Expression and Regulation of HSPB5 in the Myometrium throughout Pregnancy

A Thesis Submitted to the College of

Graduate Studies and Research

For the Degree of Master of Science

Department of Veterinary Biomedical Sciences

University of Saskatchewan

Saskatoon

By

JESSICA GRACE NICOLETTI

© Jessica Grace Nicoletti, 2015. All rights reserved.
Permission to Use

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.
Abstract

The uterine smooth muscle or myometrium goes through phases of differentiation during pregnancy to become a powerful contractile tissue at term. Small Heat Shock proteins (sHSPs) are a family of ten small molecular weight proteins in mammals that are induced by many physiological stressors such as uterine stretch. Some sHSPs act as chaperones, but also others assist in cell death regulation, cytoskeleton rearrangements, and immune system activation. We examined the spatio-temporal expression of HSPB5 protein throughout gestation via immunoblot and immunofluorescence analysis, as well as the effect of uterine distension on myometrial HSPB5 protein expression using unilaterally pregnant rat models. HSPB5 protein expression significantly increased on day (d) 17 (p<0.05; vs all other timepoints) and levels steadily decreased thereafter through to postpartum (PP). In contrast, serine-59 phosphorylated (pSer59) HSPB5 protein detection was significantly increased from d19 through to PP (p<0.05). Both HSPB5 and pSer59-HSPB5 were detected in the cytoplasm of myocytes within both uterine muscle layers mid- to late-pregnancy. In unilaterally pregnant rats, HSPB5 protein and pSer59-HSPB5 protein expression were significantly elevated in gravid uterine horns at both d19 of gestation and d23 (labour) compared to non-gravid horns. The spatial expression of total and pSer59-HSPB5 protein in a human myometrial cell line was examined by immunofluorescence analysis. Total HSPB5 co-localized with α-smooth muscle actin and pSer59-HSPB5 was co-localized with the focal adhesion protein kindlin-2 (KIN-2) and the exosomal marker CD63. Therefore, HSPB5 is highly expressed during mid- to late-pregnancy and expression appears to be regulated by uterine distension. HSPB5 may be involved in regulating actin filament dynamics and could be secreted by exosomes.
Acknowledgments

I wish to express my sincere thanks to Dr. Daniel MacPhee for giving me the opportunity to participate in his labs research as well as thanking him for sharing his expertise, both academic and personal, throughout my graduate research; whether it was a question about cell culture or vehicles you could always count on Daniel for spirited suggestions. I would also like to take this opportunity to thank all of the MacPhee lab members, past and present, for supporting me for the last two years and bearing with the occasional outburst of frustration (we all have had one – or two). Specifically, thank you to Dr. Ewa Miskiewicz for teaching me proper lab techniques and always being available to help, especially during time-trials taking place during all hours of the night! Thank you to Dr. Eiko Kawamura for setting up the confocal microscope so we could collect accurate images, the solution you came up with was ingenious! Also, thank you to Daniels previous student Dr. Bryan White who I only met once early on a Saturday morning (yes, research never rests) for conducting surgeries and collecting the tissues I used throughout my graduate research. I would like to thank all of the other VBMS (Veterinary Biomedical Sciences) students at the WCVM (Western College of Veterinary Medicine) for making the last two years ones to remember! Lastly I would like to say thank you to my family and friends back home for supporting my move from Ontario to Saskatchewan even though it was not an easy transition for all of us; their support made it possible.

This research would not have been able to be conducted if it wasn’t for an operating grant from NSERC (Natural Sciences and Engineering Research Council).
Table of Contents

Permission to Use........................................................................................................... i
Abstract ........................................................................................................................ ii
Acknowledgments ......................................................................................................... iii
Table of Contents ........................................................................................................... iv
List of Tables .................................................................................................................. vii
List of Figures ................................................................................................................ viii
List of Abbreviations ..................................................................................................... xii
Chapter 1. Literature Review ........................................................................................ 1
  1.1. Introduction ............................................................................................................. 1
  1.2. Phases of Myometrial Differentiation throughout Pregnancy.............................. 5
    1.2.1. The Proliferative Phase..................................................................................... 5
    1.2.2. The Synthetic Phase....................................................................................... 10
    1.2.3. The Contractile and Labour Phases ............................................................... 12
  1.3. Small Heat Shock Proteins (sHSPs) ..................................................................... 15
    1.3.1. Members of the sHSP Family and General Functions ................................... 15
    1.3.2. Control of Expression ................................................................................... 19
    1.3.3. Cytoskeletal Associations ............................................................................. 22
    1.3.4. Secretion via Exosomes and the Immune System .......................................... 24
  1.4. The Importance of the Immune System for Parturition ....................................... 30
  1.5. Hypotheses and Objectives .................................................................................... 37
Chapter 2. Expression of HSPB5 is Highly Induced in Rat Myometrium during Late Pregnancy and Labour and is Stimulated by Uterine Distension ........................................ 39
  2.1. Abstract .................................................................................................................. 40
  2.2. Introduction ............................................................................................................. 41
  2.3. Materials & Methods ............................................................................................ 43
    2.3.1. Animals .......................................................................................................... 43
    2.3.2. Experimental Design .................................................................................... 44
    2.3.2.1. Tissue Collection ....................................................................................... 44
    2.3.2.2. Unilaterally Pregnant Rat Model ............................................................... 45
    2.3.3. Immunoblot Analysis ..................................................................................... 45
2.3.4. Immunofluorescence Analysis ................................................................. 48
2.3.5. Data Analysis ....................................................................................... 49

2.4. Results .................................................................................................... 49
2.4.1. Expression of HSPB5 in the Myometrium throughout Gestation ............ 49
2.4.2. Uterine Stretch Induces Myometrial HSPB5 Expression ......................... 97

2.5. Discussion .............................................................................................. 97
2.5.1. Myometrial HSPB5 Expression during Pregnancy ................................. 110
2.5.2. The Influence of Uterine Distension on Myometrial HSPB5 Expression .... 111
2.5.3. Potential Roles for HSPB5 in the Myometrium .................................... 111

Chapter 3. Co-localization of HSPB5 and pSer59-HSPB5 with Cytoskeletal and Exosomal Markers ............................................................... 115

3.1. Abstract .................................................................................................. 116
3.2. Introduction ........................................................................................... 116
3.3. Materials & Methods ........................................................................... 119
3.3.1. Cell Culture of the hTERThm Cell Line ............................................... 119
3.3.2. Immunofluorescence Analysis ............................................................ 120
3.3.3. Data Analysis ..................................................................................... 122

3.4. Results .................................................................................................. 122
3.4.1. HSPB5 and Actin Co-localization ....................................................... 122
3.4.2. pSer59-HSPB5, Actin and Exosomal Protein Co-localizations ................. 122

3.5. Discussion .............................................................................................. 125
3.5.1. Implications for HSPB5 and pSer59-HSPB5 Co-localization with Actin .... 132
3.5.2. Implications for pSer59-HSPB5 Co-localization with CD63 .................... 136

Chapter 4. General Discussion .................................................................... 141
4.1. HSPB5 Expression and Potential Role with the Actin Cytoskeleton ............ 143
4.2. Stretch Regulates HSPB5 Expression ..................................................... 145
4.3. Potential Transport of HSPB5 by Exosomes and Role in Immune Activation... 146

Chapter 5. Major Conclusions & Future Directions ...................................... 148
5.1. Major Conclusions ................................................................................ 148
5.2. Regulation of HSPB5 Expression ........................................................... 149
5.3. HSPB5 and Potential Exosome-Mediated Secretion ................................. 150
5.4. Requirement of HSPB5 for Pro-Inflammatory Response in Myometrial Cells .......... 151
5.5. HSPB5 as an Impending Labour Biomarker................................................................. 151
Bibliography.......................................................................................................................... 153
List of Tables

Table 1.1. Antibodies utilized for immunoblot and immunofluorescence assays with myometrial and whole uterine tissue ................................................................. 47

Table 3.1. Antibodies utilized for immunofluorescence experiments with hTERT cells .......... 121
List of Figures

Figure 1.1. A diagrammatic representation of (A) the human simplex uterus ...................... 2

Figure 1.2. A diagrammatic representation of the phases of myometrial differentiation throughout pregnancy................................................................. 6

Figure 1.3. The organization of the human HSPB5 protein sequence .............................. 17

Figure 1.4. Phosphorylation of small heat shock proteins and their potential role in actin cytoskeletal arrangement ............................................................................. 25

Figure 1.5. Secretion of proteins by exosomes................................................................. 28

Figure 1.6. A diagrammatic representation of the phases of myometrial differentiation and the immune system throughout pregnancy............................................................... 32

Figure 2.1. Immunoblot analysis of total HSPB5 protein expression during pregnancy, parturition, and post-partum......................................................... 51

Figure 2.2. Immunoblot analysis of pSer59-HSPB5 expression during pregnancy.............. 53

Figure 2.3. Comparison of total and pSer59-HSPB5 protein expression during pregnancy.... 55

Figure 2.4. Immunofluorescence analysis of total HSPB5 protein expression in uterine longitudinal smooth muscle from NP to d17 ............................................................. 57

Figure 2.5. Immunofluorescence analysis of total HSPB5 protein expression in uterine longitudinal smooth muscle from d19 to PP .......................................................... 59

Figure 2.6. Immunofluorescence analysis of total HSPB5 protein expression in uterine circular smooth muscle from NP to d17 ........................................................................ 61
Figure 2.7. Immunofluorescence analysis of total HSPB5 protein expression in uterine circular smooth muscle from d19 to PP .......................................................... 63

Figure 2.8. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in uterine longitudinal smooth muscle layers .......................... 65

Figure 2.9. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in uterine circular smooth muscle layers .................................. 67

Figure 2.10. Immunofluorescence analysis of pSer59-HSPB5 protein expression in uterine longitudinal smooth muscle from NP to d17 ................................................................. 69

Figure 2.11. Immunofluorescence analysis of pSer59-HSPB5 protein expression in uterine longitudinal smooth muscle from d19 to PP ................................................................. 71

Figure 2.12. Immunofluorescence analysis of pSer59-HSPB5 protein expression in uterine circular smooth muscle from NP to d17 ................................................................. 73

Figure 2.13. Immunofluorescence analysis of pSer59-HSPB5 protein expression in uterine circular smooth muscle from d19 to PP ................................................................. 75

Figure 2.14. Confocal laser scanning microscopy and immunofluorescence analysis of pSer59-HSPB5 protein expression in uterine longitudinal smooth muscle layers ...................... 77

Figure 2.15. Confocal laser scanning microscopy and immunofluorescence analysis of pSer59-HSPB5 protein expression in uterine circular smooth muscle layers ...................... 79

Figure 2.16. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in the uterine longitudinal smooth muscle layer at day (d) 17 ...... 81
Figure 2.17. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in the uterine circular smooth muscle layer at day (d) 17................. 83

Figure 2.18. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in the uterine longitudinal smooth muscle layer at day (d) 23......... 85

Figure 2.19. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in the uterine circular smooth muscle layer at day (d) 23................. 87

Figure 2.20. Confocal laser scanning microscopy and immunofluorescence analysis of pSer59-HSPB5 protein expression in the uterine longitudinal smooth muscle layer at day (d) 15............ 89

Figure 2.21. Confocal laser scanning microscopy and immunofluorescence analysis of pSer59-HSPB5 protein expression in the uterine circular smooth muscle layer at day (d) 15................. 91

Figure 2.22. Confocal laser scanning microscopy and immunofluorescence analysis of pSer59-HSPB5 protein expression in the uterine longitudinal smooth muscle layer at day (d) 23............ 93

Figure 2.23. Confocal laser scanning microscopy and immunofluorescence analysis of pSer59-HSPB5 protein expression in the uterine circular smooth muscle layer at day (d) 23................. 95

Figure 2.24. Expression of total HSPB5 protein in the rat myometrium is significantly induced by uterine stretch ........................................................................................................... 98

Figure 2.25. Expression of pSer59-HSPB5 protein in the rat myometrium is significantly induced by uterine stretch ........................................................................................................... 100

Figure 2.26. Immunofluorescence analysis of total HSPB5 protein expression in longitudinal and circular muscle layers of non-gravid and gravid horns at day (d) 19 of rat gestation .......... 102
Figure 2.27. Immunofluorescence analysis of total HSPB5 protein expression in longitudinal and circular muscle layers of non-gravid and gravid horns at labour (d23)................................. 104

Figure 2.28. Immunofluorescence analysis of pSer59-HSPB5 protein expression in longitudinal and circular muscle layers of non-gravid and gravid horns at day (d) 19 of rat gestation........ 106

Figure 2.29. Immunofluorescence analysis of pSer59-HSPB5 protein expression in longitudinal and circular muscle layers of non-gravid and gravid horns at labour (d23) ......................... 108

Figure 3.1. Immunofluorescence analysis of α-smooth muscle actin and total HSPB5 protein localization in hTERThm human myometrial cells .......................................................... 123

Figure 3.2. Immunofluorescence analysis of α-smooth muscle actin and pSer59-HSPB5 protein localization in hTERThm human myometrial cells .......................................................... 126

Figure 3.3. Immunofluorescence analysis of KIN-2 and pSer59-HSPB5 protein localization in hTERThm human myometrial cells .......................................................... 128

Figure 3.4. Immunofluorescence analysis of CD63 and pSer59-HSPB5 protein localization in hTERThm human myometrial cells .......................................................... 130

Figure 3.5. A potential signaling pathway regulating function of HSPB5 ....................... 134

Figure 3.6. Secretion of HSPB5 by an exosome pathway ............................................. 137
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BrDU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAP</td>
<td>contractile associated protein</td>
</tr>
<tr>
<td>Cat #</td>
<td>catalogue number</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>Cx</td>
<td>connexin</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous-actin</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G-actin</td>
<td>globular-actin</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSE</td>
<td>heat shock element</td>
</tr>
<tr>
<td>HSF</td>
<td>heat shock transcription factor</td>
</tr>
<tr>
<td>HSPB</td>
<td>small heat shock protein B</td>
</tr>
<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>KIN</td>
<td>kindlin</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPKAPK</td>
<td>MAP kinase activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
</tbody>
</table>
mTOR -- mammalian target of rapamycin
N-terminal -- amino-terminal
NF-κB -- nuclear factor kappaB
NK -- natural killer
NOS -- nitric oxide synthase
NP -- non-pregnant
nSBs -- nuclear stress bodies
OTR -- oxytocin receptor
Pax -- paired box
PBS -- phosphate-buffered saline
PCNA -- proliferating cell nuclear antigen
PFA -- paraformaldehyde
PG -- prostaglandin
PI3K -- phosphoinositide-3-kinase
PP -- post-partum
pSer15 -- serine-15 phosphorylated
pSer59 -- serine-59 phosphorylated
RANTES -- regulated on activation, normal T-cell expression and secreted
RIPA -- radio-immunoprecipitation assay
ROS -- reactive oxygen species
RRX -- Rhodamine red-X
SDS -- sodium dodecyl sulphate
Ser59 -- serine-59
sHSP -- small heat shock protein
TBST -- Tris-buffered saline containing Tween 20
TGF -- transforming growth factor
TLR -- toll-like receptor
TNF-α -- tumour necrosis factor-α
TSG101 -- tumour-susceptibility gene 101
TUNEL -- terminal deoxynucleotidyl transferase nick end labeling
Chapter 1.

Literature Review

1.1. Introduction

The human uterus is a simplex uterus with one uterine body that consists of three separate layers: the endometrium, the myometrium, and the perimetrium (Sherwood, 2010). The myometrium consists of two indistinct muscle layers separated partially by a vascular plexus: the outer longitudinal muscle layer and the inner circular muscle layer (Chow & Marshall, 1981). Embryologically, the outer longitudinal muscle layer originates from the layer of mesenchymal tissue between the inner circular muscle layer, which originates from the paramesonephric ducts, and the peritoneum (Chow & Marshall, 1981). It is costly, time consuming, and ethically not feasible to use the human uterus as a primary experimental model, thus the rat uterus is more often used experimentally. The rat uterus has the same three uterine layers as the human uterus, as well as similar arrangements in the myometrial muscle layer, except the rat uterus is bicornuate or bipartate, having two uterine horns, whereas the human uterus has one large uterine body (Fig. 1.1 A-C; Chow & Marshall, 1981; Sherwood, 2010). It is important to understand the role of the myometrium throughout gestation since many of the mechanisms utilized by the myometrium to maintain quiescence throughout pregnancy and contraction at labour are still poorly understood.

The ability to accurately predict and effectively treat spontaneous preterm labour in clinical practice is lacking and the cost associated with preterm deliveries and complications is vast. On average in Canada the cost of a child born at full term (37 weeks of gestation or later)
Figure 1.1. A diagrammatic representation of (A) the human simplex uterus, (B) the bicornuate rat uterus, and (C) the layers of the human and rat uterus in cross section. Both the human and rat uterus are comprised of an endometrial layer, a myometrial layer, and a perimetrial layer or serosa.
will cost the health care system $1,000 and the average length of stay in a Canadian hospital is approximately two days for a single birth (Lim, et al., 2009). However, the cost and length of stay increase to approximately $9,000 and 10 days on average, respectively, for a child born prematurely (less than 37 weeks gestation), although premature children born at 30 weeks of gestation or less can cost the health care system between $50,000 and $90,000 with the length of stay increasing to anywhere between 40 and 85 days (Lim, et al., 2009). In the case of multiples, these numbers are only slightly increased (Lim, et al., 2009). Preterm birth rates in Canada have remained stable since 2006 estimated at approximately 8% of all births in Canada with the province of Saskatchewan having a preterm birth rate 8.1% (Canadian Institute for Health Information, 2013). Alberta and Newfoundland and Labrador have the highest preterm birth rates, for the provinces, of 8.3% and 8.2%, respectively (Canadian Institute for Health Information, 2013). In the territories, the premature birth rates have reached as high as 13.9% in Nunavut (Canadian Institute for Health Information, 2013).

Preterm labour can occur for various reasons and accounts for approximately 75% of all infant deaths that occur in the perinatal period (Goldenberg, et al., 2008). Two causes of preterm birth are infection and premature rupture of membranes; however, these two causes only account for approximately 55% of preterm births leaving no explanation for the other 45% of spontaneous preterm births (Goldenberg, et al., 2008). One current treatment to stop preterm labour is the administration of tocolytic agents, also known as labour represents, as a strategy to prolong pregnancy and allow for the maturation of the fetal lungs (Haas, et al., 2009; Lefevre, 1992). Without an adequate predictor of impending preterm labour many of these treatments are not as effective as they could be if labour could be predicted weeks in
advance rather than in hours. The discovery of accurate and effective biomarkers of impending labour would greatly help with predicting the onset of labour, whether it be at term or preterm, and improving the effectiveness of treatments used to repress preterm labour.

