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The role and regulation of small conductance CA2+ activated K+ channel subtype 3 in myometrial contraction and placental development

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THE ROLE AND REGULATION OF SMALL CONDUCTANCE CA^{2+} ACTIVATED K^+ CHANNEL SUBTYPE 3 IN MYOMETRIAL CONTRACTION AND PLACENTAL DEVELOPMENT

by

Stephanie Lynn Pierce

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Molecular Physiology and Biophysics
in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Professor Sarah K. England

ABSTRACT

SK3 channels contribute to membrane repolarization and hyperpolarization that leads to both relaxation of smooth muscle and vascular branching. These two distinct properties are intensified in the $SK3^{T/T}$ mice possibly influencing pregnancy by dampening uterine contractions and causing dysfunctional placental development. $SK3^{T/T}$ mice have delayed or hindered parturition, suggesting a role for SK3 channels in labor contractions (Chapter 2 & 3). Based on these findings, we hypothesized that SK3 channel expression must be reduced late in normal pregnancy to enable the uterus to produce the forceful contractions required for parturition. The mechanism(s) down-regulating this channel in the uterus during pregnancy is unknown. The SK3 gene promoter region contains two Specificity Protein (Sp) binding sites; Sp1, a transcription factor that enhances transcription of genes in response to estrogen, and Sp3, a factor that competes for the same binding motif as Sp1 to reduce gene expression (Chapter 4). SK3 channels may also be involved in the vascular remodeling that occurs during pregnancy. The SK3 channel is present in vascular endothelial cells and overexpression of this channel leads to abnormal vessel branching and an increase in vessel diameter. During pregnancy, the vascular system must adapt to accommodate dramatic increases in blood volume necessary to sustain the developing fetus. Overexpression of SK3 channels could produce abnormalities in the placental vascular network, similar to the abnormal vessel branching and vasodilatation found in the mesenteric circulation, thus leading to poor fetal outcome (Chapter 5). The aim of this research was to determine the function of the SK3 channel in pregnancy by focusing on its role in myometrial contractility in addition to identifying its role in remodeling the maternal vasculature and its impact on placental blood flow and fetal demise.

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Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

Stephanie Lynn Pierce

has been approved by the Examining Committee
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To Dad, for instilling in me the love of biology at a young age. I can only imagine the discussions we would have now. Because of your sacrifices, you gave me the opportunity to accomplish anything.

"When I had that (super-intelligence) there was too much pressure to use it. All I want out of life is to be a monkey of moderate intelligence who wears a suit. That's why I've decided to transfer to business school!"

Gunther Futurama

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SK3 channels contribute to membrane repolarization and hyperpolarization that leads to both relaxation of smooth muscle and vascular branching. These two distinct properties are intensified in the $SK3^{T/T}$ mice possibly influencing pregnancy by dampening uterine contractions and causing dysfunctional placental development. $SK3^{T/T}$ mice have delayed or hindered parturition, suggesting a role for SK3 channels in labor contractions (Chapter 2 & 3). Based on these findings, we hypothesized that SK3 channel expression must be reduced late in normal pregnancy to enable the uterus to produce the forceful contractions required for parturition. The mechanism(s) down-regulating this channel in the uterus during pregnancy is unknown. The SK3 gene promoter region contains two Specificity Protein (Sp) binding sites; Sp1, a transcription factor that enhances transcription of genes in response to estrogen, and Sp3, a factor that competes for the same binding motif as Sp1 to reduce gene expression (Chapter 4). SK3 channels may also be involved in the vascular remodeling that occurs during pregnancy. The SK3 channel is present in vascular endothelial cells and overexpression of this channel leads to abnormal vessel branching and an increase in vessel diameter. During pregnancy, the vascular system must adapt to accommodate dramatic increases in blood volume necessary to sustain the developing fetus. Overexpression of SK3 channels could produce abnormalities in the placental vascular network, similar to the abnormal vessel branching and vasodilatation found in the mesenteric circulation, thus leading to poor fetal outcome (Chapter 5). The aim of this research was to determine the function of the SK3 channel in pregnancy by focusing on its role in myometrial contractility in addition to identifying its role in remodeling the maternal vasculature and its impact on placental blood flow and fetal demise.

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LIST OF ABBREVIATIONS

ACTH - corticotropin

ATP - adenosine triphosphate

BK channel - large conductance calcium activated potassium channel

cAMP - cyclic adenosine monophosphate

CBD - calmodulin binding domain

CRE - cAMP response elements

CRH - corticotropin releasing hormone

CRHRα - CRH receptor alpha

DHEAS - dehydroepiandrosterone sulfate

DOX - doxycycline

E2 - 17β-estradiol

EC_{50} - half maximal effective concentration

EDHF - endothelium-dependent hyperpolarizing factor

ERα - estrogen receptor alpha

GAPDH - glyceraldehyde 3-phospate dehydrogenase

GTP - guanosine triphosphate

GWAS - Genome-Wide Association Study

H&E - hematoxylin and eosin staining

hMSMC – human myometrial smooth muscle cell

HPA - hypothalamic-pituitary-adrenal axis

HR - heart rate

IC_{50} - half maximal inhibitory concentration

ICAM-1 - intercellular adhesion molecule 1

IK1 - intermediate conductance calcium activated potassium channel

KCNN3 - gene for small conductance calcium activated potassium channel subtype 3

$Kcnn3^{tm1Jpad}/Kcnn3^{tm1Jpad}$ - transgenic mouse over-expressing the small conductance calcium-activated potassium channel subtype 3 (also abbreviated as $SK3^{T/T}$)

Kv Channel - *Shaker-like* voltage-gated potassium channels

L - uterine samples from human Cesarean section patients at term and in labor

L-type - Long lasting Ca^{2+} channels

LPS - lipopolysaccharide

MSMCs - myometrial smooth muscle cells

N - number of animals.

NL - uterine samples from human Cesarean section patients at term and not in labor

NP - non-pregnant

P - pregnancy day

P4 - progesterone

PG - prostaglandin

PP2 - two days postpartum

PR - progesterone receptor

PPROM - preterm premature rupture of membranes

PTD - preterm delivery

PTL - preterm labor

qPCR - real time (quantitative) PCR

RU486 - mifepristone, progesterone receptor antagonist

SBP - systolic blood pressure

SEM - standard error of the mean

SK - small conductance calcium activated potassium channel

SK1 - small conductance calcium activated potassium channel subtype one

SK2 - small conductance calcium activated potassium channel subtype two

SK3 - small conductance calcium activated potassium channel subtype three

$SK3^{DOX}$ - functional SK3 knockout mouse ($SK3^{T/T}$ fed doxycyline)

$SK3^{T/T}$ - the SK3 overexpressing transgenic mouse

SL2 - Schneider's *Drosophila* cell line 2

SMC - smooth muscle cell

Sp - Specificity Protein

Sp3li - specificity protein 3 long isoform

Sp3si - specificity protein 3 short isoform

T-type - transient Ca^{2+} channels

WT - wild-type

CHAPTER 1

INTRODUCTION

Uterine structure and growth during pregnancy

The uterus is the reproductive organ responsible for protecting the fetus as it grows and develops, along with providing the primary force necessary for labor and delivery. The uterine wall consists of three main layers, 1) the serosa on the external surface, 2) the myometrium that consists of smooth muscle, and 3) the endometrium which is the innermost layer of glandular tissue. Anatomically the uterus is divided into two regions: the fundus and the lower uterine segment. The division of these two regions becomes more visually and functionally apparent as pregnancy progresses, as the fundus becomes more contractile and the lower uterine segment remains passive (Figure 1.1). The myometrium is organized in multiple circular and longitudinal smooth muscle layers, having divergent contractile properties (133). The smooth muscle component of the myometrial layer contains thick and thin filament bundles arranged throughout the cell. This gives these cells the ability to exert force in multiple directions and provides the dynamics and strength needed to generate forceful contractions sufficient for delivery.

Throughout gestation, both high progesterone levels and mechanical stretch initiate pathways to promote uterine growth. During pregnancy the myometrium transitions through four stages of development (140). In early gestation, smooth muscle cells rapidly proliferate, thus increasing the size and thickness of the uterus. Second, the myometrium enters a synthesis phase, in which myocytes stop proliferating and begin to hypertrophy, again increasing the size of the uterus. The myometrium then transitions to a third phase when there is an increased synthesis of contractile proteins in preparation for labor. During the fourth phase, labor, the uterus begins coordinated contractions in order to deliver the fetus.

Studying mouse models of parturition

Preterm deliveries alone account for over 12.6% of all births in the U.S., and they are associated with perinatal morbidity and mortality (15). Despite increased medical intervention over the past 30 years, the rate of preterm births has risen by 28%—with most of these births occurring in women who do not have any of the known risk factors. Four distinct processes, which activate multiple signaling pathways, lead to preterm birth: early activation of the hypothalamic-pituitary-adrenal axis, uterine overdistension, decidual bleeding, and intrauterine inflammation/infection (141). Despite differences in human versus mouse parturition, both species exhibit similarities in these processes that modify pregnancy outcomes (123). With their short gestation period and convenience of genetic manipulation, mouse models are increasingly important in the study of specific genes in pregnancy. To date, genetically modified mouse models have identified genes and proteins essential for parturition to progress in mice, many affecting multiple pathways of parturition (Table 1.1).

One key argument against the use of mouse models for the study of parturition is the role of progesterone (P4). In mice, the corpus lutea provides the main source of P4 (the placenta is the main source in humans after the first trimester) and at the end of pregnancy increased prostaglandins cause the corpus lutea to be degraded. The large drop in progesterone in mice removes the inhibition of contraction-promoting pathways causing parturition to occur (147). While serum progesterone levels do not drop in humans at the time of delivery, a functional progesterone withdrawal is thought to occur via one or more mechanisms such as a decrease in progesterone receptor coactivators and/or an increase in the ratio of lower affinity P4 receptors (24, 100). Thus mouse models with altered progesterone synthesis like the cyclooxygenase-1 knockout mouse which has a delayed labor phenotype, may help elucidate the mechanisms progesterone regulates throughout gestation (48). A mouse model that targets another crucial component of parturition, cervical ripening, have been generated. In steroid 5α-reductase

type 1 deficient mice, the cervix fails to ripen at term, delaying delivery (91). An aspect of parturition gaining interest is the influence of circadian rhythms on pregnancy. Humans (and mice) frequently deliver at night arguing that circadian influence plays a role in labor initiation (58). Parturition is disrupted in the clock mutant mouse model emphasizing the importance of circadian rhythm on hormone secretion in the progression of labor (103). Study of mouse models that are resistant to infection-induced labor, such as the Toll receptor 4 mutant, provides insights into the inflammatory/infection-induced pathways involved in human preterm labor (159). In summary, genes targeted in mouse models of parturition can be used to understand the important components of labor initiation and progression that may apply to human studies.

Progesterone withdrawal, cervical ripening, circadian rhythm, and infection are all important components of preterm and full term parturition, but in order for labor to progress, the uterus must contract adequately for delivery to occur. Mouse models targeting genes that could potentially lead to an attenuation of uterine contraction include the oxytocin and oxytocin receptor gene-deleted mice. However, these mice have normal parturition suggesting that these pathways are not essential for labor in the mouse (112, 150). Other genetically altered mouse models, including the myometrial connexin43 knockout, 15-hydroxyprostaglandin dehydrogenase knockout and the SK3 channel overexpressing mouse, have significant parturition defects (123). For example, connexin 43 and gap junction formation are essential to parturition since lack of this protein results in delayed delivery (32, 155). It is likely that multiple genes contribute to the ability of the uterus to contract synchronously, and some of these genes are a factor in redundant pathways.

Uterine smooth muscle excitability

The parturition defect in the SK3 overexpressing mice is consistent with the belief that dampening myometrial cell excitability prevents preterm labor (10, 133, 142). The

regulation of myometrial smooth muscle excitability is similar to other smooth muscle where Ca^{2+} is the main determinant of membrane depolarization as well as the signaling molecule for contractile machinery (Figure 1.1) (96, 139, 163). L-type (long-lasting) Ca^{2+} channels conduct the majority of Ca^{2+} influx for myometrial depolarization (163). This induces release of Ca^{2+} from the sarcoplasmic reticulum thereby increasing intracellular Ca^{2+} levels. Calcium binds to calmodulin, which activates myosin light chain kinase (MLCK) (108). Activated MLCK phosphorylates myosin allowing it to bind to actin, resulting in contraction. Close to the time of labor, there is an upregulation of proteins, such as oxytocin and prostaglandin receptors, that drive excitation-contraction coupling forward, leading to more coordinated and stronger contractions (139, 142). Less is known about the role of the T-type (transient) Ca^{2+} channel in this process, but they are present in the uterus and could contribute to pacemaker activity of the myometrial cells (51, 164). Na^{+} channels also contribute, but to a lesser extent than Ca^{2+} channels, to membrane depolarization and action potential initiation at term. They are also theorized to be responsible for pacemaker potentials and for setting the rhythmic timing of action potentials, although this has not been demonstrated experimentally (59, 144). Following depolarization of uterine myocytes by opening of Ca^{2+} and Na^{+} channels, which produce rapid spikes on top of slow waves, Cl^{-} channels open. Cl^{-} channels have been implicated in the plateau phase of action potentials detected near term (114, 166).

While much attention is placed on the excitatory and contractile mechanisms of uterine myocytes, the buffering of cell excitability, which induces myometrial relaxation, is equally important since the uterus must remain in a relaxed and quiescent state to allow the fetus time to grow and develop. The repolarization of myometrial smooth muscle cells is predominantly due to K^{+} efflux through K^{+} channels (Figure 1.1) (75, 164). During this phase, uterine myocytes are maintained in a quiescent phase due to inhibition of contractile pathways. For example, GTP-binding proteins (Gαs) act to reduce

contractility. Activation of these GTP-binding proteins elevates the levels of cAMP and activates protein kinase A (38, 142). This also results in activation of phosphatases that inactivate myosin light chain kinase, and reduce myocyte contractions. As pregnancy progresses, these GTP-binding proteins are downregulated to allow transition from a relaxed to a contractile state at parturition (36, 37).

Myometrial excitability can change with modifications in ion channel density. During pregnancy, the membrane potential is maintained at -60 mV and eventually increases to -45 mV near the end of term (133, 134). The exact mechanisms behind this change in membrane potential are unknown although increases in both Ca^{2+} and Na^{+} channel density and activity, or decreases in K^{+} channel density and activity are distinct possibilities. Studies show that K^{+} channels decrease in density at term while Ca^{2+} channel density increases near term (27, 154). These changes in channel density are conducive for uterine myocytes to reach threshold and elicit an action potential. Depolarizations of the myometrium transition from small irregular spikes to rhythmic sustained activity at term (133, 134). This transition in electrical activity is necessary to elicit excitability changes that will lead to an increase in the intensity of myometrial contractions sufficient to induce delivery. Both Ca^{2+} and K^{+} channels are potential targets for aberrant uterine activity. This dissertation will focus on the role of K^{+} channels.

Potassium channels and myometrial relaxation

One of the main determinants of uterine quiescence is activity of K^{+} channels in the myometrium (71, 86). Potassium channels generate repolarizing and hyperpolarizing currents in myometrial smooth muscle cells (MSMCs), contributing significantly to uterine quiescence (19, 85). Accordingly, the regulation of the activities of these channels becomes inherently important as pregnancy progresses to maintain myometrial quiescence until contractions are necessary. The level of expression of K^{+} channels in

the myometrium changes dynamically throughout pregnancy (68, 86, 97, 98). The K^+ channels most intensely studied in terms of their role in pregnancy are the large-conductance calcium- and voltage-sensitive K^+ channel, the ATP-sensitive K^+ channel, the *Shaker*-like voltage-gated K^+ channels and the small conductance calcium-activated K^+ isoform 3 (KCNN3, also known as SK3) channels. Although the expression of other K^+ channels changes throughout pregnancy, SK3 channels are the first channels for which over-expression leads to a delay or cessation of parturition (8). K^+ channels are important components of uterine relaxation early in pregnancy, and their shift in function and density at term allow for contractions forceful enough for delivery.

SK family of ion channels

The SK family (SK1, SK2, and SK3) of small conductance (~10 pS) calcium activated K^+ channels are expressed in most excitable cells (7). First characterized in neuronal cells, SK channels are responsible for the after-hyperpolarization following an action potential. Activation of SK channels is dependent on intracellular Ca^{2+} (EC_{50} = 0.3 μM) and the long lasting hyperpolarization produced by these channels is consistent with the decrease in intracellular Ca^{2+} concentration following an action potential (131). This SK-induced hyperpolarization leads to inhibition of cell firing and limits burst frequency of neurons. Unlike other K^+ channels, the SK family is voltage insensitive. While SK channels share a similar structure as voltage-activated K^+ channels, they only share sequence homology with the pore region (Figure 1.2) (73). Among the family of SK channels themselves, there is extensive (80-90%) homology, excluding the N- and C-terminal regions, accounting for the differences between family members (73).

Another defining property of SK channels is their sensitivity to apamin, a peptide in bee venom, which blocks the channel. The three members of the SK family, SK1, SK2, and SK3, vary in their apamin sensitivity. SK1 is apamin insensitive, while SK2 is the most sensitive to apamin (IC_{50} = 60 pM) and SK3 is moderately sensitive to apamin

(IC_{50} = 10 nM apamin) (59). Two amino acid residues, an aspartic acid and an asparagine, within the pore region are responsible for the variation in apamin sensitivity (Figure 1.2): SK1 has neither residue making it apamin-insensitive, while SK2 contains both residues making it very sensitive. SK3 contains only the aspartic acid residue accounting for its moderate apamin-sensitivity levels (59).

Unlike the large conductance Ca^{2+} activated K^+ channel, SK channels do not contain a direct Ca^{2+}-binding region. Rather they have a calmodulin-binding domain (CBD), which is essential for trafficking of the channel to the membrane (165). When Ca^{2+} binds the CBD, the channel undergoes a conformational change, causing opening of the channel, K^+ efflux, and membrane repolarization (165). The functional significance of one of the members of this family, SK3, was identified after John Adelman's Laboratory at the Vollum Institute created the SK3 over-expressing mouse ($SK3^{T/T}$) (8).

SK3 channels and uterine function

The $SK3^{T/T}$ mouse, a transgenic knock-in animal that contains a tetracycline-based genetic cassette, reversibly eliminates expression of the SK3 channel with doxycycline (DOX) treatment (8). DOX treatment of $SK3^{T/T}$ mice generates a functional SK3 knockout, which does not result in a visible phenotypic change. $SK3^{T/T}$ mice have a ~3-fold increase in SK3 transcript levels compared to wild-type mice. SK3 channels are expressed in many tissues, including the brain, heart, kidneys, and uterus (126). Despite this, the most notable phenotype of $SK3^{T/T}$ mice was the parturition defect in 70% of mice, of which 40% had delayed delivery and 60% could not deliver at all (8). The heterozygous and DOX-treated $SK3^{T/T}$ ($SK3^{DOX}$) mice delivered all fetuses on the appropriate gestational day. Interestingly, although expressed in the myometrium, overexpression of SK2 channels does not result in abnormal parturition (Adelman, personal communication). Functionally, these channels hyperpolarize smooth muscle cells, dampening the cell's ability to produce an action potential by increasing the

electronegativity of the membrane. These mice demonstrate that SK3 channel overexpression and its functional hyperpolarizing capability play an important role in myometrial excitability and are in agreement with other studies showing that suppression of the repolarizing SK3 channel expression increases phasic contractions in urinary smooth muscle cells (53). Thus, a 2 to 3-fold increase in SK3 channels in the uterus results in reduced uterine excitability at the time of labor. These studies also indicated that if a modest increase in SK3 channel expression results in labor dysfunction, it is likely that endogenous SK3 channel expression must be downregulated in the uterus of WT mice in order for successful delivery to occur. However, the mechanism responsible for regulation of endogenous SK3 expression is unknown.

Transcriptional regulation of KCNN3

Differential expression of the SK3 channel during pregnancy may result from promoter activity of the *KCNN3*. Factors that are important in pregnancy are known to regulate SK3 expression. For example, the sex hormone 17ß-estradiol (E2), which is required for the maintenance of pregnancy, modulates SK3 transcript expression in guinea pig brain and in an *in vitro* expression system (9, 60). Promoter analysis of the *KCNN3* gene demonstrated that this estrogen-driven enhancement of transcript expression occurs despite the fact that *KCNN3* does not contain an estrogen response element. The promoter region of *KCNN3* contains two GC-rich regions located -103 to -107 and -121 to -125 bps upstream of the transcription start site (60). GC-rich regions, like those in the *KCNN3* promoter, represent binding sites for Specificity Protein (Sp) transcription factors (118, 135, 146). Estrogen receptor alpha (ERα) enhances the ability of Sp factors to bind to DNA. *KCNN3* transcription is increased in the presence of Sp1, and mutational analysis has shown that Sp binding sites on the SK3 promoter are necessary for ERα-mediated enhancement of SK3 expression (60). Sp transcription

factors and estrogen may be important components of SK3 channel regulation during pregnancy.

Sp1 and Sp3 transcription factors are expressed ubiquitously, have the same DNA binding properties, and share a similar protein structure (12, 31). Sp1 is a transcriptional activator, whereas Sp3 activates or represses transcription depending on the environment of the promoter (31, 135). Sp3 has multiple isoforms, including a short isoform (Sp3si) that is transcriptionally inactive and a long isoform (Sp3li) whose activity can change based on the promoter context. Sp3 also contains an inhibitory domain located between two glutamine-rich activation domains, which can strongly activate transcription when the inhibitory domain is mutated (31). The inhibitory domain contains three charged amino acid residues, KEE, which are essential for Sp3 to repress transcription. Acetylation of lysine residues in Sp3 regulates this inhibitory domain *in vivo* (12). In the case of transcriptional regulation of SK3, there may be a modification in acetylation of Sp3 as well as a change in the ratio of competing transcription factors, Sp1 and Sp3, to reduce expression of SK3 channels. A reduction of these channels in the myometrium near term could enhance uterine contractility.

The placenta and maternal vasculature

The generation of the transgenic SK3 channel overexpressing mouse demonstrated that SK3 channels could play a role in uterine myocyte excitability leading to dampened contractility (8, 120). However, other physiological adaptations during pregnancy require the adequate function of ion channels, including the development of the placenta. An overabundance of the SK3 channel, which is expressed only in the endothelial layer of the vasculature and not in smooth muscle, could influence the development of the maternal-fetal vasculature and alter fetal blood flow.

The placenta is a specialized organ that provides nutrients necessary to sustain the fetus during development. It is a link, as well as a barrier, between mother and baby.

Both humans and mice have hemochorial placentation (Figure 1.3), where the maternal blood bathes the chorion and molecules transverse two fetal cell layers to enter the blood stream (107, 115). The hemochorial placenta allows for transfer of nutrients without direct contact of maternal and fetal blood, which protects the fetus from rejection by the maternal immune system. This is in contrast to epitheliochorial placentas found in sheep and pigs, in which maternal and fetal blood vessels are in close proximity to each other for nutrient diffusion (115). Epitheliochorial placentation is thought to be less efficient than hemochorial because it requires much greater inflow pressure (107). The large difference in perfusion pressure that develops between the two types of placentas creates differences in arterial remodeling between these species (115).

Upon fertilization, the blastocyst forms and the first cell line to differentiate is the trophoblast layer (130). In mice the outer epithelial layer of the blastocyst and the inner cell mass, which will form the embryo, develops by P3.5 (130). When implantation of the embryo occurs on P4.5, the trophoblast layer has already begun to differentiate (130). The epithelial cells on the opposite side of the inner cell mass stop dividing but continue DNA replication in order to form trophoblast giant cells (130). The trophoblast giant cells invade the maternal endometrium for implantation. In humans, uterine invasion occurs via extravillous cytotrophoblast cells, which are also polyploid but have fewer chromosome sets than the trophoblast giant cells found in mice (Figure 1.2) (5, 130). After implantation, trophoblast giant cells form around the entire parameter of the embryo (130). By P8.5 the allantois (a sac-like structure involved in nutrition and excretion) and the chorion make contact (130), which will eventually form the fetal vascular network of the placenta. Mice and humans share similar mechanisms for implantation and invasion of the uterus, which promotes proper formation of the maternal-fetal vasculature.

The fetal labyrinth, where maternal-fetal exchange occurs, is formed early on P9 following extensive branching from the chorion (forming the chorionic villi).

Vascularization of these villi occurs simultaneously (130). In humans, branching of the chorion and vascularization of these branches are separate events (18). The mouse fetal labyrinth is identical in function to the human chorionic villi and, in both species syncytiotrophoblast cells come in direct contact with maternal blood and surround the villi. In mice and humans, villi form in rounded structures called cotyledons, and while humans have multiple cotyledons, mice only have one (central canal) making their placenta discoid (130). Following formation of the placenta, maternal blood begins to transverse the placenta into the fetal blood stream around P10.5 in mice and 10-12 weeks in humans (1, 49).

Adamson et al 2002 performed a detailed study that utilized vascular casts and histological sections of the mouse placenta from P10.5 until term in order to map out the structure and progressive changes of the maternal vasculature through the developing placenta and its interaction with the fetal vasculature (1). Branches from the uterine artery called radial arteries supply the uterus. These radial arteries enter the myometrium near an implantation site and branch into 5-10 spiral arteries (1). During placental development, these spiral arteries lose smooth muscle and elastin, which decreases vascular tone and resistance (1). These spiral arteries cross the decidua and join to form 1-4 large canals with diameters between 300-600 μm (1). Mice only have one central canal in the placenta (125), but Adamson et al found multiple canals in some cases possibly due to a variation between mouse strains or differences in histological protocol (1). Maternal blood from the central canals fills the villus spaces of the fetal labyrinth, which flows into the venous sinuses that enter veins through the decidua and into the radial and main uterine vein (1). Proper development of the maternal and fetal vasculature within the placenta is crucial to fetal survival.

During pregnancy, proper blood exchange through the placenta is essential to provide oxygen and nutrients as well as eliminate waste from the fetus. Abnormalities in the placenta can result in preeclampsia, birth defects, and spontaneous abortion. During

early stages of pregnancy the placenta develops in a low oxygen environment due to endovascular cytotrophoblast cells that block uterine spiral arterioles, limiting blood flow to the fetus (49). Unblocking of these arterioles later in pregnancy rapidly increases oxygen tension, and initiates rapid growth and differentiation of the placenta essential to maintain the fetus. Premature increases in oxygen levels can have detrimental effects on the conceptus. A premature increase in oxygen concentration can lead to damage of DNA, lipids, and proteins since the placenta contains low amounts of antioxidants during the first trimester (63). In mice, the mature placenta is established by day 10 of pregnancy (26). Dilation and loss of elastic lamina and smooth muscle of spiral arteries occurs between day 10.5 and 14.5 making this a critical point in gestation (1). In humans, premature increases in blood flow to the placenta, measured by Doppler imaging, is associated with an increased incidence of miscarriages (62). Approximately 50% of conceptions are thought to end in miscarriages, but the portion of fetal loss attributed to early placental perfusion is unknown (61, 145). A better understanding of the mechanisms responsible for development and maintenance of the vasculature in this specialized organ could reveal mechanisms underlying miscarriage and improve maternal and fetal outcomes.

K^+ channels in vascular remodeling and dilation

During pregnancy, the vascular system must adapt to accommodate dramatic increases in blood volume necessary to sustain the developing fetus. K^+ channel expression may be a contributing factor to vascular remodeling through several mechanisms (Figure 1.4). K^+ channels contribute to cell proliferation, including vascular smooth muscle proliferation in uterine arteries (102). Potassium channels are also important in mediating vascular relaxation via endothelium-dependent hyperpolarizing factor (EDHF) (42). EDHF plays a significant role in vasodilation of arteries during pregnancy. Blocking both small conductance and intermediate conductance calcium

activated K^+ channels nearly abolishes EDHF vasodilation of systemic arteries from pregnant women (42). In addition, these channels may be important targets for pregnancy related vascular diseases like preeclampsia, since patients suffering from preeclampsia have abnormal EDHF mediated vasodilation of uterine arteries (42, 67). While K^+ channels may be important in pregnancy disorders, their role in fetal demise and miscarriages are not fully characterized.

