Matrix Metalloproteinase Expression and Regulation During Pregnancy, Term Labour, and Postpartum

by

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ABSTRACT

It is widely accepted that pregnancy, spontaneous term labour (TL), and postpartum (PP) involution is associated with changes in the cellular and extracellular composition of the uterus. These changes involve metalloproteinases (MMPs) secretion by myometrial smooth muscle cells and infiltrating leukocytes. MMP activation results in collagenolysis, modulation of cellular behaviour and barrier function. I hypothesize that during late gestation, the expression and activity of myometrial MMPs and their tissue inhibitors (TIMPs) increase in preparation for TL, and are regulated by mechanical stretch. The current in vivo study demonstrated that gene and protein expression of MMP-7 and MMP-11 were upregulated during TL, suggesting labour promotion; whereas other MMPs (2, 3, 8, 9, 10, 12) were induced only during the early PP period. The sources of myometrial MMPs during TL and PP include myocytes and resident leukocytes. In vitro studies did not support the regulation of myometrial MMP expression by mechanical stretch.
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CONTRIBUTORS

The following people contributed to the results presented in this thesis:

Anna Dorogin assisted in tissue collection, immunohistochemical analysis, and *in situ* zymography for the bilaterally pregnant rat model.

Tissue collection and RT-qPCR analysis was performed in collaboration with Dr. Oksana Shynlova with the assistance of Anna Dorogin.

Tali Farine assisted with enzymatic isolation and sample collection for the *in vitro* model of mechanical stretch using primary human myometrial cells.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BM</td>
<td>Basement Membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAP</td>
<td>Contraction-Associated Protein</td>
</tr>
<tr>
<td>CBD</td>
<td>Collagen Binding Domain</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C (motif) Chemokine Ligand</td>
</tr>
<tr>
<td>CM</td>
<td>Circular Muscle Layer</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C (motif) Chemokine Ligand</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Double Deionized Water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein Diacetate</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational Day</td>
</tr>
<tr>
<td>HBSS++</td>
<td>Hank’s Buffered Salt Solution (with Calcium and Magnesium)</td>
</tr>
<tr>
<td>HBSS--</td>
<td>Hank’s Buffered Salt Solution (without Calcium and Magnesium)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunofluorescent Cytochemistry</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
<tr>
<td>IGFR</td>
<td>Insulin-like Growth Factor Receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITS-A</td>
<td>Insulin-Transferrin-Selenium-Sodium Pyruvate Supplement</td>
</tr>
<tr>
<td>LM</td>
<td>Longitudinal Muscle Layer</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil Elastase</td>
</tr>
<tr>
<td>NSM</td>
<td>Non-Stretch-Conditioned Media</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>P4</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline (without Calcium and Magnesium)</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate-Buffered Saline with Tween-20</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>Platelet-Derived Growth Factor Alpha</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PP</td>
<td>Postpartum</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl Isomerase A (Cyclophilin A)</td>
</tr>
<tr>
<td>PTL</td>
<td>Preterm Labour</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real Time Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SCM</td>
<td>Stretch-Conditioned Media</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth Muscle Actin</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA Box-binding Protein</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris Buffer Solution with 0.02% Tween-20</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-Beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-Alpha</td>
</tr>
<tr>
<td>TL</td>
<td>Term Labour</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot Analysis</td>
</tr>
<tr>
<td>ZBF</td>
<td>Zinc-Buffered Fixative</td>
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</table>
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW
1.1 Overview: Preterm and Term Labour

Preterm labour (PTL) refers to premature delivery before 37 weeks of gestation [1]. PTL is a significant clinical problem for modern obstetricians because it occurs in 5-18% of all pregnancies and is a major contributor of perinatal mortality (70%) and morbidity (75%). Preterm infants are also 40 times more likely to die than term infants, and are at an increased risk of developing cerebral palsy, blindness, deafness and respiratory illness [1-6]. However, it is difficult to define the exact causes of spontaneous PTL as many factors – such as uterine infection, premature rupture of membranes (PROM), and chronic chorioamnionitis – can contribute to the early onset of labour [7, 8]. Several studies suggest that normal term labour (TL) in humans is an inflammatory process and infection/inflammation has been seen as one of the contributing factors of PTL [9-12].

TL is a highly orchestrated process involving coordinated events in multiple tissues, which include the myometrium, decidua, fetal membranes, and cervix [13]. However past attention was mainly given to study the mechanisms that regulate the myometrium and the cervix in preparation for labour [9, 14]. Nearing the end of pregnancy, the cervix begins to soften and dilate, while the myometrium progresses to a contractile phenotype. Interestingly, both of these coordinated changes are associated with remodeling of the tissue environment, and are regulated by the endocrine and immune systems [14-17]. Current knowledge pertaining to the underlying mechanisms that regulate pregnancy associates pro-inflammatory pathways with the switch between myometrial quiescence and myometrial activation [18]. Particularly during pregnancy, myometrial quiescence is maintained – primarily by increased levels of circulating progesterone – until the switch to a contractile phenotype is required for labour initiation. Progesterone receptor signaling suppresses the expression of pro-inflammatory mediators such as chemokine CCL2,
(the major chemoattractant for monocytes), contraction-associated proteins (CAPs) such as connexin-43 (Cx43), and prostaglandin receptors (PGRs) [14, 19-23].

Inflammatory responses, which include leukocyte infiltration into uterine tissues, can spontaneously initiate processes like cervical ripening, rupture of fetal membranes, decidual and myometrial activation. Numerous studies have reported not only increases in pro-inflammatory mediators, but especially the influx of macrophages and neutrophils into several uterine compartments before and during TL [6, 10, 11, 16, 24-26]. Additionally, intrauterine infections can lead to PTL, suggesting a possible causality link between inflammation and spontaneous PTL [13, 27]. Hamilton et al. further confirmed this link, through their report of macrophages in the human and rat decidua – the pregnant endometrial layer acting as the fetal-maternal interphase – during TL and PTL, also providing evidence to suggest decidual inflammation precedes labour [26]. Interestingly, Shynlova et al. observed several differences regarding leukocyte infiltration in the myometrium and decidua between PTL and TL. During TL macrophage infiltration precedes neutrophil infiltration, whereas neutrophil infiltration specifically increased during infection-induced PTL and remained elevated PP [20, 28]. These findings indicate that particularly the myometrium, decidua, and cervix are immunoregulatory tissues capable of recruiting peripheral leukocytes through the release of different inflammatory mediators. The discovery that uterine inflammation is associated with labour onset suggests that targeting these inflammatory pathways or pro-inflammatory mediators could represent a potential approach to prevent PTL. Thus further investigation into the underlying mechanisms that regulate the labouring process may reveal more specific targets to efficiently address spontaneous PTL.
1.2 Overview: Postpartum Uterine Involution

Following parturition and shedding of the placenta, the uterus undergoes drastic physiological tissue alterations similar to wound healing, with the aim to return to a pre-pregnant state (ready for future pregnancies). This process is termed postpartum (PP) uterine involution, which involves rapid and extensive remodeling of the extracellular matrix (ECM), resulting in a dramatic reduction (at least 10-fold) in the size of the uterus in the PP period [17, 29-32]. The myometrium is the main component that undergoes reversible modification to accommodate the enlarged uterus during pregnancy, making it one of the primary sites of tissue rearrangement during uterine involution. This myometrial transition includes a reduction in number and size of the smooth muscles cells (SMCs), along with degradation of ECM components – especially the collagen and elastin deposited during pregnancy [29, 33-36]. Nishinaka et al. have reported the presence of myofibroblastic interstitial cells surrounding myometrial SMCs during the PP period for removal of degraded collagen and elastic fibers by macrophage phagocytosis [34]. Additionally Hsu et al. observed increased myometrial autophagy – degradation of cytoplasmic organelles using lysosomal machinery – was associated with reduction in SMC size [33]. Shynlova et al. also detected an increase in the expression of genes encoding insulin-like growth factor (IGF)-1, IGF binding protein (BP)-5 and IGF-1 receptor (IGF1R), associated with regeneration of uterine tissues [37]. These findings indicate that the uterine smooth muscle undergoes physiological adaptations and phenotypic differentiation during pregnancy and is followed by tissue regeneration during the involution phase.

PP involution is a complex biological process involving interactions between a variety of cells – including fibroblasts, myofibroblasts, SMCs and immune cells – and is mediated by hormones, growth factors, cytokines and chemokines [29]. In concordance, Shynlova et al.
detected high levels of macrophage infiltration during the PP period localized to the endometrium-
myometrium junction and this influx was tightly regulated by a group of chemokines including
CCL2 [22, 38]. Interestingly, chemokines released by myometrial cells not only recruit peripheral
leukocytes, but can also induce cellular proliferation, differentiation, apoptosis, and angiogenesis –
all biological processes necessary for uterine tissue rearrangement during involution [29, 39].
These findings suggest that the maternal immune system not only regulates TL but also regulates
PP uterine involution [20, 40].

1.3 The Myometrium during Pregnancy, Labour and Postpartum

1.3.1 Myometrial smooth muscle phenotypes during pregnancy

In the rodent, the myometrium is organized into two main layers: 1) the outer longitudinal
muscle layer that is oriented along the axis of the uterine horn, and 2) the sub-endometrial circular
muscle layer that is perpendicular to the uterine axis [41, 42]. The outer layer has especially been
seen to be actively associated with parturition, which suggests its primary involvement in
myometrial contractility [43, 44]. SMCs make up approximately half of the cells in the
myometrium, and are known to self-secrete basement membrane (BM) components that
encapsulate and surround each individual SMC [45-47]. These uterine SMCs physiologically
adapt to pregnancy and undergo, near term, a drastic transition from a fairly quiescent state to an
activated contractile state. In particular, the sequential phenotypic transition has been described
as follows: 1) the early proliferative phase is characterized by upregulated IGF-1 and anti-
apoptotic Bcl-2 signaling (SMC hyperplasia); 2) an intermediate synthetic phase, involving
increase in cell size (SMC hypertrophy) and induction of interstitial matrix synthesis; 3) a late
contractile phase fully committed to labour involving SMCs working as a syncytium due to Cx43
induction, with increased expression of contractile machinery proteins, and 4) PP involution following parturition, which returns the myometrium back to a pre-pregnant state (Figure 1.1) [29, 35, 48-50].

This phenotypic switch to the contractile/labour phase involves expression of well-studied pro-inflammatory mediators and CAPs [19, 21, 22]. Several studies have addressed the upregulated expression of CAPs, such as the gap junction protein Cx43 and PGRs, within the myometrium to promote labour [21, 51, 52]. These protein changes not only are necessary to increase SMC excitability and connectivity, but also to promote myometrial contractility [21, 51, 52]. Moreover, these myometrial changes are regulated by both hormonal (estrogen and progesterone) and mechanical stimuli associated with late gestation [29, 49, 53]. These findings advocate the importance of myometrial activation in labour initiation and further elucidation of the underlying mechanisms will assist in developing new therapies for PTL.
Figure 1.1 Myometrial SMC phenotypes during pregnancy, labour and postpartum.
The myometrial SMCs undergo a programmed transition between distinct phenotypes that include: 1) an early proliferative phase associated with SMC hyperplasia; 2) an intermediate synthetic phase involving SMC hypertrophy; 3) a late contractile phenotype associated with the upregulation of CAPs that will commit SMCs to synchronous labour contractions; and 4) a phase of postpartum involution involving expression of genes associated with wound repair, cell apoptosis, ECM remodeling, and tissue regeneration, which allows the uterus to resume a pre-pregnant state. Reproduced with permission from Shynlova et al.[29].
1.3.2 Extracellular matrix modifications in the myometrium during pregnancy

The ECM is an important component of all tissues, providing a stable three-dimensional environment for the growth and function of parenchymal cells. The ECM is a non-cellular structure that has a unique tissue-specific composition to support biological function and encompasses both cellular – fibroblasts that secrete matrix proteins – and non-cellular components [54, 55]. The ECM can be further divided into two types, the interstitial connective tissue matrix that provides structural support for tissues, and the basal lamina – secreted by epithelial cells and separates them from the surrounding stroma [54]. Mammalian ECM is composed of approximately 300 proteins (the core matrisome) and includes the structural proteins fibrillar collagens (type I, II and III collagen) and elastin, substrate adhesion molecules: fibronectin, laminin and collagen IV, as well as numerous glycoproteins, and proteoglycans. Additionally, the components of the ECM constantly interact with their integrin cell-surface receptors to regulate and maintain tissue integrity [35, 54]. The ECM is also constantly remodeled to maintain tissue homeostasis – such as cell proliferation, apoptosis or differentiation [56].

It is well understood that extensive remodeling of the uterine ECM throughout the course of pregnancy transpires to accommodate fetal growth and promote the SMC transition from a quiescent to a contractile phenotype (Figure 1.1) [35, 57-59]. One of the early reports of myometrial remodeling by Granstrom et al., reported extensive collagenolytic activity in the uterine body – similar to the cervical ripening process – following the onset of labour [60]. The myometrium particularly exhibits changes in the expression of ECM components as a result of hormonal and mechanical stimuli throughout pregnancy [35]. Garfield and Hayashi suggested that the degradation of the myometrial ECM facilitates the formation of gap junctions between SMCs to increase cellular connectivity for coordinated uterine contractions [61]. It is indicative that
activation of myometrial ECM remodeling is also an essential physiological process to maintain synchronized labour contractions.

Interestingly, changes in gene and protein expression of myometrial ECM components can be induced by biological mechanical stretch of the uterus in vivo using a well-established unilaterally pregnant rat model of gestation. Shynlova et al. reported significant alterations in the expression of multiple myometrial ECM genes, specifically a coordinated decrease in gene expression of fibrillar proteins (i.e. type I collagen) and an increase in elastin, fibronectin and BM proteins (laminin, and collagen IV) towards term in rats [35]. This model provided evidence that ECM modifications can be stretch-regulated, specifically in the gravid (pregnant) uterine horn to accommodate the growing fetus/es, placenta and amniotic fluid, whereas the ECM in the empty horn is not influenced by mechanical stimuli [17, 35]. It was suggested that remodeling of the ECM increases elasticity of the uterus, and supports the change in SMC phenotype in preparation for labour contractions by restoring the BM surrounding individual SMCs [35]. These findings were consistent with the immunohistochemical results from Nishinaka et al. that suggested the BM of individuals SMCs are structurally modified during late gestation to support labour contractions [34]. The organized structures of BM and focal adhesion proteins during TL further confirmed that stretched-induced modification of ECM provides cell-matrix stability for optimal labour contractions [47].

1.3.3 Myometrial inflammation

Several studies have investigated the role of immune cells and inflammatory mediators contributing to events preceding TL and PTL [10, 11, 62]. For instance, the mRNA and protein expression level of certain pro-inflammatory cytokines and chemokines were elevated before,
During, and immediately after TL in reproductive tissues [11, 17, 20, 28]. In particular, Thomson et al. first reported the predominant presence of neutrophils and macrophages accumulating in the human myometrium during spontaneous TL [10]. Osman et al. further reported increased expression of IL-1β, IL-6, and IL-8, and similar infiltration of macrophages and neutrophils, in the myometrium and cervix [11]. Our lab also recently reported that macrophage and neutrophil populations were increased in parallel with pro-inflammatory cytokine induction in the mouse myometrium and decidua during TL, which further increased during the early PP period [20, 28]. It is well understood that activated leukocytes further amplify the inflammatory signal via additional secretion of cytokines – such as IL-1β, IL-6, and TNF-α – in the targeted reproductive tissue during late gestation and TL [63]. The pro-inflammatory state that develops in reproductive tissues (i.e. in the myometrium) as a result of the increased inflammatory mediators, has been widely accepted to be essential for the induction of labour – for instance IL-1β can upregulate prostaglandin production [6, 11, 16, 24-26, 62]. Since PGRs are constantly expressed in the myometrium, the cytokine-mediated upregulation of prostaglandin production would directly promote myometrial contractions.

Interestingly, it was observed that the expression of Monocyte Chemoattractant Protein-1 (MCP-1, also known as CCL2), a chemokine involved in macrophage migration, was significantly upregulated in the pre-partum rat myometrium due to mechanical stretch from the growing fetus and was inhibited by progesterone signaling [22]. MCP-1 expression was also associated with the infiltration of macrophages into the term rat myometrium. This study links endocrine and mechanical signalling to the regulation of leukocyte infiltration by inflammatory mediators and labour induction. Lee et al. recently demonstrated that cytokines produced by mechanically stretched human myometrial SMCs were able to upregulate the expression of cell adhesion molecules (such as ICAM-1, major ligand for leukocyte integrin LFA-1) on endothelial cells.
1.4 Matrix Metalloproteinases

1.4.1 Matrix Metalloproteinases as regulators of tissue remodeling

Apart from ECM proteins and inflammatory mediators, the application of defined mechanical stimuli to different types of cells has been shown to influence gene expression and protein synthesis of a group of zinc-containing endopeptidases capable of degrading ECM proteins, known as matrix metalloproteinases (MMPs). All MMPs are grouped based on substrate preference or the organization of their domain constituents, such as collagenases (MMP-1, 8, 13), gelatinases (MMP-2 and 9), and stromelysins (MMP-3, 10, 11). However all MMPs contain a common sequence motif His-Glu-X-Gly-His-X-X-Gly-X-X-His within their catalytic site, where the three His residues coordinate the catalytic zinc ion (Zn$^{2+}$) [65]. MMPs are either secreted (as pro- or active enzymes) or anchored to the cell surface and their physiological substrates include ECM components (i.e. collagen, proteoglycans, fibronectin, and laminin), as well as non-matrix proteins (i.e. cytokines, chemokines, receptors and antimicrobial peptides) [66-69].

The majority of MMPs are secreted with a self-inhibitory pro-domain upstream of the catalytic domain, and contain a conserved Cys residue that interacts with Zn$^{2+}$ in the active site to inhibit catalytic activity. The pro-domain requires either proteolytic removal or oxidative modification of the thiol group for protease activation [54, 65, 69]. The expression and activity
level of MMPs was first believed to be regulated in two ways: 1) physiologically, by a group of proteins called Tissue Inhibitors of MMPs (TIMPs) and 2) by the α2-macroglobulin. TIMPs form non-covalent 1:1 complexes with MMPs and either inhibit or promote the enzymatic activity of MMPs; whereas α2-macroglobulin acts as a general proteinase inhibitor and regulates MMPs activity by entrapping them and leading the macroglobulin-MMP complex to be cleared by receptor-mediated endocytosis [68]. Now it is recognized that MMP gene expression can be regulated by several factors including cell-receptor signaling, growth factors, and cytokines [31, 70, 71]. Recently, a major extracellular MMP inducer, called EMMPRIN has been characterized. EMMPRIN is a member of the Ig superfamily that includes T cell receptors, major histocompatibility complex (MHC) antigens, and neural cell adhesion molecules. It was first identified as a tumor cell-surface molecule capable of stimulating collagenase expression by fibroblasts however Braudmeier et al. have shown that EMMPRIN treatment of human uterine fibroblasts also resulted in stimulation of MMP-1 and MMP-2 expression [72]. Interactions between cell-surface integrin receptors with ECM ligands such as collagen and fibronectin can also induce MMP expression to reorganize the ECM [73, 74].

