University of Nevada, Reno

Inflammatory mediators in the response to myometrial strain and the transition into labor

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
Cellular and Molecular Pharmacology and Physiology

by

Christian Copley Salem

Heather Burkin/Dissertation Advisor

December, 2019

THE GRADUATE SCHOOL

We recommend that the dissertation
prepared under our supervision by

Christian Copley Salem

Entitled

Inflammatory mediators in the response to myometrial strain and the transition into labor

be accepted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

Heather Burkin, Advisor

Iain LO Buxton, Committee Member

Cherie Singer, Committee Member

David Quilici, Committee Member

Karen Schlauch, Graduate School Representative

David W. Zeh, Ph.D., Dean, Graduate School

December-2019

Abstract

Preterm birth is one of the most costly, negative pregnancy outcomes that affects all populations worldwide. While the mechanisms that drive the transition into labor are not fully known, abnormal uterine distension is implicated in preterm birth in twins and 10% of singleton pregnancies. In Chapter 2, we hypothesized that specific phosphorylation signaling events related to the strain induced response would be upregulated in a telomerized cell culture model of human uterine smooth muscle cells. The phospho-proteome of these model cells was elucidated by mass spectrometry after cells were subjected to mechanical strain. These changes led us to hypothesize that these signaling events in the myometrium would be associated with strain induced expression of transcripts related to proteins involved in the transition into labor. Pregnant human uterine smooth muscle cells were subjected to biaxial mechanical strain for 3, 8 and 24 hours and the transcriptome probed by RNAseq. We used qPCR to investigate if p38 MAPK activation increases IL-6 transcription, and if increased IL-6 exposure can affect the transcription of contractile associated proteins Cx43 and the oxytocin receptor. Mechanical strain led to the direct activation of ERK1/2, HSP27, and MYLC9, in addition to phosphorylation of PAK2, vimentin, DOCK1, PPP1R12A, and PTPN11. These results suggest a novel network reaction to mechanical stretch and reveal proteins that participate in the activation of contractile mechanisms leading to preterm labor. In addition, at 3 and 8 hours of strain we saw increased transcripts for cytokines IL6, IL8, IL1β and CCL2 which are inflammatory mediators involved in labor. We found increases in the contractile associated proteins: oxytocin receptor, connexin45, and AP-1 subunits

at 3 and 8 hours of strain. Finally, p38 MAPK inhibition blocked transcription of IL-6 in strained myometrial cells and incubation with IL-6 increased Cx43 and oxytocin receptor transcripts suggesting a mechanism for mechanical strain to contribute to the initiation of labor.

Dedication:

To Brandon: For keeping me alive and loved

To my Mom, Dad, Stephen, and Amy: For giving me everything

To LANA Del Rio Ayanami Taco, Demon Hunter™: Because she's a dog!

Acknowledgements

Acknowledgments are meant to give credit to those people who have both supported you and contributed to your character in ways that are inseparable from who you are as a person. In reality, such a list includes everyone you have ever met. That being said, it is impossible to credit everyone, and my travels through grad school have surely been inspired by countless people who shall remain unnamed. This is a fault of my memory more than a fault of their influence. If you are reading this and I forgot to mention your contribution, Thank you!

First and foremost, I must acknowledge my mother, Sharon Yvonne Copley. Beyond the fact that I am a biological torch bearer for some of her distinctive genetic traits, I am also a product of her capacity for love and attention. Throughout my life, she would tell me that there was nothing I couldn't accomplish if I put my mind to it. This document is proof that she was right. My mother died in 2015, so she never got to see me finish graduate school, but she never stopped being proud of me, and she will never stop being my inspiration.

My father, Gary William Copley, had both a genetic and an intellectual influence on me that cannot be over stated. His love for science, technology and discovery was a constant source of inspiration for me as a child. He taught me to change the brakes on a car, and he taught me to never stop learning. He also left me with an enduring genetic legacy of borderline obsessive compulsivity that keeps my socks organized and my dishes washed, which can be said to grant me the desire to be organized and structured.

My brother, Stephen Michael Copley, who has been without a doubt the best brother a human being could ask for. His success in school motivated me, and he never fails to think of others even when he has every right to not do so. From the moment he could talk, his singular

desire was to be a pilot. Watching him achieve that goal was truly an inspiration. So much so that it inspired me to do what I always wanted to do, become a molecular biologist.

My husband, Brandon Salem Copley. Without him this journey would have not been possible. He is a pillar of emotional and financial support that has buoyed me throughout graduate school. He is intelligent, and witty, and can make me laugh, even when I don't want to. It should also be noted that he is a fantastic cook and brings joy to those around him with his creations.

My best friends Jonathon, Tobin, Beverly, and Jeffe. I would probably (definitely) not be alive today without each of them in turn. Well except maybe Tobin who followed me into almost certain death on any number of occasions. Hanging out with me was a ticket to the emergency room for him at least a couple of times. He's the smartest guy I know and my longest friend, and those are probably connected.

Beverly was less likely to follow me over a cliff, but she was much more likely to help talk me out of jumping off one myself. Her and her family are an inspiration to me and a continued source of intellectual joy, and lesbian efficiency.

Jeffe taught me to play Magic the Gathering. We are friends because we are nerds and give no apologies. He's saved my life, and endangered it, on several occasions which I will respect him by not repeating them here.

Jonathon has slowly helped rewrite the way I view myself and how I view others. He has an amazing insight into people and their rascality, and he isn't afraid to call them out. We all need a friend who tells it like they see it, and he's the best of them. It is a daily blessing to me that they continue to support and challenge me in ways that are invaluable to my well-being.

My committee. I am frequently asked, “What is the most important thing you did in graduate school,” and the answer is always “I picked the right committee.” There is nothing of greater value during graduate school than mentors that care about you and your success. To be the best that you can be is hard, and it is even harder to skillfully bring that out in others. My committee was hard on me when they needed to be and merciful when appropriate. This is exemplified by my mentor Heather Burkin who had a front row seat to my path from folly to Ph.D. candidate. She has been fun but unrelentingly critical in just the right way. As one is with their Alpha!

I want to specially acknowledge Patricia Berninsone who, like my mother, did not live to see the end of this journey. One of my first journal club experiences was in her “Post-Translational Modification” section. She was one of the most interesting and encouraging people I had the pleasure of getting to know at UNR. She will be missed.

Craig Ulrich has been an invaluable resource and friend since I started my research work in graduate school. His ideas and willingness to argue were a critical element of my growth. Let’s be honest, we argued so much that people around us thought we hated each other. This was far from the case. The truth is that sometimes, you just need to fight something out to really see all the things you need to see.

My lab manager Janet Lambert who is an unending wellspring of technical knowledge and has the magical power to proof-read like none who came before her. She is also a rather adept qPCR wizard, and without her help, much of this document would not have been possible.

Last but NOT least, I want to thank LANA Del Rio Ayanami Taco, Demon Hunter™ … because she's a dog!

Table of Contents

List of Tables

List of Figures

Chapter 1:

Preterm Birth and the Myometrium

1.1 Reproduction

The evolutionary precursors of sexual reproduction spiral outward from a variety of animal and plant lineages culminating in an almost countless diversity of processes and molecular mechanisms. From an evolutionary perspective, these mechanisms are tuned to grow and expel appropriately developed organisms, increasing survivability and reproductive potential. Even within our own class, Mammalia, there exist countless permutations of molecular regulation, maintenance, and initiation systems that facilitate sexual reproduction (Lanman, 1977). In spite of this array of regulatory checks and balances, biological and environmental variations can result in a plethora of disease states that produce negative outcomes. Unwanted outcomes include an almost endless mix of birth defects; heritable diseases; complications from preterm birth; as well as fetal, infant and maternal death. While modern advances such as antibiotics and hospital deliveries have improved human reproductive success rates (Al-Gailani and Davis, 2014), there still exist a variety of biological impediments to healthy birth.

1.2 Preterm Birth

Preterm birth is one of the most costly, negative outcomes from pregnancy that touches all regions and populations worldwide. While small variations exist in the length of most pregnancies, preterm labor is defined as the onset of labor before the 37^{th} week (Lyndon, 2013). The financial cost in the United States alone is greater than 26 billion dollars annually (Institute of Medicine, 2007), where approximately 12% of babies are born premature; shockingly, worldwide estimates of the prevalence of preterm birth are

approximately 10% or 15 million births annually ("Partnership for Maternal, Newborn and Child Health," 2016). Additionally, preterm birth is the leading cause of death among infants in the first four weeks of life (Phillips et al., 2015) and complications from preterm birth were responsible for approximately one million deaths worldwide in 2015 ("PMNCH," 2016). An inverse correlation exists between the related health issues and the time the fetus spends *in utero*. This problem is most acute in underdeveloped countries where the death rate can reach 50% of babies born before the 32nd week and 90% of babies born before 28 weeks ("PMNCH," 2016). Even when premature infants survive, they face the compounded costs of increased hospital readmissions, and are at an increased risk of a variety of long term health concerns including cerebral palsy (Arpino et al., 2010), lung disease (Greenough, 2012), necrotizing enterocolitis (Arpino et al., 2010), as well as hearing and vision impairments (Marlow et al., 2005; O'Connor et al., 2007). The average baby born prematurely in the United States accrues medical costs at least 10-fold higher than those of babies carried to term (Behrman and Butler, 2007). The high price tag of preterm birth and its prevalence across the world make it one of today's most pressing health concerns.

The socio-economic and environmental causes of preterm labor can range widely from disease exposure to a lack of healthcare. The World Health Organization reports that preterm births in disadvantaged countries often stem from a higher prevalence of diseases such as HIV and malaria, as well as reduced access to health care and an increased rate of adolescent pregnancy (Howson et al., 2013). Wealthier countries provide an increased ability to rescue previously unviable pregnancies, and to provide the

opportunity for reproductive success to populations previously cut off from these opportunities; however, one downside to this opportunity is a rise in preterm births among a population of babies that would have previously not survived. It is not surprising then that in places like the United States, preterm birth is correlated with reproductive technologies. Fertility drugs and *in vitro* fertilization increase the chance of multiple pregnancies; other correlated factors for wealthy countries include pregnancy among older women, unnecessary medical inductions, and premature Cesarean deliveries ("PMNCH," 2016). Interestingly, rates of preterm birth among women of African descent (and certain other minority populations) in the United States can be as high as 21%, even in situations where first world medical care is available (Adams et al., 2000). While this disparity suggests an underlying genetic component, these dissimilarities could also be affected by socio-economic variables present in the United States such as differential access to health care for minorities (Riley, 2012).

At the individual level, preterm birth is most commonly the result of spontaneous preterm labor, occurring in approximately 50-70% of cases (Goldenberg et al., 2008). In these cases, sociological or economic factors do not fully explain early labor and, even when they do, the underlying mechanisms remain a mystery. Our inability to deal effectively with idiopathic preterm labor is partly a result of our incomplete understanding of how labor is initiated. The problems are multiplied when one considers the variety of tissues and phenotypes that make each organ unique in function. In order to understand, treat, and prevent preterm labor, we need to understand the parameters

that govern the unique environment of the reproductive machinery, and how these systems can give rise to preterm laboring disease states.

1.3 The Uterine Myometrium (Fig. 1.1)

The human female reproductive tract consists of the uterus, the main organ responsible for successful human reproduction, the fallopian tubes, which connect the ovaries to the uterus and the cervix which marks the boundary between the uterine cavity and the vagina (Hricak et al., 1983).

The cervix is a cylindrical band that defines the opening between the vagina and the uterus. The function of the cervix during pregnancy is to maintain a barrier between the growing fetus and the outside world. Secretory cells of the cervical canal secrete a variety of mucus glycoproteins which create the cervical mucus plug (Nott et al., 2016). This plug helps protect the fetus by inhibiting bacterial mobility from the lower vagina (Hein et al., 2005) and inhibiting viral replication (Carlstedt and Sheehan, 1989).

Preterm birth can be triggered by a shortened cervix. Weakness in the cervical barrier can cause rupture of the amniotic sac that protects the fetus from bacterial infection, as well as cause spontaneous labor and birth (Nott et al., 2016).

The uterus itself consists of an internal layer called the endometrium which combines with the fetal trophoblast during pregnancy to form part of the placenta that fosters exchange of hormones and nutrients between the mother and fetus (Hricak et al., 1983). Under the endometrial layer lies a bulk of smooth muscle cells referred to as the myometrium (Hricak et al., 1983). The myometrial smooth muscle allows the expansion

of the uterus during pregnancy and provides the needed contractile force to expel the baby.

1.4 Contraction and Relaxation of Smooth Muscle

Currently, human muscle has been categorized into three distinct groups: skeletal muscle, smooth muscle, and cardiac muscle (Exeter and Connell, 2010). Skeletal muscle works to move limbs and control most voluntary actions, while smooth muscle frequently operates involuntarily and is controlled primarily, but not exclusively, from the autonomic nervous system (Sandow, 1970). Cardiac muscle is a specialized subset of striated smooth muscle that specifically controls the pumping activity of the heart and is capable of generating its own contractions (Spach and Kootsey, 1983). Smooth muscle is an integral part of hollow organs such as the bladder, blood vessels and the uterus (Webb, 2003).

Smooth muscle function is regulated by a complex system of autonomic innervation, hormones, autocrine molecules, and other local chemical signals (Beamish and He, 2010). In spite of this variety of regulatory elements, smooth muscle function is ultimately focused on its ability to contract and relax in order to regulate the size or force output of an organ or system. In blood vessels for example, smooth muscle response to chemical and mechanical signals can cause contraction to raise blood pressure or relaxation to lower pressure blood pressure (Touyz et al., 2018). In the pregnant uterus, contraction drives the birthing process while in the non-pregnant uterus contractions expel menstrual blood and can promote sperm motility and fertilization through

retrograde contractions (Bulletti et al., 2002). Thus, smooth muscle contractions play vital roles in a variety of processes across multiple organ systems.

Smooth muscle contraction is the result of calcium-activated changes in thick and thin filament interactions, namely cross-bridge cycling between actin and myosin (Webb, 2003). Actin filaments connect the cytoskeleton to the contractile machinery, and myosin, which consists of two main subunits: a regulatory light chain and a heavy chain, functions as the molecular motor which drives contraction by cross-bridge cycling and creating tension (Frearson et al., 1976; Ikebe et al., 2001). Changes in cellular calcium concentrations initiated by the opening of cell surface L-type calcium channels and the release of calcium from sarcoplasmic reticulum stores leads to the calcium dependent activation of calmodulin (Dabrowska et al., 1977; Frearson et al., 1976; Kamm and Stull, 1985). Calmodulin is a calcium binding protein that activates myosin light chain kinase (MLCK) which in turn phosphorylates the regulatory light chain of myosin (Sellers et al., 1981). Myosin light chain ATPase-mediated hydrolysis of this phosphate group provides energy for increased cross-bridge cycling and increased muscle tension (Cremo and Geeves, 1998). Thus, smooth muscle contraction is initiated by changes in intracellular calcium levels leading to upregulation of the phosphorylation (i.e., activation) of myosin regulatory light chain and an increase in myosin/actin cross-bridge cycling.

Additional regulation of smooth muscle can also occur through calcium sensitization. Ras homolog A (RhoA) directed phosphorylation and activation of the serine/threonine kinase Rho-associated protein kinase (ROCK) is responsible for down regulating phosphorylative activation of MYPT1, a phosphatase responsible for removing

the phosphate groups from myosin light chain, leading to increased calcium sensitivity (K. Kimura et al., 1996); therefore, the phosphorylation state of the myosin regulatory light chain is controlled by the opposing activities of MLCK and MYPT1 (Aguilar and Mitchell, 2010) and is regulated by a variety of inputs (Fig. 1.2A).

So, research has shown that calcium sensitization and regulation of myosin phosphorylation does not account for all smooth muscle contractions. Arterial smooth muscle has the ability to contract in the absence of any significant change in myosin light chain phosphorylation indicating that smooth muscle contraction could be regulated by a variety of signaling events (El-Yazbi et al., 2015). Scientists have yet to fully understand all the mechanisms that regulate smooth muscle contractions.

Smooth muscle relaxation occurs through a reduction in the intracellular calcium concentration and the activation of myosin light chain phosphatase (MYPT1) which acts to remove the phosphate group from the myosin light chain leading to reduced cross-bridge cycling and reduced muscle tension (Webb, 2003). A drop in stimulus closes voltage gated calcium channels, and allows for Ca2+/Mg exchangers and Na+/Ca2+ ATPases, in both the cell membrane and the sarcoplasmic reticulum, to rapidly reduce the intra cellular calcium levels (Webb, 2003). This leads to the inactivation of Calmodulin and a reduction in activity of MLCK (Saddouk et al., 2017). Additionally, MYPT1 activation in vascular smooth muscle occurs through cGMP regulation by a variety of defined and undefined mechanisms (Surks et al., 1999). Nitric oxide can mediate cGMP production through direct activation of soluble guanylyl cyclase (sGC) (Montfort et al., 2017; Norman, 1996). sGC leads to an increase of cGMP and increased activity of MYPT1 (Fig. 1.2B);

however, some data have shown that nitric oxide-induced relaxation of uterine smooth muscle tissue strips is not blocked by inhibition of sGC (Buxton et al., 2001). In addition, Ulrich et al. revealed possible mechanisms that could contribute to nitric oxide mediated relaxation, such as alteration of protein function through post-translational modification directly by nitric oxide, called nitrosation (Ulrich et al., 2013).

1.5 Pregnancy Hormones

Hormonal and structural changes are critical to successful pregnancy. Endocrine hormones such as progesterone, estrogen and human chorionic gonadotropin (hCG) play vital roles in the gestation process. In humans, progesterone and estrogen increase steadily throughout pregnancy only dropping off after delivery (Tulchinsky et al., 1972) (Fig. 1.3).

Progesterone is primarily a pro-gestation hormone that helps to maintain a quiescent myometrium until the initiation of labor. Initially produced by the corpus luteum and later by the placenta, human progesterone levels increase steadily throughout gestation and do not drop until after delivery. This is in contrast to most nonhuman animals, in which labor is signaled by dramatic drop in progesterone, termed progesterone withdrawal (Kirby et al., 2016). Progesterone's ability to maintain myometrial quiescence was recognized and named "Progesterone Block" as far back as 1956 (Csapo, 1956). Progesterone from the corpus luteum is critical in the structural remodeling necessary early in pregnancy. Additionally, progesterone induces differentiation of stromal to decidual cells and fortifies the cervix to maintain pregnancy

(Putnam et al., 1991). It is likely that the primary role for progesterone's suppression of myometrial contractility is its attenuation of the immune system. Progesterone is associated with the downregulation of nuclear factor-κB (NF-κB) and cyclooxygenase 2 (COX-2), key inflammatory proteins (Hardy et al., 2006). Additionally, regulation of the progesterone receptor (PR) and other inflammatory signals could be the result of progesterone metabolites such as 5β-dihydroprogesterone and others (Byrns, 2014; Putnam et al., 1991). Progesterone suppresses uterine contractions and helps maintain conditions for successful gestation.

hCG increases exponentially during the first trimester and is responsible for maintaining progesterone production by the corpus luteum to sustain the pregnancy during this time (LeMaire et al., 1971). hCG concentrations peak at 13 weeks and then decrease until birth (Braunstein et al., 1976). Near the end of pregnancy, the smooth muscle of the myometrium increases production of the oxytocin receptor (OXTR) and gap junction proteins that increase the electrical communication between cells, and this increased connectivity results in a syncytium, allowing the propagation of productive contractions (Wray et al., 2015). Just before the onset of labor, oxytocin secretion by the neurohypophysis is increased which potentially leads to uterine contractions (Uvnäs-Moberg et al., 2019). Prostaglandins (inflammatory signaling molecules) are released by the fetus and produced in the myometrium by cyclooxygenase (COX) enzymes which can lead to increased uterine contractions (O'Brien, 1995). In addition, strain on the myometrium and the cervix increases throughout gestation until contractions begin and

the fetus is expelled, and increased strain increases the chance of early birth (Lockwood and Kuczynski, 2001).

The transition from a quiescent to a contracting myometrium is thought to signal the end of pregnancy and the beginning of the birthing process (Shynlova et al., 2013). In cases of preterm labor, this process initiates before the 37th week of human gestation, and premature contraction of myometrial smooth muscle ultimately drives spontaneous preterm birth. This overview suggests that birth is a combination of hormonal signals and mechanical influences, however, the exact mechanisms that initiate the transition to labor have not fully been elucidated.

Myometrial smooth muscle has the ability to switch its phenotype during pregnancy based on altered environmental and hormonal signaling events (Bentley and Hershenson, 2008; Joshi et al., 2012; Zhang et al., 2016). In the smooth muscle of the myometrium, Shynlova et al proposes a three stage process where myometrial cells switch phenotype from a proliferative phenotype to a hypertrophy phase to a final "endstage" or contractile phenotype, typified by expression of the oxytocin receptor, connexin 43 (Cx43), and other contractile-associated proteins (Shynlova et al., 2009). While there is evidence that the mammalian target of rapamycin (mTOR) may regulate a transition to a hypertrophic phenotype (Jaffer et al., 2009), and progesterone withdrawal may regulate the transition to a contractile phenotype in rats (Shynlova et al., 2009), it remains unclear whether humans, whose circulating plasma progesterone concentrations remain high, are regulated by functional progesterone withdrawal (Vrachnis et al., 2012) or by other mechanisms yet to be discovered.

Oxytocin promotes uterine contractions. Historically, there was doubt whether significant increases in circulating oxytocin were seen during labor (Leake et al., 1981); however, more recent studies have shown that oxytocin levels increase during pregnancy in humans, in a pulsatile fashion, yet these pulses are not correlated directly with individual uterine contractions (Uvnäs-Moberg et al., 2019). This suggests that other factors are regulating contractions in addition to circulating oxytocin levels. It is also possible that paracrine action, such as release of oxytocin by the decidua, placenta or myometrium itself, as well as an increase in oxytocin sensitivity thought upregulation of the OXTR, has been proposed for an underlying regulatory mechanism of oxytocin-induced labor contractions (Uvnäs-Moberg et al., 2019).

1.6 Inflammation and Labor

It has been suggested that pregnancy is a complicated inflammatory event that culminates in the onset of labor and birth (Azziz et al., 1988). Yet, it is not clear whether the onset of labor drives an inflammatory response, or whether labor initiation is the result of a progressive inflammatory state or event (López Bernal et al., 1993).

Cytokines are soluble peptides that participate in intercellular communication during immune responses (Borish and Steinke, 2003). During pregnancy, sources for inflammatory mediators include the myometrium, the amniotic fluid, the cervix and decidua, and maternal circulation (Sivarajasingam et al., 2016a). Interleukin 6 (IL-6) is a pro-inflammatory cytokine that is increased in amniotic fluid and cervical connective tissue at term, and is produced by monocytes that are present in the myometrium during

pregnancy (Sivarajasingam et al., 2016a). During the second trimester of pregnancy, maternal blood samples show increased levels of circulating monocytes that are responsible for increased production of pro-inflammatory cytokines IL-6, interleukin 1β (IL-1β), and tumor necrosis factor (TNFα) (Tang et al., 2015). A dramatic increase in cervical and amniotic IL-6 among symptomatic women is a predictor of spontaneous labor (Menon, 2008). During term labor, macrophages, T lymphocytes and neutrophils invade the myometrium and produce IL-6, IL-1β, TNFα as well as matrix metalloprotease enzymes responsible for tissue remodeling and cervical ripening (Gomez-Lopez et al., 2010; Young et al., 2002). This process is likely driven by cytokine and chemokine gradients. Indeed, monocyte chemoattractant protein 1 (CCL2) is a potent macrophage recruitment molecule and is upregulated in both term and preterm laboring myometrium and in amniotic fluid from preterm laboring women (Esplin et al., 2005, 2003). Additionally, interleukin 8 (IL-8) and IL-6 are upregulated in amniotic fluid (Andrews et al., 1995) and human cervical connective tissue at term (Sennstrom et al., 2002). Interestingly, this rise in amniotic IL-8 is preceded by a rise in IL-6 suggesting that IL-6 plays a role in driving amniotic inflammatory cascades (Kemp et al., 2002). This increase in inflammatory cytokines may play a role in localized prostaglandin and MMP production leading to the initiation of labor (Sivarajasingam et al., 2016a); however, this suggests a conundrum of cyclical inflammatory responses without a clear initiating factor. Immune cell production of cytokines leads to increases in cytokine production by tissues, which then leads to immune cell production of additional cytokines. Thus, it is clear that cytokines and inflammation create a positive feedback loop that plays a role in labor progression, but it

is still not clear if or how cytokines such as IL-6 and IL-8 act as the initiators of labor (Fig. 1.4).

1.7 Matrix Metalloproteases

Matrix metalloproteases (MMP) are a large family endopeptidases that are differentially expressed in a wide variety of tissues and display broad substrate specificity (Nissinen and Kähäri, 2014). MMPs can exist as cell membrane-anchored or soluble proteinases (Klein and Bischoff, 2011). Localization and the types of MMPs produced are tissue and cell type specific and vary widely. Until now, there have been 23 different MMPs described. They are grouped into subgroups based on target cleavage sites, yet generally all contain four functional domains: signal peptide, catalytic domain, hemopexin-like domain and a propeptide (Klein and Bischoff, 2011). The catalytic domain is a Zn^{2+}-dependent site responsible for the proteinase activity, the signal peptide determine secretion and the propeptide is inhibitory and can be cleaved by other MMPs to increase activity (Klein and Bischoff, 2011).

In addition to MMP function in protease cleavage, MMPs also have the ability to act as inflammatory regulators by cleaving cytokines and chemokines which can activate or inactivate these inflammatory mediators by the production of a variety of cleaved products (Manicone and McGuire, 2008). Indeed, MMPs are capable of activating tumor necrosis factor α (TNF-α) and interleukin 1β by cleavage of their pro-form in macrophages and IL-1β-converting enzyme (caspase-1) deficient mice respectively (English et al., 2000; Mohan et al., 2002; Schönbeck et al., 1998). In the lungs of mice, MMP8 and 9 process

the murine chemokines CXCL5 and CXCL6 thereby increasing their chemoattractant activity (Van Den Steen et al., 2003). A variety of MMPs, including MMP2 and 9, are capable of cleaving stromal derived factor-1 (SDF-1) thus rendering it unable to bind its cognate receptor (McQuibban et al., 2001). In this way, it is possible for MMPs to affect chemokine gradients and regulate infiltration of tissues by monocytes. In murine lung tissue, antibody blockage of MMP function reduces inflammatory cell invasion (Greenlee et al., 2006). These data suggest that MMPs are capable of regulating inflammatory conditions in complicated and sometimes conflicting ways that are not yet fully understood.

MMP2/9 (also gelatinase A/B) are proteases that degrade extracellular matrix proteins, mainly collagen IV (Murphy et al., 1989). Additionally, much data have been produced implicating MMP2/9 activity within the uterus and during pregnancy. MMP2/9 are involved in trophoblast invasion (Isaka et al., 2003; Peng et al., 2015), the breakdown of proteins during cervical ripening (D Stygar et al., 2002), and are associated with intra-amniotic microbial infections (Maymon et al., 2000). MMP9 is elevated in the amniotic fluid of women during term and preterm parturition (Maymon et al., 2000) and MMP2 is present in the smooth muscles of the myometrium and vasculature in pregnant bitches (Beceriklisoy et al., 2007). Both MMP2's and MMP9's ability to degrade ECM is involved in cervical ripening (Denis Stygar et al., 2002). This may indicate that MMP2/9 are important mediators of both inflammation and myometrial remodeling during pregnancy.

1.8 Myometrial Distension

Fetal growth requires myometrial expansion during pregnancy. This expansion is accompanied by increases in internal pressure, yet the myometrium is able to remodel dramatically in order to accommodate fetal growth and maintain a quiescent state throughout normal pregnancy (Basford, 2002; Sivarajasingam et al., 2016a). In cases of twins, however, as well as approximately 10% of single pregnancies, abnormal uterine distension is associated with preterm birth (Lockwood and Kuczynski, 2001). Increased uterine distension has been linked to pregnancy conditions that include polyhydramnios, multiple gestation pregnancy, large for gestational age fetus, or uterine abnormality and increases the risk of preterm labor (Gardner et al., 1995; Hill et al., 1987; Lackman et al., 2001). Increasing uterine volume via a balloon catheter initiates prostaglandin secretion, myometrial contractions, and labor in women, and this process appears to occur independent of fetal signals (Yoshida and Manabe, 1988). This indicates that an increased mechanical force on the uterine wall and subsequent inflammation may play a role in increasing the risk of preterm birth (Waldorf et al., 2015).

Mechanical forces are known to activate a variety of membrane bound and intracellular response networks to regulate myometrial gene expression, cell growth, and contractility (Wu et al., 2008). In response to mechanical strain a variety of integrin proteins, including α5β1, dimerize in response to fibronectin ligand binding and form the core of the transduction machinery linking the extra cellular matrix (ECM) to the cytoskeleton through proteins vinculin, zyxin and α-actinin, among others (Guan, 1997). The interaction of integrins with these cytoplasmic proteins in response to strain is responsible for the phospho-activation of focal adhesion kinase (FAK) (Akiyama et al.,

1994). FAK activation is associated with the formation of transmembrane complexes called focal adhesions. FAK auto-phosphorylation at Y397 is responsible for the activation of Src kinase via SH2 domain interactions (Schaller et al., 1994). FAK and Src work together to regulate the cytoskeletal remodeling during cell migration and contraction by interacting with RhoA (Tomar and Schlaepfer, 2009) among others.

Previous research into the phosphorylation signaling pathways activated in response to mechanical strain of myometrial cells has been limited in scope and functional resolution (Morgan, 2014). In human myometrial tissue bath strain experiments, Li et al. demonstrated that caldesmon and mitogen-activated protein kinases (MAPK) 1/3, also known as ERK1/2, were phosphorylated in response to acute strain at five minutes (Y. Li et al., 2009). The specific MAPK pathways and their downstream targets, that respond to uterine strain are not fully known, but they may facilitate a connection between increased uterine tension and a drive towards a contractile phenotype.

Uterine distension is also directly associated with a variety of myometrial immune responses. Neutrophil attractant chemokine IL-8 mRNA expression is increased in the myometrium in term labor and in cases of preterm labor (Keelan et al., 2003). Myometrial tissue strips produced IL-8 when exposed to mechanical strain (El Maradny et al., 1996) and, in primate models, balloon catheter experiments showed an increase in IL-1β, IL-8, CCL2 with pregnant rat models confirming increases in CCL2 (Esplin et al., 2005). Additionally, *in vitro* strain experiments showed a MAPK dependent increase in IL-8 and COX-2 in human and animal myometrial cells (Sooranna et al., 2005a). Levels of MMP2/9

protein are increased in human preterm laboring myometrial samples when compared with gestational age controls (Ulrich et al., 2019) (Fig. 1.5). MMP2 can also be associated with strain in other cell types. Specifically, MMP2 is regulated by p38 MAPK activation during static strain of bone marrow mesenchymal stem cells (Yang et al., 2017). Additionally, prolonged mechanical strain of myometrial strips shows an increase in contractility and may be related to the gastrin-releasing peptide (Park et al., 2005) but the exact mechanisms involved are unknown. The precise roles and mechanisms behind the release of these inflammatory mediators is still not elucidated, but it is clear that excessive strain on the myometrium activates a variety of cell signaling and immune responses that are likely connected to the signal for the transition into labor.

1.9 Tocolytics - Current Treatment Options

Considering the overwhelming emotional and financial impact of preterm birth, it is frustrating that few interventions exist capable of delaying birth by more than seven days once labor has begun, with most treatment options delaying birth only 48 hours (Haas et al., 2009). The ultimate goal of tocolytic research is to halt labor to allow the fetus to remain in utero until at least 37 weeks. For dramatically preterm labor (around 24 weeks) this means arresting the labor process completely. The hope is to allow the fetus to remain *in utero* for as long as possible, providing time for proper development. Current clinical methods are able to reach the minimum goal of delaying birth long enough to allow time for corticosteroid treatment to help lung maturation and prevent idiopathic respiratory distress syndrome (Roberts et al., 2017). As with all drug

interventions, the tradeoff between efficacy and unwanted side effects is the main concern for physicians.

1.9.1 Betamimetics

β-adrenergic receptor agonists or betamimetics are a class of drug that mimics endogenous ligands for adrenergic receptors. A variety of betamimetic drugs such as ritodrine, terbutaline and hexoprenaline have been developed (Neilson et al., 2014). Beta receptor agonist action in the uterus results from the activation of target β-adrenergic receptors in uterine smooth muscle. Receptor activation leads to an intracellular increase in cyclic adenosine monophosphate (cAMP) via adenylyl cyclase activity and a change in sodium/calcium channel function causing increased calcium uptake into the sarcoplasmic reticulum. Ultimately this signaling cascade leads to a reduction in the phospho-activity of MLCK and relaxation through the subsequent decrease in phosphorylative activation of actin-myosin cross-bridge cycling (Scheid et al., 1979).

A meta-analysis of betamimetics, when compared to placebo controls, showed a statistically significant decrease in women giving birth prior to 48 hours after treatment; however, there was no significant change in measured fetal outcomes (perinatal death, cerebral palsy and respiratory distress syndrome) (Neilson et al., 2014). Negative maternal outcomes and cessation of treatment were significantly increased vs. placebo, with trials reporting hypokalemia, hyperglycemia, headaches, and death, related to pulmonary edema (Lamont, 2000; Neilson et al., 2014). Additionally, the capability for betamimetics to cross the placenta can lead to fetal tachycardia, and fetal hypoglycemia

after birth (Neilson et al., 2014). Thus, betamimetics have lost favor in first world settings due to the high incidence of side effects and danger to the fetus.

1.9.2 Calcium Channel Blockers

First identified by Fleckenstein around 1964, but used as early as 1021 to treat heart conditions, calcium channel blockers, or calcium channel antagonists, inhibit the influx of calcium into cells by blocking mainly L-type voltage dependent calcium channels (Fleckenstein, 1983). Voltage-dependent calcium signaling is fundamental to the contractility of smooth muscle and, in fact, all three muscle types (smooth, cardiac, and skeletal).

Similar to betamimetics, blocking calcium channels reduces intracellular calcium levels and thus inhibits active actin-myosin cross-bridge cycling and smooth muscle contraction. A comparison of the side effects between betamimetics and calcium channel antagonists reveals a dramatic decrease in the negative fetal outcomes associated with beta agonists (Flenady et al., 2013), while some risk of pulmonary edema still exist (Gáspár and Hajagos-Tóth, 2013). Thus, calcium channel blockers, like nifedipine, are preferable to beta agonists as tocolytic agents; however, nifedipine's ability to delay delivery beyond 48 hours, and therefore its benefit to long term post-partum development is not statistically significant (Houtzager et al., 2006).

1.9.3 Oxytocin Receptor Inhibitors

Oxytocin receptor inhibitors, such as Atosiban marketed by Ferring Pharmaceuticals, are proposed to block the contractile response generated by an oxytocin receptor-mediated intracellular calcium increase (Melin, 1994); however, this cannot be stated with confidence because Atosiban has a higher affinity for the vasopressin receptor than the oxytocin receptor (Akerlund et al., 1999). Clinical results from barusiban, a highly specific oxytocin receptor inhibitor, showed no tocolytic effect (Thornton et al., 2009). Additionally, Atosiban is not approved for use in the United States, partly due to a lack of adequate placebo controlled trials (Jorgensen et al., 2014), but is approved for use in European countries.

Negative outcomes from clinical trials included possible increased fetal death and low birth weight compared to placebo; additional negative reactions requiring cessation of treatment were slightly increased when compared to betamimetics, while direct comparisons with other tocolytics lacked sufficient evidence to determine outcomes (Flenady et al., 2014).

1.9.4 COX Inhibitors

Cyclooxygenase (COX-1 and COX-2) enzymes are critical to induction of a variety of inflammatory responses through the production of prostaglandins. Prostaglandins are hormones that mediate inflammation and, specifically, increase intracellular calcium leading to activation of MLCK and smooth muscle contraction. Indomethacin is the most common tocolytic representing COX-2 inhibitors, drugs designed to block the production of prostaglandins that result in uterine contractions and delay birth (Loudon et al., 2003).

A meta-analysis that compared COX inhibitors to placebo found a significant reduction in the number of babies born within 7 days of the onset of treatment and a significant reduction in birth within 48 hours of treatment when compared with betamimetics (Reinebrant et al., 2015); however, side effects for the use of COX-2 inhibitors relate to fetal development and could be the result of accumulation of the drugs in the fetal circulation. Use of indomethacin is associated with prenatal closure of the ductus arteriosus, renal dysfunction and oligohydramnios (Loudon et al., 2003).

1.9.5 Magnesium Sulfate

Magnesium sulfate was first used as a tocolytic agent in the 1960s (Rusu et al., 1966), but the first clinical trial was not reported until 1977, which showed a small decrease in likelihood of contractions for 24 hours (Reinhart, 1991). Magnesium sulfate is capable of reducing contractions in isolated myometrial tissue strips, yet the exact mechanisms of action of magnesium sulfate on the contracting uterus are not fully known (Tang et al., 2014). It is thought that the divalent magnesium ion may act to competitively block calcium channels as well as increase calcium transport out of the cells, thus reducing contractions through reduced intracellular calcium concentrations (Ramsey and Rouse, 2001).