SK3 channels in the development of the maternal vasculature of the placenta

Vascular abnormalities in $SK3^{T/T}$ mice include an increase in vessel diameter of mesenteric arteries and abnormal mesenteric branching patterns (152). Despite characterization of abnormalities in the vasculature of $SK3^{T/T}$ mice, the role of these channels in development of vascular aberrations and whether this promotes fetal demise have not been examined. The SK3 channel may contribute to the prevention of cardiovascular related disorders during pregnancy.

Although absent in vascular smooth muscle cells, SK3 channels are abundant in vascular endothelial cells under normal conditions (Figure 1.4) (14, 152). The effect of overexpressing SK3 channels on the development of other vascular networks, including the uterine artery and placenta during pregnancy, requires examination. Changes in branching and diameter of the uterine artery and vessels within the placenta could dramatically enhance blood flow to the fetus. Consequently, this increase in blood flow could occur prematurely during pregnancy causing a defect in placental development leading to fetal demise. Other genetic mouse models that exhibit fetal demise, such as BPH/5 mice (56), activin receptor-like kinase 1 null mice (57), heparan sulfate 6-O-sulfotransferase-1 null mice (50), and adrenomedullin heterozygous deficient mice (82), have implicated mechanisms responsible for the regulation of the maternal vasculature and placentation in mice. To date an increased incidence in fetal demise has not been

associated with SK3 channels in humans or mice. However, considering the impact placental development has on fetal outcome, if overexpression of SK3 channels results in an increase in spiral artery number and/or a decrease in vascular tone this could lead to fetal demise and resorption.

Figure 1.1: Uterine transition during labor. The uterus is divided into two functionally distinct sections, the upper forms the fundus, which produces contractions during labor, and the lower half of the uterus which is passive during labor to allow the fetus to be extruded. As pregnancy reaches term the physical properties of these two segments become more apparent. The cervix is located at the bottom of the uterine cavity, which ripens near term to allow enough stretch for the fetus to pass into the birth canal. Inset: Smooth muscle cells of the uterus also transition toward term. Early in pregnancy, smooth muscle cells maintain a high electronegativity via the Na^+/K^+ pump and increased expression of K^+ channels, such as the SK3 channel, which is activated by intracellular calcium. Expression of β sympathomimetic receptors is also increased early in gestation, and their activation opens K^+ channels. As pregnancy progresses myocytes transition to a contractile state with activation of calcium, sodium and chloride channels that results in an electronegativity closer to threshold. Prostaglandin $F_{2\alpha}$ and oxytocin receptors increase during pregnancy and upon ligand binding activate Ca^{2+} channels, leading to depolarization and contraction at term. Adapted from (129, 142).

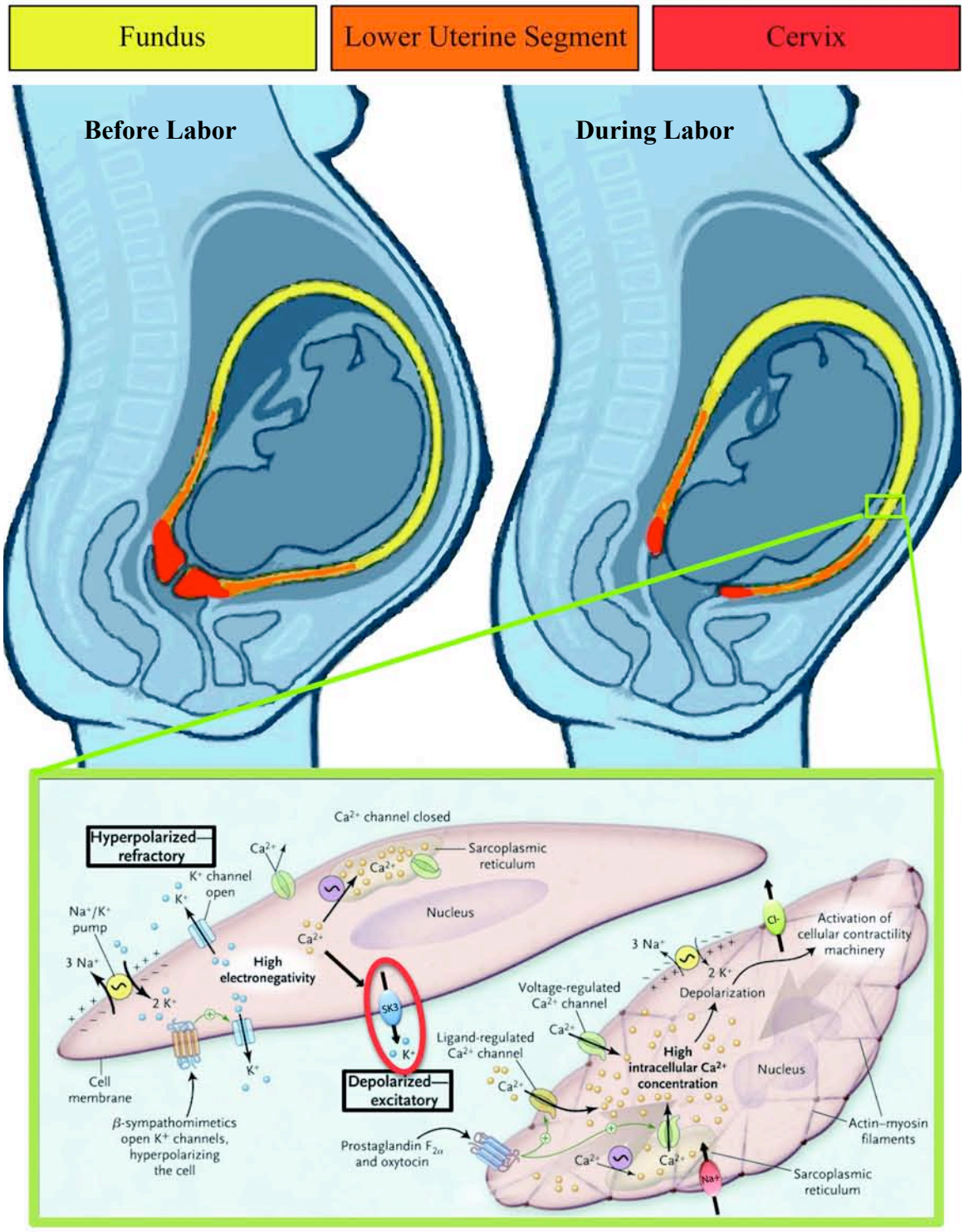

Fundus
Lower Uterine Segment
Cervix
Before Labor
During Labor
Hyperpolarized—refractory
Ca2+ channel closed
Sarcoplasmic reticulum
K+ channel open
Na+/K+ pump
3 Na+
2 K+
High electronegativity
Nucleus
Cell membrane
β-sympathomimetics open K+ channels, hyperpolarizing the cell
SK3
Depolarized—excitatory
Prostaglandin F2α and oxytocin
Ligand-regulated Ca2+ channel
Voltage-regulated Ca2+ channel
High intracellular Ca2+ concentration
Depolarization
Cl-
Activation of cellular contractility machinery
Actin–myosin filaments
Na+

Table 1.1: Genetically altered mouse models of parturition.

Genetically altered mice	Phenotype
Progesterone withdrawal	
20alpha-Hydroxyprostaglandin dehydrogenase $^{-/-}$	Delay in labor; no P4 withdrawal
PGF_{2Alpha} receptor $^{-/-}$	Fail to initiate labor; fetal demise; no P4 withdrawal
Cytoplasmic phospholipase A_2 $^{-/-}$ and COX-1$^{-/-}$	Delay in labor, fetal demise; no P4 withdrawal
Kruppel-like factor 9 $^{-/-}$	Delay in labor; aberrant PR expression
Myometrial contraction	
Oxytocin $^{-/-}$ and oxytocin receptor $^{-/-}$	No parturition phenotype
COX-1 $^{-/-}$/oxytocin $^{-/-}$	Prolonged labor initiated at normal time
Myometrial connexin43 $^{-/-}$	Delayed labor; fetal demise
15-Hydroxyprostaglandin dehydrogenase $^{-/-}$	Early labor; early PGF2Alpha increase; no progesterone withdrawal
SK3 channel overexpressor	Prolonged labor; weaker uterine contractions at term
Cervical ripening	
Relaxin and Relaxin receptor $^{-/-}$	Low penetrance nonproductive labor
Steroid 5alpha-reductase type 1 $^{-/-}$	Delayed or prolonged labor; impaired cervical ripening
Circadian influence on labor	
Clock mutant	Increased incidence of extended but nonproductive labor
Oxytocin $^{-/-}$	Phase advance or delay alters birth timing
Bacterially induced Preterm Labor	
IL-1 receptor $^{-/-}$, IL-1B $^{-/-}$,and IL-6$^{-/-}$	Susceptible to bacterially induced Preterm Labor
IL-1 receptor $^{-/-}$/TNF receptor $^{-/-}$	Decreased susceptibility to bacterially induced preterm labor
Toll-like receptor 4 mutant	Resistant to bacterially induced preterm labor

Source: Ratajczak CK, and Muglia LJ. Insights into parturition biology from genetically altered mice. *Pediatric research* 64: 581-589, 2008.

Figure 1.2: SK Channel structure. The SK channel family has six transmembrane spanning domains and subunits arrange in tetramers to form a channel, with N and C termini oriented on the cytosolic side of the plasma membrane. The channels are constitutively bound to calmodulin and their pore region contains two residues that determine their apamin sensitivity. Adapted from (7).

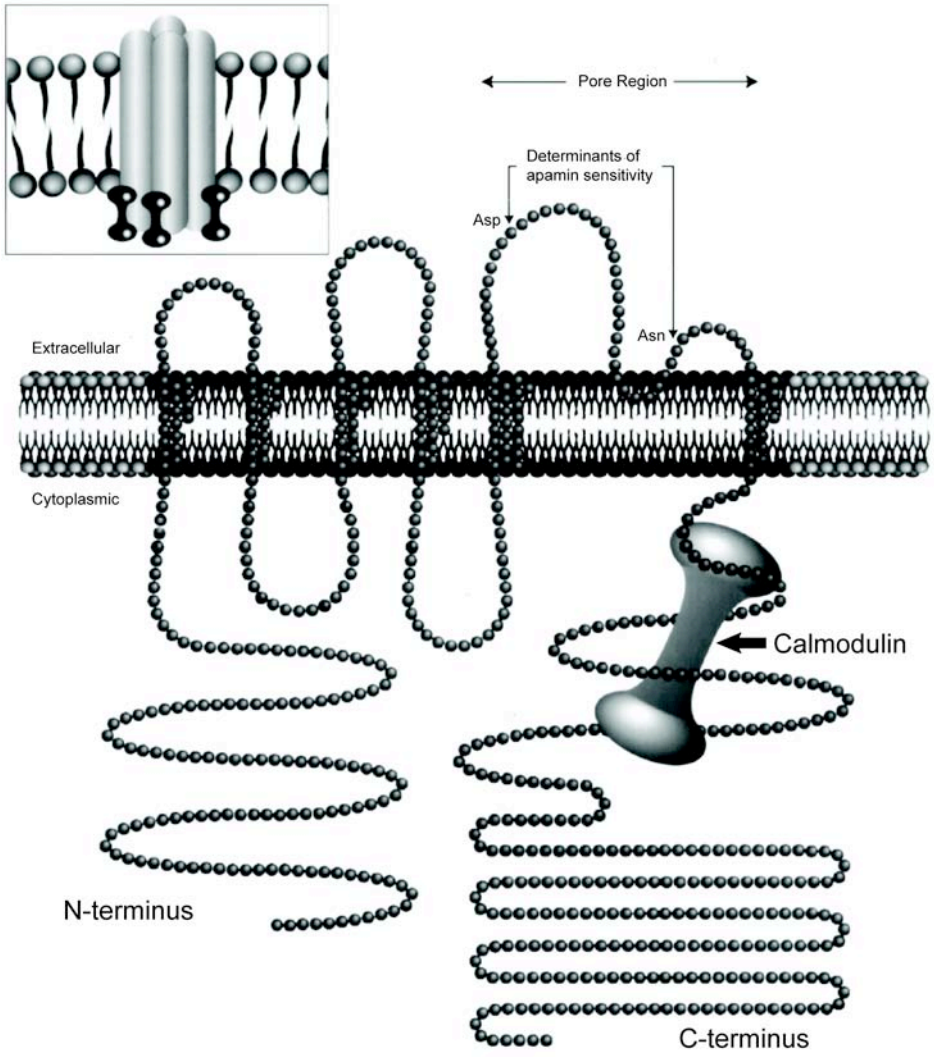

Pore Region
Determinants of
apamin sensitivity
Asp
Asn
Extracellular
Cytoplasmic
Calmodulin
N-terminus
C-terminus

Figure 1.3: Similarities and differences between human and mouse placenta. Schematic of (A) mouse and (B) human placenta illustrating overall structure, emphasizing the size and number of the invasive trophoblast and cytotrophoblast cells. (C) Schematic of human and mouse placenta demonstrating other key contributors to placental function and direction of blood flow as indicated by the arrows. Abbreviations: MD- maternal decidua, V- villi, IVS- intervillous space, S- spongiotrophblast layer, L- fetal labyrinthine, UC- umbilical cord, CMA- central maternal artery. Adapted from (65, 130).

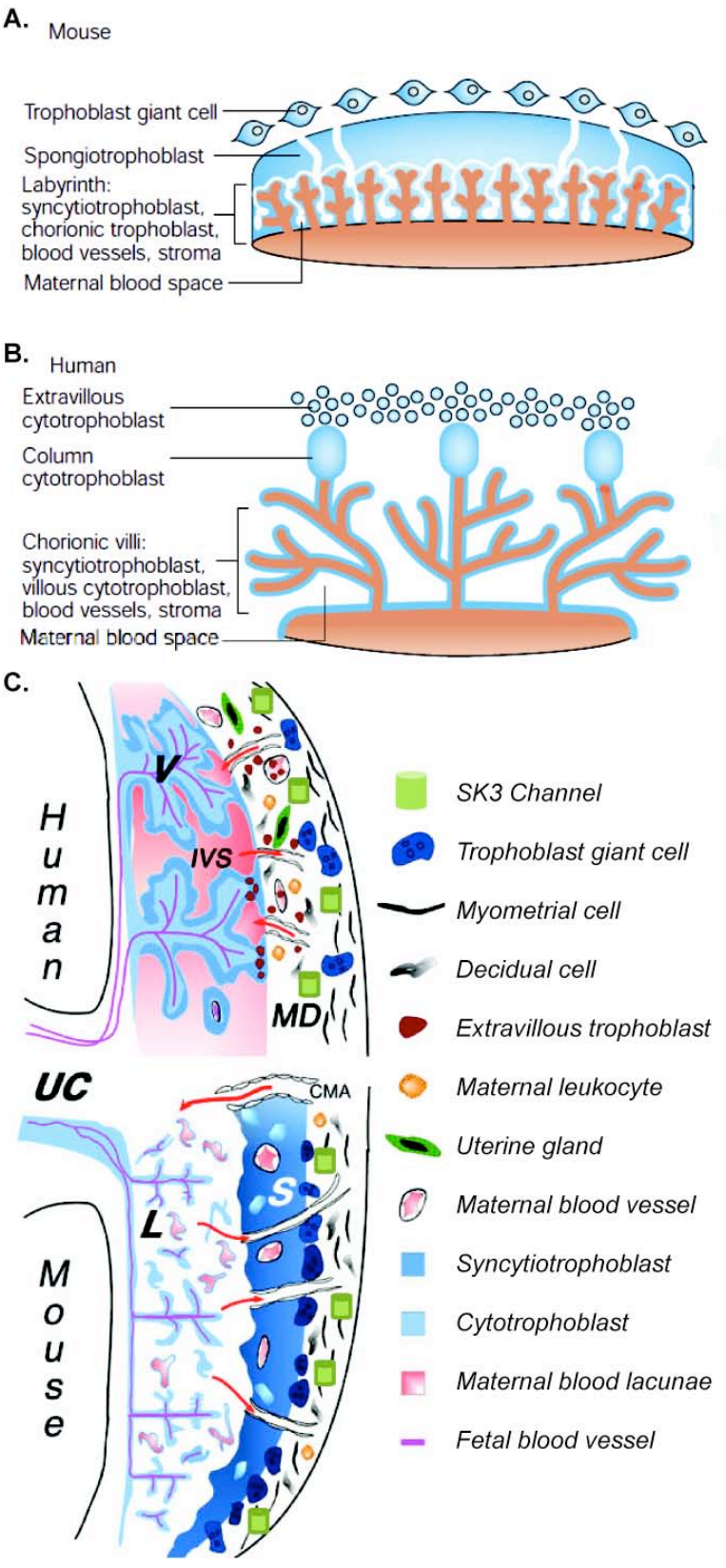
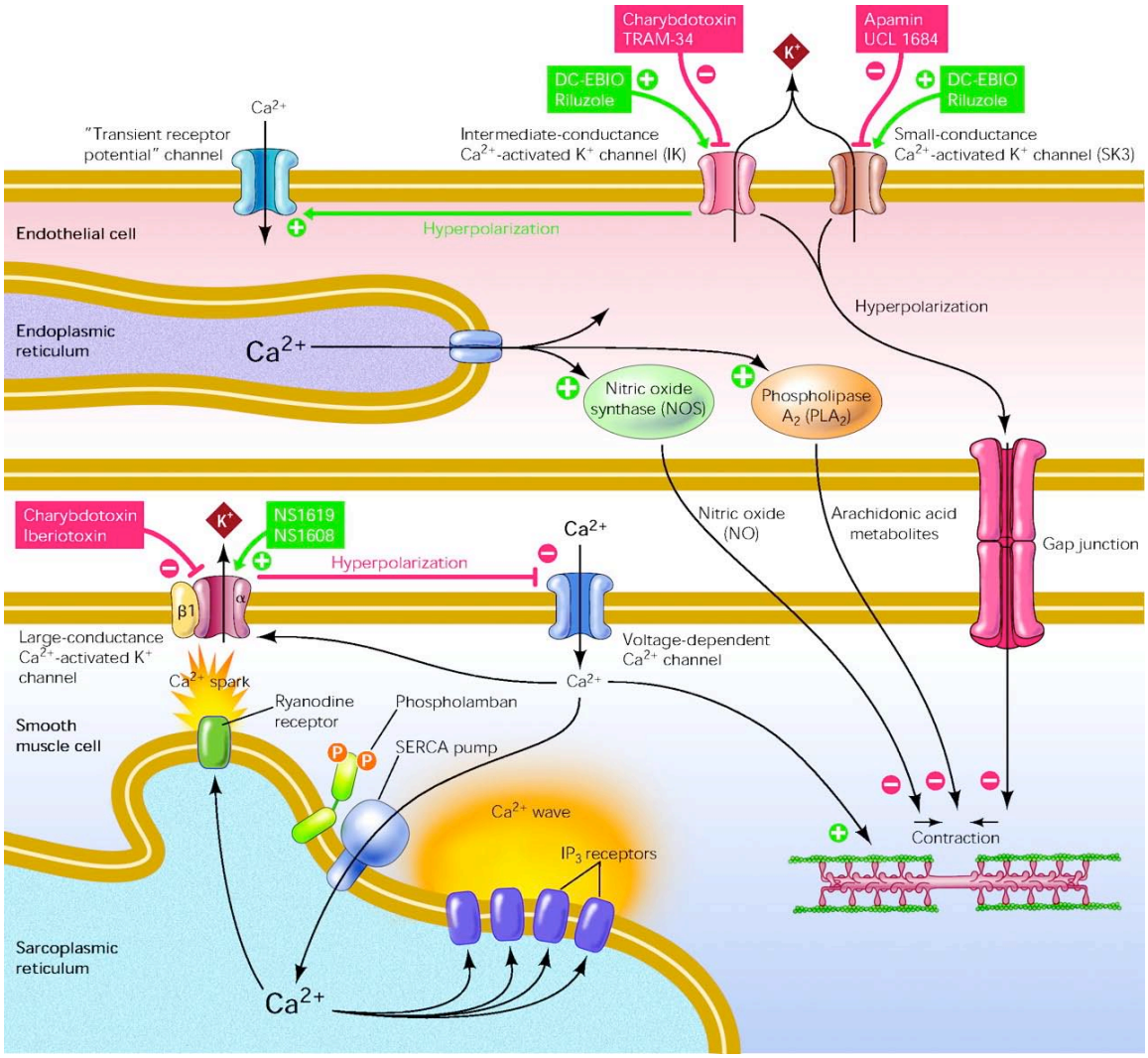

A.
Mouse
Trophoblast giant cell
Spongiotrophoblast
Labyrinth:
syncytiotrophoblast,
chorionic trophoblast,
blood vessels, stroma
Maternal blood space
B.
Human
Extravillous
cytotrophoblast
Column
cytotrophoblast
Chorionic villi:
syncytiotrophoblast,
villous cytotrophoblast,
blood vessels, stroma
Maternal blood space
C.
Human
V
IVS
MD
Mouse
UC
CMA
S
L
SK3 Channel
Trophoblast giant cell
Myometrial cell
Decidual cell
Extravillous trophoblast
Maternal leukocyte
Uterine gland
Maternal blood vessel
Syncytiotrophoblast
Cytotrophoblast
Maternal blood lacunae
Fetal blood vessel

Figure 1.4: Potassium channels in vasodilation. Schematic displaying the role potassium channels play in endothelial and smooth muscle mediated relaxation of blood vessels in response to intracellular calcium. Source: (80).


Charybdotoxin
TRAM-34
Apamin
UCL 1684
K+
DC-EBIO
Riluzole
DC-EBIO
Riluzole
Ca2+
"Transient receptor potential" channel
Intermediate-conductance Ca2+-activated K+ channel (IK)
Small-conductance Ca2+-activated K+ channel (SK3)
Endothelial cell
Hyperpolarization
Hyperpolarization
Endoplasmic reticulum
Ca2+
Nitric oxide synthase (NOS)
Phospholipase A2 (PLA2)
Charybdotoxin
Iberiotoxin
K+
NS1619
NS1608
Hyperpolarization
Ca2+
β1
α
Nitric oxide (NO)
Arachidonic acid metabolites
Gap junction
Large-conductance Ca2+-activated K+ channel
Voltage-dependent Ca2+ channel
Ca2+ spark
Ca2+
Ryanodine receptor
Phospholamban
Smooth muscle cell
P
P
SERCA pump
Ca2+ wave
IP3 receptors
Contraction
Sarcoplasmic reticulum
Ca2+

CHAPTER 2
OVEREXPRESSION OF SK3 CHANNELS DAMPENS UTERINE CONTRACTILITY TO PREVENT PRETERM LABOR IN MICE

Abstract

Mechanisms that control the timing of labor have yet to be fully characterized. In a previous study (8), over-expression of small conductance calcium-activated K^+ channel subtype 3 in transgenic mice, $Kcnn3^{tm1Jpad}/Kcnn3^{tm1Jpad}$ (also known as $SK3^{T/T}$), led to compromised parturition, which demonstrated the important role of KCNN3 in the delivery process. Based on these findings, we hypothesized that SK3 channel expression must be downregulated late in pregnancy to enable the uterus to produce the forceful contractions required for parturition. To test this hypothesis we investigated the effects of SK3 channel expression on gestation and parturition, comparing $SK3^{T/T}$ mice to WT. In WT mice, SK3 transcript and protein were significantly reduced during pregnancy. The force produced by uterine strips from P19 $SK3^{T/T}$ mice was significantly less than that measured in uterine strips from WT or $SK3^{DOX}$ mice, and this effect was reversed by application of the SK3 channel inhibitor apamin. Moreover, two treatments that induce labor in mice failed to result in delivery within 48 hours following injection on P15 in $SK3^{T/T}$ mice. Thus, stimuli that initiate parturition under normal circumstances are insufficient to coordinate the uterine contractions needed for completion of delivery when SK3 channel activity is in excess. Our data indicate that SK3 channels must be downregulated for the gravid uterus to generate labor contractions, which are sufficient for delivery, in both term and preterm mice.

Introduction

Throughout gestation the uterus is maintained in a quiescent state to allow fetal growth and development. Eventually, the relaxed uterus transitions to an active state,

with the ability to generate labor contractions in order to expel the fetus at the time of delivery. The exact mechanisms for initiation of labor have yet to be determined, but disruption of delivery can lead to complications such as preterm labor (PTL), unproductive or abnormal labor, and post-term labor. Genetic mouse models that target proteins thought to be essential for labor in mice and humans often experience normal parturition, which has ruled out several mechanisms for the regulation of the timing of labor (69). Delayed delivery due to a failure of luteolysis or cervical ripening occurs in knockout mice in which the genes encoding cyclooxygenase 1 (79), cytosolic phospholipase A_2 (157), the $PGF_{2\alpha}$ receptor (147) or 5α reductase type 1 (90) are disrupted. In some cases uterine contractility remains unaltered (90).

One determinant of uterine quiescence is the activity of K^+ channels in the myometrium (71, 86). Potassium channels generate repolarizing and hyperpolarizing currents in myometrial smooth muscle cells (SMCs), thus contributing significantly to uterine quiescence (19, 85). Accordingly, the regulation of the activation of these channels becomes inherently important as pregnancy progresses so that myometrial tranquility is maintained until the fetus is fully developed and contractions are necessary. Both the level of expression and the density of K^+ channels in the myometrium change dynamically throughout pregnancy (68, 86, 97). The K^+ channels most extensively studied in pregnancy are the large-conductance calcium- and voltage-sensitive K^+ channel, the ATP-sensitive K^+ channel, the *Shaker*-like voltage-gated K^+ channels, and the SK3 channels. Although the expression of other K^+ channels changes throughout pregnancy, SK3 channels were the first channels that over-expression led to a delay or cessation of parturition (8).

Recent studies have shown that transgenic mice over-expressing SK3 channels have compromised parturition (8). Although the underlying mechanism remains to be proven, the hyperpolarizing potential of SK3 channels, as well as the role of this current in uterine relaxation (13), support the idea that SK3 channel over-expression may

abrogate parturition by reducing the ability of the uterus to contract. The $SK3^{T/T}$ mouse is a transgenic knock-in animal that the administration of doxycycline results in a functional SK3 knockout. $SK3^{DOX}$ mice do not exhibit a detectable phenotype, whereas $SK3^{T/T}$ mice show a 2.4-fold increase in channel expression (53). Parturition is defective in 70% of $SK3^{T/T}$ mice; 40% of these cases involve delayed delivery, and 60% result in dam mortality (8). In contrast, both heterozygous and $SK3^{DOX}$ mice deliver all fetuses on the appropriate gestational day (8). These results support the hypothesis that over-expression of SK3 channels, or the failure to downregulate the expression of SK3 channels, may delay the onset of labor.

The mechanisms by which SK3 channels modify myometrial function are similar in humans and mice. In recent studies, uterine smooth muscle from non-pregnant (NP) $SK3^{T/T}$ mice showed decreased phasic contractions compared to tissue from its WT counterpart (13). In contrast, the suppression of SK3 channel expression in urinary smooth muscle increased phasic contractions (53). Furthermore, in humans SK3 transcript was lower in term pregnant myometrium than in the equivalent non-pregnant tissue (97), implicating the channel in human parturition.

Taken together, these earlier findings led us to hypothesize that SK3 channel expression is downregulated late in pregnancy in order to permit uterine contractions of the magnitude needed for parturition. The experiments undertaken in this study investigated this possibility, with respect to not only the downregulation of SK3 channels, but also the consequences of SK3 overexpression on the generation of contractions and for parturition itself.

