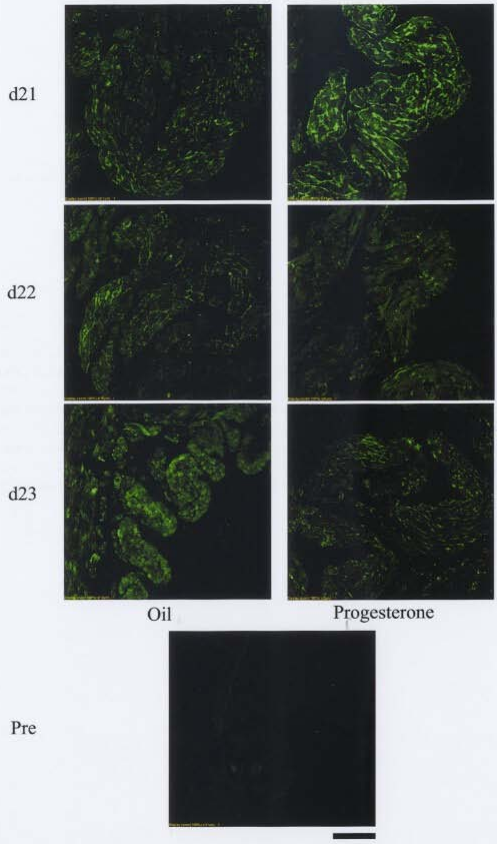

d21
d22
d23
Oil
Progesterone
Pre

Figure 3-3
Immunofluorescence detection revealed a maintenance of membrane localization of total HSPB1 in the longitudinal muscle layer with progesterone administration. A total HSPB1 rabbit polyclonal antiserum was used to detect HSPB1 in the longitudinal muscle layer of vehicle control (Oil) or progesterone treated pregnant rats. Most noticeable is the suppression of the labour associated increase in HSPB1 expression with progesterone administration. The trend of preventing translocation to the cytoplasm is also observable, between day 21 (d21) and day 23 (d23) of rat gestation, but not as pronounced as with phosphorylated HSPB1. Pre = pre-immune serum control of day 23 vehicle control. Scale bar = 50μm.

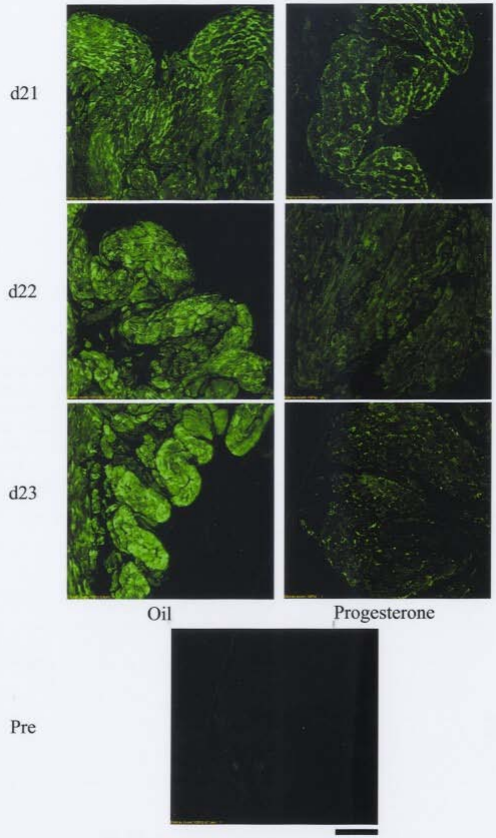

d21
d22
d23
Oil
Progesterone
Pre

Figure 3-4

Immunofluorescence detection revealed a maintenance of membrane localization of pSer HSPB1 in the circular muscle layer with progesterone administration. A pSer15 HSPB1-specific rabbit polyclonal antiserum was used to detect HSPB1 in the circular muscle layer of vehicle control (Oil) or progesterone treated pregnant rats. Expression and localization patterns were similar to that observed in the longitudinal muscle layer, albeit with a lower overall detection level. The oil control samples illustrated the normal, labour associated translocation to the cytoplasm between day 21 (d21) and day 23 (d23) of rat gestation, while the progesterone-treated samples showed a maintenance of membrane localization. Pre = pre-immune serum control of day 23 vehicle control. Scale bar = 50μm.

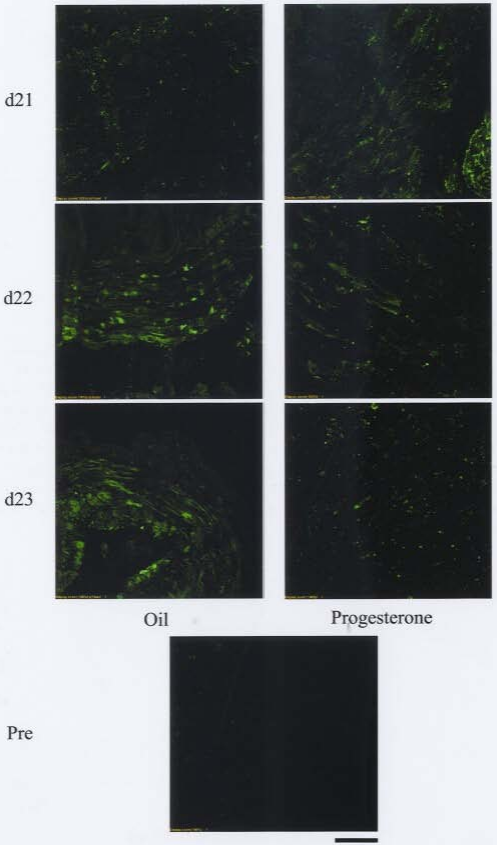
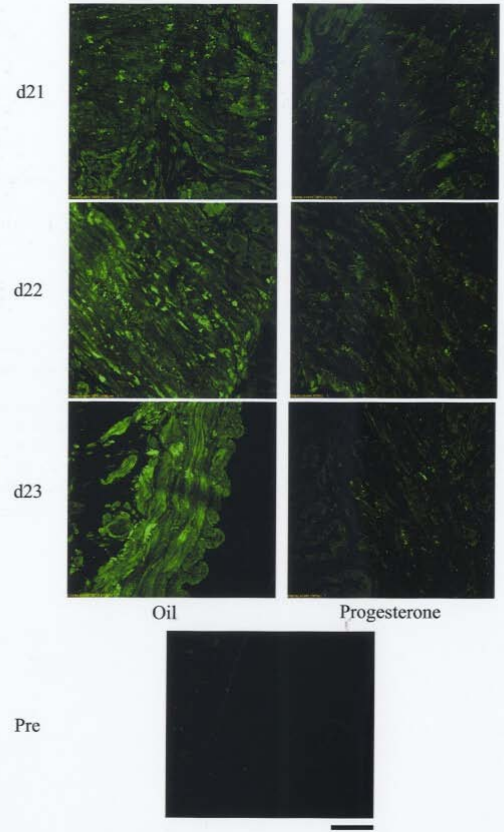

d21
d22
d23
Oil
Progesterone
Pre

Figure 3-5
Immunofluorescence detection revealed a maintenance of membrane localization of total HSPB1 in the circular muscle layer with progesterone administration. A total HSPB1 rabbit polyclonal antiserum was used to detect HSPB1 in the circular muscle layer of vehicle control (Oil) or progesterone treated pregnant rats. Expression and localization patterns were similar to that observed in the longitudinal muscle layer, albeit with a lower overall detection level. The oil control samples illustrated the the normal, labour associated translocation to the cytoplasm between day 21 (d21) and day 23 (d23) of rat gestation, while the progesterone-treated samples showed a maintenance of membrane localization. Pre = pre-immune serum control of day 23 vehicle control. Scale bar = 50μm.


d21
d22
d23
Oil
Progesterone
Pre

increase to a maximum between days 15 & 19, after which levels drop to almost baseline by labour (Pepe, 1974). As a consequence, day 19 of pregnancy was chosen for sample collection; a time when circulating levels of progesterone and *HSPB1* expression are highest. Progesterone withdrawal was facilitated in pregnant rats through injections of the progesterone receptor antagonist RU486 on d18 of pregnancy. RU486 injection induces pre-term delivery approximately 24 hours after injection when samples were collected. Northern blot analysis illustrated that progesterone withdrawal resulted in a very significant decrease in *HSPB1* mRNA expression compared to vehicle controls (Figure 3-6A). In a total of four experiments, progesterone withdrawal resulted in an average 3.6 fold decrease in mRNA expression (t-test; $p<0.05$).

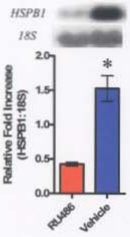
3.3.2.2 Expression and localization of HSPB1 protein.

Immunoblot analysis of total protein extracted from RU486-treated or control myometrial samples illustrated that progesterone withdrawal had no significant effect on total HSPB1 or pSer15 HSPB1 protein expression (Figure 3-6B; $p>0.05$). In contrast, localization of HSPB1 in situ was significantly altered by RU486-induced progesterone withdrawal. In the longitudinal muscle layer, both total and pSer15 HSPB1 became localized to the cytoplasm of myometrial cells on d19 compared to vehicle controls where HSPB1 remained punctate and membrane-associated (Figure 3-7). A similar but less dramatic change was also found for total and phosphorylated HSPB1 protein localization in the circular muscle layer (Figure 3-8).

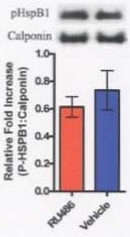
Figure 3-6

Blocking the actions of progesterone with RU486 decreases the level of *HSPB1* mRNA expression, but had no effect on HSPB1 protein expression or phosphorylation at day 19 of rat gestation. Representative A) Northern blots for *HSPB1* and ribosomal *18S* RNA, as well as protein immunoblots for B) pSer12-HSPB1, C) total HSPB1, and associated loading controls are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different (*) when $p<0.05$.

A


HSPB1
18S
Relative Fold Increase (HSPB1::18S)
2.0
1.5
1.0
0.5
0.0
*
RU486
Vehicle

B


pHspB1
Calponin
Relative Fold Increase (P-HSPB1::Calponin)
1.0
0.8
0.6
0.4
0.2
0.0
RU486
Vehicle

C

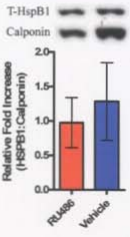

T-HspB1
Calponin
Relative Fold Increase (HSPB1::Calponin)
2.0
1.5
1.0
0.5
0.0
RU486
Vehicle

Figure 3-7
Immunofluorescence detection revealed a cytoplasmic localization of pSer HSPB1 in the myometrium with RU486 administration. A pSer HSPB1-specific rabbit polyclonal antiserum was used to detect the phosphorylated form of HSPB1 in the longitudinal (Long) and circular (Circ) muscle layers of vehicle control (Oil) or progesterone receptor antagonist (RU486) treated pregnant rats. At day 19 of rat gestation, a very marked translocation of HSPB1 from the membrane region to a more cytoplasmic/peri-nuclear localization occurred upon administration of progesterone receptor antagonist, and is easily noted in the longitudinal layer due to overall higher detection levels. Pre = pre-immune serum control for respective treatment groups. Scale bar = 50μm