Magnesium sulfate has not been shown to reduce the risk of birth before 48 hours compared to placebo (Crowther et al., 2014). Studies show an increased risk of birth before 24 hours compared with betamimetics and a decreased risk compared with COX inhibitors (Crowther et al., 2014). Magnesium sulfate slightly increased the risk of fetal,

neonatal and infant death compared to placebo, while comparison with other tocolytics produced no statistical differences (Crowther et al., 2014). Negative maternal outcomes for magnesium sulfate administration are minimal with no reported deaths, cardiac arrests, and only one respiratory arrest (Crowther et al., 2014). Based on a meta-analysis, Crowther et al. determined that there was no clinically significant value in administering magnesium sulfate as a tocolytic agent, even though it is still widely used (Crowther et al., 2014). Offering treatment that has low efficacy but also low risk may keep this drug in use, but it has not been shown to be a valuable tocolytic (Crowther et al., 2014).

1.9.6 Other

In addition, there exists a variety of potential tocolytic agents not discussed here, including ethanol and nitric oxide, which are not commonly used by physicians. None of these tocolytics are effective past 48 hours, and they each suffer from side effects and negative outcomes that make their risks outweigh their rewards.

1.9.7 Tocolytic Summary

Tocolytic research is an ongoing field that has yet to fully realize a satisfactory outcome of delaying birth until term in patients undergoing preterm labor. While some work has been done on combinations of current tocolytic agents, it is still not considered advantageous to combine agents in an attempt to delay preterm birth (Locatelli et al., 2015). New and bold innovations in our understanding of the mechanisms leading to labor will be necessary for the development of a new generation of tocolytic treatments. The

search must continue for an effective treatment that will allow for full term delivery without the introduction of clinically relevant side effects.

1.10 Methodolgies

Preterm birth is a costly problem with no solid solutions, but it is correlated with uterine distension. Thus, the work reported here was focused on understanding the cellular signaling events that are triggered by uterine distension and ways in which these events may be contributing to early birth.

Temporally speaking, one of the first signaling pathways to be activated after cellular stress (mechanical strain) are phosphorylation networks. The advent of mass spectrometry based proteomic techniques (peptide sequencing) and strong relative quantification strategies (tandem mass tag labeling) for post translational modifications made such a discovery search more economical and robust than it has been previously (see below). We took advantage of this advancement in technology and used it as a novel approach to visualize the phosphorylation changes in uterine smooth muscle cells after exposure to mechanical strain (Chapter 2).

Subsequent to our phosphoproteomic experiments, we wanted to elucidate what transcriptomic changes were activated by mechanical strain in uterine smooth muscle cells (Chapter 3). In order to do this, we utilized RNAseq. RNAseq is a powerful and robust method for RNA quantification based on Next Generation Sequencing (NGS), the advantages of which are described below (Mortazavi et al., 2008). We were able to leverage this technology in pursuit of understanding the transcriptional changes that

occur in response to mechanical strain. These technologies are described below in more detail, and they will be referenced again in the sections that describe the work based on them.

1.11 Mass Spectrometry

Mass spectrometry is an analytical tool that can measure the mass and charge of an analyte. From this fundamentally simple conceptual framework, modern mass spectrometry has grown into a powerful technology that can be used by experts in law enforcement; hospital labs and clinical settings; as well as basic chemistry and biology research laboratories. The surprising flexibility of mass spectrometry makes it an integral and valuable technology for use in measurements of masses ranging all the way from atoms to simple molecules (Finehout and Lee, 2004). Within the past few decades, this scope has been greatly expanded to include large biological molecules like proteins and nucleotides. The ability to measure the mass of proteins and to quantitate proteins within complex mixtures has dramatically increased the functionality and viability of mass spectrometry in biological contexts. Since 1999, the number of publications involving mass spectrometry has sharply increased. This increase coincides with the concomitant rise in publication of data generated from experiments in proteomics that involve using mass spectrometry to measure large amounts of proteins from complex mixtures like cell lysates (Mallick and Kuster, 2010) (Fig. 1.6). Thus, mass spectrometry remains a fast growing and powerful technology that can be used to answer an ever-increasing variety of scientific questions.

1.11.1 Development of Mass Spectrometry

The history of mass spectrometry, like many modern technologies, reaches back over a century. In 1912, Sir Joseph John Thomson constructed the first mass spectrometer as part of his research into electrons and positive ray electricity (positive ion formation) (Thomson, 1914). This machine was not capable of studying biomolecules, like proteins, as they were too large and there was no system for ionizing and injecting them into the mass analyzer. Thomson's machine was mainly used in physics to study elemental isotopes (Laeter, 2001; Willard et al., 1998). By 1956, improved mass range and the ability to vaporize different types of samples allowed organic molecules to be ionized and studied, leading to the first mass spectrometry-based analysis of the structure of nucleotides (Biemann and McCloskey, 1962). The development of fast atom bombardment, a technique to ionize larger, less volatile molecules (Barber et al., 1981), and the subsequent development of electrospray ionization laid the foundation for analysis of large biomolecules, such as proteins (Fenn et al., 1989). By 1991, the first protein structures were analyzed (Chowdhury et al., 1990). This helped in the development of peptide mass fingerprinting, a technique in which a peptide's amino acid sequence could be determined by ionization and fragmentation (Chowdhury and Chait, 1989). Recently, the development of additional techniques for quantification, such as tandem mass tags, has allowed for more targeted and quantitative approaches (McAlister et al., 2012) and pushed the field of mass spectrometry forward, broadening the range of questions that this technology is capable of answering.

1.11.2 Instrumentation

In practice, most mass spectrometers contain three integral components: An ion source, a mass analyzer, and an ion detector (Finehout and Lee, 2004) (Fig. 1.7). Additionally, computers may be used as downstream analysis and interpretation tools that are fundamental to successful work in such areas as proteomics and *de novo* protein sequencing.

The function of the ion source is to ionize and vaporize the sample. Electrospray ionization (ESI) has become one of the most widely used methods for ionizing and transforming samples of biological molecules, such as proteins, into the gas phase (Fig. 1.8). In brief, solvated molecules are passed through an electrical current producing heavily charged droplets, which are then injected into a heated vacuum chamber. The heat and low-pressure help remove the solvent, leaving gas phase ions to be passed into the mass analyzer. This process allows for conversion of large non-volatile analytes, such as proteins and peptides, into charged analytes capable of being magnetically accelerated to the detector (James, 2001). One of the main advantages of ESI over other ionization methods is its ability to be coupled to high performance liquid chromatography (HPLC). This allows for HPLC to be used as a powerful sample separation tool and analyte source for mass spectrometry. This decreases the sample complexity of each injection and thereby increases the functional resolution (Ramanathan et al., 2007).

The mass analyzer is the heart of the mass spectrometer, and a number of mass analyzers have been developed in the decades since Sir Thomson's initial creation. Two of the top mass analyzer technologies are time of flight (TOF) and the more advanced

Fourier transform ion cyclotron resonance (FTICR). In FTICR, analyte ions are trapped in a magnetic field and a current is used to excite ions whose resonance signal is digitalized and de-convoluted by Fourier transformation into a usable mass spectrum (Marshall and Verdun, 1989). In contrast, TOF analyzers accelerate ions with an electric field and measure the time it takes for each ion to reach and strike a detector that is a set distance from the origin (Wolff and Stephens, 1953). Because FTICR can trap ions and thus measure all trapped ions simultaneously, it has a higher resolving power than TOF mass spectrometers which can only read single ions directly (Marshall et al., 1998). The development of FTICR was a step forward in mass spectrometry and increased the ability to distinguish between biological molecules of very similar mass.

An offshoot from the FTICR system is the Orbitrap. In an Orbitrap analyzer, two cup-shaped end electrodes are positioned such that incoming ions circle around a third central electrode (Fig. 1.9). An electrical field is then used to create harmonic axial oscillations. These oscillations are then detected by detector plates, digitized and then Fourier-transformed into a mass spectra (just as with FTICR) (Zubarev and Makarov, 2013). Advantages to this system are not only in the cost of each machine but in size. FTICR analyzers are dependent on size for resolving due to ion phase interference and ion capacity (Marshall et al., 1998). Additionally, Orbitrap analyzers manipulate ions using electrodes and do not require large superconducting magnets to function (Zubarev and Makarov, 2013). This allows for “table top”-sized machines that have the power and resolution to analyze large biological data sets, particularly when combined with newer ion sources such as ESI and C-traps (C-traps sit between ESI systems and the Orbitrap to

regulate the flow of ion packets to the Orbitrap) used in more modern Orbitrap machines (Olsen et al., 2007) such as Thermo Fisher's LTQ Orbitrap XL™.

1.11.3 Protein Analysis

The recent advances in mass spectrometry technology have been leveraged in the discovery and quantification of large biological molecules such as nucleotides and proteins. The ability to produce large scale protein studies, called proteomics, has been historically hindered by technology. Previously, antibody-based techniques such as Western blotting were used for single protein qualification and semi-quantification. Additionally, larger scale studies of protein changes could be performed with fluorescent two-dimensional differential gel electrophoresis (2D DIGE) which separates samples in two dimensions and allows the resulting spots to be compared semi-quantitatively. Western blotting is inherently restricted to only proteins that are known and require specific antibodies to each targeted protein or isoform. 2D DIGE allows for greater throughput but is limited by resolution. Additionally, these methods can fail to differentiate between single amino acid changes and frequently struggle to separate post-translational modifications. In contrast, mass spectrometry, coupled with HPLC, ESI and electronic databases offer dramatically superior resolution with much lower sample volumes (Noordin and Othman, 2013). The clear answer to a wide variety of modern proteomic-level inquiries lies in mass spectrometry.

1.11.4 Proteomics

Protein analysis by mass spectrometry was historically limited by the size and charge states of intact proteins. TOF mass spectrometers were able to measure and report the mass of an intact protein; however, protein masses are not unique and serve as poor identifiers of proteins for later analysis (Adams et al., 1993). The introduction of two new ideas laid the groundwork for a significant leap forward in protein mass spectrometry.

Ironically, the first of these was DNA sequencing. During the 1990s, the ability to sequence cDNAs made from protein coding RNA molecules was developed (Hishiki, 2000). This combined with complete genome maps allowed for protein sequences to be determined from the amino acid sequences of these nucleic acids. The second idea was to develop algorithms capable of searching peptide sequences against a species database and identifying the proteins that produced those peptides (Mann et al., 1993). This allowed for identification of large amounts of proteins from a complex sample digested with selected proteases. It is possible to create a wide variety of different peptides from proteins by protease digestion, each with unique cut sites and resulting sequences. Trypsin has become the protease of choice in mass spectrometry for a number of reasons. First, digestion with trypsin results in a guaranteed lysine/arginine residue on each peptide. Because mass spectrometry uses electromagnetic methods of accelerating analytes, it is beneficial to have every peptide automatically carrying a positive charge; in addition, having basic residues at the C-terminal end of peptides increases detectable y-ions of the appropriate mass range, and provides increases in double charged peptides after ESI (Rosenfeld et al., 1992). Thus, trypsin-digested peptides can be identified by

mass sequencing and then searched against a species-specific database by an algorithm that matches peptides to proteins.

1.11.5 Quantification Methods

Previous quantitation methods in mass spectrometry included isotope labeling and measurement of relative abundance in simple scans of precursor ions (MS1). These methods suffer when the complexity of the sample reduce acquisition speed and correspondingly sensitivity (Karp et al., 2010; Mertins et al., 2012). The past decade has seen an explosion in new peptide labeling technologies, or tandem mass tags (TMT), allowing for 6-, 10-, and 11-plexed runs (McAlister et al., 2012).

Tandem mass tags, or TMT are amine reactive, isobaric chemical tags that can be used to label peptides before analysis by mass spectrometry. Each tag contains a reactive section that is used to label all the peptides in a single experimental condition, a reporter ion that will fragment during mass spectrometry analysis and a mass linker to maintain isobaric mass before fragmentation. Once labeled, peptides from each sample can be pooled and fractionated by HPLC, the relative intensity of these reporter ions can be quantified, and the associated MS1 scan peptide matched with its corresponding protein (Erickson et al., 2015). Here we used 6-plex (Fig. 1.10) TMT-labeling to quantify the phosphoproteome of human uterine smooth muscle cells (Chapter 2). Compared to 2D DIGE and other proteomics technologies, TMT-labeled mass spectrometry provides the most quantitative and comprehensive proteomic technology available (Erickson et al., 2015).

1.11.6 Pathway Analysis of Proteomics

All proteomic datasets were combined into pathways by Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., qiagenbioinformatics.com/products/ingenuity-pathway-analysis). IPA analysis uses literature searches combined with its own proprietary data base (Ingenuity Pathways Knowledge Base) to group proteins from a data set into associated pathways. The statistical relevance of these pathway-protein associations is calculated using the ratio of the total number of proteins from the data set that are associated with a particular pathway and divide this value by the total number of proteins that map to the canonical pathway within the literature (Krämer et al., 2014). A p-value is calculated using Fisher's exact test to determine the statistical probability that the association between the measured proteins, as part of a particular pathway, is due to change alone (Krämer et al., 2014).

1.12 RNA Sequencing (RNAseq)

A variety of methods exist for transcriptome analysis; however, until the advent of RNAseq, and other high throughput sequencing technologies, expression microarrays were the most commonly used technology for probing a large list of genes that would be unreasonable to perform one at a time by quantitative polymerase chain reactions (qPCR); however, these microarrays utilized hybridization and visualization systems which exclude alternate spliced transcripts and novel, undiscovered mRNA (Mortazavi et al., 2008). Other methodologies, such as serial analysis of gene expression (SAGE) and

massively parallel signature sequencing (MPSS) also fail to capture novel transcripts or alternative spliced variants and lack the ability to find unspecified transcripts (Brenner et al., 2000; Harbers and Carninci, 2005). RNAseq by comparison has the capacity for reading previously unknown transcripts as well as a digital output that doesn't rely on fluorescence and other less quantitative measurements (Mortazavi et al., 2008).

1.12.1 Pathway Analysis of Transcriptomics

For pathway analysis of transcriptomic data sets, we used Advaita Bio's iPathwayGuide (http://www.advaitabio.com/ipathwayguide) as both gene ontology (GO) annotation software, as well as matching of differentially expressed genes to pathways using the "Impact Analysis" approach. In brief, this method determines the up or down regulation, type of signal, and the position and function of each gene within a pathway as described in (Draghici, 2007, Tarca, 2008, Donato, 2013, Ahsan 2018). These data are then integrated into a pathway analysis report which includes all the genes associated with each pathway, as well as a p-value for each using methodology as described in (Tarca et al., 2009). This p-value represents the likelihood that a particular combination of genes would represent the activation of a particular pathway by chance (Tarca et al., 2009).

1.13 Conclusion

Pregnancy is a fascinating and complicated process that we are only now beginning to fully understand. Women ideally carry their fetuses for around 40 weeks to maximize that child's health and future success. Preterm birth is a costly worldwide

problem that carries with it increased financial and biological risks to both the mother and the child, who may suffer a lifetime of complications and developmental delays based solely on their short gestation. While social and economic factors can affect birth timing in humans, there exist underlying biological mechanisms that have yet to be fully discovered.

Some pieces of this puzzle have been firmly fixed. Inflammatory responses from both exogenous infectious agents and from indigenous mechanisms are strongly linked to early birth (Romero et al., 2007). Additionally, in both singleton and twin pregnancies, abnormal uterine distention appears to increase the risk of preterm birth (Lockwood and Kuczynski, 2001). The totality of mechanisms by which distension and inflammation may be connected and coordinated has yet to be fully mapped out. Here we take advantage of –omics technologies, including mass spectrometry and RNAseq, to map out important phosphorylation signaling and transcriptional events tied to myometrial smooth muscle distension and then investigate the connections between them. We present data in the hopes of elucidating possible connections between inflammation and distension in order to deepen our understanding of the cause of labor and add to the voices looking to find pathways and proteins that could serve as targets for a new generation of tocolytic therapies aimed at delaying preterm birth until term.

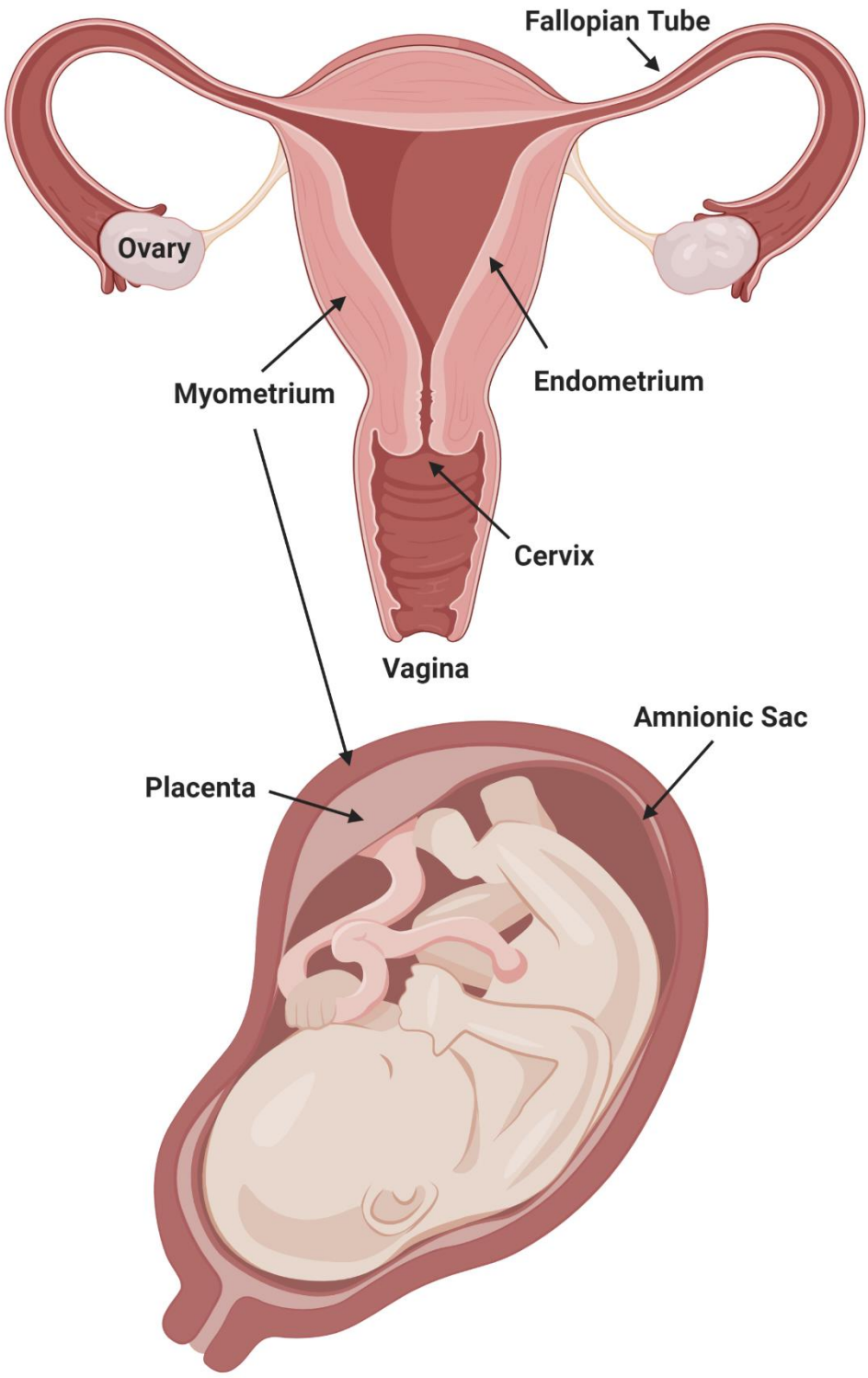

Fallopian Tube
Ovary
Myometrium
Endometrium
Cervix
Vagina
Amnionic Sac
Placenta

Figure 1.1

Female Reproductive Anatomy. The human female reproductive tract consists of the uterus, the fallopian tubes, ovaries, and the cervix which marks the boundary between the uterine cavity and the vagina. The internal layer of the uterus is called the endometrium under which lies a bulk of smooth muscle cells referred to as the myometrium. The cervix is a cylindrical band that defines the opening between the vagina and the uterus. The function of the cervix during pregnancy is to maintain a barrier between the growing fetus and the vagina. During pregnancy, the fetus is attached to the endometrium by implantation and the formation of the placenta which provides nutrients to the growing fetus. The amnion fills with fluid as pregnancy progresses allowing for protection and range of motion for the fetus.

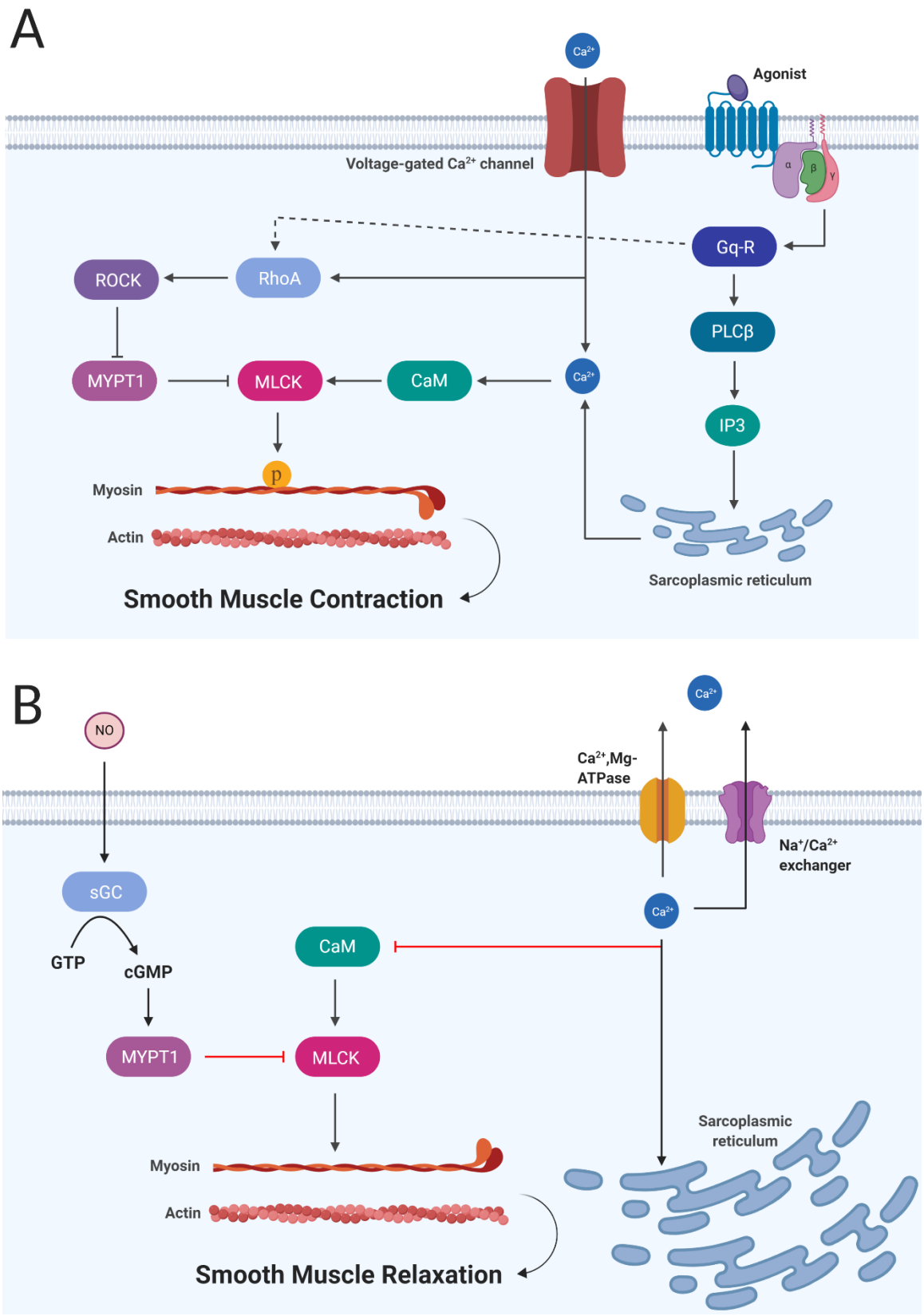

A
Ca2+
Agonist
Voltage-gated Ca2+ channel
α
β
γ
Gq-R
ROCK
RhoA
PLCβ
MYPT1
MLCK
CaM
Ca2+
IP3
p
Myosin
Actin
Sarcoplasmic reticulum
Smooth Muscle Contraction
B
NO
Ca2+
Ca2+,Mg-
ATPase
Na+/Ca2+
exchanger
sGC
Ca2+
GTP
cGMP
CaM
MYPT1
MLCK
Sarcoplasmic
reticulum
Myosin
Actin
Smooth Muscle Relaxation

Figure 1.2

A)

Smooth muscle contraction: Cellular calcium concentrations increase due to the opening of L-type calcium channels which lead to calcium-triggered calcium release from the sarcoplasmic reticulum. This activates calmodulin which activates MLCK which phosphorylates the regulatory light chain of myosin. Hydrolysis of the phosphate group provides energy for increased cross bridge cycling and contraction. RhoA based down-regulation of MYPT1 leads to increased contraction.

B)

Smooth Muscle Relaxation: When voltage gated calcium channels close, Ca^{2+}/Mg^{2+} exchangers and Na^{+}/Ca^{2+} ATPases, in both the cell membrane and the sarcoplasmic reticulum, rapidly remove calcium from the cell. This inactivates calmodulin and leads to a reduction in phosphorylation of MLCK, which leads to a reduction in contractions. Nitric oxide mediates smooth muscle relaxation by activation of sGC which activates MYPT1 leading to reduced phosphorylation of MLC9 and consequently reduced cross bridge cycling and relaxation.

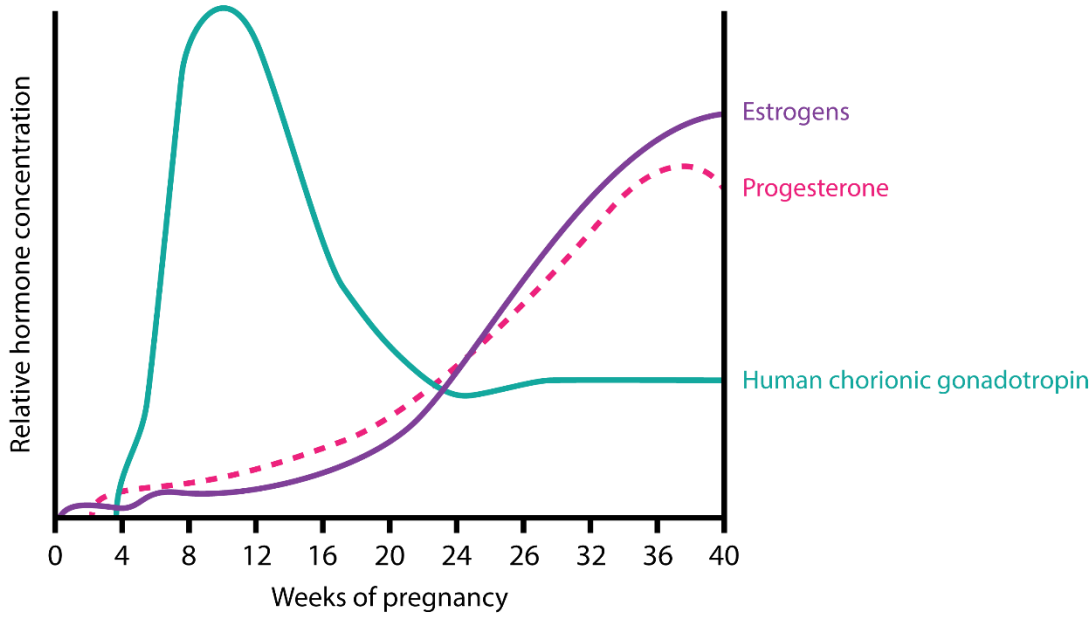

Relative hormone concentration
0
4
8
12
16
20
24
26
32
36
40
Weeks of pregnancy
Estrogens
Progesterone
Human chorionic gonadotropin

Figure 1.3

Relative hormone concentrations during pregnancy by week: Progesterone, estrogen and human chorionic gonadotropin (hCG) are endocrine hormones produced in pregnancy. In humans, progesterone (pink dashes) steadily increases until after birth. Estrogen (purple) also increases steadily throughout pregnancy only dropping off after delivery. hCG (teal) initially produced by the corpus luteum is highest during early pregnancy. After about 13 weeks, the placenta begins to take over production and the levels fall for the remainder of gestation.

Adapted from: https://courses.lumenlearning.com/suny-ap2/chapter/maternal-changes-during-pregnancy-labor-and-birth/

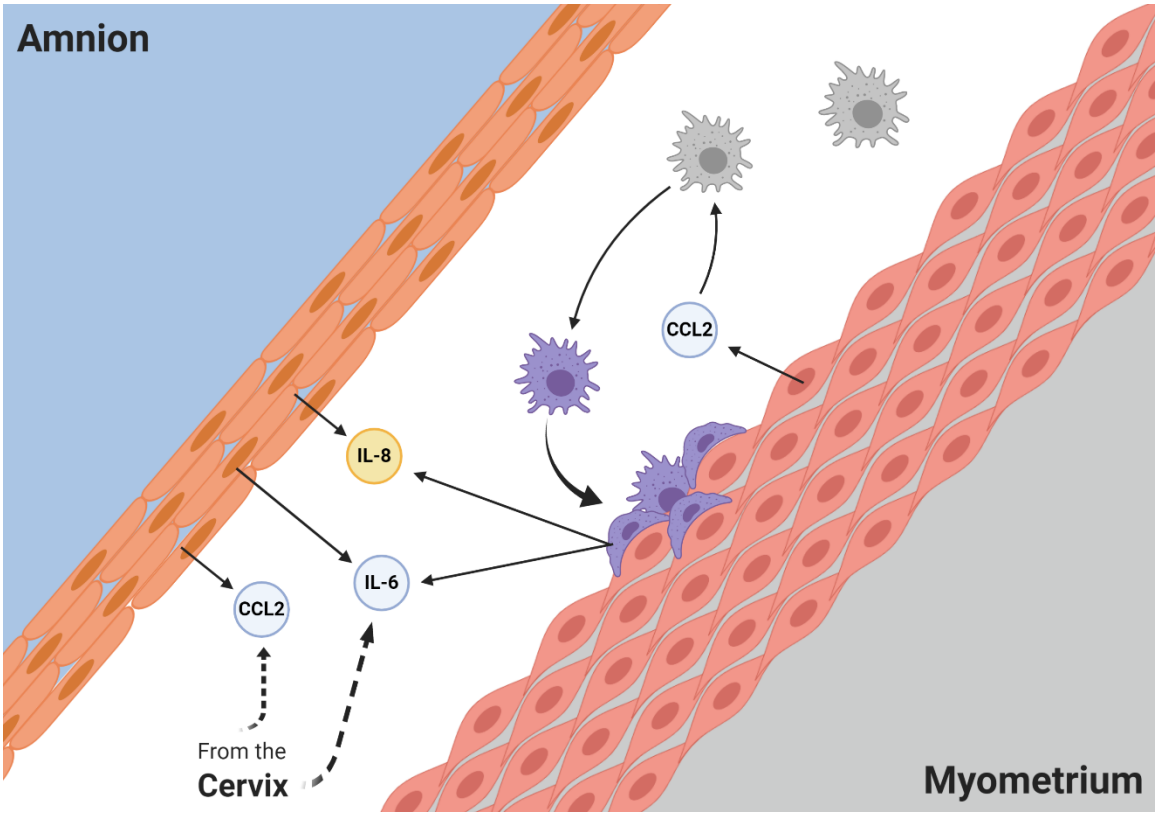
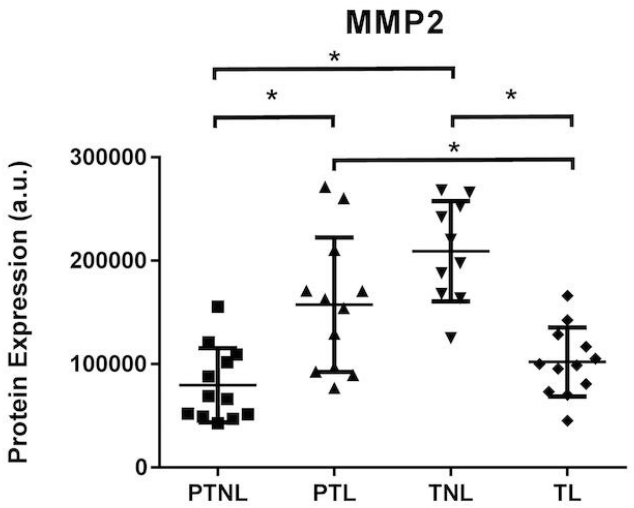
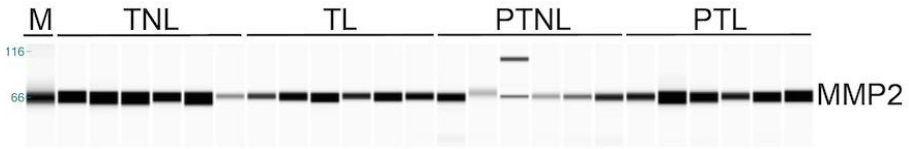
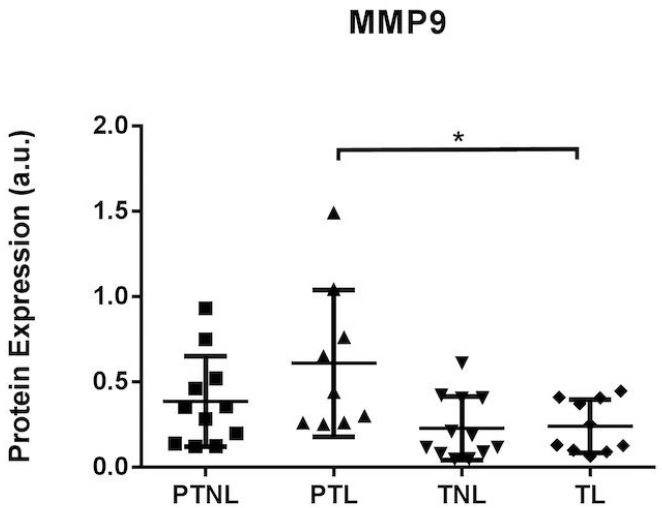

Amnion
CCL2
IL-8
IL-6
CCL2
From the
Cervix
Myometrium

Figure 1.4

Inflammatory cytokine interactions: A representative summary of key cytokines of interest produced by the myometrium, the cervix and the amnion. CCL2 functions as a recruitment molecule leading to monocyte invasion during pregnancy. The cervix produces IL-6 and CCL2. IL-8 and IL-6 are produced by the amnion and CCL2 is also produced by the myometrium. Monocytes are attracted by CCL2 and invade the myometrium, in turn producing more IL-6 and IL-8.


MMP2
Protein Expression (a.u.)
300000
200000
100000
0
PTNL
PTL
TNL
TL
*
*
*
*


M
TNL
TL
PTNL
PTL
116
66
MMP2


MMP9
Protein Expression (a.u.)
2.0
1.5
1.0
0.5
0.0
PTNL
PTL
TNL
TL
*

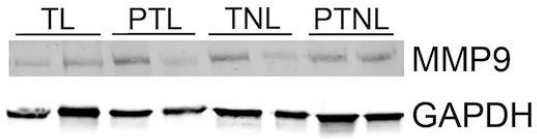

TL
PTL
TNL
PTNL
MMP9
GAPDH

Figure 1.5

Relative MMP9 and MMP2 expression is elevated in preterm laboring myometrial tissue. Samples from women in different states of pregnancy and labor (PTNL= preterm not in labor, PTL= preterm labor, TNL= term not in labor, and TL= term in labor) were subjected to SimpleWes analysis. Panel A Shows women undergoing spontaneous preterm labor (PTL) had higher MMP2 expression than gestational age matched non-laboring controls (PTNL) and term laboring (TL) controls ($p<0.05$). MMP9 expression was higher in myometrial tissue from women undergoing spontaneous preterm labor (PTL) than term laboring controls (TL; $p<0.05$). Panel B is shows representative SimpleWes* images corresponding to MMP2 and MMP9 peak quantitation experiments. This figure was generated by (Ulrich et al., 2019). Normalized group values were compared on Prism version 6 (GraphPad) with a one-way ANOVA.

*Simple Western immunoassays (SimpleWes) are a capillary based protein quantification assay. Equal volumes of each sample are drawn into the capillary and through a stacking and separation matrix which separates them by size. Target proteins are quantified by primary antibody linked to a horseradish peroxidase (HRP)-conjugated secondary antibody with a chemiluminescent substrate. Quantitation is based on relative levels of this substrate. SimpleWes assays have a wider dynamic range (3 orders of magnitude) than conventional Western blots and the capillary separation technique provides better separation and more accurate quantitation (www.proteinsimple.com).

