

**THE MODULATION OF ANDROGEN
SIGNALING BY STEROID HORMONES AND
MECHANICAL TENSION:
A NOVEL PATHWAY OF LABOR
INITIATION**

By

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A thesis submitted in conformity with the requirements

for the degree of Master of Science

Graduate Department of Physiology

University of Toronto

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**THE MODULATION OF ANDROGEN SIGNALING BY STEROID
HORMONES AND MECHANICAL TENSION:
A NOVEL PATHWAY OF LABOR INITIATION**

**Master of Science, 2011
Yunqing Li
Department of Physiology, University of Toronto**

ABSTRACT

We investigated the gestational expression of androgen receptor (AR) and defined its regulation and that of its co-repressors, PSF and p54nrb, by steroid hormones and myometrial stretch in vivo in pregnant and non-pregnant rats. Our data demonstrate that, 1) myometrial AR expression decreases prior to term; 2) AR expression is up-regulated by MPA treatment and down-regulated by mechanical stretch; (3) myometrial PSF protein expression is down-regulated by estrogen signaling and by mechanical stretch, and up-regulated by androgen signaling; (4) while myometrial PSF mRNA expression is also down-regulated by stretch, the regulation by estrogen and P4 on PSF mRNA appear to be opposite to the effects on PSF protein. We conclude that the decreased androgen signaling in late pregnancy (as a result of decreased AR and PSF expression mediated by hormonal and mechanical signals) may contribute to the mechanisms leading to labor initiation.

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CONTRIBUTIONS

The following people contributed to results presented in this thesis:

Chapter 3

- Immunohistochemical experiments shown in Figure 2.8 and 3.2 were performed in collaboration with Anna Dorogin (Dr. Lye's lab, Samuel Lunenfeld Research Institute)

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LIST OF ABBREVIATIONS

17P	17-alpha-hydroxyprogesterone
µg	micro-gram
µl	micro-litre
µM	micro-molar
AC	adenylate cyclase
AF	activation function
ANOVA	one-way analysis of variance
AP-1/2	activation protein-1/2
AR	androgen receptor
ARE	androgen response element
Ca²⁺	calcium
cAMP	cyclic adenosine monophosphate
CAPs	contraction-associated proteins
CDK2	cyclin-dependent protein kinase 2
cGMP	cyclic guanosine monophosphate
ChIP	chromatin immunoprecipitation
cm	centi-meter
COX-2	cyclooxygenase 2
CREB	cAMP-binding protein response element
Ct	cycle threshold
Cx43	connexin-43
DBD	DNA binding domain
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
E2	estradiol

EBP	enhancer binding protein
ER α/ β	estrogen receptor α / β
ERE	estrogen response element
ERK	regulated protein kinase
g	gram
GPCRs	G-protein coupled receptors
h	hour
HAT	histone acetyltransferase
HDACs	histone deacetylases
HRE	hormone response element
HSPs	heat shock proteins
ID	inhibitory domain
IL-1β/6/8	Interleukin -1 β /6/8
JNK	c-Jun NH ₂ -terminal kinase
K⁺	potassium
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
LBD	ligand binding domain
log	logarithmic
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
mg	milli-gram
mM	milli-molar
MMP-2/8/9	matrix metalloproteinase-2/8/9
MPA	medroxyprogesterone acetate
MPR	membrane progesterone receptor
NaCl	sodium chloride

NCoR	nuclear receptor co-repressor
NFκB	nuclear factor kappa B
ng	nano-gram
NP	non-pregnant
nPRs	nuclear progesterone receptors
OT	oxytocin
OTR	oxytocin receptor
OVX	ovariectomized
P/G rich region	proline/glutamine rich region
P4	progesterone
p54nrb	non-POU domain containing, octamer-binding protein
PFA	paraformaldehyde
PGDH	prostaglandin 15-hydroxy dehydrogenase
PGHs-2	prostaglandin endoperoxide H synthase -2
PGRMC1	progesterone membrane component 1
PGs	prostaglandins
PP	postpartum
PR	progesterone receptor
PRE	progesterone response element
PSF	poly-pyrimidine tract-binding protein-associated splicing factor
PTB	preterm birth
PVDF	polyvinylidene difluoride
RAL	raloxifene
ROD	relative optical density
RRM	RNA recognition motif
RU486	mifepristone
SEM	standard error of mean

SERMs	selective estrogen receptor modulators
SHBG	sex hormone-binding globulin
SHBGR	sex hormone-binding globulin receptor
SmAV	smooth muscle archvillin
SMRT	silencing mediator for retinoid and thyroid
SP-1	specificity protein 1
SRC	steroid receptor co-activator
TAM	tamoxifen
TCF-4	T-cell factor-4
TNF-α	tumor necrosis factor-alpha
Vol	volume
Wt	weight

CHAPTER 1: LITERATURE REVIEW

1.1 Pregnancy, Parturition and Premature Labor

Normal pregnancy begins with embryo implantation, goes through embryogenesis and fetal development, and ends with parturition, which is characterized by shortening and dilation of the uterine cervix, polarized contraction of uterine corpus, and progressive descent and expulsion of the fetus.