1.2. Phases of Myometrial Differentiation throughout Pregnancy

1.2.1. The Proliferative Phase

The uterine smooth musculature or myometrium goes through phases of differentiation throughout pregnancy to become a powerful contractile tissue at term. These phases have been best characterized in the rat (Fig. 1.2). The first phase, the proliferation phase, is from the start of pregnancy to day (d) 14 in the rat and is the earliest phase where there is rapid myometrial cell proliferation and an increase in the expression of anti-apoptotic factors within these cells (Orejuela, et al., 2007; Shynlova, et al., 2006). Myometrial cell hyperplasia, or cell proliferation, during the early stages of pregnancy was determined by measuring the changes in two markers of cell replication (Shynlova, et al., 2006). These two markers were: proliferating cell nuclear antigen (PCNA) and 5-bromo-2’-deoxyuridine (BrDU) incorporation (Shynlova, et al., 2006). PCNA levels increased in early pregnancy and dropped dramatically after mid-gestation which was consistent with the initiation of hypertrophy during this time period (Shynlova, et al., 2006). Samples of myometrial tissue taken from pregnant rats injected with BrDU at different time points throughout gestation also showed increased BrDU incorporation into deoxyribonucleic acid (DNA) as well as an increase in myometrial cell number during the beginning of pregnancy demonstrating that the myometrium is growing during this phase (Shynlova, et al., 2006). The activation of the insulin-like growth factor-1 (IGF-
Figure 1.2. A diagrammatic representation of the phases of myometrial differentiation throughout pregnancy. The Proliferation Phase (blue) occurs from NP to day (d) 14 and is characterized by an increase in cell number. The Synthetic Phase (pink) is from d14 to d21 and is characterized by myometrial cell hypertrophy. The Contractile Phase (yellow) is from d21 to d23 and is characterized by an increase in basement membrane protein synthesis. Finally, the Labour Phase (green) is characterized by an increase in expression of contractile associated proteins (CAPs) that initiate labour. The time between the Contractile and Labour Phase (orange) indicates an approximate time of transition from myometrial quiescence to active labour.
1)/phosphoinositide-3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling pathway by 17 beta (β)-estradiol, which is present throughout pregnancy, has been implicated in controlling myometrial hyperplasia during this time period of early gestation (Shynlova, et al., 2013).

During pregnancy, the expression of estrogen regulated growth-factor IGF-1 is up-regulated in the myometrium between d6 to d12 of rat gestation and is important for uterine cell proliferation and growth (Kahlert, et al., 2000; Shynlova, et al., 2007). Once estrogens have activated IGF-1 and IGF-1 binds its receptor there is phosphorylation of insulin receptor substrate (IRS) and PI3K is recruited as part of the IGF-1/PI3K/mTOR signaling pathway (Jaffer, et al., 2009). Therefore, estrogen in circulation can activate mTOR alone and the mTOR signaling pathway (Yu & Henske, 2006). The phosphorylation of IRS and PI3K causes a phosphorylation cascade which leads to the phosphorylation of Akt (also known as protein kinase B), a serine/threonine kinase that positively regulates mTOR (Jaffer, et al., 2009). The activation of mTOR caused the proliferation of the rat myometrium through specific stimulation of the G1 phase of the cell cycle, as well as upstream factors IRS, PI3K, and Akt which were all up-regulated during the proliferation phase (Jaffer, et al., 2009). Estrogens also regulate hyperplasia in uterine epithelia and stroma by regulating mitosis during the time of growth (Jaffer, et al., 2009).

Since the overall goal of the proliferation phase is to increase cell number, apoptosis of myometrial cells is also tightly controlled during this time period. Apoptosis of myometrial cells can happen in one of two ways: the tumor necrosis factor-α (TNF-α) pathway and the exogenous stimulus pathway (Joswig, et al., 2007; Shynlova, et al., 2006). The exogenous
stimulus pathway is a mitochondrial-dependent pathway and can be activated by environmental or physiological factors (Shynlova, et al., 2006). Environmental factors, or stimuli, could include hypoxic periods induced by decreased blood flow to the myometrium caused by pressure on blood vessels by immense fetal growth during mid-pregnancy (Shynlova, et al., 2006). The exogenous pathway of apoptosis is regulated by Bcl-2, an anti-apoptotic factor that is increased in the myometrium during the proliferation phase of the uterus to maintain uterine smooth muscle cells in high numbers (Shynlova, et al., 2006). During the process of apoptosis usually-inactive caspases are cleaved and become active leading to apoptosis of a cell; however the levels of these active caspases, such as caspase-9, in the myometrium during the proliferation phase is low (Shynlova, et al., 2006).

It is also important to consider the effects of paracrine and autocrine contraction inhibitors, like nitric oxide (NO) and relaxin, that are in high levels in the proliferation stage to prevent spontaneous uterine contraction which could lead to preterm labour (Bani, et al., 1999). During this phase there is increased production of relaxin by the corpus luteum, decidua, and trophoblast which exerts many actions on the uterus (Bani, et al., 1999). At the level of the uterus, relaxin contributes to the up-regulation of nitric oxide synthase (NOS) and, therefore, the increase in NO as well as a decrease in intracellular calcium and an increase in cyclic adenosine monophosphate (cAMP), which can all act as inhibitors of contraction (Bani, et al., 1999; Tanaka, et al., 1988). This inhibition is due to the activation of calcium-activated and adenosine triphosphate (ATP)-sensitive potassium channels, resulting in hyperpolarization of the myometrial cell membrane which will lower the contractibility and lead to muscle relaxation (Bani, et al., 1999). Although NO levels are high at the beginning of pregnancy they
dramatically drop at the time of labour allowing for powerful coordinated contractions (Bani, et al., 1999; Diejomaoh, et al., 2003).

1.2.2. The Synthetic Phase

The following phase is the synthetic phase, which spans d15 to d21 in the rat, and where smooth muscle cells increase in cell size or hypertrophy, increase their production of extracellular matrix proteins, exhibit increased expression of insulin-like growth factors-1 and -2 (IGF-1 and IGF-2), an increased protein:DNA ratio, and decrease production of Bcl-2, an anti-apoptotic factor (Shynlova, et al., 2004; Shynlova, et al., 2007; Shynlova, et al., 2009a). There are few mechanisms proposed for the regulation of myometrial hypertrophy; however, it has been shown that uterine stretch can cause a combination of hyperplasia and hypertrophy of uterine smooth muscle cells in non-pregnant rat uterine horns (Shynlova, et al., 2013). For instance, only gravid uterine horns undergo hypertrophy, increasing cell size three-fold, whereas non-gravid horns in the same animal do not undergo hypertrophy; therefore uterine stretch is required for smooth muscle cell growth in the myometrium during this phase (Shynlova, et al., 2010).

The switch from an increase in cell number to an increase in cell size in this phase allows for the increase in the overall size of the organ itself (Shynlova, et al., 2007a). Even though the cause of the switch in phenotype from hyperplasia to hypertrophy is unknown, progesterone may be a regulator of hypertrophy since when an antagonist of progesterone signaling is administered to pregnant rats on d17 of gestation myometrial hypertrophy is interrupted (Shynlova, et al., 2009a). The transition in phenotype also coincides with the apparent
activation of an intrinsic apoptotic cascade within the myometrium, which includes the increase in expression of the apoptosis initiator caspase-9 and downstream effector molecules caspases-3 and -6, as well as a decrease in the expression of anti-apoptotic protein Bcl-2 in the rat myometrium (Shynlova, et al., 2006). In high enough levels these caspases will lead to apoptosis; however, there is no apoptosis of myometrial smooth muscle cells detected in this phase by a terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay so activation of the apoptotic pathway may be due to the intermittent periods of hypoxia experienced due to the growing fetus (Shynlova, et al., 2006). To increase the size of uterine smooth muscle cells and accommodate the growing fetus, there is first an increase in synthesis of intracellular filaments (Rehman, et al., 2003). Microarray analysis has determined that in the pregnant myometrium myosin genes including myosin heavy polypeptide 11, regulatory light polypeptide 5, and myosin, increase in expression 2- to 3-fold during this phase (Rehman, et al., 2003). Caldesmon, a smooth muscle thin-filament-associated protein which inhibits generation of force by myosin when unphosphorylated, is also increased in expression almost 5-fold during this time period (Rehman, et al., 2003).

During the synthetic phase, uterine smooth muscle cells increase synthesis of extracellular matrix (ECM) proteins, like collagen I, collagen II, and elastin, to allow for anchoring of the ECM to the cell surface, which will be important in the later stages of pregnancy and labour (Shynlova, et al., 2004). This up-regulation of ECM proteins, like collagen and elastin, via c-fos transcription factor induced expression is restricted to the gravid horns of unilaterally pregnant rats; therefore, mechanical stretch does play a role in this regulation (Shynlova, et al., 2002; Shynlova, et al., 2010). The anchoring of uterine smooth muscle cells to
the ECM is likely regulated by focal adhesion kinase (FAK) and its downstream target paxillin, which is expressed at high levels in the myometrium during late-pregnancy (MacPhee & Lye, 2000). When FAK is activated by phosphorylation dense plaques in smooth muscle cells, also known as focal adhesions, are formed and actin filaments are coupled to these focal adhesions through the membrane-cytoskeletal protein vinculin (MacPhee & Lye, 2000). These cell-ECM interactions allow focal adhesions to sense mechanical forces as well as provide structural linkages and the generation of contractile force across the tissue needed at labour (Rajfur, et al., 2002).

1.2.3. The Contractile and Labour Phases

The final phases of uterine differentiation are the contractile phase and the labour phase, beginning at d21 of rat gestation and ending at d23 (Shynlova, et al., 2009a). During these two final phases there is an increase in expression of contractile-associated proteins (CAPs), such as oxytocin receptor, increased expression of integrins, and the maternal immune system becomes activated (Shynlova, et al., 2013; Williams, et al., 2005). The contractile phenotype of the myometrium is stabilized by the expression of fibronectin and its receptors – integrins – as well as increasing cell-cell communication via the CAP gap junction protein connexin 43 (Cx43; White, et al., 2005; Williams, et al., 2005). Immune system activation, cytokine production, and immune cell infiltration of the myometrium is thought to play a role in the initiation of labour at the time of the labour phase (Shynlova, et al., 2013).

During the contractile phase, hypertrophy stabilizes and expression of basement membrane (BM) proteins, including fibronectin, laminin β2, and collagen IV, are up-regulated in the myometrium from d21 through to term labour (Shynlova, et al., 2004). The functional
change from myometrial quiescence to myometrial contraction is evident in the types of BM proteins being secreted which are under endocrine and mechanical regulation (Shynlova, et al., 2004). Mechanical stimulation is required for inducing BM protein secretion since BM proteins were only secreted by gravid horns in a unilaterally pregnant rat and only in later pregnancy when distension was maximal (Shynlova, et al., 2009). However, if the non-gravid horn is artificially stretched, there is an increase in expression of these BM proteins and CAP proteins as well suggesting regulation is mainly due to uterine stretch and not solely under endocrine control (Ou, et al., 1997).

The synthetic phase to contractile phase transition is also regulated by endocrine shifts during pregnancy and in this phase is associated with a drop in circulating levels of progesterone in the rat (Shynlova, et al., 2004). When progesterone was injected the transition to the contractile phase was prevented (Shynlova, et al., 2004). Injection of a progesterone receptor antagonist at d19 of gestation in the rat can cause a pre-mature switch from the synthetic phenotype to the contractile phenotype (Shynlova, et al., 2004). Therefore, falling levels of progesterone is associated with the phenotype switch required to prepare for labour and increased BM protein expression (Shynlova, et al., 2004).

Proteins that affect calcium sensitivity in myometrial cells called caldesmon proteins also associate with contractile filaments and increased phosphorylation of these proteins at the transition to the contractile phase enhances contractility by increasing calcium sensitivity (Li, et al., 2002). In the case of the contractile phase, insulin-like growth factor binding protein-2 (IGFBP-2) expression is increased and this up-regulation of IGFBP-2 was only seen in the gravid horn and was not affected in animals pre-treated with progesterone or a progesterone receptor
antagonist supporting a role for mechanical stretch (Shynlova, et al., 2007). Therefore, some mechanisms during these phases required both endocrine and mechanical stimulation, however some mechanisms only relied on one or the other.

During the labour phase, the tension on the myometrium, as well as endocrinological factors, induce the expression of sodium-channels, oxytocin receptors (OTRs), prostaglandin (PG) receptors, and gap junction proteins to electrically couple myometrial smooth muscle cells and increase myocytes responsiveness to contractile agonists (Ou, et al., 1998). Coupling allows for depolarizing from cell-to-cell and phasic bursts of contraction of anchored myometrial cells (Blennerhassett & Garfield, 1991). During labour focal adhesion kinase (FAK) decreases and myocytes have completed ECM anchoring (Shynlova, et al., 2007a). The expression of CAPs requires low circulating progesterone levels in the rat and a decrease in FAK activity suggesting further that there are endocrine and mechanical regulatory inputs to labour (MacPhee & Lye, 2000). Stretch alone cannot increase expression of CAPs (Ou, et al., 1997). When progesterone levels are high, CAP expression cannot be induced by mechanical stretch alone, circulating levels of progesterone need to be lowered as well (Ou, et al., 1997).

During the labour phase, proper ligand-integrin interactions facilitate smooth muscle intracellular connections and focal adhesions act as major points of force transduction ensuring that the myometrial cells contract together (Shynlova, et al., 2013). The proper organization of focal adhesions relies on progesterone levels since the exogenous administration of progesterone to pregnant rats inhibits the decrease in phosphorylated FAK at labour, which allows for further remodeling and unstable focal adhesions that inhibits the process of labour (MacPhee & Lye, 2000). Therefore, through the final phase of the myometrial differentiation
during pregnancy the myometrium becomes a strong contractile organ reliant on both endocrine and mechanical stimulation.

1.3. Small Heat Shock Proteins (sHSPs)

1.3.1. Members of the sHSP Family and General Functions

Small heat shock proteins (sHSPs) in the HSPB family, previously referred to as the alpha (α)-crystallin family, are a group of ten small molecular weight proteins, between 12 and 43 kilodaltons (kDa) that are induced by many physiological stressors, such as heat shock. Mammalian HSPB proteins are labelled HSPB1 to B10 and arrange themselves in large homomeric or heteromeric complexes of about 40 to 50 subunits; however, subunit assemblies can be composed of as little as 2 subunits (Haslbeck, et al., 2005; Kappe, et al., 2003). They are ubiquitously expressed in the cytosol of most cells and tissues even in the absence of a stressor (Nover & Scharf, 1991). However, under stressful conditions the expression of mammalian sHSPs dramatically increases in a cell and it has been shown that reporter genes carrying promoter regions for small heat shock proteins increase in expression 500- to 1 200-fold after heat shock (Dreano, et al., 1986). An increase in expression levels under physiological conditions is an effect of cell growth and differentiation (Gaestel, et al., 1989). These proteins function in the formation and maintenance of native protein structure in a cell under stressful and physiological conditions as well as acting as chaperones, assisting in cell death regulation, cytoskeleton rearrangements, and immune system activation (Orejuela, et al., 2007). HSPB5 (originally known as αB-crystallin) is phosphorylated on three amino acids, serines-19, -45 and -59, whereas HSPB1 is only phosphorylated on two amino acids, serines-15 and -86 in rodents.
MAP Kinases are responsible for the phosphorylation of HSPBs, specifically, the MAP Kinase Activated Protein Kinase 2/3 (MAPKAPK 2/3) kinases are responsible for the phosphorylation of HSPB5 on serine-59 while p42/p44 MAP Kinase phosphorylates serine-45 (Rouse, et al., 1994; Stokoe, et al., 1992). sHSP phosphorylation is critical for HSPB function. For example, HSPB5 in a phosphorylated state has been found to control inter-filament interactions of actin and myosin (Wettstein, et al., 2012).

Structurally, sHSPs in the HSPB family have an amino- or N-terminal containing a hydrophobic tryptophan-aspartate/proline-phenylalanine (WD/PF) motif and phosphoserine sites followed by a conserved α-crystallin domain which is then followed by the carboxy- or C-terminal end of the protein (Kappe, et al., 2003; Theriault, et al., 2004). The N- and C-terminals are suspected to be involved in the chaperone function and complex formation of sHSP with each other and other proteins, respectively (van Montfort, et al., 2001). The C-terminal α-crystallin domain is approximately 80 to 90 amino acids in length and represents the signature motif of sHSPs since it is highly conserved (van Montfort, et al., 2001). This domain is made up of compact beta (β)-sheets composed of two layers or three and five anti-parallel strands connected by an interdomain loop, whereas the N-terminal domain is comprised of α-helixes (Kappe, et al., 2003). These α-crystallin domains can dimerize between sHSPs through formation of an intersubunit composite β-sheet (Fig. 1.3; Sun & MacRae, 2005). The C-terminal domain is also involved in stabilizing the oligomers of sHSPs (Sun & MacRae, 2005). Binding sites for the C-terminal domain are on neighbouring α-crystallin domains and this interaction is required for oligomerization (van Montfort, et al., 2001). On the other hand, the N-terminal of sHSPs is hydrophobic and highly variable in sequence and length measuring 24 to 247 residues.
long (Basha, et al., 2006; van Montfort, et al., 2001). In some species, the N-terminal region seems to be important for stabilizing oligomers of different sizes (van Montfort, et al., 2001). The N-terminal arms of two monomeric sHSP protein subunits intertwine to form knot-like structures with the hydrophobic amino acids on the inside of the oligomer and the hydrophilic amino acids on the outside (van Montfort, et al., 2001). The quaternary structure of sHSPs is variable since they form polydisperse oligomeric assemblies of up to 50 subunits (Haslbeck, et al., 2005).

sHSPs can bind denatured proteins and prevent their irreversible aggregation and insolubilization that will lead to apoptosis (Lee, et al., 1997). Bovine α-crystallin and murine HSPB1 were the first α-crystallins demonstrated to have chaperone activity (Horwitz, 1992; Merck, et al., 1993). Their specificity for proteins is unknown, but it is suggested that sHSPs are promiscuous and will bind at the N-terminal to an available denatured protein (Basha, et al., 2006). Complexes between substrates – denatured proteins – and sHSPs are very stable at physiological temperatures (Lee, et al., 1997). The WD/PF sites are hydrophobic in nature making them a good site for protein-protein interactions (van Montfort, et al., 2001). It has also been shown that deleting the N-terminal of a sHSP abolishes chaperone capacity (Plater, et al., 1996).