Materials and methods

Animals

All animal procedures complied with the guidelines for the care and use of animals set forth by the NIH. The Animal Care and Use Committee at the University of

Iowa approved all protocols. $SK3^{T/T}$ mice on a C57BL/6 background were used for this study (gift from John Adelman at the Vollum institute) (8). A tetracycline-based genetic switch inserted in the untranslated region of the SK3 gene allowed for site-specific expression and suppression of the gene upon consumption of dietary DOX (Bio-Serv, Frenchtown, NJ). DOX feed was administered at least one week before experiments were initiated, to inhibit SK3 channel expression. $SK3^{DOX}$, WT littermates and C57BL/6 mice were used as controls for comparison with $SK3^{T/T}$ mice.

Adult female mice were mated at 8 weeks of age or later. Day 0 of pregnancy was determined based on the presence of a copulatory plug. Mice were euthanized by CO_2 inhalation on particular days relative to pregnancy (non-pregnant (NP), days 7, 10, 14, 17, 19 of gestation (P7-19) and 2 days postpartum (PP2)). Uterine tissue was isolated and flash-frozen in liquid N_2.

Extraction of RNA and real-time PCR

The guanidinium isothiocyanate method was used to obtain total RNA from mouse uteri as previously described (4). Total RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA) to generate cDNA. The cDNA was amplified in triplicate, using primers specific for murine *KCNN3* channels (GGGTGTCAAGATGGAACAAA, ATCTTGGAAAGGTCCACCAG) or GAPDH (GCAGTGGCAAAGTGGAGATT, GAATTTGCCGTGAGTGGAGT), and the SYBR Green Supermix (BioRad, Hercules, CA). GAPDH served as a standard to normalize gene expression.

Electrophysiology

For electrophysiological analysis, NP mouse uterine tissue was removed, cut into 4mm x 2mm and dissociated as previously described (4). The cell suspension was allowed to settle for ~20 minutes in an external solution containing (in mM) 135 NaCl, 4.7 KCl, 1 $MgCl_2$, 10 glucose, 2 $CaCl_2$, and 5 HEPES at pH 7.4. A solution containing

(in mM) 140 KCl, 0.5 $MgCl_2$, 1 EGTA, 5 ATP, 5 HEPES and 0.5 of free Ca^{+2} at pH 7.2 was used to fill heat-polished borosilicate pipettes. Cells were patch-clamped at room temperature (~22°C). Upon achievement of a gigaohm seal (2-10 gΩ), membrane potential was clamped and series resistance compensated. Whole-cell recording was performed as previously described (11). Briefly, current was measured with a holding potential of -80 mV, and step potentials were elicited from –80 to +120 mV in 20 mV intervals by an Axopatch 200-B (Axon Instruments, Union City, CA) amplifier. Currents were measured in the absence and presence of apamin (500 nM). Commercial pClamp 9.2 software and Digidata 1322A interface (Axon Instruments) were used to acquire and digitize data. The clampfit 9.2 software program (Axon Instruments) was used to calculate mean sustained K^+ current amplitudes and normalized to cell size (pA/pF).

Isometric tension recordings

Following euthanasia of P19 mice with CO_2, uterine tissue was isolated and cut into 4mm x 8mm strips in Krebs solution containing (in mM) 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 11 glucose. Strips were mounted to a force transducer in organ baths filled with oxygenated (95% O_2, 5% CO_2) Krebs solution at 37°C, and tension was recorded by a Powerlab (ADInstruments, Castle Hill, NSW, Australia) data acquisition system. Basal tension (1 g) was applied to the tissue strips and equilibrated for 45 minutes prior to study. Apamin was added to the bath (500 nM) and tension recorded for 15 minutes. Spontaneous contractions obtained before and after addition of apamin were compared. To compare contractions between groups, the maximal tension produced by KCl (80 mM) was used to normalize contractions. KCl was used to normalize contractions because it increases extracellular K^+ leading to a rapid depolarization in excitable cells that is not affected by the outward current of SK3 channels (124). Contractions in response to KCl in WT, $SK3^{T/T}$ and $SK3^{DOX}$ mice did not significantly differ.

Tension recordings were analyzed using Hemolab Software Ver. 3.8 (78). Ten-minute traces obtained prior to and following apamin application were compared. The minimum tension was determined and subtracted from all traces to obtain a baseline response. A contraction was defined as any increase in amplitude 50% or greater than the maximal contraction. The heights of the contractions were determined and averaged.

Immunoblotting

Cell membrane fractions were isolated from mouse uterine tissue as previously described (4), separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with rabbit polyclonal anti-SK3 N-terminal primary antibody (1:100 dilution, Alomone Labs, Jerusalem, Israel) and HRP-conjugated goat anti-rabbit Fc secondary antibody (1:3,000 dilution, Pierce, Rockford, Il). To assure equal loading the blots were re-probed with anti-GAPDH primary antibody (1:1,000, Chemicon, Temecula, CA) and HRP-conjugated mouse anti-goat secondary antibody (1:3,000 dilution, Jackson ImmunoResearch, West Grove, PA). Signal was detected by chemiluminescence (ECL Western Blotting Detection Reagents, Buckinghamshire, UK) and SK3 protein expression was quantitated using densitometry (ImageJ, NIH) and normalized to GAPDH.

Induction of preterm labor

At P15, WT, $SK3^{T/T}$ and $SK3^{DOX}$ mice were injected with either lipopolysaccharide (LPS, 100 μg, *Escherichia coli*, serotype 0111:B4, Sigma, St. Louis, MO) in sterile saline solution, or RU486 (mifepristone) (100 μg) in ethanol (33, 34). Delivery was assessed after 24 hours. 48 hours post-injection, mice were sacrificed and examined for fetal remains within uterine horns, which represented a failure in delivery. Complete delivery was established by the absence of fetuses. For tension recording measurements, uteri were isolated 8 hours post-LPS injection. Progesterone withdrawal (as a consequence of LPS administration) was assessed in $SK3^{T/T}$ and WT mice using

serum samples obtained via tail vein immediately before and 8 hours after LPS injection. Serum progesterone levels were measured by ELISA (DRG Diagnostics, Germany).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significance was determined by one-way and two-way ANOVA where appropriate, followed by post-hoc comparison using Student t-tests. Success rate of delivery was analyzed using chi-square distribution. Significance was determined at $p < 0.05$. N refers to number of animals in all cases.

Results

SK3 Expression in pregnant mice

To assess the expression of *KCNN3* channel transcript during pregnancy, we isolated RNA from NP and pregnant mice at various stages of gestation (NP, P7-PP2, N=3-6). Real time PCR (qPCR) analyses revealed a decrease in SK3 transcript by P14 of gestation compared to NP, which was maintained until term (Figure 2.1A). To assess whether SK3 protein levels mimic transcript expression, immunoblotting with SK3-specfic antibodies was performed. Membranes isolated from uterine tissue of NP, P7-P19, and PP2 mice showed that SK3 protein expression decreased with progression of pregnancy, from mid-gestation onwards (Figure 2.1B). Quantification of blots using densitometry further demonstrated this downregulation of SK3 (Figure 2.1C, N=6). Thus, during normal mouse gestation, SK3 channels are downregulated from mid to late gestation.

K^+ current in myometrial smooth muscle cells

To assure that SK3 channels were present in myometrial cells and to assess the contribution of SK3 current to total myometrial cell K^+ current, whole-cell patch clamping experiments were performed. Myometrial cells isolated from NP WT and SK3

$^{T/T}$ mice were held at -70 mV and pulsed in 20 mV steps to 140 mV. At 140 mV, NP myometrial cells showed a 29% reduction in total K^+ current after apamin (500nM) administration (Figure 2.2A, N=5). In contrast, in the myometrial cells of SK3 $^{T/T}$ mice apamin reduced K^+ current by 39% (Figure 2.2B, N=2). These data indicate that the SK3 repolarizing current is increased 1.8-fold in SK3 $^{T/T}$ mice, coincident with overexpression of SK3 channels and are in agreement with other studies that show similar levels of enhancement of SK channel current in smooth muscle cells (53).

Uterine contractility in mice during late-stage pregnancy

Contractility was measured in uteri isolated from P19 WT, SK3$^{T/T}$ and SK3^{DOX} mice (Figure 2.3). Consistent with reports from other studies, we observed spontaneous uterine contractions in all three experimental groups (Figure 2.3A) (25). WT and SK3^{DOX} uterine strips produced similar peak contractions prior to apamin administration, whereas SK3$^{T/T}$ uterine strips produced reduced contractions (Figure 2.3A). Apamin (500 nM), which inhibits SK3 channels, did not affect contractions generated by WT and SK3^{DOX} uterine strips (Figure 2.3A) but led to an increase in the contraction in SK3$^{T/T}$ strips (Figure 2.3B, N=5 for all groups). Thus, over-expression of SK3 channels appears to weaken uterine contractility. Apamin did not affect oxytocin-induced contractions in WT (before 102.54±9.69, after 98.91±10.10, N=4), SK3$^{T/T}$ (before 112.21±8.68, after 87.80±6.36, N=4) or SK3^{DOX} (before 106.82±7.61, after 105.54±12.73, N=3) mice.

Deliveries after induction of preterm labor

We reasoned that, in addition to disruption of parturition at term, the reduction in contractile function of uteri from SK3$^{T/T}$ mice may prevent preterm labor (8). Although WT and SK3^{DOX} mice completely delivered within 24 hours following injection of the labor-inducing agent LPS, SK3$^{T/T}$ mice failed to complete delivery (Table 2.1). In some instances SK3$^{T/T}$ mice had a fetus lodged within the birth canal, indicating that labor had begun but fetal expulsion was not achieved.

Similar to isometric tension recordings performed with P19 mice, $SK3^{T/T}$ mice (N=7) also showed a reduction in the amount of tension produced 8 hours-post LPS injection at P15 compared to WT (N=5) and $SK3^{DOX}$ (N=5, Figure 2.4A). This further indicates that overexpression of SK3 channels dampens uterine contractility.

The onset of preterm labor in mice injected with LPS coincides with a drop in progesterone levels similar to term deliveries (39). WT (N=6), $SK3^{T/T}$ (N=5), and $SK3^{DOX}$ (N=5) mice induced to deliver preterm had similar levels of serum progesterone prior to LPS injection (Figure 2.4B), and treatment resulted in a significant (and comparable) decrease in progesterone levels in all groups. These data suggest that progesterone withdrawal necessary for induction of preterm labor occurred in the $SK3^{T/T}$ group, despite the fact that these mice were not able to complete delivery.

To eliminate the possibility that the failure of $SK3^{T/T}$ mice to deliver preterm was due to a decrease in responsiveness to LPS, preterm labor was also induced using RU486, a progesterone-receptor antagonist. As in the case of induction with LPS, WT and $SK3^{DOX}$ mice were able to complete delivery within 24 hours (Table 2.1), yet $SK3^{T/T}$ mice did not completely deliver (Table 2.1). Serum progesterone levels were not measured in this case because RU486 is a progesterone receptor antagonist, reducing receptor function rather than progesterone levels (33).

Discussion

This study addressed mechanisms that underlie the regulation of parturition by SK3 channels. Our finding that uterine tissue obtained from $SK3^{T/T}$ mice produced significantly less tension per contraction compared to WT mice, suggested that a decrease in uterine contractility was involved in the associated dysfunction of delivery. It also appeared that SK3 overexpression was responsible for the inability of uteri from $SK3^{T/T}$ mice to contract forcefully enough to complete delivery, since $SK3^{DOX}$ mice produced contractions comparable to those in WT mice. SK3 channels have previously been

shown to diminish contractility in the smooth muscle of the bladder (53) and more specifically, in the NP myometrium (13), exemplifying the role SK3 channels play in the relaxation of various types of smooth muscle tissue.

SK3 channels are widely expressed throughout tissues that are essential for pregnancy and parturition (20). For example, SK3 channels are expressed in the hypothalamus (20), where they could affect hormone secretion, thereby leading to compromised parturition. Oxytocin, a hormone that stimulates uterine contractions during labor (6), is released from neurons in the supraoptic nucleus, a region that also harbors neurons and astrocytes that express SK3 channels (3). It is thus possible that SK3 channels regulate oxytocin release. However, oxytocin knockout mice deliver at term (112), indicating oxytocin is not essential for parturition to proceed. This supports the alternative explanation that delayed delivery by $SK3^{T/T}$ mice is due to a decline of myometrial excitability.

Our data show a significant reduction in SK3 channel expression in WT mice mid-gestation, but the mechanism responsible for this downregulation has yet to be determined. Estrogens have been implicated in SK3 upregulation; SK3 mRNA levels increased in response to injection of 17β-estradiol into the rostral hypothalamus of ovariectomized guinea pigs (9). Consequently, estrogen surges are important in the regulation of pregnancy and could potentially regulate SK3 channel expression. However, the SK3 gene does not contain an estrogen response element, making a direct interaction with the estrogen receptor unlikely. However, the promoter region of the SK3 gene contains two Sp-binding motifs that are necessary for the estrogen-induced SK3 mRNA upregulation (60). Since estrogen plays a central role in the progression of pregnancy, this may be one mechanism for the regulation of SK3 expression in the uterus. This is examined in Chapter 4.

Changes in cellular content and extracellular matrix of the cervix before parturition are important for successful delivery (54). Such “ripening” of the cervix is

essential for a timely parturition in mice, as illustrated by the 5α reductase type 1 (90) and the transgenic human apolipoprotein B (162) mice, in which these proteins fail to upregulate and delivery is unsuccessful. Given the presence of SK3 channels in the cervix (20) and their hyperpolarizing influence, it seems likely these channels would contribute to cervical relaxation. Thus, regulation of cervical changes is another possible role for SK3 channels in parturition.

While the current study did not specifically examine the endothelial cells that line the myometrium, previous studies have demonstrated that endothelium-dependent relaxation in human umbilical vein is sensitive to apamin (109). Such an effect could impinge on uterine contractility, since endothelial cells are abundant in endometrium (55) and may play a role in pregnancy by contributing to relaxation of the myometrium. Additionally, a previous study using human myometrial tissue samples from NP individuals indicated that SK3 channels are necessary for the relaxant effect of nitric oxide on the uterus (106). In spite of the fact that SK3 channels are not present in vascular smooth muscle, their presence in the endothelial layer is important to maintain relaxation of resistance arteries (152). Thus, although SK3 channels are localized in myometrial smooth muscle, their presence in the endometrial layer may impact relaxation of myometrial smooth muscle. This broadens the scope of the hyperpolarizing effects of SK3 channels in smooth muscle of myometrium.

The physiological changes in the uterus that are necessary for labor at term also occur during premature labor (52). Reducing the excitability of the uterus by increasing the expression of SK3 channels could stop the progression of preterm delivery. As mentioned previously, other mouse models also fail in parturition, but many of these are caused by a failure to receive the signals required to initiate parturition (79, 147, 157). It appears that the $SK3^{T/T}$ mouse, on the other hand, is one of the few models that, despite receiving signals adequate to initiate luteolysis, are nevertheless unable to complete the process of parturition. The failure to complete preterm delivery in $SK3^{T/T}$ mice (Table 1)

suggests that SK3 channels could be targeted to prevent, or stop the progression of, preterm labor in humans. This could have a dramatic impact, as preterm delivery accounts for approximately 12.5% of all births in the U.S. (93), and premature delivery is associated with one-third of infant mortality (15). Current treatments for preterm labor remain largely ineffective and, in some cases, can be harmful to the mother or fetus. The need for a safer, more successful treatment, or the development of preventive measures for premature labor, makes SK3 channels a compelling target for further investigation.

In the study presented here, we have provided evidence that SK3 channels play a critical role in uterine contractility during pregnancy. Furthermore, we have demonstrated the ability of these channels to prevent the progression of preterm labor in mice. The next step will be to elucidate mechanisms that control this effect, which is explored in Chapter 4. Ultimately, this will allow the ability to modulate regulation of K^+ channels, which could play an important role in the treatment of preterm labor.

Table 2.1: Success rate of delivery.

	Pre -term Delivery	Total Dams
WT-LPS	100%	6
$SK3^{T/T}$-LPS	0% *	6
$SK3^{DOX}$-LPS	100%	5
$SK3^{T/T}$-RU486	0% *	5
$SK3^{DOX}$-RU486	100%	4

Note: The number of mice able to complete delivery by 48 hours after injection of 100 ug LPS or RU486. * $p < 0.05$ vs. WT-LPS.

Figure 2.1: Downregulation of SK3 in mouse uteri towards term. (A) Real-time PCR analysis of uterine SK3 transcript demonstrated downregulation at P14 and P19, relative to NP, uterine samples (N=3-6). (B) Western immunoblotting of WT mouse uteri showed downregulation of SK3 protein throughout pregnancy. (C) Densitometry analysis of western blot data for SK3 protein normalized to GAPDH protein levels (N=6, Mean ± SEM, * $p < 0.05$ vs. NP).

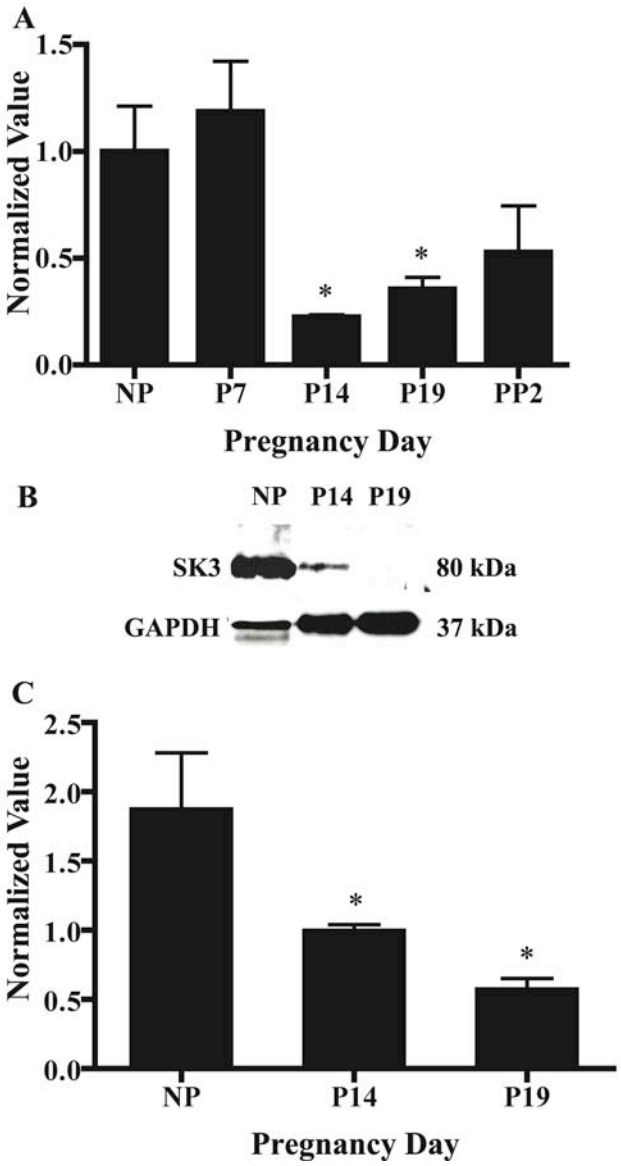

A
Normalized Value
1.5
1.0
0.5
0.0
*
*
NP
P7
P14
P19
PP2
Pregnancy Day
B
NP P14 P19
SK3
80 kDa
GAPDH
37 kDa
C
Normalized Value
2.5
2.0
1.5
1.0
0.5
0.0
*
*
NP
P14
P19
Pregnancy Day

Figure 2.2: Contribution of SK3 channels to myometrial cell K^+ current. Whole-cell patch clamping trace of (A) NP WT and (B) SK3 $^{T/T}$ myometrial cells before and after apamin (500 nM) application.

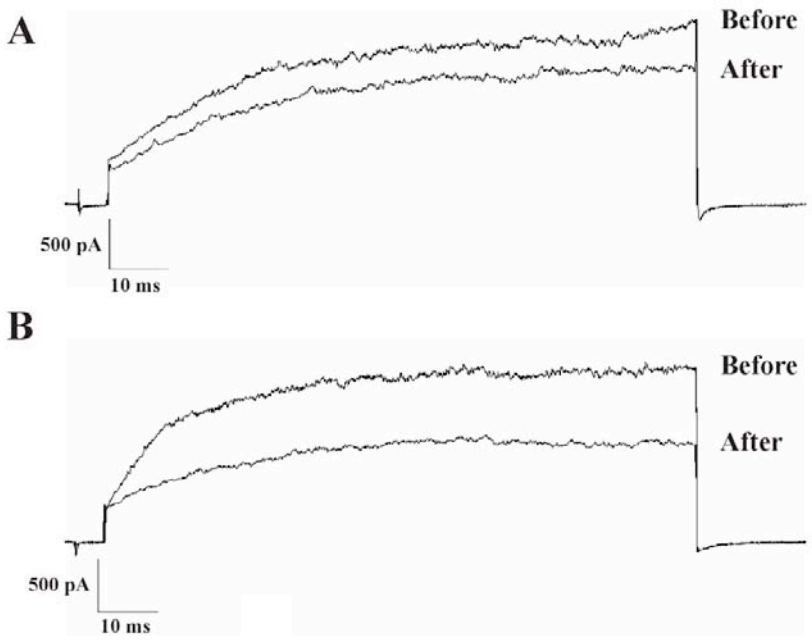

A
Before
After
500 pA
10 ms
B
Before
After
500 pA
10 ms

Figure 2.3: Spontaneous contractions of uterine strips from WT, $SK3^{T/T}$, and $SK3^{DOX}$ mice. (A) Tension development (g) of uterine strips from P19 WT, $SK3^{T/T}$, and $SK3^{DOX}$ mice before and after apamin (500 nM) application. (B) Tension development of uterine strips (percent of maximal KCl response) from P19 WT, $SK3^{T/T}$, and $SK3^{DOX}$ mice before and after application of apamin (N=5, Mean ± SEM, * $p < 0.05$ vs. $SK3^{T/T}$ after apamin, ** $p < 0.05$ vs. WT/$SK3^{DOX}$ before apamin).

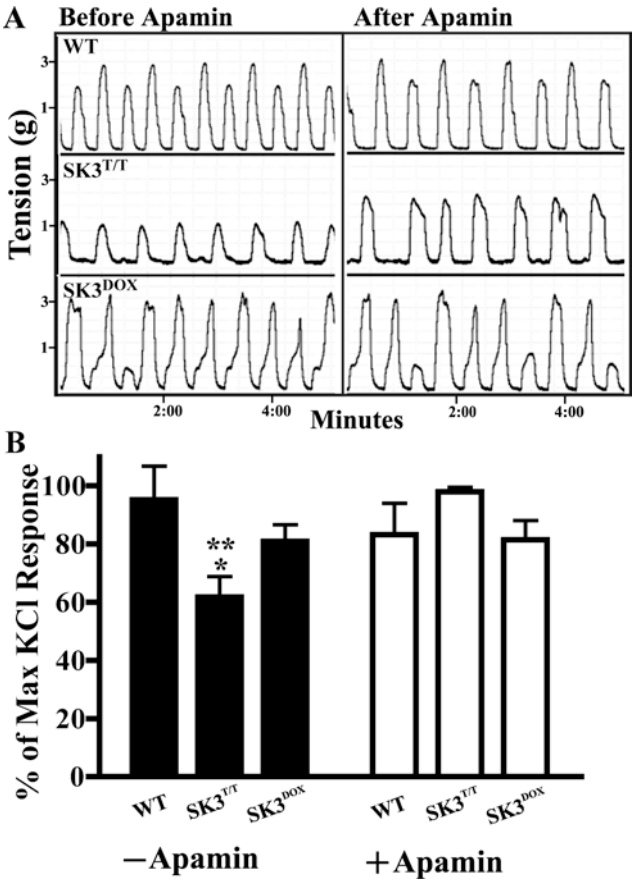
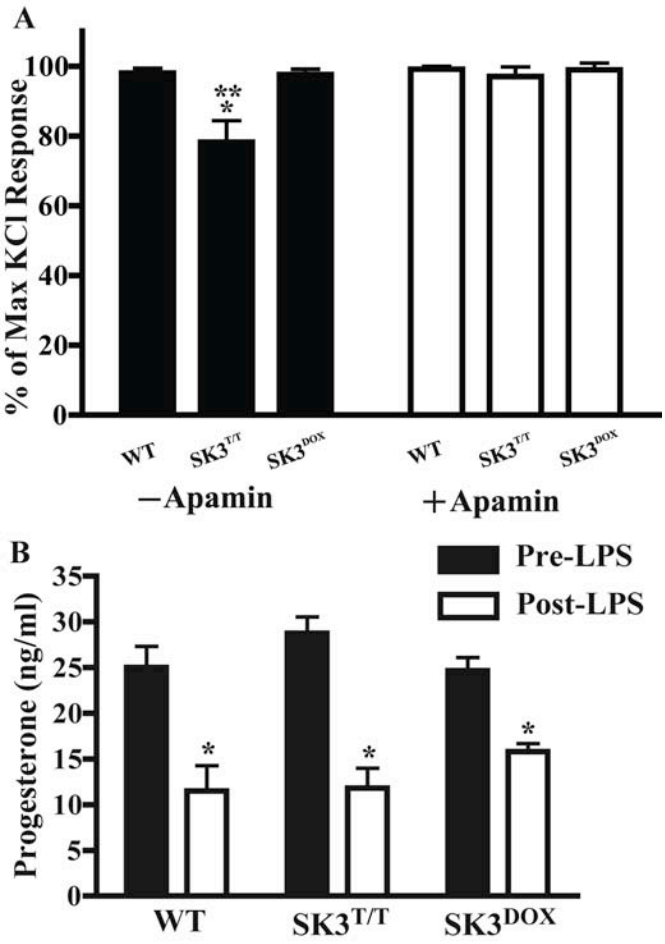

A
Before Apamin
After Apamin
WT
SK3T/T
SK3DOX
Tension (g)
3
1
2:00
4:00
Minutes
B
% of Max KCl Response
100
80
60
40
20
0
**
*
WT
SK3T/T
SK3DOX
−Apamin
+Apamin

Figure 2.4: Average tension development of uterine strips and progesterone levels after LPS injection. (A) Force of spontaneous contractions produced by uterine strips from WT, $SK3^{T/T}$, and $SK3^{DOX}$ mice (percent of maximal KCl response) 8 hours-post LPS and before and after application of apamin (N=5,7 and 5, respectively, Mean ± SEM, * $p < 0.05$ vs. $SK3^{T/T}$ after apamin, ** $p < 0.05$ vs. WT/$SK3^{DOX}$ before apamin). (B) Progesterone levels (ng/ml) were determined via ELISA in serum samples taken pre- and 8 hours post-LPS injection in WT, $SK3^{T/T}$, and $SK3^{DOX}$ mice (N=6,5,5, Mean ± SEM, * $p < 0.05$ vs. pre-LPS).


A
% of Max KCl Response
100
80
60
40
20
0
**
*
WT
SK3T/T
SK3DOX
WT
SK3T/T
SK3DOX
−Apamin
+Apamin
B
Progesterone (ng/ml)
35
30
25
20
15
10
5
0
Pre-LPS
Post-LPS
*
*
*
WT
SK3T/T
SK3DOX

CHAPTER 3

UTERINE TELEMETRIC PRESSURE MEASUREMENTS *IN VIVO*: A NEW APPROACH FOR STUDYING PARTURITION IN MOUSE MODELS

Abstract

A number of transgenic and knockout mouse models have proven useful in the study of genes necessary for parturition—including those genes that affect the timing and/or progression of labor contractions. However, taking full advantage of these models will require a detailed characterization of the contractile patterns in the mouse uterus. Currently the best methodology for this has been the *in vitro* measurement of isometric tension recordings in isolated muscle strips. However this methodology does not provide a real-time measure of changes in uterine pressure over the course of pregnancy. Recent technological advances have theoretically opened the possibility of using radiotelemetric devices to more accurately and comprehensively study intrauterine pressure *in vivo*. We set out to test the effectiveness of this technology in the mouse, in both WT mice and a mouse model of defective parturition (due to expression of a transgene encoding the SK3 channel), several days after surgical implant of telemetry transmitters into the uterine horn. Continuous recordings from day 18 of pregnancy were continued until after delivery. Calculation of average uterine pressure over the course of 12-hour light and dark cycles revealed that WT mice typically deliver during the 12-hour dark period after 19.5 days of pregnancy. In these mice, intrauterine pressure gradually increases during this period, to 3-fold greater than that measured during the 12-hour period prior to delivery. SK3-channel overexpressing mice, by contrast, exhibited lower intrauterine pressure over the same period. These results are consistent with the outcome of previous *in vitro* studies, and indicate that telemetry is an accurate method for measuring uterine contraction, and hence parturition, in mice. The use of this technology will lead to

important novel insights into changes in intrauterine pressure during the course of pregnancy.