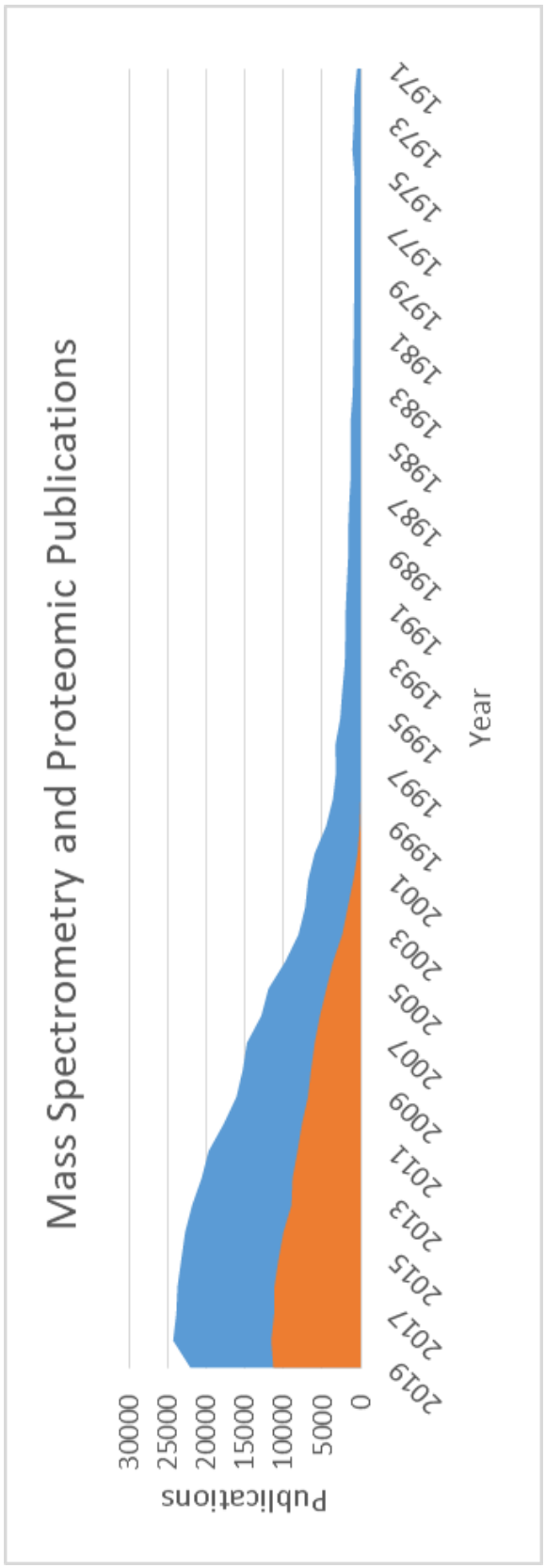

Mass Spectrometry and Proteomic Publications
Publications
30000
25000
20000
15000
10000
5000
0
Year
1971
1973
1975
1977
1979
1981
1983
1985
1987
1989
1991
1993
1995
1997
1999
2001
2003
2005
2007
2009
2011
2013
2015
2017
2019

Figure 1.6

Mass spectrometry based publications by year. A PubMed search was completed for the terms “Mass Spectrometry” (blue) and “Proteomics” (orange). The yearly distribution of publications matching those search terms is displayed graphically with the total number of matching publications on the Y-axis and the years they were published on the X-axis.

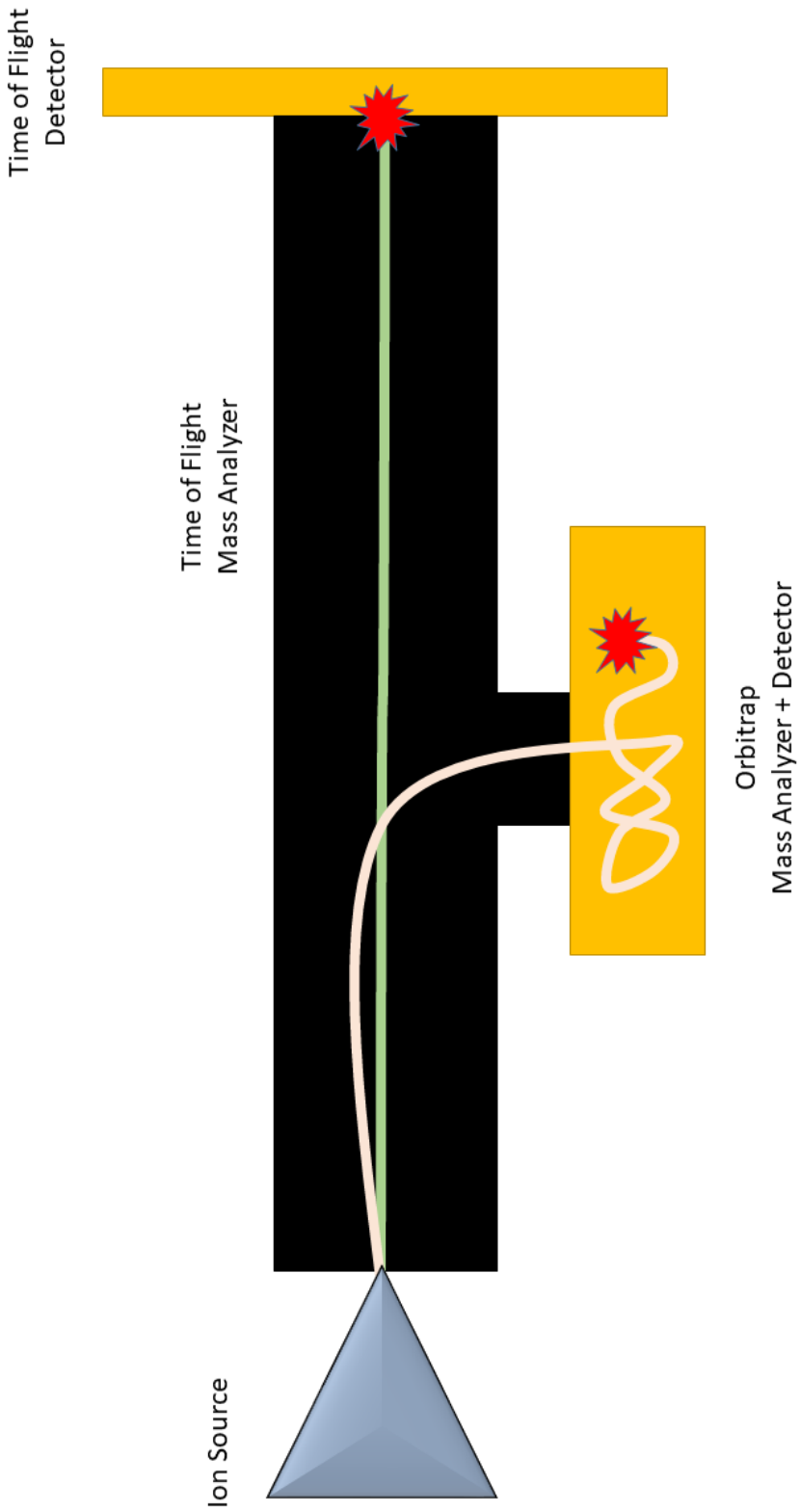

Time of Flight
Detector
Time of Flight
Mass Analyzer
Orbitrap
Mass Analyzer + Detector
Ion Source

Figure 1.7

Generalized Mass Spectrometer. All mass spectrometers consist of 1) an ion source, 2) a mass analyzer, and 3) a detector. Here are represented two different configurations for the three components of a mass spectrometer. In both cases, the ion source generates ions capable of detection by the mass spectrometer. These ions are then sent through a mass analyzer. In the case of Time of Flight, the mass and charge (m/z) of an ion determines its flight time from entry into the mass analyzer until it strikes the detector plate. Alternatively, the Orbitrap functions as both a mass analyzer and a detector. Ions travel into the Orbitrap and their m/z determines their path around a central electrode. This path is detected and used to measure the m/z of each ion.

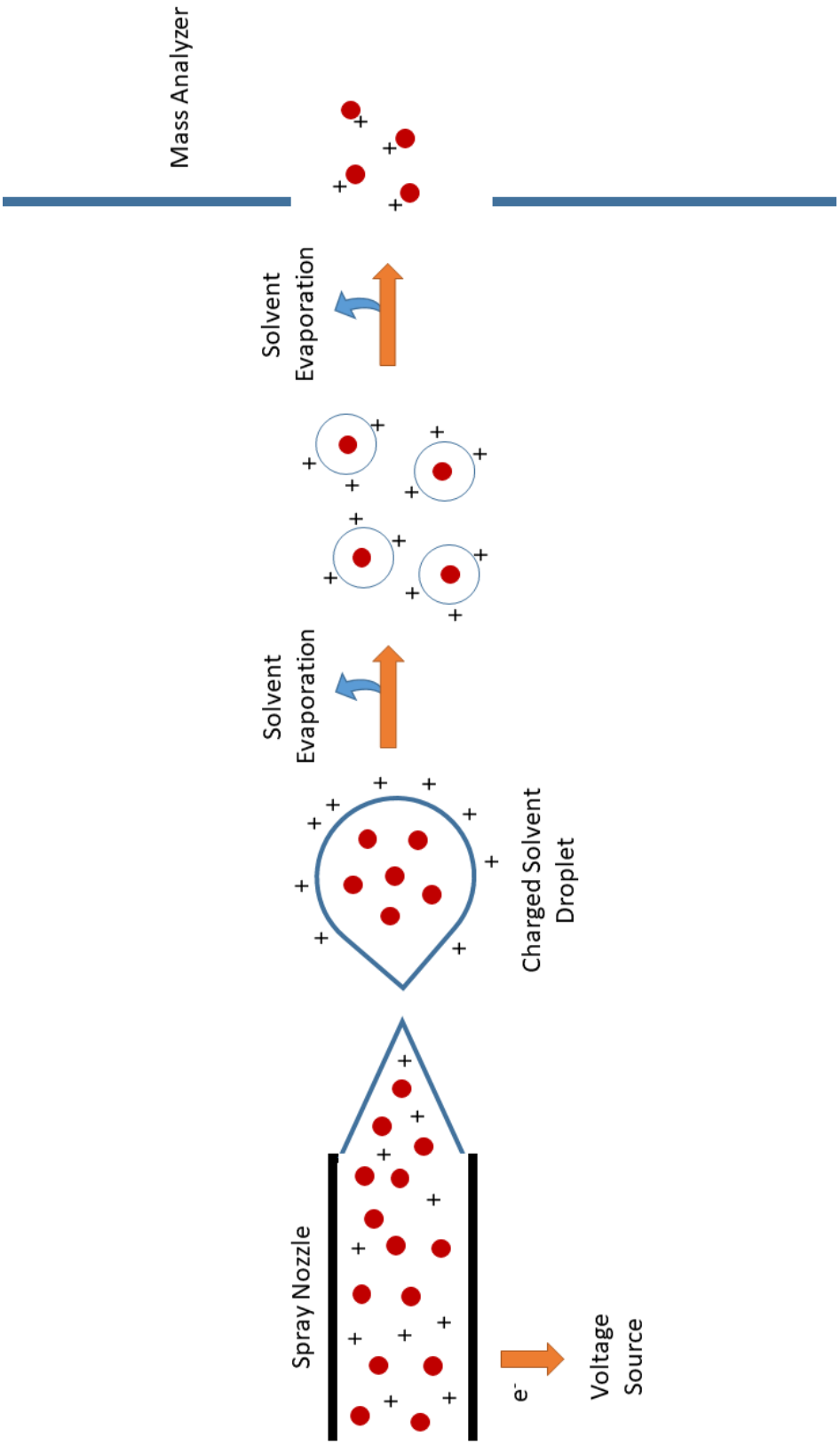

Mass Analyzer
Solvent Evaporation
Solvent Evaporation
Charged Solvent Droplet
Spray Nozzle
e-
Voltage Source

Figure 1.8

Electrospray Ionization. During electrospray ionization (ESI), solvated molecules are passed through a spray nozzle which is connected to an electrical current. This current produces charges within the solvent. Then, heavily charged droplets are ejected from the spray nozzle into a heated vacuum chamber. The heat and low pressure evaporates the solvent, leaving charged, gas phase analyte molecules to be passed into the mass analyzer.

Adapted from (Banerjee and Mazumdar, 2012)

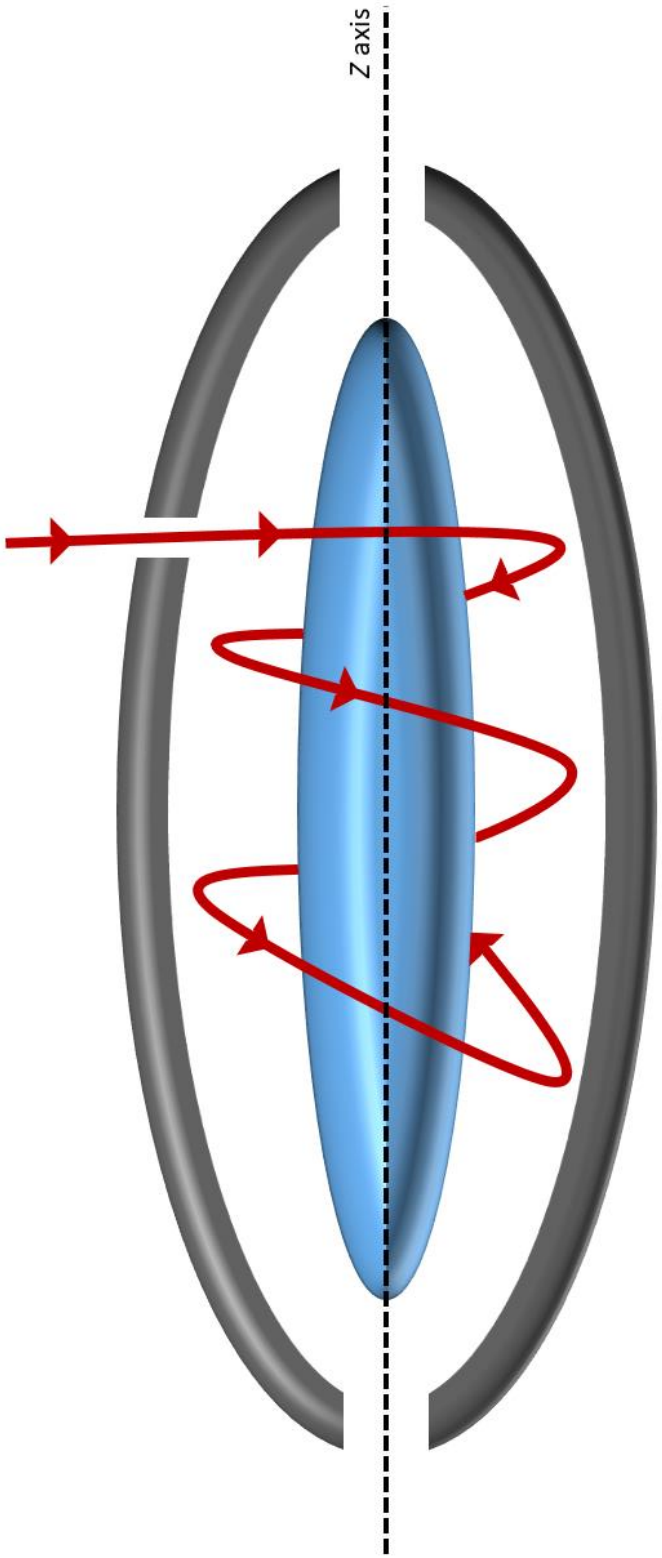

Z axis

Figure 1.9

The Orbitrap. The Orbitrap consists of two outer electrodes surrounding a central electrode. Ions are injected into the Orbitrap at an angle and velocity that is perpendicular to the Z-axis. This entry point is off center from the spindle electrode giving the ions potential energy. This energy is used by the ions to oscillate horizontally along the Z-axis. The outer electrodes keep the ions in the trap area and create a circular path around the center electrode. The specific oscillation frequency and orbital path of each ion is determined by its mass to charge ratio (m/z), which can be detected by a set of detectors around the outside of the Orbitrap.

Adapted from (Hu et al., 2005; Thermo Fisher Scientific, 2008)

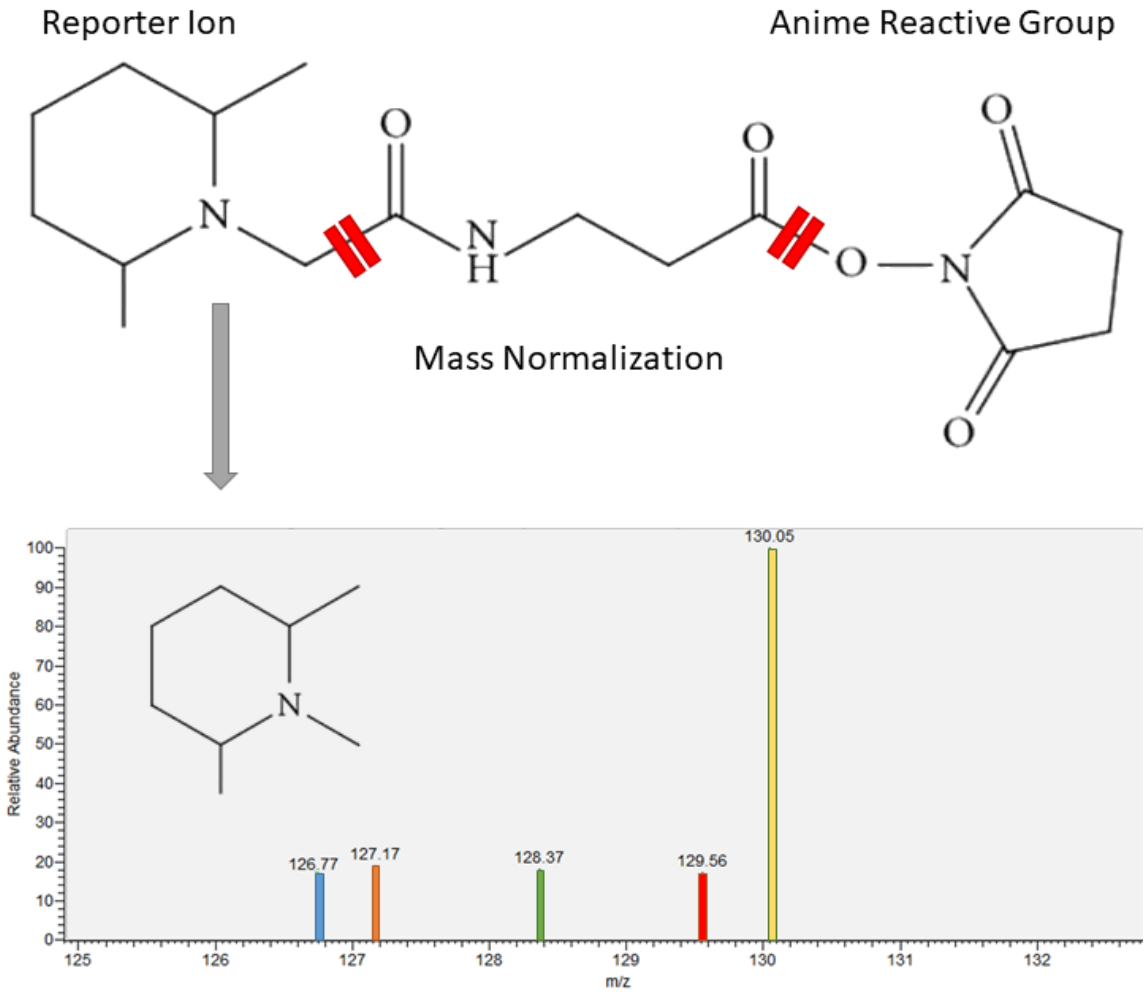

Reporter Ion
Anime Reactive Group
Mass Normalization
Relative Abundance
m/z
126.77
127.17
128.37
129.56
130.05

Figure 1.10

Tandem Mass Tags. 6-plex tandem mass tags consist of three distinct regions. 1) an amine reactive group that allows for labeling peptides at the N terminus; 2) A mass normalization group whose atoms can be substituted with isotopic variations in order to normalize the overall tag mass with the different reporter ion masses; and 3) a reporter ion with variable mass that can be detected in MS2 and MS3 experiments outside of the normal range of m/z detection. Up to 6 samples can be digested with trypsin and labeled with unique tags bearing reporter ions of specific masses ranging from 126 to 131 m/z.

Adapted from: https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-mass-spectrometry-analysis/protein-quantitation-mass-spectrometry/tandem-mass-tag-systems

Chapter 2

Mechanical strain induced phospho-proteomic signaling in uterine smooth muscle cells

Abstract

Mechanical strain associated with the expanding uterus correlates with increased preterm birth rates. Mechanical signals result in a cascading network of protein phosphorylation events. These signals direct cellular activities and may lead to changes in phenotype and calcium signaling. In this study, the complete phospho-proteome of uterine smooth muscle cells subjected to strain for 5 minutes was compared to un-strained controls. Statistically modified phosphorylation events were annotated by Ingenuity Pathway Analysis to elucidate mechanically induced phosphorylation networks. Mechanical strain lead to the direct activation of ERK1/2, HSP27, and MYLC9, in addition to phosphorylation of PAK2, vimentin, DOCK1, PPP1R12A, and PTPN11. These results suggest a novel network reaction to mechanical strain and reveal proteins that participate in the activation of contractile mechanisms leading to preterm labor.

2.1 Introduction

During pregnancy, the uterus increases in size to accommodate the growing fetus, placenta, and amniotic fluid. Despite the associated increases in intra-uterine pressure, the normal myometrium adapts to prevent increased tension and remain in a quiescent state until term (Sivarajasingam et al., 2016b; Sokolowski et al., 2010). Abnormal distension has been associated with early birth in twin (Gardner et al., 1995; Mercer et al., 1996), as well as approximately 10% of singleton (Lockwood and Kuczynski, 2001) preterm deliveries. Increasing uterine volume via a balloon catheter initiates prostaglandin secretion, myometrial contractions, and labor in women (Delaney et al., 2010; Manabe et al., 1984), and this process appears to occur independently of fetal signals (Yoshida and Manabe, 1988). These data suggest failure of the uterine myometrium to adapt to the continued expansion required during pregnancy may increase the risk of preterm birth (Waldorf et al., 2015).

In various animal models of uterine strain, changes in expression of chemokine, oxytocin receptor, connexin-43, and matrix metalloproteinases genes have been reported (Hua et al., 2012; Lee et al., 2015; Tina T.-T. Ngoc Nguyen et al., 2016; Parry and Bathgate, 2000; Shynlova et al., 2013; Waldorf et al., 2015; Yulia et al., 2016). Less data are available regarding signal transduction pathways activated by uterine stress, but ERK1/2 pathway activation is a well-established strain response in pregnant myometrial cells and tissue. Protein phosphorylation is critically important for cell signaling response pathways and mediates many important functions such as protein interactions, localization, and activity (Sharma et al., 2014). ERK1/2 pathway activation is a well-established strain response in

pregnant myometrial cells and tissue. In isolated rat myocytes biaxial strain is followed by phosphorylation of the kinases ERK1/2, JNK, and p38 MAPK (A. Oldenhof et al., 2002). *In vitro* work suggests MAPK pathways activate transcription factors such as FBJ murine osteosarcoma viral oncogene homolog B (c-FOS), resulting in increased expression of contractile associated proteins to drive the onset of labor (A. Oldenhof et al., 2002).

We hypothesized specific phosphorylation signaling events related to the strain induced integrin response would be upregulated in a telomerized cell culture model of human uterine smooth muscle cells. To test this hypothesis, we performed mechanical strain experiments on immortalized human uterine smooth muscle cells followed by phospho-peptide enrichment, identification, and quantitation.

2.2 Materials and Methods

2.2.1 Cell Culture

Telomerized pregnant human uterine smooth muscle cells (PHUSMC-HTRT) were plated on collagen coated BIOFlex plates (Flexcell INC, Burlington, NC) and grown in Hyclone DMEM (Thermo Scientific, Pittsburg, PA) supplemented with 0.11 mg/mL Sodium Pyruvate, 10% FBS (Atlantic Biologicals, Miami, FL), and 636 nM progesterone/55 nM estradiol. Media was refreshed every 3 days and cells were grown to confluence. At confluence, cells were placed in DMEM supplemented with 0.1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) supplement (Thermo Scientific, Pittsburgh, PA) in the absence of FBS for 7 days to allow for growth arrest and differentiation.

2.2.2 Strain Experiment

Growth arrested PHUSMC-HTRT cells on BIOflex collagen plates were subjected to 18% biaxial strain for 5 min on a Flexcell FX-5000 tension system (Flexcell INC, Burlington, NC). PHUSMC-HTRT cells grown on BIOflex plates not subjected to strain served as controls. Cells were lysed in 300 µL/well MAPK buffer (60 mM Tris-HCl [pH 6.8], 2 % SDS, 10% glycerol, 0.1 mM EGTA)(Singer et al., 2003) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Pittsburgh, PA). The experiment included two groups of samples, randomly placed into cohorts. Lysates were sonicated and centrifuged at 4,000 × g for 15 min. Pellets were re-suspended in MAPK buffer, precipitated in 4x volume of 100% acetone and washed 3x in 4x volume of 70% acetone. Precipitates were re-suspended in 8M urea/50mM Tris-HCl (pH 8), reduced in 5 mM DTT at 37˚C for 20 min in the dark, and alkylated in 10mM iodoacetamide (BioRad, Hercules, California) for 20 min in the dark. Samples were re-precipitated with acetone as described above and re-suspended, using sonication, in 50 mM ammonium bicarbonate buffer. Protein samples were digested with Trypsin/Lys C mix (Promega, Madison, WI) at a 75:1 protein:protease mix overnight at 37˚C , acidified in 0.1% formic acid, and desalted using C18 Sep-Pak cartridges (Waters, Milford, MA).

2.2.3 Phospho-peptide Enrichment and LC-MS^2

Samples were enriched on IMAC columns containing nickel-nitrilotriacetic acid (Ni-NTA) agarose beads charged with $FeCl_3$ and labeled with 6Plex tandem mass tags (TMT) (Thermo Scientific, Pittsburgh, PA) according to the manufacturer protocols.

Samples were pooled and then separated by hydrophilic interaction liquid chromatography fractionation as described previously (Albuquerque et al., 2008) for 60 min with a gradient change in solvent A (900 mL ACN + 100 mL ddH2O + 500 µL 10%TFA) from 0% solvent B (1L ddH2O +500 µL 10% TFA) to 90% at 45 min. 40 fractions were re-suspended in 100 µL 5% acetonitrile 0.1% formic acid for mass spectrometry analysis.

Samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) at the Mick Hitchcock, Ph.D. Nevada Proteomics Center (University of Nevada, Reno). Peptides were separated and analyzed using a Michrom Paradigm Multi-Dimensional Liquid Chromatography instrument (Michrom Bioresources Inc., Auburn, CA) coupled with a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Samples were dissolved in 100 µL of 0.1 % formic acid were loaded onto a Thermo acclaim Pepmap100 trap column (100µm x 2 cm, C18 5µm, 100 Å, Thermo Fisher Scientific, San Jose, CA), eluted from the trap, and then separated by reverse phase New Objective (New Objective Inc, Woburn, MA) Reprosil-pur C18AQ column (3 µm, 120Å, 0.075 x 105 mm) with a gradient elution using solvent A (0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min. The gradient was set from 5 % to 40 % solvent B for 90 min, increased to 80% solvent B in 10 sec and held at 80% solvent B for 1 min. MS spectra were recorded over the mass range of m/z 400-1600 with resolution of 60,000. The three most intense ions were isolated for fragmentation in the linear ion trap using collision induced dissociation (CID) with minimal signal of 500% and collision energy of 35.0% or using higher-energy collision dissociation (HCD) with minimal signal of 1000%, Collision energy of 55.0%, and an activation time of 30 ms. Dynamic

exclusion was implemented with 2 repeat counts, repeat duration of 15 sec, and exclusion duration of 90 sec. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Dennis et al., 2012) via the PRIDE partner repository with the dataset identifier PXD002538 and 10.6019/PXD002538.

2.2.3 Peptide and Protein Identification

Scaffold (version Scaffold_4.4.1.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Identification and quantification of peptides was achieved by combining CID and HCD fragmentation. CID fragmentation energy was fine tuned to avoid dissociation of phosphorylation groups. This reduced collision energy results in loss of approximately 30% of ions in the lower weight register. To maintain a high confidence of peptide identification and a valuable TMT labeled quantification signal, the top three quality peptides selected during the first mass spectrometry analysis were subjected to HCD. HCD spectra were used for quantification and integrated with the identifications obtained from CID spectra with a confidence threshold of 90%. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.0). Sequest was set up to search the ipi.HUMAN_decoy.v3.87 database, (Version 3.87, 182928 entries) assuming the digestion enzyme strict trypsin/Lys-C.

2.2.4 Bioinformatics

Sample sizes were chosen as n=3 in both cohorts, where each sample replicate was a randomly chosen combination of 72 unique cell-lysis aliquots. As we were expecting effect sizes between cohorts to be large (where effect size is defined in (Cohen, 1988), we estimated that sample sizes with n=3 would be sufficient. This is a novel experiment with no preliminary data to use as a pilot power study.

Upon generating these data, we did perform standard power studies on the 74 proteins/peptides that showed the greatest statistical significance upon hypothesis testing. Of these 74 proteins, the power of the hypothesis test in 60 (81%) reached a power of 80% or greater (80% to 99.99%), with a significance level of alpha=0.05. The power of the remaining 14 proteins ranged from 73.3% to 79.1%.

The final data set was log-transformed to follow a normal distribution, and a simple t-test was performed to test for differences in the means across the two cohorts on all peptides that contained the same PTM configuration. A multiple testing correction (Benjamini and Hochberg, 1995) was performed to adjust for the false discovery rate.

2.2.5 Gene Ontology Analysis

The functional categories of all differentially regulated phospho-proteins were classified by the Panther Gene Ontology (GO) algorithm (http://www.pantherdb.org/).

2.2.6 Ingenuity Pathway Analysis (IPA)

Mechano-transduction induced phosphorylation networks were analyzed using Ingenuity Pathway Analysis software (IPA) (Qiagen, Redwood City, CA). Annotated

phospho-proteins were included in the IPA searches based on a cut off of 1.5-fold change and p-value < 0.05. Each protein symbol was mapped to its corresponding gene and set within the context of its associated partners based on the Ingenuity Pathways Knowledge Base (IPKD).

The significance of the association between the data set and the canonical pathway was determined based on two parameters: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway and (2) a p-value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

2.2.7 Western blot analysis

Growth arrested PHUSMC-HTRT cells on BioFlexÒ collagen plates were subjected to 18% biaxial strain for 5 min on a FlexcellÒ FX-5000 tension system (FlexcellÒ INC, Burlington, NC). PHUSMC-HTRT cells grown on BioFlexÒ plates not subjected to strain served as controls. Lysates were bath sonicated for 10 min and centrifuged at 13,000g for 15 min.

Samples were electrophoresed on sodium dodecyl sulfate 4–20% gradient polyacrylamide gels and western blotted according to manufacturer instructions (BioRad, Hercules, Ca) with the following primary antibodies: p38 MAPK (D13E1) XPÒ #8690; Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XPÒ #4511; HSP27 Antibody Sampler Kit #12594 were from Cell Signaling Technology (Beverly, MA); Primary antibodies were

detected with IRDye 680LT goat-anti-rabbit (LiCor Biosciences, Lincoln, NE) or IRDye 800LT donkey anti-mouse (827-08364, Rockland Immunochemicals, Gilbertsville, PA). Band intensities were quantified with an Odyssey Infrared Imaging System (LiCor Biosciences). Phosphoprotein band intensities were normalized to total protein expression. Normalized group values were compared on Prism version 6 (GraphPad) with a one-way ANOVA.

2.3 Results

2.3.1 Peptide Identifications

We utilized mass spectrometry to reveal the phospho-proteomic changes initiated by acute 5 min strain (Fig. 2.1) in uterine smooth muscle cells. Telomerized, human, myometrial cells were exposed to 18% biaxial strain for 5 min and the phospho-proteome compared to non-strained controls. We identified 1176 phospho-sites on 1098 phospho-peptides at a 1% false discovery rate and a 90% protein confidence threshold. These phosphorylation events were comprised of 849 serine (72.2%), 264 threonine (22.4%), and 63 tyrosine (5.2%). These numbers are similar to those found in mouse tissue and HeLA cells (Huttlin et al., 2010; Zarei et al., 2011). Quantitation and statistical analysis revealed a greater than a 1.5-fold change of 204 unique protein phosphorylation sites. Increases in phosphorylation were seen at 141 phospho-sites, while decreases in phosphorylation were seen at 63 phospho-sites.

2.3.2 Gene Ontology

Proteins containing differentially phosphorylated sites were annotated, to basic cellular processes, by Panther online software (Thomas et al., 2003) into 12 functional categories. The largest category was extracellular signaling transduction (21 proteins, 25.20%) which is broken down into 7 sub-categories including extracellular matrix protein (5), transporter (4), transmembrane receptor regulatory/adaptor protein (1), cell adhesion molecule (4), membrane traffic protein (1), cell junction protein (2), and receptor proteins (4) (Fig. 2.2). The second and third most abundant categories were nucleic acid binding (13, 15.70%) and transcription factors respectively (11, 13.30%).

2.3.3 Ingenuity Pathway Analysis

Phosphorylation changes were mapped to 220 total pathways, based on a 1.5-fold change with a p-value of < 0.05, of which the top 25 were considered for further analysis (Fig. 2.3). IPA network analysis revealed that 10 of these pathways grouped into a functional network of 13 proteins collectively (Fig. 2.4). IPA then combined these proteins together to reveal an interconnected set of phospho-protein interactions (Fig. 2.5). Within this interaction network, we observed the specific phosphorylation of previously annotated sites of activation on ERK1/2 at threonine 183 and tyrosine 185, and heat shock protein 27 (HSP27) at serine 82. In addition, we detected increased phosphorylation of myosin light chain 9 (MYL9) at sites T19 and S20. These patterns are consistent with the idea that contractile mechanisms are upregulated in response to strain activation.

2.3.4 Western blot analysis

Confirmation of mass spectrometry analysis by Western blot showed increased phosphorylation of key proteins p38, ERK1/2 and HSPB1, at previously annotated sites after 5 min of 18% biaxial mechanical strain (Fig. 2.6).

2.4 Discussion

Our phospho-proteomic snapshot revealed the activation of both focal adhesion-associated and novel strain-responsive signaling pathways in human myometrial cells. We found differential phosphorylation of ERK1/2, vinculin, HSP27, protein kinase Cα (PKCα) and p21 activated kinase 2 (PAK2) which IPA revealed to be associated with the downstream activity of the integrin linked kinase pathway. In agreement with previous tissue experiments (Y. Li et al., 2009), we found increased ERK1/2 phosphorylation levels at T185 after 5 min of strain in myometrial cells. This site is one of two key regulatory sites of ERK 2 phosphorylation (Ahn et al., 1991; Anderson et al., 1990; Boulton et al., 1991). Myometrial strain has previously been shown to activate focal adhesion and ERK/MAP kinase signaling pathways (Macphee and Lye, 2000; A. D. Oldenhof et al., 2002; Sooranna et al., 2005b; Wu et al., 2008) . These data, combined with our results and the observation that the ERK inhibitor U-0126 delays preterm labor in rats treated with RU-486 (Li et al., 2004), suggest a major role for ERK activation in the regulation of gestational timing.

HSP27 is a small 27 kD heat shock protein that has been implicated in a wide variety of cellular functions including protection from heat stress, actin remodeling, and regulation of smooth muscle contraction. Cell contractility in smooth muscle cells has

been linked to phosphorylation of HSP27 on S82 (W. T. Gerthoffer and Gunst, 2001). Additionally, many of the functional roles of HSP27 are regulated by PTMs, particularly the phosphorylation of two sites (S15, S82) (Rouse et al., 1994). These data suggest HSP27 may be a key regulator of contractile phenotype and play a significant role in the induction of labor.

Phosphorylation of PAK2 and HSP27 (at site S82) were increased in response to strain suggest a mechanistic link between the mechanical strain response and the development of a contractile phenotype. PAK2 has been shown to down regulate cofilin via phosphorylation (Kosoff et al., 2015) and PKC inhibition reduces HSP27 S82 phosphorylation levels in vascular smooth muscle (Moreno-Domínguez et al., 2014). Since cofilin phosphorylation reduces actin polymerization, while HSP27 enhances polymerization, the cofilin and HSP27 S82 phosphorylation states are important regulators of smooth muscle actin polymerization and may regulate strain induced contractility.

While we found direct evidence of HSP27 activation, we did not find phosphorylation sites on PKCα and PAK2 that correlate to previously annotated sites. It is possible that the phosphorylation sites we identified represent alternative activation sites related to a particular temporal or physical pattern of cellular activity. The significance of this phosphorylation pattern needs to be further investigated.

IPA results suggest a central role for vimentin in the response to acute mechanical strain. ERK1/2 and HSP27 regulate cytoskeletal remodeling through interaction with vimentin, an intermediate filament protein that is important to cellular tensile strength

particularly in the cytoplasm (Guo et al., 2013). We saw increased phosphorylation of a number of amino acids on vimentin fibers in response to mechanical strain. Phosphorylation of vimentin at S56 is PAK1 dependent in airways smooth muscle cells responding to contractile stimulation (Tang and Gerlach, 2017); however, vimentin is regulated by a variety of kinases and phosphatases (Eriksson et al., 2004) and the long-term downstream, temporal, and mechanical consequences of such phosphorylation patterns may be related to HSP27 and PAK2 phosphorylation via mechanical activation.

Smooth muscle contraction is regulated by the kinase/phosphatase activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MYPT1) (Aguilar and Mitchell, 2010). Phosphorylation of MLCK and activation of the serine/threonine kinases are responsible for down regulating phosphorylation of MYPT1 leading to increased calcium sensitivity (Amano et al., 1996; K. Kimura et al., 1996). Focal adhesions are known activators of MLCK phosphorylation and of MYL9 which increases calcium sensitivity and force of contraction (Wray, 1993). We detected phosphorylation of MYL9 at sites T19 and S20 demonstrating that contractile mechanisms were engaged subsequent to strain activation. In addition, protein kinase C is known to phosphorylate CPI-17 leading to the phosphorylation dependent deactivation of MYPT1 and increased calcium sensitivity (Somlyo and Somlyo, 2003). The implication of the observed phosphorylation patterns suggests increased calcium sensitivity is induced by strain activation. A proposed schematic of strain induced calcium sensitization based on our data is shown (Fig. 2.7).