1.3.2. Control of Expression

Several, but not all, small heat shock protein (sHSP) family members exhibit a pattern of inducible expression in the presence of a physiological stressor (Guettouche, et al., 2005). Physiological stressors can include, but are not limited to, heat, oxidative stress, and mechanical forces, all of which have been shown to increase sHSP expression within cells and
tissues via the mammalian transactivator heat shock transcription factor 1 (HSF1; Guettouche, et al., 2005; Li, et al., 2013; Yan, et al., 2005). Recently, transcription factors other than HSF1 have been identified for the regulation of sHSP transcription, which include: paired box (Pax), SOX, and MyoD (reviewed by de Thonel, et al., 2012). The transcription factors listed have been shown to regulate sHSPs in a multitude of cell types, however, classically the activation of sHSP gene expression has been highly conserved throughout evolution and requires the conversion of HSF1 monomers to HSF1 homotrimers (de Thonel, et al., 2012; Sarge, et al., 1993). These homotrimers of HSF1 can recognize and bind to DNA motifs called heat shock elements (HSE) which are inverted repeats of ‘nGAAn’ (Kroeger & Morimoto, 1994; Sarge, et al., 1993). These inverted repeats are located in the promoter region of DNA in HSF1 responsive sHSP genes and HSF1 binds to these repeats to promote the transcription of sHSPs (Kroeger & Morimoto, 1994).

The best conserved region within the HSF family is the amino- or N-terminal DNA-binding domain (Sorger & Nelson, 1989). The HSF1 DNA-binding domain is a looped helix-turn-helix, but unlike many other looped helix-turn-helices within transcription factors, the HSF1 loop does not come in direct contact with the DNA (Harrison, et al., 1994). The HSF1 loop has been proposed to stabilize DNA-bound HSF1 trimers by protein-protein interactions (Cicero, et al., 2001). Deletion of the loop in yeast weakens the DNA-binding ability of HSF1, but does not abolish it (Cicero, et al., 2001). The oligomerization domain of HSF1 is rich in α-helices and divided into two subdomains, each having amphiphilic helices (Peteranderi & Nelson, 1992).

HSF1 is constitutively expressed in most tissues and cell types and appears to be regulated primarily through post-translation mechanisms (Baler, et al., 1993). When a cell is under physiological conditions the DNA-binding activity and transactivation capacity of HSF1 is
limited since HSF1 exists as diffuse monomers in the nucleus (Mercier, et al., 1999). Once a stress has occurred HSF1 is converted into homotrimers and DNA-binding capacity increases several-fold (Mercier, et al., 1999). After the stressor has been withdraw HSF1 returns to its monomeric state (Mercier, et al., 1999). During physiological conditions, HSF1 is shuttled in and out of the nucleus, however, after a stress has been detected by a cell, there is a nuclear accumulation of HSF1 and increased transcription of HSF1-regulated genes (Yang, et al., 2008). In human HeLa cells, activated HSF1 localizes to particular subnuclear structures in the nucleus known as nuclear stress bodies (nSBs), however the function of these nSBs is still unknown (Biamonti & Vourc’h, 2010; Cotto, et al., 1997; Mahl, et al., 1989; Sarge, et al., 1993). Activation of HSF1 transactivating capacity occurs with post-translational modifications, like phosphorylation on 12 different serine residues, yet it is unclear to what extent these modifications are needed for HSF1 activation (Guettouche, et al., 2005). For example, serine-326 phosphorylation has been determined to be important for transcriptional competence during heat stress and recently, other serine phosphorylation sites with previously no known function have been determined to play a role in the activation and repression of HSF1 (reviewed by Anckar & Sistonen, 2011; Guettouche, et al., 2005).

HSF1 is important for mammals since it controls programmed cell death, early development, and pro-inflammatory cytokine production via the production of sHSPs (Neuer, et al., 2000). For example, basal levels of HSPB1 expression were shown to be significantly down-regulated in the heart of HSF1 knockout mice when compared to wild-type mice (Yan, et al., 2002). This suggests that HSF1 might be regulated by physiological normal mechanisms, temperatures, and conditions (Yan, et al., 2002). Treatment of cells with oxidants, like...
hydrogen peroxide increases HSF1 activity through mechanisms that alter the structure of the HSF1 protein (Ahn & Thiele, 2003). Reactive oxygen species (ROS) production by mitochondrial oxidative phosphorylation can escape endogenous anti-oxidant mechanisms (West, et al., 2001). In a cell these ROS can damage macromolecules, like lipids, DNA and proteins (Cooke, et al., 2003). Redox sensing requirements of mammalian HSF1 were demonstrated to be regulated by the redox-dependent disulfide bonds in response to heat and oxidative stress (Ahn & Thiele, 2003).

Besides the classical pathways above, sHSP expression can be regulated by the muscle-specific MyoD family of transactivators – called myogenic regulatory factors – that bind to the E-box motif contained in the promoter region of some sHSPs in a murine skeletal muscle cell line (Gopal-Srivastava & Piatigorsky, 1993). Chaperone-like proteins, sHSPs, can also be up-regulated in rabbit fast-twitch skeletal muscle in response to low frequency contractile stimulation to stabilize myofibrillar components during the contraction transition process (Donoghue, et al., 2005). Previous work in the MacPhee lab has established that HSPB1, a member of the small heat shock protein family, increases in late pregnancy during myometrial hypertrophy induced by uterine stretch of the uterus caused by fetal growth (White & MacPhee, 2011).

### 1.3.3. Cytoskeletal Associations

sHSPs can undergo various post-transcriptional modifications like phosphorylation and oligomerization, however, phosphorylation is critical for their function with respect to the cytoskeleton. HSPB5 has been found to control inter-filament interactions of actin and myosin in smooth muscle cells (Webster, 2003; Launay, et al., 2010; Wettstein, et al., 2012).
Phosphorylation of HSPB1 and HSPB5 has also been shown to decrease their native chaperone function (Schmidt, et al., 2012). HSPB5 has three phosphorylation sites (serine-19, serine-45, and serine-59) and it has been suggested that HSPB5 inhibits actin fiber depolymerization and prevents their aggregation during stress (Wettstein, et al., 2012). Launay and colleagues (2006) proposed that the phosphorylation state of HSPB5 regulates the actin polymerization-depolymerization balance (Launay, et al., 2006). For example, they reported that murine myocytes treated with vinblastin and cytochalasin D, which respectively disorganize microtubules and actin microfilaments, triggered the activation of the p38/MAPKAP2 kinase pathway that lead to the phosphorylation of HSPB5 at serine-59 and that this phosphorylation allowed HSPB5 to associate and bind with actin filaments (Launay, et al., 2006). Inhibition of serine-59 phosphorylation almost completely inhibited its association with actin filaments during stress (Launay, et al., 2006).

Actin is an abundant microfilament protein found in eukaryotic cells and there are six different types in mammalian cells, four of which are expressed in muscle and two of which are expressed in non-muscle cells (Cooper, 2000). Actin filaments are dynamic structures that have an equilibrium between the monomeric globular- or G-actin form and the polymerized filamentous- or F-actin microfilament form (Cooper, 2000). The activity of actin polymerization inhibition depends on the degree of sHSP phosphorylation (Benndorf, et al., 1994). Benndorf and colleagues (1994) reported that only monomeric and nonphosphorylated murine HSPB1 could inhibit actin polymerization, whereas phosphorylated monomers had no effect (Benndorf, et al., 1994). Only nonphosphorylated mixtures of sHSP monomers and dimers were effective in suppressing actin polymerization when compared to phosphorylated ones in a
tumour cell line (Benndorf, et al., 1994). In vertebrate cells sHSPs are thought to modulate the structure of the actin cytoskeleton and this activity is linked to the protective function of sHSPs (Lavoie, et al., 1995). sHSP phosphorylation was reported to be required for actin stabilization during stress (Lavoie, et al., 1995). Therefore, sHSPs may interact with both actin monomers and filaments in a phosphorylation-dependent manner (Fig. 1.4; Benndorf, et al., 1994; Lavoie, et al., 1995).

Lengthening contraction-induced translocation of HSPB5 in skeletal muscle cells was associated with phosphorylation of these sHSPs and may trigger a protective role (Koh & Escobedo, 2004). Translocation was not seen in isometric contractions suggesting that these proteins are functioning in stabilization and repair when muscle cells are damaged during forceful contractions (Koh & Escobedo, 2004). Forceful lengthening contractions in skeletal muscle are comparable to the forceful uterine contractions at labour so it is possible that HSPB5 in smooth muscle cells could function in the same ways. HSPB5 knockout mice have been reported to develop late-onset skeletal muscle degeneration, supporting the role of HSPB5 in maintaining muscle integrity (Koh & Escobedo, 2004).

1.3.4. Secretion via Exosomes and the Immune System

Small heat shock proteins (sHSPs) in general do not contain specific signal sequences to induce their active secretion from cells; therefore, an alternate secretion-pathway must be considered. Recently the alternative secretion pathway of sHSP via exosomes has been demonstrated (van Noort, et al., 2012a). Exosomes are extracellular vesicles approximately 100nm in diameter and have been documented to contain HSP70 and HSP90 when secreted.
**Figure 1.4.** Phosphorylation of small heat shock proteins and their potential role in actin cytoskeletal arrangement. Small heat shock proteins (sHSPs) are phosphorylated in response to various contractile agonists in smooth muscle cells. Such stimuli can include: neurotransmitters, cytokines, and growth factors. sHSP phosphorylation favors stabilization of F-actin filaments by undetermined molecular mechanisms. One possibility is that sHSPs act as capping proteins and phosphorylation dissociates the cap allowing polymerization to proceed. Certain sHSPs, like HSPB5, may also sequester G-actin. Modified from Salinthone, et al. Pharmacology and Therapeutics, 119, 44-54, 2008.
HSPB5 has been found in exosomes from retinal pigment epithelial cells (Sreekumar, et al., 2010). These exosomes also contain a family of proteins known as tetraspanins which include: CD9, CD63, CD81, and CD82 (Kobayashi, et al., 2000; Thery, et al., 2002). These tetraspanin proteins are highly enriched in the membranes of exosomes from any cell type and are used as markers of these vesicles (Kobayashi, et al., 2000).

There are other markers used to detect exosomes like, tumour-susceptibility gene 101 (TSG101), which is important for the transport of membrane proteins in the endocytic pathway (Babst, et al., 2000). This suggests that exosomes may be part of the endocytic pathway; however, since most proteins detected in exosomes have no discernible signal sequence sorting of proteins into exosomes is still poorly understood.

The theory of ubiquitination has been put forward to explain how proteins are chosen for packaging into exosomes (Katzmann, et al., 2001). It is proposed that the cytosolic domain of selected membrane proteins is involved in determining how proteins are sorted into these vesicles (Katzmann, et al., 2001). This theory was proposed when ubiquitin ligase was found in T-cell derived exosomes (Blanchard, et al., 2002). Also, the ubiquitin-binding ability of vacuolar protein sorting protein 23, a yeast homologue of TSG101, is necessary for sorting proteins into internal vesicles of late endosomes which are thought to become exosomes (Katzmann, et al., 2001). Although this theory has support, other ubiquitin-independent mechanisms could also occur (Reggiori & Pelham, 2001).

Since exosomal vesicles are thought to be part of the endocytic pathway they are proposed to be formed by inward budding of endosomes (Fig. 1.5 A-G; Trajkovic, et al., 2008).
Figure 1.5. Secretion of proteins by exosomes. These exosomal vesicles are formed by (A) inward budding of large endosomal structures inside the donor cell. These vesicles then accumulate in structures called (B) multivesicular bodies. These multivesicular bodies then (C) fuse with the plasma membrane, an event which is often inducible, and the vesicle release its contents into the extracellular space. The 40–100 nm vesicles contained in the multivesicular body have now become (D) exosomes. A recipient cell can take up exosomes and their contents either by (E) direct fusion with the plasma membrane or by (F) endocytosis. In the recipient cells the exosomes either (G) release their contents or (H) become part of the endosomal compartment. Modified from van Noort, et al. The International Journal of Biochemistry & Cell Biology, 44, 1670 – 1679, 2012.
A. Donor Cell
B. Multivesicular Body
C. Inward Budding
D. Exosomes
E. Recipient Cell
F. Endosome
G. Multivesicular Body
They are then secreted by the fusion of late endocytic compartments with the plasma membrane (Booth, et al., 2006). Late endocytic vesicles fuse with the plasma membrane after activation in a calcium-dependent manner (Savina, et al., 2003). Little is known about the molecular machinery that is involved in the fusion of exosomes with the plasma membrane; however, mechanisms involving other cellular proteins have been suggested.

Different cell types use exosomes for one of two processes, either the release of intracellular proteins to the extracellular space as signals or an alternative pathway to lysosomal degradation in the case of wastes that cannot be degraded by lysosomes (Pan & Johnstone, 1983; Safaei, et al., 2005). For example, in reticulocytes it is suggested that exosomes are secreted to discard membrane proteins, like transferrin receptors, that have become useless in mature red blood cells; however, the mechanism underlying this process is not well understood (Pan & Johnstone, 1983). It has also been shown that properly characterized exosomes can induce a T-cell response, however it is possible that the final outcome of an immunological response depends on the cellular origin the exosomes and the contents of the vesicles (Thery, et al., 2002). Cancer cells have also been shown to secrete more exosomes when the lysosomal pathway has been disrupted indicating that the exosomal pathway and lysosomal pathways may be related in some cases (Safaei, et al., 2005). Overall, exosomes have some, but as of yet poorly understood, physiological functions.

1.4. The Importance of the Immune System for Parturition

Pregnancy is an anti-inflammatory condition and a shift in this condition to a pro-inflammatory state leads to parturition, but a pathological pro-inflammatory response could lead to abortion or other complications, like preterm labour (Knackstedt, et al., 2003). In order
to maintain an overall anti-inflammatory state for most of pregnancy the uterus goes through
three biological phases with respect to the immune system termed the initiation, tolerance,
and activation phases (Mor & Cardenas, 2010). The timing of these phases temporally coincide
with the differentiation phases of the myometrium and the balance of immunomodulators, like
cytokines is crucial for ensuring the correct timing of labour and delivery (Mor & Cardenas,
2010; Shynlova, et al., 2013). The initiation phase coincides with the proliferation phase, the
tolerance phase with the synthetic phase, and the activation phase with the contractile and
labour phases of the myometrium (Fig. 1.6 A-B; Mor & Cardenas, 2010; Shynlova, et al., 2013).

During the initiation phase of the immune system, which starts at the time of
implantation and placentation and ends early in the second trimester of human pregnancy, the
site of implantation resembles that of an “open wound” (Creasy, et al., 2014). Since human
blastocyst implantation forms a wound in the uterine lining – or endometrium – a strong pro-
inflammatory response is required to heal the wound (Hanna, et al., 2006). At this point in
pregnancy there is an increase in plasma concentrations of acute-phase reactants like
fibrinogen and plasminogen activator inhibitor 1, which are all required to heal wounds
(Bonnar, et al., 1969; Drew, et al., 2001). There is also an influx of leukocytes to the site of
implantation via L-selectin on trophoblast cells which help to attract leukocytes to the area and
allow for the removal of cellular debris and heal the damaged tissue (Genbacev, et al., 2003).
Natural killer (NK) cells are responsible for wound repair and removal of cellular debris found
around the site of implantation which is referred to as an allograft of maternal and fetal tissues,
part of which will become the placenta (Hanna, et al., 2006). NK cells in this area mediate
Figure 1.6. A diagrammatic representation of the phases of myometrial differentiation (B) and the immune system throughout pregnancy (A). The Proliferation Phase (blue) occurs from NP to day (d) 14 and coincides with the Initiation Phase of the immune system. The Synthetic Phase (pink) is from d14 to d21 and overlaps with the Tolerance Phase of the immune system. The Contractile Phase (yellow) is from d21 to d23 and the Labour Phase (green) are associated with the Activation Phase (orange) of the immune system.
Gestation (days)

- d12
- d6
- d15
- d17
- d19
- d21
- d22
- d23

- Cell Number
- ECM Proteins
- Hypertrophy
- Basement Membrane Proteins
- Contraction
- Labour

A. Activation
B. Proliferation

- NP
- d6
- d12
- d15
- d19
- d21
- d22
- d23
- pp
angiogenesis and trophoblast invasion which allows for placentation and communication between the mother and the fetus (Hanna, et al., 2006). During early pregnancy pro-inflammatory factors and cytokines, like interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), RANTES (regulated on activation, normal T-cell expression and secreted), and granulocyte colony-stimulating factor (G-CSF), are high to allow for wound repair; however, they decrease as pregnancy progresses and will increase again at parturition (Mor, 2008).

During the second immunological phase of the uterus, termed the tolerance phase, which is maintained until just before labour, there is rapid fetal growth and development (Shynlova, et al., 2013). The tolerance phase overlaps partially with the synthetic phase of the myometrium and is characterized by an anti-inflammatory state and ensures uterine quiescence through pro-inflammatory inhibitors like progesterone (Piccinni, et al., 1995). Progesterone plays a role in maintaining an interleukin-10 (IL-10) immunosuppressive, anti-inflammatory, cytokine profile during this phase; transforming growth factor-beta (TGF-β) is also present and aids in immunosuppression (Challis, et al., 2009). Progesterone can act as an immunomodulator and blocks lymphocyte proliferation, promotes allograft survival, reduces pro-inflammatory cytokines, and alters T-cells to favour a Th2-type cytokine profile, which includes the immunosuppressive cytokine IL-10 (Piccinni, et al., 1995; Romagnani, 2000). Progesterone also upregulates toll-like receptor-4 (TLR-4) and suppresses toll-like receptor-2 (TLR-2), which responds to infection in intrauterine tissue, so it has a protective role in preterm labour (Elovitz & Mrinalini, 2005). Progesterone also inhibits basal and cytokine-induced matrix metalloproteinases (MMPs) preventing premature cervical ripening and rupture of fetal membranes due to collagen restructuring (Oner, et al., 2008). Therefore, progesterone is
required to maintain uterine quiescence throughout this phase by regulating the maternal immune system and preventing contractions (Hendrix, et al., 1995; Oner, et al., 2008). If progesterone is injected into a rat during late labour, labour can be prolonged, pro-inflammatory cytokine expression can be blocked, and although the expression profile of the contractile-associated protein (CAP) connexin 43 (Cx43) remains the same, trafficking to the plasma membrane is prevented (Hendrix, et al., 1995; Shynlova, et al., 2008).