Introduction

Preterm deliveries alone account for over 12.6% of all births in the U.S., and are associated with a high percentage (~85%) of perinatal morbidity and mortality (15). Despite increased medical intervention over the past 30 years, preterm birth rates have risen by 28%—with most of these births occurring in women who do not have any of the known risk factors. Risk factors that have been identified are related to four distinct processes, each of which activates multiple signaling pathways and leads to preterm birth: precocious fetal endocrine activation, uterine overdistension, decidual bleeding, and intrauterine inflammation/infection (141).

Genetically modified mouse models have served as effective systems in which to investigate the signaling pathways essential to labor—both preterm and term. Despite certain differences in parturition between human and mouse, these species are similar with respect to many of the pathways that are known to modify pregnancy outcome (105). Mouse models of altered parturition include those in which myometrial contraction (Chapter 2), progesterone withdrawal, abnormal cervical ripening or the circadian rhythm is affected (123). While alterations in individual genes can affect different pathways to parturition, all processes must ultimately lead to a change in myometrial contraction. The characterization of changes in uterine contraction that are associated with abnormal pregnancy in mouse has thus far been limited to the measurement of isometric tension. Although these measurements can provide important information, they are restricted to one stage of pregnancy rather than giving a comprehensive view of contractile and pressure changes over the course of pregnancy and delivery. Furthermore, this assay requires that the animals are euthanized, and thus preclude an assessment of the innate progression of labor or its initiation. These

technical limitations have hindered the amount of information that can be obtained from these mouse models.

Recently, studies in rats demonstrated that radiotelemetry could be used to measure intrauterine pressures *in vivo* (89, 138). Although the size of the radiotelemetric transmitters initially excluded use of this technology to measure intrauterine pressure in mice, the development of a miniature transmitter (1.1 cc) that can measure physiologic pressure (in the range of -20 to 300 mmHg) has enabled us to develop a method that can be utilized to measure intrauterine pressure in mice. We have now applied this procedure to $SK3^{T/T}$ mice, in which uterine contractions are dampened, resulting in delayed or hindered delivery in 70% of dams (8, 120). In addition, we further distinguished the role that SK3 channels play in the labor process. These studies demonstrate that radiotelemetry can be effectively used to study uterine contractions, as part of the full characterization of genetic mouse models of disrupted labor.

Methods

Animals

All animal procedures complied with the guidelines for the care and use of animals set forth by the NIH. The Animal Care and Use Committee at the University of Iowa approved all protocols. $SK3^{T/T}$ mice (gift from John Adelman at the Vollum Institute) were on a C57BL/6 background. WT littermates of $SK3^{T/T}$ mice served as controls. Adult female mice were mated at 8 weeks of age or later. Day 0 of pregnancy was determined based on the presence of a copulatory plug. Animals were housed in Soft-Zorb bedding (Nepco, Warrensburg, NY) and fed standard mouse chow (Harlan Laboratories, 7913).

Surgical procedure

Originally designed for measuring blood pressure in mice (104), the PhysioTel® PA-C10 transmitter (Data Sciences International, St. Paul, MN) was used to measure intrauterine pressure during labor. Prior to implantation, PA-C10 transmitters were cleaned and sterilized according to DSI instructions. Mice were weighed and injected with buprenophrine (3 μg) prior to surgery. Animals were anesthetized with ketamine (91 mg/kg) and xylazine (9.1 mg/kg IP). Using aseptic and sterile technique, an incision approximately 1 inch long was made in the abdomen wall (Figure 3.1A). The uterus was carefully exteriorized and the number of pups in each uterine horn was counted. One horn was selected for catheter implantation, and a small incision was made at the top of the uterine horn (Figure 3.1A). Vessel cannulation forceps (World Precision Instruments, Sarasota, FL) were used to carefully thread the pressure catheter into the uterine horn, between the uterine wall and fetus (Figure 3.1B). A small drop of Vetbond (3M, St. Paul, MN) was applied to the site of insertion and allowed to dry (Figure 3.1C). The transmitter was carefully placed into the lower portion of the abdominal cavity to avoid damage to the liver, and the implanted horn was reinserted into the body cavity. The abdominal wall was then sutured closed and the mouse was monitored until fully awake. Soft food was available to animals from after surgery through to post-delivery to improve maternal outcome.

Induction of preterm labor in WT mice

To test if uterine telemetry can be successfully used to monitor labor in an induced model of preterm labor, we implanted WT mice as described above. Recordings were started on P14, and at 1:00 PM on P15 the mice were injected with RU486 (100 μg; Sigma, St. Louis, MO) in ethanol, as described previously (34, 120). Uterine pressure was measured after 24 h.

Data acquisition and statistical analysis

Telemetry recordings were performed at 500 Hz, using a Dataquest ART data acquisition system version 4.10 (Data Sciences International, St. Paul MN). Continuous recordings were begun on day P18 (P14 in preterm labor model), and continued through day P22 or until after delivery, whichever occurred first. The light and dark cycles of the mice were noted for each delivery. The changes in pressure between the 12-hour cycle in which the mouse delivered were compared to those during the 12-hour cycle preceding delivery. Hourly averages were calculated using Dataquest A.R.T. 4.2 software (Data Sciences International, St. Paul, MN), which averaged all data points from within an hour.

Data gathered by the telemetry transmitters during the 12 hour light or dark period preceding delivery and the 12-hour light or dark period in which delivery occurred were used to examine the power spectrum of the pressure, as Fast Fourier Transformations generated using Dataquest A.R.T. 4.2 software (Data Sciences International, St. Paul, MN). This allows us to capture the frequency information of the pressure oscillations and identify periodicities. All out-of-range data points (which usually result from gross body movements during the recording period) were replaced by points calculated from interpolation between adjacent points. Results from the Fast Fourier Transformation analysis ranged between 0.00039 and 0.03398 Hz (i.e. events with periods ranging from 29.4 seconds to 42 minutes, 44 seconds). The power spectrum of pressures calculated from the 12-hour period in which delivery occurred was normalized for each frequency bin, by the power calculated from the 12-hour period before delivery for each animal.

All data are presented as mean ± SEM. In all cases, N refers to the number of animals. Statistical significance was determined by one-way ANOVA, or two-way ANOVA where appropriate, followed by post-hoc comparison using Student t-tests. Significance was determined at $p < 0.05$.

Results

Intrauterine pressure increases during labor in WT mice

To determine if intrauterine pressure can be measured during labor in mice, we implanted pressure transmitters into WT mice (C57BL/6, which typically deliver at 19.5 days of gestation) on P8-9 of gestation, as this resulted in optimal fetal outcome in these animals. Continuous measurement of pressure was initiated at P18 and continued through the time of delivery. Because disturbing a mouse during parturition can cause the dam to cease labor (110), mice were monitored briefly near the beginning of each light cycle and just before each dark cycle to determine if delivery had occurred. Pressure peaks visibly increased in magnitude and number at the time coinciding with labor and delivery (Figure 3.2). $SK3^{T/T}$ mice were implanted similarly, but optimal fetal outcome was established on P13-14 of gestation. Measurements of intrauterine pressure during delivery in WT and $SK3^{T/T}$ mice were compared. This revealed that the oscillations measured by telemetry for the WT and $SK3^{T/T}$ mice (Figure 3.3A and B, respectively) mimicked those observed by phasic measurement of isometric tension in myometrial strips isolated from the same strains at P19 (Figure 3.3C and D, respectively) (9). However, the *in vivo* pressure oscillations during parturition occurred at longer intervals than the force oscillations measured by the recording of isometric tension (Figure 3.3A,B vs. Figure 3.3C,D). The observed similarities in the oscillations suggest that the intrauterine pressure readings are good indicators of uterine contractile activity. The differences in timing underscore the notion that *in vitro* measurements do not accurately reflect all aspects of these oscillations (perhaps because they are not subject to modification by endogenous factors that likely contribute to the oscillatory nature of pressure in the uterus).

To better understand how pressures change during pregnancy, we measured average pressures (on an hourly basis) before and during pregnancy. The hourly averages

calculated during the 12-hour period in which the mouse delivered were much lower than the individual pressure spikes during delivery, evident in Figure 3.2. However, the overall hourly average pressures during delivery were greater than baseline pressure, which was determined from readings during the 12-hour period preceding delivery (Figure 3.4). The average basal intrauterine pressure was 6.9±1.4 mmHg during the light or dark period preceding the period in which delivery occurred. The average intrauterine pressures in the cycle during which delivery occurred increased gradually, peaking at 25.1±5.6 mmHg, which is 3-fold greater than the maximal pressure during the preceding 12-hour period (Figure 3.4A).

Having determined, based on telemetry recordings, that average intrauterine pressure increases during delivery we next compared the shift in peak pressures generated during parturition more directly. To this end, we compared the frequency of the various pressure oscillations attained for the 12-hour period prior to and the 12-hour period during delivery. Using Dataquest A.R.T. 4.2 software (Data Sciences International, St. Paul, MN), we were able to generate a histogram binned into 10 divisions of 4 mmHg each between pressures of 0-40 mmHg, in order to assess whether there was a shift to greater pressures in the light/dark cycle in which delivery occurred. As seen in Figure 3.4B, the frequency distribution of the pressure during delivery shifted to the right, indicating that a greater number of peaks occurred at higher pressures during the cycle in which delivery occurred (Figure 3.4B). This indicates that the transmitters can measure significant pressure changes in the uterus, consistent with labor.

Induced preterm labor leads to increased intrauterine pressure

In order to determine if radiotelemetry can be used earlier in gestation with a model of induced preterm delivery, we induced labor in WT mice by injecting 100μg RU486 on P15 (120). Injection was always carried out at 1:00 pm, and all mice delivered

within 24 hours of injection. Hourly averages demonstrated that intrauterine pressure consistently began to rise approximately 8-hours post injection (Figure 3.5). Approximately 6 hours later, pressure peaked, signifying delivery, consistent with previous studies demonstrating that this is typically when mice deliver following RU486 injection (45). Uterine pressure returned to baseline following delivery. This experiment suggests that radiotelemetry can be utilized as early as P15 in mice induced to undergo labor preterm.

Mouse model of delayed labor exhibits reduced intrauterine pressure during labor cycle

Having successfully produced intrauterine recordings in WT mice, we wanted to investigate the novel information radiotelemetry could detect in a mouse model of disrupted parturition. We used $SK3^{T/T}$ mice that, in contrast to their WT counterparts, had the best fetal outcome when surgery was performed on P13-14. Recordings began on P18 and continued until delivery or P22. Since most $SK3^{T/T}$ mice do not deliver on the appropriate gestational day, we compared the time periods that coincided with typical WT delivery. Uterine pressures in the $SK3^{T/T}$ mice remained near baseline and did not show an increase in pressure like that seen in WT mice during their delivery cycle (Figure 3.6). When $SK3^{T/T}$ mice were able to deliver pups, they did not exhibit the gradual increase in pressure seen during the course of delivery in WT mice. Although several $SK3^{T/T}$ mice produced intrauterine pressures above-baseline that was sufficient for delivery, they were usually not of the same magnitude as those of their WT counterparts (Figure 3.7). The few $SK3^{T/T}$ mice that did exhibit pressures of the same magnitude as WT mice were not able to sustain these pressures, a deficiency that could have contributed to their failure to deliver in a time frame similar to that typical for WT mice (P19.5 days).

Power spectrum analysis of intrauterine pressure during pre-labor and labor in WT and SK3$^{T/T}$ mice.

To identify the frequencies of the intrauterine pressure waves that change during the 12-hour period in which parturition occurs, we performed Fast Fourier Transformations for the pressure recordings of the 12-hour light or dark period preceding delivery and the 12-hour period in which delivery occurred. In WT mice, the power spectrum of intrauterine pressure increased during the period in which delivery occurred relative to the power spectrum for the 12-hour period before delivery (Figure 3.8A). These increases in power were significantly greater in the case of contractile frequencies centered on 0.0038, 0.0065, 0.0161, 0.0238, 0.02778, and 0.0333 Hz. Although SK3$^{T/T}$ mice also exhibited an apparent increase in the power spectrum of the intrauterine pressure during parturition, this difference was not significant given the extreme variance between animals (Figure 3.8B). When comparing the power spectrum of the WT versus SK3$^{T/T}$ mice for the period of delivery, we found that WT mice generated significantly higher pressures, at frequencies of 0.0064, 0.0102, 0.0127, and 0.0263 Hz, as compared to SK3$^{T/T}$ mice, whose power peaked at similar frequencies but not to the same extent (Figure 3.8C). The rise in power at 0.005-0.010 Hz that occurred during WT labor was not produced during SK3$^{T/T}$ labor (Figure 3.8C), indicating that contractions in that range of frequencies may be dampened by the overexpression of SK3 channels.

Discussion

Most studies that investigate myometrial contractility have had to rely on *in vitro* measurements of tension. Such methods are useful, and will continue to be necessary for studying uterine contractions early in gestation (when the uterus contracts asynchronously), as well as for investigating the effects of drugs, toxins and other solutions that cannot be administered to a live animal. However, *in vivo* approaches are necessary in some contexts, for example, when investigating the relationship of

contractility to the progression of labor or the time at which labor begins. In addition, getting a complete picture of changes in uterine contractile pattern over the course of pregnancy through *in vitro* approaches would require sacrificing large numbers of animals. Furthermore, the *in vitro* approach requires that uterine strips are mounted either vertically or horizontally, which limits investigation of the contractions to those produced either by the circular or longitudinal muscle layer of the uterus; as such, removing a section from a late-gestation uterus to study its contractility *in vitro* both changes contractile fiber orientation and fails to account for most endogenous physiological neurohumoral influences. The use of uterine telemetry eliminates these obstacles, making it possible to more accurately study parturition in mouse models.

One mouse model that exhibits compromised parturition is the $SK3^{T/T}$ mouse that was used in this study (8). Using an *in vitro* approach, we previously demonstrated that uterine contractions in these mice were dampened (Chapter 2), suggesting that altered uterine function leads to dysfunctional delivery (120). Our application of a more advanced methodology, radiotelemetric technology, enabled us to more accurately study the SK3 channel's contribution to myometrial contraction. We found that whereas intrauterine pressure gradually increased 3-fold during delivery in WT animals, that in $SK3^{T/T}$ mice failed to increase to a similar extent at the same stage of gestation. Furthermore, we demonstrated that excess SK3 channels result not only in dampening of uterine contractions, but also in a reduction of the frequency of contraction in the cases when they did deliver. During delivery in $SK3^{T/T}$ mice, basal pressure did not increase gradually as in delivery in WT mice, indicating that even when these mice are able to produce pressures adequate for delivery, the overall elevation in basal pressure may not be sufficient to ensure consistent delivery on P19.5. This prior unknown function of SK3 channels in uterine activity would not have been discovered in the absence of radiotelemetry. Thus, studying uterine contraction in a live animal has given us the

capacity to obtain more precise details about the mechanisms underlying parturition, and to extract new information that will provide insights into the process of parturition.

Although radiotelemetry was successfully used in mice, one of the challenges that had to be overcome was fetal loss following implant of the telemetry transmitter. Modifying the time of implantation according to strain made successful term pregnancies more consistent, but full litters were not produced. Given that intrauterine pressure in WT mice did not correlate with delivery of larger litters versus those that delivered smaller litters, it seems that the number of pups to be birthed does not affect overall intrauterine pressure. The fact that the optimal time of surgery for $SK3^{T/T}$ mice was different for that in WT mice suggests that modifications to the surgery protocol may be necessary for each mouse model of interest. Fetal loss following telemeter implant was not completely alleviated in either the WT or the mutant strain, but fetal outcome is expected to improve as the transmitter technology is refined. This will undoubtedly advance the usefulness of this technology in studies of the mechanisms that contribute to labor.

Our *in vivo* recordings demonstrate that intrauterine pressure measurements in mouse yield data consistent with those obtained by *in vitro* tension measurements, indicating that this approach can be used to accurately quantify uterine activity. As in the telemetry-based study in rat uteri, we found that intrauterine pressure in WT mice increased at the end of pregnancy. We also discovered that not only the baseline pressure, but also the number of contractions that produce higher pressure, rise. Of further interest is the fact that the WT uterus showed a shift in the pressure produced during delivery compared to that produced during the previous12-hour period (Figure 3.8), with the maximal WT intrauterine pressure reached during delivery as high as 189±31 mmHg (Figure 3.3). These findings illustrate that the information obtained from *in vivo* uterine recordings will provide useful new details about the genes that are targeted

in mouse models of parturition, which will in turn lead to a better understanding of the mechanistic basis of labor and delivery in humans.

Genetic predisposition is one risk factor for preterm delivery (30, 121). As data from the Genome-Wide Association Study (GWAS) becomes available, multiple genes will be identified that may impact the relative risk of preterm labor. Subsequently, these genes can be targeted in mouse models in order to further study their function during parturition. Thus, technological advances that allow more precise testing of myometrial contractions in mice are expected to greatly impact the field of prevention and treatment of preterm labor by facilitating the identification of novel mechanisms underlying parturition.

Many genetically altered mice have been used to study single-gene disorders in which the regulation of parturition is defective. Notably, as pointed out by Mitchell and Taggart in a recent review, surprisingly little attention has been given to those mice that exhibit altered myometrial contraction (105). While these authors use this point to emphasize the need to understand how these mouse phenotypes relate to human parturition, we also must fully explore the mechanisms by which these models disrupt or accelerate the process of labor, even when the mechanism is presumed unrelated to uterine contraction. Thus, the field would benefit greatly from an advanced technique, such as radiotelemetry, that makes it possible to measure intrauterine pressure in mice.

Figure 3.1: Surgical procedure for implanting uterine telemetry transmitters. (A) A small cut is made at top of the uterine horn. (B) The pressure-catheter lead is carefully pushed into the uterine horn between the fetal sac and uterine wall. (C) A small drop of VetBond© is applied to the incision site to secure the catheter. The transmitter body is placed inside the lower portion of the body cavity, and uterine horn is replaced into the body cavity without disturbing the transmitter implant.

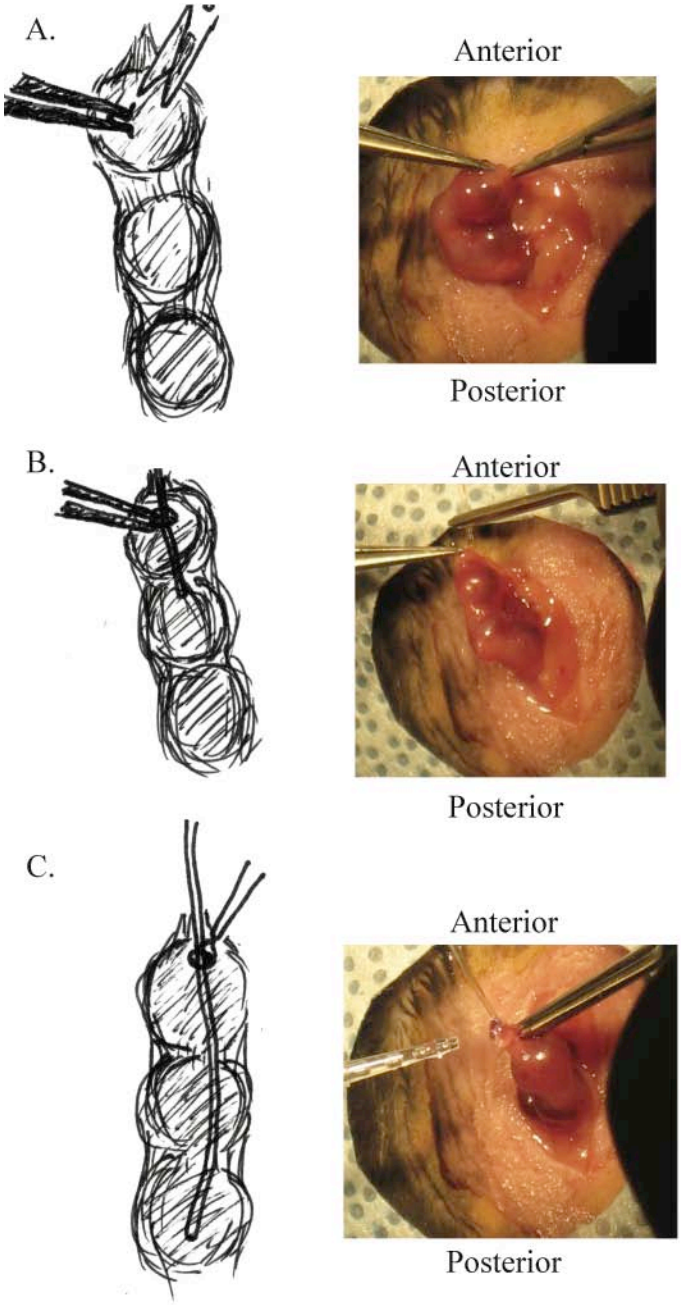

A.
Anterior
Posterior
B.
Anterior
Posterior
C.
Anterior
Posterior

Figure 3.2: Continuous recording of intrauterine pressure. The graph shows an example of WT intrauterine pressure beginning on P18.5 and continuing through P 20.5, revealing that spikes in intrauterine pressure increase in magnitude and frequency during labor. Alternating white and black bars above the graph indicate light and dark cycles, respectively. The lower black bar indicates the time of delivery.

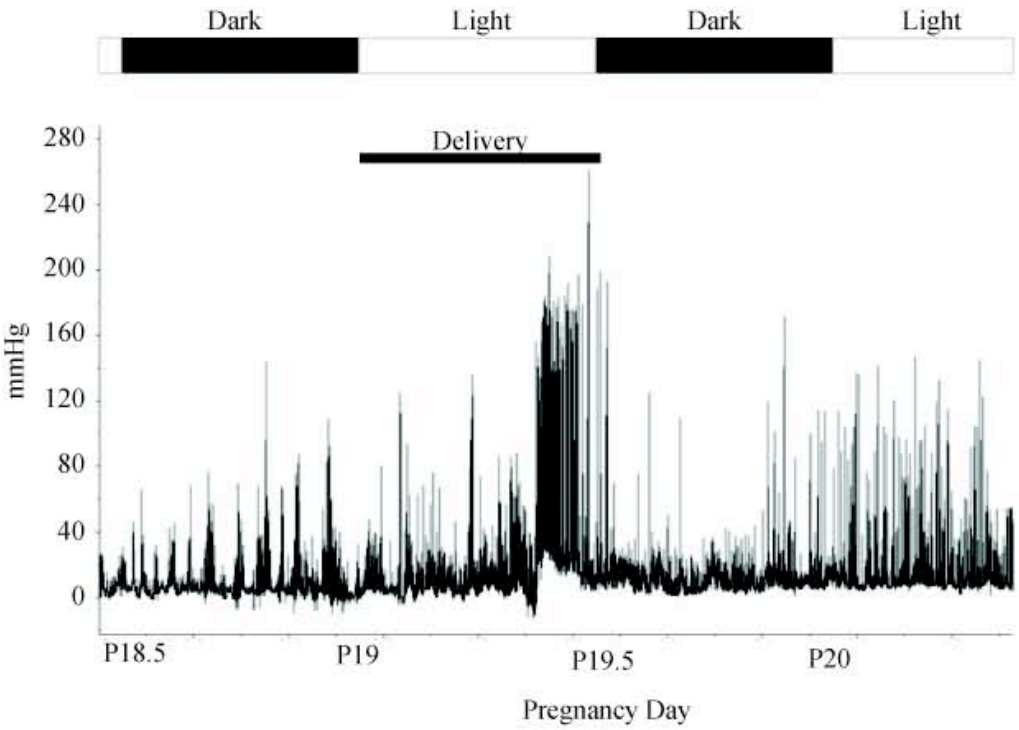

Dark
Light
Dark
Light
Delivery
280
240
200
160
120
80
40
0
mmHg
P18.5
P19
P19.5
P20
Pregnancy Day

Figure 3.3: Comparison of *in vivo* and *in vitro* recordings from mice during pregnancy. (A) Sample tracing of WT intrauterine pressure recorded *in vivo* during delivery. (B) Tracing of $SK3^{T/T}$ intrauterine pressure recorded *in vivo* during delivery, demonstrating that pressure waves are not produced as rapidly as in WT mice, and that those produced are not of the same magnitude. (C) Representative isometric tension recording from myometrial strips of a P19 WT mouse, demonstrating a wave pattern similar to that recorded by *in vivo* measurement. (D) Isometric tension recording from a P19 $SK3^{T/T}$ uterus revealed that the frequency and strength of tension peaks are reduced, consistent with results from *in vivo* recordings

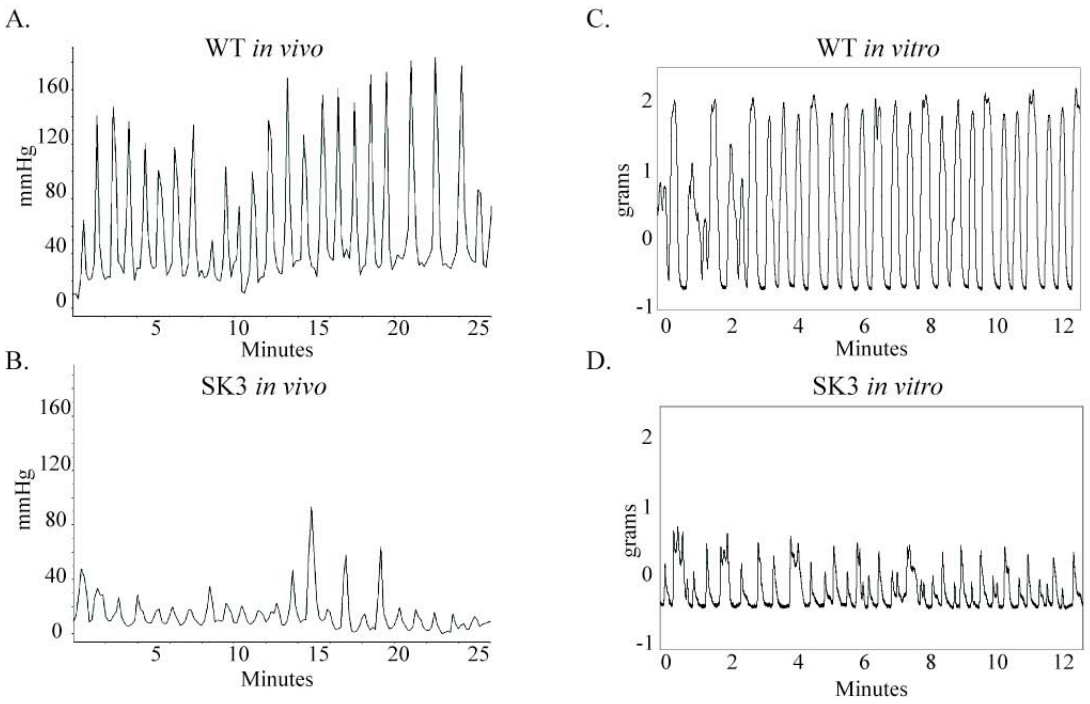

A.
WT *in vivo*
mmHg
160
120
80
40
0
5
10
15
20
25
Minutes
B.
SK3 *in vivo*
mmHg
160
120
80
40
0
5
10
15
20
25
Minutes
C.
WT *in vitro*
grams
2
1
0
-1
0
2
4
6
8
10
12
Minutes
D.
SK3 *in vitro*
grams
2
1
0
-1
0
2
4
6
8
10
12
Minutes