The outcome of pregnancy that is the health of the newborn, is affected by many factors contributed through both the maternal and external environment. The timing of the onset of labor is one determinant factor. In humans, normal term labor occurs between 37 and 42 weeks of gestation, while preterm labor is defined as delivery prior to 37 weeks of gestation. Preterm labor is associated with significant perinatal mortality and morbidity, as a result of the prematurity of fetal organs which are unable to support postnatal survival in the extra-uterine environment.

Preterm birth (PTB) is a global health issue. According to a United Nations report 2009, there are an estimated 13 million babies born prematurely worldwide, among which more than one million die each year. Africa has the highest rate of PTB at 11.9% (1), considered to result largely due to malnutrition, infectious disease and lack of prenatal health care. Of interest, the PTB rate in North America has increased 36% over the past 25 years and now ranks second to Africa. This increase in PTB rate is likely associated with a rise in average maternal age and an increase ratio of twin/multiple pregnancy due to the growing use of assisted reproduction techniques.

Despite the 25% of neonatal death (2) as consequences of PTB, prematurely born infants also have significant higher risk of chronic medical problems. A study of 241 six-year old children born between 22 and 25 weeks of gestation found 46% suffered from severe or moderate disabling conditions such as cerebral palsy, vision impairment or hearing loss and mental retardation (3).

The consequences of PTB and its highly associated mortality and morbidity are an emotional, physical and financial burden for families as well as societal medical care systems. It was estimated that in the United States PTB cost at least \$26.2 billion in 2005, including costs of medical care (\$16.9 billion), special education (\$1.1 billion), lost household/labor market productivity (\$5.7 billion) and lifetime medical care for disabling conditions (161).

To reduce peri-natal death rate and to improve neonatal health, there is a clear need to understand the underlying mechanisms of PTB in order to develop effective prevention strategies. However, while Neonatal Intensive Care has made significant progress in terms of supporting neonatal survival, causes of PTB remains elusive.

1.2 Mechanisms Involved in the Onset of Labor

The onset of labor is triggered by rhythmic and coordinated uterine contraction, based on the interaction between actin and myosin of myometrial smooth muscle cells. This contractile interaction is driven by electrical activity mediated by

oscillating intracellular calcium levels and the balance between myosin light chain kinase and myosin phosphatase activities (4).

The onset of labor results from the transformation of myometrium from a quiescent state to an activated state of increased myometrial contractility. This transformation is induced through both non-genomic and genomic pathways involving steroid hormone regulation (13), mechanical stretch and inflammatory responses (12).

1.2.1 Genomic Pathway of Labor initiation:

The up-regulation of a group of genes encoding contraction associated proteins (CAPs) is a downstream output of the genomic pathway. CAPs are categorized into several subgroups: (1) ion channels, which potentiate myometrial contractility by controlling membrane potential (K⁺ channels) or Ca²⁺ influx (Ca²⁺ channels) (59, 60, 61); (2) stimulatory uterotonic receptors such as oxytocin receptors (OTR), stimulatory prostanoid receptors and endothelin receptors (62-64); (3) uterotonic synthetic enzymes (65); (4) gap junctions which permit intercellular coupling (66).

Among all CAP genes, the gap junction protein connexin-43 (Cx43, Gja1) has attracted the most attention and is best-studied. Gap junctions are responsible for low-input resistance and coordinated contraction of myometrium. They directly connect two adjacent cells and form a hydrophilic channel between the cytoplasm of adjacent cells, which allows passage of small molecules such as ions, amino acids or short peptides, second messengers (e.g. cAMP). One gap junction channel is composed of two connexons, while six connexin monomers form one connexon. The

myometrial expression of the gap junction protein, Cx43, spikes at parturition in both mRNA and protein levels, associated with a decrease of P4/estrogen ratio in plasma (67). Administration of P4 during late gestation can prevent the dramatic increase of Cx43 expression and the onset of labor. In contrast, blocking P4 signaling and estradiol (E2) administration are both able to induce Cx43 mRNA and protein levels. Studies of the genomic sequence of Cx43 has identified several putative cis-acting elements, including PRE, estrogen response element (ERE), activator protein-1 (AP-1), activator protein-2 (AP-2), cAMP-binding protein response element (CREB). Nuclear transcription factors, Fos and Jun, bind to AP-1 site (150).