In addition to previously annotated phosphorylation sites, we discovered proteins such as PAK2, DOCK1, PPP1R12A, and PTPN11, which were phosphorylated on

unannotated sites; however, pathway signaling analysis by IPA predicts that they are activated, either by temporal separation of the signal or a novel activation from unannotated phospho-sites. The annotation of these phosphorylation sites and the temporal separation of such events still needs to be investigated.

Cultured cell models provide a valuable tool to obtain insight into the regulation of myometrial function (Mosher et al., 2013) and have the advantage of allowing analysis of the effect of strain on signal transduction events specifically in myometrial cells in the absence of complicating factors such as maternal age, race, gestational age, fetal sex, and variations in the in vivo hormone environment (Burris and Collins, 2010; Challis et al., 2013; Myatt et al., 2012). However, cultured cells are isolated from the influence of other cell types present in the parent tissue and the two-dimensional environment may not precisely approximate 3D tissue structures (Smith et al., 2017; Souza et al., 2017; Zhang et al., 2013). Cultured, non-laboring human myometrial cells undergo gene expression changes indicative of a contractile, laboring phenotype (Ilicic et al., 2017), although it is not clear if these changes correspond to protein level and phenotypic changes. Although this work provides a starting point for elucidation of molecular pathways activated by mechanical stress, future work is needed to confirm these data in tissue or animal models.

Acknowledgements –Jonathon James for graphic design and Patrick Barnett for Python script development. This work was supported by the Mountain West Clinical Translational Research-Infrastructure Network under a grant from the National Institute of General Medical Sciences (NIH award number 1U54GM104944) and a Pathway to Independence

Award from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NIH award number R00HD067342) to Heather Burkin. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.

Conflicts of Interest: We have no conflicts of interest to report and all authors have reviewed the manuscript for submission.

Chapter 2 was previously published and is reprinted here with permission of the copyright holders:

Copley Salem, C., Ulrich, C., Quilici, D., Schlauch, K., LO Buxton, I., Burkin, H., 2018. Mechanical strain induced phospho-proteomic signaling in uterine smooth muscle cells. J. Biomech. doi:10.1016/j.jbiomech.2018.03.040

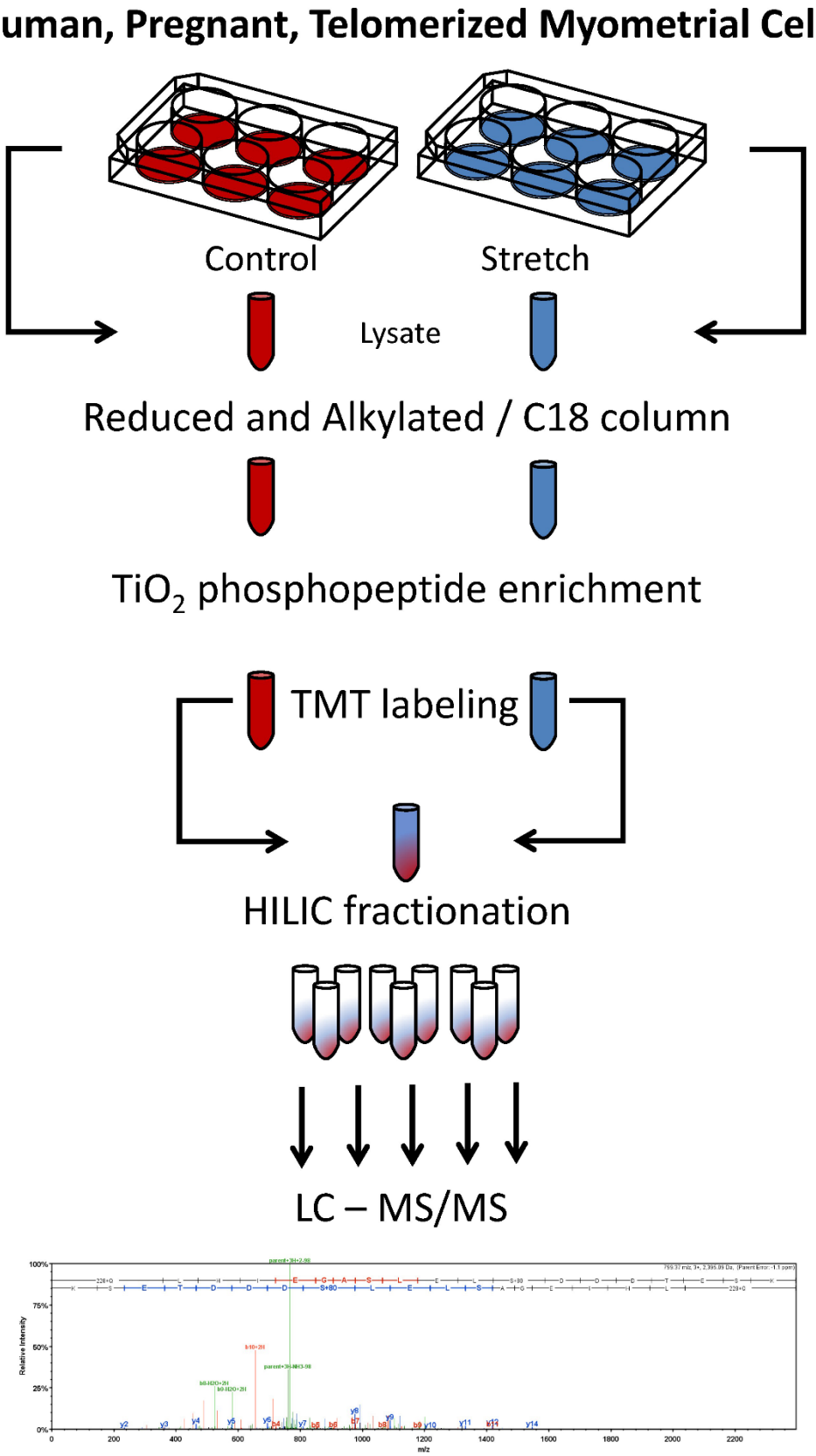

Human, Pregnant, Telomerized Myometrial Cells
Control
Stretch
Lysate
Reduced and Alkylated / C18 column
TiO2 phosphopeptide enrichment
TMT labeling
HILIC fractionation
LC – MS/MS
Relative Intensity
m/z

Figure 2.1

Myometrial cell strain experimental design. Growth arrested PHUSMC-HTRT on BIOflex plates were subjected to 18% biaxial strain for 5 min. Annotated SEQUEST peptide results were analyzed using IPA for pathway associations. Specific phospho-site annotations were taken from the Phosphosite.org database.

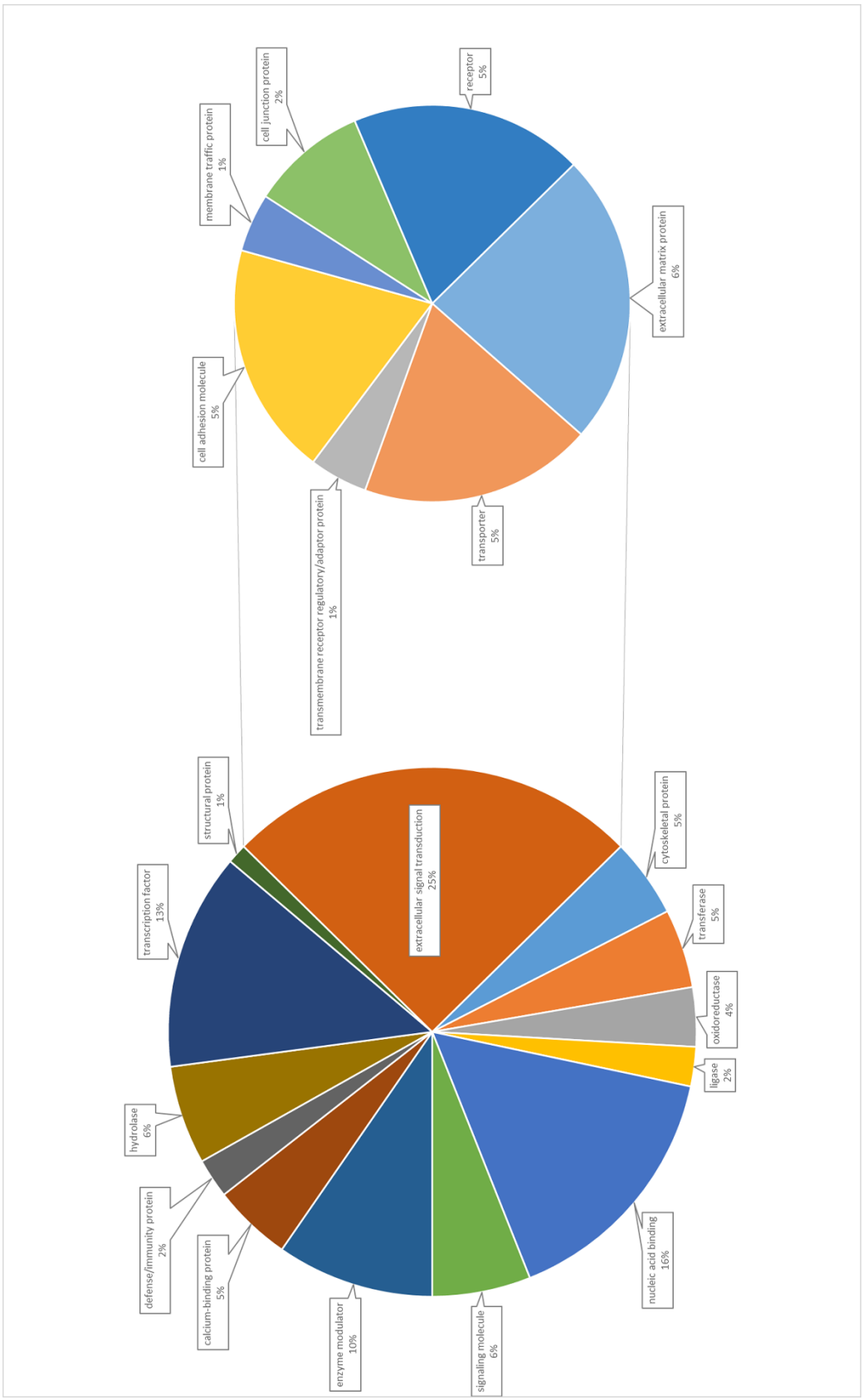

receptor 5%
cell junction protein 2%
membrane traffic protein 1%
extracellular matrix protein 6%
cell adhesion molecule 5%
transmembrane receptor regulatory/adaptor protein 1%
transporter 5%
structural protein 1%
cytoskeletal protein 5%
transcription factor 13%
extracellular signal transduction 25%
transferase 5%
oxidoreductase 4%
hydrolase 6%
ligase 2%
defense/immunity protein 2%
calcium-binding protein 5%
enzyme modulator 10%
signaling molecule 6%
nucleic acid binding 16%

Figure 2.2

Go analysis of protein functional categories represented by differentially phosphorylated proteins after exposure to 5 minutes of mechanical strain. Go annotations from the panther analysis data base revealed 19 categories of protein functions. Differentially phosphorylated proteins in each category are listed in supplementary table A.


Downregulated
No change
Upregulated
No overlap with dataset
-log(p-value)
Percentage
0
10
20
30
40
50
60
70
80
90
100
110
120
Nitric Oxide Signaling in the Cardiovascular System
113
Role of JAK family kinases in IL-6-type Cytokine Signaling
95
FAK Signaling
99
ILK Signaling
196
PAK Signaling
101
ERK/MAPK Signaling
199
Corticotropin Releasing Hormone Signaling
111
Agrin Interactions at Neuromuscular Junction
69
Assembly of RNA Polymerase I Complex
11
CXCR4 Signaling
165
Integrin Signaling
219
Antiproliferative Role of Somatostatin Receptor 2
75
Actin Cytoskeleton Signaling
228
IL-17A Signaling in Airway Cells
77
Growth Hormone Signaling
81
Renal Cell Carcinoma Signaling
81
14-3-3-mediated Signaling
130
CCR3 Signaling in Eosinophils
130
Prolactin Signaling
83
JAK/Stat Signaling
83
Signaling by Rho Family GTPases
247
Apoptosis Signaling
89
Axonal Guidance Signaling
449
Insulin Receptor Signaling
141
Death Receptor Signaling
92
0.0
0.5
1.0
1.5
2.0
2.5
3.0
-log(p-value)

Figure 2.3

Top 25 differentially regulated pathways as determined using IPA software. Over-represented pathways were sorted by log2 fold change (0.6) and the top 25 were selected for additional analysis. Red and green bars represent the percentage of pathway proteins either up (red) or down (green) regulated quantified on the top axis as compared to the total number of proteins annotated to each pathway shown as black numbers after each bar. The negative log of the p-value of each pathway enrichment is shown as a yellow line with quantification on the bottom axis.

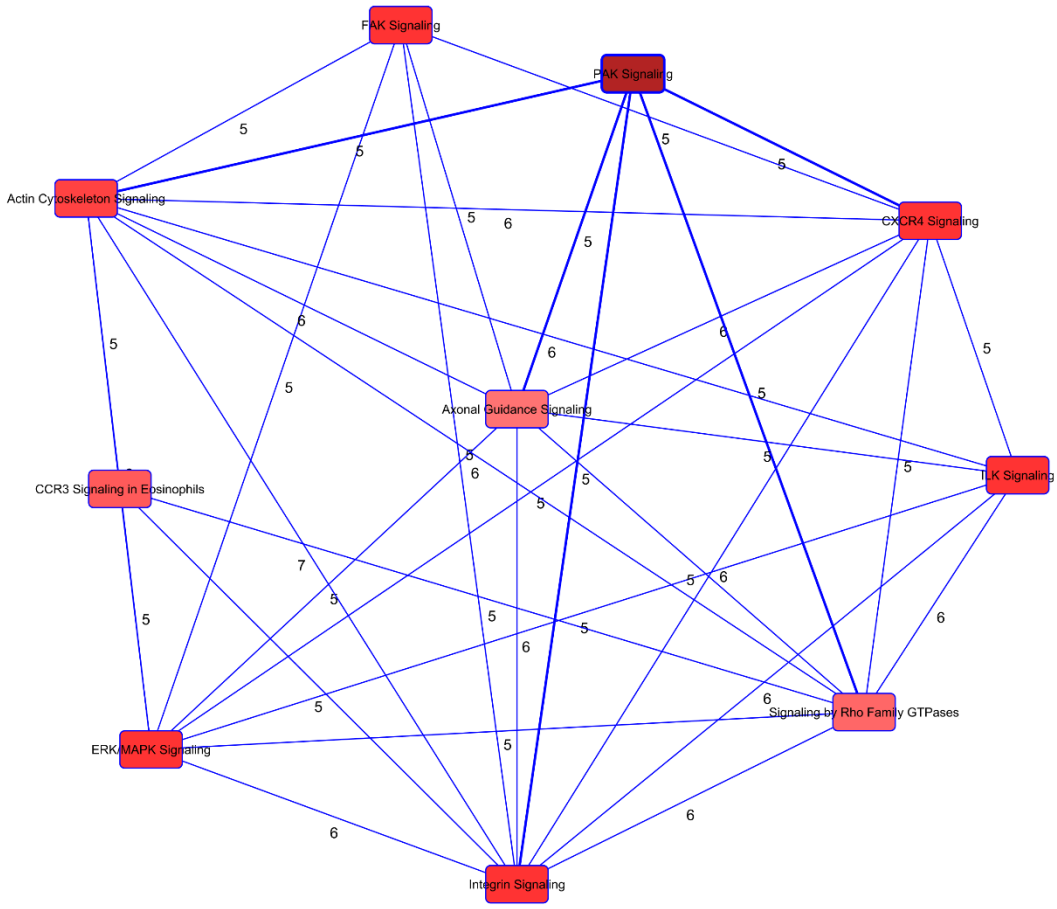

FAK Signaling
PAK Signaling
Actin Cytoskeleton Signaling
CXCR4 Signaling
Axonal Guidance Signaling
CCR3 Signaling in Eosinophils
TLK Signaling
Signaling by Rho Family GTPases
ERK/MAPK Signaling
Integrin Signaling

Figure 2.4

Overlapping Canonical Pathways generated by IPA. A pathway network was generated, from the top 25 over represented pathways determined by IPA, to reveal the most functionally active proteins with differential phosphorylation. Nodes represent pathways and edges are labeled with the number of common proteins connecting each node.

ERR Vimentin HSPB1 PAK 2

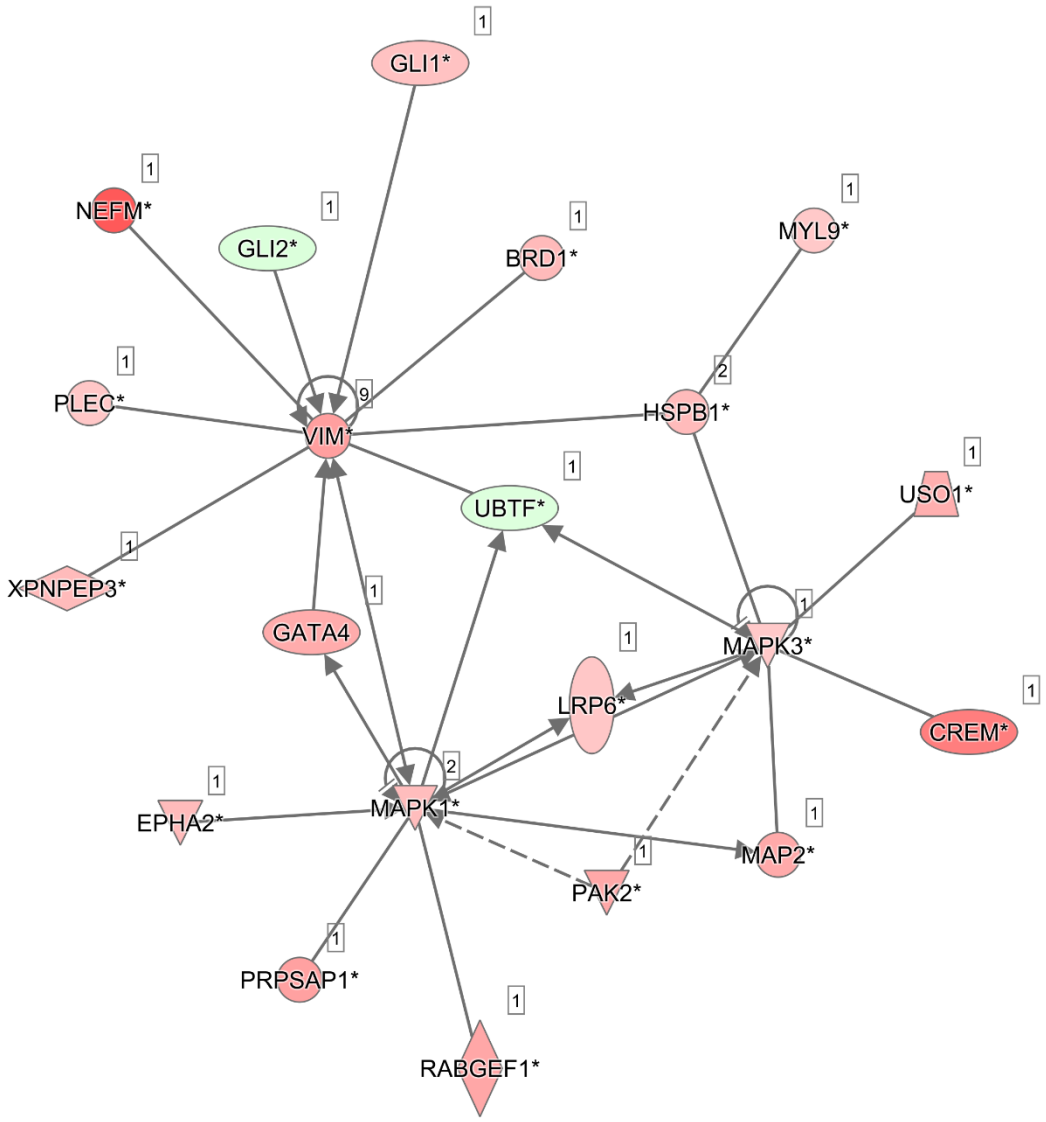


Figure 5

Network analysis of the most functionally active proteins represented by the edges of Fig. B. All unique proteins counted as part of all the edges were collected and networked by IPA. These proteins form the core of differentially expressed proteins at 5 minutes of strain. Proteins in red had increased phosphorylation and proteins in green had decreased phosphorylation.

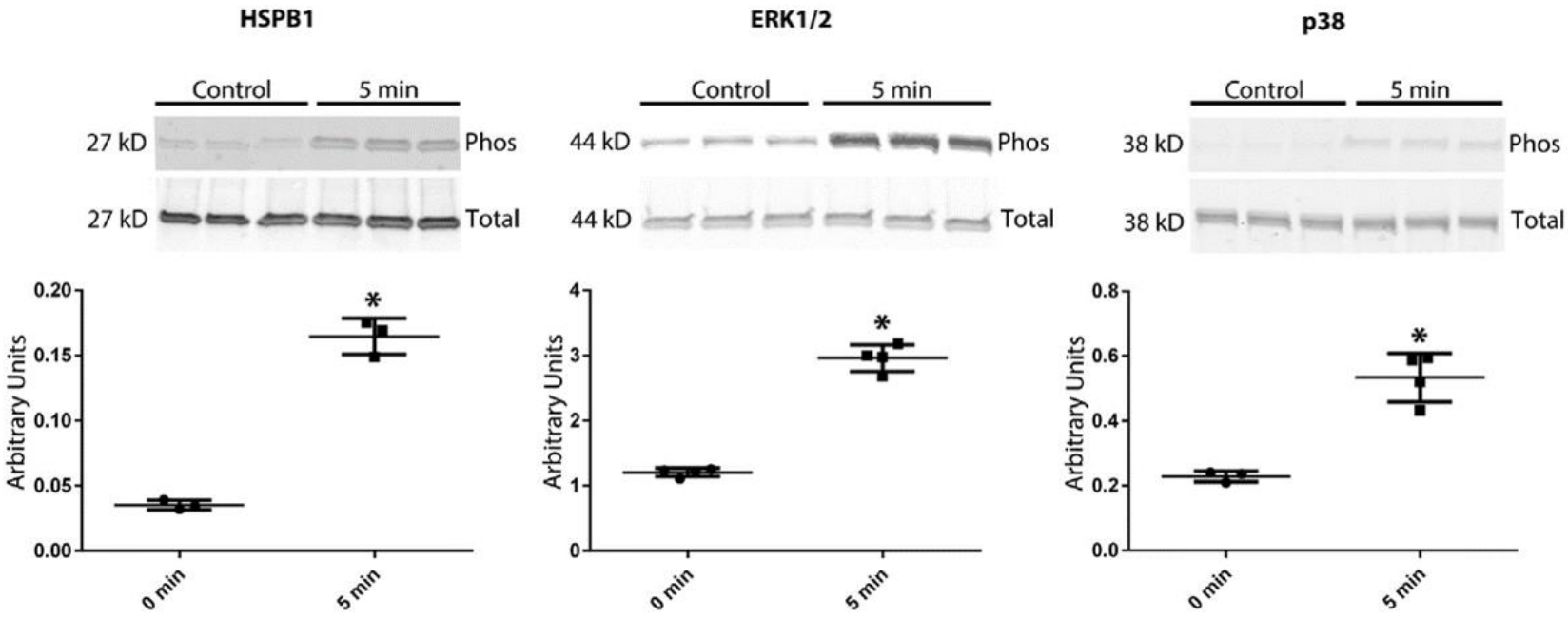

HSPB1
Control
5 min
27 kD
Phos
27 kD
Total
Arbitrary Units
0.20
0.15
0.10
0.05
0.00
*
0 min
5 min
ERK1/2
Control
5 min
44 kD
Phos
44 kD
Total
Arbitrary Units
4
3
2
1
0
*
0 min
5 min
p38
Control
5 min
38 kD
Phos
38 kD
Total
Arbitrary Units
0.8
0.6
0.4
0.2
0.0
*
0 min
5 min

Figure 6

Western blot validation of HSPB1 pathway activation. This tonic mechanical strain revealed phosphorylation of ERK1/2 and HSPB1. Additionally, we saw increased phosphorylation of p38 kinase, an activator of HSPB1 and a protein predicted by IPA to be activated. Statistical analysis of all blots was performed by normalization of phosphorylation to total protein with (*) representing a p value of <0.001.

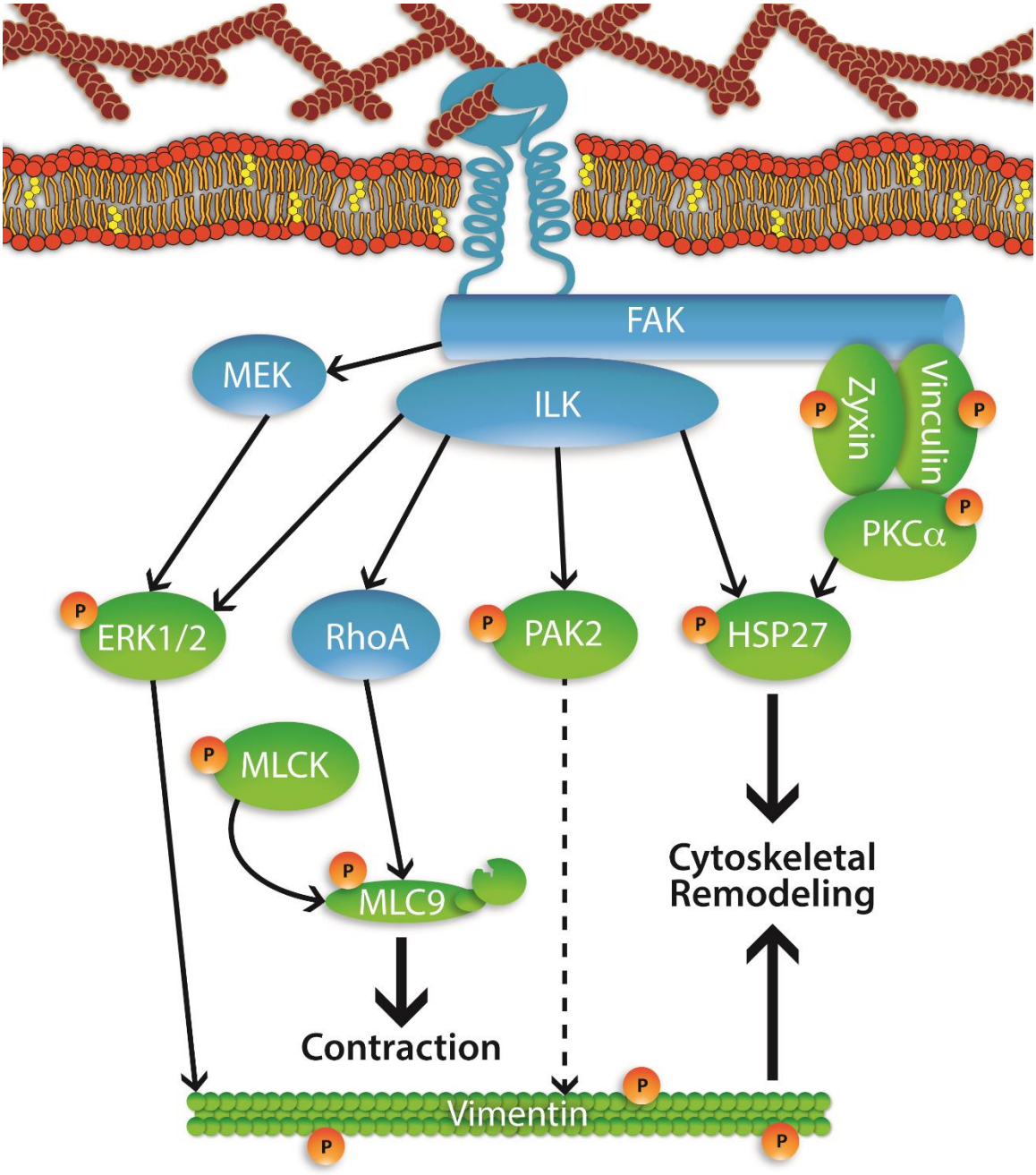

FAK
MEK
ILK
Zyxin
Vinculin
PKCα
ERK1/2
RhoA
PAK2
HSP27
MLCK
MLC9
Cytoskeletal
Remodeling
Contraction
Vimentin
P

Figure 7

Proposed strain-induced phosphorylation in myometrial cells. Experimentally determined phosphorylated proteins at 5 min (green with P) with pathway proteins (blue) that were not experimentally observed to be phosphorylated after 5 min of strain. Associations based on literature searches and phospho-site annotation.

Chapter 3

Transcriptional response to mechanical strain of the myometrium

Abstract

Abnormal distension has been associated with early birth in twin, as well as approximately 10% of singleton, preterm deliveries. We performed experiments to reveal the effect mechanical strain has on the transcriptome of pregnant human myometrial cells in the absence of complicating factors such as a changing endocrine environment or fetal influences. We hypothesized myometrial strain would be associated with increased expression of transcripts related to the transition from a quiescent to a contractile phenotype. Telomerized, pregnant human uterine smooth muscle cells were subjected to biaxial mechanical strain for 0, 3, 8 and 24 hours. At 3 and 8 hours, we saw statistically significant increases in transcripts for cytokines IL-6, IL-8, IL-1β and CCL2 which are inflammatory mediators. Additionally, we found increases in the contractile associated proteins: oxytocin receptor, Cx45, and Activating Protein 1 (AP-1) subunits. These data suggest a possible feed forward loop of increasing strain induced inflammatory signals and contractile associated proteins that could lead to the induction of labor.

3.1 Introduction

Premature entry into labor is defined as the onset of labor before the 37th week of pregnancy, and affects approximately 380,000 babies a year with an estimated annual economic impact in the United States of 30 billion dollars (Frey and Klebanoff, 2016). Increased wall tension within the expanding uterus causes stress at the cellular level, and abnormal distension has been associated with early birth in twin, as well as approximately 10% of singleton, preterm deliveries (Rebarber et al., 2001).

Failure of the uterine myometrium to adapt appropriately to the continued expansion required during pregnancy may increase the risk of preterm birth (Waldorf et al., 2015). Increasing uterine volume via a balloon catheter initiates prostaglandin secretion, myometrial contractions, and labor in women (Delaney et al., 2010; Manabe et al., 1984), and this process likely occurs independently of fetal signals (Yoshida and Manabe, 1988). In animal models with bicornate uteri, such as the rat (Hua et al., 2012; Lee et al., 2015; Shynlova et al., 2013) and wallaby (Parry and Bathgate, 2000), embryonic implantation in a single uterine horn (either naturally or due to ligation) has been used to study the effects of mechanical stress on the gravid horn. These studies identified important roles for chemokine, oxytocin receptor, and connexin-43 expression in the uterine strain response (Hua et al., 2012; Lee et al., 2015; Parry and Bathgate, 2000; Shynlova et al., 2013). In a non-human primate model, mechanical stress on the uterine wall led to increased amniotic and myometrial expression of cytokines and prostaglandins, and in several cases resulted in the onset of preterm labor (Waldorf et al., 2015). Elevated expression of the cytokines IL-6, IL-8, and CCL2 were also observed in the uterine

myometrium of women delivering twins preterm (Waldorf et al., 2015), suggesting these mechanical strain experiments may be a good model for uterine strain *in vivo.*

We performed experiments to examine the effect of mechanical strain on the transcriptome of human myometrial cells. This *in vitro* experiment allows the measurement of human myometrial gene responses to mechanical stress in the absence of complicating factors such as a changing endocrine environment or fetal influences. We hypothesized myometrial strain would be associated with increased expression of myometrial genes encoding proteins related to the inflammatory response and transition to a contractile phenotype, thus increasing the risk of preterm labor.

3.2 Materials and Methods

3.2.1 Sample preparation and RNA Sequencing

Telomerized, pregnant human uterine smooth muscle (Heyman et al., 2013) cells (hTRT) were used at passage 30 and grown at 37C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Thermo Scientific, Pittsburg, PA) supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 100 U/mL penicillin 100 µg/mL streptomycin (Pen/Strep; Thermo Scientific, Pittsburgh, PA), 636 nM progesterone (P4), and 55 nM estradiol (E4) media BioFlex-Collagen Type I-matrix-bonded growth surfaces in 6-well culture plates (FlexCell International Corporation, Burlington, NC). Cells media was replaced every three days. At 100% confluence, media was replaced with DMEM (Thermo Scientific, Pittsburg, PA) supplemented with 0.1% insulin-transferrin-

Selenium-Ethanolamine (ITS-X) (Thermo Scientific, Pittsburg, PA) and Pen/Strep + P4 and E4 as above. Cells were then allowed to differentiate for 7 days at 37C with 5% CO_2.

Differentiated cells were subjected to 18% biaxial strain for 0, 3, 8 or 24 hours in an FX-5000 Tension System (FlexCell International Corporation, Burlington, NC). Three technical replicates were prepared for each temporal state. Cells were lysed directly in TRIzol reagent (Ambion, TX) and RNA extraction performed according to the manufacturer's protocol.

3.2.2 Illumina sequencing

RNA sequencing (RNA-seq) was performed by the Nevada Genomics Center using an Illumina NextSeq 500 High Output v2 flow cell and 2x75 base-pair, paired-end sequencing. The barcode-labeled libraries were combined and sequenced in multiplex across two Illumina NextSeq 500 Mid flowcells, with all samples present on both flowcells, from the final pooled mix. Basecalling, sample demultiplexing, TruSeq adaptor masking, and fastq file generation, were performed automatically by Illumina's BaseSpace Fastq Generation bcl2fastq module (http://basespace.illumina.com).

Sequence quality control

Sequence fastq files were downloaded from BaseSpace, then trimmed and filtered for nucleotide-base quality and remaining sequencing adapters and sequencing artifacts using Trimmomatic v. 0.36,(Bolger et al., 2014) set as follows: removal of Illumina TruSeq paired-end adapter sequences and poly-G homopolymer artifacts, trimming of low-

quality (phred quality ≤ 5) and/or "N" bases from both ends of each read, further quality-trimming using a 4-base sliding window to remove regions with average Q < 5, and, lastly, exclusion of trimmed sequences with length < 35 nt in either pair member. Sequence quality metrics were visualized and inspected before and after trimming using FastQC v. 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). To verify the reverse/forward strand orientation expected of read pairs produced by TruSeq Stranded mRNA library preparation, subsets of 10,000 read pairs were randomly selected from trimmed read files and converted to FASTA format with seqtk version (https://github.com/lh3/seqtk), and compared to Ensembl release 86 human cDNA sequences (Aken et al., 2017) by BLASTN search via the command-line ncbi-blast+ (v2.5.0) application (Camacho et al., 2009). The plus/minus strand orientations of top hit results were extracted from tabular results to compare with and verify expected orientations.

3.2.4 Sequence alignment and expression quantification

To align paired sequences to the human genome, a reference was prepared from the Ensembl reference human genome (Ensembl release 86; GRCh38.p6) chromosome sequences, excluding any patches, unplaced regions and alternative loci excluded, and converted into HISAT2-format (v. 2.0.5) indices via the `hisat2-build` command (Kim et al., 2015a). Human reference genes and features were obtained from Ensembl 86 Gene Transfer Format (GTF) files that were then filtered to retain only gene features present on main reference chromosomes. Alignment of read pairs was performed using the HISAT2 aligner, with the `--rna-strandedness` option set to enforce strand-specificity, and

genomic coordinates of all transcriptional splice sites supplied to HISAT2 at time of alignment via the `--known-splicesite-infile` option. Aligned outputs were converted to the binary BAM format.(H. Li et al., 2009)

Following alignment, the raw counts of read pairs aligned to each gene were totaled using the featureCounts tool of the subread package (v. 1.5.1) (Liao et al., 2014). Reads were counted once per pair, summarized at gene loci features, and read pairs aligned to multiple genes were excluded from count totals.

3.2.5 Statistical Analyses

The technical triplicates of all four samples were multiplexed with Illumina TruSeq barcodes and yielded approximately 33 million read pairs per sample (Sims et al., 2014; Wang et al., 2011). Sequence quality was assessed using the FASTQC software tool (Babraham Institute Bioinformatics). Low quality bases and sequencing adapters were trimmed from raw reads using Trimmomatic (Bolger et al., 2014). High quality reads were aligned to the most current annotated human genome using the HISAT2 short-read mapping program (Kim et al., 2015b). Following alignment, unique transcript counts were summarized using featureCounts (Liao et al., 2014). Transcript counts were filtered using an experiment-dependent custom method and normalized using standard techniques (Love et al., 2014). Statistically significantly differentially expressed genes between temporal points were identified using DESeq2 (Love et al., 2014).

3.2.6 RNASeq Validation by qPCR

RNASeq validation was performed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) with 10 genes based on their notable differential expression across time points. RNA was extracted from triplicate samples of 3 hour strained and control cells using TRIzol reagent. One µg of RNA from each sample was used to make cDNA using the reverse transcription protocol from SuperScript™ IV VILO DNase enzyme treatment™ (Thermo Fischer, Grand Island, NY). qPCR reactions were performed in 20 µL consisting of Taqman Fast master mix and Taqman primers according to manufacturer protocol and compared to phosphodiesterase 6D (PDE6D) control (Thermo Fischer, Grand Island, NY) (Table 1). PDE6D was chosen as a control based on its consistent expression across all time points in the RNAseq data set. In order to test for correlation between the two platforms, a Spearman Rho Coefficient test was performed on all 11 genes (10 genes and 1 control) measured between RNAseq and qPCR. The Spearman Rho values can result in an r_s value of -1, 0, and 1 with zero representing no linear correlation between the data points. The results of this test were an r_s value of 1 with a p-value of $p=2.12029 \times 10^{-70}$, which indicates a strong, statistically significant correlation between qPCR relative expression values and RNAseq expression values.