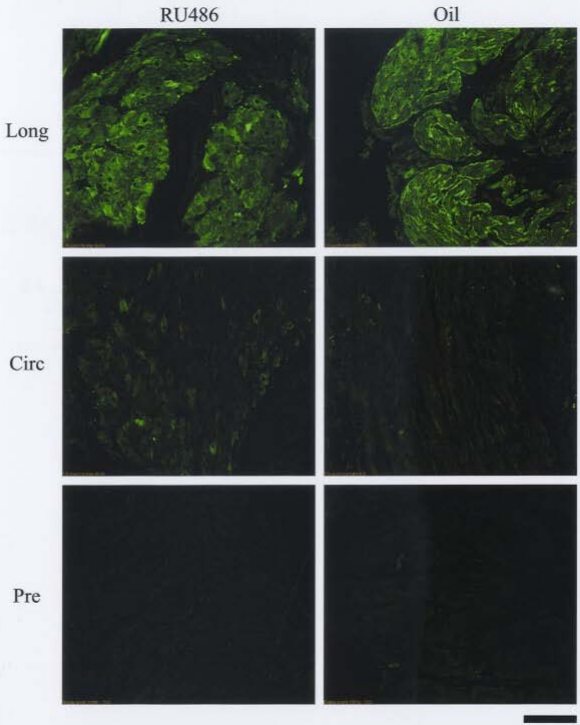
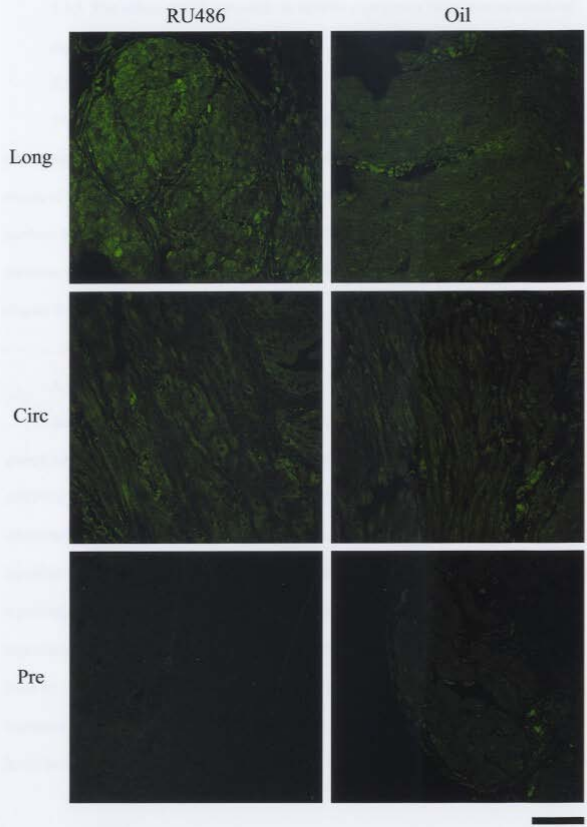

RU486
Oil
Long
Circ
Pre

Figure 3-8
Immunofluorescence detection revealed a cytoplasmic localization of total HSPB1 in the myometrium with RU486 administration. A total HSPB1 rabbit polyclonal antiserum was used to detect total HSPB1 in the longitudinal (Long) and circular (Circ) muscle layers of vehicle control (Oil) or progesterone receptor antagonist (RU486) treated pregnant rats. Expression and localization patterns were similar to that observed with pSer HSPB1, albeit with a lower overall detection level. Pre = pre-immune serum control for respective treatment groups. Scale bar = 50μm


RU486
Oil
Long
Circ
Pre

3.3.3 The effect of 17β-estradiol on HSPB1 expression in ovariectomized-rat myometrium

3.3.3.1 Expression of HSPB1 mRNA.

17β-estradiol, being the other major pregnancy-related hormone, was subsequently investigated in a hormone-minimized model. After collection of a time course of myometrial RNA samples from ovariectomized rats administered 17β-estradiol, northern blot analysis was performed to assess mRNA expression. Administration of the hormone had no significant effect on *HSPB1* mRNA expression over the time course (Figure 3-9A; Two way ANOVA; $p>0.05$).

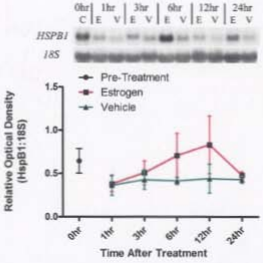
3.3.3.2 Expression of HSPB1 protein.

Immunoblot analysis demonstrated that 17β-estradiol administration significantly altered total and phosphorylated HSPB1 protein expression (Figure 3-9B; Two way ANOVA; $p<0.05$). Analysis of total HSPB1 protein expression demonstrated that administration of 17β-estradiol led to a significant increase in expression by 12 h post-injection compared to the paired vehicle control and was maintained thereafter through 24 h post-injection (Two way ANOVA; $p<0.05$). In contrast, phosphorylated HSPB1 expression responded very quickly to the 17β-estradiol stimulation. At the earliest time point of 1 hours post injection phosphorylated HSPB1 expression was significantly increased ($p<0.01$) compared to the paired vehicle control and remained higher until 24 hours post injection.

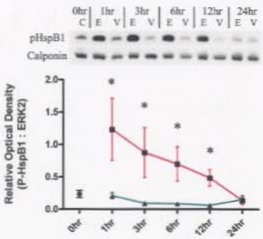
Figure 3-9

Administration of exogenous 17β-estradiol to ovariectomized rats increased expression and phosphorylation of HSPB1, but had no effect on mRNA expression. Phosphorylation of HSPB1 occurred very rapidly, while overall expression increased after 12 hours. Representative A) Northern blots for *HSPB1* and ribosomal *18S* RNA, as well as protein immunoblots for B) pSer12-HSPB1, C) total HSPB1, and associated loading controls are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different (*) from their time-matched counterparts when $p<0.05$.

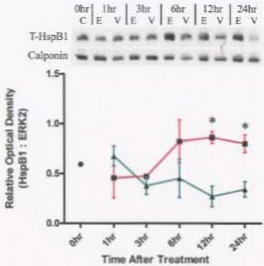
A


0hr 1hr 3hr 6hr 12hr 24hr
C E V E V E V E V E V
HSPB1
18S
Pre-Treatment
Estrogen
Vehicle
Relative Optical Density (HspB1:18S)
1.5
1.0
0.5
0.0
0hr 1hr 3hr 6hr 12hr 24hr
Time After Treatment

B


0hr 1hr 3hr 6hr 12hr 24hr
C E V E V E V E V E V
pHspB1
Calponin
Relative Optical Density (P-HspB1 : ERK2)
2.0
1.5
1.0
0.5
0.0
0hr 1hr 3hr 6hr 12hr 24hr

C


0hr 1hr 3hr 6hr 12hr 24hr
C E V E V E V E V E V
T-HspB1
Calponin
Relative Optical Density (HspB1 : ERK2)
1.5
1.0
0.5
0.0
0hr 1hr 3hr 6hr 12hr 24hr
Time After Treatment

Despite the immunoblot results, immunofluorescence analysis of the spatial localization of HSPB1 in 17β-estradiol-injected rat myometrium samples revealed that total and phosphorylated HSPB1 expression were at virtually undetectable levels in this model system using this technique.

3.4 Discussion

Through it's various means of action, progesterone proceeds to help prepare the myometrium for future contraction by directly or indirectly increasing the expression of proteins such as collagen II, integrin α5, and activated FAK, preventing an increase in contraction associated proteins such as connexin-43 (cx-43) and oxytocin receptor (OTR), and facilitating specific intracellular trafficking of various proteins such as cx-43(Hendrix *et al.*, 1995; Lye *et al.* , 1993; MacPhee & Lye, 2000; Ou *et al.*, 1997; Ou *et al.*, 1998; Shynlova *et al.*, 2004; Shynlova *et al.*, 2009; Williams *et al.*, 2005). In addition to this, progesterone interferes with normal signalling pathways of 17β-estradiol and stretch as it often prevents the 17β-estradiol/stretch-induced increase in myometrial gene expression of many proteins (Lye *et al.*, 2001; Mesiano & Welsh, 2007; Ou *et al.*, 1997; Ou *et al.*, 1998; Shynlova *et al.*, 2009). For example, Ou and colleagues (1997) found that administration of progesterone to non-pregnant rats counteracted the stretch-induced increase of Cx43 gene expression, while Petrocelli and colleagues (1993) illustrated the ability of exogenous progesterone to attenuate the induction of Cx43 expression by 17β-estradiol adminitration. In another study, Ou and colleagues (1998) also found that

progesterone administration prevented the normal late pregnancy rise in oxytocin receptor, and prevented it's pre-term labour-associated increase (Ou *et al.*, 1998)

However the eventual hormone shift, whether through a change in progesterone production levels or through changes in associated receptors and/or coactivators, allows "activation" of the muscle through expression of contraction associated proteins (CAPs) and preparations for the active contractions of labour (Allport *et al.*, 2001; Chaim & Mazor, 1998; Condon *et al.*, 2003; Dong *et al.*, 2005; Lye, 1994; McKenna *et al.*, 1999; Mesiano, 2004; Merlino *et al.*, 2007).

The role of progesterone in the modulation of HSPB1 expression was investigated through two experimental setups involving pregnant rats. One investigated the effect that addition of exogenous progesterone, and thus a delaying of labour, would have on HSPB1 expression. We also looked at a negation of the effects of progesterone through the addition of RU486 (progesterone receptor antagonist), also known as mifepristone. This drug blocks the actions of endogenous progesterone and induces pre-term labour the following day (Mao *et al.*, 1992). With most proteins associated with myometrial contraction being stimulated by 17β-estradiol and decreased by progesterone, we hypothesized that progesterone would block *HSPB1* gene expression overall. To the contrary, we found that progesterone increased expression of *HSPB1* mRNA (Figure 3-1A). Not only did the addition of progesterone elevate *HSPB1* mRNA levels on day 23 of rat pregnancy (labour), preventing the usual late pregnancy drop, but the addition of RU486 on day 18 of rat pregnancy significantly decreased *HSPB1* mRNA expression the

following day (Figure 3-1A). The timepoint chosen for RU486 administration (day 18), was the peak of *HSPB1* mRNA expression in normal gestation (White *et al.*, 2005). Therefore the significant drop in *HSPB1* mRNA expression upon RU486 administration must be attributed to a lack of stimulation by progesterone.

In contrast to *HSPB1* mRNA expression, the addition of exogenous progesterone to pregnant rats did not significantly influence total HSPB1 protein expression and prevented the increase in pSer15 HSPB1 observed previously during labour and in vehicle controls (Figure 3-1B). Further verification that progesterone did not significantly influence HSPB1 protein expression was provided in experiments with RU486 where overall total and phosphorylated HSPB1 expression levels were unaffected by progesterone receptor antagonism.

Despite the above findings, it was particularly interesting to observe the effect of progesterone on the subcellular localization of HSPB1 *in situ*. In our previous study, we found that as pSer15 HSPB1 protein expression in the myometrium increased at mid-late gestation, it was almost entirely found in a membrane-associated pattern within the myometrial cells (White *et al.*, 2005). This localization pattern remained until just prior to labour, at which point there was a shift from membrane-associated to a cytoplasmic or peri-nuclear localization pattern. Although it has been shown that intra-cellular localization is often dependent on the phosphorylation state of HSPB1 with phosphorylation leading to a membrane association, translocation does not always correlate with phosphorylation (Gusev *et al.*, 2002). This appears to be the case in the

myometrium. This localization change was thought to be associated with the late pregnancy shift in the major circulating steroid hormone from progesterone to 17β-estradiol, so we proceeded to further define its regulation. We found that regulation of HSPB1 localization within pregnant rat myometrial cells was due to the actions of progesterone. Not only did the addition of progesterone prevent the normal labour associated shift of HSPB1 to the cytoplasm (Figures 3-2 – 3-5), but blocking the actions of progesterone on day 18 with RU486 stimulated a movement from almost entirely membranous to almost entirely cytoplasmic (Figures 3-6, 3-7). As this timepoint is prior to the large surge in circulating 17β-estradiol levels in pregnant rats, it can be surmised that this shift is due to a cessation of progesterone regulatory stimuli as opposed to a cessation of the blocking of 17β-estradiol actions by progesterone. This regulatory mechanism again differs from that normally observed with CAPs, as progesterone decreases the translocation of gap junction proteins to the membrane to minimize gap junction formation (Mesiano & Welsh, 2007). One critique of this assessment could be that since RU486 has been shown to increase the levels of both 17β-estradiol and corresponding estrogen receptors in rat uterine tissue, perhaps it is the estrogen increase associated with RU486 injection that is facilitating the localization changes (Fang *et al.*, 1997). This can be refuted in two ways. First, the RU486 associated rise in 17β-estradiol reported by Fang (1997) didn't become significant until 48 hours after injection and our samples were taken after 24 hours. Additionally, if RU486 had caused an increase in circulating 17β-estradiol levels, we would have likely observed a stimulation of total and

phosphorylated HSPB1 protein expression on immunoblots following RU486 administration. HSPB1 protein expression was not significanlty altered upon RU486 administration, thus it can be surmised that any RU486-induced 17β-estradiol expression does not have a noticable effect in our experimental model. Additionally, it is possible that the action of progesterone is counteracting the default pathway of phosphorylated HSPB1 to be cytoplasmically located. Thus when the actions of progesterone are withdrawn, HSPB1 naturally migrates to the cytoplasm.