1.2.2 Non-genomic Pathway of Labor initiation:

The well-established core mechanism of the non-genomic pathway is associated with G-protein coupled receptors (GPCRs) and cyclic nucleotides (cAMP & cGMP) / Calcium (Ca²⁺). Oxytocin receptors (5), prostanoid receptors (FP and TP) and endothelin receptors (6) coupled to G_{αq} induce contraction of the myometrium by activating the phospholipase C/Ca²⁺ pathway (7), while some receptors coupled to G_{αi} potentiate uterine contractility through inhibition of cAMP generation. Meanwhile, other receptors such as prostanoid EP2 and IP coupled to G_{αs} inhibit uterine contractions by increasing myometrial cAMP levels.

1.3 Steroid Hormone Pathway:

Steroid hormones are categorized into glucocorticoids, mineralocorticoids, androgens, estrogens, and progestagens by molecular structure and biological function. In females, the natural steroid hormones are generally synthesized from cholesterol within the ovaries and adrenal glands after a series of enzyme-mediated alterations.

Transported through the circulation by sex hormone-binding globulin (SHBG) or transcortin, steroid hormones travel to their target cells and exert a variety of biological effects via two possible pathways. The first pathway is to bind to respective membrane-associated steroid hormone receptors and initiate rapid non-genomic signaling cascade (34). The second pathway is to pass through cell membranes by simple or facilitated diffusion and then bind to specific steroid hormone receptors located in the cytoplasm (type 1 nuclear receptors) and then activate the receptors by disassociating them from heat shock proteins (HSPs) followed by homo-dimerization. After that, the receptors translocate into the nucleus, bind to corresponding HREs of target genes and interact with transcription factors such as nuclear factor kappa B (NF κ B) (97), AP-1 and specificity protein 1 (SP-1) (98). Finally the complex further recruits nuclear co-regulators including co-activators such as Steroid receptor co-activator (SRC) family members and co-repressors such as nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT), with sub-recruitment of histone acetyltransferase (HAT) or histone

deacetylases (HDACs) and finally regulate target gene expression. In addition, steroid hormones can also modulate mRNA stability and translation efficiency in order to modify target gene expression (38).

Moreover, some studies show that the complex formed by SHBG and its receptor (SHBGR) activates adenylate cyclase (AC) upon binding with certain steroid hormones (35).

Steroid hormone receptors have similar structures, including a variable domain at the N-terminal, DNA binding domain, hinge region and hormone binding domain. Starting from the N-terminal, the variable domains differ in both sequence and length, and usually include a transactivation function (AF), which is a determinant of the receptor's transcriptional activity by interacting with the core transcriptional machinery, co-regulators, et al (39, 40). In the middle, the DNA binding domain recognizes and interacts with specific DNA sequence (hormone response element, HRE), while hinge region is related to receptor's translocation from cytoplasm to nucleus. The C-terminal contains the hormone binding domain, which maintains the receptor in an inactive status by binding to HSP, or facilitates ligand-dependent transactivation, dimerization and nuclear localization upon hormone binding. Phosphorylation enhances transcriptional capacity of steroid hormone receptors in both ligand-dependent and ligand-independent pathways (41, 42). Through their membrane receptors, some growth factors (such as EGF, 43) and neurotransmitters (such as dopamine, 44) activate certain steroid hormone receptors synergistically or

additively with intracellular ligands. Furthermore, chromatin structure has been shown to affect the transactivating properties of steroid hormone receptors (68).

1.3.1 Progesterone Signaling Pathway:

During pregnancy, it is well-accepted that progesterone (P4) plays a key role in maintaining the quiescence of the myometrium, while the down-regulation of P4 receptor (PR) in term pregnancy (69) in most species and the activation of estrogen signaling triggers myometrial contraction and the onset of labor. However, there has been a paradox about how labor initiates in the absence of systemic P4 withdrawal in women as is the case in other mammals. PR antagonist RU486 is widely used to terminate early pregnancy in women (70) and it is indicated by several clinical trials that 17-alpha-hydroxyprogesterone (17P; a natural progestogen derived from P4 via 17-hydroxylase) inhibits the onset of preterm labor (71). This suggests the existence of a mechanism for inducing “P4 functional withdrawal” in human myometrium. Although there are several proposed theories for P4 functional withdrawal, the mechanism remains unclear.

PR as a member of the nuclear receptor superfamily and functions mainly in a ligand-dependent manner: ligand binding, discharged from HSP, dimerization, translocation to nucleus, interaction with PRE, transformation of chromatin, recruitment of transcription factors, and regulation of target genes (Figure 1.1) (164). Nuclear PR has two main isoforms generated from two distinct estrogen-regulated promoters including PR-B (the full length form) and PR-A (the truncated form, repressor of PR-B) (72, 73), plus a second putative truncated isoform, PR-C. The