Typically, MMPs are not actively expressed in healthy tissues, but they can be detected in all diseased, inflamed, or injured tissues [75, 76]. MMPs can be produced and secreted by a variety of cells within the tissue that undergoes remodeling, which include fibroblasts, epithelial and endothelial cells [54, 76]. Once activated, MMPs initiates ECM remodeling primarily by cleavage of ECM components to modulate composition and structure, but can also release any biological molecules (i.e. growth factors) bound to ECM components [54]. Several studies investigating the phenotypes of MMP-null mice have deduced that MMPs are not only important for regulation of physiological processes including wound healing, ovulation, implantation, angiogenesis, and embryogenesis via remodeling of the ECM architecture, but also are capable of regulating cellular
behaviour – such as promoting cellular migration and increasing permeability between cell-cell
junctions [77-80]. For instance, MMPs can promote angiogenesis by degrading the vascular BM
to allow ECs to invade the surrounding tissue, releasing ECM-bound angiogenic growth factors
like VEGF, and by cleaving cell-cell adhesion molecules between ECs [79]. Interestingly,
activated leukocytes infiltrating reproductive tissues also produce several MMPs and TIMPs,
supporting the role of MMPs in regulating inflammation and reproductive function [10].

As previously mentioned, since MMPs are involved in a variety of physiological
processes, their expression must be highly regulated to maintain tissue-specific activity. MMP
synthesis and activation can be regulated for both tissue-specific remodeling (i.e. isolated wound
healing) and differential expression of specific MMPs can be required for certain functions (i.e.
MMP-9 is essential for embryo implantation) [71, 74, 81, 82]. Conversely, failure to maintain
MMP homeostasis leads to excess MMP activity and uncontrolled proteolysis, which has been
associated with impaired barrier function and several diseases – autoimmune, cardiovascular
diseases, and cancer [83]. As MMPs are synthesized and secreted as zymogens, physiological
activation becomes a checkpoint for their regulation. Plasmin, urokinase type plasminogen
activator (uPA) and tissue type plasminogen activator (tPA) have been previously implicated in
MMP activation [84]. Pro-inflammatory cytokines IL-1β and TNF-α have been shown to induce
MMP expression in numerous tissues, while progesterone and TGF-β can suppress MMP
expression [71, 85]. As was previously mentioned, the principal method of MMP regulation is
physical inhibition by TIMPs or inactivation through proteolytic degradation (by other proteases),
and/or physical clearance by α2-macroglobulin [74, 77, 81]. Furthermore, TGF-β has been shown
to induce TIMP-1 production by endometrial stromal cells to further inhibit MMP activity [71,
86]. Graham et al. have also demonstrated that TGF-β regulates MMP expression in first trimester
trophoblasts by up-regulating TIMP-1 and TIMP-2 and down-regulating plasminogen activator expression [87].

1.4.1.1 Collagenases

One of the major class of MMPs is the collagenases, named based on their preference to enzymatically process fibrillar collagens for collagenolysis. This class is comprised of MMP-1 (fibroblast collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3). The collagenases share a similar structural organization of their domains that contains a pro-peptide domain at the N-terminus, catalytic domain, a linking hinge region, and a hemopexin-like domain at the C-terminus [76, 80]. For collagenases, the hinge region coordinates the interaction between the catalytic region and the hemopexin-like domain [88]. This interaction is important for substrate recognition and positioning, necessary for their primary physiological role in remodeling of the collagenous components of the ECM [49, 89]. Interestingly, collagenases are able to cleave collagen molecules without unwinding the triple helical structure of their substrate.

Although they can cleave all fibrillar collagens, MMP-1 preferentially targets type III collagen, MMP-8 prefers type I collagen, and MMP-13 prefers type II collagen [76]. Furthermore, besides efficient degradation of collagens, the collagenases also cleave a wide variety of non-collagenous substrates, such as aggrecan, chemokines, and IGFs [76, 90]. The collagenases have been previously shown to regulate reproductive function. Specifically, MMP-1 has been associated with the breakdown of the stromal components of the uterine endometrial lining during menstruation [30]. In addition, since collagen I makes up a large portion of the uterine ECM it was suggested that collagenases may play a role in remodeling of uterine tissues during gestation to accommodate the growing fetus [35]. It is also feasible to speculate that collagenases may
become important participants during the PP uterine involution when this organ undergoes extensive remodeling to return to its pre-pregnant state [91].

1.4.1.2 Gelatinases

The gelatinases encompass MMP-2 (gelatinase A) and MMP-9 (gelatinase B), and are defined as such based on their affinity for denatured collagen (i.e. gelatin) [76]. What makes the gelatinases structurally different from the collagenases is the additional presence of a collagen binding domain (CBD), comprised of three type II fibronectin-like repeating elements inserted into the catalytic domain [80, 81, 92]. The CBD is the main domain for substrate recognition for MMP-2, and is essential for specific binding and positioning of substrates to the catalytic site for both gelatinases [76, 93, 94]. The mechanism of MMP-2 activation has been defined in detail, where one proposed model involves the interaction with TIMP-2 and the membrane-bound MT1-MMP (MMP-14) for removal of the pro-peptide [95, 96]. On the other hand, removal of the pro-peptide for MMP-9 activation can be exerted by several soluble MMPs, such as MMP-2, 3 & 7 and other proteases [94, 97].

Apart from gelatin degradation, the gelatinases also have an affinity for components of the BM, intercellular gap junctions (i.e. occludin), cytokines (i.e. IL-1β), growth factors (i.e. pro-TNFα) and ECM receptors (i.e. integrins) [66, 76, 78, 81, 98]. The expression of MMP-2&9 has been highly associated with reproductive tissues, during ovulation and implantation, making them key players in maintaining reproductive function as well as tissue remodeling within reproductive organs [30, 71, 99, 100]. Their affinity for denatured collagen and degradation of ECM components also supports their role in PP involution.
1.4.1.3 Stromelysins

This class comprises of the three stromelysins MMP-3 (stromelysin-1), MMP-10 (stromelysin-2) and MMP-11 (stromelysin-3), all of which are capable of degrading a wide range of ECM proteins including gelatin, fibronectin, and BM component laminin, with the exception of collagen I [76, 101, 102]. The stromelysins, however, can cleave other collagens (with varying affinities) such as components of the BM (i.e. collagen type IV), and MMP-3 can efficiently activate other pro-MMPs, such as pro-MMP-1 [69, 76, 102]. The stromelysins also play a role in the activation or inactivation of non-ECM proteins such as cytokines (i.e. TNF-α precursor and IL-1β) and growth factors (i.e. EGF) [76].

MMP-3 & 10 are structurally similar and contain a pro-peptide domain, a catalytic domain, and a C-terminal hemopexin-like domain responsible for substrate recognition. These two stromelysins share substrate specificity; however, MMP-3 has a higher affinity and proteolytic efficiency than MMP-10 [30, 69, 76, 80]. Whereas MMP-11 is structurally different, containing a motif for intracellular activation by furin (and other furin-like proteases) within the Golgi and is secreted extracellularly in its active form [76, 103]. Importantly, all stromelysins, especially MMP-3 &11 have been shown to be highly associated with reproductive tissues such as the uterus (during menstruation, implantation, pregnancy, labour and PP), placenta and involuting mammary gland [30, 76].

1.4.1.4 Stromelysin subgroup: Matrilysin and Metalloelastase

MMP-7 (matrilysin) and MMP-12 (macrophage metalloelastase) are two MMPs often classified in the stromelysin subgroup as they share similarities with MMP-11. MMP-7 was first described as putative uterine metalloprotease-1 (PUMP-1) but now is generally referred to as matrilysin. MMP-7 structurally is the smallest MMP as it only contains the pro-peptide and
catalytic domain, while MMP-12 contains the additional hinge region and hemopexin domain (for substrate binding and recognition) [76]. Like the stromelysins, MMP-7&12 can cleave a wide variety of ECM substrates such as collagen I&IV, gelatin, elastin, fibronectin and laminin, and have an affinity towards non-ECM proteins like cytokines (i.e. pro-TNF-α) and able to activate other pro-MMPs [69, 76, 78]. In addition, MMP-7 can also cleave cell-surface junctional molecules (i.e. VE-cadherin) to modulate tissue permeability [104, 105].

The broad range of substrates supports the notion that MMP-7&12’s main biological function is to remodel the ECM, regulate immune function and promote immune cell recruitment and migration [30, 76, 78]. MMP-7 is expressed in a variety of tissues and has been seen to be associated with reproductive tissues, especially in the uterus during the menstrual cycle, implantation, labour and PP period [30, 99, 106]. MMP-12 is essential for leukocyte transmigration into tissues, where knock-out of MMP-12 expression led to restricted macrophage infiltration [78]. These correlations suggest that MMP-7&12 may play a role in regulating tissue remodeling and inflammation within the uterus.

1.4.1.5 Regulation of MMP activity: TIMPs

The TIMPs (TIMP-1-4) are one of the endogenous forms of MMP broad spectrum regulation/inhibition factors that bind to MMPs in a 1:1 stoichiometric configuration, and like MMPs, are regulated in response to the need for tissue remodeling [69, 107, 108]. TIMPs are structurally comprised of two domains, the N- and C-terminus; the N-terminal domain can fold into a separate unit and independently inhibit MMPs [107, 108]. TIMPs normally form complexes with pro-MMPs via the interactions of their C-terminal domains with the MMP’s hemopexin domain, and since this does not involve the N-terminal domain, these complexes can further interact with a second MMP [108]. Although the four TIMPs share sequential similarities, their
differences confer specificity such that certain TIMPs have higher affinities for certain MMPs, which affects their biological function [69, 107, 108]. For instance, TIMP-1 has a more restricted inhibitory range compared to TIMP-2-4, particularly for membrane-type (MT) MMPs, but strongly inhibits MMP-3&7 [108, 109]. TIMP-2 plays an important role in the recruitment and cell-surface activation of pro-MMP-2 via MT1-MMP, while TIMP-1 cannot bind to pro-MMP-2, but instead can form an inhibitory complex with MMP-3 or pro and active MMP-9 [95, 110, 111]. TIMP-4 can also form a complex with MT1-MMP and pro-MMP-2 however, this does not lead to its activation but rather inhibits the action of MT1-MMP [112]. Interestingly, TIMP-2 and pro-MMP-2 can form a large inhibitor of MMP (LIMP) complex that can additionally inhibit MMP-2, the collagenases, and stromelysins [113]. Unlike the other TIMPs that are primarily soluble and remain diffused within tissues, TIMP-3 is also tightly bound to the ECM through its C-terminal domain [109].

In addition to MMP inhibition or activation, TIMPs can have various other biological functions including regulation of cell proliferation and migration, and be involved in angiogenic and apoptotic pathways. For example, TIMP-3 has been observed to have pro-apoptotic functions whereas, TIMP-1, 2&4 are anti-apoptotic [69, 109, 114-117]. TIMP regulation of cell signaling to promote the aforementioned processes can be either MMP-dependent, such as TIMP-1 inhibition of MMP-mediated degradation of IGF binding protein-3 (IGFBP-3), or can be through direct interactions with specific cell-surface receptors (i.e. CD63) or cell-surface integrins (i.e. α3 and β1 subunits) [109, 118]. However, it has been shown that TIMPs can either promote cell growth and survival, inhibit cell death or cell growth depending on the cell types and downstream effectors involved [109].
1.4.2 Matrix Metalloproteinases as immunoregulatory proteases

1.4.2.1 MMPs as modulators of inflammation

The involvement of MMPs in inflammatory processes has suggested their potential role to modulate leukocyte influx into inflamed tissues. For instance, in a mouse model for autoimmune skin blistering disease, MMP-9-null mice exhibited decreased neutrophil recruitment due to the lack of α1-proteinase inhibitor inactivation by MMP-9, which results in deficiency of neutrophil elastase (NE) function [119]. MMPs can also regulate the activity of cytokines and chemokines, and the formation of chemotactic gradients to affect the movement of leukocytes by being either pro-inflammatory – by activation of IL-1β and TNF-α – or anti-inflammatory – MMP-3 can degrade active IL-1β [120-123]. Several studies have shown that specific MMPs regulate chemotactic gradients by direct cleavage of chemokines or indirectly by cleaving ECM/non-ECM molecules that bind to chemokines at certain locations. For instance, Li et al. have observed MMP-7 is able to regulate the trans-epithelial efflux of neutrophils by shedding syndecan-1 bound to chemokine CXCL1 (KC) in the lung [124]. MMP-2-mediated cleavage of macrophage-derived CCL7 (MCP-3) leads to the formation of antagonistic peptides of the CC chemokine receptor [122]. MMP-9 and MMP-8 can cleave and activate CXCL8 (IL-8) and LIX (mouse equivalent of human CXCL5&6), respectively. However, proteolytic processing by MMP-9 inactivates CXCL1 and CXCL4. This provides evidence that MMPs can both amplify or reduce inflammatory signals, specifically affecting leukocyte recruitment by mediating the change in the bioavailability of local chemokines [125-128]. Processing/activating of these inflammatory mediators suggest that in addition to tissue remodeling, MMPs are involved in the labour process by regulating leukocyte infiltration into the uterus.

Leukocytes can follow two pathways of migration: 1) transcellular (through the cytoplasm) or 2) paracellular (in between ECs) [129, 130]. Several studies have indicated
leukocyte infiltration into tissues is associated with regulated modifications to increase junctional permeability while preserving endothelial barrier function [131, 132]. Specifically ECs can experience delocalization and decreased expression of their cellular junction molecules or proteolytic degradation of those complexes to disrupt and loosen the tight junctions [132-134]. It has been previously shown that leukocyte adhesion initiated intracellular signalling pathways to enhance permeability to promote migration [134, 135]. Deem et al. revealed that lymphocyte-mediated VCAM-1 crosslinking activates downstream production of NADPH oxidase and reactive oxygen species (ROS). ROS then mediates the activation of EC-associated MMPs to enable leukocyte migration; inhibition of endothelial MMPs block VCAM-1-dependent migration [135]. MMPs are suggested to modulate leukocyte migration by regulating the permeability of cell barriers. Specifically, the activity of gelatinases (MMP-2&9), are known to be responsible for the degradation of intercellular junction proteins (i.e. occludin and VE-cadherin), leading to an increase in microvascular permeability [78, 136, 137].

1.4.2.2 MMPs as modulators of pregnancy and labour

It has been well-established that MMPs are important modulators of ovulation (MMP-1), implantation of the fertilized embryo (gelatinases), and decidualization and trophoblastic invasion during early pregnancy [30, 82, 106, 138]. It has been hypothesized that late gestational changes, labour, and parturition are associated with increased expression and activation of MMPs, which would result in relaxation of uterine muscle, inhibit myometrial contraction, and/or promote tissue remodeling during PP period [139]. Yin et al. reported an increase in gelatinase gene expression but a decrease in MMP-7 in pregnant rat myometrium strips collected at mid- and late gestation, and these findings were augmented in response to prolonged mechanical stretch. These authors suggested that these changes in MMP expression inhibit contraction and induce uterine relaxation
in response to mechanical stretch imposed by the growing fetus during pregnancy [139]. Roh et al. observed that the release of pro-inflammatory cytokines (IL-1β and TNF-α) by activated macrophages and neutrophils induced the production of MMP-9 by myometrial SMCs prior to TL, suggesting a link between MMP expression and labour-associated inflammation [31]. As previously mentioned, MMP-9 is capable of cleaving 1) degraded collagen in the extracellular matrix, 2) endothelial tight junctions, and 3) activating non-matrix proteins such as IL-1β and IL-8 [31, 78].

PTL in humans has been linked to ECM degradation, caused by imbalance between MMPs and their TIMPs. Tency et al. reported that during PTL, there was an increase in the ratio of MMP-9:TIMP-1 and MMP-2:TIMP-2, favouring gelatin degradation [57]. Xu et al. also observed an increase in MMP-9 in fetal membranes (FM) and human placental (PL) tissue during TL and PTL, supporting their importance in facilitating rupture of FM and PL detachment by degrading components of the uterine ECM [58]. MMP-3, capable of activating latent forms of other MMPs (specifically MMP-2), was also significantly increased in the amniotic fluid at TL and PTL (associated with amniotic infection) [125]. MMPs may be important for the amplification of inflammatory signals in uterine tissues before and during TL and PTL; targeting premature MMP activation can be insightful in treating premature delivery. Importantly, through the use of a mouse model of inflammation-induced PTL, Koscica et al. reported that the administration of a broad spectrum MMP inhibitor (GM6001) resulted in reduction of the rate of PTL, suggesting a direct involvement of MMPs in regulating the labouring process [140].
1.5 Thesis Rationale and Hypothesis

Current PTL therapeutic strategies primarily focus on inhibiting myometrial contractions (i.e. tocolytic drugs), but have been found to ineffectively delay labour onset in the majority of women. Particularly due to these therapies targeting later stages of the labour cascade – when irreversible changes in the reproductive tissues have already occurred [141, 142]. Research aiming to characterize the exact mechanisms underlying the onset of TL will assist in understanding whether similar changes occur during PTL. Dr. Lye and colleagues have suggested a new model for labour initiation where mechanical stretch of the uterine tissues by the growing fetus increases myometrial cytokine/chemokine expression which 1) upregulates cell adhesion molecules on endothelial cells; 2) activates peripheral leukocytes and induces their infiltration into the myometrium. Here, leukocytes contribute to labour initiation by inducing myometrial contractility and play a role in decidual activation – by promoting synthesis of prostaglandins [17, 64]. Prostaglandins (i.e. PGF$_{2\alpha}$) not only stimulate myometrial contractions, they can also induce gelatinolytic activity by increasing MMP and down-regulating TIMP expression, and modulate the secretion of pro-inflammatory cytokines and chemokines [51, 143, 144].

The increasingly recognized role of MMPs as activators and regulators of inflammatory pathways suggests their potential involvement in remodeling of reproductive tissue (i.e. myometrium). My research 1) characterized the myometrial expression of MMPs and TIMPs during pregnancy, labour and postpartum, 2) to define putative mechanisms that regulate their expression and/or activity, and the contribution of MMPs to inflammatory pathways within this tissue.

I hypothesized that throughout gestation, there is an increase in myometrial MMP and a decrease in TIMP expression/activity in preparation for two major events: 1) labour contractions
to expel the fetus and 2) subsequent uterine remodeling during PP involution. I also speculate that mechanical stretch of the uterine walls by the growing fetus can regulate the expression and activity of myometrial MMPs and TIMPs.

1.6 Specific Aims and Objectives

1.6.1 Aim 1: To characterize the expression pattern of myometrial MMPs and TIMPs throughout pregnancy, labour and postpartum.

Due to a lack of pregnant human uterine tissue, I aimed to characterize the gene/protein expression of certain MMPs/TIMPs in vivo using an animal (rat) model of gestation and term labour. It was hypothesized that the gene and protein expression/activity would increase towards late gestation and culminate during the early postpartum period. It is speculated that in addition to myometrial smooth muscle cells, infiltrating leukocytes would also be the source of MMP expression. To test this, myometrial tissues were collected from pregnant (gestational day (GD) 6, 8, 10, 12, 14, 15, 17, 19, 21, 22), labouring (GD23) and postpartum (1PP and 4PP) rats. Real-Time Polymerase Chain Reaction (RT-PCR) was used to quantify changes in MMP gene expression; Western blot analysis was used to validate and quantify protein expression. This study specifically focused on studying the collagenases (MMP-1, 8, 13), gelatinases (MMP-2, 9), stromelysins (MMP-3, 10, 11), matrilysin (MMP-7), metalloelastase (MMP-12) and TIMP-1, 2, 3, and 4 mRNA expression. For protein analysis, focus was placed on 1) the expression of MMP-8 which preferentially cleaves collagen I, a major component of the uterine interstitial ECM; 2) MMP-7 known to be active in rat uterine tissue during TL and PP; 3) the major stromelysins MMP-3 and MMP-11; and 4) MMP-12 known to be highly expressed by macrophages [31, 78, 145, 146]. The activity of MMP-2 and -9 throughout gestation was also studied by gelatin
zymography and temporal/spatial localization of MMPs in rat myometrial tissue was studied using immunohistochemistry, in situ zymography, and immunofluorescence of leukocyte-specific markers.