Investigation of the effects of 17β-estradiol on HSPB1 expression was conducted through the use of an established ovariectomized, non-pregnant rat model (Ou *et al.*, 1998) Through this experimental model it was shown that 17β-estradiol has a significant effect on the expression of HSPB1 that is similar to established CAPs (Petrocelli *et al.*, 1993). We have shown that 17β-estradiol increases protein expression overall, but also very significantly, and very quickly, increases phosphorylation of HSPB1 on serine 15 (Figure 3-9). It is likely that both the "traditional" genomic actions and the quick acting non-genomic actions of 17β-estradiol are at play (Levin, 2005; 2009). It has long been shown that *HSPB1* gene expression is induced by steroid hormones, including 17β-estradiol (Edwards *et al.*, 1980). It is postulated that the genomic effects are through a combination of two mechanisms. Analysis of the *HSPB1* promoter sequence located a SP1 binding site and a half-palindromic ERE motif, implicating a direct influence from 17β-estradiol (Gaestel *et al.*, 1993; Porter *et al.*, 1996). A putative AP-1 also exists within the promoter sequence of *HSPB1*, opening the possibility of involvement of the AP-1

signalling pathway which again implicates the genomic involvement of 17β-estradiol, since 17β-estradiol receptor-α has been shown to complex with AP-1 (Assimakopoulou & Varakis, 2001; Bjornstrom & Sjoberg, 2005). Non-genomically, 17β-estradiol has been shown to activate the p38 MAPK pathway (Bjornstrom & Sjoberg, 2005; Kelly & Levin, 2001; Suzuki *et al.*, 2008). This could facilitate the very quick increase in phosphorylation of HSPB1. This increase in protein levels, but not mRNA levels can partially be explained through the observation that 17β-estradiol has been shown to increase mRNA stability and not necessarily mRNA production, allowing for an increased time in which the RNA is available for translation (Hamilton *et al.*, 1968; Ing *et al.*, 2005). Additionally, it is possible for the actions of the translational and post-translational machinery to be increased and thus act on the RNA present, regardless of any RNA changes. To further increase this effect, the greater stability of cellular proteins means a longer turnover time, thus causing an additive effect on detection levels. Therefore it is more than reasonable that protein expression be increased without an associated mRNA increase.

The observed changes in HSPB1 expression are consistent with those observed for traditional contraction associated proteins (CAPs) such as connexin-43 (Orsino *et al.*, 1996; Ou *et al.*, 1997; Petrocelli & Lye, 1993), supporting the assertion that HSPB1 is a contraction associated protein that is regulated like others, at least in part, by the increase in circulating levels of 17β-estradiol associated with late pregnancy.

3.4.1 A model for hormone regulation of HSPB1.

It is interesting to note how the results presented on the hormonal control of HSPB1 expression are not only a complex, coordinated regulation, but also correspond with two of the three proposed uterine stages of differentiation during pregnancy; the synthetic and contractile phases (Shynlova *et al.*, 2009). Progesterone levels are highest during the synthetic stage of pregnancy to sustain the quiescent uterine environment and this is a period of synthesis of ECM molecules and remodelling of cell-ECM interactions. This period also appears to be marked by increased *HSPB1* mRNA synthesis then available for subsequent translation, post-translational modification and future utilization. Existing HSPB1 occurs at membrane-associated regions where HSPB1 protein may be involved in cytoskeletal anchoring as has been proposed in White *et al.* (2005) . With the switch of circulating steroid hormones from progesterone to 17β-estradiol comes the transition from the synthetic to contractile uterine phase (Rosenblatt *et al.*, 1979; Shynlova *et al.*, 2009). Increased levels of circulating 17β-estradiol could then facilitate an overall stimulation of HSPB1 translation and particularly phosphorylation, perhaps due to an increase in p38 MAPK activation leading to the phosphorylation of HSPB1. Furthermore, 17β-estradiol stimulated HSPB1 protein expression and phosphorylation may then aid translocation into the cytoplasm where it could facilitate actin-myosin interaction and contraction of uterine smooth muscle. We also cannot rule out a role for HSPB1 as a chaperone during the contractile phase of myometrial differentiation nor can we ignore the role uterine stretch may have on *HSPB1* gene expression. It is possible that

AP-1 can serve to integrate stretch and hormonal control of HSPB1 expression in the uterus. The AP-1 family (e.g. c-fos) has been shown to respond to stretch within the uterus, but progesterone can successfully block the increased AP-1 expression (Mitchell *et al.*, 2002).

The most pertinent research required be undertaken in the future should be an examination of the role that uterine stretch plays in the regulation of HSPB1. Since the imput of mechanotransduction signalling is intertwined with many of the hormone regulated experimental designs used here, this factor needs to elucidated in order to further understand the results described here.

Additional research should be done to further classify the complex interplay of hormonal influences on HSPB1. The synergistic role of progesterone and 17β-estradiol in both pregnant and non-pregnant states, as well as examination of importance of 17β-estradiol priming in the non-pregnant state must be investigated. Additionally, co-localization studies involving other CAPs, cytoskeletal elements, and focal adhesion proteins would be beneficial. As HSPB1 patterns of expression display some similarities with cx-43 (CAP), laminin (cytoskeleton), and FAK/paxillin (focal adhesions) expression, verification of any potential co-localization would aid in further defining the role that HSPB1 plays in the uterine musculature (MacPhee & Lye, 2000; Ou *et al.*, 1998; Shynlova *et al.*, 2004).

In the end, this research provides a deeper understanding of the hormonal regulation of HSPB1 in uterine smooth muscle and additionally helps to broaden the

knowledge-base that is growing about the regulation of this small heat shock protein in smooth muscle.

Chapter 4

Distension of the Uterus Induces HSPB1 Expression in Rat Uterine Smooth Muscle.

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4.1 Introduction

The uterus during pregnancy undergoes considerable physical distension. In response to such mechanical stress, adaptation of the uterus occurs and is exemplified by increases in uterine size and weight. Before pregnancy, the human uterus weighs approximately 40-70g and can hold a volume of 10mL, whereas at labour the uterus weighs about 1100-1200g and can hold on average a volume of 5L (Monga & Sanborn, 2003). The size of human uterine smooth muscle cells have also been found to increase up to 10 times in length and 3 times in width during gestation (Monga & Sanborn, 2003; Sanborn, 1987).

During gestation the rat uterine smooth muscle, or myometrium, goes through a series of differentiation phases (Shynlova *et al.*, 2009). Early in pregnancy, the myometrium is characterized by a phase of hyperplasia marked by significantly increased mRNA expression of IGF-I and IGFBP1 as well as activation of the mTOR signalling pathway (Jaffer *et al.*, 2009; Shynlova *et al.*, 2007a). Subsequently, there is a transition to a synthetic phase with a period of hypertrophic myometrial growth that requires uterine

distension and is supported by circulating progesterone. This growth is marked by an increase in the protein:DNA ratio of the myometrium and the increased expression of interstitial matrix proteins such as collagen I (Shaw *et al.*, 2003; Shynlova *et al.*, 2006). Recently, Shynlova and colleagues (2010b) demonstrated that the significant increase in rat myometrial growth during late pregnancy was due to a threefold increase in myocyte size and confirmed that it was dependent on uterine distension. Towards the end of gestation the myometrium switches to a contractile phenotype with an increase in basement membrane matrix synthesis, marked by increased expression of fibronectin, as well as increased detection *in situ* of the fibronectin receptor protein subunits α5 and β1 integrins (Shynlova *et al.*, 2007a; Williams *et al.*, 2005; Williams *et al.*, 2010). The rat myometrium then undergoes the labour phase and becomes activated by the increased presence of contraction associated proteins (CAPs) such as the gap junction protein Cx43 and oxytocin receptors as well as decreased focal adhesion kinase activity thus marking the stabilization of myometrial cell-ECM contacts to facilitate the initiation of labour (MacPhee & Lye, 2000; Ou *et al.*, 1998; Tabb *et al.*, 1992). In this differentiation sequence, both endocrine and mechanical pathways are required for induction of CAP gene expression (Li *et al.*, 2007; Li *et al.*, 2009; Oldenhof *et al.*, 2002; Ou *et al.*, 1997; Ou *et al.*, 1998; Shynlova *et al.*, 2009).

Heat shock protein B1 (HSPB1; previously known as HSP27) is one of 11 members of the mammalian small heat shock protein (sHSP) family (Kampinga *et al.*, 2009; Laskowska *et al.*, 2010). All members of this class have a molecular weight

between 15 and 40 kDa and share a structural domain in their carboxyl-terminal halves named the α-crystallin domain that spans two putative actin binding domains (Quinlan, 2002). Furthermore, these proteins have demonstrated ATP1-independent chaperone activity (Kappe *et al.*, 2003; Laskowska *et al.*, 2010).

Phosphorylation of sHSPs is a post-translational modification that is very important for regulation of sHSP structure and function. For example, HSPB1 phosphorylation results in dissociation of large oligomers of HSPB1 and loss of chaperoning activity (Kato *et al.*, 1994b; Kostenko & Moens, 2009). Two phosphorylation sites have been reported for HSPB1 in rodents, serine (Ser)-15 and Ser-86 (homologous to Ser-82 in humans and Ser-90 in hamsters; Gaestel *et al.*, 1991). The latter site is necessary for the dissociation of large sHSP multimers, but at the cellular level it is not always sufficient (Gaestel, 2002; Kato *et al.*, 2002; Lambert *et al.*, 1999). Ser-15 phosphorylation of HSPB1 may produce a conformational change in HSPB1 that aids the direct binding of HSPB1 with actin microfilaments (Lambert *et al.*, 1999). A number of past reports have indicated a role for HSPB1 in actin polymerization, remodelling, stabilization and actin-myosin crossbridge cycling (Benndorf *et al.*, 1994; Ibitayo *et al.*, 1999; Lambert *et al.*, 1999; Lavoie *et al.*, 1993a, 1993b; Miron *et al.*, 1991). In terms of a role in smooth muscle, the facilitation of actin formation and actin-myosin interaction by HSPB1 has been shown to be essential for the contraction of colonic smooth muscle (Bitar, 2002).

Many different cellular stress signals initiate HSPB1 expression and

phosphorylation, such as heat shock and oxidative stress (Kato *et al.*, 2002; Landry *et al.*, 1992), and HSPB1 expression is reportedly induced by stretch of epithelial cells and heavy resistance training of skeletal muscle (Luo *et al.*, 2007; Murlasits *et al.*, 2006). Recently, Chaudhuri and Smith (2008) reported that cyclic mechanical stress of airway smooth muscle cells resulted in increased HSPB1 phosphorylation, yet the role of mechanical stress on HSPB1 expression within the uterine musculature during pregnancy remains unknown.

We have previously reported that *HSPB1* gene expression is highly upregulated in the rat myometrium during late pregnancy and labour (White *et al.*, 2005), which parallels the increase in mechanical stress or distension exerted on the myometrium due to growing foetuses. It was also reported that during late pregnancy Ser-15 phosphorylated HSPB1 (pSer15 HSPB1) had a predominantly membrane-associated localization *in situ* within cells of both the circular and longitudinal muscle layers. We have thus suggested that HSPB1 is a potential contraction-associated protein, but the regulatory mechanism(s) underlying myometrial HSPB1 expression during pregnancy are still unknown. Utilizing a unilaterally pregnant rat model, we examined the effect of uterine distension on myometrial HSPB1 expression. We hypothesized that this stress would increase HSPB1 expression within the uterine musculature.

4.2 Materials and Methods

4.2.1 Animals and tissue collection.

Sprague-Dawley rats were maintained in the Animal Care Unit at the Health Sciences Centre (Memorial University of Newfoundland, St. John's, Newfoundland and Labrador, Canada) under standard environmental conditions (12-h light, 12-h dark) and the institutional animal care committee approved all experiments under protocols 06-02-DM - 08-02-DM. Animals were fed Purina Rat Chow #5012 (Purina Mills, St. Louis, Missouri, USA) and water *ad libitum*. For collection of pregnant rat samples, virgin female rats (~220g) were mated with stud males. Day 1 of pregnancy was determined by the observance of vaginal plugs the next morning. Under this timing scheme the rat gestational period was 23 days.

Carbon dioxide induced asphyxiation was used for euthanisation of all animals prior to sample collection. Uterine horn samples to be used for immunocytochemical analysis were fixed in 4% paraformaldehyde in 1X PBS overnight with agitation, followed by washing in 1X PBS for 24 hours. Tissue samples were then subjected to a graded ethanol series and xylene penetration prior to embedment in paraffin wax. In other cases, excised uterine tissue was placed in ice-cold PBS (pH 7.4) and opened longitudinally for collection of the myometrium as has been previously described (White *et al.*, 2005). The endometrial layer was removed from all samples by gentle scraping with a scalpel blade prior to flash freezing in liquid nitrogen and storage at -80°C.