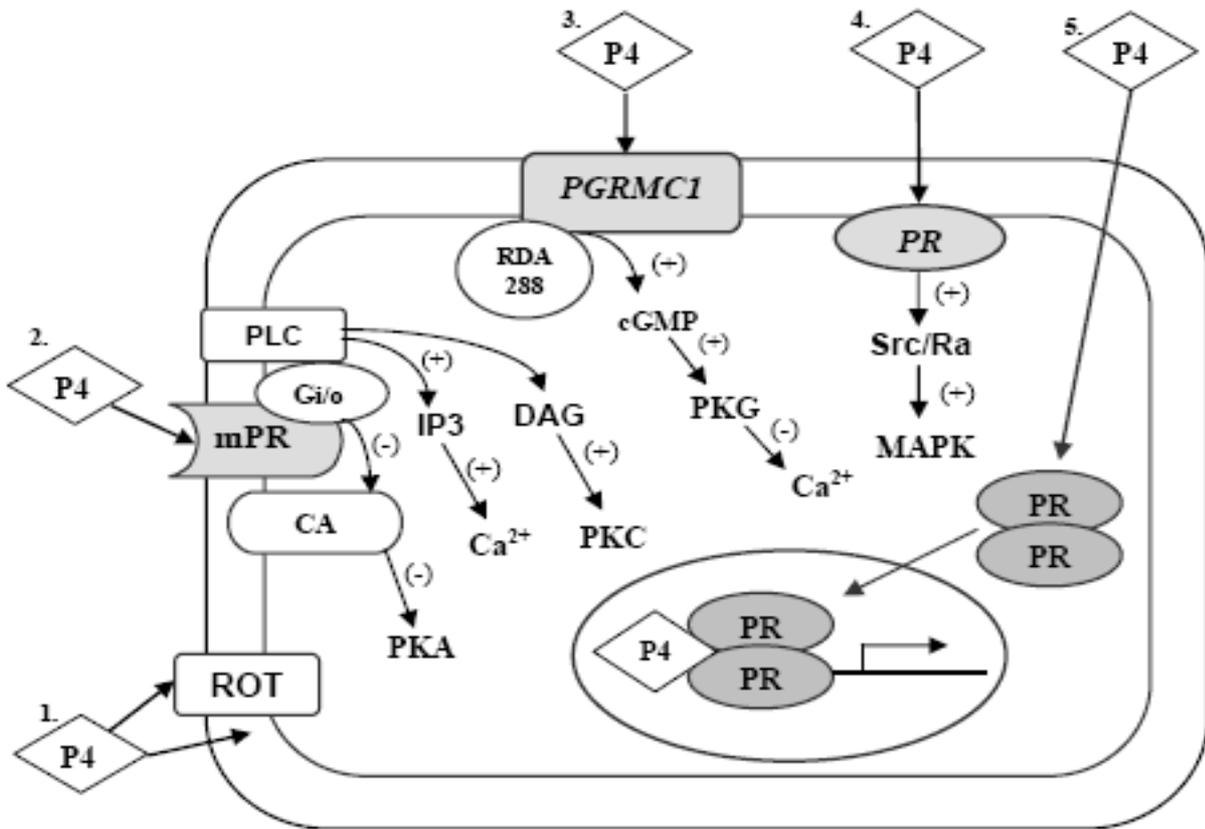


Figure 1.1 Non-genomic (1-4) and genomic (5) pathways of P4 signaling: (adapted from Rekawiecki R 2008, 164)

1. Influence on the affinity of oxytocin (OT) to oxytocin receptor (ROT),
2. Activation of membrane P4 receptor (mPR) coupled with an inhibitory G-protein, subsequently inhibiting adenylyl cyclase (CA) activity,
3. Stimulation of P4 membrane component 1 (PGRMC1),
4. Effect on intracellular P4 receptor (PR) through Src/MAPK (mitogen-activated protein kinase) signaling pathway,
5. Activation of nuclear P4 receptor (PR).

structures of PR isoforms are demonstrated in Figure 1.2 (82). The structural difference between PR-A and PR-B is the lack of activation function domains 3 (AF3) in PR-A, which is crucial for inducing maximal transactivation of PR (74). The PRA/PRB ratio in myometrium is significantly increased in human at labor, which might partially explain P4 functional withdrawal (74). The transcriptional activity of PR is assisted by the recruitment of other nuclear co-regulators, including co-activators such as SRC/p160 and CBP/p300, and co-repressors such as NCoR or SMRT and associated HDACs (79). The ligand-independent transcriptional activity of PR can also be modulated as a result of phosphorylation by cyclin-dependent protein kinase 2 (CDK2) (75).