During the last phase, the activation phase, functional progesterone withdrawal occurs in humans via a shift in progesterone receptor dynamics as a sign that the fetus has completely developed and parturition is imminent (Merlino, et al., 2007). In the rat, circulating progesterone levels decline and the decrease in progesterone is the sign that partition is imminent (Shynlova, et al., 2004). This phase of the immune system temporally relates to the contractile and labour phases of the myometrium and there is a localized inflammatory response which promotes cervical ripening, myometrial activation and contraction, membrane activation, and placental rejection (Romero, et al., 2006). These four processes are characterized by an influx of immune cells into the myometrium and placenta to promote the inflammatory process (Romero, et al., 2006). The pro-inflammatory environment causes uterine contraction, which will expel the baby, as well as promotes placental detachment (Romero, et al., 2006). During this time cervical ripening occurs via the accumulation of leukocytes – mostly neutrophils – in the cervical stroma (Bokstrom, et al., 1997). These activated leukocytes contribute to cervical remodeling by releasing proteolytic enzymes, specifically MMPs that digest the extracellular matrix (ECM) and aid in cervical dilation (Osmers, et al., 1995). Inflammatory leukocytes also infiltrate the myometrium at normal term labour
(Thomson, et al., 1999). In the myometrium and cervix the onset of labour is associated with increased expression of the chemoattractant for neutrophils – IL-8 (Osmers, et al., 1995). Neutrophils and macrophage also infiltrate the placenta as well and cause membrane rupture prior to labour (Elliott, et al., 1998; Maymon, et al., 2000; Steinborn, et al., 1998). Immune cells in the placenta are also attracted by locally produced chemokines and pro-inflammatory cytokines, like IL-8 (Elliott, et al., 1998). The cytokine interleukin-1β (IL-1β), but not interleukin-6 (IL-6) or IL-8 have been shown in myometrial cells to drive the production of prostaglandins by the myometrium which increases myometrial contractility (Todd, et al., 1996).

Increased growth of the uterus driven by fetal growth-induced distension is also thought to help promote the onset of labour. It has been shown that uterine stretch can induce small heat shock proteins (sHSPs) in vivo as well as the expression of cytokines and chemokines in vitro by the use of primary myometrial cells that were mechanically stretched (Loudon, et al., 2004; White & MacPhee, 2011). Monocyte Chemoattractant Protein-1 (MCP-1), a pro-inflammatory mediator and leukocyte chemoattractant was shown to be induced in gravid horns of unilaterally pregnancy rats, as well as in horns that were artificially stretched when compared to non-gravid horns (Shynlova, et al., 2008). Stretch induced pro-inflammatory mediators were attenuated when rat myometrial smooth muscle cells were pretreated with progesterone suggesting that mechanical as well as endocrine stimuli maybe required for the onset of labour (Shynlova, et al., 2008).

Since sHSPs have been shown to play a role in activation and repression of the immune system, which has a key role in initiation of term and pre-term labour, the secretion of HSPB5 via exosomes has been proposed for a mechanism of release from myometrial cells. It is
plausible that they can affect the immune system Th1/Th2 CD4 T-cell balance and increase the concentration of interferon-γ (IFN-γ), and subsequently pro-inflammatory cytokines, at the time of labour. HSPB5 has also been shown, in vitro, to induce an interleukin-10 (IL-10) regulatory immune response in macrophages at low concentrations (under 50ug/mL) while at higher concentrations (50ug/mL to 200ug/mL) it can increase T-cell production of IFN-γ, which activates macrophages to produce tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine (Bsibsi, et al., 2013; van Noort, et al., 2012; van Noort, et al., 2012a). HSPB5 may be the protein responsible for this induction, however in order to induce responses in varying immune cells, and eventually producing a TNF-α response from macrophages, HSPB5 must be secreted from myometrial cells. Vascular smooth muscle cells, cultured B-cells, and other cell types have been documented to secrete HSPs when stressed, but this has never been documented in myometrial cells (Clayton, et al., 2005; Martin-Ventura, et al., 2004).

1.5. Hypotheses and Objectives

Given its potential as an immune system activator and cytoskeletal associated protein, it was hypothesized that total HSPB5 and pSer59-HSPB5 protein expression in rat myometrium would be elevated during mid- to late-pregnancy when these processes occur during the contractile and labour phases of myometrial differentiation. Thus, the objectives of this research were:

**Objective 1.** To determine the spatio-temporal expression of total and pSer59-HSPB5 protein in rat myometrial tissue throughout gestation.

**Objective 2.** To determine the effects of stretch on total and pSer59-HSPB5 protein expression in a unilaterally pregnant rat model.
**Objective 3.** To determine associations of total and pSer59-HSPB5 protein with cytoskeletal filaments, as well as exosomal marker CD63 in a human myometrial cell line.
Chapter 2.

Expression of HSPB5 is Highly Induced in Rat Myometrium during Late Pregnancy and Labour and is Stimulated by Uterine Distension

Nicoletti, JG$^{1,2}$, White, BG$^3$, Miskiewicz, EI$^{1,2}$, MacPhee, DJ$^{1,2}$

$^1$Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5B4, Canada; $^2$One Reproductive Health Research Group, University of Saskatchewan, Saskatoon, SK, S7N 5B4, Canada; $^3$Okanagan College, Salmon Arm Campus, Salmon Arm, BC.

Short Title: HSPB5 expression is highly induced in pregnant rat myometrium.

Keywords: HSPB5, stress proteins, pregnancy, myometrium, uterus, labour.

Funded by the Natural Sciences and Engineering Research Council of Canada Discovery Grant #250218.

Authorship Statement:

J.G.N. performed the experiments and data analysis. E.I.M. trained J.G.N. in lab procedures including immunoblot analysis and assisted in data analysis. B.G.W. collected myometrial tissue samples used for all experiments. D.J.M. supervised J.G.N. and designed the study. J.G.N. and D.J.M. interpreted the data and wrote the manuscript.
2.1. Abstract

The uterine smooth muscle or myometrium goes through phases of differentiation throughout pregnancy to become a powerful contractile tissue at term. Small Heat Shock proteins (sHSPs) are a family of ten small molecular weight proteins that are induced by many physiological stressors such as uterine stretch. sHSPs act as chaperones, but also assist in cell death regulation, cytoskeleton rearrangements, and immune system activation upon phosphorylation. The expression of small heat shock proteins such as HSPB1 has been reported to increase during late pregnancy during the period of myometrial hypertrophy induced by fetal growth-induced uterine distension. Therefore, we examined the spatio-temporal expression of HSPB5 protein throughout gestation via immunoblot and immunofluorescence analysis, as well as the effect of uterine distension on myometrial HSPB5 protein expression using unilaterally pregnant rat models. HSPB5 protein expression significantly increased on day (d) 17 (p<0.05; vs all other timepoints) and levels steadily decreased thereafter through to postpartum (PP). In contrast, serine-59 phosphorylated (pSer59) HSPB5 protein detection was significantly increased from d19 through to PP (p<0.05). Both HSPB5 and pSer59-HSPB5 were detected in the cytoplasm of myocytes within both uterine muscle layers mid- to late-pregnancy. In unilaterally pregnancy rats, HSPB5 protein and pSer59-HSPB5 protein expression were significantly elevated in gravid uterine horns at both d19 and d23 (labour) of gestation compared to non-gravid horns. Therefore, uterine distension plays a major role in the stimulation of myometrial HSPB5 expression and increased expression of this small stress protein could be in response to the increasing uterine distension that occurs during mid- to late-pregnancy.
2.2. Introduction

The uterine smooth muscle or myometrium undergoes many structural and physiological changes throughout pregnancy to become a powerful contractile tissue at term. These changes occur in a series of four phases during pregnancy and one phase post-partum. Each phase is characterized by unique characteristics that have been well defined in the pregnant rat model (Shynlova, et al., 2013). At the beginning of rat pregnancy until day (d) 14, the myometrium enters the proliferation phase. Myometrial cells increase in number and there is an increase in the expression of anti-apoptotic factors, including Bcl-2, within these cells which contributes to an overall increase in proliferation (Shynlova, et al., 2006). The next phase is the synthetic phase from d15 to d21 where there is an increase in the protein:deoxyribonucleic acid (DNA) ratio in the myometrium, cells hypertrophy and increase their production of interstitial extracellular matrix proteins (ECM) and remodel focal adhesions (MacPhee & Lye, 2000; Shynlova, et al., 2004). In the contractile phase, there is an increase in expression of basement membrane matrix proteins such as fibronectin and associated integrin receptors (Shynlova, et al., 2004; Williams, et al., 2005). During the final phases of pregnancy, the labour phase, there is an increase in contractile associated proteins (CAPs) such as the gap junction protein connexin 43 and oxytocin receptors (Ou, et al., 1998; Tabb, et al., 1992). Immune system activation and immune cell infiltration of the myometrium are also thought to play a role in the initiation of labour (Osman, et al., 2003; Shynlova, et al., 2013; Thomson, et al., 1999). Fetal growth-induced uterine stretch may positively regulate both the production of chemokines and cytokines and chemokine-mediated infiltration of immune cells into the
myometrium during term and pre-term labour facilitating cytokine-mediated myometrial contractility (Shynlova, et al., 2008; Shynlova, et al., 2013).

The heat shock protein B (HSPB) or small heat shock protein (sHSP) family are comprised of ten small molecular weight proteins (15-40 kDa; B1-B10) that are induced by physiological stressors such as heat shock or oxidative stress. sHSPs act as adenosine triphosphate- (ATP) independent molecular chaperones, but also assist in cell death regulation, cytoskeletal rearrangements, and immune system activation (Acunzo, et al., 2012; van Noort, et al., 2012a; Wettstein, et al., 2012). The HSPB family is characterized by a conserved C-terminal region named the \( \alpha \)-crystallin domain, a more variable N-terminal sequence, and in most cases a short variable C-terminal tail (reviewed by Garrido, et al., 2012). Phosphorylation of sHSPs, particularly on serine residues, is critical for regulation of structure and function. For example, HSPB1 phosphorylation induces dissociation of large oligomers of HSPB1 and marked loss of chaperoning activity (Garrido, et al., 2012; Kato, et al., 1994).

HSPB5, previously known as \( \alpha \)B-crystallin, was discovered as a highly abundant protein in the lens of the eye where it maintains the transparency of the structure (Bloemendal & Otto, 1982; Clark, et al., 2012). To this end, it acts as a molecular chaperone to aid cytoprotection and prevent aggregation of denatured proteins (Horwitz, 1992). HSPB5 is expressed in a multitude of other tissues and mutations in HSPB5 can lead to congenital cataracts, cardiac myopathies, and neurodegenerative diseases (Acunzo, et al., 2012; Boncoraglio, et al., 2012). sHSPs appear to be important for maintaining the integrity of the cytoskeleton, particularly intermediate filaments and actin filaments (Liang & MacRae, 1997; Wettstein, et al., 2012).
fact, HSPB5 phosphorylation on serine-59 (Ser59) residues regulates HSPB5-actin interaction (Singh, et al., 2007).

As already inferred above, sHSPs can activate the immune system. HSPB5 has been shown, *in vitro*, to induce an Interleukin-10 (IL-10) regulatory macrophage immune response at low concentrations while at higher concentrations it can increase T-cell production of IFN-γ (interferon-γ), which activates macrophages (van Noort, et al., 2010; van Noort, et al., 2012a). HSPB5 can also alter the immune system by increasing the expression of endothelial cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin, that are responsible for slowing leukocyte rolling and facilitating leukocyte entry into tissues (Dieterich, et al., 2013).

The objectives of this research were to examine the spatio-temporal expression of HSPB5, including its phosphorylated form, in the rat myometrium during pregnancy and the potential regulation of HSPB5 expression by uterine distension. Due to the potential role of HSPB5 in aiding actin filament dynamics and immune cell activation, it was hypothesized that HSPB5 would be highly expressed in the myometrium during the contractile and labour phases of myometrial differentiation.

2.3. Materials & Methods

2.3.1. Animals

Sprague Dawley rats were acquired from the Mount Scio Vivarium (Memorial University of Newfoundland, St. John’s, NL, Canada) and used for all experiments. Animals were individually housed and cared for under standard environmental conditions (12 h light and 12 h
dark) in the Animal Care Unit at the Health Sciences Centre, Memorial University of Newfoundland. The rats had access to water *ad libitum* and were maintained on LabDiet Prolab RMH 3000 (PMI Nutrition International, Brentwood, MO, USA). For all experiments, virgin female rats weighing approximately 220-250g were mated with stud males. Day 1 of the gestational period was designated following the observation of a vaginal plug the morning after mating. The time of delivery under these standard conditions was d23 of the gestational period. All experiments were granted ethical approval by the institutional animal care committee under protocols 08-02-DM to 11-02-DM.

2.3.2. Experimental Design

2.3.2.1. Tissue Collection

Carbon dioxide induced asphyxiation was used for euthanization of all animals prior to sample collection. For normal gestation, samples were collected from animals at ten time points throughout gestation including: non-pregnant (NP), d6, d12, d15, d17, d19, d21, d22, d23 (labour) and 1-day post-partum (PP). For immunofluorescence detection a portion of the rat uterine horn was fixed in 4% paraformaldehyde (PFA) in 1X phosphate-buffered saline (PBS; pH 7.4) while shaking overnight at room temperature and then washed in 1X PBS for 24 h. Tissues were processed, paraffin embedded, sectioned and mounted on microscope slides by the Histology Unit of Memorial University of Newfoundland School of Medicine. Cross sections of the uterine horn were utilized for experiments and both the longitudinal and circular muscle layers of the myometrium were included in all sections. All sections were treated under identical conditions at the same time for each experiment.
For immunoblot analysis uterine horns were removed, excised and opened longitudinally, after which fetuses and placentae were discarded. Uterine tissue was then placed in ice-cold 1X PBS (pH 7.4) and a scalpel blade was used to gently scrape away the endometrial layer, as previously described (White, et al., 2005). All myometrial samples were flash-frozen in liquid nitrogen and stored at -80°C.

2.3.2.2. 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 as previously described (White & MacPhee, 2011). 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 d15, d19 and d23 (n=4 each timepoint).

2.3.3. Immunoblot Analysis

Immunoblot analysis was performed on at least 3 independent myometrium samples from normal rat gestation and unilaterally pregnant rat models. Tissue samples were homogenized in radio-immunoprecipitation assay (RIPA) lysis buffer (50mM Tris-HCl [pH 7.5], 150mM NaCl, 1% Triton-X100, 1% Sodium Deoxycholate, 0.1% SDS) plus protease and phosphatase inhibitors (Complete Mini, catalogue number (Cat #) 04693124001; PhosphoSTOP, Cat #: 04906845001; Roche Diagnostics, Laval, QC, CA). Protein concentration was determined using the Bio-Rad Bradford Protein Assay Kit per the manufacturer’s instructions (Cat #: 500-
Protein samples (40μg/lane of protein for the rat gestational profile and 15μg/lane of protein for the unilaterally pregnant rat model) were electrophoretically separated under reducing conditions in 12% Sodium Dodecyl Sulfate (SDS)-polyacrylamide gels and subsequently electroblotted to 0.2 μm nitrocellulose membranes (Cat #: 162-0097; Bio-Rad, Mississauga, ON, CA).

Membranes were stained with a Memcode Reversible Protein Stain kit, per the manufacturer’s instructions, to verify protein transfer (Cat #: 24580; Thermo Fisher Scientific Pierce, Ottawa, ON, CA). Blots were then incubated for 1 h with antisera that recognized both phosphorylated and unphosphorylated HSPB5 (total HSPB5) or HSPB5 when specifically phosphorylated on serine-59 (pSer59-HSPB5; see Table 1.1). Antisera were diluted in 5% milk in Tris-buffered saline containing Tween 20 (TBST; 20mM Tris-HCL [pH 7.6], 150mM NaCl, 0.1% Tween 20) or 5% Bovine Serum Albumin (BSA) in TBST, respectively. An appropriate Horseradish Peroxidase (HRP)-conjugated secondary antibody (see Table 1.1) diluted in 5% milk in TBST was utilized for immunoblot development. Membranes were subsequently stripped with Restore Western Blot Stripping Buffer as per the manufacturer’s instructions (Cat #: 21059; Thermo Fisher Scientific Pierce, Ottawa, ON, CA) and re-probed for Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) expression, which served as a loading control, using a rabbit polyclonal GAPDH-specific antibody (see Table 1.1) diluted in 5% milk in TBST. Total HSPB5 or pSer59-HSPB5 and GAPDH antibodies recognized proteins of predicted molecular weight; ~20-kDa for both forms of HSPB5 and 37-kDa for GAPDH. Protein detection on immunoblots was accomplished using a SuperSignal West Pico chemiluminescence substrate detection system (Cat #: 34080; Thermo Fisher Scientific Pierce, Ottawa, ON, CA) and multiple
Table 1.1. Antibodies utilized for immunoblot and immunofluorescence assays with myometrial and whole uterine tissue respectively. IB, immunoblot; IF, immunofluorescence.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Catalog Number</th>
<th>Dilution/Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total HSPB5 (Rabbit Polyclonal)</td>
<td>Enzo Life Sciences, Farmingdale, NY, US</td>
<td>ADI-SPA-223</td>
<td>1: 5 000 / IB</td>
</tr>
<tr>
<td>Total HSPB5 (Rabbit Polyclonal)</td>
<td>Sigma-Aldrich, Oakville, ON, CA</td>
<td>HPA-028724</td>
<td>1:100 / IF</td>
</tr>
<tr>
<td>pSer59-HSPB5 (Rabbit Polyclonal)</td>
<td>Enzo Life Sciences, Farmingdale, NY, US</td>
<td>ADI-SPA-227</td>
<td>1:5 000 / IB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:100 / IF</td>
</tr>
<tr>
<td>GAPDH (Rabbit Polyclonal)</td>
<td>Abcam, Cambridge, MA, US</td>
<td>Ab9485</td>
<td>1:10 000 / IB</td>
</tr>
<tr>
<td>ChromePure Rabbit IgG</td>
<td>Jackson ImmunoResearch, West Grove, PA, US</td>
<td>011-000-003</td>
<td>Variable / IF</td>
</tr>
<tr>
<td>Anti-Rabbit HRP</td>
<td>Promega, Madison, WI, US</td>
<td>W4011</td>
<td>1:10 000 / IB</td>
</tr>
<tr>
<td>Anti-Rabbit FITC</td>
<td>Sigma-Aldrich, Oakville, ON, CA</td>
<td>F7512</td>
<td>1:250 / IF</td>
</tr>
</tbody>
</table>
exposures were acquired using a Bio-Rad ChemiDoc MP digital imaging system (Bio-Rad, Mississauga, ON, CA).