4.2.2 Experimental design.

4.2.2.1 Unilaterally pregnant rat model.

Virgin female rats (~220g) were administered an intramuscular injection of anesthesia (100mg/kg ketamine, 20mg/kg xylazine; Ketaset®, Wyeth Animal Health, Guelph, ON, Canada; Rompun®, Bayer Inc., Toronto, ON, Canada) and then received a unilateral tubal ligation through bilateral flank incisions (dorsal surface), approximately 1cm distal of the spine, as described elsewhere (Ou *et al.*, 1997; Ou *et al.*, 1998). Animals were monitored post-operatively and subsequently allowed to recover for at least 5 days before matings were attempted. Samples of gravid and non-gravid horns were collected on gestational day (d) 19 and d23 (n=4).

4.2.2.2 Non-pregnant ovariectomized rat models.

To investigate the role of uterine distension on HSPB1 expression in the absence of any endocrinological contributions from the feto-placental units or ovarian steroids, we utilized a non- pregnant ovariectomized rat model. Prior to experiments using laminaria tents, female rats (~220g) were administered an intramuscular injection of anesthesia 100mg/kg ketamine, 20mg/kg xylazine; Ketaset®, Wyeth Animal Health, Guelph, ON, Canada; Rompun®, Bayer Inc., Toronto, ON, Canada). Rats were then bilaterally ovariectomized as previously described in detail (Ou *et al.*, 1997). Animals were allowed to recover for at least 5 days post-operatively before laminaria tent insertion.

To generate a dynamic uterine distension over 24h in the uterine horns of these rats, we used extra-small (2 x 50mm) laminaria tents (Catalogue #021002, MedGyn,

Lombard, IL, USA). These tents consist of dried, sterilized seaweed stems that are hygroscopic and gradually expand over 24h. Larger versions of these tents can be used clinically as disposable devices for gentle dilation and softening of the cervix. Following administration of anesthesia to the rats (as described above), bilateral flank incisions (dorsal surface) approximately 1cm distal of the spine were made to expose the uterine horns. Laminaria tents were then surgically inserted into the lumen of one of the uterine horns of the ovariectomized rats (n= 4) through a small distal incision in the horn that was then sutured closed. To control for the presence and effect of an intra-uterine device (IUD), polyethylene tubes of the same size and diameter (~2mm OD, Cat #7446, Clay Adams) as pre-expanded laminaria tents were also inserted into some uterine horns (n=4) in place of the laminaria tents. Myometrial samples were subsequently collected from empty, polyethylene tube-containing and laminaria-containing horns 24hrs post insertion.

4.2.3 Northern blot analysis.

For each experimental model studied, 4 independent sets of RNA samples (n=4) were used. TRIzol® Reagent (Invitrogen Corporation, Carlsbad, California, USA) was used for RNA extractions, and extractions were completed according to the manufacturer's instructions. Spectrophotometric analysis of RNA was performed using a Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, Ontario, Canada) to assess RNA purity and concentration before storage at -80°C.

Northern blot preparation and hybridization were performed as previously

described (See section 2.2.3: Northern Blot Analysis; White *et al.*, 2005). Briefly, RNA (10μg) was prepared, separated on a 1% agarose-formaldehyde-MOPS gel, transferred to a nylon membrane (Hybond-XL nylon membrane; GE Healthcare, Little Chalfont, Buckinghamshire, England), and stored at -20 C. Pre-hybridization of membranes for 1-2 hours at 42°C was followed by hybridization with radiolabelled (^{32}P-dCTP) probes overnight at 42°C. Probes were produced utilizing a hamster *HSPB1* cDNA template, following instructions provided in the Megaprime DNA Labeling kit (GE Healthcare, Little Chalfont, Buckinghamshire, England). Blots were subsequently washed and multiple exposures of X-ray film were produced to confirm the linearity of the film response. Following detection of *HSPB1* mRNA, northern blots were stripped and re-probed for ribosomal *18S* RNA for use as a loading control. The hamster *HSPB1* cDNA (Genbank Accession #X51747) and rabbit *18S* ribosomal cDNA (Genbank Accession #X06778) were kind gifts from Dr. J. Landry (Laval University, Quebec, Canada) and Dr. I. Skerjanc (University of Ottawa, Ontario, Canada), respectively. *18S* rRNA is constitutively expressed in rat myometrial cells and has been utilized in the past as a loading control for analysis of myometrial gene expression (Oldenhof *et al.*, 2002; Shynlova *et al.*, 2004; White *et al.*, 2005).

4.2.4 Immunoblot analysis.

Immunoblot analysis was performed on at least 4 independent sets of samples as previously described in detail (White *et al.*, 2005). Flash-frozen myometrial samples were

used to extract total protein in RIPA lysis buffer containing COMPLETE™ Mini EDTA-free protease inhibitors and PhosSTOP phosphatase inhibitors (Roche Molecular Biochemicals, Laval, Quebec, Canada). Protein concentrations were determined using the Bradford assay (Bradford, 1976). Protein samples (100ug/lane) were separated under reducing conditions in 12% SDS-polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose membranes (0.45µm, Pierce membranes, Fisher Scientific, Ottawa, ON, Canada; 0.45µm, Pall Corporation, East Hills, NY, USA).

Membranes were first probed for detection of the serine 15 phosphorylated form of HSPB1 with a pSer15 HSPB1-specific rabbit polyclonal antibody (1 ug/mL final concentration; Cat # PA1-016, Affinity BioReagents, Golden, CO, USA), then stripped with Restore western blot stripping solution (Cat# PI-21059, Fisher Scientific, Ottawa, ON, Canada) and re-probed for Calponin, which served as a loading control, using a mouse monoclonal antibody provided as an ascites fluid (0.01 ug/mL final concentration, clone hCP; Cat # C2687, Sigma-Aldrich Canada, Oakville, Ontario, Canada). The appropriate HRP-conjugated anti-rabbit (Cat #W4011, Promega Corporation, Madison, WI, USA) and anti-mouse secondary antibodies (Cat #W4021, Promega Corporation, Madison, WI, USA) were utilized in 1:10,000 and 1:150,000 dilutions respectively. The pSer15 HSPB1 and calponin-specific antisera recognized protein bands at the predicted 27 kDa and 34 kDa molecular weights, respectively. Blots were developed with the Pierce SuperSignal West Pico chemiluminescence substrate detection system (Cat# PI34080, Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer's

instructions. Multiple exposures were generated to ensure the linearity of the film exposures.

4.2.5 Immunocytochemistry.

Immunocytochemical analysis was performed on two independent sets of uterine tissue samples for each experimental design and repeated at least 2 times as previously described (White *et al.*, 2005). All paraformaldehyde fixed samples were processed, embedded, and sectioned by the Histology Unit of Memorial University of Newfoundland School of Medicine. Five micrometer thick tissue sections were adhered to silane-coated slides. Sections were de-waxed and rehydrated for immunocytochemistry as previously described in detail (White *et al.*, 2005). Briefly, the following steps were conducted at room temperature unless otherwise stated. Antigen retrieval was performed by incubating tissue sections in 0.125% Trypsin in 1XPBS for 15 minutes. Sections were then incubated in blocking solution (5% horse, 1% goat, and 1% fetal bovine serum in PBS) for 30 minutes followed by incubation in pSer15 HSPB1-specific rabbit polyclonal antiserum (5 ug/mL final concentration, Cat # PA1-018, Affinity BioReagents, Golden, CO, USA), or a pre-immune sera at the same effective concentrations for 1 hour as previously conducted (White *et al.*, 2005). After washing with PBS, sections were incubated for 1 hour with an FITC-conjugated anti-rabbit secondary antibody (20 ug/mL final concentration; Cat # F7512, Sigma-Aldrich, St. Louis, MO, USA). Following additional washes with PBT (PBS with 0.02% Tween-20), sections were mounted in Vectashield

(Vector Laboratories Inc., Burlington, Ontario, Canada) anti-fade mounting media and sealed around the edges with nail polish. Image collection was conducted using an Olympus Fluoview 300 laser scanning confocal microscope (Olympus Optical Company Ltd., Melville, New York, USA).

4.2.6 Data analysis.

Differences in expression levels between samples within each experimental group were determined through densitometric analysis of x-ray films. Following acquisition of digital scans of x-ray films with an HP Scanjet G4050 scanner, Image J (National Institutes of Health, Frederick, Maryland, USA) was used to generate raw densitometric values for each sample lane. Densitometric measurements of *HSPB1* mRNA were normalized to those of *18S* ribosomal RNA while measurements of pSer15 HSPB1 protein on immunoblots were normalized to those of calponin. Statistical significance between data sets was assessed by performing a two-tailed t-test using Graphpad Prism version 5.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com). Values with a $p < 0.05$ were considered significantly different.

4.3 Results

4.3.1 HSPB1 expression in unilaterally pregnant rat myometrium.

4.3.1.1 HSPB1 mRNA expression.

Northern blot analysis was employed to observe any change in myometrial *HSPB1*

mRNA expression due to uterine distension. Initial investigation was performed using a unilaterally pregnant model consisting of rats at d19 and d23 (labour) of gestation with one gravid (distended) and one non-gravid (empty) horn (n=4). *HSPB1* mRNA expression was significantly increased in the gravid horn myometrium from unilaterally pregnant rats on both d19 and d23 compared to samples from the non-gravid horn (Figure 4-1A, B; $p<0.05$).

4.3.1.2 pSer15 HSPB1 protein expression and localization in situ.

Total myometrial protein extracts (n=4) were utilized for immunoblot analysis to determine any distension-induced changes in HSPB1 protein expression. Investigation with pSer15 HSPB1-specific antibodies in unilaterally pregnant rats showed pSer15 HSPB1 expression in the myometrium was significantly induced with uterine distension at both d19 and d23 (Figure 4-2A,B; $p<0.05$).

To investigate changes in sub-cellular localization and detection levels of pSer15 HSPB1 between gravid and non-gravid uterine horns, immunofluorescence analysis was utilized. At day 19 the gravid horn exhibited a high level of detection of pSer15 HSPB1 in both muscle layers and localization was primarily found to be membrane-associated; however, some peri-nuclear localization was also observed (Figure 4-3). In contrast, the non-gravid horn exhibited virtually no pSer15 HSPB1 immunostaining above the pre-immune control. At d23, pSer15 HSPB1 was also highly detectable in both muscle layers of the gravid horn and localized at some membrane-associated regions, but was much

Figure 4-1
HSPB1 mRNA expression in rat myometrium is significantly induced by uterine distension. Representative northern blot analysis of *HSPB1* mRNA and *18S* rRNA expression in myometrium from non-gravid and gravid uterine horns at A) d19 and B) d23 of rat pregnancy are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Northern blot analysis was performed with an *HSPB1*-specific hamster cDNA and an *18S*-specific rabbit cDNA as templates for radiolabelled probe production. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different from one another when $p<0.05$.

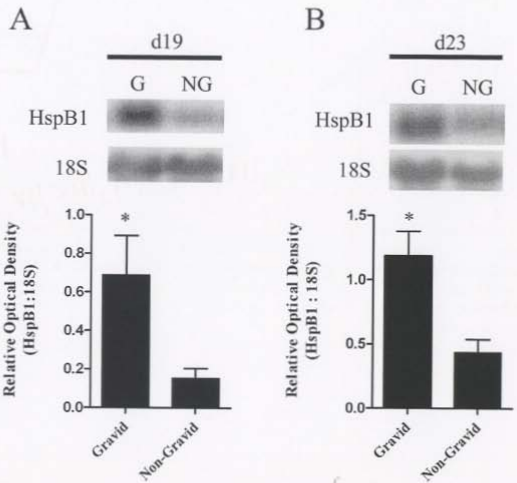

A
d19
G
NG
HspB1
18S
Relative Optical Density (HspB1:18S)
1.0
0.8
0.6
0.4
0.2
0.0
*
Gravid
Non-Gravid
B
d23
G
NG
HspB1
18S
Relative Optical Density (HspB1 : 18S)
1.5
1.0
0.5
0.0
*
Gravid
Non-Gravid

Figure 4-2
Expression of pSer15-HSPB1 protein in rat myometrium is significantly induced by uterine distension. Representative immunoblot analysis of pSer15-HSPB1 and calponin protein expression in myometrium from non-gravid and gravid uterine horns at A) d19 and B) d23 of rat pregnancy are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different from one another when $p<0.05$.