Other than regulating gene transcription, some studies show that P4 exerts a rapid, non-genomic, membrane-associated intracellular signaling effect. First of all, it is implicated that P4 initiates a rapid independent action by inducing influx of Ca^{2+} (13). Non-genomic progestin action can also be initiated via membrane progestin receptors (mPRs, ~40kDa) demonstrated in human myometrium (14) or P4 receptor membrane component 1 (PGRMC1) located at the plasma membrane (15) (Figure 1.1). Zhu et al. has identified three forms of mPRs (mPR α , mPR β and mPR γ) in several vertebrates including human (8). mPRs are well conserved among vertebrate species with seven transmembrane domains and C/N terminus on inside/outside of cells (16, 17, 18). In some species, mPRs has been proposed to be coupled with an inhibitory G-protein and subsequently decrease cAMP levels with increased phosphorylation of myosin light chain (9, 10, 21). In some other species, it is

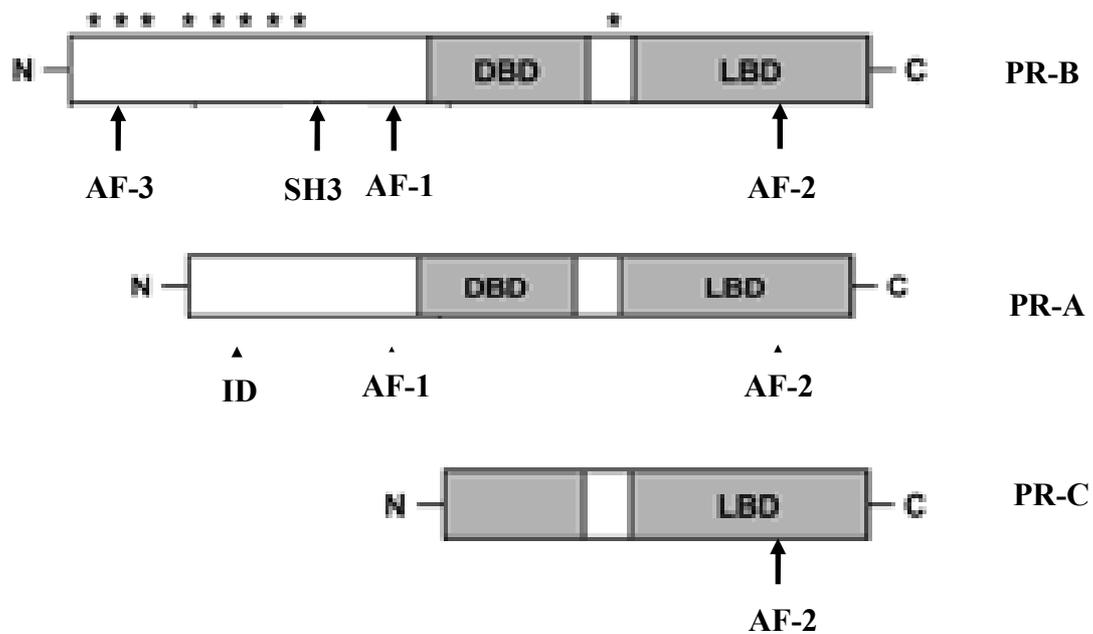


Figure 1.2 Structures of PR-B, PR-A and PR-C: (adapted from Rekawiecki R 2008, 164)

Structures of Nuclear PR isoforms (PR-B, PR-A and PR-C): common functional domains are indicated, including the DBD, LBD, AF-1 and AF-2. In PR-A, there is a special inhibitory domain (ID) instead of the extra AF domain (AF-3) in PR-B (83). The proline-rich region, SH3 domain interaction motif is underlined (84), and phosphorylation sites are indicated by asterisks (85).

suggested that mPR coupled with a stimulatory G-protein could be activated by P4 and induce an increase in cAMP levels (11). The PGRMC1 contains 194 amino acids, an N-terminal transmembrane domain and a ligand-binding domain (19). It is demonstrated that PGRMC1 decreases at term or preterm labor in human myometrium and pretreatment with PGRMC1 antibody inhibits the relaxation effect by P4 (80). Interestingly, besides the well-defined transcriptional activity, nuclear P4 receptors also participate in the non-genomic progestin signaling pathway (20). This action might be mediated by the interaction between PR and Src through its SH3 domain interaction motif followed by the activation of Src/MAPK (mitogen-activated protein kinase) signaling pathway (81).

RU486 (Mifepristone) is a P4 antagonist that has been widely used in clinical application. RU486 binds to LBD of PR with higher affinity than PR agonists (76). Different from PR agonists, RU486 causes transformation of the C-terminal of PR which inhibits AF-2 recruitment of co-activators (77), and impairs the physical interaction between the C-terminus and N-terminus of PR (78).

1.3.2 Estrogen Signaling Pathway:

In contrast to PR, the estrogen receptor (ER) isoforms, ER- α and ER- β are encoded by separate genes. Expression of ER- α , but not ER- β , is significantly elevated in the myometrium during labor (87). Estrogen alters the rate of target gene transcription not only by direct binding of ligand-bound receptor dimers to ERE, but also by

indirectly modifying the synthesis of nuclear transcription factors including Fos, Jun, Myc et al (88, 89, 90).