1.6.2 Investigate the in vivo regulation of MMP and TIMP expression by mechanical stretch.

Previous reports have suggested that mechanical stretch can influence gene/protein expression of MMPs and TIMPs in other tissues [147, 148]. It has been previously shown by colleagues in the Lye laboratory using a unilaterally pregnant rat model, that the gravid (pregnant) uterine horn experiences greater changes (across gestation) in the expression of ECM components compared to the non-gravid (empty) horn [35]. It is hypothesized that mechanical stretch from the growing fetus upregulates the expression of myometrial MMPs compared to the empty horn. Analysis of this well-established rat model, may reveal differences in the expression and activity of major MMPs in the gravid and empty uterine horns in comparison with normal bilaterally pregnant rats. Myometrial tissues were collected from GD 6, 12, 15, 17, 19, 21, 22, labouring (GD23) and 1 day PP rats. Similar to Aim 1, RT-qPCR was used to quantify changes in MMP gene expression, Western blot and gelatin zymography was used to validate and quantify protein expression and activity. These experiments provide insight on the regulation of MMP expression by in vivo biological mechanical stretch from the growing fetus.

1.6.3 Investigate the in vitro regulation of MMP and TIMP expression by mechanical stretch.

Although the in vivo unilaterally pregnant rat model provides insight on the regulation of myometrial MMP expression, it does not provide confirmation whether mechanical stretch directly regulates MMP expression. To investigate the sole effect of mechanical stretch – without
other potential regulatory factors such as signals from the fetus and placenta – an *in vitro* cell model will be studied. It was recently shown by Lee *et al.* that media collected from stretched myometrial cell line hTERT-HM 1) showed an increase in multiple pro-inflammatory cytokines and chemokines, and 2) promoted an increase in permeability of human endothelial cells in culture [17]. In addition, Loudon *et al.* observed that mechanical stretch of primary human myometrial cells increased mRNA and protein levels of CXCL8 [149]. However, the role of MMPs was not considered in these studies. It is hypothesized that applying static mechanical stretch would simulate the stretch of the growing fetus and upregulate the expression of MMPs by myometrial SMCs. To investigate the direct effect of static mechanical stretch on the synthesis and secretion of MMPs/TIMPs *in vitro*, two different approaches were employed.

First, primary rat myocytes were enzymatically isolated from the uterine tissues. These myocytes were mechanically stretched using a computer-driven *in vitro* stretch system (FX-5000, Flexcell Int). Total RNA and protein were extracted from these cells and stretch-conditioned media (SCM) or media conditioned by the non-stretched cells (NCM) were collected to compare the gene/protein expression level of MMPs, TIMPs using RT-PCR and gelatin zymography analysis, correspondingly. However, results from this *in vitro* model were inconclusive.

Secondly, primary human myometrial cells were enzymatically isolated and mechanically stretched for 24 hours using the FX-5000 stretch system, and the expression of MMPs (both mRNA and protein) as well as NCM/SCM were analyzed. Using human myometrial cells produced from tissue biopsies instead of primary rat myometrial cells will better correlate the findings from our animal studies for future human applications.
CHAPTER 2

MATERIALS AND METHODS
Chapter 2: Materials and Methods

2.1 INTRODUCTION

This chapter describes the materials and detailed methodologies used for the thesis experiments. The buffers that were used such as phosphate buffered saline (PBS) and Hank’s buffered salt solution (HBSS), were prepared and autoclaved in-house by the sterilization and Media Prep department at the Lunenfeld-Tanenbaum Research Institute (LTRI) unless otherwise specified.

2.2 RAT GESTATIONAL PROFILE OF MMP EXPRESSION

2.2.1 Bilaterally Pregnant Rat Animal Model of Gestation

The in vivo myometrial mRNA gestational expression profile of MMPs and TIMPs was investigated using a bilaterally pregnant rat model of gestation, term labour (TL) and postpartum (PP). Virgin female Wistar rats (12-15 weeks, 225-250g weight) and male Wistar rats (250-300g weight) were purchased from Charles River Laboratories (St. Constance, QC, Canada) and housed in the MaRS Toronto Medical Discovery Tower Animal Research Facility (Toronto, ON, Canada). The research ethics board approved these animal studies (AUP #2379). Rats were maintained on standard rat chow and water in a 12:12 hour light-dark cycle. The rats were mated and the day when a vaginal plug was detected was considered day 1 of gestation. Animals were euthanized by carbon dioxide inhalation and uterine samples were collected on gestational day (GD) 6, 8, 10, 12, 14, 15, 17, 19, 21, 22, 23 (TL), 1 day PP and 4 day PP.
2.2.2 Unilaterally Pregnant Rat Model of Gestation

The in vivo effect of mechanical stretch from the growing fetuses was investigated using a unilateral horn pregnancy model. Virgin female Wistar rats were anesthetized and tubal ligation was performed through a flank incision. This ensured that the animals would become pregnant in only one uterine horn [150]. Prior to mating, animals were allowed to recover from surgery for at least 7 days. Uterine tissues from the gravid and empty horns were collected separately on GD 6, 12, 15, 19, 21, 23L, and 1PP.

2.2.3 Tissue Collection and Preparation

Whole uteri were excised from the pregnant rats, and fat and blood vessels were carefully removed.

For biochemical studies: Placentae and pups were removed from the pregnant uteri, the myometrium was separated from the decidua basalis and decidua parietalis (endometrium) by mechanical scraping on ice which we have previously shown removes the entire luminal epithelium and the majority of the uterine stroma [151]. Tissue was rinsed in ice-cold PBS (Sigma-Aldrich Canada Co., Oakville, ON, Canada) and flash frozen in liquid nitrogen.

For immunohistochemical, immunofluorescence and in situ zymography analysis: The intact uterine horns were placed in ice-cold PBS, cut into 3-10-mm segments using a scalpel blade and fixed immediately in 10% neutral-buffered formalin (NBF; VWR International, Mississauga, ON, Canada) solution at 4°C for 24 hours or overnight in zinc buffered fixative (ZBF; 100mM Tris buffer pH 7.4, 3mM calcium acetate, 27mM zinc acetate, 37mM zinc chloride; see Table 2.2 [14;17]) while shaking at RT. Following fixation, the whole uteri were rinsed with PBS overnight. NBF-fixed tissue were dehydrated in 70%, 80%, 90%, 95%, 100% ethanol series for 30 minutes
Chapter 2: Materials and Methods

each, cleared in xylene three times for 15 minutes, heated in paraffin wax three times for 30 minutes and embedded into paraffin blocks. ZBF-fixed tissue were dehydrated in 70%, 80%, 90% and 100% (twice) ethanol baths for one hour each, cleared in xylene three times for 15 minutes, heated in paraffin wax three times for 30 minutes and embedded into paraffin blocks.

2.2.4 Real-Time Polymerase Chain Reaction (RT-PCR) Analysis of mRNA expression

The frozen myometrium was homogenized using the TissueLyser II (Qiagen Inc., Mississauga, ON, Canada) to isolate total RNA using Qiagen RNeasy Universal Mini kit (Qiagen Inc.). Total myometrial RNA was reverse transcribed using the iScript supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON, Canada) to cDNA (1µg of RNA in 20µL reaction). A primer mastermix (2.5µL of Sigma Scientific SYBR green, 1µL of RNase-free water, and 0.5µL of 3µM forward and reverse primers) was combined with 1µL of the 5 ng/µL cDNA product for a final volume of 5 µL per well in a 384-well PCR plate. The plate was then subjected to Real-Time PCR using the CFX384 system (Bio-Rad). Transcript levels of collagenases (MMP-1, 8, 13), gelatinases (MMP-2, 9), stromelysins (MMP-3, 10, 11), matrilysin (MMP-7), metalloelastase (MMP-12) and TIMP-1, 2, 3, 4 were examined using set of specific primers designed using free NCBI Primer Blast software and produced by Eurofins Genomics (Eurofins MWG Operon LLC, Huntsville, AL, USA), (see Table 2.1).
Table 2.1 Primer pair information for Rat (Rattus Norvegicus) genes examined using RT-PCR

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Accession Number</th>
<th>Primers (5’-3’)</th>
<th>Amplicon Size (base pairs)</th>
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</thead>
</table>
| MMP-1†       | Interstitial /Fibroblast Collagenase       | NM_001134530.1   | F: ACGCAGATTTAGCCTCCGAA  
R: TGACTTGTGTAATGGGTGTC | 97             |
| MMP-8        | Neutrophil Collagenase                    | NM_022221.1      | F: GGAGTGTGACCACCTCACTGGTACC  
R: ACCATGGCTCTTACGAGACGAAAGCA | 86             |
| MMP-13       | Collagenase 3                             | NM_133530.1      | F: GCCTTAGAGTGGACTGGCAA  
R: CATTTGAGTGTTCAGGGAGGA | 113            |
| MMP-2        | Gelatinase A                              | NM_031054.2      | F: TGCCGAGACAGTACACAGC  
R: TGGGCTCTGATACACGTGC | 105            |
| MMP-9        | Gelatinase B                              | NM_031055.1      | F: TTCTCTGGGCGCAAAGAATGTCG  
R: ACGGAGAGGAGGAGGAGGAG | 114            |
| MMP-3        | Stromelysin 1                             | NM_133523.3      | F: AGATCCAGAGGAGGAGGAG  
R: AGGAAACCCTTCATGGAGGAC | 136            |
| MMP-10       | Stromelysin 2                             | NM_133514.1      | F: TGCTGCTGGCTCCTTCGGA  
R: AGCAAGATCCATGGTGGAGG | 80             |
| MMP-11       | Stromelysin 3                             | NM_012980.2      | F: TGCTGCTTTCCAGGAGTGAGG  
R: AAGTCGGGACCTATGGGCGAG | 120            |
| MMP-7        | Matrilysin (PUMP-1)                       | NM_012864.2      | F: CCTGTCTCCCCCCATGGTGTC  
R: TAATCTGCGCTGCTTCCCA | 102            |
| MMP-12       | Macrophage Metalloelastase                | NM_053963.2      | F: TCCTGCTGGCTCTCCATCTCTG  
R: TCCTGCTCTCAATTCATCC | 116            |
| TIMP-1       | Tissue Inhibitor of Metalloproteinase 1    | NM_053819.1      | F: CTGCCCTCGCTCTCTGCTCC  
R: AGGGAAACCACCTCACTGGGAC | 105            |
| TIMP-2       | Tissue Inhibitor of Metalloproteinase 2    | NM_021989.2      | F: AGATCCAGGCTCGCCCTATG  
R: TGCTGCTTCCATGGAGGAC | 97             |
| TIMP-3       | Tissue Inhibitor of Metalloproteinase 3    | NM_012886.2      | F: TGCTGCTGGCTGCTTCCATAG  
R: CCGCTGCGCTGCTTCCATAG | 93             |
| TIMP-4       | Tissue Inhibitor of Metalloproteinase 4    | NM_001109393.1   | F: TTGCTGCTGCTGCTGCTGCTG  
R: TGCGCTGCTGCTGCTGCTG | 93             |
| IGF1R        | Insulin-like Growth Factor 1 Receptor      | NM_000875.4      | F: GCATCTGACATTGCTCAGC  
R: GGGCCATCTGGTTATCC | 103            |
| PPIA         | Peptidylprolyl Isomerase A (Cyclphilin A)  | NM_017101.1      | F: TATCTGACATTGCTCAGC  
R: CCACAATGTGTCTCTTCTTCT | 80             |
| PDGF-A       | Platelet-derived Growth Factor Alpha       | NM_012801.1      | F: AGCGACTGGCTCGGAAGTC  
R: CTCAAGGAATCCATCCACTG | 89             |
| TBP          | TATA Box-Binding Protein                   | NM_0001004198    | F: AGAAACATCCAGAGCAGC  
R: GGAACTTACATCACAGTCC | 120            |

† Genes excluded from statistical analysis; either not detected or n < 4 after exclusion.
Following RT-qPCR, a dissociation curve was constructed by increasing temperature from 65°C to 95°C for detection of PCR product specificity. In addition, a no-template control (H₂O instead of cDNA) was analyzed for possible contamination in the master-mix. A cycle threshold (Ct) value was recorded for each sample. The Ct value is defined as the number of amplification cycles required for detection of a fluorescent signal (SYBR green) that exceeds background levels. Ct values are inversely proportional to mRNA levels within each sample. PCR reactions were set up in technical triplicates and the mean of the 3 Ct values was calculated by the software (CFX Manager, Bio-Rad). A comparative Ct method (ΔΔCt method) was applied to the raw Ct values to find a relative gene expression. The expression levels of MMPs and TIMPs were normalized to the geometric mean of three housekeeping genes for bilaterally pregnant rat samples: cyclophilin/peptidylprolyl isomerase A (PPIA), platelet-derived growth factor alpha (PDGF-A), and TATA box-binding protein (TBP). For unilaterally pregnant animals’ MMP and TIMP gene expression was normalized to the three housekeeping genes PPIA, PDGF-A and insulin-like growth factor 1 receptor (IGFR1). All housekeeping genes used exhibited stable mRNA levels throughout gestation. Results were displayed as fold-change relative to GD6 (bilaterally pregnant rats) or relative to a corresponding GD6 in the empty horn (unilaterally pregnant rats) within each experimental run.

2.2.5. Gelatin Zymography and Western Blot Analysis of Protein Expression

Total protein was extracted from frozen myometrial tissue homogenized using a bicene salt lysis buffer. The homogenate was centrifuged at 20,800 g for 15 minutes at 4⁰C. The supernatant was collected and protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). For Western blot analysis of
MMP-8, MMP-7, MMP-3, and MMP-11 protein expression, 30 µg (for MMP-8 analysis) or 70 µg of total protein (for MMP-7, 3 & 11 analysis) were added to sample buffer (NuPAGE LDS 4x Sample Buffer, Novex) with β-merceptoethanol solution (Sigma-Aldrich) and heated for 10 minutes at 95°C. The protein was then loaded on 12% bis-acrylamide electrophoresis gels and ran for 2 hours at 100V at room temperature with 1x Tris-Glycine SDS running buffer diluted from the 10x stock (Wisent Inc., St-Bruno, QC, Canada). Following gel electrophoresis, proteins were transferred onto PVDF membranes (0.2 µm Trans-Blot® Turbo™ Midi PVDF Transfer Packs (Bio-Rad), using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were quickly washed with TBS-T, blocked in 5% non-fat milk for 1 hour at room temperature, and incubated with primary antibodies (Table 2.2) overnight at 4°C with gentle shaking. Next, membranes were washed 3 times 5 minutes in TBS-T prior to incubation with HRP-biotinylated secondary antibody (Table 2.2) for 1 hour with gentle shaking at room temperature. Membranes were washed 3 times for 5 minutes in TBS-T and then developed with Luminata Crescendo Western HRP substrate (Millipore (Canada) Ltd., Etobicoke, ON, Canada) or SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific Inc.), depending on the intensity of the signal. Images were taken using the VersaDoc™ Imaging System (Bio-Rad) and analysed using Image Lab software (Bio-Rad). Protein expression levels were normalized to the calcium-binding protein calponin used as the housekeeping protein, and expressed relative to GD6.
Table 2.2 Information of antibodies used for Rat-specific experiments.

<table>
<thead>
<tr>
<th>Target</th>
<th>Host</th>
<th>Company</th>
<th>Used for Method</th>
<th>Antibody Type</th>
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<tr>
<td>MMP-9</td>
<td>Mouse</td>
<td>Millipore</td>
<td>IHC</td>
<td>Primary</td>
<td>1:200, 1:200</td>
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<tr>
<td>MMP-8</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>WB</td>
<td>Primary</td>
<td>1:500</td>
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<tr>
<td>MMP-3</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>WB</td>
<td>Primary</td>
<td>1:500</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>WB, IHC</td>
<td>Primary</td>
<td>1:500, 1:150</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>WB, IHC</td>
<td>primary</td>
<td>1:200, 1:150</td>
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<td>MMP-12</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>WB</td>
<td>primary</td>
<td>1:1000</td>
</tr>
<tr>
<td>Calponin</td>
<td>Mouse</td>
<td>Sigma</td>
<td>WB</td>
<td>Primary</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD45</td>
<td>Mouse</td>
<td>Bio Legend</td>
<td>IHC</td>
<td>Primary</td>
<td>1:200</td>
</tr>
<tr>
<td>CD68</td>
<td>Mouse</td>
<td>AbD Serotec</td>
<td>IF</td>
<td>Primary</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-rabbit-HRP</td>
<td>Donkey</td>
<td>GE Healthcare</td>
<td>WB</td>
<td>Secondary</td>
<td>1:2000</td>
</tr>
<tr>
<td>Anti-mouse biotinylated</td>
<td>Goat</td>
<td>Vector</td>
<td>IHC</td>
<td>Secondary</td>
<td>1:300</td>
</tr>
<tr>
<td>Anti-rabbit biotinylated</td>
<td>Goat</td>
<td>Vector</td>
<td>IHC</td>
<td>Secondary</td>
<td>1:200</td>
</tr>
<tr>
<td>Alexa 546 anti-mouse</td>
<td>Donkey</td>
<td>Life Technologies</td>
<td>IF</td>
<td>Secondary</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Western Blot (WB), Immunohistochemistry (IHC), Immunofluorescence (IF)

For analysis of enzymatic gelatinase activity, gelatin zymography was applied: 50 µg of total protein from myometrial tissue homogenates were added to sample buffer (1:2, Novex, Life Technologies Inc., Burlington, ON, Canada) prior to loading onto Novex pre-cast 12% tris-glycine gelatin gels (Novex, Life Technologies Inc.). Gel electrophoresis was run for approximately 4 hours at 110V at 4°C (to prevent denaturation of gelatinases) with 1x Novex Tris-Glycine SDS running buffer (Life Technologies Inc.) using the Novex X-Cell II Mini Cell system (Life Technologies Inc.). Following electrophoresis, gels were incubated for 30 minutes in 1x renaturing buffer (Novex) to remove SDS and renature the gelatinases and then for 30 minutes in 1x developing buffer (Novex) with gentle shaking at room temperature. Next gels were incubated in developing buffer overnight at 37°C. Gels were then washed 3 times with double
deionized water (ddH₂O, Milli-Q, Millipore (Canada) Ltd.) to remove excess developing buffer prior to staining with Simply Blue stain (Novex) for 1 hour with gentle shaking at room temperature. Gels were rinsed with ddH₂O to clearly see the contrast between the blue-stained gel and gelatinase activity (white bands). Images were taken using the VersaDoc™ Imaging System (Bio-Rad) and analysed using Image Lab software (Bio-Rad). Gelatinase activity levels were calculated relative to GD6.