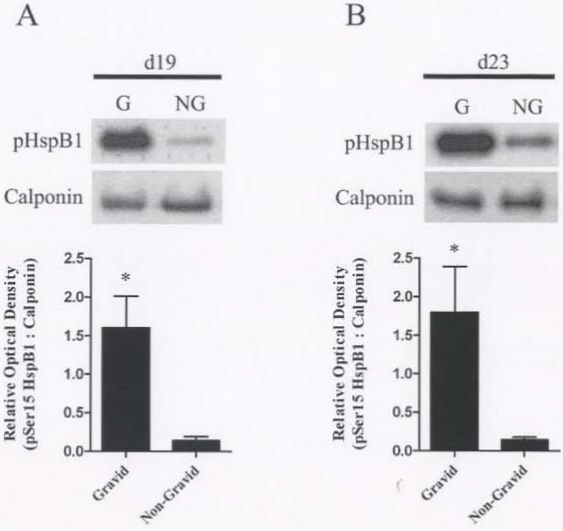
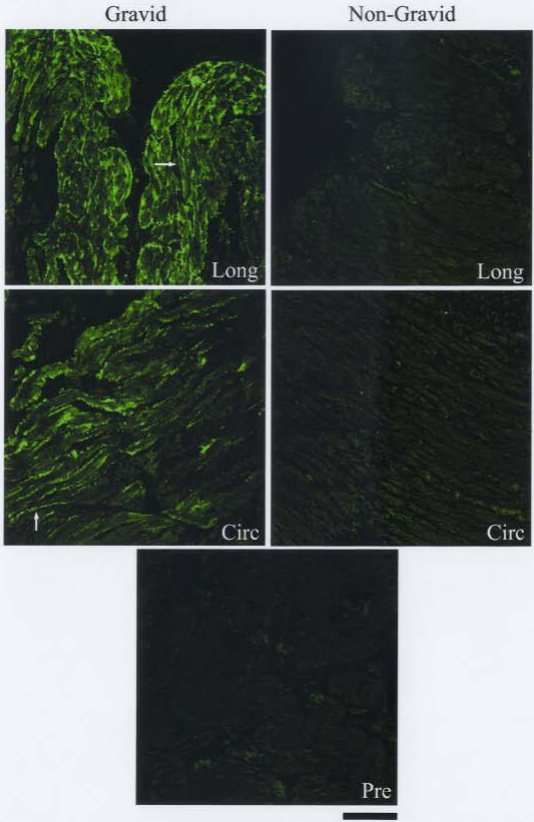

A
d19
G
NG
pHspB1
Calponin
Relative Optical Density
(pSer15 HspB1 : Calponin)
2.5
2.0
1.5
1.0
0.5
0.0
*
Gravid
Non-Gravid
B
d23
G
NG
pHspB1
Calponin
Relative Optical Density
(pSer15 HspB1 : Calponin)
2.5
2.0
1.5
1.0
0.5
0.0
*
Gravid
Non-Gravid

Figure 4-3
Immunofluorescence detection of pSer15-HSPB1 in rat myometrium was markedly increased by uterine distension at d19 of pregnancy. A pSer15 HSPB1-specific rabbit polyclonal antiserum was used to detect this phosphorylated form of HSPB1 in longitudinal and circular muscle layers of distended gravid uterine horns and non-gravid horns. The gravid horns exhibited a high level of detection of pSer15 HSPB1 in both muscle layers, compared to the non-gravid horns, and localization was primarily found to be membrane-associated (arrows). Pre = pre-immune serum control. Scale bar = 50μm.


Gravid
Non-Gravid
Long
Long
Circ
Circ
Pre

more detectable in the cytoplasm of myometrial cells (Figure 4-4). The non-gravid horn exhibited virtually no pSer15 HSPB1 immunostaining above the pre-immune control.

4.3.2 HSPB1 expression in non-pregnant rat myometrium.

4.3.2.1 HSPB1 mRNA expression.

It was possible that the increased myometrial HSPB1 expression observed in the gravid horns during pregnancy could be due, at least in part, to fetal or placental paracrine influences absent in the non-gravid horns. Thus, to more precisely confirm the induction of HSPB1 expression by uterine distension, in the absence of any underlying influence from the fetoplacental unit or even ovarian steroids, a non-pregnant ovariectomized (OV) rat model was also employed for experiments. In this model, laminaria tents were surgically inserted into one of the two uterine horns of ovariectomized female rats for 24h. The result was the production of uterine distension stress in the laminaria-distended horn compared to the contralateral empty horn (Figure 4-5A). In other experiments a polyethylene tube of similar length and diameter to the pre-expanded laminaria tents (i.e. intra-uterine device control) was surgically inserted in one of the horns while the contralateral horn was empty.

Northern blot analysis (n=4) revealed a significant increase in myometrial *HSPB1* mRNA expression in the laminaria-distended horn of ovariectomized rats compared to empty horns (Figure 4-5B; $p<0.05$). There was no significant difference observed in myometrial *HSPB1* mRNA expression between uterine horns containing a polyethylene

Figure 4-4
Immunofluorescence detection of pSer15-HSPB1 protein in rat myometrium was markedly increased by uterine distension at labour. A pSer15 HSPB1-specific rabbit polyclonal antiserum was used to detect pSer15-HSPB1 in longitudinal and circular muscle layers of distended gravid uterine horns and non-gravid horns. pSer15 HSPB1 was highly detectable in both muscle layers of the gravid horns compared to the non-gravid horns and localized at some membrane-associated regions, but was much more detectable in the cytoplasm of myometrial cells (arrows). Pre = pre-immune serum control. Scale bar = 50μm.

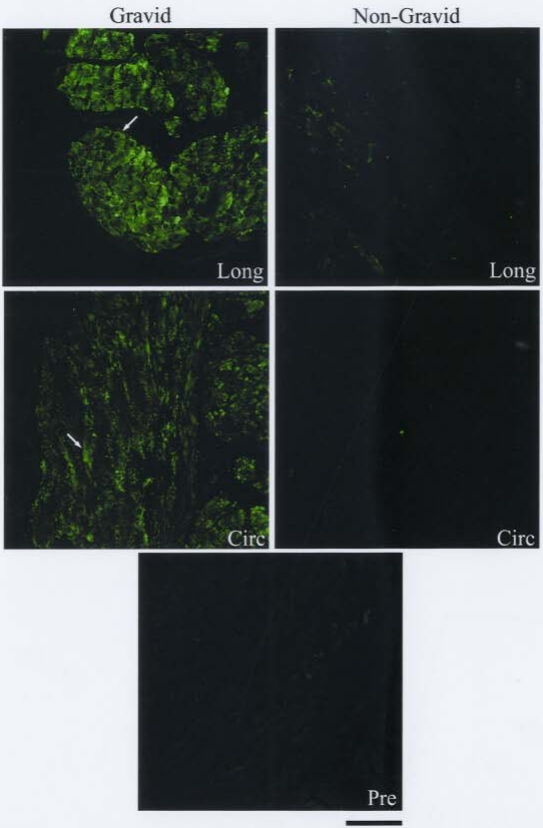
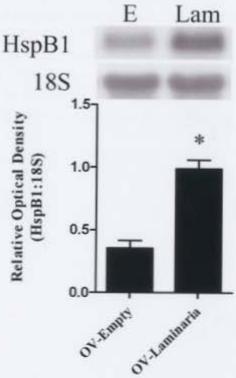

Gravid
Non-Gravid
Long
Long
Circ
Circ
Pre

Figure 4-5
HSPB1 mRNA expression in myometrium from non-pregnant, ovariectomized (OV) rats is significantly elevated in uterine horns exposed to stretch with laminaria tents.
A) Hematoxylin and Eosin-stained tissue sections of an empty rat uterine horn or horns after removal of Laminaria tents or polyethylene tubes demonstrating uterine diameter differences.
B) and C) Northern blot analysis. Laminaria tents (B) or polyethylene tubes of the same size and diameter as pre-expanded laminaria tents (C) were surgically inserted into one of the uterine horns of non-pregnant, ovariectomized rats. Myometrial mRNA samples were collected from empty (E), polyethylene tube-containing (P) and laminaria-containing (Lam) horns 24hrs post insertion. Northern blot analysis was performed with an *HSPB1*-specific hamster cDNA and an *18S*-specific rabbit cDNA as templates for radiolabelled probe production. Representative northern blot analysis of *HSPB1* mRNA and *18S* rRNA expression in myometrium are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different from one another when $p<0.05$. Scale Bar = 250μm.

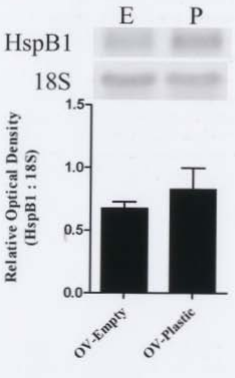
A


Laminaria
Empty
Polyethylene Tube

B


E
Lam
HspB1
18S
Relative Optical Density (HspB1:18S)
1.5
1.0
0.5
0.0
*
OV-Empty
OV-Laminaria

C


E
P
HspB1
18S
Relative Optical Density (HspB1 : 18S)
1.5
1.0
0.5
0.0
OV-Empty
OV-Plastic

tube and empty uterine horns (Figure 4-5C).

4.3.2.2 pSer15 HSPB1 protein expression.

Following laminaria tent insertion, immunoblot analysis (n=4) also demonstrated that the expression of pSer15 HSPB1 in the myometrium of the distended horn was significantly increased compared to the empty horn (Figure 4-6A; $p<0.05$). In contrast, there were no significant differences in myometrial pSer15 HSPB1 protein expression between uterine horns containing a polyethylene tube, mimicking a pre-expanded tent, and empty uterine horns demonstrating a lack of any intra-uterine device effect on protein expression (Figure 4-6B).

4.3.2.3 pSer15 HSPB1 localization in situ.

Overall we observed a decrease in detectable pSer15 HSPB1 immunolocalization as compared to detection in the pregnant state. However, there was an increase in pSer15 HSPB1 detection in the myometrium of laminaria distended horns compared to detection in the myometrium of empty horns, particularly in the longitudinal muscle layer (Figure 4-7A). Localization of pSer15 HSPB1 in the distended horn appeared to be more cytoplasmic and peri-nuclear as compared to the membrane-associated pattern of detection observed in the unilaterally pregnant rat model at d19 of gestation. As expected, there were no observable changes in myometrial pSer15 HSPB1 detection levels or localization between the uterine horns containing a polyethylene tube and empty uterine horns (Figure 4-7B).

Figure 4-6

Myometrial expression of pSer15-HSPB1 protein is significantly induced in uterine horns of non-pregnant, ovariectomized rats (OV) exposed to stretch with laminaria tents. Laminaria tents (A) or polyethylene tubes of the same size and diameter as pre-expanded laminaria tents (B) were surgically inserted into one of the uterine horns of non-pregnant, ovariectomized rats. Samples were subsequently collected from empty (E), polyethylene tube-containing (P) and laminaria-containing (Lam) horns 24hrs post insertion. Representative immunoblot analyses of pSer15-HSPB1 (pHSPB1) and calponin protein expression in myometrium are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different from one another when $p<0.05$.