1.3.3 Androgen Signaling Pathway:

There is substantial evidence implying that androgen signaling is also involved in parturition and preterm labor. This evidence is listed below:

(1) Serum levels of androgen show a similar trend with P4 levels throughout gestation in both human and rat. (Figure 1.3; 91, 92) In human, there is a progressive increase in serum levels of androgen and P4 during pregnancy. Meanwhile, in rodents, during early pregnancy androgen/P4 levels steadily increase, peak on day 16 or 17 of gestation, and start decreasing until the onset of labor. Interestingly, administration of RU486 elevates androgen serum levels during clinical interruption of human pregnancy (128).

(2) The structure of PR and AR has some similarity (Figure 1.4, 93, 36). There are two main isoforms of both PR and AR, although other androgen receptor (AR) splicing variants have been identified in prostate cancer cells and may function in a ligand-independent manner (37).

(3) A microarray study of human myometrial genes reveals that AR is one of the 77 genes down-regulated significantly in both preterm (0.4 fold) and term labor (0.3 fold) (95).

(4) Androgen causes inhibition of spontaneous contractile activity in human myometrium and inhibits contractions induced by high concentration of potassium

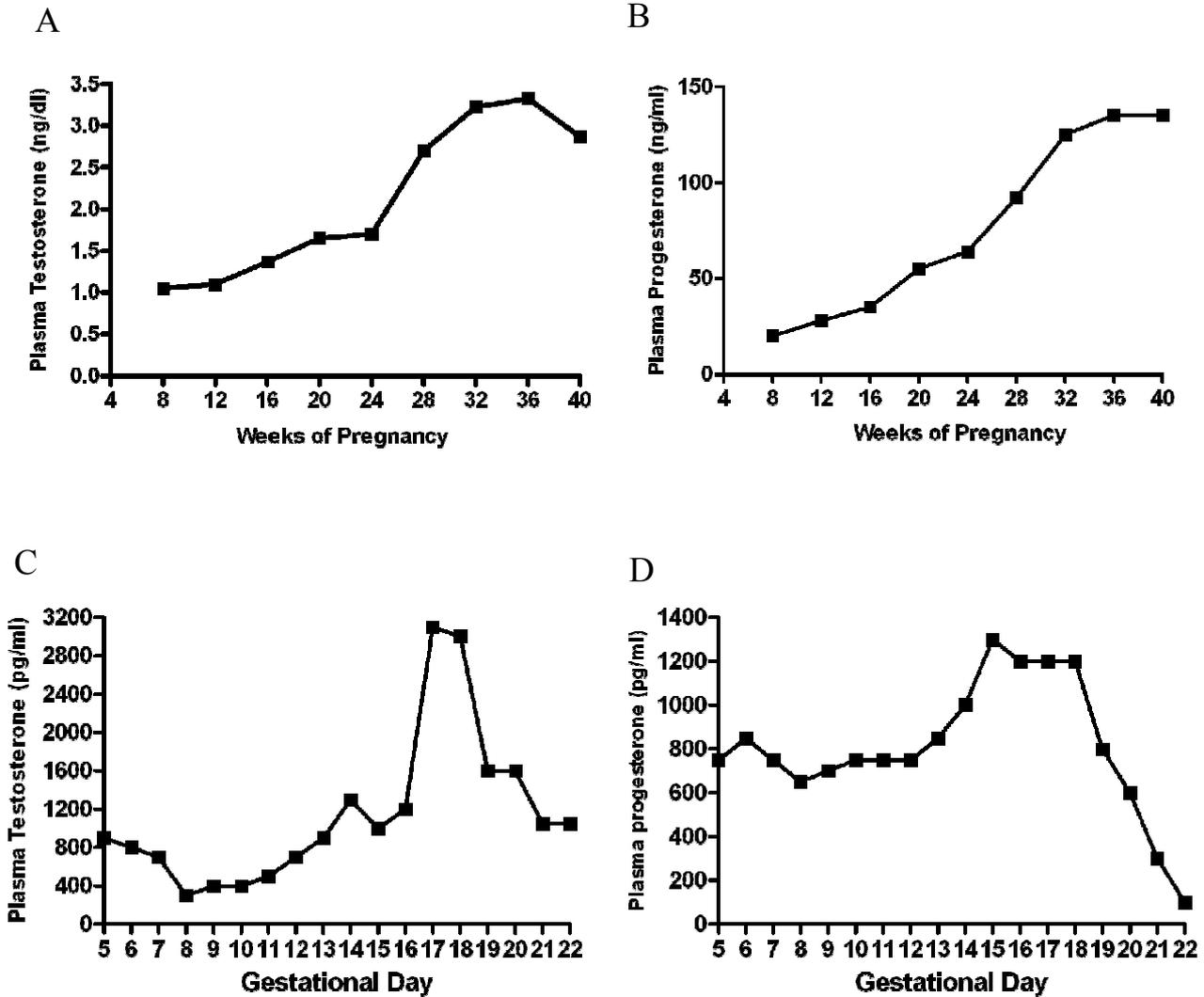


Figure 1.3 Gestational profiles of serum hormone levels in humans and rats (91, 92)

(A) androgen and (B) P4 levels in human: there is a progressive increase in serum levels of both androgen and P4 during pregnancy.