2.3.4 Immunofluorescence Analysis

Tissue sections from 3 independent sets of rat uterine tissue samples from normal rat gestation and unilaterally pregnant rat models were used for experiments. Tissue sections were deparaffinized in xylene three times for 5 min each and then rehydrated in a descending series of ethanol (100%, 95%, 90%, 80%, 70%, and 50% ethanol). The tissues sections were subsequently washed once in PBS for 10 min and then marked off on the slide using an ImmEdge Hydrophobic Barrier Pen (Cat #: H-4000; Vector Laboratories, Burlington, ON, CA). Epitope retrieval was then conducted on all tissue sections. Heat-induced epitope retrieval was conducted once with boiling 0.1M Sodium Citrate buffer [pH 6.0] for 10 min and then sections allowed to cool for 20 min. Following a 10 min wash in PBS, enzyme-induced epitope retrieval was performed with 1 mg/mL trypsin solution (4mM CaCl2, 200 mM Tris, pH 7.7; Cat #: T7168; Sigma Chemical Co, Oakville, ON, CA) at room temperature for 10 min. Sections were then incubated for 30 min in a blocking solution consisting of 5% normal goat serum, 1% normal horse serum, and 1% fetal bovine serum.

Tissue sections were incubated overnight at 4°C with either total HSPB5 or pSer59-HSPB5-specific antisera (see Table 1.1) diluted in blocking solution. Non-specific rabbit IgG (see Table 1.1), used at the same concentration as primary antisera, was utilized as a negative control in all experiments. Sections were washed with PBS then incubated with fluorescein isothiocyanate (FITC) conjugated sheep anti-rabbit IgG. Following two washes in ice cold 0.02% Tween-20 in PBS, sections were then mounted in ProLong Gold Anti-Fade Reagent with DAPI
Images were collected using an Olympus BX51 microscope with widefield epi-fluorescence capabilities and a DP70 Olympus digital camera (Olympus, Richmond Hill, ON, CA). Representative timepoints throughout gestation were also imaged using a Leica TCS-SP5 laser scanning confocal microscope, equipped with Leica LAS AF imaging software (Leica, Scarborough, ON, CA). A z-series of microscope images were also taken at timepoints of interest using this confocal microscope and immunostaining was assessed in XZ/ZY axes using Image J software (Schindelin, et al., 2012).

**2.3.5. Data Analysis**

Densitometric analysis was performed on immunoblot data with the use of Image Lab software (Bio-Rad, Mississauga, ON, CA). Densitometric measurements of total HSPB5 protein or pSer59-HSPB5 protein were normalized to the densitometric measurements of GAPDH protein expression on immunoblots. Statistical analysis was performed using GraphPad Instat version 6.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Data from normal gestational profiles were subjected to a one-way Analysis of Variance and Tukey-Kramer multiple comparisons tests. Data sets from unilateral pregnancies were assessed by performing a two-tailed t-test. Values with a p<0.05 were considered significantly different. GraphPad Prism version 5.0 (GraphPad Software) was utilized to graph immunoblot data.

**2.4. Results**

**2.4.1. Expression of HSPB5 in the Myometrium throughout Gestation**

The temporal expression pattern of total and pSer59-HSPB5 protein were determined via immunoblot analysis. Total HSPB5 protein expression significantly increased on d17
compared to NP, d6, d12, d23 and PP timepoints (Fig. 2.1; p<0.05). In contrast, pSer59-HSPB5 expression was significantly increased from d19 through to PP (Fig. 2.2; p<0.05). Specifically, pSer59-HSPB5 expression was significantly increased at d21, d22 and d23 vs NP (*), d19, d21, d22, and d23 vs d6 (**), d22 and d23 vs d12 (***) , and at d22 vs d15 and d17 (#). The temporal relationship of total and pSer59-HSPB5 protein expression over gestation was also examined and it was clear that serine phosphorylation of HSPB5 occurred subsequently after the significant increase in total HSPB5 protein expression at d17 (Fig. 2.3).

The spatial expression of total HSPB5 and pSer59-HSPB5 was examined by immunofluorescence analysis. Both forms of HSPB5 were virtually undetectable from NP to d6 of gestation in myometrial cells from longitudinal and circular muscle layers. Total HSPB5 was particularly detectable in the cytoplasm of longitudinal and circular muscle cells at d15 through to PP and detected at a low level in stromal cells around muscle bundles (Figs. 2.4-2.7). Confocal analysis of the longitudinal and circular muscle layers at NP, d17, and d23 also showed and confirmed total HSPB5 detection in the cytoplasm of myocytes at d17 and d23, however total HSPB5 was not readily detectable in a NP state (Figs. 2.8, 2.9). pSer59-HSPB5 was also detected from d15 to PP in both longitudinal and circular muscle layers, but with prominent immunolocalization from d21 to d23 (Figs. 2.10-2.13). Expression was restricted to the cytoplasm of myocytes in both muscle layers with minimal immunodetection in the stroma of the tissue as confirmed by confocal analysis of pSer59-HSPB5 at time points NP, d15, and d23 (Figs. 2.14, 2.15). A z-series analysis of total HSPB5 and pSer59-HSPB5, as well as analysis of the XY and XZ planes further confirmed the localization of these proteins to primarily the cytosol of myocytes (Figs. 2.16-2.23).
Figure 2.1. Immunoblot analysis of total HSPB5 protein expression during pregnancy, parturition, and post-partum. (A) Representative immunoblots from analysis of total HSPB5 and GAPDH expression during gestation. (B) Densitometric analysis illustrating the dramatic increase in detection of total HSPB5 during pregnancy. The expression of HSPB5 on day (d) 17 of pregnancy was significantly (*; p<0.05) greater than expression on NP, d6, d12, d23, and PP timepoints. Values are the means from four independent experiments (n=4) and error bars represent the SEM. NP, non-pregnant; PP, 1 day post-partum; HSPB5, total HSPB5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.
A. 

HSPB5

GAPDH

B. 

Relative Optical Density
HSPB5:GAPDH

Day of Gestation
Figure 2.2. Immunoblot analysis of serine-59 phosphorylated (pSer59) HSPB5 expression during pregnancy, parturition, and post-partum. (A) Representative immunoblots from analysis of pSer59-HSPB5 and GAPDH expression during gestation. (B) Densitometric analysis illustrating the dramatic increase in detection of pSer59-HSPB5 during pregnancy and parturition. Values on d21 - d23 were significantly (*; p<0.05) increased compared with the NP timepoint, values on d19 - d23 were significantly (***; p<0.05) increased compared with the d6 timepoint, values on d22 and d23 were significantly (**; p<0.05) increased compared with the d12 timepoint, and expression on d22 was significantly elevated when compared with d15 and d17 (#; p<0.05). Values plotted are means from four independent experiments (n=4) and error bars represent the SEM. NP, non-pregnant; PP, 1 day post-partum; pSer59-HSPB5, serine-59 phosphorylated HSPB5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.
A. pSer59-HSPB5

B. Relative Optical Density pSer59-HSPB5:GAPDH

Day of Gestation
Figure 2.3. Comparison of total HSPB5 (blue) and serine-59 phosphorylated (pSer59) HSPB5 (pink) protein expression during pregnancy. pSer59-HSPB5 expression became elevated following elevation of total HSPB5 indicating temporal-specific post-translational modification of HSPB5 occurred. Values plotted represent means from 4 independent experiments and error bars represent the SEM. HSPB5, total HSPB5; pSer59-HSPB5, serine-59 phosphorylated HSPB5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.
Figure 2.4. Immunofluorescence analysis of total HSPB5 protein expression in uterine longitudinal smooth muscle. Spatiotemporal expression of HSPB5 was assessed in longitudinal muscle layers of non-pregnant (NP) and pregnant rat myometrium between day 6 (d6) and day 17 (d17) of gestation using a rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. Immunofluorescent detection was notably higher at d15 and d17. Representative images are shown. Cntrl, IgG control. Scale bar = 50µm.
Figure 2.5. Immunofluorescence analysis of total HSPB5 protein expression in uterine longitudinal smooth muscle. Spatiotemporal expression of HSPB5 was assessed in longitudinal muscle layers of pregnant rat myometrium between day 19 (d19) of gestation and 1 day post-partum (PP) using a rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. Immunofluorescent detection was prominent from d19 to PP. Representative images are shown. Cntrl, IgG control. Scale bar 50µm.
**Figure 2.6.** Immunofluorescence analysis of total HSPB5 protein expression in uterine circular smooth muscle. Spatiotemporal expression of HSPB5 was assessed in circular muscle layers of non-pregnant (NP) and pregnant rat myometrium between day 6 (d6) and day 17 (d17) of gestation using a rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. Representative images are shown. Cntrl, IgG control. Scale bar = 50µm.
Figure 2.7. Immunofluorescence analysis of total HSPB5 protein expression in uterine circular smooth muscle. Spatiotemporal expression of HSPB5 was assessed in circular muscle layers of pregnant rat myometrium between day 19 (d19) of gestation and 1 day post-partum (PP) using a rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. Representative images are shown. Cntrl, IgG control. Scale bar = 50µm.
Figure 2.8. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in uterine longitudinal smooth muscle layers. Representative images of non-pregnant (NP) and pregnant rat myometrium at day 17 (d17) and labour at day 23 (d23) are shown. The experiments were conducted using a rabbit polyclonal anti-human HSPB5 specific antisera, 5µm thick tissue sections, and images collected using a Leica TCS-SP5 laser scanning confocal microscope. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. Cntrl, IgG control; DAPI, 4’,6-diamidino-2-phenylindole. Scale bar = 50µm.
**Figure 2.9.** Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in uterine circular smooth muscle layers. Representative images of non-pregnant (NP) and pregnant rat myometrium at day 17 (d17) and labour at day 23 (d23) are shown. The experiments were conducted using a rabbit polyclonal anti-human HSPB5 specific antisera, 5µm thick tissue sections, and images collected using a Leica TCS-SP5 laser scanning confocal microscope. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. Cntrl, IgG control; DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 50µm.
Figure 2.10. Immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in uterine longitudinal smooth muscle. Spatiotemporal expression of pSer59-HSPB5 was assessed in longitudinal muscle layers of non-pregnant (NP) and pregnant rat myometrium between day 6 (d6) and day 17 (d17) of gestation using a rabbit polyclonal anti-human pSer59-HSPB5 specific antisera and 5µm thick tissue sections. pSer59-HSPB5 immunostaining became particularly noticeable starting at d15. Representative images are shown. Cntrl, IgG control. Scale bar = 50µm.
**Figure 2.11.** Immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in uterine longitudinal smooth muscle. Spatiotemporal expression of pSer59-HSPB5 was assessed in longitudinal muscle layers of pregnant rat myometrium between day 19 (d19) of gestation and 1 day post-partum (PP) using a rabbit polyclonal anti-human pSer59-HSPB5 specific antisera and 5µm thick tissue sections. Predominant immunodetection was observed from d21 to d23. Representative images are shown. Cntrl, IgG control. Scale bar = 50µm.
Figure 2.12. Immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in uterine circular smooth muscle. Spatiotemporal expression of pSer59-HSPB5 was assessed in circular muscle layers of non-pregnant (NP) and pregnant rat myometrium between day 6 (d6) and day 17 (d17) of gestation using a rabbit polyclonal anti-human pSer59-HSPB5 specific antisera and 5µm thick tissue sections. pSer59-HSPB5 became markedly detectable after d6. Representative images are shown. Cntrl, IgG control. Scale bar = 50µm.
**Figure 2.13.** Immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in uterine circular smooth muscle. Spatiotemporal expression of pSer59-HSPB5 was assessed in circular muscle layers of pregnant rat myometrium between day 19 (d19) of gestation and 1 day post-partum (PP) using a rabbit polyclonal anti-human pSer59-HSPB5 specific antisera and 5µm thick tissue sections. pSer59-HSPB5 was highly detectable throughout the timespan, but particularly prominent from d21 to d23. Representative images are shown. Cntrl, IgG control. Scale bar = 50µm.
Figure 2.14. Confocal laser scanning microscopy and immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in uterine longitudinal smooth muscle layers. Representative images of non-pregnant (NP) and pregnant rat myometrium at day 15 (d15) and labour at day 23 (d23) are shown. The experiments were conducted using a rabbit polyclonal anti-human HSPB5 specific antisera, 5µm thick tissue sections, and images collected using a Leica TCS-SP5 laser scanning confocal microscope. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. Cntrl, IgG control; DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 50µm.
Figure 2.15. Confocal laser scanning microscopy and immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in uterine circular smooth muscle layers. Representative images of non-pregnant (NP) and pregnant rat myometrium at day 15 (d15) and labour at day 23 (d23) are shown. The experiments were conducted using a rabbit polyclonal anti-human HSPB5 specific antisera, 5µm thick tissue sections, and images collected using a Leica TCS-SP5 laser scanning confocal microscope. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. Cntrl, IgG control; DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 50µm.
Figure 2.16. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in the uterine longitudinal smooth muscle layer at day (d) 17. The experiments were conducted using rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. A z-series of images were collected using a laser scanning confocal microscope and the results assessed in the XZ and YZ axes using Image J. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 10µm.
**Figure 2.17.** Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in the uterine circular smooth muscle layer at day (d) 17. The experiments were conducted using rabbit polyclonal anti-human HSPB5 specific antisera and 5μm thick tissue sections. A z-series of images were collected using a laser scanning confocal microscope and the results assessed in the XZ and YZ axes using Image J. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 10μm.
**Figure 2.18.** Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in the uterine longitudinal smooth muscle layer at day (d) 23. The experiments were conducted using rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. A z-series of images were collected using a laser scanning confocal microscope and the results assessed in the XZ and YZ axes using Image J. Mounting media containing DAPI was utilize to visualize nuclei. Arrow indicates cytoplasmic staining. DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 10µm.
Figure 2.19. Confocal laser scanning microscopy and immunofluorescence analysis of total HSPB5 protein expression in the uterine circular smooth muscle layer at day (d) 23. The experiments were conducted using rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. A z-series of images were collected using a laser scanning confocal microscope and the results assessed in the XZ and YZ axes using Image J. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 10µm.
Figure 2.20. Confocal laser scanning microscopy and immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in the uterine longitudinal smooth muscle layer at day (d) 15. The experiments were conducted using rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. A z-series of images were collected using a laser scanning confocal microscope and the results assessed in the XZ and YZ axes using Image J. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 10µm.
Figure 2.21. Confocal laser scanning microscopy and immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in the uterine circular smooth muscle layer at day (d) 15. The experiments were conducted using rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. A z-series of images were collected using a laser scanning confocal microscope and the results assessed in the XZ and YZ axes using Image J. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 10µm.
Figure 2.22. Confocal laser scanning microscopy and immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in the uterine longitudinal smooth muscle layer at day (d) 23. The experiments were conducted using rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. A z-series of images were collected using a laser scanning confocal microscope and the results assessed in the XZ and YZ axes using Image J. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. DAPI, 4',6-diamidino-2-phenylindole. Scale bar = 10µm.
**Figure 2.23.** Confocal laser scanning microscopy and immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in the uterine circular smooth muscle layer at day (d) 23. The experiments were conducted using rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections. A z-series of images were collected using a laser scanning confocal microscope and the results assessed in the XZ and YZ axes using Image J. Mounting media containing DAPI was utilized to visualize nuclei. Arrow indicates cytoplasmic staining. DAPI, 4′,6-diamidino-2-phenylindole. Scale bar = 10µm.
2.4.2. Uterine Stretch Induces Myometrial HSPB5 Expression

Since uterine distension, due to the effects of fetal growth, is a powerful inducer of protein expression, uterine tissue and myometrial protein extracts were collected from unilaterally pregnant rats at d15, d19, and d23 for immunoblot and immunofluorescence analysis. Immunoblot analyses demonstrated that both total and pSer59-HSPB5 protein expression were markedly increased in myometrial lysates from gravid horns compared to non-gravid horns at d19 and d23 of gestation. In addition, total HSPB5 protein expression was significantly higher in gravid horns at d15 (Figs. 2.24, 2.25). Immunofluorescence analysis supported the immunoblot results as immunodetection of total and pSer59-HSPB5 was also markedly increased in the cytoplasm of myometrial cells from gravid horns at d19 and d23, in both muscle layers, compared to contralateral non-gravid uterine horns (Figs. 2.26-2.29).

2.5. Discussion

The HSPB family of small stress proteins are ATP-independent chaperones that also have important roles in processes such as cell death regulation and cytoskeletal organization (Acunzo, et al., 2012; Wettstein, et al., 2012). Several family members have been reported to be dynamically expressed in the myometrium during pregnancy (Cross, et al., 2007; MacIntyre, et al., 2008; Marsh, et al., 2015; White, et al., 2005; White & MacPhee, 2011). HSPB5, previously known as αB-crystallin, was originally discovered in the eye lens, but has never been thoroughly examined in the myometrium throughout pregnancy. Since HSPB5 appears to aid actin filament dynamics and immune cell activation, it was hypothesized that HSPB5 would be highly expressed in the myometrium during the contractile and labour phase of myometrial
Figure 2.24. Expression of total HSPB5 protein in the rat myometrium is significantly induced by uterine stretch. Representative immunoblots of total HSPB5 and GAPDH protein expression in myometrium from non-gravid and gravid uterine horns at day (d) 15, d19 of rat gestation, and d23 (labouring) uterine horns are shown. Densitometric analyses illustrated that total HSPB5 expression significantly increased (*; p<0.05) in gravid horns compared to non-gravid horns at all timepoints (A-C). Values shown are means from a minimum of three independent experiments (n=3) and error bars represent the SEM. NP, non-pregnant; PP, 1 day post-partum; HSPB5, total HSPB5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.
**Figure 2.25.** Expression of serine-59 phosphorylated (pSer59) HSPB5 protein in the rat myometrium is significantly induced by uterine stretch. Representative immunoblots of pSer59-HSPB5 and GAPDH protein expression in myometrium from non-gravid and gravid uterine horns at day (d) 19 of rat gestation and d23 (labouring) uterine horns are shown. Densitometric analyses illustrated pSer59-HSPB5 protein expression significantly increased in myometrium from gravid uterine horns compared to non-gravid horns at d19 and d23 (*; p<0.05) (A, B). Values shown are means from a minimum of three independent experiments (n=3) and error bars represent the SEM. NP, non-pregnant; PP, 1 day post-partum; pSer59-HSPB5, serine-59 phosphorylated HSPB5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.
A. d19

<table>
<thead>
<tr>
<th></th>
<th>NG</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSer59-HSPB5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. d23

<table>
<thead>
<tr>
<th></th>
<th>NG</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSer59-HSPB5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.26. Immunofluorescence analysis of total HSPB5 protein expression in longitudinal and circular muscle layers of non-gravid and gravid horns at day (d) 19 of rat gestation. A rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections were utilized for experiments. Total HSPB5 expression was highly detectable in the cytoplasm of myocytes within both muscle layers of gravid horns when compared to non-gravid horns. Representative images are shown. Long, longitudinal; Circ, circular; Cntrl, IgG control. Scale bar = 50µm.
Figure 2.27. Immunofluorescence analysis of total HSPB5 protein expression in longitudinal and circular muscle layers of non-gravid and gravid horns at labour (d23). A rabbit polyclonal anti-human HSPB5 specific antisera and 5µm thick tissue sections were utilized for experiments. Total HSPB5 expression was highly detectable in both muscle layers of gravid horns when compared to non-gravid horns at d23 of rat gestation. Representative images are shown. Long, longitudinal; Circ, circular; Cntrl, IgG control. Scale bar = 50µm.
Figure 2.28. Immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in longitudinal and circular muscle layers of non-gravid and gravid horns at day (d) 19 of rat gestation. A rabbit polyclonal anti-human pSer59-HSPB5 specific antisera and 5µm thick tissue sections were utilized for experiments. pSer59-HSPB5 expression was highly detectable in both muscle layers of gravid horns when compared to non-gravid horns at d19 of rat gestation. Representative images are shown. Long, longitudinal; Circ, circular; Cntrl, IgG control. Scale bar = 50µm.
**Figure 2.29.** Immunofluorescence analysis of serine-59 phosphorylated (pSer59) HSPB5 protein expression in longitudinal and circular muscle layers of non-gravid and gravid horns at labour (d23). A rabbit polyclonal anti-human pSer59-HSPB5 specific antisera and 5µm thick tissue sections were utilized for experiments. pSer59-HSPB5 expression was highly detectable in both muscle layers of gravid horns when compared to non-gravid horns at d23 of rat gestation. Representative images are shown. Long, longitudinal; Circ, circular; Cntrl, IgG control. Scale bar = 50µm.
differentiation.