2.2.6 Localization of Myometrial MMPs by immunohistochemistry

Paraffin embedded formalin-fixed rat uteri were sectioned at 5 µm. Sections were baked at 37⁰C overnight, deparaffinized and rehydrated in xylene, 100%, 95%, 80%, and 70% ethanol baths for 5 minutes each and quenched with 3% H₂O₂ in methanol for 20 minutes. Slides were washed 3 times with PBS and antigen retrieval was performed by microwaving the slides in DAKO (Burlington, ON, Canada) Target Retrieval Solution (pH 9, diluted 1:10 in ddH₂O). Slides were first microwaved at power level 6 for 5 minutes in a closed container and then left to cool on ice for 20 minutes. The microwaving process was repeated but at power level 6 for 3 minutes in an open container and then cooled on ice for 15 minutes. Sections were washed three times for 5 minutes with PBS and isolated on the glass slide (Fisher Scientific, Ottawa, ON, Canada) using a wax pen (DAKO). Additional permeabilization was performed using 0.01% Triton X-100 (Sigma-Aldrich) diluted in PBS for 10 minutes. Slides were then blocked for 20 minutes at room temperature using protein blocking solution (DAKO, 1:1 diluted in PBS) and incubated with primary antibody (Table 2.2) for MMP-9, MMP-7 and MMP-11 – rabbit IgG (1:200, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used as the negative control – diluted in a PBS solution (1/3 DAKO antibody diluent, 2/3 PBS⁻) at 4⁰C overnight in a covered humidified box.
The following day, sections were washed three times with PBS and incubated with biotinylated secondary anti-rabbit antibody (diluted in PBS, Table 2.2) for 1 hour at room temperature in a humidified box followed by incubation with streptavidin-HRP solution (DAKO) for 1 hour at room temperature. Sections were then washed three times with PBS and developed with a DAB kit (DAKO), counterstained with Gill’s No.1 Accustain® Hematoxylin (Sigma-Aldrich), and were mounted with Surgipath Micromount® mounting media (Leica Microsystems Inc., Concord, ON, Canada). For the assessment of staining intensity, myometrial cells from each of the two sets of tissues were observed on the Olympus BX61 Motorized Microscope and MicroSuite™ system (Olympus America Inc., Melville, New York, USA). A minimum of five fields were examined for each gestational day and uterine horn for each set of tissue, and representative tissue sections were photographed with a Olympus DP72 Microscope Digital Camera (Olympus America Inc.).

2.2.7 Localization of Myometrial MMP expression and activity by in situ zymography

ZBF-fixed, paraffin-embedded whole uteri were sectioned at 5 µm, baked at 37°C for 1 hour (to adhere the sections onto glass slides), deparaffinized and rehydrated in xylene, 100%, 95%, 80%, and 70% ethanol for 5 minutes each. Sections were then incubated with fluorescent substrates: DQ gelatin, collagen I, or collagen IV (Life Technologies Inc.) in a reaction buffer (1/50 dilution) for 2 hours at 37°C in a humidified box. For a negative control (NEG) sections were pre-incubated for 1 hour at 37°C with 0.02M EDTA and 0.02M EDTA + substrate for 2 hours at 37°C. Following incubation with the fluorescent substrates, sections were post-fixed in a 4% NBF solution for 5 min, washed three times for 5 min with PBS, counterstained with DAPI.
(1:1000, Sigma-Aldrich) for 10 min, and mounted with a coverslip using Immu-Mount mounting media (Thermo Fisher Scientific Inc.).

2.2.8 Localization of Leukocyte MMP expression and activity by in situ zymography

Immunolocalization of MMP expression by leukocytes and specifically macrophages was defined using a mouse anti-CD45 (leukocyte common antigen) and anti-CD68 (macrophage-specific marker) specific antibodies. Immediately following incubation with DQ gelatin, collagen I or collagen IV fluorescent substrates and 4% NBF post-fixation for in situ zymography, sections were blocked using a protein blocking solution (DAKO) for 1 hour at room temperature, then incubated with primary anti-CD45 (BioLegend, San Diego, CA, USA) and anti-CD68 (AbD Serotec, Bio-Rad, Raleigh, NC, USA) antibody overnight at 4°C in a humidified box. Sections were washed three times for 5 minutes with PBS, cell nuclei were counter-stained using DAPI (1:1000) in conjunction with the secondary antibody (donkey anti-mouse Alexa Fluor 546, Life Technologies Inc.) for 1 hour at room temperature. Slides were washed once with PBS and mounted with a coverslip using Immu-Mount mounting media (Thermo Fisher Scientific Inc.). Images for regular and in situ zymography were taken using a spinning disc confocal Leica DMI6000 B Inverted Microscope for Biomedical Research system (Leica Microsystems Inc.) and the Volocity (Version 6.3) 3D Image Analysis software (PerkinElmer, R&D, Woodbridge, ON, Canada).
2.3 EFFECT OF IN VITRO MECHANICAL STRETCH ON MMP EXPRESSION

2.3.1 In vitro primary human myometrial cell model of mechanical stretch

In addition to rat primary myometrial cells I used human primary myometrial cells to investigate the direct effect of static mechanical stretch on MMP/TIMP expression and secretion. Tissue biopsies were acquired from term-not-in-labour women undergoing elective caesarian section and enzymatically digested. The uterine sample was placed in HBSS++ containing 1x Pen-Strep-AmpB (Lonza, VWR International) and 50 µg/mL of Normocin™ (InvivoGen, San Diego, CA, USA), and brought to a biological safety cabinet. Any remaining fat and blood vessels were carefully excised and the sample was rinsed with fresh HBSS++. The sample was then cut into approximately 1mm x 2mm pieces, washed 3 times for 5 minutes with HBSS and 5mL of warm enzyme mix were added, transferred to a 50mL tube (Greiner Bio-One, VWR International), and incubated in a water bath (37°C) for 1 hour. Following 1 hour of enzymatic digestion, 5mL of dissociation mix were added (to stop digestion) and the tissue solution was pipetted, passed through a 100µm metal cell strainer, and the collected primary cells were placed on ice. The leftover tissue was put into a clean 50mL tube and 5mL of fresh warm enzyme mix was added. The tissue was again incubated for 1 hour in a 37°C water bath; the enzymatic digestion was repeated 4 times or until all of the uterine tissue was digested.

The collected cell suspension was centrifuged for 15 minutes at 250 g at 4°C. The supernatant was aspirated and the pellet was resuspended in 5mL fresh 10% FBS DMEM (with Pen-Strep-AmpB and Normocin) and the cell suspension was passed through a 23G needle. Cells were counted using the CASY Cell Counter and analyzer system (Roche Innovatis AG, Roche Diagnostics, Laval, QC, Canada), plated into 10 cm diameter tissue culture dishes with 10% FBS DMEM and left to grow in a 5% CO₂, 37°C incubator. Cells were washed daily with HBSS− (with
Pen-Strep-AmpB and Normocin) and DMEM was changed. Once the plates were 90-100% confluent, cells were harvested by trypsinization with 0.5% Trypsin-EDTA (Gibco®, Life Technologies Inc.). Cells were collected into 15mL tubes (Greiner Bio-One, VWR International) and centrifuged at 180 g for 5 minutes at 4°C to pellet the cells and the supernatant was aspirated. Fresh warm 20% FBS DMEM were then added to resuspend the pellet and cells were passaged.

At passage 4, 200,000 cells in 3mL/well were plated onto collagen I-coated 6-well Flexcell plates (Flexcell International Corp.) and allowed to grow until 75-80% confluency. When confluent, cells were serum starved for 24 hours with 5mL/well of serum-free DMEM with a insulin-transferrin-selenium-sodium pyruvate supplement (ITS-A, Life Technologies Inc.) and subjected to a static mechanical stretch using a computer-driven in vitro stretch system (FX-5000, Flexcell International Corp.) in a 5% CO₂, 37°C incubator for 24 hours at 25% elongation, or were stretched for 24 hours and then left for another 24 hours without stretch to simulate postpartum relaxation following pregnancy (total of 48 hours). Control plates were left unstretched in the same incubator to provide similar environmental conditions.

2.3.2 Assessment of cell viability following in vitro static mechanical stretch

The assessment of cell viability following static stretch and/or additional 24 hours of relaxation was employed using propidium iodide (PI) and fluorescein diacetate (FDA) staining (Sigma-Aldrich). The principle behind this method is that FDA is taken up by live cells, and the non-fluorescent dye is converted to the green fluorescent fluorescein metabolite. Whereas PI is a nuclear staining dye that can pass through permeated cell membranes of dead cells and intercalates with DNA to fluoresce red [152]. Immediately after stretch, cells were washed twice and then stained with PI and FDA by incubating with 1mL of PBS, 500µL of FDA (20µg/mL) and 150µL
of PI (20µg/mL) for 3 minutes at room temperature. The solution was aspirated and the flexible membranes of the Flexcell plate containing the stained cells were carefully cut out with a blade and immediately mounted onto a glass slide (Fisher Scientific) without mounting media. The slides were viewed and images were taken immediately using a spinning disc confocal microscope Leica DMI6000 B Inverted Microscope for Biomedical Research system (Leica Microsystems Inc.) and the Volocity 3D Image Analysis software (PerkinElmer, R&D) at 100x and 200x magnification.

2.3.3 Characterization of primary human myometrial cells by immunocytochemistry

To characterize the origin of enzymatically isolated cells, a small aliquot of the cell suspension (~100-1000 cells) at passage 0, 1, 2, 4, 6 and 8 was plated onto sterile microscope cover glass (22x30, Fisher Scientific) and left to attach in the 37°C incubator overnight. Cells were washed twice in PBS, fixed in 4% PFA (diluted in PBS) for 20 minutes. Plates were rinsed once for 5 minutes with PBS and stored in the 4°C fridge for at least 24 hours until further processing. Next, coverslips were rinsed with fresh PBS, permeabilized with PBS-T (250µL of Tween-20 in 500mL of PBS−) for 15 minutes, then washed with PBS and blocked in 5% FBS blocking solution (46.5mL of PBS, 2.5mL of FBS, 1mL of donkey serum) for 30 minutes at room temperature. Next, coverslips were transferred to a plate covered in aluminum foil, outlined with a wax pen (DAKO), the primary antibody (diluted in blocking solution) was applied (150-200µL per coverslip) and incubated overnight at 4°C (Table 2.3). Following incubation, coverslips were washed twice with PBS-T and incubated with secondary antibody (table 2.2) for 1 hour at room temperature in the covered plate. Coverslips were washed again with PBS-T, incubated for 10 minutes with DAPI (1:1000 diluted in PBS−) and carefully
mounted with Shandon Immu-Mount™ (Thermo Fisher Scientific Inc.) onto glass slides. Images were taken using a spinning disc confocal microscope Leica DMI6000 B Inverted Microscope for Biomedical Research system (Leica Microsystems Inc.) and the Volocity 3D Image Analysis software (PerkinElmer, R&D) at 100x and 200x magnification.

**Table 2.3** Information of antibodies used for immunocytochemistry

<table>
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<tr>
<th>Target</th>
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<th>Antibody Type</th>
<th>Dilution</th>
</tr>
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<tr>
<td>Smooth Muscle Actin (SMA)</td>
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<td>DAKO</td>
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<td>Mouse</td>
<td>Sigma</td>
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**2.3.4 Sample collection, preparation and RT-PCR analysis of mRNA expression**

Similarly as section 2.4.2, non-stretched (NSM) and stretched (SCM) conditioned media was collected and stored at -20°C, total RNA was isolated using TRIzol and purified using RNeasy MinElute Cleanup kit. Purified RNA was stored at -80°C or reverse transcribed to cDNA and analyzed using RT-qPCR (see section 2.2.3). Changes in human MMPs and TIMPs mRNA expression were examined using a specifically designed set of primers (Eurofins Genomics, Table 2.4). Expression levels were normalized to the geometric mean of three human housekeeping genes: cytochrome C-1 (CYC1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and TATA box-binding protein (TBP).
Table 2.4 Primer pair information for Human (Homo sapien) genes examined using RT-PCR

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Accession Number</th>
<th>Primers (5'-3')</th>
<th>Amplicon Size (base pairs)</th>
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<td>F: GCCCTCTACTGGAAGGACT&lt;br&gt;R: TGCGTATGGAAGCACAGCA</td>
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</tr>
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<td>Stromelysin 3</td>
<td>NM_005940.3</td>
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<td>Matrilysin (PUMP-1)</td>
<td>NM_002423.3</td>
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<td>Macrophage Metalloelastase</td>
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<td>NM_003254.2</td>
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<td>NM_003255.4</td>
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<td>Tissue Inhibitor of Metalloproteinase 4</td>
<td>NM_003256.3</td>
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† Genes excluded from statistical analysis; either not detected or n < 4 after exclusion
‡ Primers designed based on human MMP primer sequences from [153]
2.3.5 Analysis of conditioned media for secreted MMPs and TIMPs’ protein expression using human Luminex assays

The Bio-Plex Pro™ Human 9-plex MMP (MMP-1, 2, 3, 7, 8, 9, 10, 12, and 13) and 4-plex TIMP (TIMP-1-4) Luminex assays (Bio-Rad) were used to assess MMP secretion in NSM and SCM. This assay allows for simultaneous detection of multiple analytes within a sample in one well of a 96-well plate, through the use of magnetic beads coupled to individual monoclonal antibodies specific for the analytes of interest. The Bio-Plex® system then detects and quantifies the specific targets based on the spectral signatures (bead region) assigned to the magnetic beads (xMAP Technology, Luminex Corporation, USA) and the amount of fluorescent signal from the biotin-labeled detection antibodies and fluorescent streptavidin reporter complex bound to the analytes in each sample.

Five milliliters of the NSM and SCM were analyzed for MMP/TIMP protein expression. Prior to use, the frozen media was slowly defrosted at 4°C to prevent degradation of enzymes and kit reagents were also brought to room temperature. Secreted MMP and TIMP protein concentrations in the CM were assessed in 50 µL of the undiluted NSM and SCM samples with 0.5% BSA (in duplicate). The assays were performed according to the provided instruction manuals. The plates were washed with the Hydroflex™ microplate washer (Tecan Group Ltd., Switzerland) and data acquisition was performed and analyzed using a Bio-Plex® 200 system and Bio-Plex Manager™ 5.0 software (Bio-Rad).
2.3.6 In vitro model of mechanical stretch using primary rat myometrial cells

The direct effect of static mechanical stretch on the synthesis and secretion of MMPs/TIMPs by myometrial SMCs in vitro was also investigated using a primary rat myometrial cell model. Virgin female Wistar rats (12-15 weeks, 225-250g weight) were purchased from Charles River Laboratories (Wilmington, MA) and housed in the MaRS Toronto Medical Discovery Tower Animal Research Facility (Toronto, ON). They were maintained on standard rat chow and water in a 12:12 hour light-dark cycle. The rats were subcutaneously injected with 50µg of 17β-estradiol 24 hours prior to tissue collection.

Rats were euthanized by CO₂ inhalation and whole uteri were collected and placed in HBSS⁺⁺ (with calcium and magnesium) supplemented with 1.25mL of Penicillin-Streptomycin-Amphotericin B (Pen-Strep-AmpB) (Lonza BioWhittaker™, VWR International). Uteri were placed inside a biological safety cabinet, rinsed with fresh HBSS⁺⁺ (with Pen-Strep-AmpB), fat and blood vessels were removed. The uteri were then cut into approximately 1mm-thick rings into a 125mL Erlenmeyer flask and washed 3 times for 5 minutes with HBSS⁻⁻(without calcium and magnesium). Myocytes were enzymatically isolated by collagenase digestion: 10 ml of warm enzyme mix were added and incubated with gentle shaking at 37⁰C for one hour. 10 mL of dissociation mix were added to the flask and the mixture was pipetted 30 times to mechanically disrupt the tissue. Next the entire solution was passed through a cell strainer (pore size 40 µm) to separate the isolated myometrial cells and incompletely digested tissue. The strained cells were placed on ice and 10mL of fresh warm enzyme mix were added to the incompletely digested tissue. The flask was incubated with gentle agitation at 37⁰C for 30 minutes and the process was repeated until all tissue was digested (5-6 times).
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The collected cells were then centrifuged for 15 minutes at 4°C and 250 g, and the supernatant was discarded. Cell pellets were resuspended with warm 10% FBS in high glucose Dulbecco’s Modified Eagle Medium (DMEM, Gibco®, Life Technologies Inc.) and strained through a 70µm cell strainer (BD Falcon™, BD Biosciences, Mississauga, ON, Canada). To separate fibroblasts from primary rat myocytes, the cell suspension was plated on 15cm plastic plates (~20 mL/plate) and incubated at 37°C for 30 minutes. Myocytes were collected, stained to check their viability using 0.4% Trypan Blue solution (Lonza BioWhittaker™, VWR International), counted using a hemocytometer (50µL of Trypan Blue and 50µL of cell suspension) and plated on collagen I-coated 6-well Flexcell (Flexcell International Corp., Burlington, NC, USA) plates (2.2 million cells per well) with 3mL of DMEM. Cells were cultured for 2-3 days until 75-80% confluent, then serum starved for 24 hours with 5mL of serum-free DMEM with ITS-A (Life Technologies Inc.). Primary myocytes underwent a static mechanical stretch for 2, 8, and 24 hours (25% elongation) using a computer-driven in vitro stretch system (FX-5000, Flexcell International Corp.) in a 5% CO₂, 37°C incubator. Control non-stretched plates were left in the same incubator to provide similar environmental conditions.

2.3.7 Sample collection from rat culture plates, preparation and RT-PCR analysis of mRNA expression

After 2, 8, and 24 hours media conditioned by non-stretched (NSM) and stretched (SCM) cells were collected and plates were washed twice with ice-cold PBS. 1mL (per 3 wells of the sample treatment) of TRIzol (Life Technologies Inc.) was added to 3 wells, cells were scraped with cell scrapers (Sarstedt Inc., Montreal, QC, Canada) and TRIzol mix was transferred to a 1.5mL tubes. Similar to Section 2.3.4, total RNA was isolated, reverse
transcribed to cDNA, and subjected to RT-qPCR analysis using the same primers as the *in vivo* study (Table 2.1).