Figure 3.4: Fold change in baseline intrauterine pressure, as measured by telemetry, in WT mice during pregnancy. (A) An hourly moving average was calculated for both the 12 hours before delivery (filled circle) and the 12 hours in which delivery occurred (filled square) (N=7). (B) A histogram comparing the frequency of a given pressure for the12-hour period before delivery and for the 12-hour period in which delivery occurred. Pressure shift to the right was seen in the 12 hours during delivery (N=7). Mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

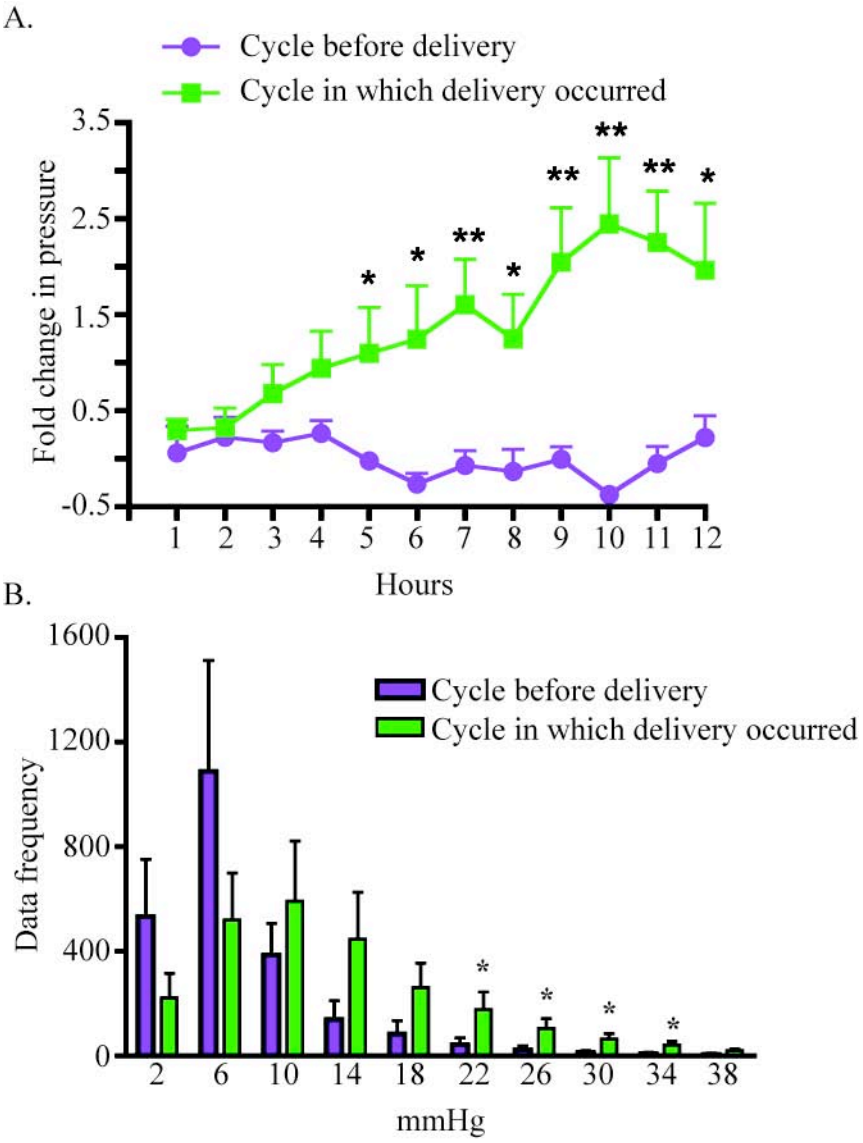

A.
Cycle before delivery
Cycle in which delivery occurred
Fold change in pressure
3.5
2.5
1.5
0.5
-0.5
1 2 3 4 5 6 7 8 9 10 11 12
Hours
B.
Cycle before delivery
Cycle in which delivery occurred
Data frequency
1600
1200
800
400
0
2 6 10 14 18 22 26 30 34 38
mmHg

Figure 3.5: Intrauterine pressure (hourly average) during induced preterm labor in WT mice. Recordings began at 11:00AM and mice were injected with RU486 at 1:00PM (indicated by the arrow). Intrauterine pressure began to increase approximately 8 hours post-injection and peaked 6 hours later.

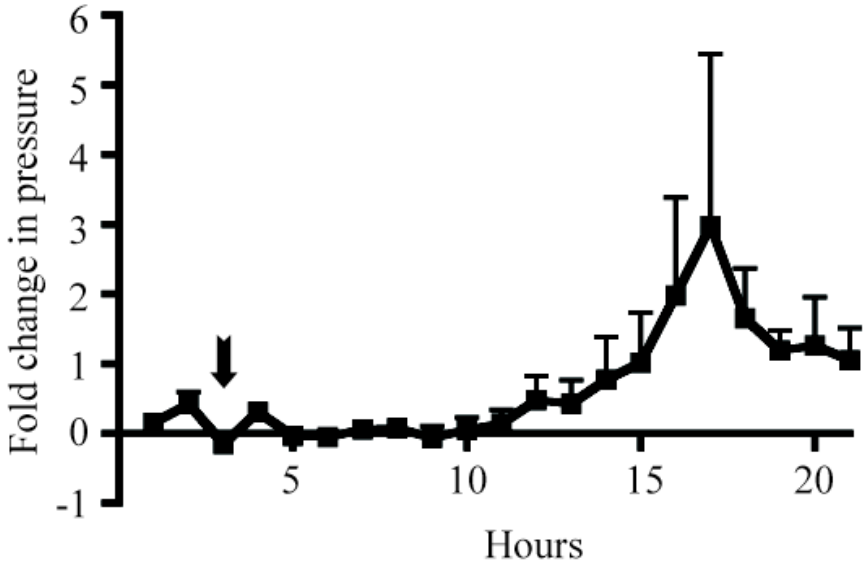

Fold change in pressure
6
5
4
3
2
1
0
-1
5
10
15
20
Hours

Figure 3.6: Baseline intrauterine pressure (hourly average) during pregnancy in $SK3^{T/T}$ versus WT mice. In $SK3^{T/T}$ mice that were not able to deliver by P19.5, basal intrauterine pressure did not increase according to the dark cycle in which delivery occurred as it does in WT mice (WT data duplicated from Figure 3.4). $SK3^{T/T}$ mice maintained an intrauterine pressure consistent with the baseline pressure seen in WT mice during the 12-hour cycle before delivery. (WT N=7, SK3 N=3).

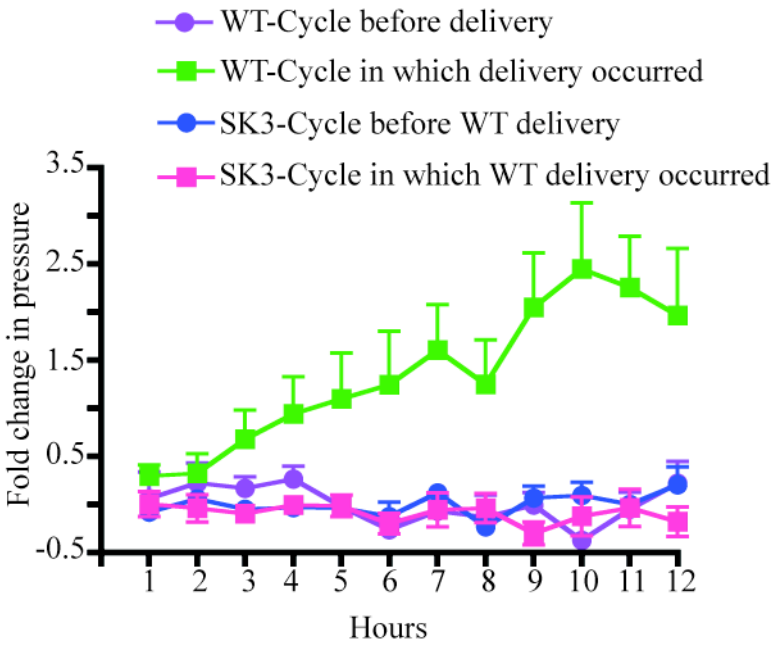

WT-Cycle before delivery
WT-Cycle in which delivery occurred
SK3-Cycle before WT delivery
SK3-Cycle in which WT delivery occurred
Fold change in pressure
3.5
2.5
1.5
0.5
-0.5
1
2
3
4
5
6
7
8
9
10
11
12
Hours

Figure 3.7: Hourly averages of $SK3^{T/T}$ intrauterine pressure during delivery. Baseline intrauterine pressure (hourly average) increased above baseline in $SK3^{T/T}$ uteri during delivery, but not to the same extent as that seen during WT delivery (WT data duplicated from Figure 3.4). Baseline intrauterine pressure in $SK3^{T/T}$ mice during delivery also was not gradually elevated as seen in WT mice during delivery. (WT=7, SK3 N=4). Mean ± SEM. * $p < 0.001$ vs. WT-Cycle before delivery, † $p < 0.05$ vs. WT-Cycle in which delivery occurred.

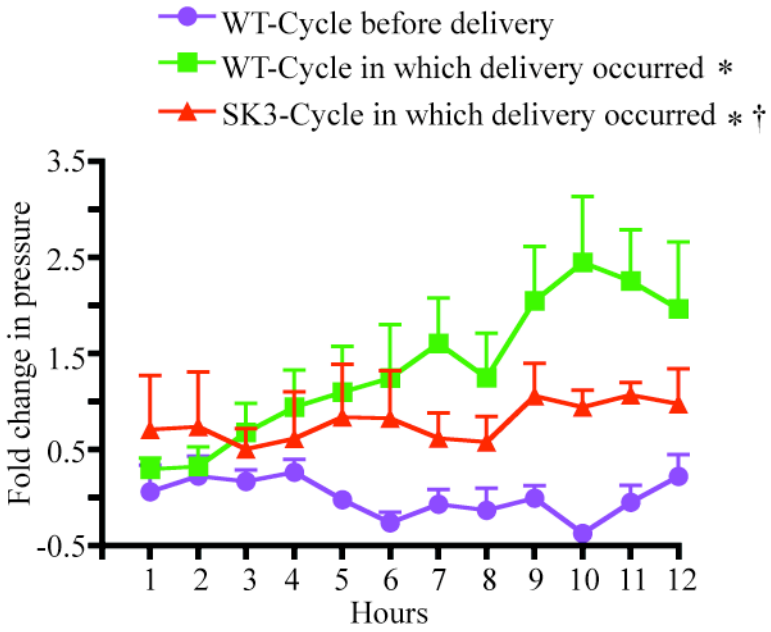
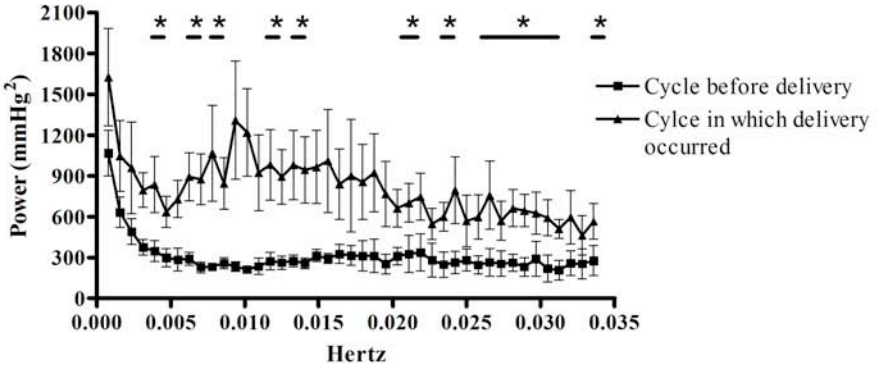
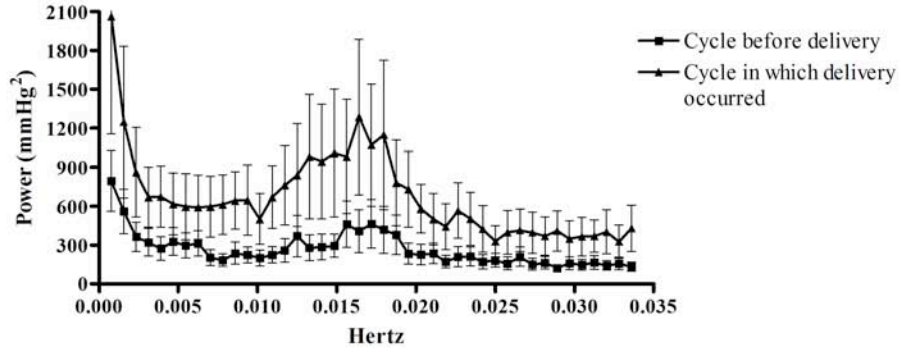

WT-Cycle before delivery
WT-Cycle in which delivery occurred ∗
SK3-Cycle in which delivery occurred ∗ †
Fold change in pressure
3.5
2.5
1.5
0.5
-0.5
1 2 3 4 5 6 7 8 9 10 11 12
Hours

Figure 3.8: Power spectrum of intrauterine pressure during the 12 hours before delivery and the 12 hours in which delivery occurred. (A) The power spectrum of WT baseline intrauterine pressure produced in the 12-hour cycle during delivery increased above the baseline frequency measured during the 12-hour cycle before delivery. Mean ± SEM. (N=4), * $p < 0.05$ for period of pre-delivery vs. period in which delivery occurred. (B) The power spectrum generated from $SK3^{T/T}$ intrauterine pressure during the 12-hour cycle in which delivery occurred was not significantly elevated above that for the 12-hour cycle before delivery. (C) Comparison of the normalized power spectra of WT and $SK3^{T/T}$ intrauterine pressure during the 12-hour cycle in which delivery occurred, demonstrating that at certain frequencies more power was produced during WT delivery. Mean ± SEM. (N=4 for both WT and $SK3^{T/T}$), * $p < 0.05$ vs. WT-Cycle in which delivery occurred.

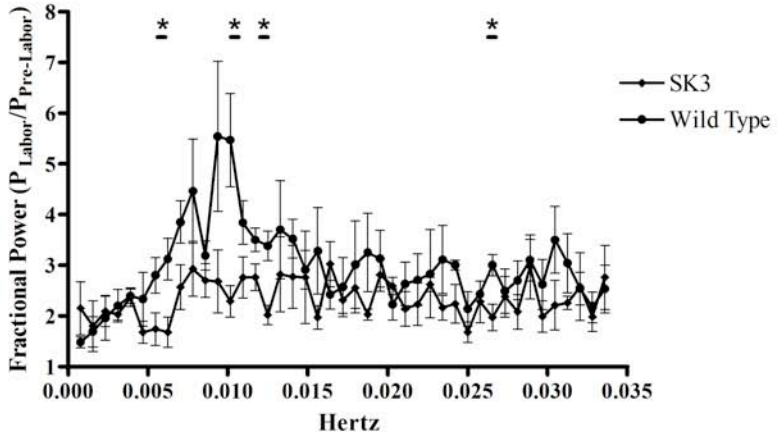
A.


Power (mmHg^2)
2100
1800
1500
1200
900
600
300
0
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.035
Hertz
Cycle before delivery
Cylce in which delivery occurred

B.


Power (mmHg^2)
2100
1800
1500
1200
900
600
300
0
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.035
Hertz
Cycle before delivery
Cycle in which delivery occurred

C.


Fractional Power ($P_{Labor}/P_{Pre-Labor}$)
8
7
6
5
4
3
2
1
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.035
Hertz
SK3
Wild Type

CHAPTER 4
SK3 CHANNEL EXPRESSION DURING PREGNANCY IS REGULATED THROUGH ESTROGEN AND SP FACTOR-MEDIATED TRANSCRIPTIONAL CONTROL OF THE *KCNN3* GENE

Abstract

Overexpression of the SK3 in transgenic mice compromises parturition, suggesting that SK3 channels play a role in pregnancy. In WT mouse myometrium, expression of SK3 transcript and protein is significantly reduced during pregnancy, but the mechanism(s) responsible for this attenuation of channel expression is unknown. The promoter region of the SK3-encoding mouse *KCNN3* gene contains two binding sites for Sp transcription factors, two of which are expressed in the uterus: Sp1, which enhances gene transcription in response to estrogen; and Sp3, which competes for the same binding motif as Sp1 and can repress gene expression. We investigated the hypothesis that Sp1 and Sp3 regulate SK3 channel expression during pregnancy. In mouse myometrium, Sp1 expression was reduced during late gestation, whereas Sp3 expression levels were constant throughout pregnancy. Using a reporter system, Sp1 overexpression significantly increased SK3 promoter activation and Sp3 co-transfection reduced promoter activation to basal levels. These findings indicate that Sp3 outcompetes Sp1 to decrease SK3 transcription. To determine if high levels of estrogen *in vivo* could affect Sp factors regulation of SK3 protein levels, ovariectomized mice were implanted with a 17ß-estradiol or placebo pellet for three weeks. Estrogen-treated mice had reduced uterine SK3 protein expression compared to placebo-treated. In human myometrial cells overexpressing Sp1, estrogen treatment stimulated expression of SK3 transcript. Overall, our findings indicate that Sp1 and Sp3 compete to regulate SK3 channel expression during pregnancy, in response to stimulation by estrogen.

Introduction

Current tocolytics, medications used to stop preterm labor, are either ineffective or prolong gestation for only 24-48 hours, emphasizing the need for improved methods of preventing preterm labor. Potassium (K^+) channels are logical targets for tocolytics since their activation produces repolarizing and hyperpolarizing currents that relax myometrium. Additional investigation is needed, however, to confirm that manipulating K^+ channel function can prevent premature labor contractions. One channel of particular interest is the SK3 (105). Seventy percent of mice that overexpress SK3 channels have a parturition defect that results in either delayed or failed delivery (8). When $SK3^{T/T}$ mice were induced to deliver prematurely with lipopolysaccharide or RU-486, they were unable to fully expel all fetuses, unlike their WT and functional knockout counterparts (120). Our laboratory has shown that uteri of SK3 mice produce less forceful contractions *in vitro*, which may contribute to their inability to deliver (Chapter 2) (120).

In uterine tissue from WT mice, the expression of SK3 channel transcript and protein decreases as pregnancy progresses (Chapter 2) (120), suggesting that downregulation of the SK3 gene, *KCNN3*, may be necessary for parturition. However, the mechanism responsible for the down-regulation of SK3 expression is unknown. A change in the promoter activity of the *KCNN3* gene is one mechanism that could account for the down-regulation of SK3 expression in the uterus prior to delivery. E2, a sex hormone required for the maintenance of pregnancy, enhances SK3 transcript expression despite the fact that *KCNN3* does not contain an estrogen response element (9, 60). Rather, the promoter region of *KCNN3* contains two GC-rich regions that represent binding sites for Sp transcription factors (118, 135, 146). Of this class of transcription factors, Sp1 and Sp3 are expressed ubiquitously, have the same DNA binding properties, and share a similar protein structure (12, 31). Sp1 is a transcriptional activator, whereas Sp3 activates or represses transcription depending on the environment of the promoter (31, 135). Sp3 has multiple isoforms, including short isoforms (Sp3si) that are

transcriptionally inactive and long isoforms (Sp3li) whose transcriptional activity can change based on the promoter context. ERα enhances the ability of Sp1 and Sp3 to bind to DNA. *KCNN3* transcription is increased in the presence of Sp1, and mutational analysis has shown that Sp binding sites on the SK3 promoter are necessary for ERα-mediated enhancement of SK3 expression (60). However, the mechanism underlying transcriptional regulation of this channel—which is a key contributor of myometrial quiescence during pregnancy—has not been identified. Here we assessed whether Sp factors regulate transcriptional activity and expression of SK3 channels during pregnancy.

Materials and methods

Animals and breeding protocol

All animal procedures used in this study complied with the guidelines for the care and use of animals set forth by the NIH, and were approved by the Animal Care and Use Committee at the University of Iowa. Adult C57BL/6 female mice were mated between 8 weeks to 8 months of age. Day 0 of pregnancy was determined based on the presence of a copulatory plug. Mice were euthanized by CO_2 inhalation on specific days relative to pregnancy duration (NP, P7, P14, P18 and PP2).

Human tissue collection

Human myometrial tissue from the lower uterine segment was obtained from patients who, in the absence (non-labor (NL)) or presence (laboring (L)) of labor contractions (spontaneous or induced), underwent Cesarean section during late pregnancy (38-40 wk gestation) while under spinal anesthesia. NP tissue was isolated from uteri removed for hysterectomies. All patients signed written consent forms approved by the University of Iowa's Internal Review Board (approval no. 199809066).

Immunoblotting

Mouse and human uterine tissues were isolated, flash frozen, and homogenized, and whole cell lysates were prepared as previously described (4). Following protein quantitation by BCA, equal amounts of protein were separated by SDS-PAGE. Immunoblots using mouse protein were probed with rabbit polyclonal anti-Sp1 or anti-Sp3 (1:250 dilution, Santa Cruz Biotechnology Inc, Santa Cruz, CA) and HRP-conjugated goat anti-rabbit secondary antibody (1:3000 dilution, Pierce, Rockford, Il). Immunoblots using human protein were probed with rabbit polyclonal anti-SK3 N-terminal primary antibody (1:100 dilution, Alomone Labs, Jerusalem, Israel) and HRP-conjugated goat anti-rabbit Fc secondary antibody (1:3000 dilution, Pierce, Rockford, Il). To assure equal loading, the blots were re-probed with anti-GAPDH primary antibody (1:1000, Chemicon, Temecula, CA) and HRP-conjugated mouse anti-goat secondary antibody (1:3000 dilution, Jackson ImmunoResearch, West Grove, PA). Signal was detected by chemiluminescence (ECL Western Blotting Detection Reagents, Buckinghamshire, UK or SuperSignal West Femto Maximum Sensitivity Substrate Thermo Scientific, Rockford, IL). Sp1/Sp3 and SK3 protein expression was quantitated using densitometry (ImageJ, NIH) and normalized to GAPDH.

Design of SK3 promoter construct

An 875 bp fragment (containing both Sp binding sites) of the 5' promoter region of the *KCNN3* gene was prepared by PCR amplification of mouse uterine genomic DNA, using a sense primer containing a KpnI restriction site and an antisense primer containing a SacI restriction site (5' CAGGAGTGGGTCCCTTCTGC 3', 5' TTGGGGCCTGGCTGAGTT 3'). The PCR product was purified by agarose gel electrophoresis, extracted from gel slices (QIAquick; Qiagen Inc., Chatsworth, CA) and cloned into the pCRII vector (Invitrogen, San Diego, CA). Following restriction digestion with KpnI and SacI, the SK3 promoter fragment was directionally cloned into

the pGL3-Basic luciferase reporter vector (Promega, Madison, WI) between unique KpnI and SacI sites, to generate the SK3/luciferase reporter construct. The plasmid constructs pPac, pPac Sp1, pPac Sp3 Complete (short isoform, Sp3si), and pPac Sp3 New (long isoform, Sp3li) were a kind gift from Dr. Guntram Suske.

SL2 transfection and bioluminescence imaging

Schneider's *Drosophila* cell line 2 (SL2) (Invitrogen, Carlsbad, CA) was cultured in Schneider's insect medium supplemented with 10% heat-inactivated fetal bovine serum and 50 units/mL penicillin/streptomycin at room temperature. SL2 cells (3×10^6 cells per well in 6-well plate) were seeded the day before transfection. Cells were transfected with the SK3/luciferase construct and the pPac, pPac Sp1, pPac Sp3 short isoform (si), or pPac Sp3 long isoform (li) using the calcium phosphate method. The medium was changed 24 hours post transfection, and 1×10^5 cells of each transfection were analyzed with the Luciferase Assay System (Promega, Madison, WI) for bioluminescence, using an IVIS 100 imaging system (Caliper Life Sciences, Hopkinton, MA).

Transfection of adherent human myometrial cells

Human myometrial tissue from non-laboring patients were used to isolate hMSMCs within 1-3 h of collection, as previously described (75). For these experiments, adherent cells were trypsinized, plated in fresh dishes, and grown for two additional days until they reached 70-80% confluency, at which point they were transfected with constructs pN3 and pN3-Sp1FL-Complete (gifts from Dr. Guntram Suske) using Lipofectamine LTX and PLUS reagent (Invitrogen, Carlsbad, CA), following the instructions provided.

RNA Isolation and qPCR analysis

The Total RNA mini kit (BioRad, Hercules, CA) was used to obtain total RNA from human uterine tissue and cultured hMSMCs. Total RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA) to generate cDNA. The cDNA was amplified in triplicate with SYBR Green Supermix (BioRad, Hercules, CA), using primers specific for human *SK3* channels (5' GAGCGTCAAGATGGAACAGA 3', 5' ATCTTGGAAAGGTCCACCAG 3'), Sp1 (5' GCTACCTTGACTCCCAGCTC 3', 5' TGGAACTGGCTTGTGATGAT 3') or GAPDH (5' TCAAGAAGGTGGTGAAGCAG 3', 5' CGCTGTTGAAGTCAGAGGAG). GAPDH served as a standard to normalize gene expression.

Ovariectomy and E2 pellet implantation of C57BL/6 mice

Mice were anesthetized with IP ketamine (91 mg/kg) xylazine (9.1 mg/kg IP) mix and ovariectomized as previously described (41). Twenty-one day release 17ß-estradiol 0.5 mg pellets or placebo pellets (Innovative Research, Sarasota, FL) were implanted subcutaneously at the time of the surgery. Three weeks post-surgery, mice were euthanized by CO_2 and blood extracted for measurement of serum estradiol, using the EIA kit (Cayman Chemical, Ann Arbor, MI). The uterus was removed, flash frozen in liquid nitrogen and stored at -80ºC for protein isolation (for placebo treated mice, 3 uteri were pooled together per sample) and immunoblotting.

Statistical analysis

All data are presented as mean ± SEM. Statistical significance was determined by one-way and two-way ANOVA where appropriate, followed by post-hoc comparison using Student t-tests. Non-parametric data analysis was used to evaluate hMSMC transfection experiments, with "0" assigned for a less than 10% change in expression, "+" for a 10% increase in transcript expression, and "-" for a 10% decrease in transcript

expression compared to control vector. Significance was determined at $p < 0.05$. N refers to the number of animals in all cases.

Results

The Sp1:Sp3 protein ratio shifts during mouse gestation.

We began our study by assessing whether the levels of Sp1 and Sp3 protein change during pregnancy. Western blotting showed that the expression of Sp1 protein was prominent in NP and P7 myometrium but decreased significantly by P14 and remained low until after delivery (Figure 4.1A). In contrast, Sp3si levels remained at the level in NP tissue throughout gestation. We did not detect Sp3li in the mouse uterus by Western blotting (data not shown). Densitometry-based quantitation of immunoblots confirmed that Sp1 was significantly downregulated by P14 compared to NP levels, while Sp3 levels remained constant (Figure 4.1B) (N=3-7). Thus, in the mouse uterus, Sp1 protein decreased from levels detected in NP tissue throughout mid-gestation and remained low throughout pregnancy, whereas Sp3si expression was sustained at levels in uteri from NP mice.