2.5.1. Myometrial HSPB5 Expression during Pregnancy

Immunoblot analysis demonstrated a significant increase in total HSPB5 protein at d17 of pregnancy; however, a significant increase of pSer59-HSPB5 protein expression was observed from d19 to d23. The increase in the phosphorylated form of HSPB5 followed the significant increase in total HSPB5 levels suggesting HSPB5 is produced in large amounts in the synthetic phase of myometrial programming and then subsequently phosphorylated during the later phases. HSPB5 is phosphorylated on Ser59 by the p38 MAPK pathway (Kato, et al., 1998). Oldenhof and colleagues (2002) reported that activated (phosphorylated) p38 MAPK was readily expressed in rat myometrium during late pregnancy (Oldenhof, et al., 2002). Thus, this pathway is likely involved, in part, in the increased expression of pSer59-HSPB5 during late pregnancy and labour. Our observed significant increase in expression of total HSPB5 protein at d17 compared to timepoints including d23 (labour) is also notable as MacIntyre and colleagues (2008) reported that HSPB5 protein expression decreased 71% in myometrium from laboring women compared to non-laboring women (MacIntyre, et al., 2008). However, these authors did not examine the phosphorylated form of HSPB5 and our results indicate the importance of assessing the expression of phosphorylated forms of sHSPs.

Both total and pSer59-HSPB5 were detected from d15 to PP in both longitudinal and circular uterine smooth muscle layers, with expression throughout the cytoplasm of myocytes. In contrast, MacIntyre and colleagues (2008) only detected total HSPB5 in the myometrium of non-laboring patients from 37-40 weeks of gestation with localization in perinuclear regions of
myocytes (MacIntyre, et al., 2008). Detection of total HSPB5 was barely above background levels in myometrium from laboring patients within the same timeframe. This may reflect species-specific differences in total HSPB5 expression and localization in the myometrium at labour. As stated above pSer59-HSPB5 immunolocalization was not examined by the authors.

### 2.5.2. The Influence of Uterine Distension on Myometrial HSPB5 Expression

Both total and pSer59-HSPB5 protein expression and immunolocalization were markedly increased in the myometrium upon uterine distension at d19 and d23 of pregnancy, as well as total HSPB5 expression at d15. To our knowledge, this is the first demonstration of mechanical forces inducing HSPB5 expression in myometrial smooth muscle. In terms of pSer59-HSPB5 expression, Oldenhof and colleagues (2002) demonstrated that activated p38 MAPK expression was significantly increased at d22 in myometrium from gravid rat uterine horns compared to myometrium from non-gravid horns (Oldenhof, et al., 2002). Again, this implicates the p38 MAPK pathway in the serine phosphorylation of HSPB5. It is clear that HSPB5 has a broad interactome, but it is well reported that HSPB5 can readily interact and form a functional complex with HSPB1 (reviewed by Arrigo, 2013). Both total and pSer15-HSPB1 expression are also readily induced in the rat myometrium by uterine distension at d19 and d23 of pregnancy (White & MacPhee, 2011). Thus, both of these sHSPs may be working together during this period of gestation in response to uterine distension.

### 2.5.3. Potential Roles for HSPB5 in the Myometrium

The serine phosphorylation of HSPB5, like serine phosphorylation of HSPB1, leads to production of small oligomers from very large complexes and decreased chaperone function
As a result, both HSPB1 and HSPB5 can interact with actin fibres (Arrigo, et al., 2007; Arrigo & Gilbert, 2013; Wettstein, et al., 2012). For example, Singh and colleagues (2007) showed that HSPB5 can interact directly with actin in rat H9C2 cardiomyoblasts, regulate actin filament dynamics, and that the association is dependent, in part, on Ser59 phosphorylation (Singh, et al., 2007). Two specific regions of the HSPB5 molecule encompassing amino acids 113-120 and 131-142 have also been found to be responsible for promoting actin polymerization (Ghosh, et al., 2007; Launay, et al., 2006).

The dynamic modulation of actin microfilament formation plays a large role in smooth muscle contraction (Taggart & Morgan, 2007). For example, Shaw and colleagues (2003) reported that agonist induced constriction of non-pregnant rat myometrium was reduced by inhibition of actin polymerization with cytochalasin D (Shaw, et al., 2003). Focal adhesions, also known as smooth muscle dense plaques, are sites on the plasma membrane where clusters of integrins, signaling molecules and adapters such as focal adhesion kinase (FAK) can provide a structural link between the ECM and the actin cytoskeleton. Focal adhesion signaling is important for promoting myometrial cell contraction in late pregnancy (Li, et al., 2007). Due to the increase in pSer59-HSPB5 expression during late pregnancy and labour and induction by uterine distension, we speculate that pSer59-HSPB5 may be part of a mechano-adaptive response, perhaps in partnership with pSer15-HSPB1, to modulate actin polymerization dynamics at focal adhesions in the myometrium during late pregnancy and to facilitate phasic labour contractions.

In contrast to a role with the cytoskeleton, sHSPs have also been shown to be extracellular signaling molecules that can be secreted via exosomes (Clayton, et al., 2005;
Exosome production has been demonstrated in vascular smooth muscle and recently implicated in myometrium (Cretoiu, et al., 2013; Liao, et al., 2000; Martin-Ventura, et al., 2004). The uptake of exosomes by macrophages through phagocytosis and release of contents has been demonstrated (Feng, et al., 2010). In this manner, HSPB5 and other HSPB family members can signal to macrophages or macrophage-like cells and induce innate immune responses (Bhat & Sharma, 1999; van Noort, et al., 2010; Wu, et al., 2001). Interestingly, van Noort and colleagues (2010) have demonstrated that HSPB family members appear to promote activation of macrophages into an immune regulatory state that stimulates tissue repair and attenuates inflammation (van Noort, et al., 2010). This likely depends on the local concentration of HSPB5 as high local concentrations have been reported to stimulate a pro-inflammatory immune response (van Noort, et al., 2010; van Noort, et al., 2012; van Noort, et al., 2012a). The maternal immune system during pregnancy and labour goes through an immunological transformation from initiation to tolerance and then activation in concert with myometrial programming (Shynlova, et al., 2013). Thus, HSPB5 may have chronologically specific anti-inflammatory and pro-inflammatory roles in the myometrium during pregnancy to aid this immunological transformation.

Overall, the spatial and temporal expression of HSPB5 changes dynamically in the myometrium during late pregnancy and labour and is regulated by uterine distension. HSPB5 could play an important role in facilitating the contractility of myometrial smooth muscle cells by regulating actin filament dynamics at focal adhesions and/or help regulate immune responses within the myometrium during late pregnancy and labour. Further investigation at
the cellular and molecular level are required to understand whether or not HSPB5 associates with the actin cytoskeleton and vesicles destined to become exosomes for intercellular signaling.
Chapter 3.

Co-localization of HSPB5 and pSer59-HSPB5 with Cytoskeletal and Exosomal Markers

Nicoletti, JG\textsuperscript{1,2}, Miskiewicz, EI\textsuperscript{1,2}, MacPhee, DJ\textsuperscript{1,2}

\textsuperscript{1}Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5B4, Canada; \textsuperscript{2}One Reproductive Health Research Group, University of Saskatchewan, Saskatoon, SK, S7N 5B4, Canada.

Short Title: Co-localization of HSPB5 and pSer59-HSPB5 with cytoskeletal and exosomal markers.

Keywords: HSPB5, stress proteins, myometrium, actin, kindlin-2, CD63.

Funded by the Natural Sciences and Engineering Research Council of Canada Discovery Grant #250218.

Authorship Statement:

J.G.N. performed the experiments and data analysis. E.I.M. trained J.G.N. in lab procedures including cell culture protocols and data analysis. D.J.M. supervised J.G.N. and designed the study. J.G.N. and D.J.M. interpreted the data and wrote the manuscript.
3.1. Abstract

Small Heat Shock proteins (sHSPs) are a family of ten small molecular weight proteins that are induced by many physiological stressors such as uterine stretch. sHSPs act as chaperones, but also assist in cell death regulation, cytoskeleton rearrangements, and immune system activation upon phosphorylation. sHSPs, specifically HSPB5 and serine-59 phosphorylated (pSer59) HSPB5, have been shown to assist in cytoskeletal rearrangements by working with cytoskeletal filaments, like actin, to potentially prepare muscle cells for contraction. Therefore, we examined the spatial expression of total HSPB5 protein in a human myometrial cell line via immunofluorescence analysis and its potential co-localization with α-smooth muscle actin. Furthermore, the potential co-localization of pSer59-HSPB5 with α-smooth muscle actin and the focal adhesion protein kindlin-2 (KIN-2) was also examined in the cell line. Since HSPB5 has also been shown to activate and/or attenuate the immune system, depending on concentration, by exosome-mediated secretion the potential co-localization of pSer59-HSPB5 with CD63, an exosomal marker, was also determined. Total HSPB5 co-localized with α-smooth muscle actin while pSer59-HSPB5 co-localized with KIN-2 and CD63; however, even though pSer59-HSPB5 and α-smooth muscle actin were located in relatively close proximity no true co-localization was observed.

3.2. Introduction

At the time of labour the myometrium needs to be a strong and contractile organ to expel a fetus from the uterus, thus the establishment of strong cell-extracellular matrix (ECM) and strong cell-cell interactions are essential (MacPhee & Lye, 2000). The establishment of cell-ECM interactions depends on the integrin family of receptor proteins (Tu, et al., 2003). There
are 24 proteins in the integrin family and they are responsible for cell-ECM interactions and critical for the development of multicellular organisms (Hynes, 2002). Integrins are heterodimeric glycoproteins with alpha (α) and beta (β)-subunits and are comprised of extracellular, transmembrane, and cytoplasmic domains (Montanez, et al., 2008). Kindlins are a novel family of adaptor proteins that are recruited to integrin-containing adhesion sites termed focal adhesions (Montanez, et al., 2008; Rogalski, et al., 2000; Tu, et al., 2003). There are three proteins in this family, kindlin-1 (KIN-1), specific to epithelial cells, kindlin-2 (KIN-2), ubiquitous to all cell types, and kindlin-3 (KIN-3), expressed in hematopoietic cells only (He, et al., 2011). KIN-1 and KIN-2 are essential for mediating cell-ECM adhesion and spreading (He, et al., 2011). Since HSPB5 has been shown to interact with different cytoskeletal proteins, like desmin, it is hypothesized that serine-59 phosphorylated (pSer59) HSPB5, the active form of HSPB5, could potentially associate with KIN-2, which is ubiquitous to all cell types, and help stabilize and establish cell-ECM interactions required for the formation of a powerful, contractile organ (Wettstein, et al., 2012).

Phosphorylation of HSPB5 has also been shown to decrease their native chaperone function (Schmidt, et al., 2012). HSPB5 has three phosphorylation sites (serine-19, serine-45, and serine-59) and it has been suggested that HSPB5 inhibits actin fiber depolymerization and prevents their aggregation during stress (Wettstein, et al., 2012). The phosphorylation state of HSPB5 regulates the actin polymerization-depolymerization balance (Launay, et al., 2006). For example, Launay and colleagues (2006) reported that serine-59 phosphorylation (pSer59) was up-regulated after cytoskeletal stress and that this phosphorylation allowed HSPB5 to associate and bind with actin filaments (Launay, et al., 2006). Inhibition of serine-59 phosphorylation
(pSer59) almost completely inhibited its association with actin filaments during stress (Launay, et al., 2006). Some researchers suggest that HSPB5 must be phosphorylated on the serine-59 residue to co-localize with the cytoskeleton (Launay, et al., 2006). Since it is known that HSPB5 can interact with actin, as well as intermediate filament proteins such as desmin, the potential co-localization of HSPB5 and pSer59-HSPB5 with actin filaments in the human myometrial cell line, hTERThm, will be studied. Previous work (see Chapter 2) has demonstrated that pSer59-HSPB5 is highly expressed in the rat myometrium at late-pregnancy and labour where it could be involved in regulating actin dynamics. It is hypothesized that HSPB5 and its phosphorylated form will both co-localize with α-smooth muscle actin.

CD63 is a tetraspanin family protein that resides in late endosomal membranes as well as exosomal membranes (Kobayashi, et al., 2000; Kore & Abraham, 2014). Since HSPB5 has been shown to induce varying immunologic responses dependent on phosphorylation state, the secretion of HSPB5 or its phosphorylated forms via exosomes, could induce an immunogenic response (van Noort, et al., 2012a). It is well known that the immune system plays an important role in the activation and progression of labour, however the exact triggering mechanisms are not well understood (Mor & Cardenas, 2010). If HSPB5 was involved in stimulating the increase in pro-inflammatory cytokines at the time of labour it would have to induce responses in various immune cells following secretion from myometrial cells. Small heat-shock proteins (sHSPs) in general do not contain specific signal sequences to induce their active secretion; therefore, an alternate secretion-pathway must be considered. Recently the alternative secretion of sHSP via exosomes has been demonstrated and confirmation of the co-localization of CD63 and pSer59-HSPB5 in myometrial cells would add to this evidence (van
Noort, et al., 2012a). Thus, it is hypothesized that pSer59-HSPB5 will co-localize with exosomal marker CD63.

Overall, the characterizations of these proteins in the hTERThm, human myometrial cell line, would be a significant addition to the growing body of knowledge on sHSP expression, localization in cells and potential functions in the myometrium during pregnancy.

3.3. Materials & Methods

3.3.1. Cell Culture of the hTERThm Cell Line

The hTERThm cell line was established via stable transfection of cells with expression vectors containing the human telomerase reverse transcriptase (hTERT) which maintains telomere length and immortalizes cells (Condon, et al., 2002). Myometrial cells immortalized with hTERT retain smooth muscle and myometrium cell characteristics demonstrated by the expression of smooth muscle and myometrial cell markers including calponin, smoothelin, and oxytocin receptor proteins (Condon, et al., 2002). hTERThm cell cultures were maintained at 37°C in 95% air-5% CO2 in DMEM/F12 media with L-glutamine and 15mM HEPES (catalogue number (Cat #): 11330-032; Life Technologies, Burlington, ON, CA) plus 10% fetal bovine serum (FBS) and 1% penicillin- streptomycin (Cat #: 15140-122; Life Technologies, Burlington, ON, CA). Medium was refreshed every 24 hours (h). Cells were passaged when cells reached ~90% confluence using trypsin-EDTA solution (0.05% trypsin in EDTA, Cat #: 15400-054; Life Technologies, Burlington, ON, CA).
3.3.2. Immunofluorescence Analysis

For immunofluorescence analysis, cells were cultured in T75 tissue culture plates, collected by trypsinization, and counted with a Bio-Rad TC20 cell counter. 0.1 x 10^6 cells were seeded onto 22mm x 22mm sterile glass coverslips in six well plates. After allowing 24 h for attachment and proliferation in DMEM/F12 media (as described above) cells were fixed with 4% paraformaldehyde (PFA) in 1X phosphate-buffered saline (PBS; pH 7.4) for 5 min at room temperature and then washed with PBS. Cells were then treated with PBS containing 0.1% Triton X-100 for 15 min at room temperature and subsequently with blocking solution (5% normal goat serum, 1% normal horse serum, and 1% fetal bovine serum) for 30 min at room temperature. A minimum of 4 sets of proliferating cells at 24 h and 48 h post seeding were probed with four combinations of primary antibodies. Primary antibodies specific for total HSPB5, pSer59-HSPB5, KIN-2, α-smooth muscle actin, and CD63 were used in the following pairs: total HSPB5 and α-smooth muscle actin (rabbit polyclonal), pSer59-HSPB5 and α-smooth muscle actin (mouse monoclonal), pSer59-HSPB5 and KIN-2, and pSer59-HSPB5 and CD63 (mouse monoclonal; see Table 3.1). The dilution of control mouse IgG or rabbit IgG used in place of the primary antisera (see Table 3.1), was determined by matching the control IgG concentration to the concentration of the primary antibodies. A fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG and a Rhodamine red-X (RRX) conjugated anti- mouse IgG (see Table 3.1) were utilized as a secondary antibodies. Slides were then mounted in ProLong Gold Anti-Fade Reagent with DAPI (Cat #: P36931; Life Technologies, Burlington, ON, CA) as per the manufacturer’s instructions.
Table 3.1. Antibodies utilized for immunofluorescence experiments with hTERT cells. IF, immunofluorescence.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Catalog Number</th>
<th>Dilution/Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total HSPB5 (Mouse Monoclonal)</td>
<td>Stressmarq Biosciences, Victoria, BC, CA</td>
<td>SMC-159</td>
<td>1:100 / IF</td>
</tr>
<tr>
<td>Kindlin-2 (Mouse Monoclonal)</td>
<td>Millipore, Etobicoke, ON, CA</td>
<td>MAB2617</td>
<td>1:100 / IF</td>
</tr>
<tr>
<td>α-Smooth Muscle Actin (Rabbit Polyclonal)</td>
<td>Abcam, Cambridge, MA, US</td>
<td>Ab5694</td>
<td>1:20 / IF</td>
</tr>
<tr>
<td>α-Smooth Muscle Actin (Mouse Monoclonal)</td>
<td>Sigma-Aldrich, Oakville, ON, CA</td>
<td>A2547</td>
<td>1:1 000 / IF</td>
</tr>
<tr>
<td>CD63 (Mouse Monoclonal)</td>
<td>Thermo Fisher Scientific Pierce, Ottawa, ON, CA</td>
<td>MA5 11501</td>
<td>1:20 / IF</td>
</tr>
<tr>
<td>ChromePure Rabbit IgG</td>
<td>Jackson ImmunoResearch, West Grove, PA, US</td>
<td>011-000-003</td>
<td>Variable / IF</td>
</tr>
<tr>
<td>ChromePure Mouse IgG</td>
<td>Jackson ImmunoResearch, West Grove, PA, US</td>
<td>015-000-003</td>
<td>Variable / IF</td>
</tr>
<tr>
<td>Anti-Rabbit FITC</td>
<td>Sigma-Aldrich, Oakville, ON, CA</td>
<td>F7512</td>
<td>1:250 / IF</td>
</tr>
<tr>
<td>Anti-Mouse RRX</td>
<td>Jackson ImmunoResearch, West Grove, PA, US</td>
<td>715-295-150</td>
<td>1:150 / IF</td>
</tr>
</tbody>
</table>
3.3.3. Data Analysis

Images were collected with an Olympus BX51 microscope equipped for widefield epifluorescence and a digital colour DP70 camera. Images were merged to determine co-localizations of: total HSPB5 and α-smooth muscle actin (rabbit polyclonal), pSer59-HSPB5 and α-smooth muscle actin (mouse monoclonal), pSer59-HSPB5 and KIN-2, and pSer59-HSPB5 and CD63 (mouse monoclonal). A z-series of images were also collected for each antibody pair using a Leica TCS-SP5 laser scanning confocal microscope and Leica LAS AF imaging software (Leica, Scarborough, ON, CA). Immunofluorescence was also assessed in the XZ and YZ axes using Image J software (Schindelin, et al., 2012).