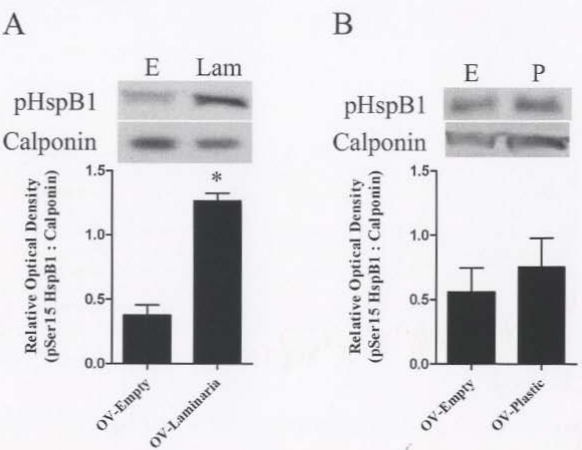
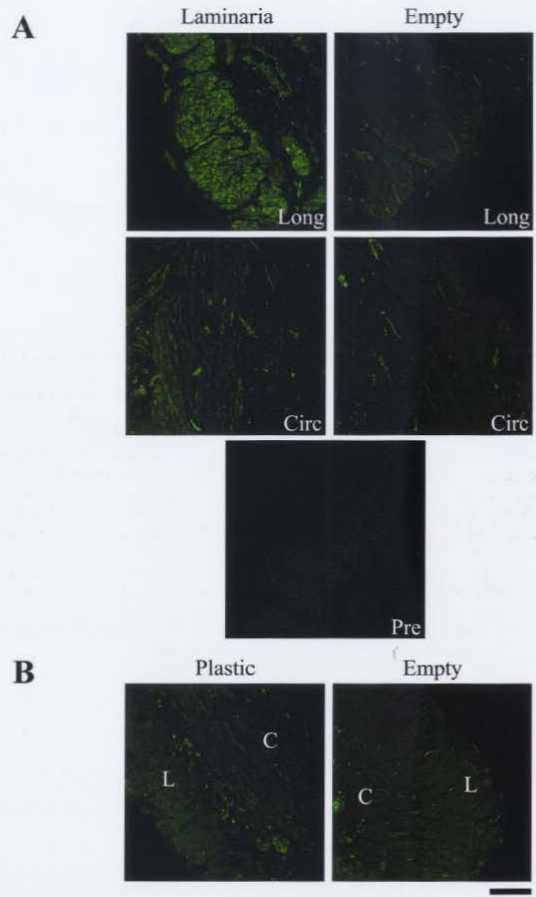

A
E
Lam
pHspB1
Calponin
Relative Optical Density
(pSer15 HspB1 : Calponin)
1.5
1.0
0.5
0.0
*
OV-Empty
OV-Laminaria
B
E
P
pHspB1
Calponin
Relative Optical Density
(pSer15 HspB1 : Calponin)
1.5
1.0
0.5
0.0
OV-Empty
OV-Plastic

Figure 4-7
Immunofluorescence detection of pSer15-HSPB1 in ovariectomized, non-pregnant rat myometrium was increased in laminaria-distended uterine horns. Laminaria tents (A) or polyethylene tubes of the same size and diameter as pre-expanded laminaria tents (B) were surgically inserted into one of the uterine horns of non-pregnant, ovariectomized rats. Samples were subsequently collected from empty, polyethylene tube-containing and laminaria-containing horns 24hrs post insertion.
A) An increase in pSer15 HSPB1 detection in the myometrium of laminaria-distended horns compared to detection in the myometrium of empty horns was observed, particularly in the longitudinal muscle layer. Localization of pSer15 HSPB1 in the distended horn appeared to be more cytoplasmic and peri-nuclear as compared to the membrane-associated pattern of detection observed in the unilaterally pregnant rat model at d19 of gestation.
B) pSer15 HSPB1 detection was very low within circular and longitudinal muscle layers and no observable changes were noted in the muscle layers from empty uterine horns compared to horns containing polyethylene tubes (plastic). Pre = pre-immune serum control, C = circular muscle layer, L = longitudinal muscle layer. Scale bar = 50μm.


A
Laminaria
Empty
Long
Long
Circ
Circ
Pre
B
Plastic
Empty
C
L
C
L

4.4 Discussion

In the myometrium during pregnancy, uterine distension has been reported to increase the expression and/or post-translational modification of contraction-associated (CAP) proteins, focal adhesion proteins and signaling kinases such as connexin-43, FAK, and extracellular signal-regulated kinases (ERK), respectively (Li *et al.*, 2007; Li *et al.*, 2009; Oldenhof *et al.*, 2002; Ou *et al.*, 1997; Ou *et al.*, 1998). While investigation has been undertaken into the effects of stretch on the larger class of heat shock proteins in smooth muscle such as HSP70, research on sHSPs is still emerging (Bornfeldt, 2000). In particular, there has still been limited investigation of the response of HSPB1 to mechanical stress. Chaudhuri and Smith (2008) showed that cyclic strain of airway smooth muscle cells resulted in significant and rapid increases in expression of phosphorylated HSPB1, although the exact phosphorylated form studied was not specified. Thus, we took the opportunity to investigate the effects of uterine distension or mechanical stress on myometrial HSPB1 expression in the unilaterally pregnant rat model that at the same time might advance knowledge and stimulate research with additional types of smooth muscle.

4.4.1 Uterine distension induces expression of *HSPB1* mRNA and pSer15 HSPB1 protein.

We have previously reported that *HSPB1* mRNA expression was significantly elevated in the pregnant rat myometrium at d19 then significantly decreased

by d23 (labour) while pSer15 HSPB1 expression was significantly increased from d19 to d23 compared to earlier points in pregnancy (White *et al.*, 2005). Our results presented here show that uterine distension induced expression of *HSPB1* mRNA and pSer15 HSPB1 protein levels at both d19 and d23 demonstrating the important role of distension or mechanical stress on the regulation of expression regardless of the period of late pregnancy. However, the increased expression in the gravid horns was observed at times when circulating levels of progesterone and 17β-estradiol are reported to be elevated in the rat at d19 and d23, respectively (Rosenblatt *et al.*, 1988). As a result, it was still possible that these steroids as well as other contributions from the fetoplacental unit could contribute to the observed increases in HSPB1 expression during late pregnancy. Examination of HSPB1 expression in ovariectomized, non-pregnant rats subjected to uterine distension with laminaria tents clearly showed that *HSPB1* mRNA and pSer15 HSPB1 protein expression could be potently induced by uterine distension *in vivo* in the absence of fetal, placental paracrine or ovarian steroid influences. However, we cannot exclude the likelihood that a substance(s) could leach out of the laminaria tents or that intrauterine and interstitial fluid could be excessively absorbed by the tents and contribute signals, in addition to uterine distension, leading to the observed increase in HSPB1 expression.

Despite our findings, hormonal influences cannot be excluded as a regulatory mechanism underlying HSPB1 expression during pregnancy. We did observe an overall decrease in detectable pSer15 HSPB1 immunolocalization in ovariectomized, non-

pregnant rats compared to detection in the pregnant state. This could be a result of the loss of the specific hormone environment of late pregnancy. Lastly, marked differences in levels of pSer15 HSPB1 immunolocalization were also observed between the two uterine muscle layers following laminaria distension in ovariectomized, non-pregnant rats compared to detection in gravid uterine myometrium. Laminaria tents cannot completely simulate the timing and extent of fetal growth-induced stretch of the uterus during pregnancy and it is conceivable that, as a result, there are different uterine muscle layer-specific responses to the distension produced by the laminaria tents compared to growing foetuses.

It was previously reported that the rat *HSPB1* gene promoter contains overlapping Sp-1 and AP-2 transcription factor binding sites and a heat shock element (HSE; Oesterreich *et al.*, 1996). Thus, it is possible that that Sp-1, AP-2 and/or Heat Shock Factor 1 or 2 (HSF1, 2) could induce transcription of *HSPB1* mRNA in response to uterine distension. Importantly, HSF1 has been found to be necessary for stress induced *HSPB1* upregulation in mouse embryonic fibroblasts (Zhang *et al.*, 2002) and Xu and colleagues (Xu *et al.*, 2000) have reported that HSF1 activation was induced by cyclic mechanical stress in vascular smooth muscle cells. However, the expression of HSF-1 has yet to be determined in pregnant rat myometrium.

HSPB1 is a substrate for phosphorylation by the p38 MAPK pathway, utilizing members of the MAPK-activated protein kinase (MK) family, although phosphorylation of Ser-15 and Ser-86 does not appear to occur in any specific obligate order (Kostenko &

Moens, 2009; Larsen *et al.*, 1997; Stokoe *et al.*, 1992b). Static stretch of primary cultures of rat myometrial smooth muscle cells *in vitro* strongly induces activated p38 MAPK expression and uterine distension also led to increased p-p38 MAPK expression beginning ~d19 of pregnancy in the rat myometrium reaching significantly elevated levels by d22 (Oldenhof *et al.*, 2002). Thus, the increased expression of pSer15 HSPB1 protein at d19 and d23 may be due, at least in part, to some contributions from the p38 MAPK signaling pathway.

4.4.2 HSPB1 and the actin cytoskeleton.

The dynamic modulation of actin microfilament formation likely plays a large role in smooth muscle contraction (Taggart & Morgan, 2007). For example, Shaw *et al.* (2003) reported that agonist induced constriction of non-pregnant rat myometrium was reduced by inhibition of actin polymerization with cytochalasin D. All three actin isoforms (α, β, γ) are expressed in rat myometrium, but only γ-actin appears to undergo increased expression and changes in localization as term approaches (Shynlova *et al.*, 2005). Analysis of term pregnant mouse uterus demonstrated that actin microfilaments were densely packed and ran parallel to the longitudinal axis of uterine smooth muscle cells (Taggart & Morgan, 2007). Smooth muscle dense plaques, or focal adhesions, are sites on the plasma membrane where clusters of integrins, signalling molecules and adapters such as FAK and vinculin can provide a structural link between the ECM and the actin cytoskeleton. FAK activation is highly induced in rat myometrium during late

pregnancy where focal adhesion signalling may be necessary to remodel cell-ECM adhesion during myometrial hypertrophy (MacPhee & Lye, 2000). Shynlova et al (2010b) recently demonstrated, using a stereological approach, that there were significant increases in uterine smooth muscle cell sizes at late pregnancy (d19) that were not significantly decreased until post partum. Myometrial hypertrophy was also significantly higher in gravid uterine horns compared to non-gravid horns at d19 and at term in unilaterally pregnant rats. Therefore, in this study the increased expression of pSer15 HSPB1 protein levels in distended uterine horns at d19 and labour, and the immunolocalization of the protein to membrane-associated regions indicate pSer15 HSPB1 could be part of a mechano-adaptive response to regulate actin cytoskeleton dynamics at focal adhesion sites and support hypertrophy-induced focal adhesion reorganization during late pregnancy. Evidence to support this possibility can be found in recent reports. During *et al.* (2007) demonstrated that HSPB1 is a G-actin sequestering protein and that HSPB1 phosphorylation enhances actin filament assembly. Jia *et al.* (2010) have also shown, with mass spectrometry analysis, that phosphorylated HSPB1 co-immunoprecipitated with members of the actin regulatory complex Arp2/3.

Since our results demonstrated significantly induced pSer15 HSPB1 levels at d23 (labour) upon uterine distension and immunolocalization of pSer15 HSPB1 to the cell cytoplasm at this time, we cannot rule out a role for this protein in myometrial contraction. Increased myometrial stretch, as a result of multiple gestation pregnancies for instance, has been suggested as one factor that could lead to the increased incidence of

premature uterine contractions (Challis & Lye, 2004). The facilitation of actin formation and actin-myosin interaction by HSPB1 has been shown to be essential for the contraction of colonic smooth muscle (Bitar, 2002) and it is postulated that the mechanism involves phosphorylated HSPB1-mediated modulation of caldesmon association with tropomyosin, a thin filament protein critical for actin-myosin interaction (Bitar, 2002; Somara & Bitar, 2006). Taken with the recent findings of stretch inducing phosphorylation of caldesmon in the myometrium (Li *et al.*, 2007), a specific association or relationship of HSPB1, caldesmon and stretch in uterine smooth muscle contraction may exist.

4.4.3 Perspectives and significance.

Cellular stress signals such as heat shock and oxidative stress are known to initiate HSPB1 expression and phosphorylation (Kato *et al.*, 2002; Landry *et al.*, 1992). The results of this investigation provide novel insights into the mechanism of regulation of HSPB1 expression in uterine smooth muscle during pregnancy and demonstrate that mechanical forces have a major role in regulating HSPB1 expression. The data presented also add to a limited literature on the importance of mechanical stress in inducing *HSPB1* mRNA and phosphorylated protein expression in smooth muscle *per se*. The increasing identification of proteins, such as HSPB1, that are highly expressed and regulated by uterine distension in the myometrium during late pregnancy also provides new potential targets for the development of effective therapeutic strategies to mitigate premature

myometrial contraction. Further identification of the exact mechanism(s) of action of HSPB1 on the myometrial cell actin cytoskeleton and on contraction will require a molecular approach at the cellular level.

Chapter 5

Summary and Future Directions

5.1 Knowledge Gained

There are several significant findings that can be derived from this thesis, due in part to the novelty of the research presented. They include:

1. Expression of HSPB1 during pregnancy changes in association with late pregnancy and labour. mRNA detection of *HSPB1* increases mid-late gestation (d17-22), several days before a late-gestation/labour associated rise in total and phosphorylated HSPB1 protein expression (d19-d23). Also observed from this gestational profile through immunocytochemistry, was a specificity of sub-cellular localization in a time and muscle layer-dependent manner. Temporally, we observed a shift in HSPB1 localization near labour from a specific membrane-associated localization toward a cytoplasmic/peri-nuclear localization. This was observed across both myometrial layers, but was more pronounced in the longitudinal layer. The circular layer displayed increased membrane-associated localization later in pregnancy, and with less immunofluorescent detection. The same patterns were observed with both phosphorylated and total HSPB1 protein detection. Therefore, as a result of the role hypothesized for HSPB1 in smooth muscle contraction, these findings implicate it as a possible contraction associated

protein (CAP) in the myometrium during pregnancy.