### 2.5 STATISTICAL ANALYSIS

For gestational profiles of the bilaterally pregnant rat model, analysis of statistically significant differences between groups was performed on the natural log (ln) transformation of the normalized data (relative to a corresponding GD6), unless otherwise stated, using one-way ANOVA with the Newman-Keuls post-hoc test for multiple comparisons. Specifically for samples collected from the unilaterally pregnant rat model, unless otherwise stated, statistical analysis was performed on the normalized data (relative to a corresponding GD6e) using Two-way ANOVA with Bonferroni’s post-hoc test for multiple comparisons. For *in vitro* studies, statistical analysis was performed on the fold-change data (relative to a corresponding 24 hours not-stretched sample) using one-way ANOVA and Newman-Keuls multiple comparisons test. The level of significance was set at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***)). Significant outliers within each data set were determined by the Grubb’s test (GraphPad Software, Inc., CA, USA) and removed from analysis. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc.).
3.1 INTRODUCTION

It is widely accepted that pregnancy is associated with changes in the cellular characteristics and the extracellular composition of the uterus [14, 35, 82]. As it has been previously reported, uterine SMCs in the myometrium and activated infiltrating immune cells can produce extracellular matrix-degrading MMPs before TL and during the PP period [30, 31, 78, 82, 139]. This can result in restricted collagenolysis and can affect the function of cellular barriers due to the broad variety of MMP substrates available in the uterus [30, 78, 99, 134, 136]. The increasingly recognized role of MMPs as activators and regulators of inflammatory pathways suggests their potential role as key immunoregulatory enzymes in reproductive tissues, specifically in the myometrium [31, 75, 78, 82, 99, 124]. Therefore, we hypothesize that during late pregnancy, the expression and activity of myometrial MMPs increases in preparation for two major events: 1) term labour and 2) PP uterine involution. Our aim was to 1) characterize the myometrial expression of MMPs and TIMPs during pregnancy, TL and PP, 2) define putative mechanisms that regulate their expression and/or activity. Our findings will contribute to defining the involvement of MMPs in inflammatory pathways within uterine tissue and will provide insight on the mechanism responsible for labour induction.
3.2 GESTATIONAL REGULATION OF MMP GENE EXPRESSION IN THE RAT MYOMETRIUM

Gestational regulation of MMP gene expression was assessed using two rat models of pregnancy. Six sets of rat myometrial tissue were collected from bilaterally pregnant rats throughout gestation (GD6, 8, 10, 12, 14, 15, 17, 19, 21, 22), during active term labour (GD23), and the PP period (1PP and 4PP). In addition, four sets of rat myometrial tissue were collected from both the empty (e) and gravid (g) uterine horns throughout gestation (GD6, 12, 15, 17, 19, 21, 23, 1PP) from unilaterally pregnant rats. mRNA levels of ten specific soluble MMPs and four TIMPs was analyzed using RT-qPCR and normalized against three housekeeping genes: PPIA, PDGF-A, and TBP or IGF1R (for bilaterally or unilaterally pregnant samples respectively). Specific focus was placed on analysing gestational changes in mRNA expression observed for collagenases (MMP-1, 8, 13), gelatinases (MMP-2, 9), stromelysins (MMP-3, 10, 11), matrilysin (MMP-7), metalloelastase (MMP-12) and TIMP-1, 2, 3, 4. The results acquired from RT-qPCR analysis were expressed as a fold-change relative to GD6 (early gestation) to picture the change in MMPs’ mRNA expression throughout gestation; data for MMP-1 was excluded due to the low expression of mRNA transcripts observed (Ct values > 33). Statistical analysis for the bilaterally pregnant tissues was performed on the non-transformed relative expression data (determined with the computed ΔΔCT method) using one-way ANOVA and the Newman-Keuls multiple comparisons test. Statistical analysis for the bilaterally pregnant tissues was performed on the transformed relative fold-change data (relative to GD6e) using two-way ANOVA and Bonferroni’s multiple comparisons test.
3.2.1 Myometrial MMP mRNA expression in the bilaterally pregnant rat model

mRNA levels of the well-studied class of soluble MMPs, the gelatinases (*MMP-2&9*), determined by RT-qPCR were relatively stable throughout bilaterally pregnant rat gestation and labour, with an increase in mRNA expression 1 day PP (Figure 3.1). Furthermore, *MMP-2* mRNA levels were consistently high throughout gestation (Ct values approximately 22-25) and were upregulated 2-3-fold in the PP period (Ct values approximately 20-22) supporting a role of MMP-2 in tissue remodeling 1) to accommodate the growing fetus and 2) for PP involution. In contrast, mRNA levels for *MMP-9* were lowly expressed throughout gestation as depicted by Ct values of approximately 31-33. Similarly, mRNA levels of the collagenases (*MMP-8&13*) and two stromelysins (*MMP-3&10*) were low throughout gestation compared to a sharp increase observed 1 day PP compared to GD6.

Most interesting is that the myometrial gene expression of *MMP-7* (Figure 3.4) in bilaterally pregnant rats was very low throughout gestation (GD6-GD21) but was dramatically upregulated in the term rat myometrium (GD22, 200-fold increase) and further increased in the labouring myometrium at GD23 (350-fold increase). Importantly, *MMP-7* remained elevated 1 day PP (150-fold increase) and 4 day PP (20-fold increase). The drastic changes observed for *MMP-7* gene expression at term on GD22 could reflect a role for MMP-7 during the preparation of the uterus for the active labouring process. The continuous expression of mRNA during GD23 and 1PP supports the role of this enzyme in tissue remodeling to support active labour contractions and involution for transitioning the uterus back to a pre-pregnant state. Similar to *MMP-7*, *MMP-11* (stromelysin-3) exhibited sustained elevation of transcript levels during term, active labour and PP which may indicate that this specific stromelysin is also important for promoting the labour process and PP involution alongside MMP-7. Both genes displayed very similar gestational
profiles; however, only changes for MMP-7 were found to be significant during labour. Whereas mRNA levels for MMP-12 were significantly elevated during GD22, they immediately decreased during active labour (GD23) – suggesting the existence of a protective mechanism stabilizing the myometrium during active labour contractions – and was again induced during the PP period. This may suggest the observed changes to MMP-12 gene expression is contributed by immune cells infiltrating the myometrium prior to labour and during PP involution.

Evidently, all MMPs studied experienced a significant increase in mRNA expression 1 day PP (1PP), and this highest level of mRNA expression validates their role for promoting tissue remodeling during PP involution. In regards to their endogenous tissue inhibitors, TIMP-1 and TIMP-2 gene expression was elevated at mid-gestation compared to the PP period, whereas TIMP-3 and TIMP-4 expression was constant during gestation but elevated during the PP period (Figure 3.5). This correlates with the suggested role of TIMP-1 and TIMP-2 in suppressing MMP activity during pregnancy whereas TIMP-3 and TIMP-4 may play more of a role in regulating tissue remodeling in the PP period.
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Figure 3.1: Gestational expression of Gelatinases’ mRNA in bilaterally pregnant, laboring and postpartum (PP) rat myometrium.
Transcript levels of MMP-2 (A) and MMP-9 (B) were both significantly upregulated in the rat myometrium during 1PP and 4PP compared to the rest of gestation supporting their role in PP involution. mRNA expression was normalized to three housekeeping genes, Cyclophilin A (PPIA), platelet-derived growth factor A (PDGF-A), and TATA-binding protein (TBP). Statistical analysis (one-way ANOVA and the Newman-Keuls multiple comparisons test) was performed on the ln-transformed data and are presented as fold-changes relative to a corresponding GD6 within each run. The bars represent mean±SEM (n=5-6/GD). Bars with different letters are statistically different from each other. p<0.001.
Figure 3.2: Gestational expression of Collagenases’ mRNA in bilaterally pregnant, laboring and postpartum rat myometrium.

Transcript levels of Collagenases: *MMP-8* (A) and *MMP-13* (B) were both highly upregulated in the rat myometrium during 1PP compared to the rest of gestation supporting their role for promoting tissue remodeling in PP involution. mRNA expression was normalized to three housekeeping genes, *PPIA*, *PDGF-A*, and *TBP*. Statistical analysis (one-way ANOVA and the Newman-Keuls multiple comparisons test) was performed on the ln-transformed data and are presented as fold-changes relative to a corresponding GD6 within each run. The bars represent mean±SEM (n=5-6/GD). Bars with different letters are statistically different from each other. *p*<0.001.
Figure 3.3: Gestational expression of Stromelysin's mRNA in bilaterally pregnant, laboring and postpartum rat myometrium.

Transcript levels of MMP-3 (A), MMP-10 (B) and MMP-11 (C) were significantly upregulated in the rat myometrium during 1PP compared to the rest of gestation supporting their role in PP involution. mRNA expression was normalized to three housekeeping genes, PPIA, PDGF-A, and TBP. Statistical analysis (one-way ANOVA and the Newman-Keuls multiple comparisons test) was performed on the ln-transformed data and are presented as fold-changes relative to a corresponding GD6 within each run. The bars represent mean±SEM (n=5-6/GD). Bars with different letters are statistically different from each other. $p<0.001$. 

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Figure 3.4: Gestational expression of Matrilysin (MMP-7) and Macrophage Metalloelastase’s (MMP-12) mRNA in bilaterally pregnant, laboring and postpartum rat myometrium.

Transcript levels of MMP-7 (A) was induced in the rat myometrium during late gestation (GD22), further increased during term labour (GD23), and remained elevated during the PP period. MMP-12 (B) expression was induced during GD22, decreased during active labour (GD23) but increased during the PP period. mRNA expression was normalized to three housekeeping genes, PPIA, PDGF-A, and TBP. Statistical analysis (one-way ANOVA and the Newman-Keuls multiple comparisons test) was performed on the ln-transformed data and are presented as fold-changes relative to a corresponding GD6 within each run. The bars represent mean±SEM (n=5/GD). Bars with different letters are statistically different from each other. p<0.001.
Figure 3.5: Gestational expression of TIMP mRNA in bilaterally pregnant, laboring and postpartum rat myometrium.

mRNA expression of *TIMP-1* (A) and *TIMP-2* (B) remained constant throughout gestation while transcript levels of *TIMP-3* (C) and *TIMP-4* (D) increased during the PP period. mRNA expression was normalized to three housekeeping genes, *PPIA*, *PDGF-A*, and *TBP*. Statistical analysis (one-way ANOVA and the Newman-Keuls multiple comparisons test) was performed on the ln-transformed data and are presented as fold-changes relative to a corresponding GD6 within each run. The bars represent mean±SEM (n=5/GD). Bars with different letters are statistically different from each other. *TIMP-1 &2 p<0.01, TIMP-3&4 p<0.001*
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A

Comparative Fold Change

Gestational Day

B

Comparative Fold Change

Gestational Day

C

Comparative Fold Change

Gestational Day

MMP-10
MMP-3
MMP-11

MMP-7
MMP-12

MMP-8
MMP-13

6 8 10 12 14 16 18 20 22 24 1PP 4PP
Figure 3.6: Comparative characteristics of MMP and TIMP gene expression in the pregnant, term labour, and postpartum rat myometrium.
A) Relative gene expression of stromelysins (MMP-10,3 & 11), B) matrilysin (MMP-7) and metalloelastase (MMP-12), C) collagenases (MMP-8 & 13), D) gelatinases (MMP-2 & 9), and E) TIMPs (TIMP-1-4). Normalized expression of mRNA levels (as calculated by the ΔΔCT method) were expressed as a fold-change relative to MMP-10’s (lowest expression among all MMPs and TIMPs) gene expression on GD6, which was chosen as a calibrator for a comparative study.
Total mRNA expression throughout gestation, TL and PP for MMPs and TIMPs was further analyzed in a comparative study. The normalized expression calculated by the ΔΔCT method (Bio-Rad CFX Manager software) were compared to visualize the relative gestational changes of the MMPs and TIMPs genes studied (Figure 3.6). MMP-10’s gene expression was lowest among all MMP and TIMP therefore MMP-10’s expression on GD6 was chosen as a calibrator. The data were analyzed and grouped by MMP classes and revealed that all (the stromelysins, matrilysin, metalloelastase, and collagenases) exhibited low mRNA abundance throughout gestation. In contrast, the gelatinases were highly expressed throughout gestation relative to other MMP subgroups, especially MMP-2, showing at least a 200-fold relative expression compared to MMP-10. Relative to MMP-10, the other stromelysins (MMP-3 & 11), collagenases and gelatinases showed the highest increase 1PP (at least 350-fold), whereas MMP-7 and MMP-12 show weaker increases in mRNA on 1PP (150-fold). Furthermore MMP-7 displayed the highest level of gene expression (at least 550-fold relative expression) during active TL compared to all MMPs and TIMPs analyzed. This comparative study further suggests the abundance of MMP mRNA is MMP-specific during TL to regulate myometrial function, and all MMPs are upregulated in the PP period to promote post-labour remodeling of the uterus.

Interestingly, the TIMPs’ mRNA levels were also highly expressed compared to the majority of MMPs (except for the gelatinases) throughout gestation, supporting their involvement in regulating MMP activity. TIMP-2 exhibited the highest level of TIMP expression throughout gestation, while TIMP-1 & 3 had a similar pattern of expression similar to that of the gelatinases. These findings support the inhibitory role of TIMP-2 for the majority of MMPs, and potential interaction between TIMP-1 & 3 with MMP-2 & 9 to form inhibitory complexes in the rat myometrium throughout gestation. Importantly, TIMP-3 & 4 were highly upregulated PP (450-fold), suggesting their role in regulating uterine involution.
3.2.2 Myometrial MMP mRNA expression in the unilaterally pregnant rat model

To assess the effect of biological uterine stretch on the expression of MMPs/TIMPs in vivo, a unilaterally pregnant rat model was used (in collaboration with Dr. O. Shynlova). Myometrial tissues from empty (non-pregnant) and gravid (pregnant) uterine horns were collected at different gestational days. Gene expression was normalized to three housekeeping genes (PPIA, PDGF-A, and IGFR1) and expressed as fold-changes relative to a GD6 empty (e) horn sample. As expected, gene expression of all MMPs was very low in the empty horn myometrium during gestation and term labour. Analysis of the full spectrum of specific soluble MMPs in the gravid uterine horn (described above for bilaterally pregnant rats) showed a very similar expression profile during pregnancy, labour and the PP involution period. There were no statistically significant differences (by Bonferroni’s multiple comparisons test) in mRNA expression between the empty and gravid horn during gestation and active labour (GD23) of the gelatinases (MMP-2 and 9) and the majority of the MMPs studied (MMP-3, 8, 10, 11, 12).

However, MMP-3, 8, 9, 10, and 12 all exhibited a significantly higher expression of transcript levels in the gravid horn compared to the empty horn on 1 day PP (Figure 3.7-3.9). A dramatic increase in mRNA expression of the stromelysin MMP-11 was observed in both the empty and gravid horns 24 hours after term labour, possibly indicating that expression of this gene during the PP period was regulated by hormonal changes rather than by the release of mechanical stretch of the uterus after the expelling of the fetuses. Furthermore, the upregulation of certain MMP mRNA 1 day PP specifically in the gravid horn and not the empty shows a correlation with remodeling the pregnant tissue back to a non-pregnant state.
Figure 3.7: Expression of Gelatinases MMP-2 (A) and MMP-9 (B) genes in the myometrium of unilaterally pregnant rats during gestation, term labour and postpartum.

Transcript levels in empty (white bars) and gravid (gray bars) uterine horns were normalized to three housekeeping genes, PPIA, PDGF-A and IGFR1 levels and expressed in fold-changes relative to a GD6 empty sample. The bars represent mean±SEM (n=3-4/GD). Statistical analysis was performed on the natural log-transformed data using two-way ANOVA and the Bonferroni’s multiple comparisons test. A significant difference between the gravid and empty horn of the same gestational day is indicated by ***(p<0.001)***. Gestational days with different letters (gravid horn) or Greek symbols (empty horn) are statistically different from each other within the respective horns; *p*<0.05. Gestational days without letters or Greek symbols are not statistically different from each other by two-way ANOVA.
Figure 3.8: Expression of Stromelysins *MMP-3* (A), *MMP-10* (B) and *MMP-11* (C) genes in the myometrium of unilaterally pregnant rats during gestation, term labour and postpartum.

Transcript levels in empty (white bars) and gravid (gray bars) uterine horns were normalized to three housekeeping genes, *PPIA*, *PDGF-A*, and *TBP* and expressed in fold-changes relative to a GD6 empty sample. The bars represent mean±SEM (n=3-4/GD). Statistical analysis was performed on the relative fold-change data using two-way ANOVA and the Bonferroni’s multiple comparisons test. A significant difference between gravid and empty horn of the same gestational day is indicated by *** (*p*<0.001). Gestational days with different letters (gravid horn) or Greek symbols (empty horn) are statistically different from each other within the respective horns; *p*<0.05. Gestational days without letters or Greek symbols are not statistically different from each other by two-way ANOVA.
Figure 3.9: Expression of Collagenase (MMP-8, A), Matrilysin (MMP-7, B) and Metalloelastase (MMP-12, C) genes in the myometrium of unilaterally pregnant rats during gestation, term labour and postpartum.

Transcript levels in empty (white bars) and gravid (gray bars) uterine horns were normalized to three housekeeping genes (PPIA, PDGF-A, and TBP) expressed in fold-changes relative to a GD6 empty sample. The bars represent mean±SEM (n=3-4/GD). Statistical analysis was performed on the relative fold-change data (MMP-3&7) or ln-transformed data (MMP-12) using two-way ANOVA and the Bonferroni’s multiple comparisons test. A significant difference between gravid and empty horn of the same gestational day is indicated by *(p<0.05), **(p<0.01), ****(p<0.001). Gestational days with different letters (gravid horn) or Greek symbols (empty horn) are statistically different from each other within the respective horns; p<0.05. Gestational days without letters or Greek symbols are not statistically different from each other by two-way ANOVA.
Interestingly, in the case of *MMP-7*, we observed upregulation of mRNA in the gravid (pregnant) horn as compared to the empty horn during GD23, suggesting an effect of either mechanical stretch or the presence of the fetal/placental unit on *MMP-7*’s transcript levels (Figure 3.9). More specifically, the induction of uterine contractions during labour and the removal of mechanical stretch after expelling of fetuses may be involved with the upregulation of *MMP-7*. This phenomenon was also observed for *MMP-12*, which also displayed higher mRNA levels within the gravid horn during late gestation in addition to TL and PP – beginning from GD19 – compared to the empty horn, suggesting a correlation between *MMP-12* mRNA expression and myometrial transition to a contractile phenotype in preparation for active TL. On the contrary, we also detected a significant upregulation of *MMP-7* in the empty uterine horn during active labour contractions suggesting mechanical stretch is not the sole regulator of gene expression. These findings support the hypothesis of 1) MMPs having a dual role in the myometrium as key regulators of tissue remodeling during labour and PP involution and 2) different regulatory mechanisms exists in the transcriptional activation of different MMP genes.
3.3 GESTATIONAL REGULATION OF MMP PROTEIN EXPRESSION IN THE RAT

MYOMETRIUM

3.3.1 Western blot analysis of MMP levels and gelatin zymography of the myometrial tissue from bilaterally pregnant rats

To investigate whether the upregulated expression of mRNA transcripts resulted in the production and secretion of MMPs by the myometrium, gestational changes in protein levels were further assessed by WB. Specific interest was on the well-studied gelatinases (MMP-2&9), the collagenase (MMP-8), the major stromelysins (MMP-3&11), and the uterine matrilysin (MMP-7) to identify any correlation between mRNA gestational changes with protein expression. Statistical analysis was performed on the ln-transformed relative fold-change data using one-way ANOVA and Newman-Keuls multiple comparisons test.

MMP-8 is a major collagenase known to be secreted mainly by neutrophils and has a high affinity for fibrillar collagen I, a major component of the uterine ECM. MMP-8 protein expression was highly expressed with no significant changes throughout gestation, TL, and PP. This, however, did not correlate with transcript levels that exhibited a dramatic increase 1 day PP.
Figure 3.10: Protein levels of Collagenase MMP-8 in the rat myometrium throughout gestation, labour and post-partum.

Representative Western blots (A) and densitometric analysis of Neutrophil Collagenase (B, MMP-8) protein expression levels were normalized to Calponin. Statistical analysis (one-way ANOVA) was performed on the ln-transformed data and are presented as fold-changes relative to a corresponding GD6 within each run. Bar graphs showing the mean±SEM (n=4/GD). Any differences between gestational days were not found to be statistically significant by one-way ANOVA.
Figure 3.11: Protein levels of Stromelysins in the rat myometrium throughout gestation, labour and postpartum.