(C) androgen and (D) P4 levels in rats: during early pregnancy androgen/P4 levels steadily increase, peak on day 16 or 17 of gestation, and start decreasing until the onset of labor.

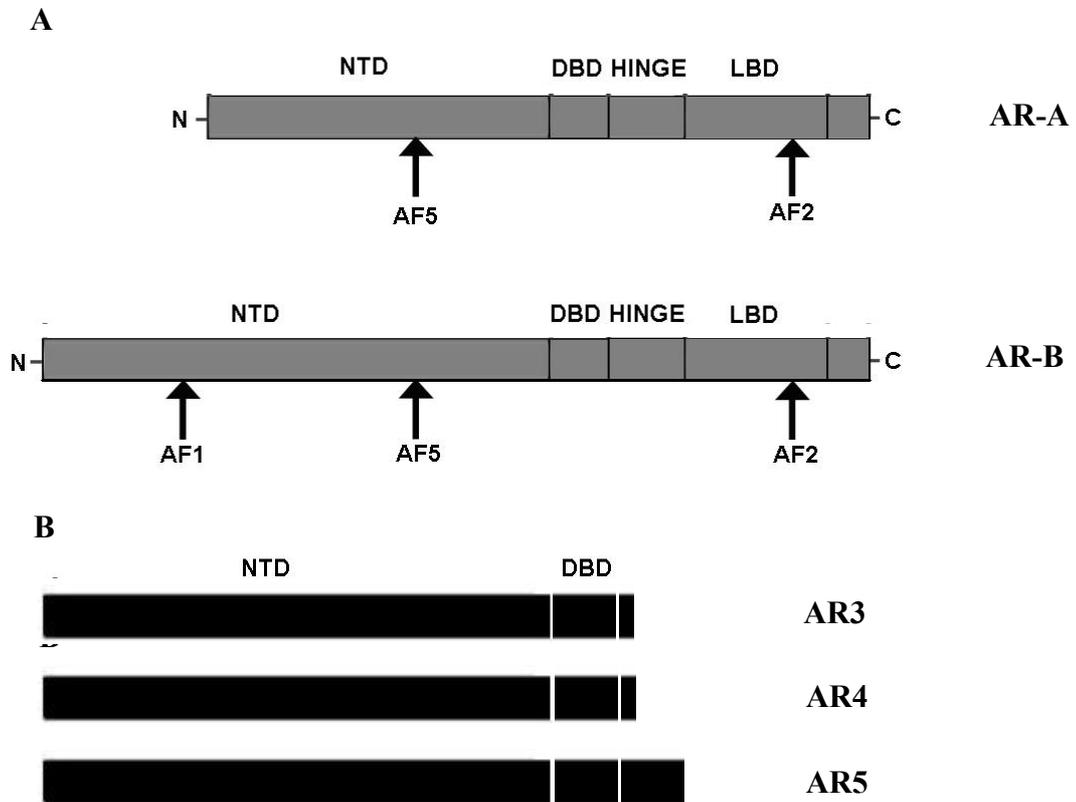


Figure 1.4 Structures of main isoforms of androgen receptor (AR) in human (36, 37 and 93).

A, structures of AR-A and AR-B;

B, structures of other AR splicing variants

AF = activation function; LBD = ligand binding domain; DBD = DNA binding domain.

chloride (KCl) solution through a non-genomic pathway. The effect is rapid, unaffected by specific antiandrogen (Flutamide) and involves the blockade of L-type calcium channels (94).

(5) In rat ventral prostate tissue, castration is shown to induce a dramatic increase of Cx43 mRNA and protein, which is abolished after administration of the non-aromatized androgen, dihydrotestosterone (DHT) (96).

To date, however, little is known about the role of androgen signaling during pregnancy or parturition.

1.3.4 Nuclear Co-repressors (PSF and p54nrb):

Our laboratory has identified two PR co-repressors, poly-pyrimidine tract-binding protein-associated splicing factor (PSF) and non-POU domain containing, octamer-binding protein (p54nrb). Both proteins can negatively regulate AR transcriptional activity in a ligand-independent manner (99).