2. Progesterone control of HSPB1 expression was investigated through addition of exogenous progesterone and removal of it's functional effects during pregnancy. The former strategy prevented the late pregnancy associated decline in circulating P_4 levels, while the latter strategy led a functional withdrawal of the effects of progesterone. Two important conclusions were elucidated from these experiments. First, progesterone had a direct stimulatory effect on *HSPB1* mRNA expression. Second, progesterone did not seem to have a direct regulatory effect on protein levels of HSPB1, although sub-cellular localization of HSPB1 expression was affected by progesterone administration. A maintenance of membrane association of HSPB1 in myometrial cells upon progesterone administration, and a switch to cytoplasmic localization upon functional progesterone withdrawal with RU486 was observed. This confirmed our earlier findings that with a switch from progesterone to 17β-estradiol just prior to labour, there was also a switch from membrane association to cytoplasmic localization of HSPB1 (White *et al.*, 2005).

3. Administration of 17β-estradiol had no significant effect on *HSPB1* mRNA expression, but significantly increased both total HSPB1 protein levels and pSer15 HSPB1 levels. However, the temporal responses of total HspB1 and pSer15-HspB1 expression to 17β-estradiol were markedly different, with

pSer15-HspB1 detection more rapidly increasing compared to total HspB1 levels. This could be due to differing mechanisms of action of 17β-estradiol. The faster response may be a membrane receptor signalling mechanism resulting in rapid phosphorylation of HSPB1 via the p38 MAPK signalling pathway, while the slower response may be as a result of the traditional nuclear receptor genomic response leading to an increase in the levels of *HSPB1* transcription and subsequent translation.

4. Uterine stretch had a significant effect on myometrial HSPB1 expression, but the response was dependent on the underlying hormonal state. Overall, stretch of the myometrium due to gravidity in pregnant rats, or the presence of laminaria tents in non-pregnant rats, increased HSPB1 mRNA and protein expression compared to unstretched myometrium. Interestingly, it seems that the signalling cascade leading to HSPB1 phosphorylation is more stretch responsive than that facilitating HSPB1 translocation. Immunocytochemical analysis verified the increase in HSPB1 expression due to stretch, but also revealed that stretch-induced changes in HSPB1 localization are also dependent on the underlying endocrine environment. Stretch of ovariectomized, non-pregnant rat uteri resulted in a primarily cytoplasmic/nuclear localization of HSPB1 in the myometrium. Therefore stretch has a significant effect on HSPB1 expression and localization, but that it may act in cooperation with the underlying hormonal state.

5.2 Preliminary Results

Near the end of the above summarized experiments, a set of preliminary experiments were undertaken in order to better determine the role of HSPB1 in contraction of myometrial smooth muscle cells. The basic premise of this experiment was to compare the contractility of myometrial cell lines overexpressing heat shock protein B1 with cells expressing endogenous HSPB1 alone. The design of the experiment was based on work reported by Devost & Zingg (2007) and earlier work by Dallot (2003), with the human myometrial M11 cell line. This cell line was originally derived from primary human myometrial cells by repeated passaging without a immortalization agent. It was utilized due to its characteristic uterine smooth muscle phenotype, and generously provided by Dr. John A. Copland of the Mayo clinic. To summarize, collagen "plugs" were prepared in a 24 well plate at a concentration of 1.5mg/mL. After polymerization at 37 C, M11 cells were plated (20,000/well) on the plugs and allowed to attach for 2 hours. Cells were then infected with lentiviruses, developed and generously provided by Dr. Karen Mearow (Faculty of Medicine, Memorial University of Newfoundland), containing various *HSPB1* constructs (Table 5-1). The following morning, the collagen plugs were separated from the chamber wall using an extra-small spatula. Over time, the myometrial cells generate tension and contract, thus decreasing the diameter of the plug. This was examined and overall contraction levels were compared for the M11 cells expressing different forms of HSPB1.

M11 cells plated in normal 6 well plates and infected with the various lentivirus

Table 5-1: Lentiviral Mutant HSPB1 Constructs

Symbol	Type of HSPB1	Characteristic
WT *HSPB1*	Wild Type	Wild type hamster HSPB1
Δ	WDPF domain removed	Hamster HSPB1 with aa's 5-23 removed
E	Empty Vector	Lentivirus with no construct added
EE	Constitutively Phosphorylated	Hamster HSPB1 with Ser-15 & Ser-86 replaced with glutamine residues
AA	Constitutively Un-phosphorylated	Hamster HSPB1 with Ser-15 & Ser-86 replaced with alanine residues
All constructs were bi-cistronic, containing hamster *HSPB1* genes joined to orange fluorescence protein. These were developed and generously provided by Dr. Karen Mearow.		

constructs were first examined through immunoblotting, to determine whether M11 cells naturally expressed HSPB1 and whether infection resulted in an increase in the different forms of the protein (Figure 5-1). It was found that M11 cells had minimal HSPB1 expression, and that a multiplicity of infection (MOI) equal to 10 was sufficient to induce detectable increases in even the least expressing *HSPB1* construct. Detection of expressed proteins was increased in correlation with MOI changes. Also relevant to note, was that the empty vector showed no expression of HSPB1 and the Δ form was of lower molecular mass, as expected. Overall, it was determined that with a MOI of 100, virtually all of the smooth muscle cells would be expressing the various exogenous proteins, with minimal damage to their viability (no change in phenotype and minimal observable cell death). Using this experimental design, a contraction timeline was completed to determine the most appropriate contraction time for cells before imaging (Figure 5-2). It was determined that 9 hours contraction resulted in clear contraction of collagen plugs and was used in subsequent experiments. Additionally, this experiment allowed a first glimpse at any contraction difference between wild-type HSPB1-infected and uninfected cells, and showed that the wild-type HSPB1 expressing cells did indeed contract with greater magnitude. Following this, an experiment was conducted (n=4) using wild type and empty vector viruses, as well as uninfected cells (Figure 5-3A). It was observed, that there was a significant increase in contraction of M11 cells overexpressing WT *HSPB1* compared to the empty vector control, and a trend of an increase in contraction as compared to uninfected M11 cells although not reaching statistical significance

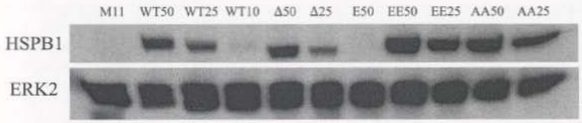
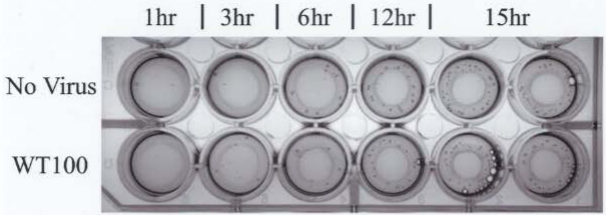

M11
WT50
WT25
WT10
Δ50
Δ25
E50
EE50
EE25
AA50
AA25
HSPB1
ERK2

Figure 5-2
Collagen plug contraction time line. Initial experiment done to determine the appropriate time to allow for contraction of subsequent experiments. Contraction of M11 cells infected with wild type HSPB1 construct was compared to the contraction of regular, uninfected M11 cells. Nine hours was chosen at the timepoint to be utilized. Collagen concentration was 1.5mg/mL, with 20,000 cells plated per well. 24 well culture plates were used.
WT or WT100 = wild type HSPB1 lentiviral construct at a MOI of 100.


1hr
3hr
6hr
12hr
15hr
No Virus
WT100

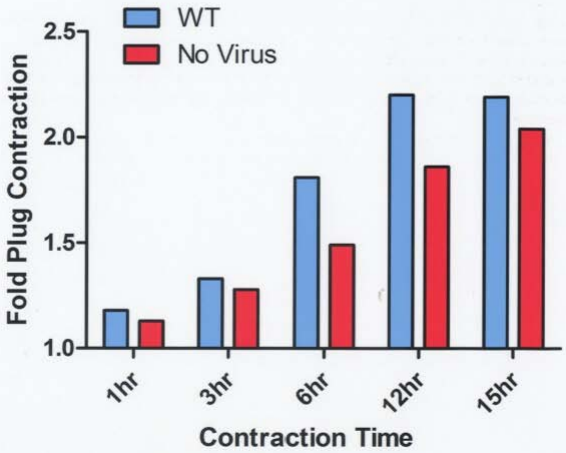
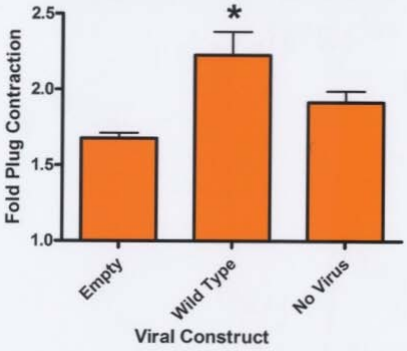

WT
No Virus
Fold Plug Contraction
2.5
2.0
1.5
1.0
1hr
3hr
6hr
12hr
15hr
Contraction Time

Figure 5-3
Collagen plug contraction assay performed in replicate (n=4). Analysis revealed a significant increase in contraction with increased HSPB1 expression (ANOVA, $p<0.05$).

A

B


2.5
2.0
1.5
1.0
Fold Plug Contraction
*
Empty
Wild Type
No Virus
Viral Construct

(Figure 5-3B). Due to time constraints, contraction experiments using the constitutively phosphorylated and non-phosphorylatable forms (EE & AA) were not performed.

Overall, these results indicate that HSPB1 may play an important role in uterine smooth muscle contraction, and that if it is increased in myometrial cells, it is likely that the contractility of those cells is also increased. This further adds supports to the model of the involvement of HSPB1 in facilitation of actin-myosin interaction and hence contraction, developed in other smooth muscle types.

5.3 Significance of Research

Premature labour, and hence preterm birth, remains a major problem today, with 5-10% of all pregnancies occurring preterm (Challis, 2001; Lye & Olson, 1996). The underlying cause of many of these cases has not been determined. These preterm births account for up to 75% of neonatal morbidity and death and can lead to multiple health problems later in life (Challis, 2001; Gibb & Challis, 2002; Lye & Olson, 1996; Lyndon, 2006; Marlow *et al.*, 2007; Public Health Agency of Canada, 1999). As such, this is a major cost to healthcare organizations and governments. Even though there has been much research into developing an understanding of pregnancy, the underlying mechanisms that induce or regulate uterine contractions during labour remain poorly understood. Some recent findings implicate an underlying infection-like reaction as the initiating factor, but exactly what initiates labour and the underlying contractile mechanisms are still incomplete (Challis *et al.*, 2002; Gibb & Challis, 2002). Therefore

any research that expands the knowledge base concerning mechanisms of uterine smooth muscle contraction or the factors causing or aiding the initiation of labour is of utmost importance in helping to prevent preterm birth.

Although there have been some studies performed investigating HSPB1 in human endometrium, cervical tissue, and cancer derived cell lines, prior to this thesis there was no research involving HSPB1 in the myometrium (Ciocca, 1983; Edwards *et al.*, 1981; Mendelsohn *et al.*, 1991; Porter *et al.*, 1996; White *et al.*, 2005). With the knowledge of the proposed crucial role of HSPB1 in colonic smooth muscle contraction (Somara & Bitar, 2006), it was imperative that it be investigated with respect to myometrial smooth muscle. The initial published research findings in this thesis provided the research community with a profile of HSPB1 expression and localization changes in the rat myometrium during pregnancy and labour (White *et al.*, 2005). Following publication of this research, a study by MacIntyre (2008) confirmed these results using human myometrial tissue samples from caesarean section patients. They found that at labour there was an increase in phosphorylated HSPB1 in human myometrium, with pSer15-HSPB1 showing a 3 fold increase. In addition, labour stimulated a shift in localization from peri-nuclear localization to association with the actin cytoskeleton, with α-actin coimmunoprecipitating with HSPB1. The researchers also found through an *in vitro* study that stimulation of myometrial contraction with oxytocin stimulated an increase in serine-15 phosphorylation of HSPB1. This study demonstrated that basic research into mechanism of smooth muscle function in rats using the uterus as a model

for smooth muscle containing organs can have relevance to human physiology.