KCNN3 promoter activation in mouse is regulated by the Sp1:Sp3 protein ratio

To investigate how Sp1 and Sp3 regulate expression of endogenous SK3 channels, we generated a luciferase reporter plasmid driven by the mouse *KCNN3* promoter. In this system, fold-changes in luciferase activity, as measured by bioluminescence imaging, correspond to changes in SK3 channel expression. The reporter plasmid was co-transfected with plasmids expressing Sp1, Sp3si, or Sp3li into *Drosophila* SL2 cells, which lack endogenous Sp transcription factors. Although both the short and long isoforms of Sp3 bind the promoter and reduce promoter activation (Figure 4.2A), only the short isoform was detected in mouse myometrium by

immunoblotting, and was pursued in these experiments (data not shown). As illustrated in Figure 4.2B, Sp1 enhanced luciferase expression nearly 3.5-fold by 24 hours after transfection, and Sp3si alone reduced it 2.6-fold (Figure 4.2A), with respect to luminescence detected in the vector control. Furthermore, in the presence of Sp1, Sp3si suppressed SK3 transcriptional activation in a concentration dependent manner (N=6). Thus, Sp1 expression enhanced SK3 promoter activation, whereas Sp3si reduced Sp1-induced SK3 promoter activation.

SK3 expression decreases in estrogen treated mice

Estrogen levels increase during pregnancy and may contribute to the down-regulation of SK3 channels by modulating the binding of Sp1/Sp3 to the SK3 promoter. We used an *in vivo* approach to test whether estrogen is an endogenous regulator. Mice were ovariectomized and implanted with placebo or a 17ß-estradiol pellet to assess whether estrogen treatment regulates expression of the SK3 channel protein in the uterus. Three weeks after ovariectomy and pellet implantation, serum samples were taken and uteri were isolated from mice and total lysates were prepared. Estradiol levels were 961 ± 76 pg/mL in 17ß-estradiol treated mice (N=6) while estradiol levels were below detectable levels (<20 pg/mL) in placebo treated mice (N=11). Immunoblotting showed that while SK3 channel protein was present in the placebo-treated mice, it was nearly absent in the estradiol-treated mice (Figure 4.3A). Quantitation demonstrated that SK3 protein was decreased by 42% in the uteri of the estradiol-treated mice (Figure 4.3B). Hence estrogen reduced SK3 protein expression in mouse uteri *in vivo*.

Uterine SK3 channel expression decreases during pregnancy in humans

To determine if the observed decrease in mouse SK3 channel expression during pregnancy represents a pattern shared with the human uterus, we isolated RNA from NP, NL or L tissue (N=3). qPCR analyses confirmed that SK3 transcript was reduced in NL

and L tissue compared to NP myometrium (Figure 4.4A). To assess whether the levels of SK3 protein mimic transcript expression, we carried out immunoblotting with SK3-specfic antibodies. Total cell lysates extracted from NP, NL, and L myometrium showed that SK3 protein expression in humans decreases by late gestation compared to NP (Figure 4.4B). Quantitation of the blots by densitometry verified that SK3 protein is down-regulated in human uterine samples from late gestation (Figure 4.4C) (N=4). Thus, consistent with our findings in mice, SK3 channels in the human uterus are down-regulated during late gestation.

SK3 channel expression increases in estrogen-treated hMSMCs overexpressing Sp1

Similar to the mouse *KCNN3* promoter, the human promoter contains two Sp binding sites, and the expression of SK3 in human uteri is diminished during pregnancy (Figure 4.4). We determined whether estrogen enhances SK3 channel expression in hMSMCs when Sp1 levels are elevated. Human MSMCs were transfected with Sp1 or the control vector. Cells were treated with vehicle or 17ß-estradiol (0.1nM-10nM) for 48 hours, and SK3 transcript was measured by qPCR. Due to variability between patient uterine samples, non-parametric data analysis was carried out by assigning a value of "0" for no change in transcript expression, "+" for an increase in transcript expression, and "-" for a decrease in transcript expression compared to levels produced in cells transfected with the control vector in the absence of E2 (0nM E2) within each experiment. As seen in Table 4.1, SK3 transcript increased consistently in cells transfected with Sp1, even in the absence of E2 (0nM E2). This result demonstrated that Sp1 overexpression alone is sufficient to increase SK3 expression. Treatment with 10nM and 5nM E2 also enhanced SK3 transcript expression in hMSMCs when transfected with Sp1. This implies that although estrogen may lead to SK3 down-regulation during pregnancy, it upregulates SK3 in hMSMCs provided that Sp1 is in abundance.

Discussion

The uterus is a dynamic organ that switches from a relaxed state early in pregnancy to a powerful contractile state in late pregnancy in order to facilitate expulsion of the fetus. One determinant of uterine quiescence is the activity of myometrial K^+ channels (69, 86). Generating both repolarizing and hyperpolarizing currents in MSMCs, these channels contribute significantly to uterine quiescence (19, 85). Accordingly, their activities must be regulated as pregnancy progresses, so that myometrial quiescence is maintained until labor. As such, in the myometrium, the level of expression, the density, and the properties of K^+ channels change dynamically throughout pregnancy (68, 86, 120). The K^+ channels most intensely studied during pregnancy and parturition are the ATP-sensitive K^+ channel, *Shaker*-like voltage-gated K^+ channels, the large-conductance calcium- and voltage-sensitive K^+ channel, and the small conductance calcium-activated K^+ channel.

To date, the SK3 channel is the only one in which overexpression in transgenic mouse models was reported to delay or impede parturition (8). Despite the interesting parturition phenotype of SK3-overexpressing mice, little information previously existed on the mechanisms by which SK3 channels are regulated in normal pregnancy, or on how this leads to a modification of myometrial function. SK3-channel function can be altered through transcription from alternate promoters, resulting in the expression of dominant-negative forms of the channel that can attenuate current and significantly enhance cell excitability (74, 158). Previous transcriptional studies using Cos7 cells demonstrated that Sp1 can augment SK3 channel expression (60). We were able to demonstrate that whereas Sp1 activated the SK3 promoter, Sp3si reduced this *KCNN3* promoter activation in a dose-dependent matter. In addition, we showed that Sp1 protein was remarkably diminished in pregnant myometrium while Sp3si levels remained constant, suggesting that the physiological balance between Sp1 and Sp3 during pregnancy may underlie the regulation of SK3 channels and modulate the excitability of myometrial cells during

pregnancy. These results suggest that the constant Sp3si protein levels seen during pregnancy may partially account for the reduction in the SK3 channel expression in mouse uterus, and that they may aid in the transition to a laboring state. However the mouse *KCNN3* promoter contains multiple additional transcription factor binding sites that can potentially influence SK3 channel expression. For example, the cyclic AMP response elements (CRE), the Nkx-2 homeobox protein, and GATA transcription factors, are all expressed in the uterus and may influence transcription of the SK3 channel. In particular, levels of the CRE binding protein were reduced in both human laboring uterine tissue and mouse uterine tissue at term (24), and estrogen also enhances the CRE binding protein (16). Further investigation is needed to understand the complex nature of transcriptional regulation of the *KCNN3* promoter during pregnancy.

Hormones may contribute to SK3 changes during pregnancy. Hormones and other second messengers modulate the transcription of multiple ion-channel genes, including those encoding the voltage-gated K^+ channels Kv4.2 and Kv1.5 (40, 64, 167) and the BK channel-encoding mouse *slo* gene (76). While many of these channel genes contain hormone-response elements within the promoter, others contain transcription factor binding sites that either complement these elements or themselves provide hormone-responsiveness. For example, many genes regulated by progesterone do not contain progesterone response elements (105). For the SK3 channel in particular, injecting 17β-estradiol into the rostral hypothalamus of ovariectomized guinea pigs increases SK3 mRNA levels (9), indicating that estrogen may contribute to SK3 regulation in myometrium during pregnancy. Previous studies showed that estrogen increases SK3 channel expression despite the fact that the *KCNN3* promoter does not contain an estrogen response element; thus Sp binding sites play an important role in the genomic regulation by estrogen (60). Our lab has shown that SK3 channel expression decreases during pregnancy, occurring simultaneously with a rise in estrogen levels. In the mouse, estrogen levels range from 390 pg/ml in NP tissue to as high as 1801 pg/ml

during pregnancy (169). We hypothesized that if estrogen contributes to down-regulation of uterine SK3 channels during mouse pregnancy, 17ß-estradiol treatment could mimic this response in NP animals. Our *in vivo* approach using ovariectomized mice implanted with a 17ß-estradiol pellet exhibited estrogen levels similar to those in pregnancy reduce SK3 channel expression. This indicates that, although estrogen can stimulate Sp1 activation of the *KCNN3* gene, the physiological balance between Sp factors likely regulates SK3 channel expression *in vivo*.

While SK3 channel expression plays an important role in mouse parturition, its regulation in humans has not been explored previously. In this study we showed that, as is the case in mice, in humans SK3 transcript and protein were downregulated in myometrium during late pregnancy (120). In addition, both mouse and human contained two Sp binding sites in the *KCNN3* promoter, suggesting that the regulatory mechanism was conserved. When Sp1 was overexpressed in human MSMCs that were then treated with 17ß-estradiol, there was a trend toward an increase in SK3 channel expression. However, there was considerable variability in the magnitude of this effect. This variability in human samples could be a consequence of reduced expression of estrogen receptors in cultured myometrial cells, or variability between levels of estrogen in the patient samples (23, 137). Changes in the density of the estrogen receptor in hMSMCs could greatly influence variability in these experiments, since the experiments involve indirect binding of the estrogen receptor to the promoter region of SK3. However, this dilemma was avoided by comparing expression levels within each primary culture experiment. We concluded that estrogen reduces SK3 expression in the uterus *in vivo* (Figure 4.3), but can also promotes SK3 channel expression in the presence of overexpressed Sp1 (Table 1), as found in previous studies (9, 60).

In conclusion, Sp1 was decreased in the uterus of the mouse during pregnancy, whereas Sp3si remained at a constant level. With an abundance of Sp3si in comparison to Sp1, Sp3si is more likely to bind to Sp binding sites and reduce the expression of

transcripts such as *KCNN3*. Estrogen enhanced the binding of Sp factors and contributed to Sp3si binding of the SK3 promoter during pregnancy to further diminish expression levels. Although multiple mechanisms may contribute to the regulation of SK3 channels during pregnancy, the reported studies demonstrated that an ion channel important to myometrial excitability during pregnancy was controlled via transcriptional regulation.

Figure 4.1: Western blot analysis of whole-cell lysates from mouse uteri. Protein was extracted at different stages of pregnancy (NP, P7, P14, P18 and PP2) and separated by SDS-PAGE. Blots were probed with antibodies against Sp1, Sp3 and GAPDH, which served as a loading control. (A) Sp1 protein was prominent only in NP and P7 tissue, decreasing by P14, whereas Sp3 protein levels were similar in all tissue. (B) Quantitation of Sp1 and Sp3 protein levels, with data normalized to GAPDH protein levels (N=3-7). Mean ± SEM. * $p < 0.05$ vs. NP, † $p < 0.05$ vs. Sp1.

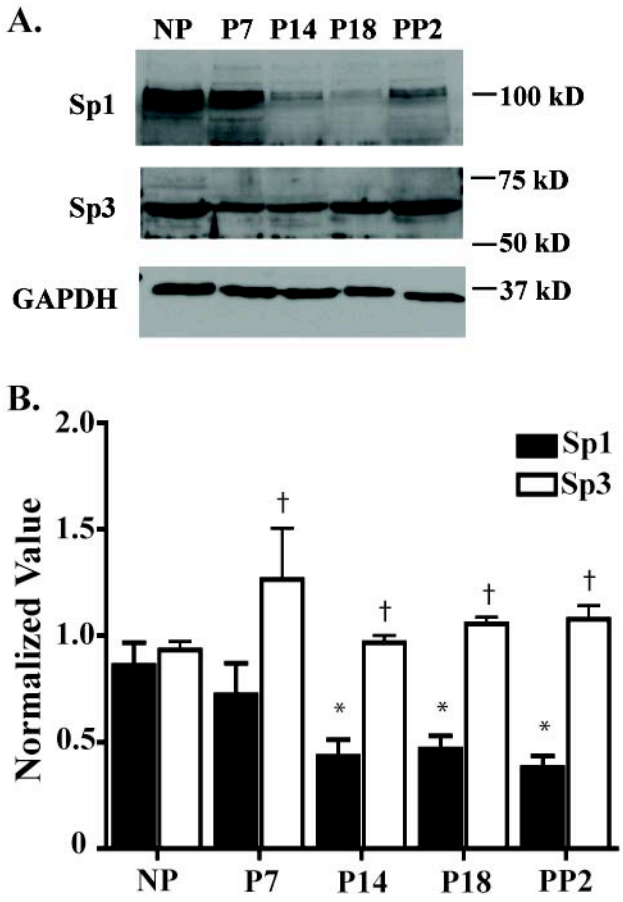

A.
NP P7 P14 P18 PP2
Sp1
100 kD
Sp3
75 kD
50 kD
GAPDH
37 kD
B.
Normalized Value
2.0
1.5
1.0
0.5
0
Sp1
Sp3
NP
P7
P14
P18
PP2

Figure 4.2: Sp3 competes with Sp1 to decrease SK3 promoter activation. *Drosophila* SL2 cells were co-transfected with an SK3 promoter-driven luciferase gene and (A) the Pac vector (control), Sp3si, Sp3li, or (B) Sp1 plus Sp3si at various concentrations. Bioluminescence was quantified, verifying that Sp3si and Sp3li alone can reduce SK3 promoter activation (N=4-6). Mean ± SEM. * $p<0.05$ vs. Pac. (B) Sp3si dose-dependently reduced Sp1 activation of the SK3 promoter (N=6). Mean ± SEM. * $p<0.05$ vs. Sp1.

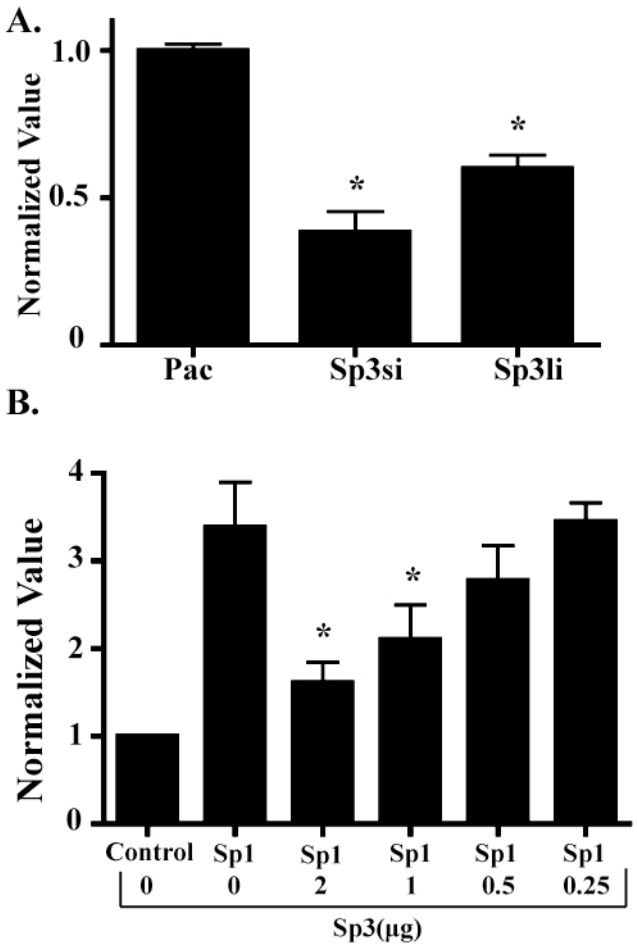

A.
Normalized Value
1.0
0.5
0
*
*
Pac
Sp3si
Sp3li
B.
Normalized Value
4
3
2
1
0
*
*
Control
0
Sp1
0
Sp1
2
Sp1
1
Sp1
0.5
Sp1
0.25
Sp3(µg)

Figure 4.3: Estrogen depletion reduces SK3 channel expression in mouse uteri. Uterine whole-cell lysates were prepared 3 weeks after ovariectomy and pellet implantation (17ß-estradiol or placebo), and proteins were separated by SDS-PAGE. Blots were probed with antibodies against SK3 and GAPDH (loading control). (A) SK3 protein diminished in the presence of estrogen, but was augmented with placebo treatment. (B) Quantification of SK3 protein levels normalized to GAPDH loading control (N=6). Mean ± SEM. * $p < 0.05$ vs. placebo.

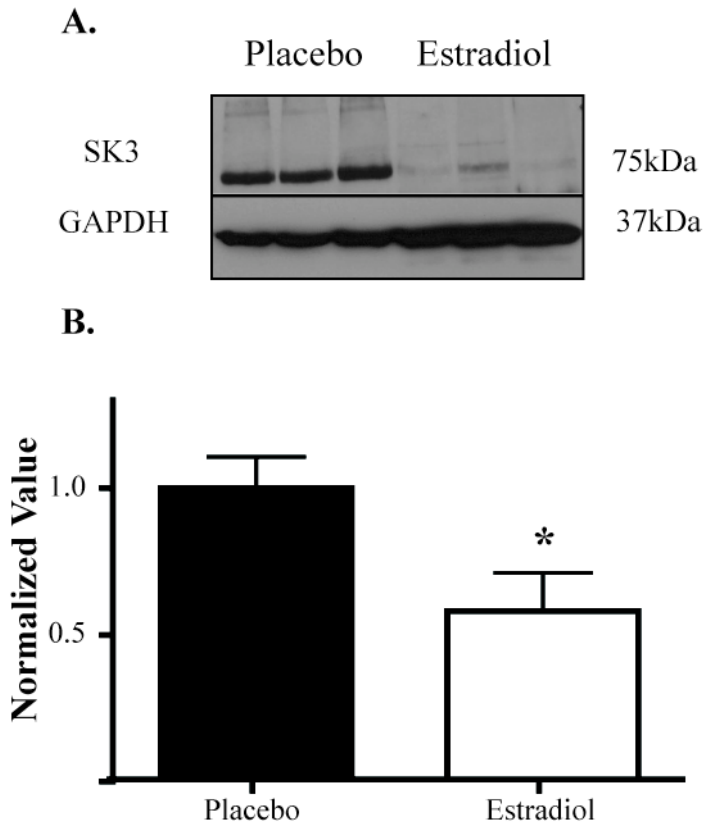

A.
Placebo
Estradiol
SK3
GAPDH
75kDa
37kDa
B.
Normalized Value
1.0
0.5
*
Placebo
Estradiol

Figure 4.4: Decrease in SK3 channel expression in human uteri at term. (A) qPCR analysis of SK3 transcript demonstrated a decline at term in both NL and L tissue compared to NP uterine samples (N=3). (B) Western immunoblotting of lysates from human uteri confirmed that SK3 protein was down-regulated at term. (C) Quantification of SK3 protein levels normalized to GAPDH protein (N=4). Mean ± SEM. * $p < 0.05$ vs. NP.

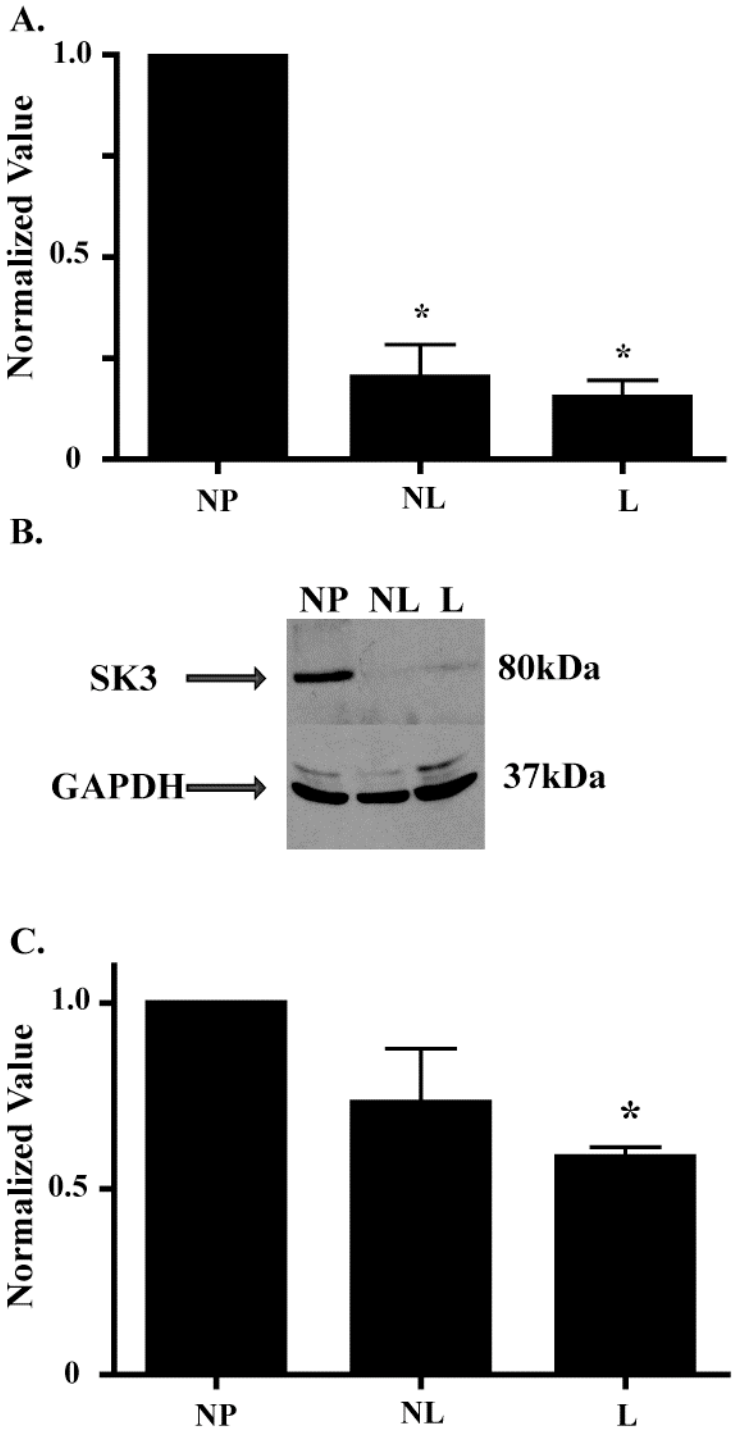

A.
1.0
0.5
0
Normalized Value
*
*
NP
NL
L
B.
NP NL L
SK3
80kDa
GAPDH
37kDa
C.
1.0
0.5
0
Normalized Value
*
NP
NL
L

Table 4.1: SK3 channel levels increase with Sp1 overexpression and E2 treatment.

Construct	Vector	Vector	Sp1 *	Sp1 *	Sp1 *	Sp1	Sp1	Sp1
Estradiol	0nM	10nM	0nM	10nM	5nM	1nM	0.5nM	0.1nM
Patient 1	0	+	-	+	+	0	0	-
Patient 2	0	0	+	+	+	+	+	-
Patient 3	0	+	+	+	+	+	+	+
Patient 4	0	0	+	-	+	0	-	-
Patient 5	0	-	+	+	+	+	+	0
Patient 6	0	0	+	+	0	0	0	+
Patient 7	0	0	+	+	+	+	+	+
Patient 8	0	+	+	+	+	+	+	+

Note: Cultured hMSMCs were transfected with vector control or Sp1-containing vector. Cells were treated with 17ß-estradiol (10nM-0.1nM) or vehicle control. Cells were collected after 48 hours, and transcript analysis was carried out using qPCR and normalizing to GAPDH. Due to variability in human uterine patient samples, each experiment was assigned a nominal value with reference to control-treated cells, to indicate a decrease of less than 10% (-), no change (0) or an increase of more than 10% (+) in the expression of SK3 transcript (N=8). * $p < 0.05$ vs. control.

CHAPTER 5
OVEREXPRESSION OF SK3 CHANNELS INDUCES MATERNAL VASCULAR AND PLACENTAL ABNORMALITIES LEADING TO FETAL DEMISE

Abstract

During pregnancy, the vasculature adapts to accommodate the dramatic increases in blood volume that are necessary to sustain the developing fetus. Potassium channels that promote vasodilation and proliferation are required for this pregnancy-associated vascular remodeling. Overexpression of the SK3 channel leads to abnormal vessel branching and increased vessel diameter in the mesenteric circulation of $SK3^{T/T}$ mice. Thus, vascular remodeling may contribute to our recent finding that $SK3^{T/T}$mice have fewer pups per litter than WT (WT=6.7±0.73, SK3=3.58±0.45). We sought to determine if overexpression of SK3 channels in mice results in placental abnormalities that could lead to fetal demise and resorption accounting for the decreased litter size. While systolic blood pressure was normal in non-pregnant and pregnant $SK3^{T/T}$ mice, heart rate was elevated, implicating a role for this channel in cardiovascular regulation. The same branch order of arteries (isolated and pressurized at 40mmHg) from $SK3^{T/T}$ mice had a larger basal diameter compared to their WT counterparts (WT=231.8±25.4 μm vs. SK3=342.6±12.3 μm), suggesting that basal tone was decreased in these transgenic mice. Constriction of uterine arteries from $SK3^{T/T}$ mice in response to thromboxane A_2 mimetic U46619 was reduced compared to WT mice. Placental weight and morphology differed between $SK3^{T/T}$ and WT mice, with an increased vessel number, diameter, and areas of necrosis and rarefaction observed in $SK3^{T/T}$ placentas. Finally, oxidative stress in placentas from $SK3^{T/T}$ dams was normal early in gestation (P9-10), but there was a trend of elevated levels late in gestation (P18). These data demonstrate that the SK3 channel

contributes to the development and maintenance of the maternal vasculature and placenta, and emphasizes the importance of K^+ channels in placentation.

Introduction

During pregnancy, proper blood flow through the placenta is essential to provide oxygen and nutrients as well as eliminate waste from the fetus. Abnormalities in the placenta can result in preeclampsia, birth defects, and spontaneous abortion. During early stages of pregnancy the placenta develops in a low oxygen environment (49) due to endovascular cytotrophoblast cells that block uterine spiral arterioles, limiting blood flow to the fetus. The opening of these arterioles later in pregnancy rapidly increases oxygen tension, and initiates angiogenesis of the placental vasculature to maintain the fetus. To date, approximately 50% of conceptions are thought to end in miscarriages (61, 145). A better understanding of the mechanisms responsible for the development and maintenance of the vasculature in this specialized organ could reveal mechanisms underlying miscarriage and ultimately improve both maternal and fetal outcomes.

During pregnancy, the vascular system must adapt to accommodate dramatic increases in blood volume necessary to sustain the developing fetus. A contributing factor to vascular remodeling is K^+ channel expression, which contributes to vessel dilation, proliferation, and angiogenesis (102). One particular channel, the SK3 channel, is absent in vascular smooth muscle cells but is abundant in vascular endothelial cells under normal conditions (14, 152). Overexpression of this channel causes abnormal vessel branching and an increase in vessel diameter in mesenteric arteries of $SK3^{T/T}$ mice (152). Similar changes in branching and diameter of the uterine artery and vessels within the placenta could dramatically affect blood flow to the fetus. SK3 channels are expressed in atrial and ventricular myocytes and the SK3 gene was recently linked to lone atrial fibrillation (35, 156). To date fetal demise has not been associated with SK3 channels in humans or mice. However considering the impact placental development has

on fetal outcome, overexpression of SK3 channels could result in an increase in the number of spiral arteries and a decrease in vascular tone with the end result of fetal demise and resorption.

Methods

Mouse breeding

All animal procedures complied with the guidelines for the care and use of animals set forth by the NIH. All protocols were approved by the Animal Care and Use Committee at the University of Iowa. $SK3^{T/T}$ mice on a C57BL/6 background were used for this study (8). Adult female mice were mated at 8 weeks of age or later with an adult WT male to ensure a heterozygous offspring and control for the maternal contribution to placentation. Day 0 of pregnancy was determined based on the presence of a copulatory plug.