3.2.7 Pathway Analyses

Processed RNA-seq data were analyzed using Advaita Bio's iPathwayGuide (http://www.advaitabio.com/ipathwayguide). This software analysis tool implements the 'Impact Analysis' approach that takes into consideration the direction and type of all

signals on a pathway, including the position, role and type of every gene, as described (Donato et al., 2013; Draghici et al., 2007).

iPathwayGuide analysis thresholds were set at 0.05 for statistical significance (p-value) and a log fold change of expression with absolute value of at least 0.6. These data were analyzed in the context of pathways obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 81.0+/0120, Jan 17) (Kanehisa et al., 2000; Kanehisa et al., 2002), gene ontologies from the Gene Ontology Consortium database (2016Sep26) (Ashburner et al., 2000; Gene Ontology Consortium, 2001), miRNAs from the miRBase (Release 21) and TARGETSCAN (Targetscan version: 7.1) databases (Agarwal et al., 2015; Nam et al., 2014; Griffiths Jones et al., 2008; Kozomara and Griffiths Jones, 2014; Friedman et al., 2009; Grimson et al., 2007), and diseases from the KEGG database (Release 81.0+/0120, Jan 17) (Kanehisa et al., 2000; Kanehisa et al., 2002).

3.3 Results

Pregnant human uterine smooth muscle cells were subjected to biaxial mechanical strain for 3, 8 and 24 hours and compared to an unstrained (0 time point) control. RNA was extracted for analysis by RNAseq and validated by qPCR (Table 1). Based on comparative analysis of each condition, we identified 6,414 total transcripts that were differentially expressed across all conditions and statistically significantly based on an adjusted p-value of <0.05 and a $\log_2$-fold change of greater than 0.06. Transcript changes at each strain time point were quantitated relative to the zero-strain control. The total amount of transcripts with differential expression compared to control decreased for

each time condition. Three hours of strain revealed the highest number of statistically significant transcripts, with 3431 from a total of 16723 displaying differential expression. After 8 hours of strain, 2848 genes were statistically significant and differentially regulated out of 17058 total detected transcripts. A dramatic decrease in statistically significantly differentially regulated genes compared to the other time points were found after 24 hours of strain, with only 135 genes being differentially regulated out of 16723 total transcripts detected (Table 2).

Combining gene ontology (GO) analysis with detailed protein annotation, an iPathway meta-analysis revealed that all statistically significant, differentially expressed transcripts were clustered in 27 enriched pathways across all conditions (with an iPathwayGuide adjusted p-value of $p<0.05$). Pathways were evaluated for potential relevance to smooth muscle contraction and parturition relative to a smooth muscle phenotypic change. The top relevant pathways were cytokine-cytokine receptor interactions (Fig. 3.1) and MAPK signaling pathways (Fig. 3.2).

Within iPathway guide annotations, we found specific proteins associated with uterine contractility and a laboring state. At 3 and 8 hours we saw statistically significant increased expression in transcripts of cytokines IL-6, IL-8, IL-1β and CCL2 compared to the unstrained control. IL-6, IL-8, IL-1β and CCL2 are inflammatory mediators known to be involved in a wide variety of differentially regulated molecular pathways, and are active in pregnant tissues (Park et al., 2005).

After 3 hours of strain, connexin 45 (Cx45) transcripts were upregulated by 3-fold and AP-1 associated proteins, Fos Proto-Oncogene, AP-1 Transcription Factor Subunit

(FOS), FOSB, FOSL1, transcripts were increased at 8 hours. Connexin 43/45 form gap junctions allowing myometrial syncytium formation and are regulated by the transcription factor AP-1 (Mitchell and Lye, 2001). Additionally, another AP-1 regulated transcript, the oxytocin receptor was increased at 3 and 8 hours of mechanical strain (Mitchell and Lye, 2001). OXTR is the target of oxytocin which is capable of producing contractions in myometrial smooth muscle (Arrowsmith and Wray, 2014; Du Vigneaud et al., 1953).

3.4 Discussion

Here we performed experiments to probe the global transcriptional effects of mechanical strain on cultured pregnant human myometrial cells (Buxton et al., 2011). This *in vitro* approach allows for control of many factors that may influence birth timing distinct from mechanical strain such as changing hormone levels and influence from other cell and tissue types within the uterus.

In order to specifically probe the changes in gene transcript levels induced by mechanical strain on myometrial cells, we performed an RNAseq analysis of cells exposed to 0, 3, 8 and 24 hours of 18% biaxial strain along with identically cultured unstrained controls. 23,040 unique transcripts were sequenced and identified across all three time conditions with a combined total of 6,414 statistically significant, differentially expressed transcripts. The greatest increases in altered transcripts were detected between 3 hours and unstrained control, followed by 8 hours vs control. 24-hour strain showed a return to

the control baseline for most of the transcript differences seen at 3 and 8 hours with the exception of a variety of inflammatory transcripts. Inflammation and cellular stresses often cause perturbations in cellular homeostasis but cells seek to regain that stasis (Chovatiya and Medzhitov, 2014), therefore it is not surprising that at 24 hours of strain we see reduced amounts of transcripts compared to more immediate time points. Our data reveal a variety of transcripts that are differentially regulated during acute strain of human myometrial cells.

3.4.1 Upregulation of Inflammatory Pathways

The initiation of labor is tied to the transition of the myometrium from a quiescent to a contractile phenotype (Shynlova et al., 2013). Cytokine expression and the activation of inflammatory pathways likely play key roles in the transition of myometrial smooth muscle to a contractile state (Bollopragada et al., 2009; Sivarajasingam et al., 2016b) and subsequently the transition into labor (Shynlova et al., 2009).

We saw increases in the inflammatory mediators IL-6, IL-8, IL-1β and CCL2. IL-8 mRNA expression is elevated in myometrium in term labor and in cases of preterm labor (Keelan et al., 2003). Human myometrial tissue strips produce IL-8 when exposed to mechanical strain (El Maradny et al., 1996) and experiments show an increase in IL-1β, IL-8, and CCL2 in primate models of strain (Esplin et al., 2005). Additionally, a dramatic increase in cervical and amniotic IL-6 concentrations in women is a predictor of spontaneous labor (Menon, 2008). CCL2 is a potent attractor of monocytes, which produce additional IL-6, IL-8 and other regulatory cytokines which are capable of driving

inflammation responses in the myometrium (Esplin et al., 2005, 2003; Gomez-Lopez et al., 2010; Young et al., 2002). Up- and down-regulation of genes from strain may reach a threshold with the help of monocytes and additional inflammatory signals to overcome myometrial resistance to contractions.

3.4.2 Altered Expression of Gap Junction Transcripts

Gap junction proteins allow electrical signals to pass between cells rapidly and are a fundamental part of the spread of contraction signals through the myometrium during labor (Lefebvre et al., 1995). Gap junctions are made from specialized proteins (connexins) that combine to form a variety of hemi channels (connexons) that join across the intercellular space to create a connected channel between cells that allows for the exchange of signaling molecules and the propagation of electrical signals (Kumar and Gilula, 1992).

Connexin 43 and Cx45 are gap junction proteins that form connexons allowing electrical coupling between myometrial smooth muscles which then form a syncytium that is capable of producing synchronized and productive contractions to expel the fetus (Desplantez, 2017). Differences between connexin proteins are manifest in their unique responses to differential voltage between connected cells (Sheldon et al., 2014). Connexons composed of Cx45 are more sensitive to voltage and may not allow for proper syncytium formation; however, in the myometrium during labor, the ratio of Cx43/45 may change such that increases in Cx43 protein levels are seen just prior to the onset of labor (Sheldon et al., 2014).

Connexin 45 transcripts were upregulated here in response to strain while Cx43 transcript changes remained below the chosen threshold for consideration. The upregulation of Cx45 and the lack of increased Cx43 in response to strain could be a cellular response designed to maintain myometrial quiescence during the stressful conditions present during pregnancy. However, transcripts for AP-1 subunit proteins (FOS, FOSB, and FOSL1) were increased at the 8-hour time point which may suggest that increases of Cx43 protein could occur outside the scope of this experiment. IL-6 exposure in bladder smooth muscle dramatically increases the Cx43/45 ratio (Heinrich et al., 2011). Additionally, our strain data show an increase in Cx45 which promotes a more quiescent phenotype; however, at some point, increased IL-6 recruitment of monocytes could lead to the increase of production of IL-6, IL-8 and other cytokines in a feed-forward loop that could begin to drive Cx43 expression at a certain threshold and begin the push towards active gap junction formation and a laboring phenotype. More research is necessary to determine exactly what signals may result in the switch in the Cx43/45 ratio seen at the onset of labor.

3.4.3 Increased Oxytocin Receptor Transcription

We found increased expression of the oxytocin receptor. OXTR is a G-protein coupled receptor that binds the nonapeptide oxytocin (Du Vigneaud et al., 1953). In uterine smooth muscle, this receptor plays a complex role in the development of contractions, mainly through increased calcium release and calcium sensitization (Arrowsmith and Wray, 2014). In tissue taken from laboring women, the human OXTR

increases in density and transcription at the onset of labor (T. Kimura et al., 1996). Enhanced OXTR expression at the onset of labor may increase myometrial sensitization to oxytocin and promote contractions (Yulia and Johnson, 2014). In addition, oxytocin exposure in the baboon corpus luteum up-regulates the expression of Cx43, likely by the oxytocin mediated activation of AP-1 transcription factors (Khan-Dawood et al., 1998). Interestingly, in mice and other animals with a bicornuate uterus, upregulation of OXTR is only found in the pregnant horn suggesting mechanical strain is required to increase OXTR expression (Wu et al., 1999). These data strongly support the idea that OXTR upregulation during pregnancy in humans is due in part to increased mechanical strain on the myometrium.

3.4.4 Increased Transcripts for AP-1 Associated Genes

AP-1 is actually a diverse set of transcription factors composed of homo and hetero dimeric combinations of Jun, FOS, and/or ATF (activating transcription factor) proteins that bind the AP-1 DNA binding sequence (Karin et al., 1997). The AP-1 DNA binding site (TGAC) exists as a sub-sequence within a variety of other promoter response sequence elements (Hai and Curran, 1991; Kim et al., 1997; Rauscher et al., 1988), and these are preferentially bound by AP-1 based on the different variety of dimers that can exist. For example, Jun-ATF dimers preferentially bind cyclic adenosine monophosphate (cAMP)-responsive elements, and Jun-FOS dimers bind the phorbol 12-O-tetradecanoate-13-acetate (TPA)-response elements (Hai and Curran, 1991; Karin et al., 1997). As such, AP-1 is capable of regulating a wide variety of genes with different response elements.

Activation and regulation of AP-1 is carried out at that level of transcription and by post translational modification (Karin et al., 1997). MAPK pathways such as Jun kinase (JNK) and ERK are responsible for the phosphorylation of the AP1, Activator protein 1 (Jun), FOS and ATF subunits with AP-1 dimers; the different phosphorylation configurations can result in either activation and inhibition of activity leading to increases or decreases in particular dimers and thus binding affinities (Karin, 1996). Thus, regulation of AP-1 activity is multilayered and complex.

In this experiment, transcription of several genes encoding proteins that participate in the formation of the AP-1 leucine zipper complex, FOS, FOSB and FOSL1 were increased after 8 hours of mechanical strain. Interestingly, AP-1 DNA binding sites are found in the promoter regions of the OXTR and Cx43 genes (Mitchell and Lye, 2001). In addition, *in vitro* strain of human myometrial cells results in increased COX-2 mRNA levels that are associated with activation of AP-1 (Sooranna et al., 2004). As such, it is possible that AP-1 activation can contribute to increased prostaglandin production through COX-2 and an increase in labor associated proteins including the oxytocin receptor and Cx43 that can help drive the pregnant myometrium increasingly towards a contractile phenotype.

3.5 Conclusions

Much like cancer, preterm birth likely results from a variety of causes although the broad outcomes are similar. It is possible that inflammatory pathways are triggered by different stimuli in some cases, or that biological conditions exist such that non-

inflammatory responses are sometimes involved in the development of preterm labor (Gotsch et al., 2008). Based on previous data, it is known that mechanical strain is associated with premature birth in a subset of patients. Myometrial smooth muscle remains quiescent until a set of signals brings about the onset of labor (Shynlova et al., 2013). Data shows a strong correlation between strain, cytokine expression and the activation of inflammatory pathways that are likely involved in the transition of myometrial smooth muscle to a contractile, laboring state (Bollopragada et al., 2009; Shynlova et al., 2009; Sivarajasingam et al., 2016b).

However, it is difficult to separate mechanical strain from fluctuating hormonal signals during pregnancy. Additionally, it is likely that a variety of combinations of mechanical and hormonal states may produce a contractile state within the smooth muscle of the myometrium. Here, we attempt to isolate strain as much as possible from changes in uterine conditions to help understand strain's unique contribution to such a phenotypic transition.

During pregnancy, mechanical strain alone is likely not sufficient to bring about a transition in phenotype. In rats, the smooth muscle cells of the uterus undergoes hyperplasia, hypertrophy and an extensive remodeling of the extra cellular matrix (T. T.-T. N. Nguyen et al., 2016). This remodeling of the myometrial tissue may alleviate some issues of mechanical strain. However, in extreme cases such as twins, the remodeling may not be enough to rescue the cells from the effects of mechanical forces. Our data indicate a variety of contractile associated genes that display altered transcription in response to mechanical strain (Fig. 1.3).

The combination of an increase in inflammatory molecules such as IL-6, IL-8 and CCL2, along with increases in the OXTR, and AP-1 binding activity may work synergistically to promote the development of the myometrial contractile phenotype. For example, the OXTR promoter region contains an IL-6 reactive interleukin binding element (NF-IL6) that binds the NF-IL6 transcription factor and may promote transcription of the OXTR in response to strain-induced inflammatory signaling (Gimpl and Fahrenholz, 2001). From previous work, it is clear that oxytocin can produce contractions in the myometrium (Dale, 1909), but it is not clear how changes in oxytocin or in OXTR levels affect the pregnant myometrium (Fuchs et al., 1995). OXTR protein levels in the myometrium are significantly elevated in laboring tissue (T. Kimura et al., 1996), and it is possible that an increase in OXTRs, combined with an increase in Cx43 may help prime the myometrium to respond to increased inflammation from both myometrial secretions of IL-6, IL-8 and CCL2, and the increased inflammatory mediators produced by the monocytes they attract. While our results show no increase in Cx43, Cx43 is increased in the empty uterine horn of rats when exposed to strain during pregnancy (Ou et al., 1997). The increase in monocyte invasion, triggered by strain induced increases in IL-6 and CCL2 may induce Cx43 transcription. This suggests a possible feed-forward loop of increasing strain producing inflammatory signals which in turn recruit monocytes and increase cellular responses to these signals until a threshold is reached and the smooth muscle transitions to a contractile phenotype leading to the induction of labor.

Gene ID	Fold Change (log2)	RNAseq	TaqMan™Gene Expression Assay ID
PDE6D	-0.01	1.00	Hs01062025_m1
MFSD2A	4.50	22.77	Hs00293017_m1
UBA3	0.02	1.02	Hs01091470_m1
MAFF	4.46	22.06	Hs00544822_m1
OXTR	2.40	5.29	Hs00168573_m1
FOSB	7.38	166.80	Hs00171851_m1
TCF7	5.06	33.44	Hs01556515_m1
ERRFI1	4.84	28.69	Hs00219060_m1
DUSP5	3.89	14.84	Hs00244839_m1
LIF	6.21	74.51	Hs01055668_m1
LIMS3	3.80	14.01	Hs00414460_m1

Table 3.1

qPCR validation of RNAseq: The relative expression of 10 genes was evaluated by qPCR and compared to the control gene PDE6D. PDE6D was selected as a control based on its flat expression across all time points in the RNAseq data. Log_2-fold values are shown for qPCR and RNAseq for comparison. These values had a Spearman Rho Coefficient (r_s) value of 1 with a p-value of $p=2.12029 \times 10^{-70}$.

Gene abbreviations:

PDE6: phosphodiesterase 6D, **MFSD2A**: major facilitator superfamily domain containing 2A, **UBA3**: ubiquitin like modifier activating enzyme 3, **MAFF**: MAF bZIP transcription factor F, **OXTR**: oxytocin receptor, **FOSB**: FosB proto-oncogene, AP-1 transcription factor subunit, **TCF7**: transcription factor 7 (T-cell specific, HMG-box), **ERRFI1**: ERBB receptor feedback inhibitor 1, **DUSP5**: dual specificity phosphatase 5, **LIF**: leukemia inhibitory factor, **LIMS3**: LIM zinc finger domain containing 3.

Length of Stretch	Differential Transcripts	Total Transcripts
0 hours	N/A	20340
3 hours	3431	16723
8 hours	2848	17058
24 hours	135	16723

Table 3.2

Differential Genes Expression in hTRT cells: Table contains total number of statistically significant and differentially expressed transcripts measured by RNAseq at 0, 3, 8 and 24 hours in strained hTRT cells compared with total number of transcripts measured. Each gene at each time point was measured in technical triplicate, which were used to calculate transcript changes and statistical significance for the 3, 8, and 24 hour time points by comparison to the 0 time point.

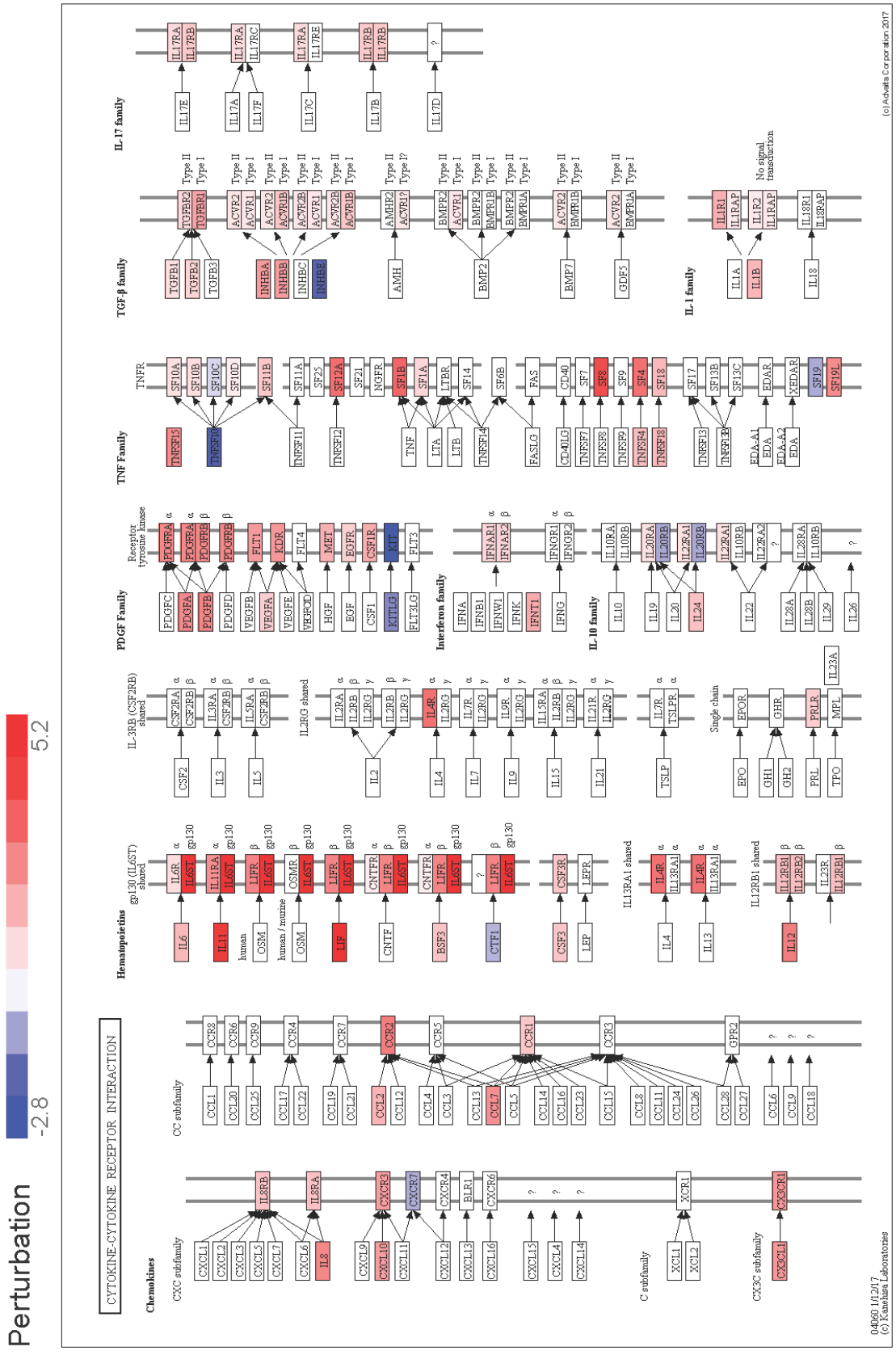

Perturbation
5.2
-2.8
CYTOKINE-CYTOKINE RECEPTOR INTERACTION
Chemokines
CXC subfamily
CC subfamily
C subfamily
CX3C subfamily
Hematopoietins
gp130 (IL6ST) shared
IL-3RB (CSF2RB) shared
IL2RG shared
IL13RA1 shared
IL12RB1 shared
Single chain
PDGF Family
Receptor tyrosine kinase
Interferon family
IL-10 family
TNF Family
TNFR
TGF-β family
IL-1 family
IL-17 family
human
human / murine
No signal transduction
04060 1/12/17
(c) Kanehisa Laboratories
(c) Advaita Corporation 2017

Figure 3.1

Cytokine-cytokine receptor interaction iPathway Guide analysis of three hour mechanical strain transcript pathway (KEGG 04060): Differentially expressed genes at 3 hours were evaluated by iPathwayGuide. Genes are arranged in cytokine families based on their associations within the literature. Perturbation of each gene's measured fold change is calculated by the iPathwayGuide algorithm such that it accounts for accumulated changes in any upstream genes. Negative perturbations are in dark blue and positive perturbations are in dark red. The gradient is described in the legend. Genes are not restricted to one area of the diagram but can appear linked with other genes from multiple pathways. For each association of a gene, its total perturbation is reported in the color displayed for that gene. A graphical representation of the most highly enriched perpetration pathway, cytokine-cytokine interactions, is overlaid with the computed perturbation of each gene.

Adapted from iPathwayGuide (https://www.advaitabio.com/ipathwayguide)

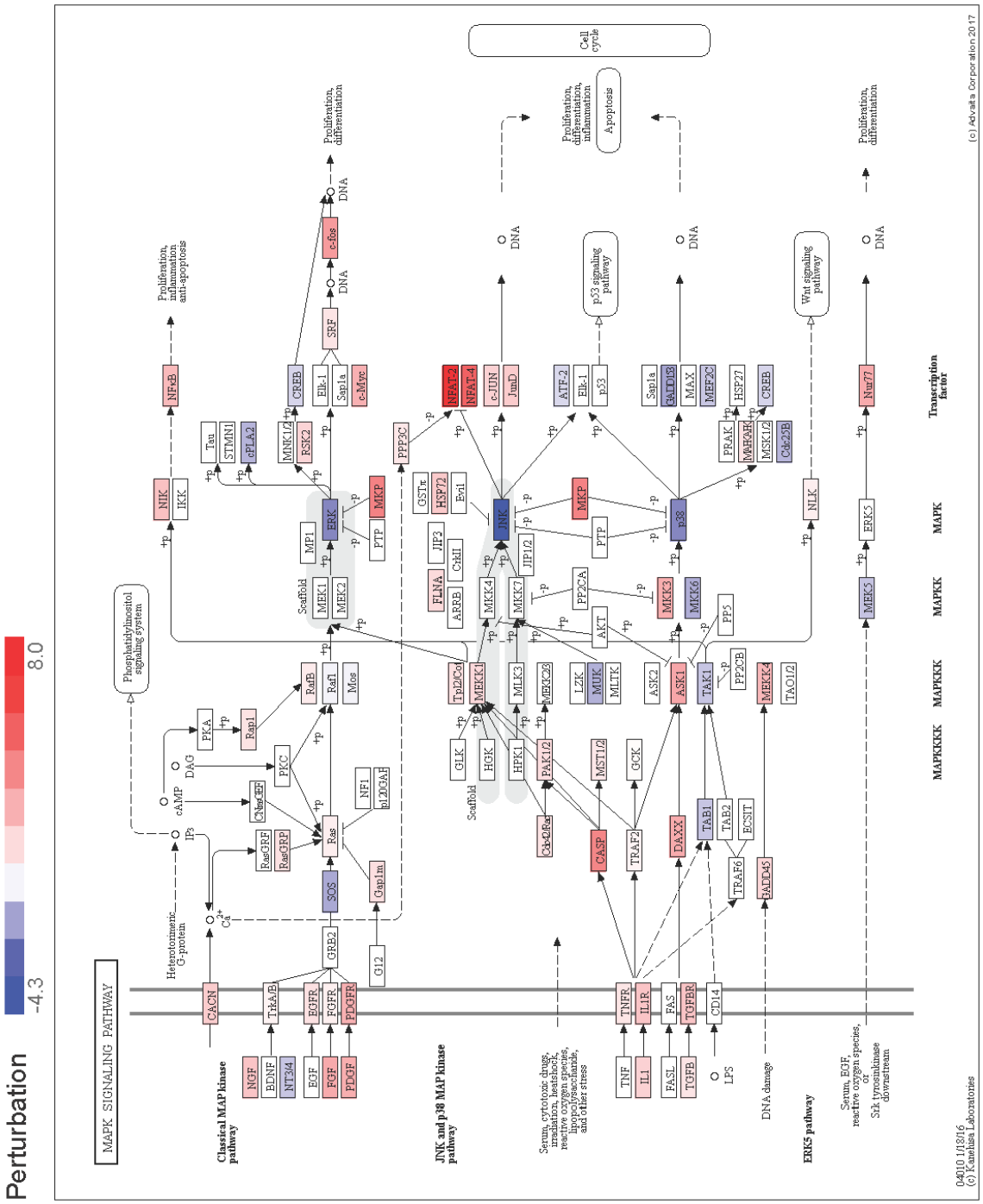

Perturbation
-4.3
8.0
MAPK SIGNALING PATHWAY
Classical MAP kinase pathway
JNK and p38 MAP kinase pathway
ERK5 pathway
MAPKKKK
MAPKKK
MAPKK
MAPK
Transcription factor
Cell cycle
Apoptosis
p53 signaling pathway
Wnt signaling pathway
Proliferation, differentiation
Proliferation, inflammation anti-apoptosis
Proliferation, differentiation, inflammation
Phosphatidylinositol signaling system
Heterotrimeric G-protein
Scaffold
DNA
LPS
DNA damage
Serum, cytotoxic drugs, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress
Serum, EGF, reactive oxygen species, Src tyrosinkinase downstream
(c) Kanehisa Laboratories
04010 1/18/16
(c) Advaita Corporation 2017

Figure 3.2

MAPK signaling pathway iPathway Guide analysis of three-hour mechanical strain transcript pathway (KEGG 04010): Differentially expressed genes at 3 hours were evaluated by iPathwayGuide. The perturbation of each gene's measured fold change is calculated by the iPathwayGuide algorithm such that it accounts for accumulated changes in any upstream genes. Negative perturbations are in dark blue and positive perturbations are in dark red. The gradient is described in the legend. Genes are not restricted to one area of the diagram but can appear linked with other genes from multiple pathways. For each association of a gene, its total perturbation is reported in the color displayed for that gene. A graphical representation of the second most highly enriched perpetration pathway, MAPK signaling, is overlaid with the computed perturbation of each gene.

Adapted from iPathwayGuide (https://www.advaitabio.com/ipathwayguide)

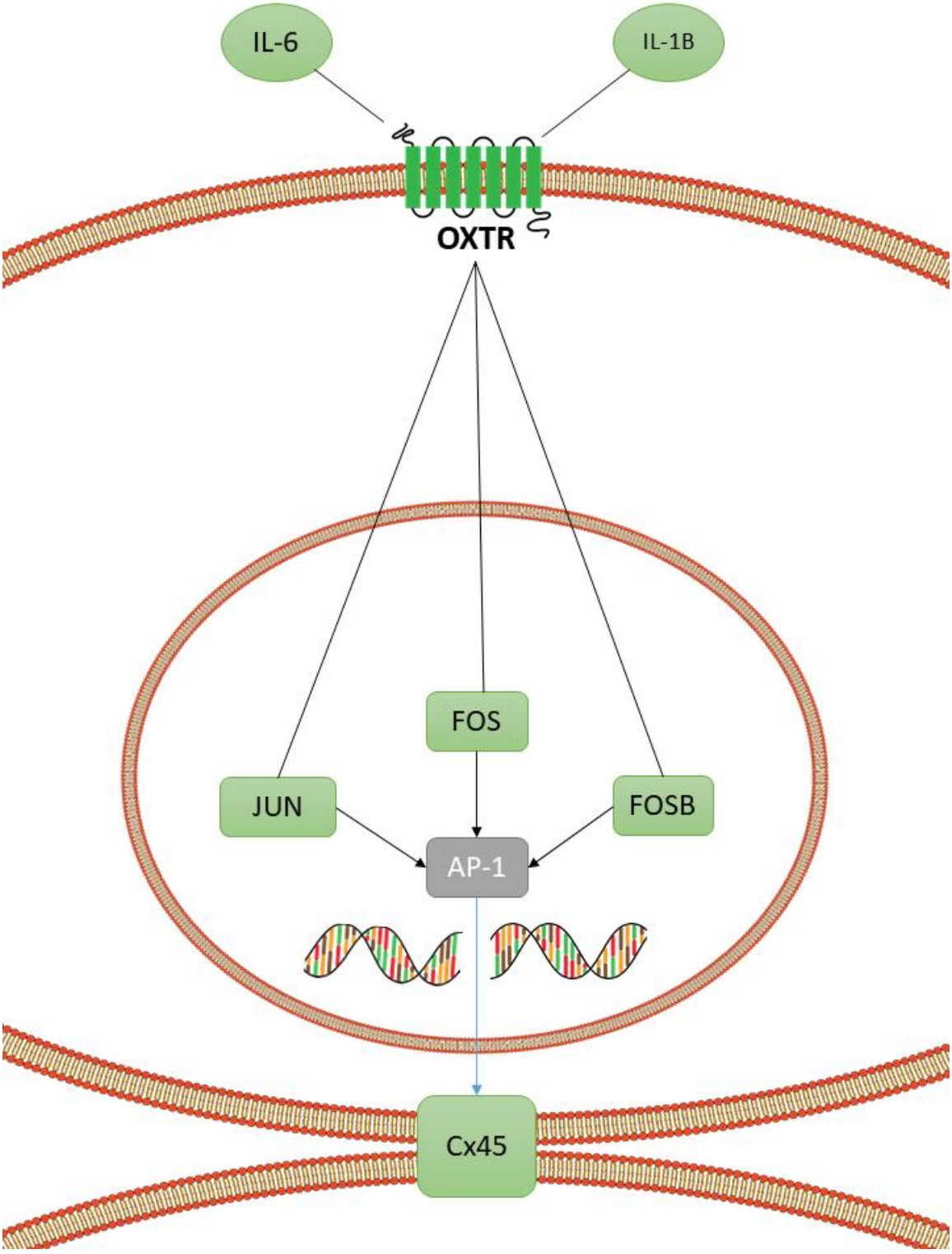

IL-6
IL-1B
OXTR
FOS
JUN
FOSB
AP-1
Cx45

Figure 3.3

Oxytocin network: Genes involved in contractile associated pathways that were also upregulated at 3 and/or 8 hours in the RNAseq data set of strained hTRT cells. Network generated by iPathway analysis of upregulated genes and literature searches based on input of contractile associated proteins. Transcripts in green were upregulated in our data set, and proteins in grey are implied by network connections but not reflected in our data.

Chapter 4

p38 MAPK regulation of IL-6 and contractile associated transcripts

Abstract

Uterine smooth muscle has the ability to transition between phenotypes in response to external signals and mechanical perturbation. During pregnancy, the myometrium remains largely quiescent until the onset of labor. Abnormal uterine distension is associated with early birth in twins and 10% of singleton births. Previous studies have identified mechanical strain-induced increases in transcription of inflammatory mediators interleukin 6 and 8 as well as activation of the p38 MAPK pathway. Additionally, matrix metalloprotease 2 protein expression is upregulated in preterm laboring tissue. We used qPCR to investigate whether p38 MAPK activation increases IL-6 transcription, and whether increased IL-6 exposure can affect the transcription of contractile-associated proteins Cx43 and the oxytocin receptor. Inhibition of p38 MAPK blocked transcription of IL-6 in strained myometrial cells but did not inhibit increases in MMP2 transcripts. Incubation with IL-6 increased Cx43 and oxytocin receptor transcripts suggesting a mechanism for mechanical strain to contribute to the initiation of labor.

4.1 Introduction

Smooth muscle is unique among muscle types due to its ability to transition between phenotypes in response to external signals and mechanical perturbation (Shynlova et al., 2013). During pregnancy, the uterus is challenged by a variety of changes in hormone levels, inflammatory molecules, as well as mechanical strain related to the growth of the fetus. However, in cases of twins, as well as approximately 10% of single pregnancies, abnormal uterine distension is associated with increased risk of preterm birth (Lockwood and Kuczynski, 2001).

Mechanical strain of the myometrium increases inflammatory transcripts, IL-6, IL-8, IL-1β, CCL2 and MMP2 (Copley Salem et al., 2018; Ulrich et al., 2019) (Chapter 3). IL-6 is a cytokine that is known to be increased in amniotic fluid and cervical connective tissue at term, and is produced by monocytes that are present in the myometrium during labor (Sivarajasingam et al., 2016a). In amniotic fluid, IL-8 production is preceded by IL-6 elevation indicating that IL-6 may be capable of driving an increase in immune responses (Kemp et al., 2002). CCL2 increases in the myometrium are likely responsible for attracting monocytes and thus producing an additional increase in local IL-6, IL-8, and TNFα concentrations (Gomez-Lopez et al., 2010; Young et al., 2002). These data suggest that IL-6 production is tied to downstream gene regulation and the formation of an inflammatory cascade that could drive labor.

In addition to the upregulation of inflammatory mediators, strain also increased transcription of the important contractile associated proteins: the OXTR, Cx45, and FOS proteins which are subunits of the transcription factor activator protein 1 (AP-1) (Chapter

3). The human OXTR promoter region contains two nucleofactor interleukin-6 (NF-IL6) consensus binding sequences, suggesting that strain-induced production of IL-6 during pregnancy could promote the expression of OXTR thereby enhancing the myometrial response to circulating oxytocin (Gimpl and Fahrenholz, 2001). Upregulation of IL-6 and corresponding downstream effectors could provide a mechanistic explanation for the strain induced inflammatory responses in the myometrium seen at the end of gestation.

Mechanical strain of myometrial smooth muscle cells increased the phosphorylation of HSP27 at serine 82 (S82) suggesting a potential role for HSP27 in the regulation of the uterine mechanical stress response (Copley Salem et al., 2018). HSP27 is a small 27 kD heat shock protein that has been implicated in a wide variety of cellular functions including protection from heat stress, actin remodeling, and regulation of smooth muscle contraction (W. Gerthoffer and Gunst, 2001). HSP27 protein-protein interactions, and thus activity, are regulated by its phosphorylation state (Bruey et al., 2000; Lambert et al., 1999). HSP27 phosphorylation patterns vary between different states of pregnancy. In pregnant myometrial smooth muscle cells exposed to strain, HSP27 phosphorylation at site S82 is upregulated, yet after the onset of labor phosphorylation of site S15 dominates (MacIntyre et al., 2008). Phosphorylation of S15 is associated with stabilization of the actin cytoskeleton while phosphorylation at S82 is involved in the polymerization of actin (W. Gerthoffer and Gunst, 2001).

HSP27 is the target of MAPK activated protein kinase 2 (MAPKAPK2), itself a substrate of stress activated p38 MAPK (Larsen et al., 1997). p38 MAPK is the key component in one of four well characterized MAPK pathways (p38 MAPK, ERK1/2, JNK

and ERK5) and is regulated a variety of external stimuli including inflammatory signals, growth factors and external stressors such as mechanical strain (Copley Salem et al., 2018; Cuadrado and Nebreda, 2010; Cuenda et al., 1995; Rouse et al., 1994). p38 MAPK is activated by phosphorylation on the dual sites of Thr (180) and Tyr (182) by mitogen-activated protein kinase kinases 3,4, and 6 (MKK3,4, 6) as well as weakly activated by Protein Kinase C (PKC) and receptor tyrosine kinases (RTK) (Chichger et al., 2015; Raingeaud et al., 1996). The p38 MAPK strain response is linked to microfilament remodeling in airway smooth muscle (Chaudhuri and Smith, 2008) and increases in smooth muscle α-actin expression (Tock et al., 2003). Thus, p38 MAPK is an important signaling hub that is activated in uterine smooth muscle cells undergoing mechanical strain (Copley Salem et al., 2018) and capable of fostering downstream changes including transcriptional regulation.

Interestingly, p38 MAPK has been linked to mRNA stabilization of inflammatory cytokine transcripts (Neininger et al., 2002). Activation of MKK6 and MKK1 stabilized IL-8 transcripts in a p38 MAPK → MAPKAPK2 dependent manner (Winzen et al., 1999). Additionally, stabilization of AU-rich elements of transcripts in HeLa cells is p38 dependent as well as sensitive to HSP27 activation (Brook et al., 2000). mRNA stabilization is an important regulatory step in the control of a variety of inflammatory genes such as IL-8 and IL-6 (Elias and Lentz, 1990) and are likely controlled by p38 MAPK activation of HSP27.