Although there has been research published on hormonal regulation of HSPB1 expression, none of the work has been conducted in uterine smooth muscle. It has been shown in other cell types that 17β-estradiol stimulated the production of HSPB1 through an interaction of nuclear estrogen receptors (ERs) with an SP1 site and a half-palindromic 17β-estradiol response element (ERE) separated by 10 nucleotides that exists in the *HSPB1* promoter, thus increasing overall HSPB1 levels (Edwards *et al.*, 1981; Mendelsohn *et al.*, 1991; Porter *et al.*, 1996). Experiments in vascular smooth muscle and breast cancer cell lines also showed that 17β-estradiol not only increases HSPB1 expression, but HSPB1 also interacts directly with the nuclear estrogen receptors (ERα & ERβ), facilitating their trafficking to the plasma membrane (Al-Madhoun *et al.*, 2007; Chen *et al.*, 2008; Edwards *et al.*, 1981; Levin, 2005; Miller *et al.*, 2005; Rayner *et al.*, 2008; Razandi *et al.*, 2010). In addition, HSPB1 can potentially even associate directly with, and sequester, estrogen (Al-Madhoun *et al.*, 2007). Research into the role of stretch and mechanotransduction in regulation of HSPB1 is lacking as well. Recently, several investigators have shown an increase of HSPB1 expression in skeletal and smooth muscle due to mechanical stress. Chaudhuri and Smith (2008) showed that cyclical strain of airway smooth muscle promoted an increase in phosphorylation of HSPB1 and in formation and organization of actin microfilaments, while Murlastis and colleagues (2006) showed that resistance training, regardless of age, facilitated an increase in HSPB1 levels in skeletal muscle.

Both stretch and mechanotransduction likely play a critical role in the control of pregnancy and the initiation of labour. The research presented in this thesis further defines the regulatory factors affecting the expression and subcellular localization of HSPB1. Although this has been investigated in uterine smooth muscle, it may be applicable across various species and smooth muscle containing organs, and significantly adds to the knowledge base concerning HSPB1.

This research further defines the synergistic role that endocrinology and mechanotransduction may play during pregnancy. First, the high levels of progesterone at mid gestation were important in increasing mRNA expression of *HSPB1*, however progesterone must decrease late in pregnancy to allow increased production and phosphorylation of HSPB1 protein. This was observed due to the fact that progesterone administration prevented the late pregnancy increase in HSPB1 protein expression associated with an increase in estrogen and stretch signalling. However, once the effects of progesterone decrease, 17β-estradiol and mechanotransduction work together to facilitate an increase in pSer15 HSPB1. Stretch stimulated an overall increase in *HSPB1* gene transcription and translation, while 17β-estradiol facilitated an increase in pSer15 HSPB1 production. It was also observed that it was the interplay of steroid hormones that regulated the subcellular trafficking of HSPB1. While progesterone was the dominant hormone, a membrane localization was observed, however the switch to 17β-estradiol production led to a movement of HSPB1 to a cytoplasmic and peri-nuclear localization. In addition, this localization doesn't seem to be directed by stretch as the increase in

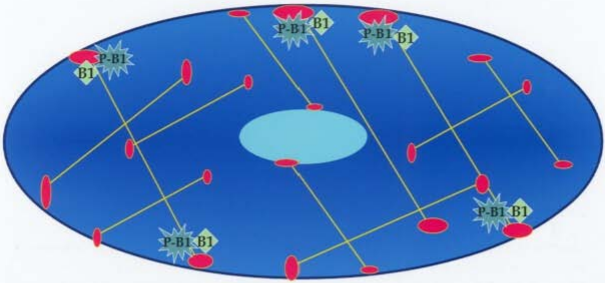
HSPB1 due to stretch seemed to have a diffuse cytoplasmic localization. Thus is can be seen that there is a complex interplay that directs the regulation of transcription, translation, and subcellular localization within the myometrium. In this way, these factors not only work together to promote proper gestation length and fetal development, but also act as a check and balance system.

Based on the above expression, localization, and regulation findings, we suggest that the model of action of HSPB1 in myometrium may be similar to that observed in colonic smooth muscle and defined by two temporally specific events. None of our experimental findings contradict an adoption of that model to help explain myometrial smooth muscle function at this time. In late pregnancy, HSPB1 expression is increased due to mechanical stretch of the uterus, and is located at the cellular membrane of myometrial cells due to the indirect or direct interplay of steroid hormones (Figure 5-4). During this time, HSPB1 may act to aid in the preparation and stabilization of focal adhesion sites. Through capping F-actin, HSPB1 may stabilize the actin cytoskeleton at or near attachment sites for F-actin at the cell membrane, enabling them to bind with the focal adhesion machinery, leading to the creation of a myometrial syncytium (Gerthoffer, 2005 ; An *et al.*, 2004; Bitar 2002). This is the state where force can be effectively transmitted across uterine muscle through specific organization of the cytoskeleton and membrane connection points (Kuo & Seow, 2004). Following the decrease in progesterone, and subsequent increase in estrogenic signalling, there is a distinct change in the localization of HSPB1 and this may indicate a distinct change in HSPB1 function.

Figure 5-4
Diagram of proposed functional localization of HSPB1 in the myometrium during late pregnancy and labour. In late pregnancy, HSPB1 expression and phosphorylation is upregulated and localized to the plasma membrane. The proposed role of HSPB1 during this timepoint is as an actin capping molecule, leading to formation of actin filaments, and to facilitate interaction of these filament with focal adhesions. This allows the focal adhesion molecules to secure actin to the membrane in preparation for contraction. During labour, HSPB1 translocates to the cytoplasm and peri-nuclear regions where is facilitates actin-myosin interaction and thus contraction of the uterine smooth muscle. We cannot however, rule out a possible chaperone activity during this time as the myometrium is under considerable stress. The light blue structure in the centre is the nucleus, the red circles represent focal adhesions or dense plaques, while the yellow lines represent actin filaments. B1 = unphosphorylated HSPB1, P-B1 = Serine-15 phosphorylated HSPB1.

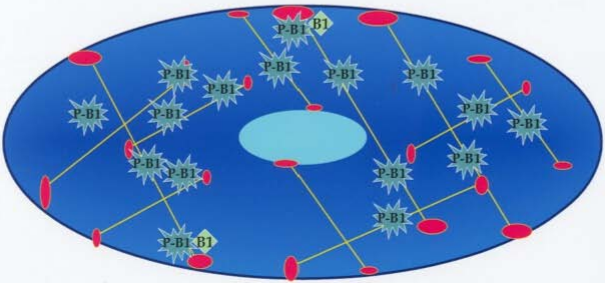
Late Pregnancy

Actin Capping and FA Anchoring



Labour

Mediator of Contraction and/or Chaperone



With the hormonal switch, HSPB1 moves into the cytoplasm of myometrial cells in preparation for labour (Figure 5-4). Through research into other smooth muscle types such as colonic smooth muscle, and through the preliminary experiments (See section 5.2: Preliminary Results), we propose that during labour HSPB1 may be involved with the facilitation of actin-myosin interaction, and thus smooth muscle contraction (Somara & Bitar, 2004, 2006). HSPB1 may thus act both as a focal adhesion protein and CAP, responding to two differing stimuli at different times. This data support the findings in other smooth muscle types that HSPB1 is essential for contraction, and presents the first model of the role of HSPB1 in uterine smooth muscle during pregnancy, which may be critical for pregnancy and labour.

5.4 Future Research

As with any viable and productive research project, it inevitably concludes with having more unanswered questions to drive future research. Overall, there are three areas for future research to focus on; hormonal regulation of HSPB1, co-localization of HSPB1 with focal adhesion proteins, and the role of HSPB1 in myometrial contraction.

5.4.1 Hormonal regulation studies.

Due to the immense complexity that exists within steroid hormone signalling during pregnancy, our study has only preliminarily delved into steroid regulation of HSPB1 expression. It can clearly be observed through our results, that hormonal

regulation of HSPB1 is an intricate interplay of the pregnancy hormones present in the myometrium. Therefore HSPB1 should be investigated utilizing a mixed hormone approach as observed in hormonal control experimental models published by the Lye lab (Ou *et al.*, 1997; Petrocelli & Lye, 1993). In these studies, varying levels and timing of both estrogen and progesterone injections were employed. Future experimental design should take into consideration the interplay of both of these hormones, and the effect on HSPB1 expression. This includes the effect of estrogen priming on research animals, the syncronization of oestrous cycle in non-pregnant laboratory animals, the administration of estrogen and progesterone concurrently and in differing ratios, and further defining whether localization is controlled by progesterone or simply due to an inhibition of the effects of 17β-estradiol. Additionally, the specific routes of signalling of these two hormones on HSPB1 should be investigated, with further research into whether the hormonal signalling is through the associated membrane receptors, or traditional nuclear receptors. This is a research topic that recently has had much debate, and further investigation into this within the uterine environment would aid the entire research community (Arreguin-Arevalo & Nett, 2006; Chamniansawat & Chongthammakun, 2009; Gellersen *et al.*, 2009; Hogan *et al.*, 2009; Karteris *et al.*, 2006).

5.4.2 Co-localization studies.

Since HSPB1 exhibits many of the characteristics of late pregnancy-related focal adhesion proteins, as well as labour associated contraction associated proteins, this dual

role must be further investigated. One method that should be employed is a co-localization study. The relationship/interaction of HSPB1 with focal adhesion proteins such as FAK and paxillin, dense plaques, actin and/or myosin molecules, and CAPs such as cx-43, integrins, and OTR should be determined in the myometrium by immunoprecipitation studies, co-immunolocalization studies, and perhaps immunogold electron microscopy. Additionally, changes in these associations should be assessed over time to develop an understanding of temporal specific associations. In this way, determination of the proteins that HSPB1 associates with and/or has a direct interaction with can help to further define the role of HSPB1 within the uterus and uterine contractions.

5.4.3 Contraction studies.

One of the most obvious areas of future research should be the continuation of the collagen plug contraction assays to specifically determine the role of HSPB1 in uterine smooth muscle contraction. These experiments should also be expanded to include the additional lentiviral *HSPB1* mutant constructs available in the laboratory (Table 5-1). Also, this test should be further developed to not only assess natural contractility of M11 cells, but also to assess agonist-induced contractability. Lastly, these experiments should be investigated in M11 cells, as well as other myometrial cell cultures such as OM-1, hTERT-HM, SHM and PHM1, as well as primary cultures to assess consistency of results (Ciarmela *et al.*, 2009; Dallot *et al.*, 2003; Devost & Zingg, 2007; Mitchell & Lye, 2005; Monga *et al.*, 1999).

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Appendix 1

Co-Authorship Statement

I, Bryan G White, am the first author for published manuscripts included in this thesis (Chapter 2), and will be first author for the subsequent unpublished manuscripts that are prepared for submission (Chapters 3 & 4).

Chapter 2 - Small heat shock protein 27 (Hsp27) expression is highly induced in rat myometrium during late pregnancy and labour

All experimental work and data analysis pertaining to this manuscript was completed as part of my PhD research. Dr. Kerri Highmore completed preliminary experiments that led to the conception of this project, while Dr. Selena Joy Williams provided assistance with sample collection. Dr. Daniel J. MacPhee provided the initial conceptual design for this experiment, invaluable guidance throughout, and constant assistance throughout the various stages of writing.

Chapter 3 - Progesterone and 17β-Estradiol Differentially Regulate the Expression and Phosphorylation of the Small Heat Shock Protein HSPB1 in the Rat Myometrium.

All experimental work and data analysis pertaining to this manuscript was completed as part of my PhD research, providing first authorship. Dr. Selena Joy Williams, Mr. Brandon Cross, and Ms. Mandy Peach provided assistance with sample collection and animal care. Dr. Daniel J. MacPhee provided assistance with initial design

conception for this experiment, provided invaluable guidance throughout, and continues to provide assistance throughout the various stages of writing.

Chapter 4 - Uterine Stretch Regulates the Expression and Phosphorylation of the Small Heat Shock Protein HSPB1 in the Rat Myometrium.

All experimental work pertaining to this manuscript was completed as part of my PhD research. Both myself and Dr. Daniel MacPhee performed data analysis, interpreted the results of the experiments, prepared figures, drafted the manuscript, and approved the final version of the manuscript. Dr. Daniel MacPhee was was responsible for conception and design of the research project, and edited/revised the manuscript.

Appendix 2

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