3.4. Results

3.4.1. HSPB5 and Actin Co-localization

Cytoskeletal marker protein α-smooth muscle actin showed characteristic actin filament immunofluorescence detection in proliferating hTERThm cells. HSPB5 was also localized to these areas as seen by co-immunofluorescence staining (Fig. 3.1 A-C). Since HSPB5 co-localized with α-smooth muscle actin there is potential for HSPB5 to inhibit actin fibre depolymerization and protect actin filament organization (Wettstein, et al., 2012).

3.4.2. pSer59-HSPB5, Actin and Exosomal Protein Co-localizations

Focal adhesion marker protein KIN-2 was localized to membrane associated regions of proliferating hTERThm cells. The actin cytoskeletal marker protein α-smooth muscle actin also showed characteristic actin filament localization in proliferating hTERThm cells. pSer59-HSPB5
Figure 3.1. Immunofluorescence analysis of alpha (α)-smooth muscle actin and total HSPB5 protein localization in hTERThm human myometrial cells. Representative images are shown of (A) α-smooth muscle actin and total HSPB5 protein localization and (B) IgG controls of hTERThm cells. (C) Represents a z-series of images that were collected using a laser scanning confocal microscope. Immunofluorescence was also assessed in the XZ and YZ axes using Image J. An α-smooth muscle actin and total HSPB5 specific antisera were utilized. Mounting media containing DAPI was utilized to visualize nuclei. Actin, α-smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; HSPB5, total HSPB5; Merge, α-smooth muscle actin, HSPB5, and DAPI; Cntrl, IgG control. (A, B) Scale bar = 50μm. (C) Scale bar = 10 μm.
co-localized with both KIN-2, but not appreciably with α-smooth muscle actin filaments (Figs. 3.2 A-C, 3.3 A-C). Since the function of HSPB5 is regulated by phosphorylation the co-localization of pSer59-HSPB5 with KIN-2 must have some physiological significance (Wettstein, et al., 2012). The association of pSer59-HSPB5 with KIN-2 suggests that pSer59-HSPB5 might stabilize actin connections at cell-ECM and cell-cell interactions at focal adhesions which are important for the stability of the myometrium as a contractile unit. Actin filaments are also very active at focal adhesion locations, remodeling and anchoring the plasma membrane to the ECM and to other cells (Launay, et al., 2006).

Exosomal marker protein CD63 showed characteristic immunofluorescence detection in perinuclear regions in proliferating hTERThm cells. pSer59-HSPB5 was also co-localized to these areas (Fig. 3.4 A-C). This suggests that pSer59-HSPB5 may be localized in vesicles destined to be released as exosomes from hTERThm cells. Secretion of pSer59-HSPB5 from exosomes might explain the activation of the immune system at the initiation of labour (Shynlova, et al., 2008; Shynlova, et al., 2009a; Shynlova, et al., 2013; van Noort, et al., 2012a).

3.5. Discussion

The HSPB family of small stress proteins are adenosine triphosphate (ATP)-independent chaperones that also have important roles in cell processes such as cell death regulation and cytoskeletal organization (Acunzo, et al., 2012; Wettstein, et al., 2012). The localization of HSPB5, previously known as αB-crystallin, has been examined in vitro in various cell types, such as neurons, but has not been investigated in myometrial cells in vitro (Schmidt, et al., 2012). In particular, the specific immunolocalization of phosphorylated forms of HSPB5 have not been
**Figure 3.2.** Immunofluorescence analysis of alpha (α)-smooth muscle actin and serine-59 phosphorylated (pSer59) HSPB5 protein localization in hTERThm human myometrial cells. Representative images are shown of (A) α-smooth muscle actin and pSer59-HSPB5 protein localization and (B) IgG controls of hTERThm cells. (C) Represents a z-series of images that were collected using a laser scanning confocal microscope. Immunofluorescence was also assessed in the XZ and YZ axes using Image J. An α-smooth muscle actin and pSer59-HSPB5 specific antisera were utilized. Mounting media containing DAPI was utilized to visualize nuclei. Actin, α-smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; pSer59-HSPB5, serine-59 phosphorylated HSPB5; Merge, α-smooth muscle actin, pSer59-HSPB5, and DAPI; Cntrl, IgG control. (A, B) Scale bar = 50µm. (C) Scale bar = 10 µm.
Figure 3.3. Immunofluorescence analysis of kindlin-2 (KIN-2) and serine-59 phosphorylated (pSer59) HSPB5 protein localization in hTERThm human myometrial cells. Representative images are shown of (A) KIN-2 and pSer59-HSPB5 protein localization and (B) IgG controls of hTERThm cells. (C) Represents a z-series of images were collected using a laser scanning confocal microscope. Immunofluorescence was also assessed in the XZ and YZ axes using Image J. A KIN-2 and pSer59-HSPB5 specific antisera were utilized. Mounting media containing DAPI was utilized to visualize nuclei. KIN-2, kindlin-2; DAPI, 4',6-diamidino-2-phenylindole; pSer59-HSPB5, serine-59 phosphorylated HSPB5; Merge, KIN-2, pSer59-HSPB5, and DAPI; Cntrl, IgG control. (A, B) Scale bar = 50µm. (C) Scale bar = 10 µm.
**Figure 3.4.** Immunofluorescence analysis of CD63 and serine-59 phosphorylated (pSer59) HSPB5 protein localization in hTERThm human myometrial cells. Representative images are shown of (A) CD63 and pSer59-HSPB5 protein localization and (B) IgG controls of hTERThm cells. (C) Represents a z-series of images that were collected using a laser scanning confocal microscope. Immunofluorescence was also assessed in the XZ and YZ axes using Image J. A CD63 and pSer59-HSPB5 specific antisera were utilized. Nuclei were stained blue by a DAPI nuclear counterstain in the mounting media. Mounting media containing DAPI was utilized to visualize nuclei. DAPI, 4',6-diamidino-2-phenylindole; pSer59-HSPB5, serine-59 phosphorylated HSPB5; Merge, CD63, pSer59-HSPB5, and DAPI; Cntrl, IgG control. (A, B) Scale bar = 50µm. (C) Scale bar = 10 µm.
thoroughly investigated; however, phosphorylation of HSPB5 can determine whether or not actin filaments will be polymerizing or depolymerizing (Wieske, et al., 2001). Since HSPB5 has been shown to aid in actin filament dynamics in various cell types, it was hypothesized that HSPB5 would co-localize with actin in myometrial cells (Wieske, et al., 2001). Actin polymerization occurs at the plasma membrane, or plus end, of the filament and actin filaments attach to focal adhesions at the plasma membrane. Thus, co-immunolocalization of serine-59 phosphorylated (pSer59) HSPB5 with the focal adhesion protein kindlin-2 (KIN-2) was investigated.

HSPB5 can also activate the immune system and to do so it needs to be released from cells (van Noort, et al., 2012a). HSPB5 can be released from cells via exosomes (Sreekumar, et al., 2010). Thus it was also hypothesized that pSer59-HSPB5 would co-localize with an exosomal marker, CD63, in cultured myometrial cells.

3.5.1. Implications for HSPB5 and pSer59-HSPB5 Co-localization with Actin

sHSPs have been shown to bind partially denatured proteins and prevent their denaturation and aggregation, however recently they have been shown to also be involved in other processes like the modulation of the actin cytoskeleton and immune cell activation or attenuation (Arrigo, 2013; Horwitz, 1992; Jakob, et al., 1993; Mehlen, et al., 1997). Since the function of HSPB5 is regulated by phosphorylation the co-localization of phosphorylated and non-phosphorylated HSPB5 might have a significant physiological purpose. Phosphorylation of sHSPs decreases their chaperone function and leads to localization to different cellular compartments, for example, in neurons pSer59-HSPB5 is localized to the punctate regions along
the plasma membrane, whereas non-phosphorylated HSPB5 was dispersed throughout the cytoplasm of culture neuronal cells (Schmidt, et al., 2012). In the hTERThm human myometrial cell line similar localizations of pSer59-HSPB5 and non-phosphorylated HSPB5 were observed.

HSPB5 co-localized with α-smooth muscle actin, however pSer59-HSPB5 was virtually unassociated with α-smooth muscle actin and more localized to focal adhesion-like structures at the plasma membrane were actin filaments can end. Localization of pSer59-HSPB5 at focal adhesions was determined by co-immunolocalization of pSer59-HSPB5 with KIN-2. KIN-2 is a focal adhesion protein that resides near the plasma membrane and can be in close proximity to the minus end of actin filaments (Cattelino, et al., 1999). Focal adhesions are also thought to respond to tension, similar to that of pSer59-HSPB5, and focal adhesion signalling in the myometrium increases during mid- to late-pregnancy and terminated at labour due to focal adhesion stabilization (MacPhee & Lye, 2000; Ou, et al., 1997). Myometrial pSer59-HSPB5 protein expression was also dramatically increased during this period. The association of pSer59-HSPB5 with KIN-2 suggests that pSer59-HSPB5 may stabilize cell-ECM and cell-cell interactions at focal adhesions which are important for the stability of the myometrium as a contractile unit. Actin filaments are also very active at focal adhesion locations, remodeling and helping to anchor the plasma membrane to the ECM and to other cells (Launay, et al., 2006).

HSPB5 functions in similar ways to HSPB1 and it is thought that non-phosphorylated sHSPs, in this case specifically HSPB5, sequester monomeric G-actin and when phosphorylated these sHSPs release G-actin at the plasma membrane and allow for the polymerization of actin filaments at the minus end (Fig. 3.5; Doshi, et al., 2010; Piotrowicz & Levin, 1997; Wieske, et al., 2001). HSPB1 has been shown to inhibit actin polymerization by capping the plus end of actin
Figure 3.5. A potential signaling pathway regulating function of HSPB5. The small heat shock protein HSPB5 is phosphorylated in response to various contractile agonists in smooth muscle cells, specifically the myometrium. Such stimuli can include: neurotransmitters, cytokines, and growth factors. HSPB5 phosphorylation favors stabilization of F-actin filaments by undetermined molecular mechanisms. One possibility is that HSPB5 acts as capping proteins and phosphorylation dissociates the cap allowing polymerization to proceed. HSPB5 may also sequester G-actin and release it for polymerization following HSPB5 phosphorylation. Modified from Salinthone, et al. Pharmacology and Therapeutics, 119, 44-54, 2008.
Integrin

GPCR

Contractile Agonist

Kinase Cascade

Cytokines

Growth Factors

HSPB5

Talin/Kindlin-2

FAK

HSPB5

G Actin

Actinin
filaments and, therefore, participating in the regulation of actin assembly (Doshi, et al., 2010). This capping activity of HSPB1 protects the plus end and might favour the depolymerization of the minus end, allowing for growth of uncapped filaments (Doshi, et al., 2010). Slight conformational changes occur in phosphorylated sHSPs, like disulfide-linked dimerizations and oligomerizations, that might prevent actin and sHSPs from interacting and abolishing the capping ability of sHSPs, like HSPB1 (Mounier & Arrigo, 2002). This transformation could be required for HSPB5 at the time of labour and delivery where it is necessary for the myometrium to become a powerful contractile tissue to expel the fetus at term.

3.5.2. Implications for pSer59-HSPB5 Co-localization with CD63

Small heat shock proteins (sHSPs) in general do not contain specific signal sequences to induce their active secretion from cells; therefore, an alternative secretion-pathway via exosomes has been considered (van Noort, et al., 2012a). Exosomes are extracellular vesicles approximately 100nm in diameter and have been documented to contain HSP70 and HSP90 from cell types such as reticulocytes, human peripheral blood mononuclear cells, and some white blood cells (Harding, et al., 1983; Lancaster & Febbraio, 2005). Retinal epithelia cells have also been shown to secrete exosomes containing HSPB5 (Sreekumar, et al., 2010). Exosomes are identified by their membrane components since they are highly enriched with tetraspanin proteins like CD9, CD63, CD81, and CD82; however, late endosomes can also contain tetraspanins since exosomal vesicles are thought to be formed by inward budding in the endocytic pathway (Fig. 3.6 A-G; Kobayashi, et al., 2000; Thery, et al., 2002; Trajkovic, et al., 2008). This makes the absolute identification and characterization of exosomes difficult and, therefore, techniques such as electron microscopy are typically performed to confirm the
Figure 3.6. Secretion of HSPB5 by an exosome pathway. Without any known signal sequence, exosomes have been proposed as the secretion pathway for small heat shock proteins, specifically (A) HSPB5 from myometrial cells. These exosomal vesicles are formed by (A) inward budding of large endosomal structures inside the donor cell. These vesicles then accumulate in structures called (B) multivesicular bodies. These multivesicular bodies then (C) fuse with the plasma membrane, an event which is often inducible, and the vesicle release its contents into the extracellular space. The 40–100 nm vesicles contained in the multivesicular body have now become (D) exosomes. A recipient cell can take up exosomes and their contents either by (E) direct fusion with the plasma membrane or by (F) endocytosis. In the recipient cells the exosomes either (G) release their contents or (H) become part of the endosomal compartment. Modified from van Noort, et al. The International Journal of Biochemistry & Cell Biology, 44, 1670 – 1679, 2012.
existence of exosomes. Since sHSPs have been shown to play a role in activation and repression of the immune system which has a key role in initiation of term and pre-term labour the secretion of HSPB5 via exosomes could be a possible mechanism of release from the myometrial cells to initiate immune activation and labour.

The investigation of pSer59-HSPB5 and CD63 co-localization to determine the existence of exosomes in myometrial cells was conducted. pSer59-HSPB5 and CD63 together were seen in perinuclear areas of human myometrial cells as punctate staining which suggests that they may be closely associated with one another and that pSer59-HSPB5 has the potential to be found within vesicles coated with CD63. It cannot be ruled out, however, that pSer59-HSPB5 co-localization with CD63 reflects a pool of HSPB5 destined for degradation. Importantly, vascular smooth muscle cells, cultured B-cells, and other cell types have been documented to secrete sHSPs when stressed, but this has never been documented in myometrial cells (Clayton, et al., 2005; Martin-Ventura, et al., 2004). In fact, exosomes have not yet been isolated from myometrial cells. Thus, the identification of exosomes from myometrial cells needs to be confirmed by ultracentrifugation and electron microscopy.

HSPB5 has been shown previously, in vitro, to induce an interleukin-10 (IL-10) regulatory immune response in macrophages at low concentrations (under 50ug/mL) while at higher concentrations (50ug/mL to 200ug/mL) it can increase T-cell production of IFN-γ, which activates macrophages to produce tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine (Bsibsi, et al., 2013; van Noort, et al., 2012; van Noort, et al., 2012a). In the myometrium exosomes containing pSer59-HSPB5 could be responsible for activating the immune system during labour since pregnancy is, in part, an anti-inflammatory condition and a
shift in this condition to a pro-inflammatory state by unknown processes leads to parturition (Knackstedt, et al., 2003). In the early stages of pregnancy, during implantation, pro-inflammatory factors and cytokines, like interleukin-8 (IL-8), are high to allow for wound repair; however, they decrease as pregnancy progresses and will increase again at parturition (Mor, 2008). As pregnancy progresses there is an interleukin-10 (IL-10) immunosuppressive, anti-inflammatory, cytokine profile, including high levels of transforming growth factor-beta (TGF-β; Challis, et al., 2009). During this point of pregnancy leading up to labour, there is a localized inflammatory response which promotes cervical ripening, myometrial activation and contraction, membrane activation, and placental rejection (Romero, et al., 2006). The pro-inflammatory environment causes uterine contraction by inflammatory leukocyte infiltration into the myometrium at normal term labour and potentially pre-term birth as well (Thomson, et al., 1999). The increase in pSer59-HSPB5 during the days preceding labour in the rat, coupled with HSPB5's ability to maintain different immunological environments based on concentration as well as the relative proximity of pSer59-HSPB5in the cytoplasm and potentially exosomes suggest that pSer59-HSPB5 could aid in the onset of labour (Bsibsi, et al., 2013; van Noort, et al., 2012; van Noort, et al., 2012a).
Chapter 4.