Based on observations that p38 MAPK is phosphorylated on activating sites and it's potential to regulate mRNA stability (Copley Salem et al., 2018; Miyazawa et al., 1998),

combined with an increase in available IL-6 transcripts (Chapter 3), as well as MMP2 protein expression being elevated in preterm laboring samples (Ulrich et al., 2019), we hypothesized that IL-6 and MMP2 transcription was regulated by p38 MAPK activation. To test this, we measured changes in transcription levels of IL-6 and MMP2 in response to mechanical strain of hTRT cells in the presence of a specific inhibitor of p38 MAPK by qPCR. We then hypothesized that increased IL-6 exposure regulates the production of transcripts for contractile-associated proteins OXTR and Cx43; therefore, we measured changes in the transcript levels of OXTR and Cx43 in hTRT cells, by qPCR, after culture with human recombinant IL-6 for 8 and 24 hours.

4.2 Methods and Materials

4.2.1 Cellular Growth Media

Dulbecco's Modified Eagle Medium (DMEM; Thermo Scientific, Pittsburg, PA) supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 100 U/mL penicillin 100 µg/mL streptomycin (Pen/Strep; Thermo Scientific, Pittsburgh, PA), 636 nM progesterone, and 55 nM estradiol.

4.2.2 Differentiation Media

Differentiation media consisted of DMEM (Thermo Scientific, Pittsburg, PA) with 0.1% insulin-transferrin-Selenium-Ethanolamine (ITS-X) (Thermo Scientific, Pittsburg, PA), 100 U/mL penicillin 100 µg/mL streptomycin (Pen/Strep; Thermo Scientific, Pittsburgh, PA), 636 nM progesterone, and 55 nM estradiol.

4.2.3 Cell Culture

Telomerized pregnant human uterine smooth muscle cells (Heyman et al., 2013) (hTRT, passage 30) were cultured at 37^{o}C with 5% CO_2 in cellular growth media on BioFlex-Collagen Type I-matrix-bonded growth surfaces in 6-well culture plates (FlexCell International Corporation, Burlington, NC). Cell media was replaced every 3 days. At 100% confluence, media was replaced with differentiation media. Cells were then allowed to differentiate for 7 days prior to the strain experiment.

4.2.4 Strain Experiment

Media in two experimental cell plates was refreshed with 4 mL/well Differentiation Media + 4 µL of SB203580, resulting in a final concentration of 25 µM SB203580 + 0.1% dimethyl sulfoxide (DMSO) vehicle. Media for the 4 control cell plates was refreshed with 4 mL/well of Differentiation Media + 0.1% DMSO vehicle and all conditions were incubated at 37^{o}C with 5% CO_2 for 1 hour. After one hour, cells were exposed to 18% biaxial strain for 0 (2 vehicle plates), or 3 hours (2 vehicle control and 2 drug plates) on a FX-5000 Tension System (FlexCell International Corporation, Burlington, NC). Three replicates were prepared for each temporal state. At the end of each time point, RNA was extracted as described below.

4.2.5 OXTR and Connexin Experiment

Cell culture was performed as described above except cells were plated on 12 cell culture-treated 6-well plates (83.3920; Sarstedt, Nümbrecht, Germany). After differentiation, media was replaced with Differentiation Media supplemented with 10 ng/mL recombinant human IL-6 (Invitrogen, Carlsbad, Ca) in three plates for 24 hours and three plates for 8 hours. Three control plates were refreshed with standard Differentiation Media. RNA was extracted from all plates as described below.

4.2.6 RNA Extraction and Preparation

RNA was extracted from TRIzol reagent (Thermo Scientific, Pittsburg, PA) according to the manufacturer's protocol. Extracted RNA was resuspended in 20 µL RNase/DNase free water. RNA concentrations were determined by measuring absorbance at 260 nm on a GE NanoVue Plus spectrophotometer (Thermo Scientific, Pittsburg, PA). Reverse transcribed complimentary DNA (cDNA) was generated with SuperScript™ IV VILO™ Master Mix with ezDNase enzyme treatment (Thermo Scientific, Pittsburg, PA) using manufacturer's protocol with 2.5 µg of RNA per sample on a Bio Rad iCycler thermocycler (Bio Rad, Hercules, CA).

4.2.7 qPCR Analysis

Quantitative polymerase chain reaction (qPCR) was conducted in a 20 µL reaction volume consisting of Applied Biosystems™ TaqMan™ Fast Advanced Master Mix (Thermo Scientific, Pittsburg, PA), primers, and 2 µL of template DNA for 40 cycles of 95°C for 5 s and 60°C for 30 s on an Applied Biosystems™ 7900HT Fast Real-Time PCR System (Thermo

Scientific, Pittsburg, PA). TaqMan™Gene Expression Assay (FAM) based primers were used for IL-6 (Hs00174131_m1), MMP2 (Hs01548727_m1), OXTR (Hs00168573_m1), and GJA1 (Hs04194727_g1) normalized to the 18s (Hs99999901_s1) control. Fold change was calculated for each normalized transcript in relation to unstrained vehicle control expression using the $2^{-\Delta\Delta CT}$ method.

4.2.8 Statistical Analysis

All qPCR comparisons were performed on the ΔCT values before $\log_2$ transformation to fold-change values. All experimental data was verified to have normal distribution using Kolmogorov-Smirnov Test of Normality and so all p-values were calculated using a two tailed student's t-test.

4.3 Results

We performed experiments to determine if p38 MAPK inhibition with SB203580 treatment could attenuate the transcription of inflammatory regulators IL-6 and MMP2 in response to strain in immortalized human uterine smooth muscle cells. We saw a 1.35-fold increase in IL-6 transcription in response to strain compared to unstrained controls. However, in the presence of 25 μM SB203580, there was a statistically significant reduction of IL-6 transcript (51.8 %) after exposure to biaxial strain (Fig. 4.1). We saw a 1.45-fold increase in MMP2 transcription in response to strain compared to unstrained controls but this increase was not statistically significantly affected by p38 MAPK inhibition (Fig. 4.2).

Next, we considered whether these strain-induced increases in IL-6 could affect downstream gene transcription. We probed the transcript levels of contractile-associated proteins Cx43 and the OXTR from myometrial cells after 8 and 12 hours of incubation with recombinant human IL-6 by qPCR. After 8 hours of IL-6 exposure, we saw a statistically significant 5.60-fold increase in OXTR and a 4.78-fold increase in Cx43 expression. After 24 hours of IL-6 exposure, we saw a statistically significant 3.16-fold increase in OXTR and a 5.32-fold increase (Fig. 4.3) in Cx43 expression (Fig. 4.4).

4.4 Discussion

Mechanical strain in the myometrium is linked to preterm birth in humans (Waldorf et al., 2015). Inflammatory mediators such as IL-6 and MMP2 are associated with a transition into labor and potentially the contractility of the myometrium (Shynlova et al., 2013; Ulrich et al., 2019). We investigated the connection between mechanical strain and IL-6 production in uterine smooth muscle cells and found that strain-induced p38 MAPK activation is at least partly responsible for increases in IL-6 transcripts (Fig. 4.1). p38 MAPK's ability to drive IL-6 production during mechanical strain could be a significant component of the transition into labor. IL-6 drives IL-8 production in amniotic fluid and IL-8, along with CCL2, attracts monocytes such as neutrophils to invade the tissue (Sivarajasingam et al., 2016a). Myometrial production of IL-6 and CCL2 could form a feedback loop with invading monocytes to drive inflammation (Fig. 4.5).

We observed an increase of in MMP2 transcripts in response to mechanical strain in cultured uterine myometrial cells, however, p38 MAPK does not appear necessary to

drive this increased expression. *In vivo* MMP2 levels are increased in preterm laboring myometrial samples (Ulrich et al., 2019). Failure of p38 MAPK inhibition to regulate MMP2 transcription is interesting considering that we saw a strain mediated increase in AP-1 associated protein transcripts (Chapter 3). The transcription of FOS genes, proteins necessary for the formation of AP-1, is regulated by p38 MAPK and AP-1 binds the promoter sequences for MMP2 and MMP9 (Chapter 3) (Huang et al., 2014); however, the upstream regulation of MAPKs by MAPKK is complicated and there is not a one to one relationship between a MAPKK and a target MAPK (Derijar et al., 1995). MKK3 and MKK6 have been shown to directly activate p38 MAPK, but MKK4 activates p38 MAPK and JNK pathways while MKK5 and MEKK3 activate ERK5 (Derijar et al., 1995; Lin et al., 1995). PKC can activate p38 MAPK and ERK1/2 pathways, while MEK1/2 activates ERK1/2, but not directly p38 MAPK or JNK (Chichger et al., 2015). Indeed, ERK1/2 is phosphorylated in response to myometrial strain experiments in cultured, uterine myocytes (Copley Salem et al., 2018; Y. Li et al., 2009); however, we did not detect strain-induced phosphorylation of the canonical PKC activation sites (Copley Salem et al., 2018). In gastric adenocarcinoma, the p38 MAPK>AP-1>MMP2/9 axis promotes invasion and metastasis, and p38 MAPK inhibition down-regulates MMP2's ability to promote astrocyte migration (Huang et al., 2014; Zhang et al., 2018). Thus, it is possible that ERK1/2 is involved in the activation of AP-1 and is either sufficient for strain-induced MMP2 transcript increases or can compensate for p38 MAPK inhibition.

IL-6 is thought to have pro-contractile effects in myometrium, however, there is no evidence of direct interactions between IL-6 and contractile proteins. We found that

incubation of cultured hTRT cells with IL-6 dramatically increased both OXTR and Cx43 transcripts (Fig. 4.3, 4.4). This is not surprising as both OXTR and Cx43 have NF-IL6 binding regions in their promoters. However, it is interesting that while both OXTR and Cx43 genes contain AP-1 binding sites, the Cx43 transcript was not upregulated after 8 hours of strain (Chapter 3) but the OXTR was. It is possible that AP-1 sites on Cx43 are blocked during exposure to mechanical stain, but NFIL-6 sites may be available to drive expression while with OXTR both sites are exposed. Indeed, this has explanatory power because the myometrial ability to form a syncytium is predicated on an increased ratio of Cx43/45 (Sheldon et al., 2014). After acute mechanical strain, we observed that the expression ratio decrease due to increased Cx45, possibly as a quiescence promoting mechanism (Chapter 3) (Fig. 4.6).

These data, combined with increased OXTR expression and oxytocin's ability to promote contraction, could provide a mechanistic explanation of the contribution of strain to the transition into a laboring phenotype (Arrowsmith and Wray, 2014). However, if long term strain is able to generate an IL-6 feedback loop, this would allow for increasing amounts of Cx43, thereby overcoming the potentially quiescent effect of strain-induced Cx45 (Chapter 3). Additionally, alternative MAPK pathways could be interacting with other repressors or enhancers to help block Cx43 expression during strain. It is still unclear how the interaction of Cx43 and 45 relate to the contractile state of the myometrium. Cx43 can form gap junctions and create a contractile syncytium to promote coordinated and productive contractions, and it is unlikely that Cx45 can do this; however, Cx43 hemichannels may promote quiescence by acting as calcium-sensitive, calcium-leak

channels and holding the intracellular calcium levels low (De Bock et al., 2012). Both the phosphorylation and nitrosation states of both connexin hemichannels may also contribute to myometrial quiescence or contractility and labor. Much work still needs to be completed to answer these questions and provide a complete picture of how mechanical strain leads to preterm labor.

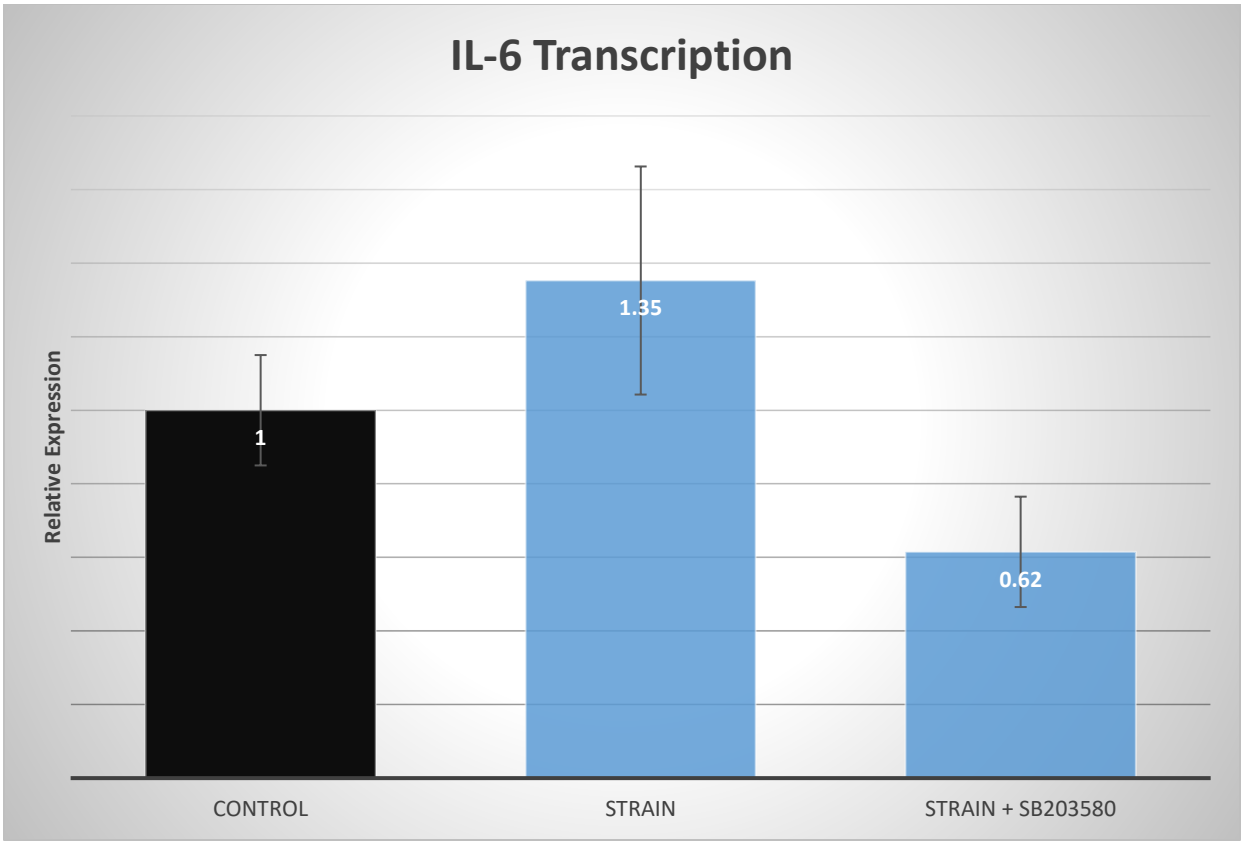

IL-6 Transcription
Relative Expression
1
1.35
0.62
CONTROL
STRAIN
STRAIN + SB203580

Figure 4.1

p38 regulation of IL-6 transcription: IL-6 transcription was measured in the presence of a specific p38 MAPK inhibitor (SB203580). Cells were exposed to mechanical strain for 0 and 3 hours with and without 25 μM SB203580. Transcript quantity was determined by qPCR and normalized to 18S ribosomal RNA. IL-6 transcript level was 1.35-fold increase in response to strain compared controls. IL-6 transcript levels were 51.8 percent lower in the SB203580 treatment condition after 3 hours of mechanical strain (p-value 0.041). All experimental data was tested for normal distribution using Kolmogorov-Smirnov Test of Normality and p-values were calculated using a two tailed student's t-test. Error bars were calculated using the formula 2^X where: X= the average ΔCT value for each condition ± one standard deviation.

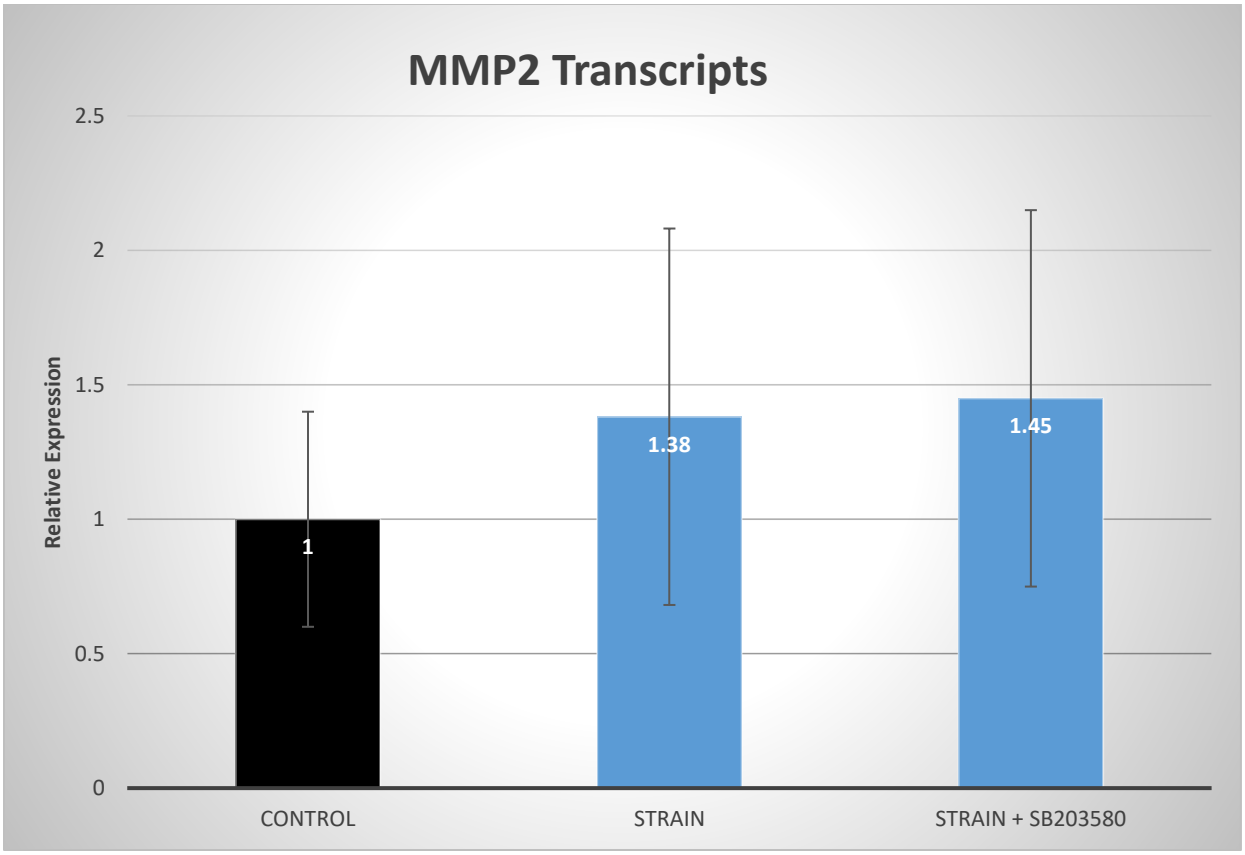

MMP2 Transcripts
Relative Expression
2.5
2
1.5
1
0.5
0
1
1.38
1.45
CONTROL
STRAIN
STRAIN + SB203580

Figure 4.2

p38 MAPK regulation of MMP2 transcription: MMP2 transcription was measured by qPCR in the presence of the specific p38 MAPK inhibitor SB203580. Cells were exposed to mechanical strain for 0 and 3 hours with and without 25 µM SB203580. Transcript quantity was determined by qPCR and normalized to 18S ribosomal RNA. MMP2 transcript level was 1.38-fold increase in response to strain compared controls. Transcript levels were 1.45-fold increased in the SB203580 treatment condition after 3 hours of mechanical strain. None of the comparisons were statistically significant. All experimental data was tested for normal distribution using Kolmogorov-Smirnov Test of Normality and p-values were calculated using a two tailed student's t-test. Error bars were calculated using the formula 2^X where: X= the average ΔCT value for each condition ± one standard deviation.

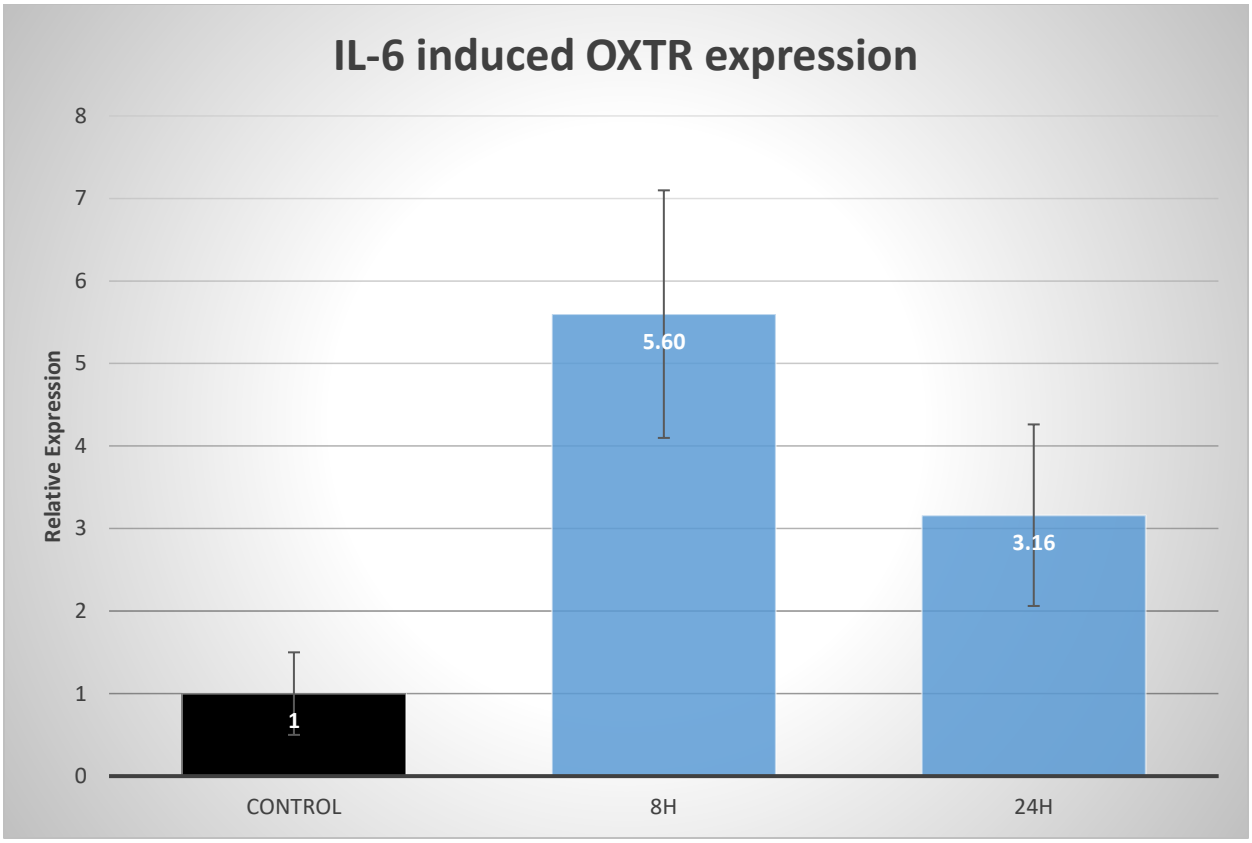

IL-6 induced OXTR expression
Relative Expression
8
7
6
5
4
3
2
1
0
1
5.60
3.16
CONTROL
8H
24H

Figure 4.3

IL-6 induced transcription of the oxytocin receptor: Transcript levels of the oxytocin receptor were measured by qPCR after 8 and 24 hours of incubation with 10 ng/mL of recombinant human IL-6 protein. Transcripts increased 5.60-fold after 8 hours (p-value 0.035) and 3.16-fold after 24 hours (p-value 0.009). All experimental data was tested for normal distribution using Kolmogorov-Smirnov Test of Normality and p-values were calculated using a two tailed student's t-test. Error bars were calculated using the formula 2^X where: X= the average ΔCT value for each condition ± one standard deviation.

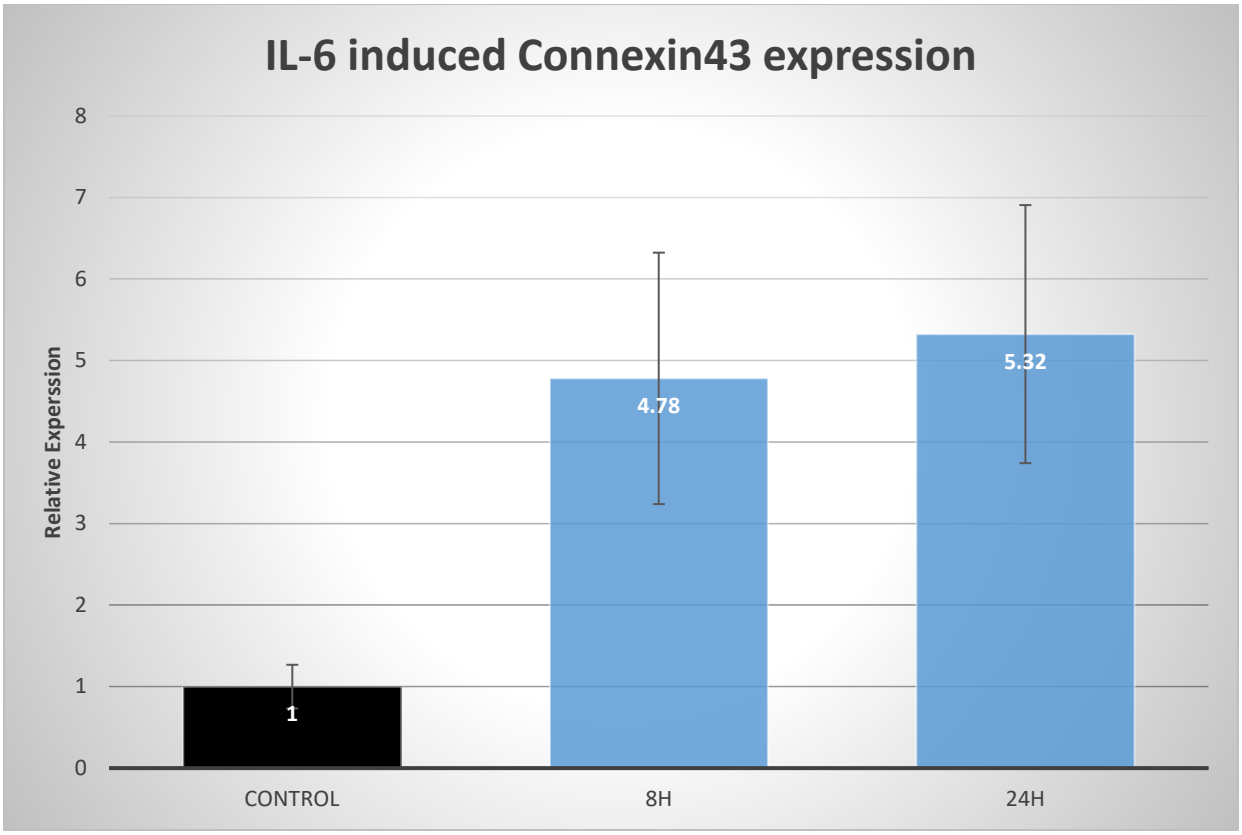

IL-6 induced Connexin43 expression
Relative Expersion
8
7
6
5
4
3
2
1
0
1
4.78
5.32
CONTROL
8H
24H

Figure 4.4

IL-6 induced transcription of Cx43: Transcript levels of Cx43 were measured by qPCR after 8 and 24 hours of incubation with 10 ng/mL recombinant human IL-6 protein. Transcripts increased 4.78-fold (p-value 0.0048) after 8 hours and 5.32-fold (p-value 0.00589) after 24 hours. All experimental data was tested for normal distribution using Kolmogorov-Smirnov Test of Normality and p-values were calculated using a two tailed student's t-test. Error bars were calculated using the formula 2^X where: X= the average ΔCT value for each condition ± one standard deviation.

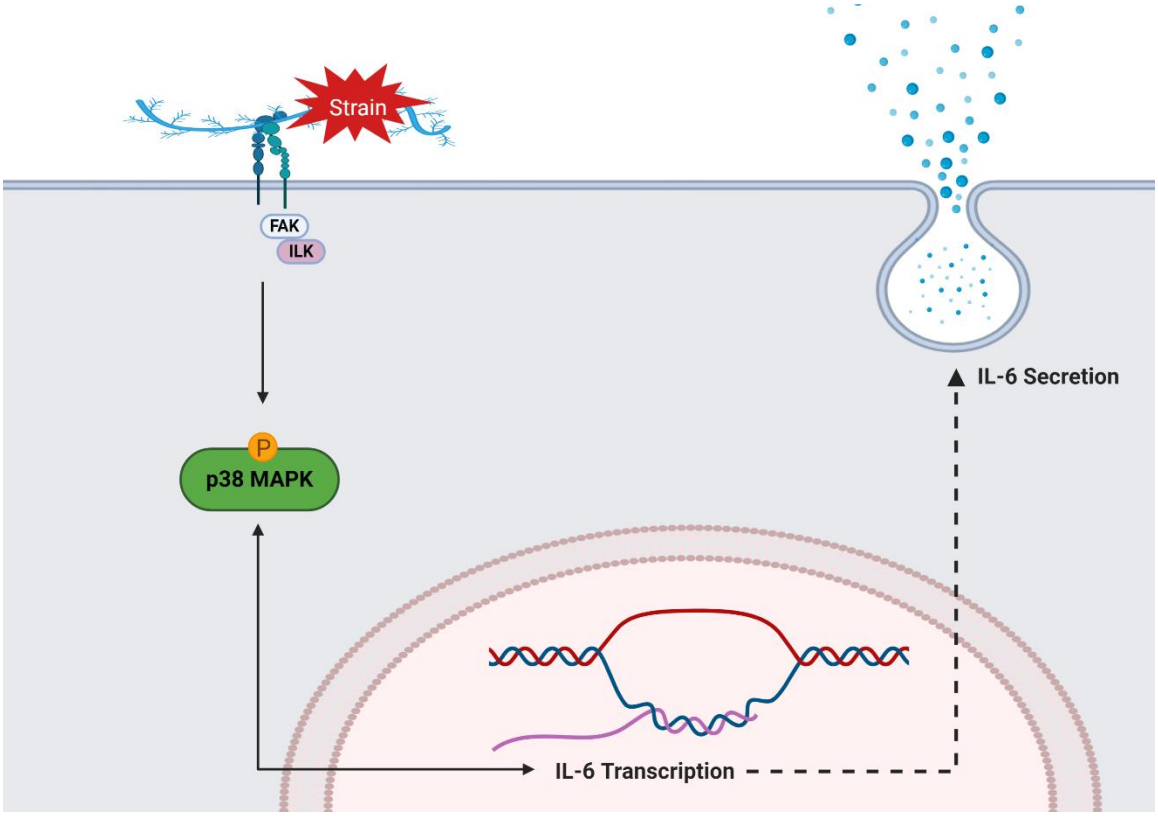

Strain
FAK
ILK
P
p38 MAPK
IL-6 Secretion
IL-6 Transcription

Figure 4.5

Inflammatory feedback loop: IL-6 transcripts increased by p38 MAPK activation could lead to an increase in secretion of IL-6 by the myometrium. This could initiate a feedback loop where IL-6 concentrations rise, along with existing myometrial secretions of CCL2 and amnion production of IL-8, which could increase monocyte invasion of the myometrium. Invading monocytes would produce additional IL-6 and IL-8 in a positive feedback loop. This model is based on strain induced p38 MAPK regulation of IL-6 and reviews of the literature on myometrial inflammation during pregnancy.

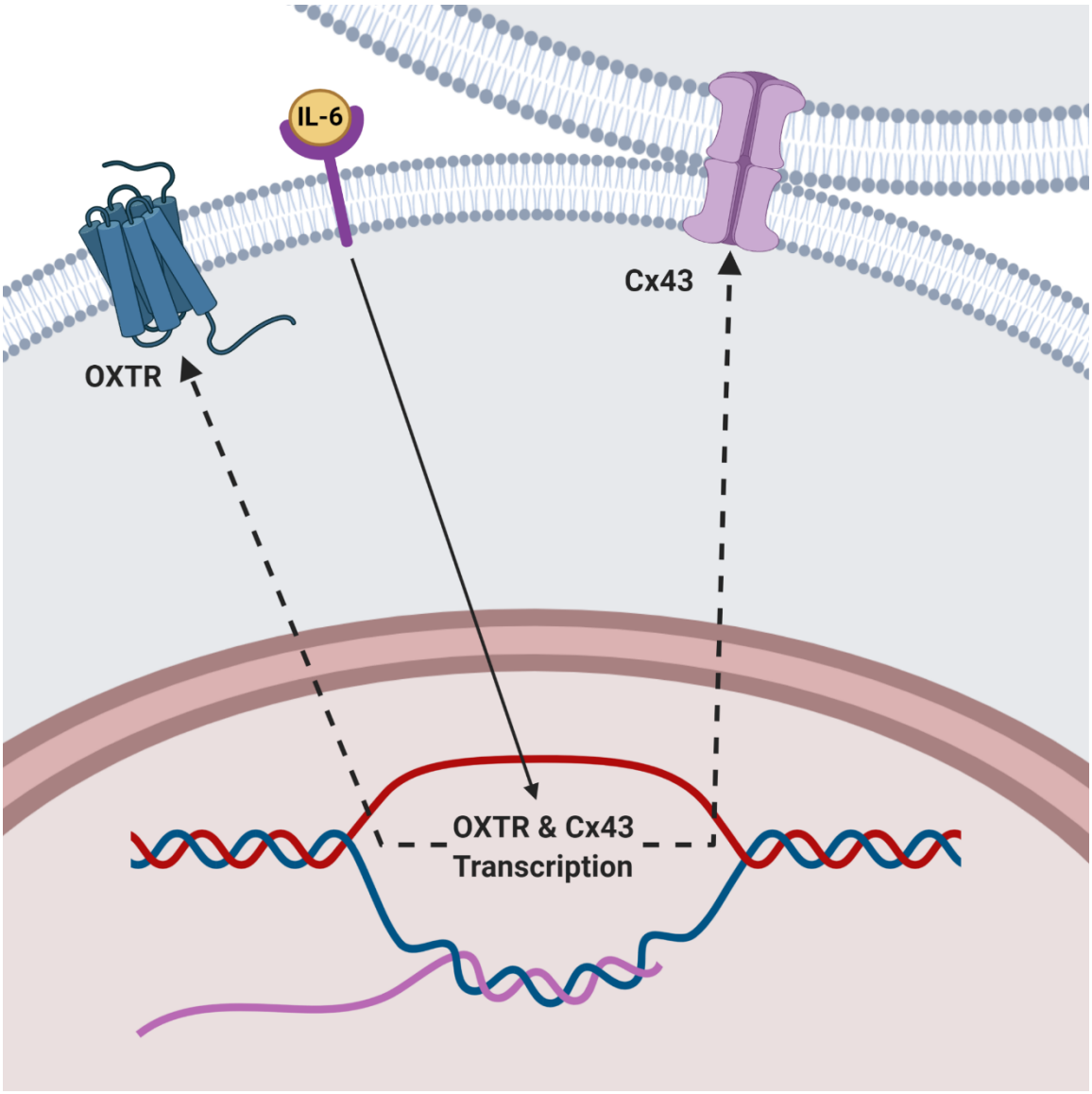

IL-6
Cx43
OXTR
OXTR & Cx43
Transcription

Figure 4.6

IL-6 mediate increase of contractile proteins: IL-6 increases, driven by p38 MAPK activation in response to myometrial strain, could feed back onto the myometrium leading to the activation of contractile proteins. p38 MAPK is activated by mechanical strain and its activation promotes the production of IL-6 transcripts. IL-6 increases transcription of Cx43 and the oxytocin receptor. Monocytes attracted to the myometrium by inflammatory signals such as CCL2 and IL-8 produce additional IL-6 among other cytokines. This could result in a positive feedback loop and lead to a dramatic increase in local IL-6 concentrations which may act as a signal for the transition into labor.

Chapter 5

Conclusions and Future Directions

5.1 Conclusions

Preterm birth, like cancer, is not a singular problem. While there may exist a "magic bullet" that can stop cancer or shut down preterm laboring tissue, it is more likely that a variety of causes and mechanisms lead to what society views as a single disorder. Socio-economic solutions as well as biochemical ones are being actively studied. Genetic, hormonal and mechanical systems are all players that must be isolated and investigated for a true understanding of the chemistry of preterm birth, and the ability to stop it. Here we have sought to understand but a small piece of the biochemical puzzle that is preterm birth and how it could be caused by abnormal uterine distension and inflammation.

Abnormal levels of uterine distension correlates strongly with preterm birth (Lockwood and Kuczynski, 2001). Here we provide evidence that inflammation and distension may work together in driving the myometrium to a contractile state. IL-6 plays an important role in this story. Previous work has shown that IL-6 expression can drive IL-8, IL-1β and CCL2 production (Kemp et al., 2002). These work synergistically to promote the invasion of myometrial tissue by monocytes that in turn produce additional inflammatory markers including IL-6, IL-1β and TNFα (Gomez-Lopez et al., 2010; Young et al., 2002). This has the potential to create an inflammatory feedback loop. Based on our work, this feedback loop's ability to increase IL-6 production could drive the production of OXTR and Cx43 within myometrial tissues. This suggests a mechanism by which abnormal amounts of uterine distension could prematurely drive myometrial smooth muscle into a contractile phenotype.