Representative Western blots (A) and densitometric analysis of Stromelysins (B, MMP-3 and C, MMP-11). MMP protein expression levels were normalized to Calponin. Statistical analysis (one-way ANOVA and Newman-Keuls multiple comparisons test) was performed on the ln-transformed data and are presented as fold-changes relative to a corresponding GD6 within each run. Bar graphs showing the mean±SEM (n=4/GD). Bars with different letters are statistically different from each other (MMP-11 p<0.01). Gestational days without letters are not statistically different from each other by one-way ANOVA.
Figure 3.12: Protein levels of Matrilysin and Metalloelastase in the rat myometrium throughout gestation, labour and post-partum.
Representative Western blots (A) and densitometric analysis of Matrilysin (B, MMP-7) and Metalloelastase (C, MMP-12). MMP protein expression levels were normalized to Calponin. Statistical analysis (one-way ANOVA and Newman-Keuls multiple comparisons test) was performed on the ln-transformed data and are presented as fold-changes relative to a corresponding GD6 within each run. Bar graphs showing the mean±SEM (n=4/GD). Bars with different letters are statistically different from each other (MMP-7 $p<0.001$ and MMP-12 $p<0.05$). Gestational days without letters are not statistically different from each other by one-way ANOVA.
Stromelysins, known to cleave a variety of ECM substrates, mRNA levels were dramatically upregulated during PP involution, however, the protein immunoblot analysis of the major stromelysin MMP-3 showed stable protein levels throughout gestation, labour and PP. On the contrary, active stromelysin MMP-11 protein showed a dramatic upregulation at late gestation (starting from GD17) and a significant increase in protein expression on GD23, as compared to early gestation. The gestational profile for protein expression of MMP-7 and MMP-12 were also analyzed using WB analysis. MMP-7 (29 kDa) was significantly upregulated on GD23 (Figure 3.12) and depicts a similar pattern as transcript levels of *MMP-7* (Figure 3.4). MMP-12 was highly and relatively stable expressed throughout gestation, with a slight increase in protein expression in the early PP period (Figure 3.12). This MMP-12 protein expression pattern was different to *MMP-12* gene, which was increased 150-fold on GD22 (Figure 3.4) but this upregulation was not seen at the protein level. The observed increase in protein levels of MMP-7 and MMP-11 on GD23 suggest their involvement in tissue remodeling of the myometrium to promote labour contractions.

Protein activity observed for active MMP-2 using gelatin zymography (Figure 3.13) showed a significant induction at 1 day PP, a profile similar to the expression of *MMP-2* gene. Active MMP-9 protein activity detected by gelatin zymography was very low and could not be accurately analysed.
Figure 3.13: Representative Gelatin Zymography (A) and Densitometric analysis (B), (C). Gelatinase activity of total protein (50µg/well) in rat myometrial tissues throughout gestation, labour and postpartum (visualized as light bands and presented as fold-change relative to a corresponding GD6). MMP-2 activity is observed predominantly at 68 kDa (latent pro-form) and 62 kDa (active form). MMP-9 at 92 kDa (latent pro-form) and 87 kDa (active form) was very low throughout gestation. MMP-9 was observed in the decidua conditioned media used as a positive control (+ve). (B) Densitometric analysis of the gelatin zymography; bars represent the relative density of MMP-2. The results shown are the mean±SEM (n=4/GD). Statistical analysis (one-way ANOVA and Newman-Keuls multiple comparisons test) was performed on the fold-change data. Significant differences between gestational days were indicated by different letters (pro-MMP-2 $p<0.01$ and active MMP-2 $p<0.001$).
3.3.2 Western blot analysis of MMP levels and gelatin zymography of the myometrial tissue from unilaterally pregnant rats

We examined whether the differences in MMP-7 mRNA expression between the empty and gravid horn observed during GD23 and 1PP, resulted in similar changes in protein levels. Visually, there were no differences in MMP-7 protein expression between the gravid and empty horns during labour and 1PP. Yet statistical analysis (two-way ANOVA and Bonferroni’s multiple comparisons test) revealed that MMP-7 protein expression in the gravid horn was statistically significantly different from the empty horn. This phenomenon is due to the inexplicable dramatic decrease in the expression of the housekeeping protein calponin (that we used as a loading control) in the gravid horn myometrium 1 day PP (Figure 3.14). Furthermore, calponin was only detectable in three of the four data sets for gravid 1PP thus the only optical density detected for 1PP was applied to the remaining three data sets for normalization. This decrease in expression was also observed in the bilaterally pregnant rat model (Figure 3.10-12), however, for unknown reason, the down-regulation was more dramatic in the unilaterally pregnant model. Interestingly, this correlates with Brodt-Eppley and Myatt, who also used calponin for normalization and detected a slightly lower expression of calponin during early PP in the bilaterally pregnant rat myometrium [154].

Surprisingly, immunoblot analysis of MMP-12 protein expression revealed an increase in active MMP-12 expression (not detected in the bilaterally pregnant myometrium) within the gravid horn compared to the empty horn (Figure 3.14). These results further confirm that MMP expression is not exclusively regulated by mechanical stretch of the rat myometrium, but rather other mechanisms, potentially endocrine factors or activated leukocytes (known sources of MMP secretion) might also be responsible for the difference in MMP expression within the empty and
gravid uterine horns [78, 85]. For instance, the PP MMP-12 could be secreted by infiltrating macrophages [20, 30, 82].

Gelatin zymography revealed a similar expression profile between pro-MMP-2 protein and MMP-2 mRNA throughout gestation, depicting no differences between the empty and gravid uterine horns (Figure 3.15). In contrast, starting from early gestation until term active MMP-2 was higher in the empty horn compared to the gravid horn. This suggests that during gestation and labour, MMP-2 activity in the gravid horn is suppressed by the presence of fetal/placental unit and it is activated during PP for the remodeling/involution process, when the fetuses are expelled. We speculate that in the empty horn, this suppression is absent due to a lack of factors produced by placenta and/or fetus.
Figure 3.14: Protein levels of Matrilysin and Macrophage Metalloelastase in the unilaterally pregnant rat myometrium throughout gestation, labour and postpartum.

Representative Western blots (A) and densitometric analysis of Matrilysin (B, MMP-7) and MMP-12 (C, pro-MMP-12 and D, active MMP-12). MMP protein expression levels were normalized to Calponin and expressed as fold-change relative to a GD12 empty horn (e) sample. Bar graphs showing the mean±SEM (n=4/GD). Statistical analysis was performed on the relative fold-change data using two-way ANOVA and the Bonferroni’s multiple comparisons test. A significant difference between the gravid and empty horn of the same gestational day is indicated by *** (p<0.001). Gestational days with different letters (gravid horn) or Greek symbols (empty horn) are statistically different from each other within the respective horns; p<0.05. Gestational days without letters or Greek symbols are not statistically different from each other by two-way ANOVA.
Figure 3.15: Representative Gelatin Zymography (A) and Densitometric analysis (B), (C). Gelatinase activity of total protein (50µg/well) in unilaterally pregnant rats presented as empty (e) vs. gravid (g) (visualized as light bands). Densitometric analysis was presented as fold-change relative to the GD6 empty horn (6e). MMP-2 activity is observed predominantly at 68 kDa (latent pro-form) and 62 kDa (active form). MMP-9 at 92 kDa (latent pro-form) and 87 KDa (active form) was undetectable throughout gestation similar to the bilaterally pregnant rats. (B) Densitometric analysis of the gelatin zymography; bars represent the relative density of MMP-2. The results shown are the mean±SEM (n=4/GD). Statistical analysis was performed on the relative fold-change data using two-way ANOVA and the Bonferroni’s multiple comparisons test. A significant difference between the gravid and empty horn of the same gestational day is indicated by *(p<0.05), **(p<0.01), and *** (p<0.001). Gestational days with different Greek symbols are statistically different from each other within the empty horn; p<0.05. Gestational days without letters or Greek symbols are not statistically different from each other by two-way ANOVA.
3.4 TEMPORAL AND SPATIAL LOCALIZATION OF MMPs IN THE RAT MYOMETRIUM

3.4.1 Tissue localization of MMP in the rat myometrium by immunohistochemistry

Temporal localization of MMP proteins was first assessed in formalin-fixed and paraffin-embedded tissue sections collected during mid-gestation (GD15), late gestation (GD21), term labouring (GD23) and 1 day PP using antibodies specific to MMP-7 (Figure 3.16), MMP-9 (Figure 3.17), and MMP-11 (Figure 3.18). MMPs were localized to both longitudinal (LM) and circular (CM) myometrial layers throughout gestation. We noticed a difference in the spatial localization of MMPs: during mid- and late-gestation myometrial MMP expression was predominantly intracellular within the perinuclear region of SMCs, however during TL and PP involution there was a shift to extracellular localization.

Interestingly, MMP-7 and 9 were highly expressed at term (GD21) and during labour (GD23) but only MMP-7 remained elevated PP. Immunostaining results suggests that MMP-9 protein is expressed within the rat myometrium during gestation although the enzyme is not active (as revealed by gelatin zymography, Figure 3.13). MMP-11 expression was seen primarily intracellular throughout gestation with highest extracellular levels during 1PP. Altogether we observed that intracellular and extracellular MMP protein expression in the rat uterus is increased as gestation progresses towards term labour and peaked during PP involution. In addition, MMP expression was not only observed in myometrial SMCs but very often much stronger staining was detected in cells morphologically distinct from SMCs – suggestive of infiltrating immune cells – localized especially around myometrial blood vessels and in the vascular plexus, the area between longitudinal and circular myometrial layers.
Figure 3.16: Temporal and spatial localization of MMP-7 in pregnant, labouring and postpartum rat uterus.
Tissue sections from GD 15, GD21, labouring (GD23), and 1 day PP (1PP) rat uteri were immunostained for MMP-7 (brown immunostain) and counterstained with haematoxylin. Positive immunostaining was observed in both the circular myometrial layer (CM), longitudinal myometrial layer (LM) and decidua (Dec) throughout gestation. Single cell staining (red arrow) is suggestive of MMP-7 expression by infiltrating immune cells while perinuclear staining suggests expression by SMCs. Intracellular and extracellular expression of MMP-7 increased drastically in GD23 and 1PP animals. No staining was detected in the negative control slide (1PP) using rabbit IgG. Magnification: 100x
Figure 3.17: Temporal and spatial localization of MMP-9 in pregnant, labouring and postpartum rat uterus.
Tissue sections from GD 15, GD21, labouring (GD23), and 1 day PP (1PP) rat uteri were immunostained for MMP-9 (brown immunostain) and counterstained with haematoxylin. Positive immunostaining for MMP-9 was observed in the longitudinal myometrial layer (LM), the circular myometrial layer (CM), and the decidua throughout gestation. Expression was predominantly perinuclear during mid and late gestation (GD15, GD21) and PP by smooth muscle cells. Increased extracellular expression of MMP-9 was primarily observed in the LM during labour, with some expression present in non-SMC cells (red arrow) suggestive of infiltrating immune cells. MMP-9 expression began to decrease in PP animals. No staining was detected in the negative control slide (GD23) using rabbit IgG. Magnification: 100x
Figure 3.18: Temporal and spatial localization of MMP-11 in pregnant, labouring and postpartum rat uterus.
Tissue sections from GD 15, GD21, labouring (GD23), and 1 day PP (1PP) rat uteri were immunostained for MMP-11 (brown immunostain) and counterstained with haematoxylin. Positive immunostaining was observed in both the circular myometrial layer (CM), longitudinal myometrial layer (LM) and decidua (Dec) throughout gestation. Expression was predominantly perinuclear in SMCs and present in non-SMC single cells (red arrow) suggestive of infiltrating immune cells. Some extracellular expression of MMP-11 was observed in the LM during late gestation and labour, and further increased in the CM during GD23 and 1PP. No staining was detected in the negative control slide (GD23) using rabbit IgG. Magnification: 100x
3.4.1 Tissue localization of MMP activity in the rat myometrium by in situ zymography

Spatial distribution/localization of MMP enzymatic activity was performed using in situ zymography with different ECM substrates (Figure 3.19). Four sets of rat uteri were collected at GD15, 21, 22, 23 and 1 day PP, fixed in a zinc-buffered fixative and paraffin-embedded. This methodology was adapted from Hadler-Olsen et al., who reported that zinc fixation and paraffin embedding was able to preserve tissue morphology and enzymatic activity better than that of frozen sections [155]. Tissue localization of MMP activity was detected in the uterine sections incubated with fluorescent substrates (1) DQ-gelatin, (2) DQ-type I collagen and (3) DQ-type IV collagen. These substrates were chosen based on a study by Gonzales et al. that reported these three ECM proteins are actively degraded by major MMPs in mice [102, 156]. Increased fluorescence indicated increased gelatin and collagen degradation by MMPs. To verify that the observed fluorescence is due to protease activity, negative control sections were pre-incubated with buffer containing 10 mM EDTA to inhibit MMPs which was followed by substrate application.
Figure 3.19: Representative images for in situ zymography using fluorescent MMP substrates: DQ gelatin (second column), DQ collagen type I (third column) and DQ collagen type IV (right column) for tissue localization of MMP activity.

Increased green fluorescence (increased gelatin, collagen I and IV degradation) was localized intracellularly to the perinuclear region of the myometrial cytoplasm during late gestation (D21&22) and term labour (D23), however during 1 day PP (1PP) fluorescence was mostly detected extracellularly in the myometrial parenchyma. Sections were pre-incubated with 0.02M EDTA for 1 hour, then incubated with EDTA+substrate as the negative control (EDTA+gelatin) of fluorescence. Slides were counter-stained with DAPI (nuclear staining). Magnification: 100x.
In situ zymography indicated high levels of MMP activity for gelatin and collagen I degradation were localized to the perinuclear region of individual myometrial SMCs at term (GD22) and during labour (GD23). Importantly, MMP activity was highly upregulated in the myometrial parenchyma on 1 day PP as shown by increased fluorescence, depicting degradation of gelatin, collagen I and collagen IV. Similar to Immunohistochemical analysis, numerous single cells morphologically different from SMCs were also observed in myometrial tissue in a close proximity to blood vessels. We speculated that in addition to SMCs, immune cells may also be a source of MMP expression. This hypothesis was tested by performing immunofluorescent staining for CD45 (leukocyte common antigen) and CD68 (macrophage marker) immediately following in situ zymography.

CD45 immunofluorescent staining in combination with in situ zymography depicted co-expression of CD45 (red fluorescence) and protease activity (green fluorescence – gelatin degradation) by activated immune cells (Figure 3.20). These data support the notion that infiltrated leukocytes within the myometrium expresses MMPs either to aid in their migration by increasing EC permeability, or to remodel the myometrium to promote the labouring process and PP involution. Specific subgroup of leukocytes, CD68+ macrophages, have been previously shown to infiltrate into uterine tissues (myometrium and decidua) before TL and during PP involution [20, 28]. Further investigation of immunofluorescent co-staining for CD68 and in situ zymography indicated that macrophages are one of the leukocytes responsible for myometrial MMP expression. Importantly, macrophages show expression of gelatin, collagen I and collagen IV-degrading proteases as seen by co-staining of green (protease activity) and red fluorescence (CD68). (Figure 3.21-23), which suggests that they are partially responsible for total MMP gene and/or protein expression detected in myometrium.
Figure 3.20: Gelatinase activity of rat myometrium and tissue leukocytes during late gestation (GD21, 22), term labour (D23) and postpartum (1PP).
Representative images showing co-localization of *in situ* gelatin zymography (green) and immunofluorescence of the leukocyte common antigen CD45 (red). Co-immunostaining indicates that leukocytes (arrows) recruited into the myometrium during gestation, labour, and PP expressed gelatinases along with smooth muscle cells. Slides were counter-stained with DAPI (blue nuclear staining). Images were acquired using Volocity software and a spinning-disc confocal microscope at 400x magnification.
Figure 3.21: Representative images from in situ gelatin zymography and CD68 immunofluorescence.
Co-immunostaining for CD68 (red) following in situ zymography indicated that macrophages (arrows) infiltrated into the myometrium also expressed gelatinases along with smooth muscle cells. Slides were counter-stained with DAPI (blue nuclear staining). Images were acquired using Volocty software and a spinning-disc confocal microscope at 400x (GD15 and GD21) and 600x (GD23 and 1PP) magnification.
Figure 3.22: Representative images from *in situ* collagen I zymography and CD68 immunofluorescence.

Co-immunostaining for CD68 (red) following *in situ* zymography indicated that macrophages (arrows) infiltrated into the myometrium also expressed collagen I-degrading enzymes along with smooth muscle cells. Slides were counter-stained with DAPI (blue nuclear staining). Images were acquired using Volocity software and a spinning-disc confocal microscope at 400x magnification.
Figure 3.23: Representative images from *in situ* collagen IV zymography and CD68 immunofluorescence.

Co-immunostaining for CD68 (red) following *in situ* zymography indicated that macrophages (arrows) infiltrated into the myometrium also expressed collagen IV-degrading enzymes along with smooth muscle cells. Slides were counter-stained with DAPI (blue nuclear staining). Images were acquired using Volocity software and a spinning-disc confocal microscope at 400x magnification.
Chapter 3: Results

3.5 IN VITRO PRIMARY CELL MODEL OF MECHANICAL STRETCH

3.5.1 In vitro primary rat myometrial cell model of mechanical stretch

Numerous studies indicate that MMP expression and activity is regulated by mechanical stretch in different cell types [157-160]. To examine the direct effect of mechanical stimuli on the expression of MMPs, we developed an in vitro culture system using primary myometrial cells. Primary myocytes were enzymatically isolated from 10 uteri collected from virgin estrogenized rats and plated on 6-well flexplates coated with collagen I (n=3). The number of isolated cells differed significantly between 3 consecutive cell cultures. To simulate biological mechanical stretch of the uterine horn by growing fetus, primary myometrial cells were artificially stretched for 2, 8 and 24 hours (25% elongation) and the expression of different MMPs and TIMPs was analyzed using specific rat primers. Stretch-related changes in mRNA expression were minimal and the expression was highly variable between three cultures, possibly due to a difference in characteristics of the isolated primary cells.

We also studied the activity of the protein secreted by those cells into the SCM. We found that protein expression of secreted MMP-7 and MMP-11 were undetectable in the collected NCM and SCM when compared with an in vivo rat myometrial GD15 sample as positive control. Pro- and active-MMP-2 (69 and 62 kDa) and active MMP-9 (84kDa) were detected by zymography, however, there was no effect of stretch (S) compared to non-stretch (NS) on protein expression and activity (data not shown). Thus as an alternative approach, the effect of in vitro static mechanical stretch on MMP expression was assessed using a primary human myometrial cell culture model.
3.5.2 *In vitro primary human myometrial cell model of mechanical stretch*

Human primary myometrial cells enzymatically isolated from term non-labouring tissue biopsies were characterized by immunofluorescent cytochemistry (ICC) to assess their phenotype at passage 0, 1, 2 and 4. ICC assessment suggests that cultured primary myometrial cells are myofibroblasts characterized by the co-expression of fibroblastic (Vimentin) and smooth muscle (Desmin, SMA, and H-Caldesmon) differentiation markers (Figure 3.24).