Measurement of uterine artery reactivity

Vascular responses were measured as described previously (77) in uterine arteries from WT and $SK3^{T/T}$ mice in response to apamin (SK3 blocker) and agonist induced constrictions. Non-pregnant WT and $SK3^{T/T}$ mice were euthanized with sodium pentobarbital (150 mg/kg IP). The uterus was excised and placed in ice-cold Krebs buffer. The main uterine artery was dissected of fat and connective tissue and tied with ophthalmic suture (10-O) onto a glass micropipette filled with Krebs (in mM: 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, 5 d-glucose, pH 7.4) in an oxygenated organ bath. The vessel was secured to the pipette with 10-O ophthalmic suture and the bath was continuously circulated with oxygenated Krebs (5% CO_2, 20% O_2, balance N_2). The diameter of the vessel was measured using a video dimension analyzer system. Vessels were equilibrated for 30 minutes before study. To ensure viability of vessels, constriction to increasing amounts of KCl (25-75 mM) was measured

in each vessel. The thromboxane A_2 mimetic U46619 (1-5 μM) was added to compare vasoconstrictor responses between WT and $SK3^{T/T}$ uterine arteries. After U46619 induced constriction, acetylcholine (1 μM) was added to test endothelial function. After washing, apamin (200-500 nM) was added to determine SK3 contribution to basal tone of the uterine arteries in WT and $SK3^{T/T}$ mice.

Litter counts and ultrasound imaging of fetal viability

Ultrasonography was performed to determine fetal viability. Imaging was performed with the HP Sonos 5500 ultrasound (Philips, Bothell, WA) with a 15 MHz linear array oscillator (resolution ~0.1 mm) on P14. After shaving of the abdomen, midazolam was administered (0.30 mg; SQ) to reduce animal movement during imaging. Fetal death was determined by an abundance of homogeneous fluids in fetal sac, absence of fetuses when compared to prior images, or lack of fetal heartbeat (28). Litter size was determined by counting the number of pups at delivery or by excising the uterus after euthanasia of the mouse on P7-9, P13-14 and P18-19.

Measurement of heart rate and blood pressures

Heart rate (HR) and systolic blood pressure (SBP) were measured by a computerized tail-cuff procedure (BP-2000 system; Visitech Systems, Apex, NC). Cardiovascular measurements were taken for 5 days for habituation prior to breeding. Measurements of blood pressures were restarted 6-7 days after identification of a copulatory plug and continued everyday throughout gestation. The mean of 20 measurements per day was obtained for each animal.

Measurement of fetal and placental weights

WT and $SK3^{T/T}$ fetuses were excised from the uterus on P18 and all fetal membranes were removed. Placentas were also detached from the uterine wall and

separated from fetal membranes. Weights were recorded for each pup and placenta separately.

Histological analysis of placental development

WT and $SK3^{T/T}$ mice were euthanized during gestation (P9, 10, 14, 18). The uterus was removed and cut into sections between fetal sacs, fixed in 4% paraformeldehyde (24 hours), and embedded in paraffin wax. Placentas were oriented either vertically or horizontally to determine vascularization from both angles. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) to observe any gross morphologic changes in the placental layers, and alterations in placental density and necrosis.

Measurement of oxidative stress in placenta

Oxidative stress was measured in placental tissue using an 8-Isoprostane EIA kit (Cayman Chemicals, Ann Arbor, MI). Placentas from P9, 10 and 18 mice were excised and homogenized promptly and kit instructions were followed accordingly to determine the levels of excessive oxidative stress in $SK3^{T/T}$ mouse placenta compared to WT by measuring 8-isoprostane levels using a microplate reader at a wavelength of 405 nm.

Results

Larger basal diameter and reduced vasoconstriction in $SK3^{T/T}$ uterine arteries

The maternal vasculature of the placenta plays an important role in the regulation of blood flow to the fetus. Morphologic vascular abnormalities were reported in mesenteric arteries from $SK3^{T/T}$ mice (152). Based on these findings, uterine arteries may undergo similar malformations. To test this hypothesis, the main uterine arteries from NP WT and $SK3^{T/T}$ mice were removed and vascular reactivity was measured. Baseline diameters of $SK3^{T/T}$ uterine arteries were significantly larger compared to WT

(Figure 5.1A). Apamin (200 nM), an inhibitor of SK3 channels, caused a greater increase in basal tone in arteries from $SK3^{T/T}$ mice compared to WT but this trend did not reach significance (Figure 5.1B). Constriction of uterine arteries from WT and $SK3^{T/T}$ to KCl (25 and 50 mM) was similar (Figure 5.1C) suggesting that contractile responses were intact. However constriction to the thromboxane mimetic, U46619, was attenuated in SK3 mice compared to WT (Figure 5.1D). These studies indicate overexpression of SK3 channels in the vasculature resulted in a dampened constriction in both basal and post-agonist stimulation. This would suggest that these mice would have enhanced blood flow through the uterine artery.

$SK3^{T/T}$ mice yield fewer pups per litter

Vascular abnormalities in $SK3^{T/T}$ mice (152) may in part contribute to fetal demise considering the dependence of the developing fetus on the maternal vasculature. An additional phenotype of $SK3^{T/T}$ mice that was discovered in our laboratory was a decrease in litter size (Figure 5.2B). The mechanism(s) behind the reduction in litter size is unknown. If overexpression of SK3 channels was able to produce vascular abnormalities in the placenta similar to the mesenteric circulation, this could result in resorption of the fetus. Doppler ultrasound analysis of $SK3^{T/T}$ mice at P14 showed an increased occurrence of fetal sacs with dark, homogeneous-fluid, indicative of fetal resorption in these animals (Figure 5.2C). When we counted the number of pups in WT vs. $SK3^{T/T}$ mice at various stages of pregnancy, litter size was significant decreased at P13-14, while earlier in pregnancy (P7-9) pup number was similar to WT (Figure 5.2A). This indicates that cardiovascular changes that occur after P9 may result in fetal demise.

Normal blood pressure measurements but increased heart rate in transgenic mice

During pregnancy maternal blood volume increases 45-50% in humans and the vascular system must adapt to these volumes without dramatic changes in blood pressure.

Low or high blood pressure during gestation can result in poor fetal outcome (66, 168). Non-pregnant $SK3^{T/T}$ mice have normal blood pressure when compared to WT mice (152). To determine whether pregnancy induces abnormal blood pressure in $SK3^{T/T}$ mice, tailcuff plethysmography was used to measure heart rate and systolic pressures in NP mice and pregnant mice throughout gestation (Figure 5.3). Systolic blood pressures were similar between WT and $SK3^{T/T}$ mice throughout gestation, demonstrating that pregnancy did not cause abnormal changes in blood pressure in $SK3^{T/T}$ mice. However, heart rate was elevated in NP $SK3^{T/T}$ mice at baseline (Figure 5.3A) and during gestation (Figure 5.3B) compared to WT mice.

Placental weight increased but fetal weight normal in $SK3^{T/T}$

Based on the changes in uterine artery constriction and heart rate in $SK3^{T/T}$ compared to WT mice, we assessed whether these alterations resulted in abnormal growth of the fetus and placenta. To investigate whether pups that survive to term have abnormal growth or development, fetuses and placentas were weighed on P18 to test for differences between WT and $SK3^{T/T}$ dams. Fetal weights were the same between the two groups (Figure 5.4B), but placental weight increased an average of 32.9±8 mg in $SK3^{T/T}$ dams (Figure 5.4A). Increases in placental weight could be associated with a change in morphology of the placenta.

Morphologic abnormalities detected in $SK3^{T/T}$ placentas

In order to determine differences in morphology of WT versus $SK3^{T/T}$ placentas, placental tissue was isolated from viable fetuses throughout gestation, fixed, embedded, sectioned and stained with H&E. There were marked structural differences between the placentas observed in these mice. The decidua of the $SK3^{T/T}$ placenta appeared thin in comparison to WT (Figure 5.5A and B) suggesting SK3 channels affect the process of decidualization and placenta formation. Similar to the mesenteric vasculature, there

appeared to be an increased number of maternal vessels entering the placenta of $SK3^{T/T}$ mice compared to WT (Figure 5.5C and D). Further observation showed that the blood vessels of the $SK3^{T/T}$ placenta were enlarged, which may be related to the decreased basal tone and agonist-induced constriction measured in isolated vessels. However, there was also a notable degree of necrosis of the vessel wall in both the maternal and fetal portions of the placenta from SK3 mice. The placenta also showed reduced tissue density in areas of the decidua from $SK3^{T/T}$ mice (Figure 5.5F). Even though the fetuses survived up to P18 (the day before delivery), there were numerous morphologic deficiencies noticed in the placenta.

Oxidative stress levels were similar between WT and $SK3^{T/T}$ mice

A premature delivery of oxygen to the placenta could lead to an increase in oxidative stress before protective mechanisms have developed in the fetus. This may contribute to a decreased littersize in $SK3^{T/T}$ mice. To test whether oxidative stress was elevated at P9, P10, and P18 in $SK3^{T/T}$ mice, a marker for oxidative stress, 8-isoprostane, was measured by enzyme immunoassay (Figure 5.6). There was no evidence of enhanced oxidative stress since levels of isoprostane were similar in placentas from P9 (Figure 5.6A) and P10 (Figure 5.6B) WT and $SK3^{T/T}$ mice. However, placentas tested from P18 mice showed a trend toward elevated oxidative stress in $SK3^{T/T}$ mice (Figure 5.6C). These results would suggest that an increase in oxidative stress early in pregnancy was not the cause for fetal demise in the $SK3^{T/T}$ mice.

Discussion

The discovery that $SK3^{T/T}$ mice exhibit abnormal vascular morphology in the mesenteric circulation begged the question whether these abnormities were restricted to this vascular bed, or if SK3 channels influenced morphology in other organs or vascular beds. We found that basal diameters of uterine arteries from $SK3^{T/T}$ mice were increased

53% compared to WT. This decrease in basal tone was likely related to the dilating properties of K^+ channel activation. K^+ channels are essential regulators of placental vasculature tone and blood flow to the placenta (160). Overexpression of SK3 channels also reduced receptor-mediated vasoconstriction to thromboxane A_2 mimetic U46619 in uterine arteries. These data suggest that along with an increase in diameter, uterine arteries from $SK3^{T/T}$ mice also lacked the ability to constrict to receptor-mediated agonists to the levels measured in arteries from WT mice. In contrast, arteries constricted normally to KCl, likely because KCl elicits non-receptor mediated depolarization of the membrane and activation of voltage-gated calcium channels (124). This implies that uterine arteries from $SK3^{T/T}$ mice constrict normally when activated by direct membrane depolarization rather than G-protein coupled pathways. How these changes in uterine artery function affect pregnancy outcomes was unclear.

Despite abnormalities in the vasculature of $SK3^{T/T}$ mice, the possible role of these channels in promoting fetal demise has not been examined. The reduction in the number of pups per litter from $SK3^{T/T}$ mice suggested that these transgenic mice were consistently resorbing approximately 50% of their pups. At P7-9, $SK3^{T/T}$ mice had litter sizes similar in size to WT mice, but as gestation progressed, litter sizes were reduced by more than half in the $SK3^{T/T}$ mice, often exhibiting fetal demise and resorption. With the increased diameter and reduced agonist response of uterine arteries in $SK3^{T/T}$ mice, a premature increase of oxidative stress was a suspected cause of resorption. We measured 8-isoprostane from P9-10, a critical point in placental development and near the time fetal demise occurs in these mice, but our results confirmed that oxidative stress early in gestation was not the source of fetal death. Placentas taken from P18 mice showed a trend for increased oxidative stress but at this point any remaining pups are most likely to survive. The altered tone of uterine arteries in $SK3^{T/T}$ dams may contribute to an increase in blood flow and fetal demise in these animals.

Regulation of SK3 channel expression may prevent cardiovascular-related disorders during pregnancy. Blood pressure regulation in $SK3^{T/T}$ mice was normal, but the increased cardiac demands of pregnancy could contribute to abnormalities in vascular morphology and heart rate (152). Consistent with previous studies, blood pressures of pregnant $SK3^{T/T}$ mice were comparable to WT mice. However, heart rates were elevated before and during pregnancy in $SK3^{T/T}$ mice. While blood pressure was normal, the elevated heart rate could be compensating for other cardiovascular defects not yet detected. Expression of SK3 channels in atrial and ventricular pacemaker regions may contribute to the rise in heart rate, which may lead to an increase in cardiac output but cardiac output was not determined in $SK3^{T/T}$ mice (156). The role of SK3 channels in the heart is unknown, but the closely related SK2 channel, which is predominately expressed in atrial myocytes, contributes to myocyte depolarization and fibrillation (83). Recently, genome wide association studies have identified an association between *KCNN3* (SK3 channel gene) and lone atrial fibrillation in humans (35). The region of the *KCNN3* gene that was linked to patients with lone atrial fibrillation has not been identified, but supports a role for SK3 channels in cardiovascular regulation that could potentially affect pregnancy outcomes.

K^+ channels and endothelial cells are known contributors to regulation of angiogenesis and proliferation of blood vessels. Recently the intermediate conductance calcium activated channel (IK1), closely related to the SK3 channel, was directly linked to angiogenesis of human umbilical veins (47). Blockade of the IK1 resulted in a reduction of vessel outgrowth *in vivo*. Also, EDHF, another factor that regulates angiogenesis (101), was completely blocked by apamin and TRAM-1 treatment (SK3 and IK1 blockers respectfully) (22). SK3 channels also regulated cell migration in breast cancer (122), which could contribute to endothelial cell migration, an essential step of angiogenesis (127). With the abnormal branching produced in mesenteric arteries and the maternal portion of the placenta, defects in blood vessel development may be a

generalized phenomenon in $SK3^{T/T}$ mice. Future studies are needed to determine whether SK3 channels play a role in angiogenesis, which could greatly influence many organ systems and pathologies, including abnormal development of the maternal vasculature of the placenta. With a better understanding of SK3 channel contribution to vessel formation and growth we can improve our understanding of the key players in placental development to prevent fetal loss.

Figure 5.1: Responses of uterine arteries from NP WT and $SK3^{T/T}$ mice. (WT =green, $SK3^{T/T}$ = orange). (A) Basal diameters of uterine arteries from $SK3^{T/T}$ mice were significantly larger than in WT mice. (B) Uterine arteries from $SK3^{T/T}$ mice constricted more to apamin (200 nM) compared to WT. (C) % Constriction to KCl was similar in uterine arteries from WT and $SK3^{T/T}$ mice. (D) % Constriction to thromboxane A2 mimetic U46619 was greater in uterine arteries from $SK3^{T/T}$ mice compared to WT. (For all experiments: mean ± SEM; N = 4-12 * $p < 0.05$ vs. WT, ** $p = 0.0004$ vs. WT).

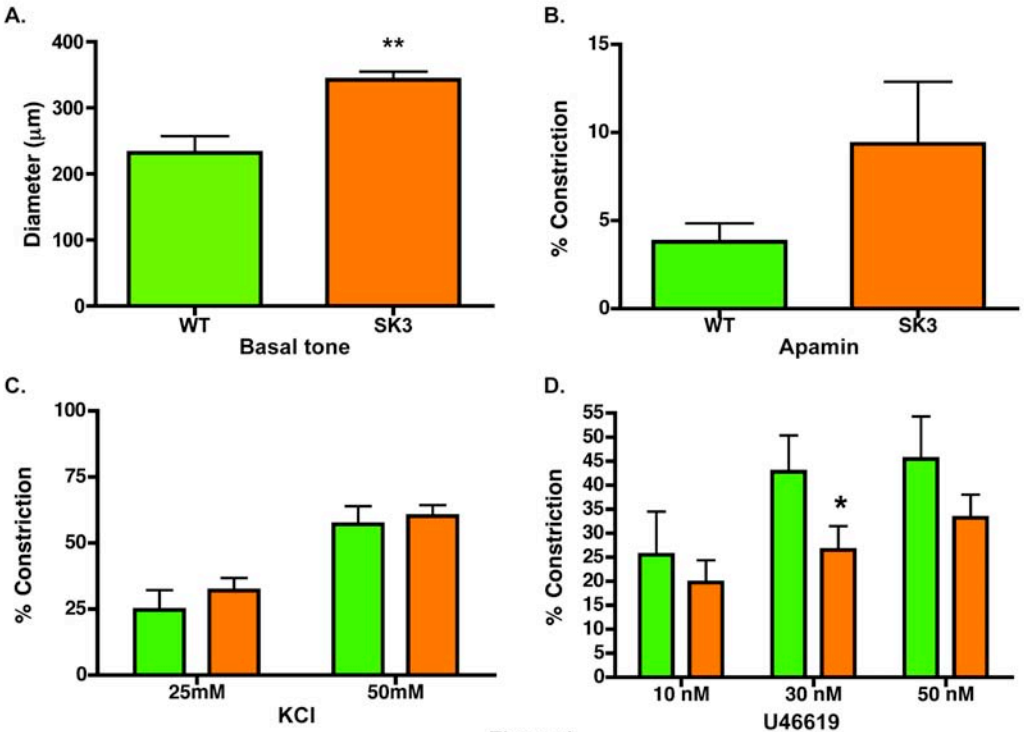

A.
Diameter (μm)
400
300
200
100
0
**
WT
SK3
Basal tone
B.
% Constriction
15
10
5
0
WT
SK3
Apamin
C.
% Constriction
100
75
50
25
0
25mM
50mM
KCl
D.
% Constriction
55
50
45
40
35
30
25
20
15
10
5
0
*
10 nM
30 nM
50 nM
U46619

Figure 5.2: Litter size of WT and $SK3^{T/T}$ mice during different stages of pregnancy. (WT = green, $SK3^{T/T}$ = orange). (A) Average pups per litter at P7-9, P13-14 and P18-19. (Mean ± SEM; N=29, 8, 10, 6, 25, 17 respectively; * $p < 0.05$ vs. WT all gestations, ** $p < 0.001$ vs. WT at same gestation stage, † $p < 0.05$ vs. SK3 P7-9). (B) Ultrasound imaging of mid-gestation mice. Red circle and arrows highlight a viable fetus in the womb of a SK3 heterozygous mouse on P14. (C) Red circles and arrows indicate two empty, homogenous fluid filled sacs, which are the result of fetal demise in an $SK3^{T/T}$ mouse, P14.

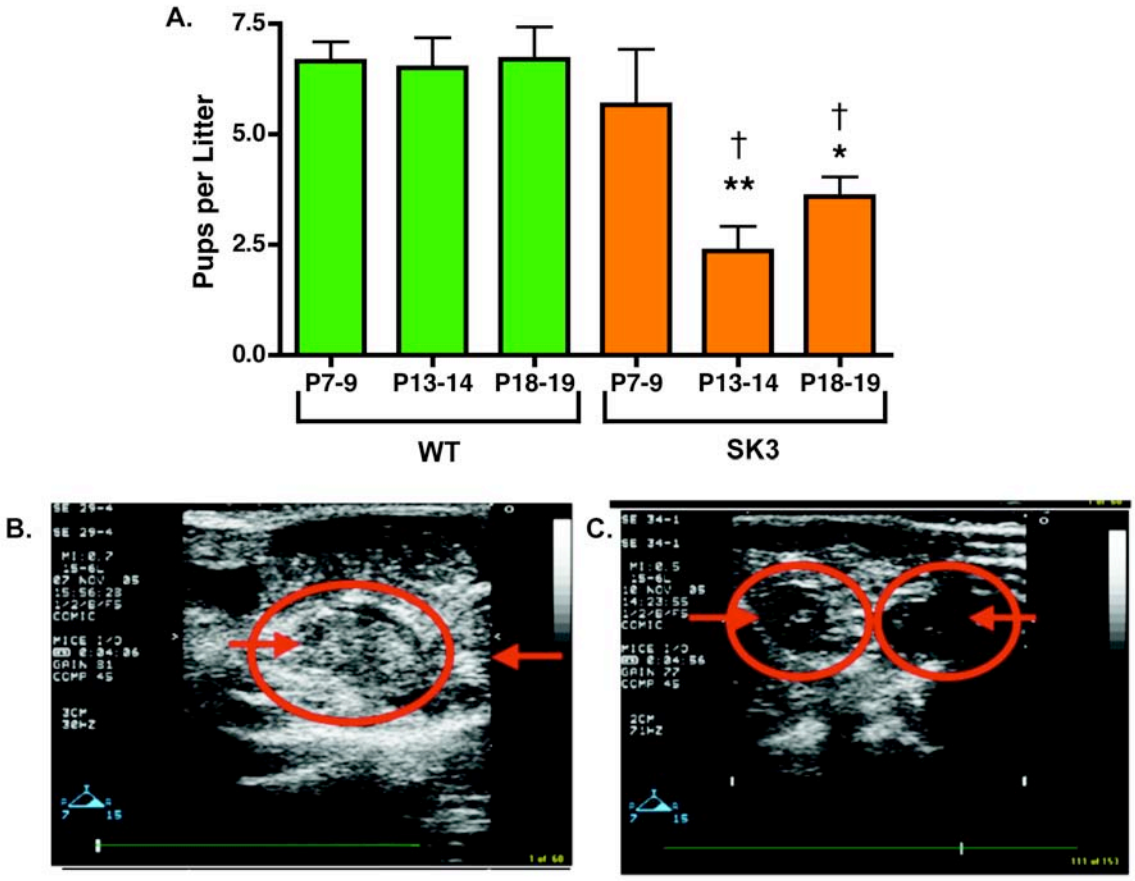

A.
7.5
5.0
2.5
0.0
Pups per Litter
†
**
†
*
P7-9
P13-14
P18-19
P7-9
P13-14
P18-19
WT
SK3
B.
C.

Figure 5.3: Measurements of heart rate and blood pressure by tailcuff in NP and during gestation in WT and $SK3^{T/T}$ mice. (WT =green, $SK3^{T/T}$ = orange). (A) Heart rates in NP $SK3^{T/T}$ mice were elevated compared to WT mice (WT N=6, $SK3^{T/T}$ N=14) (B) Heart rates were increased in $SK3^{T/T}$ mice on P13 and P15 compared to WT mice (n=3-7). (C) Systolic blood pressure of WT and $SK3^{T/T}$ mice were not different throughout gestation (WT n=3-7; * $p < 0.05$ vs WT).

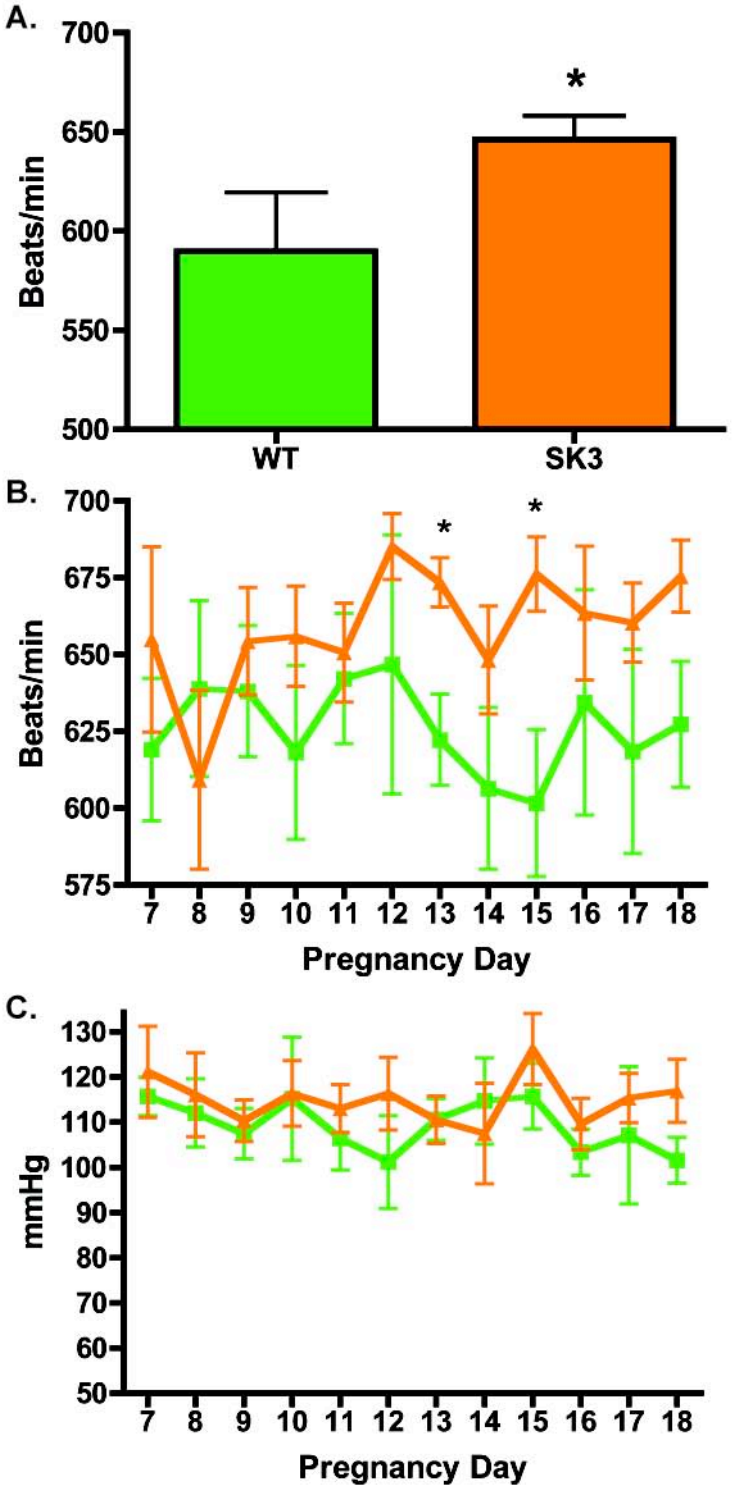

A.
700
650
600
550
500
Beats/min
*
WT
SK3
B.
700
675
650
625
600
575
Beats/min
*
*
7 8 9 10 11 12 13 14 15 16 17 18
Pregnancy Day
C.
130
120
110
100
90
80
70
60
50
mmHg
7 8 9 10 11 12 13 14 15 16 17 18
Pregnancy Day

Figure 5.4: Placental and fetal weights at P18. (A) Placentas collected from $SK3^{T/T}$ dams weighed significantly more than placentas from WT mice (WT N=8, $SK3^{T/T}$ N=10; * $p < 0.05$ vs WT). (B) Fetal weights were comparable between the two groups. (WT N=16, $SK3^{T/T}$ N=10).

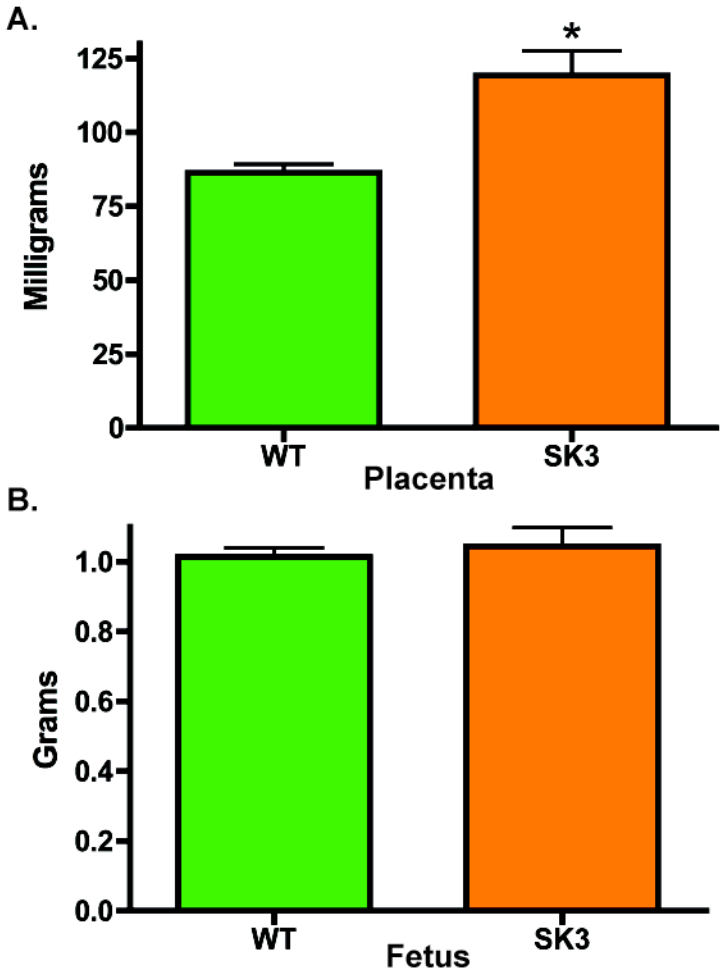

A.
*
125
100
75
50
25
0
Milligrams
WT
SK3
Placenta
B.
1.0
0.8
0.6
0.4
0.2
0.0
Grams
WT
SK3
Fetus

Figure 5.5: H&E staining of placenta from P18 WT and $SK3^{T/T}$ mice (vertical cross-sections unless indicated). (A) Placenta from P18 WT mouse showed normal morphology (dotted line represents width of labyrinth and solid line represents width of decidua; 20x; N=3). (B) Placenta from P18 $SK3^{T/T}$ mouse tended to have smaller decidua compared to WT (solid line; 20x; N=3). (C) Horizontal section of decidua from placenta of P18 WT (arrows indicate maternal blood vessels entering the placenta, 40x). (D) Horizontal section of decidua from placenta of P18 $SK3^{T/T}$ mouse had an increased number of maternal vessels (40x). (E) Decidual portion of placenta from P18 WT mouse showed normal morphology (100x; N=3). (F) Placental decidua from P18 $SK3^{T/T}$ mouse showed abnormal morphology. Triangle indicates enlarged vessels, arrow shows area of thrombi, pentagon points to necrosis, and star indicates rarefaction (100x; N=3).