General Discussion

Currently, the detailed signalling mechanisms behind preterm labour and even term labour are poorly understood; however, a consensus has been reached that both mechanical and endocrine factors play a role in preterm and term birth. The myometrium plays an important role in maintaining quiescence throughout pregnancy and consists of two distinct muscle layers of different embryological origins called: the outer longitudinal muscle layer and the inner circular muscle layer (Chow & Marshall, 1981). These are separated by a vascular plexus (Chow & Marshall, 1981). It is costly, time consuming, and ethically not feasible to use the human uterus as a primary experimental model, thus the rat uterus was used. The rat uterus has the same three uterine layers as the human uterus, as well as similar arrangements in the myometrial muscle layer (Chow & Marshall, 1981; Sherwood, 2010). To understand and subsequently treat preterm labour effectively the biochemical processes regulating term labour with relation to the differentiation and contraction of the myometrium must be understood.

The costs associated with preterm deliveries and the subsequent health complications are vast. On average in Canada the cost of a child born prematurely (less than 37 weeks gestation) can cost up to ninety times more than a child born at term (37 weeks of gestation or later) and the length of hospital stay for a pre-mature child can be upwards of forty times as long compared to a single birth (Lim, et al., 2009). In the case of multiple births, these numbers are only slightly increased (Lim, et al., 2009). Preterm birth rates in Canada have remained stable since 2006; however, without an adequate predictor of impending preterm labour many
of the available treatments for premature infants are not as effective as they could be if labour could be predicted weeks in advance rather than hours (Canadian Institute for Health Information, 2013). Therefore, knowledge of the expression of potential regulators of myometrial function such as HSPB5, would be useful to help determine if a child will be born prematurely.

Small heat shock proteins (sHSPs) in the HSPB family are a group of ten small molecular weight proteins, between 12 and 43 kilodaltons (kDa) that are induced by many physiological stressors, such as heat shock. Mammalian HSPB proteins are labelled HSPB1 to B10 and arrange themselves in large homomeric or heteromeric complexes of about 40 to 50 subunits; however, subunit assemblies can be composed of as little as 2 subunits (Haslbeck, et al., 2005; Kappe, et al., 2003). A number of sHSPs, including HSPB1, HSPB6 and HSPB8, have already been investigated in the rat myometrium during pregnancy, however the expression of HSPB5, including its phosphorylated forms, is unknown in the myometrium during pregnancy (Marsh, et al., 2015; White, et al., 2005). It is important to determine the expression pattern of HSPB5 throughout gestation, since it plays a role in cytoskeletal rearrangements as well as immune system activation, which are key players in the onset of labour.

The expression pattern of total, as well as serine-59 phosphorylated (pSer59) HSPB5 was investigated in the rat myometrium throughout gestation. Furthermore, the effects of stretch on total HSPB5 and pSer59-HSPB5, were investigated to determine whether mechanical stimulation plays a role in its regulation, like that of HSPB1 (White & MacPhee, 2011). The spatial expression of total HSPB5 protein in a human myometrial cell line and its potential co-localization with α-smooth muscle actin was also investigated since sHSPs may play a role in the
regulation of cytoskeletal filaments in the myometrium and this is important for contraction during labour. Furthermore, the potential co-localization of pSer59-HSPB5 with α-smooth muscle actin and the focal adhesion protein kindlin-2 (KIN-2) was also examined in the myometrial cell line. An exosomal marker, CD63, was also examined for co-localization with pSer59-HSPB5 to suggest a secretion mechanism via exosomes for immune system activation preceding labour, since HSPB5 has also been shown to activate and/or attenuate the immune system.

4.1. HSPB5 Expression and Potential Role with the Actin Cytoskeleton

The first objective of this thesis was to determine the protein expression patterns of total HSPB5 and pSer59-HSPB5 in the myometrium during pregnancy. During the synthetic and contractile phases of pregnancy there are marked cytoskeletal rearrangements, like actin reorganization at focal adhesions, necessary for future generation of powerful contractions during the labour phase (MacPhee & Lye, 2000; Shynlova, et al., 2007a). HSPB5 protein expression increased mid- to late-pregnancy, whereas pSer59-HSPB5 was increased during late-pregnancy and labour when the majority of cytoskeletal rearrangement in the myometrium during pregnancy occurs. Phosphorylation of sHSPs has been shown to alter the function of sHSP proteins, for example, Launay and colleagues (2006) demonstrated that myocytes treated with cytochalasin D, which disorganizes actin microfilaments, triggered the phosphorylation of HSPB5 at serine-59 (Launay, et al., 2006). This phosphorylation allowed HSPB5 to associate and bind with actin filaments (Launay, et al., 2006). Wang and Spector (1996) showed similar results in the eye lens, demonstrating that actin microfilaments were protected by non-phosphorylated HSPB5 when cells were exposed to cytochalasin D (Wang & Spector, 1996).
They also reported that phosphorylation of HSPB5 decreased their protective effect in the lens; however in the absence of cytochalasin D both forms of HSPB5 protein showed protective qualities (Wang & Spector, 1996). Although there are some conflicting views above on phosphorylated HSPB5-actin dynamics, it is agreed upon that HSPB5 can bind actin in *in vitro* cultured cells (Gopalakrishnan & Takemoto, 1992). Wang and Spector (1996) also suggest that phosphorylation of HSPB5 may contribute to changes in actin structure that are observed during cytoskeletal remodeling which we know occurs in late-pregnancy and labour at the time when significant pSer59-HSPB5 protein expression was observed (Wang & Spector, 1996).

From a cellular standpoint, a human myometrial cell line (hTERThm) and laser scanning confocal microscope with Image J analysis software was used to determine the cellular associations of total HSPB5 and pSer59-HSPB5 with the cytoskeletal protein actin, the focal adhesion protein kindlin-2 (KIN-2), and the exosomal marker and tetraspanin CD63. HSPB5 associated with actin in the hTERThm cell line. Based on the literature, this suggests a role in the sequestering of actin monomers throughout gestation. During and colleagues (2007) showed that non-phosphorylated HSPB1 can sequester monomeric actin in human neutrophils and HeLa cells infected with *Listeria monocytogenes*, blocking actin-based motility (During, et al., 2007). Since HSPB1 and HSPB5 have similar actions *in vitro* and *in vivo*, it is possible that HSPB5 is capable of sequestering actin monomers in myometrial cells during the synthetic phase where the myometrium is preparing for the upcoming contractile phase in which cytoskeletal reorganization will occur to make the uterus a powerful contractile organ to expel the fetus during labour. Interestingly, During and colleagues (2007) showed that phosphorylated HSPB1 lost the ability to bind actin monomers and, therefore, the authors
proposed that actin-based motility is controlled through phosphorylation and dephosphorylation of HSPB1, shuttling actin monomers to sites of actin polymerization (During, et al., 2007).

During and colleagues (2007) results could explain why an association between pSer59-HSPB5 and actin filaments in the cytosol of hTERThm cells was not observed, but why associations were found at the end of actin filaments in membrane associated regions of the cell where actin polymerization occurs. pSer59-HSPB5 did associate with the focal adhesion protein KIN-2 at focal adhesion structures which contain the minus ends of actin filaments. These results support the notion that pSer59-HSPB5 functions in actin polymerization at these focal adhesion sites. They also correlate with results that unphosphorylated mouse and chicken HSPB1 inhibit actin polymerization in vitro, but phosphorylation of HSPB1 favours the formation of filamentous- or F-actin and stabilizes focal adhesions during stress (Benndorf, et al., 1994; Lavoie, et al., 1993; Lavoie, et al., 1993a; Miron, et al., 1991; Schneider, et al., 1998). The formation of F-actin is important since G-to-F-actin transformation is required for subsequent contraction (Jones, et al., 1999; Mehta & Gunst, 1999). Overall, the findings in this thesis indicate that phosphorylation of HSPB5 on serine-59 could control actin cytoskeletal dynamics in the myometrium during late-pregnancy and labour.

4.2. Stretch Regulates HSPB5 Expression

The effect of stretch, or uterine distension, on the expression of HSPB5 and pSer59-HSPB5 protein was conducted using a unilaterally pregnant rat model. Using immunoblot analysis it was shown that both HSPB5 and pSer59-HSPB5 protein expression were up-regulated
at day (d) 19 of gestation and d23 (labour) in the gravid horns when compared to non-gravid horns. This demonstrated that HSPB5 and pSer59-HSPB5 expression were induced by uterine distension during late-pregnancy and at labour. The results correlate with previous findings in the myometrium. Although endocrinological factors may play a role in HSPB1 expression throughout gestation, mechanical stretch of the myometrium in the absence of pregnancy hormones was shown to induce HSPB1 expression in ovariectomized non-pregnant rat myometrium distended with laminaria tents compared with empty horns (White & MacPhee, 2011). Marsh and colleagues (2015) also showed another sHSP, HSPB8, could be induced by mechanical stretch of the myometrium in a unilaterally pregnant rat model at d15 of gestation (Marsh, et al., 2015). Interestingly, other smooth muscle organs have been shown to exhibit increased expression of sHSPs in response to mechanical stretch (Batts, et al., 2006). For example, HSPB6 protein expression was higher due to mechanical stress in partially obstructed rat bladders when compared to non-obstructed animals suggesting that mechanical stress can increase sHSPs in various types of smooth muscle organs (Batts, et al., 2006).

**4.3. Potential Transport of HSPB5 by Exosomes and Role in Immune Activation**

The exosomal marker and tetraspanin CD63 was also shown to be co-localized with pSer59-HSPB5 in the hTERThm cell line, which suggests that pSer59-HSPB5 could be secreted via exosomes into the extracellular space and eventually into the blood stream (Caby, et al., 2005). The process of exosomal-mediated sHSP secretion occurs in other cell types, like vascular smooth muscle cells, retinal pigment cells, and have recently been implicated in the myometrium (Cretoiu, et al., 2013; Liao, et al., 2000; Martin-Ventura, et al., 2004; Sreekumar, et al., 2010). It has also been shown that the phagocytic uptake of exosomes from cultured
fibroblast cells by macrophages will result in the release of granular contents and the induction of innate immune responses (Bhat & Sharma, 1999; Feng, et al., 2010; van Noort, et al., 2010; Wu, et al., 2001). It is important to note that multiple investigators used HSP70 as an exosomal marker and that exosomes are known to contain various sHSPs, further supporting the notion that HSPB5 could be one of these sHSPs (Feng, et al., 2010). Since these studies demonstrated that it is possible to activate the immune system via phagocytosis of exosomes, it is possible that myometrium-produced exosomes containing HSPB5 could function in a similar way during late pregnancy and labour. Activation of the immune system at labour promotes a localized inflammatory response that stimulates cervical ripening, myometrial activation and contraction, membrane activation, and placental rejection (Romero, et al., 2006). The pro-inflammatory environment causes uterine contraction and the infiltration of inflammatory leukocytes into the myometrium at normal term labour (Romero, et al., 2006; Thomson, et al., 1999). The inflammatory cytokine interleukin-1β (IL-1β) has been shown in myometrial cells to drive the production of prostaglandins by the myometrium, which increases myometrial contractility (Todd, et al., 1996).

In summary, my results show that HSPB5 protein expression is markedly increased from mid-pregnancy to labour and that uterine distension can regulate the expression of both total HSPB5 and pSer59-HSPB5 protein. My results also show that HSPB5 can co-localize with actin filaments and, that the pSer59-HSPB5 form can co-localize with focal adhesion proteins and exosomal markers. The results suggest a possible role for HSPB5 in regulating actin filament dynamics and immune activation in the myometrium during late-pregnancy and labour.
Chapter 5.

Major Conclusions & Future Directions

5.1. Major Conclusions

Given its potential as an immune system activator and cytoskeletal associated protein, it was hypothesized that total HSPB5 and pSer59-HSPB5 protein expression in rat myometrium would be elevated during mid- to late-pregnancy when these processes occur during the contractile and labour phases of myometrial differentiation. Thus, the objectives of this research and the subsequent conclusions are:

Objective 1. To determine the spatio-temporal expression of total and pSer59-HSPB5 protein in rat myometrial tissue throughout gestation.

HSPB5 protein expression in the myometrium increased mid- to late-pregnancy, whereas pSer59-HSPB5 was increased during late-pregnancy and labour when the majority of cytoskeletal rearrangement in the myometrium occurs during pregnancy. Both HSPB5 and pSer59-HSPB5 were detected in the cytoplasm of myocytes within both uterine muscle layers.

Objective 2. To determine the effects of stretch on total and pSer59-HSPB5 protein expression in a unilaterally pregnant rat model.

Both total and pSer59-HSPB5 protein expression were markedly increased in myometrial lysates from gravid horns compared to non-gravid horns at d19 and d23 of gestation. Immunofluorescence analysis supported the immunoblot results as immunodetection of total and pSer59-HSPB5 was also markedly increased in the cytoplasm of myometrial cells from
gravid horns at d19 and d23, in both muscle layers, compared to contralateral non-gravid uterine horns.

**Objective 3.** To determine associations of total and pSer59-HSPB5 protein with cytoskeletal filaments, as well as the exosomal marker CD63 in a human myometrial cell line.

Total HSPB5 co-localized with α-smooth muscle actin filaments while pSer59-HSPB5 co-localized with the focal adhesion protein Kindlin-2 at the ends of actin filaments near the plasma membrane. pSer59-HSPB5 did not appreciably co-localize with α-smooth muscle actin filaments. The exosomal marker protein CD63 showed characteristic immunofluorescence detection in perinuclear regions in proliferating hTERThm cells and pSer59-HSPB5 was also co-localized to these areas.

**5.2. Regulation of HSPB5 Expression**

Further investigation into the endocrinological and mechanical regulation of HSPB5 gene expression is required to determine if mechanical stretch is the predominant regulator of expression or if endocrinological factors, like 17β-estradiol, may play a significant role in regulation of expression (White & MacPhee, 2011). Interestingly, 17β-estradiol circulating levels increase dramatically just prior to labour (Challis, et al., 2000). This coincides in part with the dramatic increase in pSer59-HSPB5 and, therefore, 17β-estradiol could play a role in regulating a signal transduction pathway that induces phosphorylation of HSPB5 prior to labour. Similarly to White and MacPhee (2011), expression of HSPB5 as well as pSer59-HSPB5, should be examined in non-pregnant, ovariectomized rats that contain a cervical dilator in one horn while the contralateral horn remains empty. It was found that there is greater HSPB1
expression in uterine horns that contain a cervical dilator in non-pregnant, ovariectomized rats when compared to the untouched horn (White & MacPhee, 2011). This would allow the effects of stretch to be studied in the absence of ovarian endocrinological factors and shed light on the regulatory mechanisms controlling HSPB5 expression in vivo.

5.3. HSPB5 and Potential Exosome-Mediated Secretion

Further investigation on the capabilities of myometrial cells to secrete exosomes, both in vitro and in vivo would be ideal to determine if pSer59-HSPB5 is contained within them. It has already been reported that HSPB5 is contained in exosomes secreted by other cell types so it is possible myometrial cells could secrete them as well (Sreekumar, et al., 2010). The characterization of these vesicles from myometrial cells could be accomplished in several ways. Traditionally, ultracentrifugation utilizing a sucrose gradient has been the standard for exosome isolation; however, these procedures can take upwards of 20 hours (Gangalum, et al., 2011). Life Technologies (Burlington, ON, CA) and other biotechnology companies have exosome isolation kits available which are gaining popularity since they contain all reagents needed to isolate exosomes and the protocol can be performed in a shorter time frame than traditional ultracentrifugation. Both techniques should be used in this case to confirm exosomes could be produced by myometrial cells in vitro. It is critical to determine whether or not exosomes containing HSPB5 can be released from myometrial cells since HSPB5 has the ability to enhance NF-κB (nuclear factor kappaB) signalling, which increases pro-inflammatory cytokines as well as aids in the recruitment of leukocytes (Dieterich, et al., 2013). NF-κB has also been implicated in regulating the expression of pro-inflammatory cytokines from the myometrium which we know is part of initiating labour, therefore if pSer59-HSPB5 can be released from myometrial cells in
exosomes, they have the potential to recruit leukocytes to the myometrium as well as enhance NF-κB signalling within myometrial cells, increasing pro-inflammatory mediators (Cookson & Chapman, 2010).

5.4. Requirement of HSPB5 for Pro-Inflammatory Response in Myometrial Cells

Human myometrial cells should also be cultured in the absence or presence of different concentrations of lipopolysaccharide (LPS), a bacterial constituent that is known to induce strong pro-inflammatory responses that can lead to pre-term birth (Shynlova, et al., 2013a). Consequently, the levels of total and pSer59-HSPB5 protein in LPS-treated cells compared to non-treated cells should be examined by immunoblot analysis. Conditioned media should be collected and examined for cytokine production by myometrial cells in the presence or absence of LPS to verify pro-inflammatory activation. The characterization of cytokines released into this media will help determine whether or not myometrial cells can secrete the inflammatory cytokines associated with labour. If LPS exposure increases the levels of total and pSer59-HSPB5 protein, as well as the expression of inflammatory cytokines the next step would be to knockdown HSPB5 expression in human myometrial cells via siRNA, for example, and then determine if the cytokine profile in the media is altered upon LPS exposure. This would determine if HSPB5 played a role in the activation of cytokine production and their active secretion or if it could be a response to LPS exposure alone.

5.5. HSPB5 as an Impending Labour Biomarker

_in vivo_ studies are also required to determine whether or not HSPB5 can be used as a biomarker for impending labour. Since pSer59-HSPB5 levels are highest one day pre-labour in
the rat, which equates to approximately twelve days prior to labour in the human female, it
would be advantageous to know whether or not these proteins can be detected in the serum of
pregnant women. It has already been demonstrated that small heat shock proteins (sHSPs) can
be detected in extracellular fluids, like plasma (Cherneva, et al., 2012), so it would be
advantageous to know if higher levels could be detected before the onset of preterm and term
labour. Thus a longitudinal exploratory study should be conducted examining serum and
plasma levels of HSPB5 in pregnant women throughout the second and third trimester of
pregnancy. It might also be prudent to include women carrying multiples, since they are at
higher risk for going into preterm labour, to determine if HSPB5 in blood serum can in fact be
used to accurately predict impending labour. The samples could be analysed using
commercially available enzyme-linked immunosorbent assay kits. In summary, these future
investigations could lead to potential advancements in neonatal medicine as well as the survival
of pre-mature infants.
Bibliography


Canadian Institute for Health Information, 2013. *Highlights of 2011–2012 selected indicators describing the birthing process in canada*, Ottawa, Ont: Canadian Institute for Health Information.


Piccinni, M. et al., 1995. Progesterone favours the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *The Journal of Immunology*, 155, pp. 128-133.


Yang, J., Bridges, K., Chen, K. & Liu, A., 2008. Riluzole increases the amount of latent HSF1 for an amplified heat shock response and cytoprotection. *PLOS One*, 3(8), pp. e2864-e2864.