![Figure 3.24: Representative immunocytochemical fluorescent images of primary human myometrial cells at passage 0, 2 and 4.](image)

Cells were characterized for expression of differentiation markers for fibroblasts and smooth muscle cells. Primary myometrial cells were plated directly after enzymatic isolation (passage 0), and following the 2nd and 4th passages; they expressed markers for differentiated fibroblasts (Vimentin) and some (arrows) expressed markers for differentiated smooth muscle cells (Desmin, Smooth Muscle Actin, H-Caldesmon). Images were acquired using Velocity software and a spinning-disc confocal microscope at 200x magnification.
As seen in Figure 3.23, cells enzymatically isolated from pregnant human uterine tissue at passage 0 are primarily fibroblasts expressing Vimentin, with some cells expressing smooth muscle differentiation markers. Following the 1\textsuperscript{st} (not shown) and 2\textsuperscript{nd} passage, these cultured myometrial cells begin to increasingly differentiate towards a myofibroblast phenotype by up-regulating SMA and H-Caldesmon expression, while still retaining expression of the fibroblast marker Vimentin. By passage 4, the majority of cells in culture were myofibroblasts, by simultaneously expressing of Vimentin, Desmin, SMA and H-Caldesmon. Since this \textit{in vitro} primary human cell culture model is phenotypically variable from cells present in the \textit{in vivo} environment, I suggest that these cells may behave differently in response to static mechanical stretch.

After the 4\textsuperscript{th} passage, approximately 200,000 myometrial cells were plated on collagen I-coated 6-well flex (containing flexible membranes) plates (Flexcell Inc., n=4) and left to grow to confluency (75-80\%) prior to serum starvation for 24 hours. Immediately following serum starvation, static mechanical stretch (25\% elongation) was applied to the flex plates for 24 hours (24h S) or 24 hours of stretch followed by 24 hours of relaxation (24h S + 24h R). For both groups (S and S+R) there were non-stretched cells incubated for the same time interval (24h NS and 48h NS). Cell viability after stretch and stretch with relaxation was assessed using PI-FDA staining and confirmed that stretch did not induce cell death since there was no significant increase in PI-positive cells after stretch as compared to static non-stretched cells (Figure 3.25).
Figure 3.25: Representative images from assessment of cell viability following mechanical stretch of primary human myometrial cells.
The assessment of cell viability following 24 hours static stretch and additional 24 hours for relaxation, was employed using propidium iodide (PI) and fluorescein diacetate (FDA) staining. FDA (green) revealed live cells while PI (red) revealed cell death. There was minimal strain of static mechanical stretch on the cell viability. Images were acquired using the Volocity software and a spinning-disc confocal microscope at 200x magnification.
3.5.3 *In vitro mechanical stretch does not influence myometrial MMPs gene expression*

Total RNA was extracted from the myometrial cells collected with TRIzol® (Life Technologies) and the mRNA was reversed transcribed to cDNA for RT-qPCR analysis. The entire set of MMPs and TIMPs was assessed using human primers (similar to section 3.2), however, only MMP-1, 2, 3, 11 and TIMP-1 & 2 were expressed (Ct values ≤ 33) by cultured primary human myometrial cells (Figure 3.26-3.28). The data suggests that there was no significant effect of stretch on MMP-1, 2, 3, 11 and TIMP-1 & 2 mRNA expression. Additionally, it was observed that 24 hour static mechanical stretch followed by an additional 24 hours in culture without stretch (to simulate the relaxation observed in the PP period) modified mRNA expression for MMP-1, 2, 3 and 11 as compared to cells stretched for 24 hours. However, this trend was similar for both non-stretched and stretch plates, indicating that there is an indirect effect of time in culture rather than stretch. We also noticed that in non-stretched myometrial cultures MMP-1, and MMP-3 exhibited a decrease in mRNA expression, while MMP-2, MMP-11 and TIMP-2 exhibited an increase after being in culture for 48 hours as compared to 24 hours group.
Figure 3.26: Expression changes in mRNA levels of MMPs as an effect of in vitro static mechanical stretch of primary human myometrial cells.

Transcript levels of MMP-1 (A) and MMP-2 (B) expressed by 24 hour non-stretched (24h NS), 24 hour stretched (24h S), 48 hour non-stretched (48h NS) and 24 hour stretch + 24 hour relaxation (24h S + 24h R), were normalized to three housekeeping genes, TATA-binding protein (TBP), cytochrome C1 (CYC1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold-change relative to 24 hours Non-Stretch. The bars represent mean±SEM (n=3-4 at each time point). Statistical analysis (one-way ANOVA and Newman-Keuls multiple comparisons test) was performed on the relative fold-change data. Significant differences between gestational days are indicated by asterisks, * (p<0.05), ** (p<0.01).
Figure 3.27: Expression changes in mRNA levels of MMPs as an effect of *in vitro* static mechanical stretch of primary human myometrial cells.

Transcript levels of *MMP-3* (A) and *MMP-11* (B) expressed by 24 hour non-stretched (24h NS), 24 hour stretched (24h S), 48 hour non-stretched (48h NS) and 24 hour stretch + 24 hour relaxation (24h S + 24h R), were normalized to three housekeeping genes, TATA-binding protein (*TBP*), cytochrome C1 (*CYC1*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and expressed as fold-change relative to 24 hours Non-Stretch. The bars represent mean±SEM (n=3-4 at each time point). Statistical analysis (one-way ANOVA and Newman-Keuls multiple comparisons test) was performed on the relative fold-change data. Significant differences between gestational days are indicated by asterisks, * (p<0.05), ** (p<0.01), and *** (p<0.001).
Figure 3.28: Expression changes in mRNA levels of TIMPs as an effect of in vitro static mechanical stretch of primary human myometrial cells.

Transcript levels of TIMP-1 (A) and TIMP-2 (B) expressed by 24 hour non-stretched (24h NS), 24 hour stretched (24h S), 48 hour non-stretched (48h NS) and 24 hour stretch + 24 hour relaxation (24h S + 24h R), were normalized to three housekeeping genes, TATA-binding protein (TBP), cytochrome C1 (CYC1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold-changes relative to 24 hours Non-Stretch. The bars represent mean±SEM (n=3-4 at each time point). Statistical analysis (one-way ANOVA and Newman-Keuls multiple comparisons test) was performed on the relative fold-change data. Significant differences between gestational days are indicated by asterisks, * (p<0.05).
3.5.4 Secreted myometrial MMPs proteins detected by Luminex assay

The undiluted NSM and SCM were screened by the Bio-Plex Pro™ Human MMP and TIMP Luminex assays (Bio-Rad) and MMP-1, 2, 3, & 10 and TIMP-1 & 2 were detected. For the analysis, some calculated MMP/TIMP concentrations were excluded if detection values were extrapolated below the lowest concentration of the standard curve. In addition, if some values were extrapolated beyond the highest concentration of the standard curve, that particular data point was assigned the value of the highest standard concentration. Based on the aforementioned criteria, secreted MMPs/TIMPs that were detected in less than four of NSM/SCM data sets were excluded from further analysis. Therefore only MMP-1 & 2 and TIMP 1 & 2 underwent statistical analysis (Figure 3.29).

Cultured primary human myometrial cells secreted MMP-1 (105-8400 pg/mL), MMP-2 (1220-33,000 pg/mL), TIMP-1 (3600-18,000 pg/mL) and TIMP-2 (11,500-19,500 pg/mL) in the NSM and SCM. Importantly, there was no effect of static mechanical stretch, and only secreted MMP-2 levels exhibited a 10-fold increase after culturing for 48 hours as compared to 24 hours incubation in serum-free media. This further suggests that in vitro culture conditions can influence the expression of MMPs and the underlying mechanisms that regulate myometrial MMP expression and secretion need to be further elucidated.
Figure 3.29: Changes in MMP and TIMP protein secretion primary human myometrial cells as an effect of *in vitro* mechanical stretch.

Changes in protein secretions detected in the conditioned medium of myometrial cell culture collected from non-stretched and stretched flex plates after 24 hours and 48 hours of incubation. The bars represent mean±SEM (n=4-5 at each time point) expressed in fold-change relative to 24 hours Non-Stretch Conditioned Medium (NSM). Statistical analysis (one-way ANOVA and Newman-Keuls multiple comparisons test) was performed on the relative fold-change data. Significant differences between gestational days are indicated by asterisks, * (p<0.05).
CHAPTER 4

DISCUSSION
4.1 GESTATIONAL REGULATION OF MMP GENE AND PROTEIN EXPRESSION IN THE RAT MYOMETRIUM

My results show that: 1) throughout gestation the majority of myometrial MMP genes displayed low and stable expression that was highly upregulated PP. 2) Myometrial MMP-7 (matrilysin), MMP-11 (stromelysin-3), and MMP-12 (macrophage metalloelastase) genes and proteins were induced at term. Particularly, on GD22 (term) MMP-7, MMP-12, and MMP-11 showed 200-fold, 150-fold and 15-fold increase respectively compared to early gestation (GD6), which remained elevated during TL and PP. 3) Gelatin, collagen I, and collagen IV-degradation mediated by MMP activity in the pregnant, labouring and PP myometrium can be attributed to two cell types: myometrial SMCs and resident leukocytes (specifically CD68+ macrophages). These in vivo results suggest a dual role for myometrial MMPs in regulating processes involved in 1) promoting TL and 2) PP uterine involution.

During late gestation and TL the expression of certain MMP genes was significantly different between gravid and non-gravid (empty) uterine horns of unilaterally pregnant rats. This difference suggests that in vivo mechanical stretch or the presence of the fetus and/or placenta may play a role in regulating MMP expression. To examine the potential regulatory role of mechanical stretch, I performed in vitro studies using primary myometrial SMCs in combination with Flexcell stretch system. My data indicate that there is not a direct effect of static mechanical stretch on both MMP gene and secreted protein expression by human myometrial cells.

4.1.1 The Role of MMPs during Gestation, Term Labour, and Postpartum Involution

The myometrium undergoes dramatic changes in its cellular phenotypes and ECM composition throughout pregnancy, TL and PP. During gestation, specific molecular mechanisms
must exist to regulate myometrial ECM remodeling to accommodate the growing fetus in a quiescent environment, and yet be able to activate labour contractions at term [20, 35, 37]. Following parturition, the uterus is remodeled to a pre-pregnant state, which is accompanied by a dramatic reduction in size which primarily involves rapid and extensive MMP-mediated ECM degradation and cessation of cellular hypertrophy [31, 32, 35, 161, 162]. MMP activity during PP involution has been studied extensively in rodents, undeniably correlating it with the dramatic regeneration of uterine tissue including neo-angiogenesis, ECM transformation and modulation of cellular phenotype [17, 29, 30, 34, 35]. MMP-7 was first identified by Woessner et al. due to its high expression in the early PP rat uterus, whereas MMP-13 collagenase activity was also recognized for its role in uterine involution [145, 161, 163-166]. However, little is known about the role of other myometrial MMPs during gestation, active TL or PP.

Throughout gestation, myometrial SMCs change their phenotype from hyperplastic (proliferative) to hypertrophic (or synthetic). Both stages of myometrial growth are associated with ECM modifications to accommodate the increase in SMC number and size [49]. Therefore, the major structural component of the uterine ECM, fibrillar collagen, must be constantly reorganized for pregnancy maintenance [35]. Comparative analysis of overall MMP gene expression (Figure 3.6) indicates that the collagenases (MMP-8 & 13) and gelatinases, especially MMP-2, were highly (yet relatively stable) expressed throughout pregnancy with mRNA levels being 100-300 fold higher in comparison to the lowest expressed MMP-10. In addition, the results obtained from gelatin zymography indicate that throughout gestation, MMP-2 is constantly active, whereas western blot analysis revealed persisting high levels of MMP-8 protein expression (Figure 3.10), suggestive of restricted collagenolysis. Compared to the other collagenases MMP-1 & 13, MMP-8 is known to preferentially cleave collagen type I, the most abundant collagen type in the uterus [76]. Through speculation, it is plausible that similar to keratinocytes [167, 168],
when SMCs proliferate or expand in size, their cell-surface collagen integrin receptors (such as \( \alpha 2\beta 1 \)) come into contact with the surrounding collagen I molecules, which may induce intracellular signaling and increase expression of MMP-8. These findings point to the role of both collagenases and gelatinases, in the modification of the ECM environment during pregnancy to increase elasticity of the myometrium. This action is necessary to accommodate the growing fetus/es, in which the collagenases would cleave fibrillar collagens into fragments that would be denatured into gelatin. Furthermore, the gelatinases would then degrade gelatin into small peptide fragments for rapid removal by the infiltrating immune cells possibly macrophages [162].

Although \textit{MMP}-9 was highly expressed throughout gestation (Figure 3.6), MMP-9 protein activity was not detected by gelatin zymography. This phenomenon has been previously reported, suggesting that mRNA and protein abundances in cells correlate poorly due to different post-transcriptional intracellular regulatory mechanisms, for instance microRNA (miRNA) can either upregulate or silence gene expression [169-171]. The silencing is mediated by miRNA binding to mRNA by complimentary alignment to 1) inhibit protein translation or 2) induce mRNA degradation via RISC (RNA induced silencing complex) [172]. Several studies have revealed a variety of miRNAs that target MMP mRNA, either by upregulating gelatinase expression (i.e. miR-29b directly targets MMP-2) to directly promote cell proliferation and migration, or by down-regulating gelatinase expression (miR-9 negatively modulates MMP-2 and -9 expression indirectly by targeting their upstream NF-\( \kappa \)B1 signaling) [171, 173-175]. It is also plausible to speculate that MMP-9 function during TL is species- or tissue-specific, as was previously shown by Roh \textit{et al.} on the lack of progesterone-mediated suppression of MMP-9 production by human myometrial cells, the effect that was previously observed in cultured rabbit cervical fibroblasts [31].
My zymography results are in concordance with preceding data by Yin et al., which reported prominent active and pro-form of MMP-2 and less intense MMP-9 bands in uterine tissue homogenate from pregnant rats at mid-gestation (GD12) and late gestation (GD19). Unlike our experiments that were done solely on the myometrium throughout rat gestation, they detected weak MMP-9 activity in the uterine tissue, possibly due to the presence of the decidua, which was not separated from the myometrium in their study [139]. In contrast, our immunohistochemical examination of pregnant rat uterus detected prominent myometrial MMP-9 protein expression (probably in its pro-MMP form). During late gestation (GD21) MMP-9 expression was localised mostly intracellular, particularly in the perinuclear region of myocytes (i.e. in the endoplasmic reticulum and Golgi bodies), but increased extracellularly during active TL (Figure 3.17). This confirmed previous results by Roh et al. that reported in vivo expression of MMP-9 by SMCs, interstitial fibroblasts and inflammatory cells in the term non-labouring and labouring human myometrium samples [31].

My analysis revealed generally steady levels of MMPs-7, 11 and 12 genes throughout gestation with upregulation at term. This profile correlates well with the second switch in the myometrial phenotype during late gestation from synthetic to contractile [15] to support the synchronized contractions of labour [34]. The stromelysins (MMP-3, 10, 11) and the related MMPs (MMP-7 & 12) are capable of degrading elastin, cell adhesion molecules, proteoglycans, fibronectin and components of the BM, laminin and collagen type IV [54, 99], which can modify the ECM structural environment for active labour contractions. Although uterine MMP-7 was first identified during PP involution [54, 161, 166], these findings – in which a strong correlation was observed between gene and protein expression – suggest functionality of this enzyme during late gestation and TL. In addition, active MMP-11 protein also showed a dramatic upregulation at late gestation and active TL, as compared to the early gestation which corresponds well with its gene
expression (Figure 3.11). Protein expression for MMP-7, 11 & 12, and especially enzymatic activity of the major gelatinase MMP-2 were all dramatically upregulated during PP, consistent with their involvement in uterine involution and ECM remodeling resulting in reduced uterine size. Additionally, due to the upregulation of CX-43 protein observed during TL to promote SMC contractility, the ability of MMP-7 to degrade these junction proteins suggests its possible role in controlling SMC connectivity after birth [176].

Similar to the temporal and spatial localization of MMP-9, MMP-7 & 11 proteins were expressed by SMCs in both the longitudinal and circular myometrial layers. Their expression was predominantly intracellular (in the perinuclear region) during mid and late-gestation, however, a shift to extracellular MMP-7 protein localization occurred earlier during labour compared to MMP-11 and was upregulated PP. Remarkably, positive staining indicated that MMP-9, MMP-7 and MMP-11 proteins were expressed not only by uterine myocytes but also by single cells morphologically distinct from SMCs, localizing outside the muscle bundles. In accordance with numerous publications, it is speculated that those cells are activated leukocytes recruited to the myometrium to express pro-inflammatory cytokines and chemokines [11, 133]. Previous studies have identified that macrophages were the predominant immune cells in the myometrium during late gestation, TL and PP [11, 20, 62]. Consistent with these findings, our lab has previously reported induction of MCP-1 (CCL2, chemokine involved in macrophage migration), and confirmed an influx of CD68+ macrophages before and during TL in the rat myometrium [22]. Uterine leukocytes, especially macrophages can support active labour contractions by secreting cytokines like IL-1β, IL-6, and TNF-α to promote the induction of CAPs [63]. Infiltrating leukocytes are themselves known sources of MMPs, and additional MMPs produced by other cells can further promote leukocyte migration into inflamed tissue [67, 75, 78]. In situ zymography analysis suggests that uterine matrix remodeling by SMCs and leukocytes is MMP-
mediated as seen by degradation of fluorescent-labelled gelatin, collagen I, and collagen IV substrates, known to be actively cleaved by MMPs [76]. This suggests that the increase in MMP production by the rat myometrium correlates with leukocyte infiltration to promote term labour and PP involution. Therefore, myometrial ECM degradation may actually be a combined result of MMP secretion by both myometrial cells and infiltrating peripheral or resident leukocytes.

The role of MMP-11 in the myometrium, is not well defined. Previous studies have indicated that MMP-11 can regulate BM stability and levels of fibronectin, collagen IV, and laminin (BM components) in the ECM [76, 177, 178]. I speculate that the substantial increase in MMP-11 during active labour (GD23) suggests a role in degrading BM surrounding individual SMCs to increase SMC connectivity for coordinated myometrial contractions, and to facilitate leukocyte migration within the myometrium [34, 35, 78, 178]. MMP-11 has also been described to readily cleave α1-proteinase inhibitor (also known as α1-antitrypsin) that predominantly inhibits neutrophil elastase (NE)-mediated degradation of ECM components, and α2-macroglobulin – a general proteinase inhibitor that induces MMP removal by endocytosis [68, 179-181]. Based on the literature, MMP-11 can potentially maintain proteolytic activity of other MMPs by preventing their inhibition during late gestation, TL and PP. Conversely, MMP-12 protein levels were also highly expressed throughout gestation and were further upregulated in the rat myometrium PP (Figure 3.12). MMP-12 is expressed primarily by macrophages and is important for their migration [78]. Previous reports have indicated an increase in the monocyte/macrophage population in the rat and mouse myometrium prior to term, that remained elevated during TL and PP. This sustained elevation suggests that in addition to myometrial SMCs or fibroblasts (as skin-derived myofibroblasts/fibroblasts have been previously shown to be a source of MMP-12), resident or infiltrating immune cells might be responsible for the observed
MMP-12 expression – especially higher mRNA levels within the gravid horn (compared to the empty horn) during late gestation [20, 26, 182].