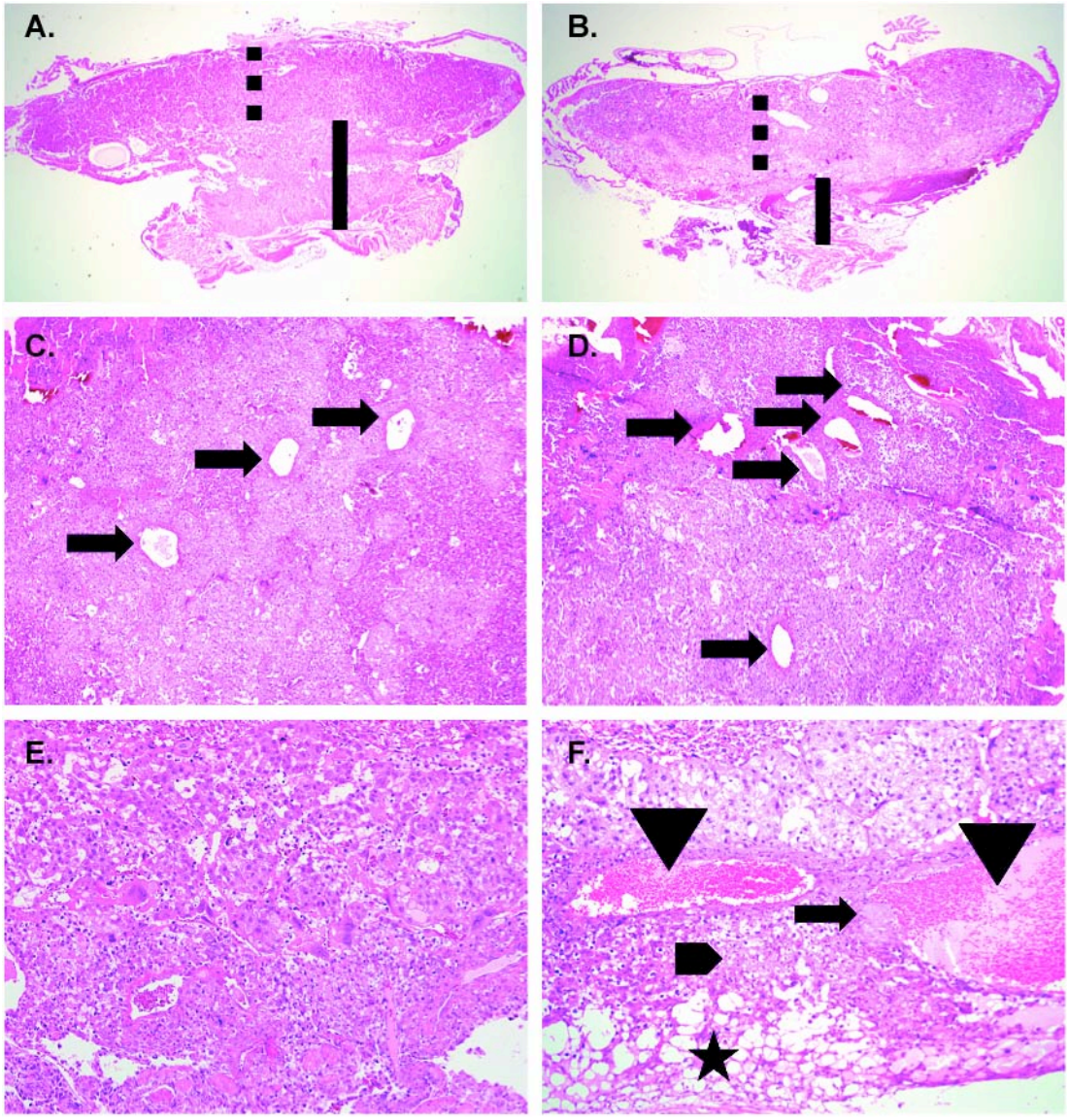
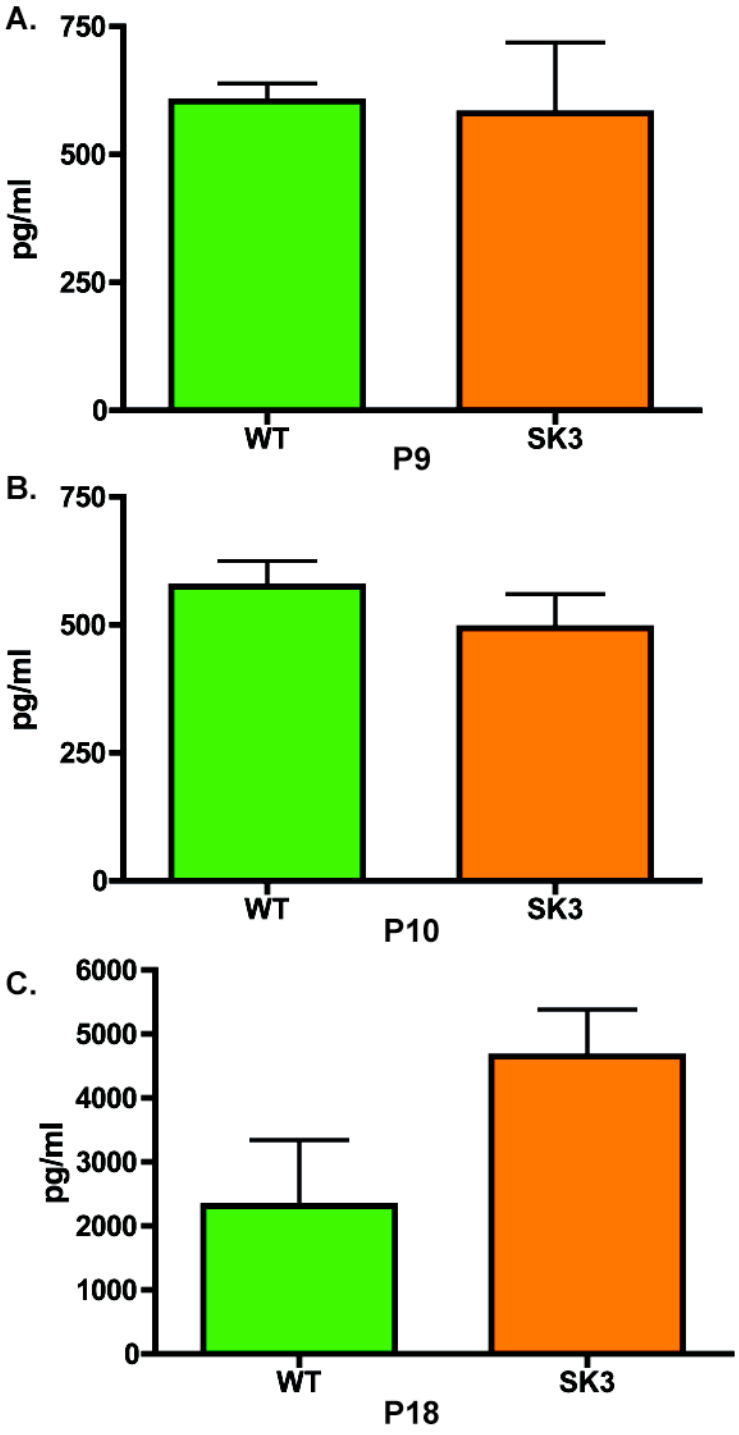

A.
B.
C.
D.
E.
F.

Figure 5.6: Measurement of oxidative stress in placenta from WT and $SK3^{T/T}$ mice. An index for oxidative stress, 8-isoprostane, was measured in placental homogenates at P9 (A), P10 (B, both critical time points in placental development) and P18 (C, the day before delivery). Levels of oxidative stress were similar between placentas from WT and $SK3^{T/T}$ mice at P9 and P10. 8-Isoprostane levels were elevated in $SK3^{T/T}$ placentas at P18 compared to WT, but did not reach significance ($p = 0.058$; N=4 for all groups).


A.
750
500
250
0
pg/ml
WT
SK3
P9
B.
750
500
250
0
pg/ml
WT
SK3
P10
C.
6000
5000
4000
3000
2000
1000
0
pg/ml
WT
SK3
P18

CHAPTER 6

CONCLUSIONS

SK3 channels play a direct role in uterine contraction. However their widespread expression in various tissues suggests that this channel contributes to multiple mechanisms and pathways essential for maintenance of pregnancy and parturition. The mechanisms responsible for the initiation of labor are not fully understood, however many of the pathways implicated in the initiation of term labor may also be activated in preterm labor. The current belief is that four distinct processes, each of which activates multiple signaling pathways, lead to preterm birth: activation of the hypothalamic-pituitary-adrenal axis, decidual bleeding, uterine overdistension, and intrauterine inflammation/infection. The possible role of the SK3 channel in each of these processes will be discussed (Figure 6.1).

The HPA axis and the timing of labor

In humans, secretion of hormones from the hypothalamic-pituitary-adrenal (HPA) axis has been suggested to initiate labor. The HPA axis secretes hormones (including corticotropin releasing hormone (CRH) from the hypothalamus, corticotropin (ACTH) from the pituitary, and cortisol from the adrenals) that stimulate receptors in the fetus, fetal membranes, the uterus, and the placenta but also produces a positive feedback loop on the HPA axis itself (95). During the course of pregnancy, the maternal pituitary increases by about one third in size but continues to function normally despite this growth (94). The maternal pituitary secretes ACTH, which stimulates secretion of cortisol from the adrenal glands of both the mother and the fetus. The release of cortisol from the mother in turn inhibits secretion of CRH from the hypothalamus. During pregnancy, the placenta also produces CRH. In contrast to the hypothalamus, cortisol stimulates the placenta to produce more CRH (111). In humans, throughout the course of pregnancy, the levels of CRH continue to rise, reaching levels of a thousand-fold more than non-

pregnant states (94, 113). This gradual rise in CRH during gestation is thought to act as the "placental clock" that signals when labor should begin (99).

Early in gestation, CRH relaxes the uterus. The myometrium expresses CRH receptor alpha (CRHRα) and when bound by CRH, adenylyl cyclase is activated and intracellular cAMP increases, which leads to relaxation of the uterus (142). As pregnancy progresses, a shift in CRH receptor subtype expression transitions CRH from causing uterine relaxation to instead causing uterine contraction (46, 142). The exact mechanism for this transition is not fully understood. In addition, CRH and corticotropin can stimulate secretion of dehydroepiandrosterone sulfate (DHEAS) from the maternal and fetal adrenal glands. DHEAS is converted to estrogen, which promotes uterine contraction (94, 142). Thus, multiple theories are proposed to explain how the CRH surge towards the end of gestation initiates labor in humans. While CRH gradually rises during term pregnancies, studies demonstrated that CRH levels rise quickly in preterm labor (99). Absolute levels of CRH are not an adequate indicator of potential preterm labor, however the rate at which CRH increases in a pregnant individual does correlate with preterm birth (81).

The HPA axis is an important component of pregnancy, contributing to both uterine quiescence and contractility. To date, SK3 channels have not been implicated in this pathway, despite the fact that SK3 channels are expressed in both the hypothalamus and adrenal glands (20). SK3 channels are present in the rostral portion of the hypothalamus, which includes the nuclei that produce both gonadotropin-releasing hormone and oxytocin. Secretion of gonadotropin-releasing hormone is needed for the menstrual cycle but does not contribute to pregnancy. In contrast, oxytocin is a strong uterotonic thought to contribute to labor initiation and progression (153). Overexpression of SK3 channels could influence release of oxytocin from nuclei in the hypothalamus by affecting membrane potential and attenuating uterine contractile activity. While gene depletion of oxytocin and the oxytocin receptor does not delay parturition in mice, we

cannot eliminate the possibility that SK3 channels influence uterine contraction via this process. The function of SK3 channels in adrenal glands also needs to be investigated. These channels could influence secretion of hormones that are synthesized in the adrenals and are necessary to maintain uterine quiescence. Dampening of cellular excitability via SK3 channels could affect the hormone secretions of the HPA axis, and alter the timing of labor initiation.

The decidua and rupture of membranes

The decidua is an important contributor to maintenance of uterine quiescence during pregnancy. As described in earlier sections, the uterus is composed of several layers, including the endometrium, which becomes the decidua (87). Under the appropriate conditions, this glandular tissue provides a receptive surface for implantation of the embryo. During the menstrual cycle in humans, estrogen primes the endometrial stromal cells, which then transition into the decidua following a rise in progesterone (132). In other animals, this process of decidualization does not occur until implantation begins. The process of decidualization prepares the uterus for invasion by the trophoblast cells, which are the cells on the perimeter of the embryo (87). As growth continues, angiogenesis of the uterus near the implantation site occurs with the decidua contributing to the maternal portion of the placenta.

Term labor is theorized to initiate following activation of the decidua, causing a release of $PGF_{2\alpha}$ (92). Preterm labor is typically related to an early rupture of fetal membranes after decidual dysfunction or early decidual activation (43). This dysfunction or early activation tends to be associated with uterine bleeding or infection (43). An increase in the generation of cytokines can occur without uterine infection, and these cytokines directly influence the decidua. Once cytokine levels rise in the decidua, it leads to production of a cascade of labor-inducing factors, including cyclooxygenase-2 and prostaglandin receptors (21). Production of these stimulating factors causes rupture of

membranes, cervical ripening and uterine contraction. Additionally, bleeding in the decidua promotes thrombin production, which upregulates proteases that also lead to rupture (136). Further investigation into ways to prevent decidual dysfunction could decrease the risk of preterm delivery.

SK3 channels are expressed in the endometrium (20), but a role for these channels in the decidua has yet to be elucidated. In porcine endometrial epithelial cells, SK3 channels contribute to calcium-dependent potassium ion secretion (117), a necessary process for fertilization and proper implantation (17). Given the importance of SK3 channels in endometrium, it seems likely that these channels also contribute to the function of the decidua when it is transformed during pregnancy. Also, estrogen is an important regulator of decidual function, and as described in Chapter 3, estrogen can regulate SK3 channel expression through a shift in the Sp1/Sp3 ratios. As estrogen rises during pregnancy this change in the Sp1/Sp3 ratio could influence expression of SK3 channels in the decidua. The decidua is a fundamental component of pregnancy that contributes to the delicate balance of signaling pathways. Thus ion channels like SK3 could mediate secretion of factors important in pregnancy maintenance and labor initiation.

Overdistension and uterine stretch

The uterus is regulated by many factors during pregnancy, including stretch of the myometrial smooth muscle. As the fetus develops and expands, the uterus undergoes stretch without eliciting a subsequent contraction. As pregnancy nears term, the uterus transitions and responds to stretch by eliciting contractions (88). The process in which the uterus converts to a stretch-induced contractile organ is most likely regulated by progesterone (88). The transition from a quiescent to a contractile state occurs when smooth muscle cells attach to the basement membrane via integrins allowing for forceful contractions (84). Uterine stretch also upregulates connexin 43, cyclooxygenase 2, and

oxytocin receptors, which contribute to the syncitial nature of the uterus allowing coordinated contractions essential for delivery (116, 143, 153).

Overdistension of the uterus, or too much stretch, can lead to preterm labor and delivery. The most common case of overdistension occurs in pregnancy with multiple fetuses. The risk of preterm birth in twins is 60% compared to about 12% in all pregnancies, and preterm birth is almost unavoidable in pregnancy with more than two fetuses (43). Also greater stretch is exerted on the uterus in the cases of macrosomia (abnormally high fetal weight) and polyhydramnios (excess amniotic fluid), both of which typically result in preterm labor (161). The involvement of novel mechanisms induced by overdistention makes stretch a valuable tool in the study of preterm labor.

SK3 channels play an integral role in uterine smooth muscle physiology as demonstrated in Chapters 2-4. As pregnancy progresses, stretch exerted on the myometrium activates pathways that lead to contraction toward term. K^+ channels, in general, activate upon stretch in the stomach, colon, arteries and bladder (72, 119, 151, 170). However, the effect of stretch on SK3 channel expression and activation remains uncharacterized. Previous studies implicated SK3 channels in smooth muscle response to distension upon bladder filling (53), and while shear stress is distinct from stretch, this form of mechanical stimulation activates SK3 channels (148). The higher expression of SK3 channels in early gestation compared to late gestation (120), indicates that activation of SK3 channels following stretch could dampen contractile activity during the quiescent phase of the uterus. Evidence of stretch activation of SK3 channels in myometrium would further emphasize the role of this channel in myometrial relaxation and could be a target for preventing preterm labor.

Intrauterine infection and the inflammatory pathway

Infection contributes to about 25-40% of preterm births, but intrauterine infection can be difficult to detect (43). Low virulence pathogens, not uncommon in the flora of

the reproductive tract, can cause preterm labor and this low virulence may account for why pregnant women frequently lack symptoms of intrauterine infection (44). Intrauterine infection is associated with 80% of early (21-24 weeks) preterm labor (128) and is a serious risk factor for preterm delivery.

Inflammation pathways also contribute to labor at term, with recruitment and activation of cytokines that lead to upregulation of contraction-inducing agents (21). In the case of preterm labor, upon infection, the immune system activates prematurely, most likely by activation of toll-receptors (43, 44, 128). Toll receptors, in turn, recruit cytokines to the uterine cavity. These signaling molecules initiate secretion of prostaglandins that stimulate uterine contractions and proteases that cause rupture of fetal membranes, as described in the previous section. Once these pathways are initiated prematurely, available tocolytics help little to prevent labor.

There is little information linking the SK3 channel to infection, or infection-induced preterm labor. Sultan et al established a possible link when they determined that SK3 channels are necessary for the increase in intercellular adhesion molecule 1 (ICAM-1) following increases in shear stress in the saphenous vein (148). The endothelium of veins responds to increases in flow with rapid upregulation of ICAM-1 (2) which can stimulate leukocyte adhesion and infiltration (149). The activation of SK3 channels could lead to increased transcript expression of ICAM-1 via changes in membrane potential. While SK3 and SK2 channels are both present in endothelium of the saphenous veins, SK3 channel expression is higher (148). Endothelial SK channels are activated by increases in intracellular calcium following increased shear stress of the vein. Activation of the SK channels could induce dilatation of veins to reduce shear stress (148). Consequently, with an upregulation of ICAM-1 due to SK3 channel activation, promotion of leukocyte adhesion and infiltration could occur. In the case of preterm labor, upon infection, leukocytes migrate to the decidua, increasing cytokine production that leads to initiation of pathways that promote rupture of membranes and

contraction (21). As described in the previous section, mechanical forces, such as stretch and shear stress, could activate SK3 channels. Once these channels are activated following an increase in intracellular calcium, they could promote infiltration of leukocytes via ICAM-1, promoting the inflammatory pathway leading to preterm labor.

Implantation and the decidua

Implantation occurs following formation of the blastocyst, which embeds into the stromal cells of the decidua via trophoblast cells (87). In order for implantation to be successful and for the fetus to develop, the blastocyst must properly attach to the uterine wall. This process relies on several factors including hormone secretion from the maternal HPA axis, adequate decidual activation to prevent over invasion of the blastocyst into the uterine wall (70), and initiation of inflammatory pathways to recruit cytokines and leukocytes for remodeling the endometrium. Once implantation starts, the placenta also begins to form. Implantation defects can result in miscarriage, preeclampsia and intrauterine growth restriction (87). They may also promote preterm labor because of the long term effects that placental establishment and dysfunction have on the likelihood of membrane rupture (70).

During the course of experiments, it was noticed that the distribution of the embryos in $SK3^{T/T}$ uteri early in gestation appeared abnormal (unpublished observation) (Figure 6.2). In WT mice, embryos typically spread out evenly along the uterine horns, forming a "string of pearls" (29). In contrast, embryos in $SK3^{T/T}$ dams tended to implant closer together. The mechanism for this was unclear, however SK3 channels could be involved in the release of chemoattractants from the decidua that guide blastocysts to a more evenly distributed orientation. With an overexpression of SK3 channels in the decidua, dampened cell excitability could reduce the secretion of factors that attract an embryo to a particular site in the uterus. The uneven distribution could lead to overcrowding and contribute to the fetal demise found in the $SK3^{T/T}$ dams.

The role of the SK3 channel in the maternal vasculature during placental development (as described in Chapter 5) could be further complicated by the effects of overexpression of the SK3 channel on the developing embryo. While vascularization occurs following implantation, abnormal blastocyst attachment and subsequent trophoblast invasion could hinder proper formation of vessels from the maternal decidua to the placenta. Alterations in the pathways that lead to proper formation of the maternal-fetal interface can greatly influence maternal and fetal health. Continued delineation of the SK3 channel's contribution to the physiology of multiple aspects of pregnancy will support an improved understanding to prevent pathologies.

Impact

To date, the SK3 channel is the only ion channel for which overexpression in transgenic mouse models was reported to delay or impede parturition. Before this project, little information existed on the mechanisms of SK3 channels regulation in normal pregnancy, or how SK3 channels contribute to myometrial function. We determined that SK3 channels were downregulated in the uterus mid-gestation in both humans and mice, to allow for enhanced contractile response. We further demonstrated that estrogen stimulated Sp1 activation of the SK3 gene, possibly by altering the physiological balance between Sp1 and Sp3 expression that determined SK3 channel expression in the uterus during pregnancy. Furthermore, we demonstrated that overexpression of SK3 channels prevented the progression of preterm labor in mice and successfully developed methods to measure intrauterine pressures in mice using radiotelemetry. Using this approach we characterized the role SK3 channels play in the labor process in $SK3^{T/T}$ mice. Thus, we have provided evidence that SK3 channels play a critical role in uterine excitability by dampening contractions. Additionally we discovered a novel phenotype in the $SK3^{T/T}$ mice, fetal demise, which had not been associated with SK3 channels in humans or mice. We demonstrated the contribution of

SK3 channels to vessel formation and growth. With the results from studies described in this thesis, we found novel information on the relationship of SK3 channels in the regulation of pregnancy.

Figure 6.1: Schematic of the four intersecting pathways to preterm delivery. Lockwood CJ Unpublished 2002.

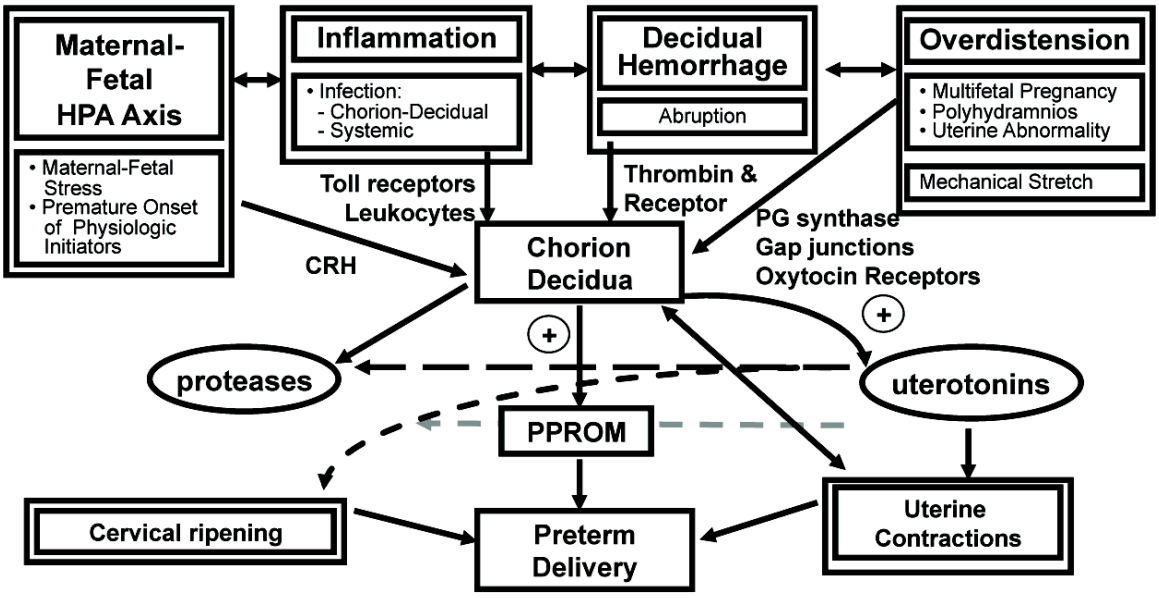
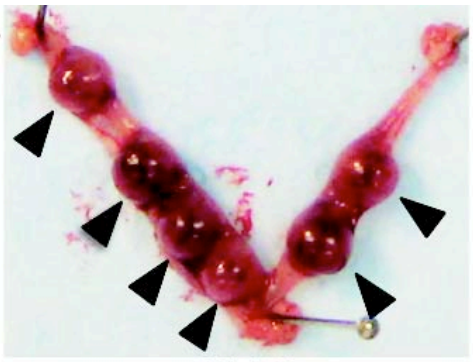

Maternal-Fetal HPA Axis
• Maternal-Fetal Stress
• Premature Onset of Physiologic Initiators
Inflammation
• Infection:
- Chorion-Decidual
- Systemic
Decidual Hemorrhage
Abruption
Overdistension
• Multifetal Pregnancy
• Polyhydramnios
• Uterine Abnormality
Mechanical Stretch
Toll receptors
Leukocytes
Thrombin & Receptor
PG synthase
Gap junctions
Oxytocin Receptors
CRH
Chorion Decidua
+
+
+
proteases
uterotonins
PPROM
Cervical ripening
Preterm Delivery
Uterine Contractions

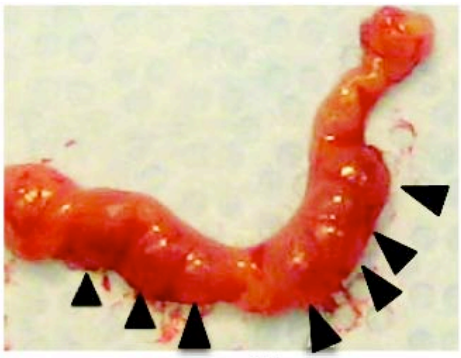
Figure 6.2: Overcrowding of fetuses in $SK3^{T/T}$ uterus. Arrows point to the fetal sacs. (A) The fetal sacs in the P9 WT uterus are distinctly separated from each other, (B) while the fetal sacs from the P9 $SK3^{T/T}$ uterus do not appear as distinct from one another, especially in the right horn.

A.



WT P9

B.



$SK3^{T/T}$ P9

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