5.2 Future Directions

mRNA production is only loosely correlated with protein production, and there are a variety of additional regulatory layers that may affect the amount of cytokine secretion in response to transcriptional activation (Liu et al., 2016). It is possible that p38 MAPK regulation of IL-6 transcription may not lead to an increase in IL-6 protein secretion. The mechanism through which p38 MAPK works to create the observed increase in IL-6 transcripts is still not fully elucidated. It is possible that p38 MAPK activation of HSP27 could work to regulate IL-6 transcript stability (Brook et al., 2000); however, this stabilization may or may not affect translation in uterine smooth muscle cells.

New mass spectrometry technologies could be leveraged here. Triggered by Offset, Multiplexed, Accurate mass, High resolution, and Absolute Quantitation (TOMAHAQ) is a newly developed method which uses prefabricated precursor ions labeled with a super heavy TMT label allowing for triggered acquisition of data from sample peptides matching that spectra (Erickson et al., 2017). The lower complexity of cell media would improve functional resolution. In addition, IL-6 specific peptides can be visualized in a mass spectrometry experiment and are registered in the Peptide Atlas database (Deutsch, 2010). Peptide Atlas collects data from previous mass spectrometry experiments and provides statistics and data on which peptides are seen, how frequently they are seen, and under what conditions they may be seen.

It will be important to determine the ratio of protein produced vs transcripts produced. A disparity in the production of protein would ask questions about mRNA and translational regulation. P38 and ERK1/2 could drive layers of translational regulation that have yet to be

discovered. Additionally, the lack of JNK or ERK 5 activation could fail to relieve a basal level of repression.

Throughout this work, we have used pregnant, human myometrial cells that were cultured in progesterone and estrogen at physiologically relevant levels to maintain the cells in a pregnant phenotype. However, it is unknown if these hormones have regulatory effects on any of the transcripts studied here. It is possible that progesterone concentrations could explain the disparity of regulation between the two genes Cx43 and Cx45, each with similar AP-1 promoter sequences, and could possess functional relevance to myometrial quiescence or contractility.

Beyond these concerns, *in vivo* testing of the models proposed here will be necessary. Animal models of preterm labor are flawed because of progesterone withdrawal not experienced by humans (Csapo and Resch, 1979). This is true regardless of possible "functional" progesterone withdrawal in humans as the mechanisms underlying these differences have likely diverged (Byrns, 2014). However guinea pigs are a potential animal model that do not experience progesterone withdrawal. Additionally, human tissue and primary cell cultures could also provide more human centric answers.

Preterm birth is a concern that transcends race and culture, yet is linked to both. Thousands of years ago, the writers of ancient Semitic literature recognized that pregnancy was both an amazing and critical function of human reproduction, as well as a terrifying event that often brought with it incredible suffering, and death. They viewed this as a primordial god's punishment for disobedience. Today, we are also able to understand the values and dangers inherent to pregnancy but through the lens of scientific discovery. This tasks us to walk the long

and complicated road of unraveling the biochemical processes necessary, not just for the perpetuation of our species, but for the ability to do so without suffering.

Funding

This work was supported by the Mountain West Clinical Translational Research-Infrastructure Network under a grant from the National Institute of General Medical Sciences (NIH award number 1U54GM104944), a Pathway to Independence Award from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NIH award number R00HD067342), a Nevada Women's Health Initiative Grant, an Illumina sequencing award from the Nevada Genomics Center, and an award from the Nevada INBRE Bioinformatics Core to Heather Burkin. This manuscript was made possible by a grant from the National Institute of General Medical Sciences (GM103440) from the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the INBRE or the National Institute of Health.

REFERENCES

Adams, M., Elam-Evans, L.D., Wilson, H.G., Gilbertz, D.A., 2000. Rates of and Factors Associated With Recurrence of Preterm Delivery. Am. Med. Assoc. 283, 1591–1596.

Adams, M.D., Kerlavage, A.R., Fields, C., Venter, J.C., 1993. 3,400 new expressed sequence tags identify diversity of transcripts in human brain. Nat. Genet. 4, 256–67. doi:10.1038/ng0793-256

Aguilar, H.N., Mitchell, B.F., 2010. Physiological pathways and molecular mechanisms regulating uterine contractility. Hum. Reprod. Update 16, 725–744. doi:10.1093/humupd/dmq016

Ahn, N.G., Seger, R., Bratlien, R.L., Diltz, C.D., Tonks, N.K., Krebs, E.G., 1991. Multiple components in an epidermal growth factor-stimulated protein kinase cascade. J. Biol. Chem. 266, 4220–4227.

Aken, B.L., Achuthan, P., Akanni, W., Amode, M.R., Bernsdorff, F., Bhai, J., Billis, K., Carvalho-Silva, D., Cummins, C., Clapham, P., Gil, L., Girón, C.G., Gordon, L., Hourlier, T., Hunt, S.E., Janacek, S.H., Juettemann, T., Keenan, S., Laird, M.R., Lavidas, I., Maurel, T., McLaren, W., Moore, B., Murphy, D.N., Nag, R., Newman, V., Nuhn, M., Ong, C.K., Parker, A., Patricio, M., Riat, H.S., Sheppard, D., Sparrow, H., Taylor, K., Thormann, A., Vullo, A., Walts, B., Wilder, S.P., Zadissa, A., Kostadima, M., Martin, F.J., Muffato, M., Perry, E., Ruffier, M., Staines, D.M., Trevanion, S.J., Cunningham, F., Yates, A., Zerbino, D.R., Flicek, P., 2017. Ensembl 2017. Nucleic Acids Res. 45. doi:10.1093/nar/gkw1104

Akerlund, M., Bossmar, T., Brouard, R., Kostrzewska, A., Laudanski, T., Lemancewicz, A., Gal, C.S.-L., Steinwall, M., 1999. Receptor binding of oxytocin and vasopressin antagonists and inhibitory effects on isolated myometrium from preterm and term pregnant women. BJOG An Int. J. Obstet. Gynaecol. 106, 1047–1053. doi:10.1111/j.1471-0528.1999.tb08112.x

Akiyama, S.K., Yamada, S.S., Yamada, K.M., LaFlamme, S.E., 1994. Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. J. Biol. Chem. 269, 15961–15964.

Al-Gailani, S., Davis, A., 2014. Introduction to “Transforming pregnancy since 1900.” Stud. Hist. Philos. Sci. Part C Stud. Hist. Philos. Biol. Biomed. Sci. 47, 229–232. doi:10.1016/j.shpsc.2014.07.001

Albuquerque, C.P., Smolka, M.B., Payne, S.H., Bafna, V., Eng, J., Zhou, H., 2008. A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol. Cell. Proteomics 7, 1389–1396. doi:10.1074/mcp.M700468-MCP200

Amano, M., Ito, M., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., Kaibuchi, K., 1996. Phosphorylation and Activation of Myosin by Rho-associated Kinase (Rho Kinase). J. Biol. Chem. 271, 20246–20249. doi:10.1074/jbc.271.34.20246

Anderson, N.G., Maller, J.L., Tonks, N.K., Sturgill, T.W., 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature

343, 651–653. doi:10.1038/343651a0

Andrews, W.W., Hauth, J.C., Goldenberg, R.L., Gomez, R., Romero, R., Cassell, G.H., 1995. Amniotic fluid interleukin-6: Correlation with upper genital tract microbial colonization and gestational age in women delivered after spontaneous labor versus indicated delivery. Am. J. Obstet. Gynecol. 173, 606–612. doi:10.1016/0002-9378(95)90290-2

Arpino, C., Compagnone, E., Montanaro, M.L., Cacciatore, D., De Luca, A., Cerulli, A., Di Girolamo, S., Curatolo, P., 2010. Preterm birth and neurodevelopmental outcome: A review. Child's Nerv. Syst. 26, 1139–1149. doi:10.1007/s00381-010-1125-y

Arrowsmith, S., Wray, S., 2014. Oxytocin: Its mechanism of action and receptor signalling in the myometrium. J. Neuroendocrinol. 26, 356–369. doi:10.1111/jne.12154

Azziz, R., Cumming, J., Naeye, R., 1988. Acute myometritis and chorioamnionitis during cesarean section of asymptomatic women. Am. J. Obstet. Gynecol. 159, 1137–1139. doi:10.1016/0002-9378(88)90431-0

Banerjee, S., Mazumdar, S., 2012. Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the Analyte. Int. J. Anal. Chem. 2012, 1–40. doi:10.1155/2012/282574

Barber, M., Bordoli, R.S., Sedgwick, R.D., Tyler, A.N., 1981. Fast atom bombardment of solids as an ion source in mass spectrometry. Nature 293, 270–275.

Basford, J.R., 2002. The Law of Laplace and its relevance to contemporary medicine and rehabilitation. Arch. Phys. Med. Rehabil. 83, 1165–1170. doi:10.1053/apmr.2002.33985

Beamish, J., He, P., 2010. Molecular regulation of contractile smooth muscle cell phenotype: implications for vascular tissue engineering. Tissue Eng. 16. doi:10.1089/ten.teb.2009.0630

Beceriklisoy, H.B., Walter, I., Schäfer-Somi, S., Miller, I., Kanca, H., Izgür, H., Aslan, S., 2007. Matrix metalloproteinase (MMP)-2 and MMP-9 activity in the canine uterus before and during placentation. Reprod. Domest. Anim. 42, 654–659. doi:10.1111/j.1439-0531.2006.00838.x

Behrman, R.E., Butler, A.S., 2007. Prematurity at Birth: Determinants, Consequences, and Geographic Variation, Preterm birth: causes, consequences and prevention. doi:10.1080/01443610802243047

Benjamini, Y., Hochberg, Y., 1995. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57, 289–300. doi:10.2307/2346101

Bentley, J.K., Hershenson, M.B., 2008. Airway smooth muscle growth in asthma: Proliferation, hypertrophy, and migration. Proc. Am. Thorac. Soc. 5, 89–96. doi:10.1513/pats.200705-063VS

Biemann, K., McCloskey, J.A., 1962. Application of Mass Spectrometry to Structure Problems. VI. Nucleosides. J. Am. Chem. Soc. 84, 2005–2007. doi:10.1021/ja00869a048

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. doi:10.1093/bioinformatics/btu170

Bollopragada, S., Youssef, R., Jordan, F., Greer, I., Norman, J., Nelson, S., 2009. Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. Am. J. Obstet. Gynecol. 200, 104.e1-104.e11. doi:10.1016/j.ajog.2008.08.032

Borish, L.C., Steinke, J.W., 2003. 2. Cytokines and chemokines. J. Allergy Clin. Immunol. 111, 460–475. doi:10.1067/mai.2003.108

Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radzlejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., Yancopoulos, G.D., 1991. ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65, 663–675. doi:10.1016/0092-8674(91)90098-J

Braunstein, G.D., Rasor, J., Adler, D., Danzer, H., Wade, M.E., 1976. Serum human chorionic gonadotropin levels throughout normal pregnancy. Am. J. Obstet. Gynecol. 126, 678–681. doi:10.1016/0002-9378(76)90518-4

Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D.H., Johnson, D., Luo, S., McCurdy, S., Foy, M., Ewan, M., Roth, R., George, D., Eletr, S., Albrecht, G., Vermaas, E., Williams, S.R., Moon, K., Burcham, T., Pallas, M., DuBridge, R.B., Kirchner, J., Fearon, K., Mao, J.I., Corcoran, K., 2000. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. Nat. Biotechnol. 18, 630–634. doi:10.1038/76469

Brook, M., Sully, G., Clark, A.R., Saklatvala, J., 2000. Regulation of tumour necrosis factor α mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. FEBS Lett. 483, 57–61. doi:10.1016/S0014-5793(00)02084-6

Bruey, J.M., Paul, C., Fromentin, a, Hilpert, S., Arrigo, a P., Solary, E., Garrido, C., 2000. Differential regulation of HSP27 oligomerization in tumor cells grown in vitro and in vivo. Oncogene 19, 4855–4863. doi:10.1038/sj.onc.1203850

Bulletti, C., Ziegler, D. De, Polli, V., Del Ferro, E., Palini, S., Flamigni, C., 2002. Characteristics of uterine contractility during menses in women with mild to moderate endometriosis. Fertil. Steril. 77, 1156–1161. doi:10.1016/S0015-0282(02)03087-X

Burris, H.H., Collins, J.W., 2010. Race and preterm birth--the case for epigenetic inquiry. Ethn. Dis. 20, 296–299.

Buxton, I.L.O., Heyman, N., Wu, Y., Barnett, S., Ulrich, C., 2011. A role of stretch-activated potassium currents in the regulation of uterine smooth muscle contraction. Acta Pharmacol. Sin. 32, 758–64. doi:10.1038/aps.2011.62

Buxton, I.L.O., Kaiser, R.A., Malmquist, N.A., Tichenor, S., 2001. NO induced relaxation of labouring and non labouring human myometrium is not mediated by cyclic GMP. Br. J. Pharmacol. 134, 206–214.

Byrns, M.C., 2014. Regulation of progesterone signaling during pregnancy: Implications for the

use of progestins for the prevention of preterm birth. J. Steroid Biochem. Mol. Biol. 139, 173–181. doi:10.1016/j.jsbmb.2013.01.015

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., Madden, T.L., 2009. BLAST plus: architecture and applications. BMC Bioinformatics 10, 1. doi:Artn 421\nDoi 10.1186/1471-2105-10-421

Carlstedt, I., Sheehan, J.K., 1989. Structure and macromolecular properties of cervical mucus glycoproteins. Symp. Soc. Exp. Biol. 43, 289–316.

Challis, J., Newnham, J., Petraglia, F., Yeganegi, M., Bocking, A., 2013. Fetal sex and preterm birth. Placenta 34, 95–99. doi:10.1016/j.placenta.2012.11.007

Chaudhuri, S., Smith, P.G., 2008. Cyclic strain-induced HSP27 phosphorylation modulates actin filaments in airway smooth muscle cells. Am. J. Respir. Cell Mol. Biol. 39, 270–278. doi:2007-0263OC [pii]10.1165/rcmb.2007-0263OC

Chichger, H., Vang, A., O'Connell, K.A., Zhang, P., Mende, U., Harrington, E.O., Choudhary, G., 2015. PKC δ and βII regulate angiotensin II-mediated fibrosis through p38: A mechanism of RV fibrosis in pulmonary hypertension. Am. J. Physiol. - Lung Cell. Mol. Physiol. 308, L827–L836. doi:10.1152/ajplung.00184.2014

Chovatiya, R., Medzhitov, R., 2014. Stress, Inflammation, and Defense of Homeostasis. Mol Cell 54, 281–288. doi:doi:10.1016/j.molcel.2014.03.030

Chowdhury, S.K., Chait, B.T., 1989. Chapter 27. Recent Developments in the Mass Spectrometry of Peptides and Proteins. Annu. Rep. Med. Chem. 24, 253–263. doi:10.1016/S0065-7743(08)60549-7

Chowdhury, S.K., Katta, V., Chait, B.T., 1990. Electrospray ionization mass spectrometric peptide mapping: A rapid, sensitive technique for protein structure analysis. Biochem. Biophys. Res. Commun. 167, 686–692. doi:10.1016/0006-291X(90)92080-J

Cohen, J., 1988. Statistical power analysis for the behavioral sciences. Lawrence Erlbaum Associates, Mahwah, pp. 567. doi:10.1234/12345678

Copley Salem, C., Ulrich, C., Quilici, D., Schlauch, K., LO Buxton, I., Burkin, H., 2018. Mechanical strain induced phospho-proteomic signaling in uterine smooth muscle cells. J. Biomech. doi:10.1016/j.jbiomech.2018.03.040

Cremo, C.R., Geeves, M.A., 1998. Interaction of actin and ADP with the head domain of smooth muscle myosin: Implications for strain-dependent ADP release in smooth muscle. Biochemistry 37, 1969–1978. doi:10.1021/bi9722406

Crowther, C.A., Brown, J., Mckinlay, C.J.D., Middleton, P., 2014. Magnesium sulphate for preventing preterm birth in threatened preterm labour. Cochrane Database Syst. Rev. 2014. doi:10.1002/14651858.CD001060.pub2

Csapo, A., 1956. Progesterone block. Am. J. Anat. 98, 273–291. doi:10.1002/aja.1000980206

Csapo, A.I., Resch, B.A., 1979. Induction of preterm labor in the rat by antiprogesterone. Am. J. Obstet. Gynecol. 134, 823–827. doi:10.1016/0002-9378(79)90954-2

Cuadrado, A., Nebreda, A.R., 2010. Mechanisms and functions of p38 MAPK signalling. Biochem. J. 429, 403–417. doi:10.1042/BJ20100323

Cuenda, a., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., Lee, J.C., 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. 364, 229–233. doi:10.1016/0014-5793(95)00357-F

Dabrowska, R., Aromatorio, D., Sherry, J.M., Hartshorne, D.J., 1977. Composition of the myosin light chain kinase from chicken gizzard. Biochem. Biophys. Res. Commun. 78, 1263–1272.

Dale, H.H., 1909. The Action of Extracts of the Pituitary Body. Biochem. J. 4, 427–447. doi:10.1042/bj0040427

De Bock, M., Wang, N., Bol, M., Decrock, E., Ponsaerts, R., Bultynck, G., Dupont, G., Leybaert, L., 2012. Connexin 43 hemichannels contribute to cytoplasmic Ca 2+ oscillations by providing a bimodal Ca 2+-dependent Ca 2+ entry pathway. J. Biol. Chem. 287, 12250–12266. doi:10.1074/jbc.M111.299610

Delaney, S., Shaffer, B.L., Cheng, Y.W., Vargas, J., Sparks, T.N., Paul, K., Caughey, A.B., 2010. Labor induction with a Foley balloon inflated to 30 mL compared with 60 mL: a randomized controlled trial. Obstet. Gynecol. 115, 1239–45. doi:10.1097/AOG.0b013e3181dec6d0

Dennis, M.K., Field, A.S., Burai, R., Ramesh, C., Whitney, K., Bologa, C.G., Oprea, T.I., Yamaguchi, Y., Hayashi, S., Sklar, L. a, Hathaway, H.J., Arterburn, J.B., Prossnitz, E.R., 2012. ProteomeXchange provides globally co-ordinated proteomics data submission and dissemination. Nat Biotechnol 127, 358–366. doi:10.1016/j.jsbmb.2011.07.002.Identification

Derijar, B., Raingeaud, J., Davis, R., Ulevitch, R., Han, J., Wu, I., Barrett, T., 1995. Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. Science (80-.). 267, 682–685.

Desplantez, T., 2017. Cardiac Cx43, Cx40 and Cx45 co-assembling: Involvement of connexins epitopes in formation of hemichannels and Gap junction channels. BMC Cell Biol. 18, 1–13. doi:10.1186/s12860-016-0118-4

Deutsch, E.W., 2010. The PeptideAtlas Project. Methods Mol. Biol. 604, 285–296. doi:10.1093/nar/gkj040

Donato, M., Xu, Z., Tomoiaga, A., Granneman, J.G., Mackenzie, R.G., Bao, R., Than, N.G., Westfall, P.H., Romero, R., Draghici, S., 2013. Analysis and correction of crosstalk e ff ects in pathway analysis. Genome Res. 1–23. doi:10.1101/gr.153551.112

Draghici, S., Khatri, P., Tarca, A., Amin, K., Done, A., Voichita, C., Georgescu, C., Romero, R.,

2007. A systems biology approach for pathway level analysis. Genome Res. 1537–1545. doi:10.1101/gr.6202607.1

Du Vigneaud, V., Ressler, C., Trippett, S., 1953. The Sequence of Amino Acids in Oxytocin, with a Proposal for the structure of Oxytocin. J. Chem. Inf. Model. 53, 1689–1699.

El-Yazbi, A.F., Abd-Elrahman, K.S., Moreno-Dominguez, A., 2015. PKC-mediated cerebral vasoconstriction: Role of myosin light chain phosphorylation versus actin cytoskeleton reorganization. Biochem. Pharmacol. 95, 263–278. doi:10.1016/j.bcp.2015.04.011

El Maradny, E., Kanayama, N., Halim, A., Maehara, K., Terao, T., 1996. Stretching of fetal membranes increases the concentration of interleukin- 8 and collagenase activity. Am. J. Obstet. Gynecol. 174, 843–849. doi:10.1016/S0002-9378(96)70311-3

Elias, J.A., Lentz, V., 1990. IL-1 and tumor necrosis factor synergistically stimulate fibroblast IL-6 production and stabilize IL-6 messenger RNA. J. Immunol. 145, 161–6.

English, W.R., Puente, X.S., Freije, J.M.P., Knäuper, V., Amour, A., Merryweather, A., López-Otín, C., Murphy, G., 2000. Membrane type 4 matrix metalloproteinase (MMP17) has tumor necrosis factor-α convertase activity but does not activate pro-MMP2. J. Biol. Chem. 275, 14046–14055. doi:10.1074/jbc.275.19.14046

Erickson, B.K., Jedrychowski, M.P., McAlister, G.C., Everley, R.A., Kunz, R., Gygi, S.P., 2015. Evaluating multiplexed quantitative phosphopeptide analysis on a hybrid quadrupole mass filter/linear ion trap/orbitrap mass spectrometer. Anal. Chem. 87, 1241–1249. doi:10.1021/ac503934f

Erickson, B.K., Rose, C.M., Braun, C.R., Erickson, A.R., Knott, J., McAlister, G.C., Wühr, M., Paulo, J.A., Everley, R.A., Gygi, S.P., 2017. A Strategy to Combine Sample Multiplexing with Targeted Proteomics Assays for High-Throughput Protein Signature Characterization. Mol. Cell 1–10. doi:10.1016/j.molcel.2016.12.005

Eriksson, J.E., He, T., Trejo-Skalli, A. V, Härmälä-Braskén, A.-S., Hellman, J., Chou, Y.-H., Goldman, R.D., 2004. Specific in vivo phosphorylation sites determine the assembly dynamics of vimentin intermediate filaments. J. Cell Sci. 117, 919–932. doi:10.1242/jcs.00906

Esplin, M.S., Peltier, M.R., Hamblin, S., Smith, S., Fausett, M.B., Dildy, G.A., Branch, D.W., Silver, R.M., Adashi, E.Y., 2005. Monocyte chemotactic protein-1 expression is increased in human gestational tissues during term and preterm labor. Placenta 26, 661–71.

Esplin, M.S., Romero, R., Chaiworapongsa, T., Kim, Y.M., Edwin, S., Gomez, R., Gonzalez, R., Adashi, E.Y., 2003. Amniotic fluid levels of immunoreactive monocyte chemotactic protein-1 increase during term parturition. J. Matern. Neonatal Med. 14, 51–56. doi:10.1080/jmf.14.1.51.56

Exeter, D., Connell, D., 2010. Skeletal Muscle: Functional Anatomy and Pathophysiology. Semin. Musculoskelet. Radiol. 14, 097–105. doi:10.1055/s-0030-1253154

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., Whitehouse, C.M., 1989. Electrospray ionization for mass spectrometry of large biomolecules. Science (80-.). 246, 64–71. doi:10.1126/science.2675315

Finehout, E.J., Lee, K.H., 2004. An Introduction to Mass Spectrometry Applications in Biological Research. Biochem. Mol. Biol. Educ. 32, 93–100. doi:10.1002/bmb.2004.494032020331

Fleckenstein, A., 1983. History of calcium antagonists. Circ. Res. 52, I3-16.

Flenady, V., Papatsonis, D.N.M., Stock, O., Middleton, P., Murray, L., Carbonne, B., 2013. Calcium channel blockers for inhibiting preterm labour: A cochrane systematic review. J. Paediatr. Child Health 49, 117. doi:10.1002/14651858.CD002255.pub2.www.cochranelibrary.com

Flenady, V., Reinebrant, H.E., Liley, H.G., Tambimuttu, E.G., Papatsonis, D.N.M., 2014. Oxytocin receptor antagonists for inhibiting preterm labour. Cochrane Database Syst. Rev. 2014. doi:10.1002/14651858.CD004452.pub3

Frearson, N., Focant, B.W.W., Perry, S. V., 1976. Phosphorylation of a light chain component of myosin from smooth muscle. FEBS Lett. 63, 27–32. doi:10.1016/0014-5793(76)80187-1

Frey, H.A., Klebanoff, M.A., 2016. The epidemiology, etiology, and costs of preterm birth. Semin. Fetal Neonatal Med.

Fuchs, A.R., Fields, M.J., Freidman, S., Shemesh, M., Ivell, R., 1995. Oxytocin and the timing of parturition. Influence of oxytocin receptor gene expression, oxytocin secretion, and oxytocin-induced prostaglandin F2 alpha and E2 release. Adv. Exp. Med. Biol. 395, 405–20.

Gardner, M.O., Goldenberg, R.L., Cliver, S.P., Tucker, J.M., Nelson, K.G., Copper, R.L., 1995. The origin and outcome of preterm twin pregnancies. Obstet. Gynecol. 85, 553–557. doi:10.1016/0029-7844(94)00455-M

Gáspár, R., Hajagos-Tóth, J., 2013. Calcium channel blockers as tocolytics: Principles of their actions, adverse effects and therapeutic combinations. Pharmaceuticals 6, 689–699. doi:10.3390/ph6060689

Gerthoffer, W., Gunst, S., 2001. Invited review: focal adhesion and small heat shock proteins in the regulation of actin remodeling and contractility in smooth muscle. J. Appl. Physiol. 91, 963–972.

Gerthoffer, W.T., Gunst, S.J., 2001. Invited review: focal adhesion and small heat shock proteins in the regulation of actin remodeling and contractility in smooth muscle. J Appl Physiol 91, 963–972.

Gimpl, G., Fahrenholz, F., 2001. The oxytocin receptor system: Structure, function, and regulation. Physiol. Rev. 81, 629–683. doi:10.1152/physrev.2001.81.2.629

Goldenberg, R.L., Culhane, J.F., Iams, J.D., Romero, R., 2008. Epidemiology and causes of preterm birth. Lancet 371, 75–84. doi:10.1016/S0140-6736(08)60074-4

Gomez-Lopez, N., Laresgoiti-Servitje, E., Olson, D.M., Estrada-Gutiérrez, G., Vadillo-Ortega, F., 2010. The Role of Chemokines in Term and Premature Rupture of the Fetal Membranes: A Review1. Biol. Reprod. 82, 809–814. doi:10.1095/biolreprod.109.080432

Gotsch, F., Romero, R., Kusanovic, J.P., Erez, O., Espinoza, J., Kim, C.J., Vaisbuch, E., Than, N.G., Mazaki-Tovi, S., Chaiworapongsa, T., Mazor, M., Yoon, B.H., Edwin, S., Gomez, R., Mittal, P., Hassan, S.S., Sharma, S., 2008. The anti-inflammatory limb of the immune response in preterm labor, intra-amniotic infection/inflammation, and spontaneous parturition at term: a role for interleukin-10. J Matern Fetal Neonatal Med 21, 529–47. doi:10.1080/14767050802127349

Greenlee, K.J., Corry, D.B., Engler, D.A., Matsunami, R.K., Tessier, P., Cook, R.G., Werb, Z., Kheradmand, F., 2006. Proteomic Identification of In Vivo Substrates for Matrix Metalloproteinases 2 and 9 Reveals a Mechanism for Resolution of Inflammation. J. Immunol. 177, 7312–7321. doi:10.4049/jimmunol.177.10.7312

Greenough, A., 2012. Long term respiratory outcomes of very premature birth (<32 weeks). Semin. Fetal Neonatal Med. 17, 73–76. doi:10.1016/j.siny.2012.01.009

Guan, J.L., 1997. Role of focal adhesion kinase in integrin signaling. Int. J. Biochem. Cell Biol. 29, 1085–1096. doi:10.1016/S1357-2725(97)00051-4

Guo, M., Ehrlicher, A.J., Mahammad, S., Fabich, H., Jensen, M.H., Moore, J.R., Fredberg, J.J., Goldman, R.D., Weitz, D. a., 2013. The role of vimentin intermediate filaments in cortical and cytoplasmic mechanics. Biophys. J. 105, 1562–1568. doi:10.1016/j.bpj.2013.08.037

Haas, D.M., Imperiale, T.F., Kirkpatrick, P.R., Klein, R.W., 2009. Tocolytic Therapy. Am. Coll. Obstet. Gynecol. 113, 1–10.

Hai, T., Curran, T., 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc. Natl. Acad. Sci. U. S. A. 88, 3720–3724. doi:10.1073/pnas.88.9.3720

Harbers, M., Carninci, P., 2005. Tag-based approaches for transcriptome research and genome annotation. Nat. Methods 2, 495–502. doi:10.1038/nmeth768

Hardy, D.B., Janowski, B.A., Corey, D.R., Mendelson, C.R., 2006. Progesterone receptor plays a major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-κB activation of cyclooxygenase 2 expression. Mol. Endocrinol. 20, 2724–2733. doi:10.1210/me.2006-0112

Hein, M., Petersen, A.C., Helmig, R.B., Uldbjerg, N., Reinholdt, J., 2005. Immunoglobulin levels and phagocytes in the cervical mucus plug at term of pregnancy. Acta Obstet. Gynecol. Scand. 84, 734–742. doi:10.1111/j.0001-6349.2005.00525.x

Heinrich, M., Oberbach, A., Schlichting, N., Stolzenburg, J.U., Neuhaus, J., 2011. Cytokine effects on gap junction communication and connexin expression in human bladder smooth muscle cells and suburothelial myofibroblasts. PLoS One 6. doi:10.1371/journal.pone.0020792

Heyman, N.S., Cowles, C.L., Barnett, S.D., Wu, Y.-Y., Cullison, C., Singer, C.A., Leblanc, N., Buxton, I.L.O., 2013. TREK-1 currents in smooth muscle cells from pregnant human myometrium. AJP Cell Physiol. 305, C632–C642. doi:10.1152/ajpcell.00324.2012

Hill, L.M., Breckle, R., Thomas, M.L., Fries, J.K., 1987. Polyhydramnios: ultrasonically detected prevalence and neonatal outcome. Obstet. Gynecol.

Hishiki, T., 2000. BodyMap: a human and mouse gene expression database. Nucleic Acids Res. 28, 136–138. doi:10.1093/nar/28.1.136

Houtzager, B.A., Hogendoorn, S.M., Papatsonis, D.N.M., Samsom, J.F., Van Geijn, H.P., Bleker, O.P., Van Wassenaer, A.G., 2006. Long-term follow up of children exposed in utero to nifedipine or ritodrine for the management of preterm labour. BJOG An Int. J. Obstet. Gynaecol. 113, 324–331. doi:10.1111/j.1471-0528.2006.00851.x

Howson, C.P., Kinney, M. V, McDougall, L., Lawn, J.E., 2013. Born Too Soon: Preterm birth matters. Reprod. Health 10, 1–9. doi:10.1097/00002341-199612000-00005

Hricak, H., Alpers, C., Crooks, L.E., Sheldon, P.E., 1983. Magnetic Imaging Pelvis : Resonance of the Female Experience. AJR Am J Roentgenol 141, 1119–1128.

Hu, Q., Noll, R.J., Li, H., Makarov, A., Hardman, M., Cooks, R.G., 2005. The Orbitrap: A new mass spectrometer. J. Mass Spectrom. 40, 430–443. doi:10.1002/jms.856

Hua, R., Pease, J.E., Sooranna, S.R., Viney, J.M., Nelson, S.M., Myatt, L., Bennett, P.R., Johnson, M.R., 2012. Stretch and inflammatory cytokines drive myometrial chemokine expression via NF-??B activation. Endocrinology 153, 481–491. doi:10.1210/en.2011-1506

Huang, Q., Lan, F., Wang, X., Yu, Y., Ouyang, X., Zheng, F., Han, J., Lin, Y., Xie, Y., Xie, F., Liu, W., Yang, X., Wang, H., Dong, L., Wang, L., Tan, J., 2014. IL-1β-induced activation of p38 promotes metastasis in gastric adenocarcinoma via upregulation of AP-1/c-fos, MMP2 and MMP9. Mol. Cancer 13, 1–15. doi:10.1186/1476-4598-13-18

Huttlin, E.L., Jedrychowski, M.P., Elias, J.E., Goswami, T., Rad, R., Beausoleil, S. a., Villén, J., Haas, W., Sowa, M.E., Gygi, S.P., 2010. A tissue-specific atlas of mouse protein phosphorylation and expression. Cell 143, 1174–1189. doi:10.1016/j.cell.2010.12.001

Ikebe, M., Komatsu, S., Woodhead, J.L., Mabuchi, K., Ikebe, R., Saito, J., Craig, R., Higashihara, M., 2001. The Tip of the Coiled-coil Rod Determines the Filament Formation of Smooth Muscle and Nonmuscle Myosin. J. Biol. Chem. 276, 30293–30300. doi:10.1074/jbc.M101969200

Ilicic, M., Butler, T., Zakar, T., Paul, J.W., 2017. The expression of genes involved in myometrial contractility changes during ex situ culture of pregnant human uterine smooth muscle tissue. J. Smooth Muscle Res. 53, 73–89. doi:10.1540/jsmr.53.73

Institute of Medicine, 2007. Preterm Birth. National Academies Press, Washington, D.C. doi:10.17226/11622

Isaka, K., Isaka, K., Usuda, S., Usuda, S., Ito, H., Ito, H., Sagawa, Y., Sagawa, Y., Nakamura, H.,

Nakamura, H., Nishi, H., Nishi, H., Suzuki, Y., Suzuki, Y., Li, Y.F., Li, Y.F., Takayama, M., Takayama, M., 2003. Expression and activity of matrix metalloproteinase 2 and 9 in human trophoblasts. Placenta 24, 53–64.

Jaffer, S., Shynlova, O., Lye, S., 2009. Mammalian target of rapamycin is activated in association with myometrial proliferation during pregnancy. Endocrinology 150, 4672–4680. doi:10.1210/en.2009-0419

James, P., 2001. Proteome Research: Mass Spectrometry, Principles and Practice. Springer Berlin Heidelberg, Berlin, Heidelberg. doi:10.1007/978-3-642-56895-4

Jorgensen, J.S., Weile, L.K.K., Lamont, R.F., 2014. Preterm labor: Current tocolytic options for the treatment of preterm labor. Expert Opin. Pharmacother. 15, 585–588. doi:10.1517/14656566.2014.880110

Joshi, S.R., Comer, B.S., Mclendon, J.M., Gerthoffer, W.T., 2012. MicroRNA regulation of smooth muscle phenotype. Mol. Cell. Pharmacol. 4, 1–16. doi:10.4255/mcpharmacol.12.01

Kamm, K.E., Stull, J.T., 1985. The Function of Myosin and Myosin Light Chain Kinase Phosphorylation in Smooth Muscle. Ann Rev Pharmacol Toxicol 25, 593–620.

Karin, M., 1996. The regulation of AP-1 activity by mitogen-activated protein kinases. Philos. Trans. R. Soc. B Biol. Sci. doi:10.1098/rstb.1996.0008

Karin, M., Liu, Z.G., Zandi, E., 1997. AP-1 function and regulation. Curr. Opin. Cell Biol. 9, 240–246. doi:10.1016/S0955-0674(97)80068-3

Karp, N.A., Huber, W., Sadowski, P.G., Charles, P.D., Hester, S. V., Lilley, K.S., 2010. Addressing accuracy and precision issues in iTRAQ quantitation. Mol. Cell. Proteomics 9, 1885–1897. doi:10.1074/mcp.M900628-MCP200

Keelan, J.A., Blumenstein, M., Helliwell, R.J.A., Sato, T.A., Marvin, K.W., Mitchell, M.D., 2003. Cytokines, Prostaglandins and Parturition—A Review. Placenta Trophobl. Res. 24, 33–46. doi:10.1053/plac.2002.0948

Kemp, B., Winkler, M., Maas, A., Maul, H., Ruck, P., Reineke, T., Rath, W., 2002. Cytokine concentrations in the amniotic fluid during parturition at term: Correlation to lower uterine segment values and to labor. Acta Obstet. Gynecol. Scand. 81, 938–942. doi:10.1034/j.1600-0412.2002.811007.x

Khan-Dawood, F.S., Yang, J., Dawood, M.Y., 1998. Hormonal regulation of connexin-43 in baboon corpora lutea. J. Endocrinol. 157, 405–414. doi:10.1677/joe.0.1570405

Kim, D., Langmead, B., Salzberg, S.L., 2015a. HISAT: a fast spliced aligner with low memory requirements Daehwan. Nat Methods 12, 357–360. doi:10.1177/0963721412473755.Surging

Kim, D., Langmead, B., Salzberg, S.L., 2015b. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360. doi:10.1038/nmeth.3317

Kim, H., Pennie, W.D., Sun, Y., Colburn, N.H., 1997. Differential functional significance of AP-1 binding sites in the promoter of the gene encoding mouse tissue inhibitor of metalloproteinases-3. Biochem. J. 324, 547–553. doi:10.1042/bj3240547

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, a, Kaibuchi, K., 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273, 245–248. doi:10.1126/science.273.5272.245

Kimura, T., Takemura, M., Nomura, S., Nobunaga, T., Kubota, Y., Inoue, T., Hashimoto, K., Kumazawa, I., Ito, Y., Ohashi, K., Koyama, M., Azuma, C., Kitamura, Y., Saji, F., 1996. Expression of oxytocin receptor in human pregnant myometrium. Endocrinology 137, 780–785. doi:10.1210/endo.137.2.8593830

Kirby, M.A., Heuerman, A.C., Custer, M., Dobyns, A.E., Strilaeff, R., Stutz, K.N., Cooperrider, J., Elsissy, J.G., Yellon, S.M., 2016. Progesterone Receptor-Mediated Actions Regulate Remodeling of the Cervix in Preparation for Preterm Parturition. Reprod. Sci. 23, 1473–1483. doi:10.1177/1933719116650756

Klein, T., Bischoff, R., 2011. Physiology and pathophysiology of matrix metalloproteases. Amino Acids 41, 271–290. doi:10.1007/s00726-010-0689-x

Kosoff, R.E., Aslan, J.E., Kostyak, J.C., Dulaimi, E., Chow, H.Y., Prudnikova, T.Y., Radu, M., Kunapuli, S.P., Mccarty, O.J.T., Chernoff, J., 2015. Pak2 restrains endomitosis during megakaryopoiesis and alters cytoskeleton organization. Blood 125, 2995–3006. doi:10.1182/blood-2014-10-604504.The

Krämer, A., Green, J., Pollard, J., Tugendreich, S., 2014. Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics 30, 523–30. doi:10.1093/bioinformatics/btt703

Kumar, N.M., Gilula, N.B., 1992. Molecular biology and genetics of gap junction channels. Semin. Cell Biol. 3, 3–16. doi:10.1016/S1043-4682(10)80003-0

Lackman, F., Capewell, V., Richardson, B., DaSilva, O., Gagnon, R., 2001. The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. Am. J. Obstet. Gynecol. 184, 946–953. doi:10.1067/mob.2001.111719

Laeter, J.R., 2001. Applications of Inorganic Mass Spectrometry, 1st ed. John Wiley & Sons, Inc., New York, New York.