Analysis of TIMP expression in the rat myometrium (Figure 3.5 and 3.6) provides potential explanation of the constant low expression and activity of MMPs throughout gestation. Comparative analysis revealed that TIMP-2 exhibited the highest expression level from all TIMPs, while TIMP-1 and TIMP-3 were also highly expressed throughout gestation, and their expression pattern was similar to the gelatinases. This pattern of TIMP protein expression is similar to our lab’s recent results in the human vagina [183]. It is known that TIMP-1 and TIMP-2 can form specific complex with MMP-9 and MMP-2 (via their C-terminus), respectively, and further interact with other MMPs via their N-terminus to potentially prevent their activation [107, 108]. These findings support the inhibitory role of TIMP-2 for the majority of MMPs, and the potential interaction between TIMP-1 & 3 with MMP-2 & 9 to form inhibitory complexes in the rat myometrium throughout gestation. Interestingly, a recent study that sought to compare the total myometrial transcript repertoire between term and spontaneous TL (in humans) by high-throughput RNA sequencing reported that TIMP-1 was the gene most highly upregulated in association with labour [184]. Furthermore, in regards to the PP period, TIMP-3 & 4’s gene expressions were highly upregulated (450-fold) suggesting their role in regulating PP involution (Figure 3.5 and 3.6). Apart from MMP inhibition/activation, TIMPs are also known to have other biological functions which include regulation of cell proliferation, migration, angiogenesis and apoptosis. Since TIMP-3 has been observed to have pro-apoptotic function, while TIMP-4 is anti-apoptotic, this correlates with the observed increase in expression for these two TIMPs during PP, which may promote apoptosis of hypertrophied SMC and stimulate new cell growth and regeneration respectively [69, 109, 114-117].
It has been previously reported that MMP-mediated degradation of ECM components can result in biologically active peptides. Interestingly, these particular chemoattractive peptides can originate from ECM components – such as elastin, laminin and fibronectin – that are known to be cleavable substrates for MMP-7, 11 & 12. Elastin abundance is vital for the elastic recoil of the uterine tissue and provides resilience when exposed to external stimuli. This property of elastin is important in the reproductive tract to accommodate its enormous expansion in pregnancy [35]. However, elastin degradation can result in the generation of elastin-derived peptides with chemotactic properties for monocyte recruitment [185]. MMP-12 indirectly promotes additional elastin destruction by degrading the serine proteinase inhibitor α1-antitrypsin (which potentiates NE activity) [179, 186]. Cleavage of the laminin α5 chain results in a chemotactic peptide that has been shown to recruit leukocytes and induce MMP-9 production, both in vitro and in vivo [187]. MMP-9-mediated hydrolysis of collagen can also result in a chemotactic fragment (Pro-Gly-Pro) that is homologous to the major chemoattractant for neutrophils CXCL1/GROα/KC and can activate the CXCL1 receptor to induce neutrophil chemotaxis in airway inflammation [78, 127, 188]. Moreover, fibronectin and hyaluronan fragments have been shown to stimulate macrophage cytokine and chemokine production [189, 190]. Fibronectin fragments have also been observed to stimulate transendothelial migration of HIV-1 infected leukocytes by upregulating the expression of TNF-α which induces the expression of adhesion molecules like selectins, ICAMs and VCAM-1 [191, 192]. This correlates with previous in vitro studies in our lab that observed an upregulation of ICAM-1 in response to mechanical human myometrial stretch-induced cytokines, which promoted leukocyte transendothelial migration [64]. Apart from increasing chemoattraction, MMP-7 has also been shown to actively cleave VE-cadherin – one of the junctional proteins that maintains vascular barrier function [193]. These findings additionally
suggest the MMP-specific production observed during late gestation and TL promotes leukocyte recruitment and infiltration by upregulating chemotaxis and vascular EC permeability.

4.1.2 The Role of MMPs in Uterine Inflammation: Mutual Regulation

Labour and PP involution-associated pathways – which includes ECM remodeling – are known to be largely stimulated by the expression of cytokines secreted by myometrial cells and the active release of cytokines and growth factors already present in the ECM [194, 195]. It is anticipated that the influx of leukocytes further amplifies the inflammatory signal to induce parturition and PP involution through the release of leukocyte-derived cytokines (such as IL-1β and TNF-α) and MMPs [17, 22, 29, 63, 64, 196]. Not surprisingly, monocytes/macrophages have been identified to have the ability to secrete all soluble MMPs that were studied – MMP-1, 2, 3, 7, 8, 9, 10, 11, 12, and 13, as well as the four TIMPs [197]. Neutrophils, however, have been reported to primarily secrete MMP-8, MMP-9 and MMP-12 [127, 196, 198]. Several cytokines (i.e. TNF-α, and IL-1β) and growth factors (i.e. IGF-1 and TGF-β) have been previously shown to regulate transcriptional activation of MMP genes in a variety of cells including monocytes/macrophages in atherosclerotic plaques, glomerular mesangial cells, and the human colonic epithelium [197, 199, 200]. Binding of cytokines and growth factors to their cell-surface receptors results in upregulation of intracellular signaling (such as the mitogen-activated protein kinases (MAPK) and NF-κB pathways) inducing MMP expression [200-203]. More importantly, although these pro-inflammatory cytokines and growth factors can regulate MMP expression, MMPs can also regulate their bioavailability by cleavage of their binding proteins [71, 204, 205].

As previously mentioned, MMP-mediated cleavage of ECM peptides can result in the formation of chemotactic gradients, but can also regulate cytokine and chemokine release [67, 75, 78]. TNF-α is a potent pro-inflammatory cytokine that can be expressed as a membrane-bound
protein on the surface of macrophages, or secreted and bound to fibronectin and laminin [203, 206, 207]. MMP-mediated cleavage (especially MMP-7 and MMP-3) releases active TNF-α to generate a chemoattractive gradient for macrophage recruitment and induce expression of labour-associated genes (like prostaglandins) in the myometrium [208-210]. Another potent pro-inflammatory cytokine IL-1β, can also be activated by MMP-mediated cleavage of its precursor via MMP-2, 3 and 9. Interestingly, MMP-3 can degrade active IL-1β therefore suggesting a dual role for MMPs in cytokine regulation [211]. Similar to MMP-3 inactivation of IL-1β, MMP-mediated cleavage of CCL7 (by MMP-2) and MCP-1 (by MMP-1, -3 and -13) results in a truncated peptide that is unable to bind to the CC chemokine receptor. MMP-9 processing of mature chemokine IL-8/CXCL8 has been reported to result in a more effective chemoattractant for neutrophil recruitment [127]. Furthermore, previous studies have reported that MMP-null mice displayed diminished leukocyte infiltration into the targeted tissue – such as MMP-7 knockout demonstrated impaired neutrophil efflux into the alveolar space due to the lack of MMP-7 mediated shedding of syndecan-1 and release of CXCL1 to generate a chemotactic gradient [124]. These findings suggest that MMPs identified in the rat myometrium show a possible involvement in the regulation of cytokine and chemokine bioavailability and activity. This further reinforces the role of MMPs in regulating leukocyte migration and leukocyte-mediated inflammation.

The family of insulin-like growth factors, in particular IGF-1 and IGF-II are known to stimulate cell proliferation, collagen synthesis, and muscle growth/regeneration, therefore making their bioavailability important to assist PP uterine involution to a non-pregnant state [212-214]. The IGF-signaling system is tightly regulated by IGFBPs which prevent IGF-1 and IGF-II from binding to the IGF-1 receptor [194, 215]. MMP-7-mediated cleavage of IGFBP-5 is capable of liberating IGF-II to function as an autocrine myofibroblast growth factor [215]. Interestingly,
Shynlova et al. previously reported an increase in IGF-1, IGFBP-5 and the IGF-1 receptor in the rat myometrium during TL and PP involution [37]. In accordance, the observed increase in MMP-7 can conceivably suggest a potential role for this enzyme in upregulating IGF-1 signaling in the myometrium. Another growth factor, TGF-β is secreted in its inactive form which can be associated with the interstitial ECM through latent TGF-β binding proteins (LTBPs) [194, 216-218]. Active TGF-β can also bind to collagen IV, fibronectin and heparin-sulfate proteoglycans (HSPGs). MMP-mediated release of TGF-β bound to ECM components results in a potent chemoattractant for monocytes and macrophages, and further stimulates their expression of bFGF and TNF-α [204]. These reports together with the data provide evidence to support that MMP expression in the PP rat myometrium mediates ECM remodeling and also promotes tissue regeneration through the liberation of growth factors.

4.1.3 Regulation of Myometrial MMP Expression: Potential Mechanisms

Yin et al. had previously reported that stretch of myometrium strips isolated from virgin rats was associated with an increase in gelatinase expression and activity, which was further enhanced following hormonal treatment with 17-β estradiol and progesterone. This study suggested that MMP expression could be regulated by mechanical stretch of the uterine walls by growing fetuses [139]. As a result, a unilaterally pregnant rat model was employed to thoroughly investigate the potential mechanisms of myometrial MMP activation [35]. It was observed that mRNA levels of MMP-7, 11 and 12 in the gravid uterine horn was greater during labour as compared to the empty uterine horn, however, protein levels did not exhibit similar differences. Furthermore, gelatin zymography revealed a stable expression profile for pro-MMP-2 protein throughout gestation, which depicts no differences between empty and gravid uterine horns (Figure 3.13). Surprisingly, starting from early gestation until term, active MMP-2 was higher in

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the empty horn compared to the gravid horn. Based on my results from bilaterally pregnant rats, which exhibited dramatic increase in activated MMP-2 during PP (when the fetus is expelled), these findings suggest that during gestation and labour, MMP-2 activity in the gravid horn is suppressed by the presence of fetal/placental units (possibly inhibins or TGF-β capable of down-regulating MMP expression) while this suppression is absent in the empty uterine horn [219].

It can be speculated that since MMP expression can be cytokine-mediated, the observed difference in gene expression is a result of activation by cytokines derived from infiltrating leukocytes, the placenta, and fetal membranes present in the gravid horn [10, 20, 78, 197, 200, 220, 221]. In accordance, the mRNA expression of *MMP*-3, 8 and 11 was also dramatically upregulated in the gravid uterine horn 24 hours after the delivery of the fetus due to tissue remodeling during PP involution; and was not stretch-dependent [17, 22, 29, 33, 35, 64, 82]. Additionally, active MMP-12 protein was greatly detected in the gravid horn (compared to the empty horn) of the PP uterus, which may indicate the presence of MMP-secreting immune cells [78]. These findings suggest that mechanical stretch may not play a direct role in the regulation of MMP expression, but rather is regulated by factors from the fetal/placental units.

One of the possible molecular mechanisms of MMP gene regulation before labour is the activator protein (AP)-1 family of transcription factors (Jun and Fos) that are known to transcriptionally activate a variety of myometrial genes involved in the labouring process [222, 223]. Previous studies found that many ECM, CAPs, MMPs and TIMPs genes contain AP-1 sites in their promoter regions [223, 224]. In particular, all the MMPs that were highly regulated in the rat myometrium contained the AP-1 promoter region – MMP-9, 7, 12 and 11 (AP-1-like region) [74]. It was also reported that mRNA levels of cFos, FosB and JunB transcription factors are increased in the rat myometrium during TL, implicating their role in regulating parturition [223, 225]. I suggest that at term, upregulated myometrial AP-1 proteins that promote the expression of
ECM components, CAPs, and cytokines, can also influence the expression of certain MMPs. The direct mechanism is currently unknown; however, given that IL-1β and TNF-α is expressed in the labouring myometrium and can stimulate AP-1 transcriptional activity, it can be speculated that myometrial MMPs might be regulated by cytokine-mediated induction of AP-1 transcription factors [17, 74, 226, 227].

4.2 CHANGES OF MYOMETRIAL MMPs ARE NOT AN EFFECT OF MECHANICAL STRETCH (IN VITRO PRIMARY CELL MODEL)

Results from the in vivo experiments using isolated rat myocytes did not allow us to conclude whether myometrial MMP expression is regulated by mechanical stretch. However, this contradicts the previously observed increase in gelatinases as a result of mechanically stretch rat myometrial strips [139]. Particularly, cyclic mechanical stretch of reproductive cells in vitro resulted in augmented expression of MMP-1 in both cultured human uterine cervical fibroblasts cells and human decidual cells [147, 228]. To further investigate the potential mechanical regulation of MMP expression, in vitro stretch experiments were performed using primary human myometrial cells. Overall, the in vitro experiments using primary human cells are consistent with the results from the in vivo rat studies, and indicated that there was no effect of static mechanical stretch on MMP-1, 2, 3, 11 and TIMP-1&2 gene expression. Additionally, the analysis of secreted MMP and TIMP proteins within the conditioned media demonstrated that in vitro mechanical stretch also had no significant effect on MMP secretion by primary human myometrial cells. However, 24 hour static mechanical stretch that was followed by an additional 24 hours of relaxation (mimicking the release of stretch in the PP period following labour) resulted in a change in mRNA expression for human MMP-1, 2, 3 and 11.
One possible explanation for this observation is that prolonged serum-starvation (72 hours in total) – media containing ITS-A (Life Technologies Inc.) supplement instead of FBS – may influence the expression of multiple MMPs. In addition, our in vitro results contradict the findings of Zhao et al., who previously reported that cyclic stretch augmented MMP-1 production in human uterine SMCs [229]. I noticed that the recorded increase in MMP expression in other cultured primary uterine cells – human decidua, cervical fibroblasts, and myometrial SMCs – were always a result of cyclic mechanical stretch, while our study employed static mechanical stretch to simulate continuous stretch by the growing fetus during pregnancy [17, 64, 147, 228, 229]. It is suggestive that our model of biologic static stretch mimics a quiescent pregnancy environment and was not able to stimulate MMPs, while cyclic stretch, which simulates active labour contractions, might show direct effect on MMP expression. Based on these findings, the mechanisms that regulate myometrium MMPs need to be further elucidated.

4.3 CONCLUSION: A PROPOSED MODEL FOR THE ROLE OF MYOMETRIAL MMPS DURING LATE GESTATION, TERM LABOUR, AND POSTPARTUM INVOLUTION

In summary, the main findings from the in vivo studies using the bilaterally pregnant rat model clearly suggests two distinct roles for MMPs within the myometrium. Expression of specific MMPs by myometrial SMCs is 1) constantly expressed throughout gestation to remodel the ECM and to increase elasticity of the tissue to accommodate the growing fetus, and 2) upregulated during late gestation to promote leukocyte infiltration to support active labour contractions. In addition, infiltrating leukocytes express MMPs to remodel the ECM and amplify the inflammatory signal. MMP expression is further upregulated during the PP period to 1)
remodel the ECM back to a pre-pregnant state and 2) promote tissue regeneration following birth. Based on our findings from characterizing the gestational profile of soluble MMP expression within the rat myometrium, I propose the following model to explain the role of myometrial MMPs (Figure 4.1).

Results from my studies suggest that expression/activity of specific MMPs (MMP-7, 11 and 12) may be important for mediating the transition from term to active labour. Therefore MMP-7, 11 and 12 can act as potential targets for the development of novel therapeutic strategies for prevention of PTL. For instance, instead of the use of a broad spectrum MMP inhibitor (i.e. GM6001), an inhibitor specific to MMP-7, 11 or 12 may provide favourable outcomes for women at risk for PTL – such as synthetic MMP Inhibitor II or III (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA). Moreover, in vitro static mechanical stretch (which resembles uterine stretch during gestation) has no effect of MMP gene and protein expression in primary human myometrial cells. Further studies must be performed to elucidate 1) the possible regulatory role of cyclic mechanical stretch on the expression of the MMPs studied and 2) the functional role of myometrial MMPs.
Figure 4.1. Evidence-based model for the role of myometrial MMPs during labour and postpartum involution.

Based on our data and previous studies in the literature, I suggest that near term, hormonal changes and expression of pro-inflammatory cytokines 1) induces the secretion of multiple MMPs by myometrial cells. 2) By degrading ECM components, myometrial MMPs would increase leukocyte chemoattraction and vascular permeability to 3) facilitate peripheral leukocyte recruitment and transendothelial migration into the uterine smooth muscle. Within the myometrium, these activated leukocytes can secrete pro-inflammatory cytokines and MMPs to 4) amplify the inflammatory signal and promote the laboring process and 5) promote ECM remodeling during postpartum involution to return the uterus back to a pre-pregnant state.
CHAPTER 5

FUTURE DIRECTIONS
5.1 FUTURE DIRECTIONS

The major findings from the two in vivo rat models of pregnancy suggested the role of MMPs during late gestation and TL is to regulate ECM remodeling to accommodate uterine contractions and promote leukocyte infiltration, possibly by increasing vascular permeability. In situ zymography revealed that CD45+ leukocytes in the myometrium, especially CD68+ macrophages expressed gelatin, collagen I and collagen IV-degrading enzymes (Figure 3.20-3.23), consistent with MMP expression. To confirm our results, fluorescent immunohistochemical studies should be performed for co-expression of markers of different immune cells (i.e. CD68, NE, CD3 etc.) and MMP-specific antibodies to identify specific leukocyte populations expressing MMPs. A previous study by Koscica et al. reported that the administration of broad-spectrum MMP inhibitor GM6001 resulted in reduced rates of PTL in mice [140]. Therefore further animal studies could be performed to characterize the MMP expression profile in the GM6001-mediated PTL attenuation model (with PTL induced by RU-486 and LPS) and investigate the effect of more specific MMP inhibitors – that would target specific MMP subgroups – on the rate of PTL.

Current results from both in vivo and in vitro models suggest that there is no effect of mechanical stretch on MMPs expression. Zhao et al. however reported increased expression of MMP-1 by primary human myometrial cells in response to cyclic mechanical stretch. Based on the aforementioned in vitro studies, it is also possible that applying cyclic stretch – instead of or following static stretch – to simulate active labour contractions may better induce expression of the major MMPs studied (MMP-7 and MMP-11) to resemble the changes observed in the in vivo rat models, and better define the regulation of MMP expression (by mechanical stretch) during TL [147, 228, 229].
It has been shown that hormones (estrogen and progesterone), pro-inflammatory cytokines (i.e. IL-1β), growth factors (i.e. IGF-1), and cell-adhesion molecules (i.e. VCAM-1 with LFA-1) affect MMP expression [31, 135, 230, 231]. Modification to the cell culture system by treatment of myometrial cells with 1) mifepristone (the progesterone antagonist RU486) or 2) cytokines and/or growth factors may allow for better understanding of MMP regulated expression [64, 71, 85]. Alternatively, co-culture of myometrial cells (either hTERT-HM cell line or primary myometrial cells) with other cells such as ECs and certain leukocytes populations (i.e. human monocytic cell line THP-1), may reveal expression changes of MMPs in vitro that would better simulate in vivo conditions (i.e. cellular interactions) and provide potential evidence of upregulating MMPs to promote TL and PP involution.

We suggest that the major role of myometrial MMPs is to promote transendothelial migration of leukocytes into the myometrium by upregulating vascular permeability – based on the substrates that certain MMPs are able to cleave include BM components, gap junction and cell adhesion proteins [67, 134, 193]. To investigate the functional role of myometrial MMPs on endothelial cell permeability, a transendothelial permeability assay can be employed using the human uterine microvascular endothelial cell line (hUtMVEC-Myo, Lonza, currently used in the Lye lab) [64]. EC barrier function can be assessed by testing the ability of a fluorescein probe to pass from the upper to lower chamber of the endothelium-covered inserts with/without the broad spectrum MMP inhibitor GM6001, or MMP inhibitors more specific to MMP-7 and 11.
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