Lambert, H., Charette, S.J., Bernier, A.F., Guimond, A., Landry, J., 1999. HSP27 multimerization mediated by phosphorylation-sensitive intermolecular interactions at the amino terminus. J Biol Chem 274, 9378–9385. doi:10.1074/jbc.274.14.9378

Lamont, R.F., 2000. The pathophysiology of pulmonary oedema with the use of beta-agonists. BJOG An Int. J. Obstet. Gynaecol. 107, 439–444. doi:10.1111/j.1471-0528.2000.tb13259.x

Lanman, J.T., 1977. Parturition in nonhuman Primates. Biol. Reprod. 16, 28–38.

doi:10.1093/biolreprod/16.1.28

Larsen, J.K., Yamboliev, I. a, Weber, L. a, Gerthoffer, W.T., 1997. Phosphorylation of the 27-kDa heat shock protein via p38 MAP kinase and MAPKAP kinase in smooth muscle. Am. J. Physiol. 273, L930–L940.

Leake, R.D., Weitzman, R.E., Glatz, T.H., Fisher, D.A., 1981. Plasma oxytocin concentrations in men, nonpregnant women, and pregnant women before and during spontaneous labor. J. Clin. Endocrinol. Metab. 53, 730–733. doi:10.1210/jcem-53-4-730

Lee, Y.-H., Shynlova, O., Lye, S.J., 2015. Stretch-induced human myometrial cytokines enhance immune cell recruitment via endothelial activation. Cell. Mol. Immunol. 12, 231–42. doi:10.1038/cmi.2014.39

Lefebvre, D.L., Piersanti, M., Bai, X.H., Chen, Z.Q., Lye, S.J., 1995. Myometrial transcriptional regulation of the gap junction gene, connexin-43. Reprod. Fertil. Dev. 7, 603–11. doi:10.1071/rd9950603

LeMaire, W.J., Conly, P.W., Moffett, A., Spellacy, W.N., Cleveland, W.W., Savard, K., 1971. Function of the human corpus luteum during the puerperium: its maintenance by exogenous human chorionic gonadotropin. Am. J. Obstet. Gynecol. 110, 612–8. doi:10.1016/0002-9378(71)90239-0

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079. doi:10.1093/bioinformatics/btp352

Li, Y., Je, H.-D., Malek, S., Morgan, K.G., 2004. Role of ERK1/2 in uterine contractility and preterm labor in rats. Am J Physiol Regul Integr Comp Physiol 287, R328–R335. doi:10.1152/ajpregu.00042.2004\n00042.2004 [pii]

Li, Y., Reznichenko, M., Tribe, R.M., Hess, P.E., Taggart, M., Kim, H.R., DeGnore, J.P., Gangopadhyay, S., Morgan, K.G., 2009. Stretch activates human myometrium via ERK, caldesmon and focal adhesion signaling. PLoS One 4, e7489. doi:10.1371/journal.pone.0007489

Liao, Y., Smyth, G.K., Shi, W., 2014. FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930. doi:10.1093/bioinformatics/btt656

Lin, A., Minden, A., Martinetto, H., Claret, F.X., Lange-Carter, C., Mercurio, F., Johnson, G.L., Karin, M., 1995. Identification of a dual specificity kinase that activates the jun kinases and p38-Mpk2. Science (80-.). 268, 286–290. doi:10.1126/science.7716521

Liu, Y., Beyer, A., Aebersold, R., 2016. On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell 165, 535–550. doi:10.1016/j.cell.2016.03.014

Locatelli, A., Consonni, S., Ghidini, A., 2015. Preterm Labor: Approach to Decreasing Complications of Prematurity. Obstet. Gynecol. Clin. North Am. 42, 255–274.

doi:10.1016/j.ogc.2015.01.004

Lockwood, C.J., Kuczynski, E., 2001. Risk stratification and pathological mechanisms in preterm delivery. Paediatr. Perinat. Epidemiol. 15 Suppl 2, 78–89.

López Bernal, A., Watson, S.P., Phaneuf, S., Europe-Finner, G.N., 1993. Biochemistry and physiology of preterm labour and delivery. Baillieres. Clin. Obstet. Gynaecol. 7, 523–52.

Loudon, J.A.Z., Groom, K.M., Bennett, P.R., 2003. Prostaglandin inhibitors in preterm labour. Best Pract. Res. Clin. Obstet. Gynaecol. 17, 731–744. doi:10.1016/S1521-6934(03)00047-6

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 1–34. doi:Artn 550\rDoi 10.1186/S13059-014-0550-8

Lyndon, A., 2013. Preterm Labor and Birth. J. Perinat. Neonatal Nurs. 20, 82–84. doi:10.1097/00005237-200601000-00024

MacIntyre, D. a., Tyson, E.K., Read, M., Smith, R., Yeo, G., Kwek, K., Chan, E.C., 2008. Contraction in human myometrium is associated with changes in small heat shock proteins. Endocrinology 149, 245–252. doi:10.1210/en.2007-0662

Macphee, D.J., Lye, S.J., 2000. Focal adhesion signaling in the rat myometrium is abruptly terminated with the onset of labor. Endocrinology 141, 274–283.

Mallick, P., Kuster, B., 2010. Proteomics: A pragmatic perspective. Nat. Biotechnol. 28, 695–709. doi:10.1038/nbt.1658

Manabe, Y., Mori, T., Yoshida, Y., 1984. Decidual morphology and F prostaglandin in amniotic fluid in stretch-induced abortion. Obstet. Gynecol. 64, 661–665.

Manicone, A., McGuire, J., 2008. Matrix metalloproteinases as modulators of inflammation. Semin. Cell Dev. Biol. 19, 34–41. doi:10.1016/j.semcdb.2007.07.003

Mann, M., Højrup, P., Roepstorff, P., 1993. Use of mass spectrometric molecular weight information to identify proteins in sequence databases. Biol. Mass Spectrom. 22, 338–45. doi:10.1002/bms.1200220605

Marlow, N., Wolke, D., Bracewell, M., Samara, M., 2005. Neurologic and Developmental Disability at Six Years of Age after Extremely Preterm Birth. N. Engl. J. Med. 352, 9–19.

Marshall, A.G. (Alan G., Verdun, F.R., 1989. Fourier transforms in NMR, optical, and mass spectrometry : a user's handbook. Elsevier.

Marshall, A.G., Hendrickson, C.L., Jackson, G.S., 1998. Fourier transform ion cyclotron resonance mass spectrometry: A primer. Mass Spectrom. Rev. 17, 1–35. doi:10.1002/(SICI)1098-2787(1998)17:1<1::AID-MAS1>3.0.CO;2-K

Maymon, E., Romero, R., Pacora, P., Gervasi, M.T., Gomez, R., Edwin, S.S., Yoon, B.H., 2000. Evidence of in vivo differential bioavailability of the active forms of matrix metalloproteinases 9 and 2 in parturition, spontaneous rupture of membranes, and intra-

amniotic infection. Am. J. Obstet. Gynecol. 183, 887–894. doi:10.1067/mob.2000.108878

McAlister, G.C., Huttlin, E.L., Haas, W., Ting, L., Jedrychowski, M.P., Rogers, J.C., Kuhn, K., Pike, I., Grothe, R.A., Blethrow, J.D., Gygi, S.P., 2012. Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses. Anal. Chem. 84, 7469–7478. doi:10.1021/ac301572t

McQuibban, G.A., Butler, G.S., Gong, J.H., Bendall, L., Power, C., Clark-Lewis, I., Overall, C.M., 2001. Matrix Metalloproteinase Activity Inactivates the CXC Chemokine Stromal Cell-derived Factor-1. J. Biol. Chem. 276, 43503–43508. doi:10.1074/jbc.M107736200

Melin, P., 1994. Development of an oxytocin antagonist atosiban. Res Clin Forums 16, 155–168.

Menon, R., 2008. Spontaneous preterm birth, a clinical dilemma: etiologic, pathophysiologic and genetic heterogeneities and racial disparity. Acta Obstet. Gynecol. Scand. 87, 590–600. doi:791847679 [pii]10.1080/00016340802005126

Mercer, B.M., Goldenberg, R.L., Das, A., Moawad, A.H., Iams, J.D., Meis, P.J., Copper, R.L., Johnson, F., Thom, E., McNellis, D., Miodovnik, M., Menard, M.K., Caritis, S.N., Thurnau, G.R., Bottoms, S.F., Roberts, J., 1996. The preterm prediction study: a clinical risk assessment system. Am. J. Obstet. Gynecol. 174, 1885.

Mertins, P., Udeshi, N.D., Clauser, K.R., Mani, D.R., Patel, J., Ong, S.E., Jaffe, J.D., Carr, S.A., 2012. iTRAQ labeling is superior to mTRAQ for quantitative global proteomics and phosphoproteomics. Mol. Cell. Proteomics 11, 1–12. doi:10.1074/mcp.M111.014423

Mitchell, J.A., Lye, S.J., 2001. Regulation of connexin43 expression by c-fos and c-jun in myometrial cells. Cell Commun. Adhes. 8, 299–302.

Miyazawa, K., Mori, A., Miyata, H., Akahane, M., Ajisawa, Y., Okudaira, H., 1998. Regulation of interleukin-1beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. J. Biol. Chem. 273, 24832–8. doi:10.1074/jbc.273.38.24832

Mohan, M.J., Seaton, T., Mitchell, J., Howe, A., Blackburn, K., Burkhart, W., Moyer, M., Patel, I., Waitt, G.M., Becherer, J.D., Moss, M.L., Milla, M.E., 2002. The tumor necrosis factor-α converting enzyme (TACE): A unique metalloproteinase with highly defined substrate selectivity. Biochemistry 41, 9462–9469. doi:10.1021/bi0260132

Montfort, W.R., Wales, J.A., Weichsel, A., 2017. Structure and Activation of Soluble Guanylyl Cyclase, the Nitric Oxide Sensor. Antioxidants Redox Signal. 26, 107–121. doi:10.1089/ars.2016.6693

Moreno-Domínguez, A., El-Yazbi, A.F., Zhu, H.L., Colinas, O., Zhong, X.Z., Walsh, E.J., Cole, D.M., Kargacin, G.J., Walsh, M.P., Cole, W.C., 2014. Cytoskeletal reorganization evoked by Rho-associated kinase- and protein kinase C-catalyzed phosphorylation of cofilin and heat shock protein 27, respectively, contributes to myogenic constriction of rat cerebral arteries. J. Biol. Chem. 289, 20939–20952. doi:10.1074/jbc.M114.553743

Morgan, K.G., 2014. The importance of the smooth muscle cytoskeleton to preterm labour. Exp. Physiol. 99, 525–9. doi:10.1113/expphysiol.2013.072876

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628. doi:10.1038/nmeth.1226

Mosher, A.A., Rainey, K.J., Bolstad, S.S., Lye, S.J., Mitchell, B.F., Olson, D.M., Wood, S.L., Slater, D.M., 2013. Development and validation of primary human myometrial cell culture models to study pregnancy and labour. BMC Pregnancy Childbirth 13, 1–14. doi:10.1186/1471-2393-13-S1-S7

Murphy, G., Ward, R., Hembry, R.M., Reynolds, J.J., Kuhn, K., Tryggvason, K., 1989. Characterization of gelatinase from pig polymorphonuclear leucocytes. A metalloproteinase resembling tumour type IV collagenase. Biochem. J. 258, 463–472. doi:10.1042/bj2580463

Myatt, L., Eschenbach, D.A., Lye, S.J., Mesiano, S., Murtha, A.P., Williams, S.M., Pennell, C.E., 2012. A standardized template for clinical studies in preterm birth. Reprod. Sci. 19, 474–82.

Neilson, J.P., West, H.M., Dowswell, T., 2014. Betamimetics for inhibiting preterm labour. Cochrane Database Syst. Rev. 2014. doi:10.1002/14651858.CD004352.pub3

Neininger, A., Kontoyiannis, D., Kotlyarov, A., Winzen, R., Eckert, R., Volk, H.D., Holtmann, H., Kollias, G., Gaestel, M., 2002. MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. J. Biol. Chem. 277, 3065–3068. doi:10.1074/jbc.C100685200

Nguyen, Tina T.-T. Ngoc, Shynlova, O., Lye, S.J., 2016. Matrix Metalloproteinase Expression in the Rat Myometrium During Pregnancy, Term Labor, and Postpartum. Biol. Reprod. 95, 1–14. doi:10.1095/biolreprod.115.138248

Nguyen, T. T.-T. N., Shynlova, O., Lye, S.J., 2016. Matrix Metalloproteinase Expression in the Rat Myometrium During Pregnancy, Term Labor, and Postpartum. Biol. Reprod. 95, 24–24. doi:10.1095/biolreprod.115.138248

Nissinen, L., Kähäri, V., 2014. Matrix metalloproteinases in inflammation. Biochim. Biophys. Acta 1840, 2571–2580. doi:10.1016/j.bbagen.2014.03.007

Noordin, R., Othman, N., 2013. Proteomics technology - a powerful tool for the biomedical scientists. Malays. J. Med. Sci. 20, 1–2.

Norman, J., 1996. Nitric oxide and the myometrium. Pharmacol.Ther. 70, 91–100.

Nott, J.P., Bonney, E.A., Pickering, J.D., Simpson, N.A.B., 2016. The structure and function of the cervix during pregnancy. Transl. Res. Anat. 2, 1–7. doi:10.1016/j.tria.2016.02.001

O'Brien, W.F., 1995. The role of prostaglandins in labor and delivery. Clin. Perinatol. 22, 973–84.

O'Connor, A.R., Wilson, C.M., Fielder, A.R., 2007. Ophthalmological problems associated with preterm birth. Eye 21, 1254–1260. doi:10.1038/sj.eye.6702838

Oldenhof, A., Shynlova, O., Liu, M., Langille, B., Lye, S., 2002. Mitogen-activated protein kinases mediate stretch-induced c-fos mRNA expression in myometrial smooth muscle cells. Am J Physiol Cell Physiol 283, C1530--C1539. doi:10.1152/ajpcell.00607.2001

Oldenhof, A.D., Shynlova, O.P., Liu, M., Langille, B.L., Lye, S.J., 2002. Mitogen-activated protein kinases mediate stretch-induced c-fos mRNA expression in myometrial smooth muscle cells. Am. J. Physiol. Physiol. 283, C1530–C1539.

Olsen, J. V, Macek, B., Lange, O., Makarov, A., Horning, S., Mann, M., 2007. Higher-energy C-trap dissociation for peptide modification analysis. Nat. Methods 4, 709–712. doi:10.1038/nmeth1060

Ou, C.W., Orsino, A., Lye, S.J., 1997. Expression of connexin-43 and connexin-26 in the rat myometrium during pregnancy and labor is differentially regulated by mechanical and hormonal signals. Endocrinology 138, 5398–5407.

Park, J.S., Park, C.W., Lockwood, C.J., Norwitz, E.R., 2005. Role of cytokines in preterm labor and birth. Minerva Ginecol. 57, 349–366.

Parry, L.J., Bathgate, R.A., 2000. The role of oxytocin and regulation of uterine oxytocin receptors in pregnant marsupials. Exp. Physiol. 85, 91S-99S.

Peng, B., Zhu, H., Klausen, C., Ma, L., Wang, Y.L., Leung, P.C.K., 2015. GnRH regulates trophoblast invasion via RUNX2-mediated MMP2/9 expression. Mol. Hum. Reprod. 22, 119–129. doi:10.1093/molehr/gav070

Phillips, J.B., Abbot, P., Rokas, A., 2015. Is preterm birth a human-specific syndrome? Evol. Med. Public Heal. 2015, 136–148. doi:10.1093/emph/eov010

PMNCH [WWW Document], 2016. . WHO. URL https://www.who.int/pmnch/knowledge/publications/preterm_birth_report/en/index3.html (accessed 8.6.19).

Putnam, C.D., Brann, D.W., Kolbeck, R.C., Mahesh, V.B., 1991. Inhibition of Uterine Contractility by Progesterone and Progesterone Metabolites: Mediation by Progesterone and Gamma Amino Butyric AcidA Receptor Systems. Biol. Reprod. 45, 266–272. doi:10.1095/biolreprod45.2.266

Raingeaud, J., Whitmarsh, A.J., Barrett, T., Dérijard, B., Davis, R.J., 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol. Cell. Biol. 16, 1247–1255. doi:10.1128/mcb.16.3.1247

Ramanathan, R., Zhong, R., Blumenkrantz, N., Chowdhury, S.K., Alton, K.B., 2007. Response Normalized Liquid Chromatography Nanospray Ionization Mass Spectrometry. J. Am. Soc. Mass Spectrom. 18, 1891–1899. doi:10.1016/j.jasms.2007.07.022

Ramsey, P.S., Rouse, D.J., 2001. Magnesium sulfate as a tocolytic agent. Semin. Perinatol. 25,

236–247. doi:10.1053/sper.2001.27546

Rauscher, F.J., Voulalas, P.J., Franza, B.R., Curran, T., 1988. Fos and Jun bind cooperatively to the AP-1 site: reconstitution in vitro. Genes Dev. 2, 1687–1699.

Rebarber, A., Carreno, C.A., Lipkind, H., Funai, E.F., Maturi, J., Kuczynski, E., Lockwood, C., 2001. Cervical length after multifetal pregnancy reduction in remaining twin gestations. Am J Obs. Gynecol 185, 1113–1117. doi:S0002-9378(01)41205-1 [pii]10.1067/mob.2001.117683

Reinebrant, H.E., Pileggi-Castro, C., Romero, C.L.T., dos Santos, R.A.N., Kumar, S., Souza, J.P., Flenady, V., 2015. Cyclo-oxygenase (COX) inhibitors for treating preterm labour. Cochrane Database Syst. Rev. 2015. doi:10.1002/14651858.CD001992.pub3

Reinhart, R.A., 1991. Clinical correlates of the molecular and cellular actions of magnesium on the cardiovascular system. Am. Heart J. 121, 1513–1521. doi:10.1016/0002-8703(91)90160-J

Riley, W.J., 2012. Health disparities: gaps in access, quality and affordability of medical care. Trans. Am. Clin. Climatol. Assoc. 123, 167–174.

Roberts, D., Brown, J., Medley, N., Dalziel, S.R., 2017. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane Database Syst. Rev. 2017. doi:10.1002/14651858.CD004454.pub3

Romero, R., Espinoza, J., Gonçalves, L., Kusanovic, J., Friel, L., Hassan, S., 2007. The Role of Inflammation and Infection in Preterm Birth. Semin. Reprod. Med. 25, 021–039. doi:10.1055/s-2006-956773

Rosenfeld, J., Capdevielle, J., Guillemot, J.C., Ferrara, P., 1992. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal. Biochem. 203, 173–9. doi:10.1016/0003-2697(92)90061-b

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., Nebreda, A.R., 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78, 1027–1037. doi:10.1016/0092-8674(94)90277-1

Rusu, O., Lupan, C., Baltescu, V., 1966. Magneziul seric in sarcina normala la termen si nasterea permatura. Rolul maneziterapiei in combatera nasterii premature. Obstet. Si Ginecol. 14, 215–224.

Saddouk, F.Z., Ginnan, R., Singer, H.A., 2017. Ca2 +/Calmodulin-Dependent Protein Kinase II in Vascular Smooth Muscle. Adv. Pharmacol. 78, 171–202. doi:10.1016/bs.apha.2016.08.003

Sandow, A., 1970. Skeletal Muscle. Annu. Rev. Physiol. 32, 87–138.

Schaller, M.D., Hildebrand, J.D., Shannon, J.D., Fox, J.W., Vines, R.R., Parsons, J.T., 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. Mol. Cell. Biol. 14, 1680–1688. doi:10.1128/MCB.14.3.1680.Updated

Scheid, C.R., Honeyman, T.W., Fay, F.S., 1979. Mechanism of B-adrenergic relaxation of smooth muscle. Nature 277, 32–36.

Schönbeck, U., Mach, F., Libby, P., 1998. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J. Immunol. 161, 3340–6.

Sellers, J.R., Pato, M.D., Adelstein, R.S., 1981. Reversible phosphorylation of smooth muscle myosin, heavy meromyosin, and platelet myosin. J. Biol. Chem. 256, 13137–13142.

Sennstrom, M.B., Ekman, G., Westergren-Thorsson, G., Malmström, A., Byström, B., Endresen, U., Mlambo, N., Norman, M., Stabi, B., Brauner, A., 2002. Human cervical ripening, an inflammatory process mediated by cytokines. Mol. Hum. Reprod. 6, 375–381. doi:10.1093/molehr/6.4.375

Sharma, K., D'Souza, R.C.J., Tyanova, S., Schaab, C., Wiśniewski, J.R., Cox, J., Mann, M., 2014. Ultradeep Human Phosphoproteome Reveals a Distinct Regulatory Nature of Tyr and Ser/Thr-Based Signaling. Cell Rep. 8, 1583–1594. doi:10.1016/j.celrep.2014.07.036

Sheldon, R.E., Mashayamombe, C., Shi, S.Q., Garfield, R.E., Shmygol, A., Blanks, A.M., Van Den Berg, H.A., 2014. Alterations in gap junction connexin43/connexin45 ratio mediate a transition from quiescence to excitation in a mathematical model of the myometrium. J. R. Soc. Interface 11. doi:10.1098/rsif.2014.0726

Shynlova, O., Lee, Y.H., Srikhajon, K., Lye, S.J., 2013. Physiologic uterine inflammation and labor onset: integration of endocrine and mechanical signals. Reprod. Sci. 20, 154–167. doi:10.1177/1933719112446084

Shynlova, O., Tsui, P., Jaffer, S., Lye, S.J., 2009. Integration of endocrine and mechanical signals in the regulation of myometrial functions during pregnancy and labour. Eur. J. Obstet. Gynecol. Reprod. Biol. 144 Suppl, S2-10. doi:10.1016/j.ejogrb.2009.02.044

Sims, D., Sudbery, I., Ilott, N.E., Heger, A., Ponting, C.P., 2014. Sequencing depth and coverage: key considerations in genomic analyses. Nat. Rev. Genet. 15, 121–32. doi:10.1038/nrg3642

Singer, C. a, Baker, K.J., McCaffrey, A., AuCoin, D.P., Dechert, M. a, Gerthoffer, W.T., 2003. p38 MAPK and NF-kappaB mediate COX-2 expression in human airway myocytes. Am. J. Physiol. Lung Cell. Mol. Physiol. 285, L1087–L1098. doi:10.1152/ajplung.00409.2002

Sivarajasingam, S.P., Imami, N., Johnson, M.R., 2016a. Cytokines and Myometrial Signalling in Human Labour. J. Endocrinol. 231, R101–R119. doi:10.1530/JOE-16-0157

Sivarajasingam, S.P., Imami, N., Johnson, M.R., 2016b. Myometrial cytokines and their role in the onset of labour. J. Endocrinol. 231, R101–R119. doi:10.1530/JOE-16-0157

Smith, I., Silveirinha, V., Stein, J.L., Torre-ubieta, L. De, Farrimond, J.A., Williamson, E.M., Whalley, B.J., 2017. Human neural stem cell-derived cultures in three- dimensional substrates form spontaneously functional neuronal networks. J Tissue Eng Regen Med 11, 1022–1033. doi:10.1002/term.2001

Sokolowski, P., Saison, F., Giles, W., McGrath, S., Smith, D., Smith, J., Smith, R., 2010. Human uterine wall tension trajectories and the onset of parturition. PLoS One 5, e11037. doi:10.1371/journal.pone.0011037

Somlyo, A.P., Somlyo, A. V, 2003. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. 83, 1325–1358. doi:10.1152/physrev.00023.2003

Sooranna, S.R., Engineer, N., Loudon, J.A.Z., Terzidou, V., Bennett, P.R., Johnson, M.R., 2005a. The mitogen-activated protein kinase dependent expression of prostaglandin H synthase-2 and interleukin-8 messenger ribonucleic acid by myometrial cells: The differential effect of stretch and interleukin-1β. J. Clin. Endocrinol. Metab. 90, 3517–3527. doi:10.1210/jc.2004-1390

Sooranna, S.R., Engineer, N., Loudon, J.A.Z., Terzidou, V., Bennett, P.R., Johnson, M.R., 2005b. The mitogen-activated protein kinase dependent expression of prostaglandin H synthase-2 and interleukin-8 messenger ribonucleic acid by myometrial cells: The differential effect of stretch and interleukin-1?? J. Clin. Endocrinol. Metab. 90, 3517–3527. doi:10.1210/jc.2004-1390

Sooranna, S.R., Lee, Y., Kim, L.U., Mohan, A.R., Bennett, P.R., Johnson, M.R., 2004. Mechanical stretch activates type 2 cyclooxygenase via activator protein-1 transcription factor in human myometrial cells. Mol. Hum. Reprod. 10, 109–113. doi:10.1093/molehr/gah021

Souza, G.R., Tseng, H., Gage, J.A., Mani, A., Desai, P., Leonard, F., Liao, A., Longo, M., Refuerzo, J.S., Godin, B., 2017. Magnetically bioprinted human myometrial 3D cell rings as a model for uterine contractility. Int. J. Mol. Sci. 18, 1–10. doi:10.3390/ijms18040683

Spach, M.S., Kootsey, J.M., 1983. The nature of electrical propagation in cardiac muscle. Am. J. Physiol. - Hear. Circ. Physiol. 13.

Stygar, D, Wang, H., Vladic, Y.S., Ekman, G., Eriksson, H., Sahlin, L., 2002. Increased level of matrix metalloproteinases 2 and 9 in the ripening process of the human cervix. Biol. Reprod. 67, 889–894.

Stygar, Denis, Wang, H., Vladic, Y.S., Ekman, G., Eriksson, H., Sahlin, L., 2002. Increased Level of Matrix Metalloproteinases 2 and 9 in the Ripening Process of the Human Cervix1. Biol. Reprod. 67, 889–894. doi:10.1095/biolreprod.102.005116

Surks, H.K., Mochizuki, N., Kasai, Y., Georgescu, S.P., Tang, K.M., Ito, M., Lincoln, T.M., Mendelsohn, M.E., 1999. Regulation of myosin phosphatase by a specific interaction with cGMP- dependent protein kinase Iα. Science (80-.). 286, 1583–1587. doi:10.1126/science.286.5444.1583

Tang, D.D., Gerlach, B.D., 2017. The roles and regulation of the actin cytoskeleton , intermediate filaments and microtubules in smooth muscle cell migration. Respir. Res. 18, 1–12. doi:10.1186/s12931-017-0544-7

Tang, M.X., Hu, X.H., Liu, Z.Z., Kwak-Kim, J., Liao, A.H., 2015. What are the roles of macrophages

and monocytes in human pregnancy? J. Reprod. Immunol. 112, 73–80. doi:10.1016/j.jri.2015.08.001

Tang, Y.Y., Du, Y., Ni, J., Ma, Y.S., Lin, X.M., Zhou, J., 2014. Relaxant effects of metoclopramide and magnesium sulfate on isolated pregnant myometrium: An in vitro study. Int. J. Obstet. Anesth. 23, 131–137. doi:10.1016/j.ijoa.2013.11.004

Tarca, A.L., Draghici, S., Khatri, P., Hassan, S.S., Mittal, P., Kim, J.-S., Kim, C.J., Kusanovic, J.P., Romero, R., 2009. A novel signaling pathway impact analysis. Bioinformatics 25, 75–82. doi:10.1093/bioinformatics/btn577

Thermo Fisher Scientific, 2008. LTQ Orbitrap XL ETD ™.

Thomas, P.D., Campbell, M.J., Kejariwal, A., Mi, H., Karlak, B., 2003. PANTHER: A Library of Protein Families and Subfamilies Indexed by Function. Genome Res. 13, 2129–2141. doi:10.1101/gr.772403

Thomson, J., 1914. Rays of Positive Electricity and their Application to Chemical Analysis. Nature 92, 549–550. doi:10.1038/092549a0

Thornton, S., Goodwin, T.M., Greisen, G., Hedegaard, M., Arce, J.C., 2009. The effect of barusiban, a selective oxytocin antagonist, in threatened preterm labor at late gestational age: a randomized, double-blind, placebo-controlled trial. Am. J. Obstet. Gynecol. 200, 627.e1-627.e10. doi:10.1016/j.ajog.2009.01.015

Tock, J., Van Putten, V., Stenmark, K.R., Nemenoff, R.A., 2003. Induction of SM-α-actin expression by mechanical strain in adult vascular smooth muscle cells is mediated through activation of JNK and p38 MAP kinase. Biochem. Biophys. Res. Commun. 301, 1116–1121. doi:10.1016/S0006-291X(03)00087-1

Tomar, A., Schlaepfer, D.D., 2009. Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. Curr Opin Cell Biol. 21, 676–683. doi:10.1016/j.ceb.2009.05.006.Focal

Touyz, R.M., Alves-Lopes, R., Rios, F.J., Camargo, L.L., Anagnostopoulou, A., Arner, A., Montezano, A.C., 2018. Vascular smooth muscle contraction in hypertension. Cardiovasc. Res. 114, 529–539. doi:10.1093/cvr/cvy023

Tulchinsky, D., Hobel, C.J., Yeager, E., Marshall, J.R., 1972. Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. Am. J. Obstet. Gynecol. 112, 1095–1100. doi:10.1016/0002-9378(72)90185-8

Ulrich, C., Quilici, D.R., Schlauch, K.A., Buxton, I.L., 2013. LC/MS/MS data analysis of the human uterine smooth muscle S-nitrosoproteome fingerprint in pregnancy, labor, and preterm labor. Am J Physiol Cell Physiol 305, C803-16. doi:10.1152/ajpcell.00198.2013

Ulrich, C.C., Arinze, V., Wandscheer, C.B., Copley Salem, C., Nabati, C., Etezadi-Amoli, N., Burkin, H.R., 2019. Matrix metalloproteinases 2 and 9 are elevated in human preterm laboring uterine myometrium and exacerbate uterine contractility†. Biol. Reprod. 100, 1597–1604.

doi:10.1093/biolre/ioz054

Uvnäs-Moberg, K., Ekström-Bergström, A., Berg, M., Buckley, S., Pajalic, Z., Hadjigeorgiou, E., Kotłowska, A., Lengler, L., Kielbratowska, B., Leon-Larios, F., Magistretti, C.M., Downe, S., Lindström, B., Dencker, A., 2019. Maternal plasma levels of oxytocin during physiological childbirth – a systematic review with implications for uterine contractions and central actions of oxytocin. BMC Pregnancy Childbirth 19, 1–17. doi:10.1186/s12884-019-2365-9

Van Den Steen, P.E., Wuyts, A., Husson, S.J., Proost, P., Van Damme, J., Opdenakker, G., 2003. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. Eur. J. Biochem. 270, 3739–3749. doi:10.1046/j.1432-1033.2003.03760.x

Vrachnis, N., Malamas, F.M., Sifakis, S., Tsikouras, P., Iliodromiti, Z., 2012. Immune aspects and myometrial actions of progesterone and CRH in labor. Clin. Dev. Immunol. 2012. doi:10.1155/2012/937618

Waldorf, K.M.A., Singh, N., Mohan, A.R., Young, R.C., Ngo, L., Das, A., Tsai, J., Bansal, A., Paolella, L., Herbert, B.R., Sooranna, S.R., Gough, G.M., Astley, C., Vogel, K., Baldessari, A.E., Bammler, T.K., MacDonald, J., Gravett, M.G., Rajagopal, L., Johnson, M.R., 2015. Uterine overdistention induces preterm labor mediated by inflammation: Observations in pregnant women and nonhuman primates. Am. J. Obstet. Gynecol. 213, e1–e19. doi:10.1016/j.ajog.2015.08.028

Wang, Y., Ghaffari, N., Johnson, C.D., Braga-Neto, U.M., Wang, H., Chen, R., Zhou, H., 2011. Evaluation of the coverage and depth of transcriptome by RNA-Seq in chickens. BMC Bioinformatics 12, S5. doi:10.1186/1471-2105-12-S10-S5

Webb, R.C., 2003. Smooth muscle contraction and relaxation. Am. J. Physiol. - Adv. Physiol. Educ. 27, 201–206.

Willard, H.H., L. L. Merritt, J., Dean, J.A., 1998. Instrumental Methods of Analysis, 7th ed. Wadsworth Publishing Co, Belmont, California.

Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C.Y.A., Shyu, A. Bin, Müller, M., Gaestel, M., Resch, K., Holtmann, H., 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. EMBO J. 18, 4969–4980. doi:10.1093/emboj/18.18.4969

Wolff, M.M., Stephens, W.E., 1953. A Pulsed Mass Spectrometer with Time Dispersion. Rev. Sci. Instrum. 24, 616–617. doi:10.1063/1.1770801

Wray, S., 1993. Uterine contraction and physiological mechanisms of modulation. Am. J. Physiol. 264, 53–54. doi:10.1016/S0140-6736(01)00669-9

Wray, S., Burdyga, T., Noble, D., Noble, K., Borysova, L., Arrowsmith, S., 2015. Progress in understanding electro-mechanical signalling in the myometrium. Acta Physiol. 213, 417–431. doi:10.1111/apha.12431

Wu, W.X., Ma, X.H., Yoshizato, T., Shinozuka, N., Nathanielsz, P.W., 1999. Differential expression of myometrial oxytocin receptor and prostaglandin H synthase 2, but not estrogen receptor α and heat shock protein 90 messenger ribonucleic acid in the gravid horn and nongravid horn in sheep during betamethasone-induced labor. Endocrinology 140, 5712–5718. doi:10.1210/endo.140.12.7201

Wu, X., Morgan, K.G., Jones, C.J., Tribe, R.M., Taggart, M.J., 2008. Myometrial mechanoadaptation during pregnancy: Implications for smooth muscle plasticity and remodelling. J. Cell. Mol. Med. 12, 1360–1373. doi:10.1111/j.1582-4934.2008.00306.x

Yang, Z., Wu, B., Jia, S., Zhao, Y., Hou, R., Liu, X., Wang, X., Chen, L., Yang, X., Lei, D., Wang, L., 2017. The mechanically activated p38/MMP-2 signaling pathway promotes bone marrow mesenchymal stem cell migration in rats. Arch. Oral Biol. 76, 55–60. doi:10.1016/j.archoralbio.2017.01.017

Yoshida, Y., Manabe, Y., 1988. Stretch-induced delivery is independent of the functional fetal role and dysfunction of the amnion and decidua: A morphologic and enzyme cytochemical study. Am. J. Obstet. Gynecol. 159, 1293–1298. doi:10.1016/0002-9378(88)90466-8

Young, A., Thomson, A.J., Ledingham, M., Jordan, F., Greer, I.A., Norman, J.E., 2002. Immunolocalization of Proinflammatory Cytokines in Myometrium, Cervix, and Fetal Membranes During Human Parturition at Term1. Biol. Reprod. 66, 445–449. doi:10.1095/biolreprod66.2.445

Yulia, A., Johnson, M.R., 2014. Myometrial oxytocin receptor expression and intracellular pathways. Minerva Ginecol. 66, 267–80.

Yulia, A., Singh, N., Lei, K., Sooranna, S.R., Johnson, M.R., 2016. Cyclic AMP effectors regulate myometrial oxytocin receptor expression. Endocrinology 157, 4411–4422. doi:10.1210/en.2016-1514

Zarei, M., Sprenger, A., Metzger, F., Gretzmeier, C., Dengjel, J., 2011. Comparison of ERLIC-TiO(2), HILIC-TiO(2), and SCX-TiO(2) for Global Phosphoproteomics Approaches. J. Proteome Res. 10, 3474–3483. doi:10.1021/pr200092z

Zhang, D., Shadrin, I., Lam, J., Xian, H.-Q., Snodgrass, R., Bursac, N., 2013. Tissue-engineered Cardiac Patch for Advanced Functional Maturation of Human ESC-derived Cardiomyocytes. Biomaterials 34, 5813–5820.

Zhang, L., Ma, P., Guan, Q., Meng, L., Su, L., Wang, L., Zhao, J., Ji, S., 2018. Protein phosphatase 2A regulates the p38 signaling pathway to affect the migration of astrocytes. Mol. Med. Rep. 18, 4328–4334. doi:10.3892/mmr.2018.9425

Zhang, Y. nan, Xie, B. dong, Sun, L., Chen, W., Jiang, S.L., Liu, W., Bian, F., Tian, H., Li, R.K., 2016. Phenotypic switching of vascular smooth muscle cells in the “normal region” of aorta from atherosclerosis patients is regulated by miR-145. J. Cell. Mol. Med. 20, 1049–1061. doi:10.1111/jcmm.12825

Zubarev, R.A., Makarov, A., 2013. Orbitrap mass spectrometry. Anal. Chem. 85, 5288–5296.

doi:10.1021/ac4001223