PSF and p54nrb are two highly homologous multi-functional polypeptides (Figure 1.5, 45). They contain similar structural elements including an RNA recognition motif (RRM) at the C-terminus and regions rich in proline (P) and/or glutamine (Q). RRM are associated with localization to sub-nuclear foci. P/G rich regions might potentiate protein-protein interactions (46). In contrast to p54nrb, PSF has an RNA-binding RGG element, while p54nrb contains a potential DBD. PSF protein (100kDa) is expressed in the nucleoplasm with diffuse and punctate distribution (30, 31), mostly associated with the nuclear matrix or binding to polypyrimidine tract-binding protein

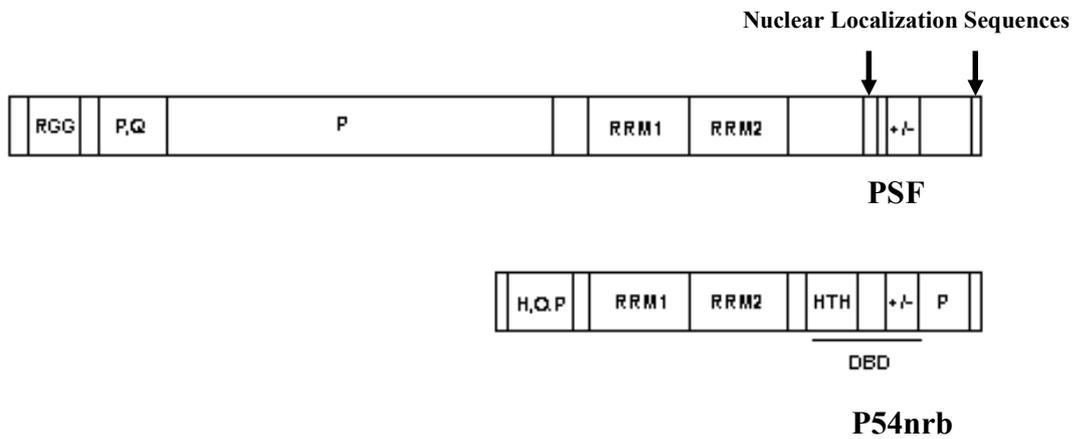


Figure 1.5 Structures of human nuclear co-repressors: PSF and p54nrb (45)
 Homologous elements are identified as RNA recognition motifs (RRMs) at C-terminus, and regions rich in proline (P) and/or glutamine (Q). Different from p54nrb, PSF has an RNA-binding RGG element and nuclear localization sequences, while there is a potential DBD in p54nrb instead.

(32, 33). In addition to co-localization with PSF in nucleus (47) and association with the nuclear membrane (48), p54nrb is also located in paraspeckles (49).

PSF can form heterodimers with its C-terminal homologue, p54nrb. Besides forming a hetero-dimerized complex, PSF and p54nrb can function as monomers as well by binding to RNA or DNA, with or without recruitment of other nuclear factors.

PSF interacts with RNA in the process of pre-mRNA splicing: binding to polypyrimidine tract within introns and defining splicing site for 3' terminus. Moreover, PSF and p54nrb also trap defective RNAs inside of the nucleus (50).

PSF exerts its inhibitory effect on transcription of target genes by either binding to certain sequences within promoters, such as the insulin-like growth factor response element, or binding to the DBD of nuclear hormone receptors (51). Meanwhile, p54nrb enhances the interaction between some transcriptional factors and their specific DNA binding sequences (54). In addition, p54nrb can also directly interact with certain transcriptional factors (55). When the PSF/p54nrb complex binds to promoters of target genes, it further recruits other nuclear regulators such as SF-1 and the HDAC/mSin3A complex enhancing repression of target gene expression. This repression can be abolished by cAMP stimulation, dephosphorylation of SF-1 controlled by phosphatase activity, and the release of HDAC/mSin3A from the promoters (52, 53). Furthermore, the PSF/p54nrb complex is also involved in DNA unwinding mediated by topoisomerase I. PSF alone participates in DNA paring which is an important step involved in DNA recombination, DNA repair, DNA replication etc (162).

PSF regulates PR and AR transactivation by preventing receptors from binding to PRE and the androgen response element (ARE). In addition, PSF enhances PR degradation through the proteasomal pathway (100) but does not affect AR protein stability (101).

It was reported that myometrial PSF protein expression in the human is significantly up-regulated as pregnancy progresses and levels remain elevated in labor (103). However, in the rat both myometrial PSF and p54 protein levels decrease dramatically at the approach to parturition (102). The expression of PSF and p54nrb are also modified during myeloid (56) and neural differentiation (57), and in human breast cancer cells (58).

1.4 Mechanical Stretch Pathway

By term pregnancy, the human uterus expands approximately 250 times in size. Mechanical stretch of the uterine tissue by the growing fetus has been implicated in the stimulation of myometrial contraction and is considered as one risk factor for preterm labor, as exemplified by the known association between multi-fetal pregnancy or polyhydramnios and PTB.

Animal experiments indicate that the expression of CAP genes including Cx43 (105), OTR (106) and cyclooxygenase 2 (COX-2) (107) is up-regulated by uterine stretch. In vitro experiments shows that stretch of human uterine myocytes collected from pregnant women before labor results in up-regulation of OTR mRNA expression and activation of the OTR promoter along with increased recruitment of AP-1 and CCAAT/enhancer binding protein (C/EBP) (108).

Furthermore, stretch can also induce expression of AP-1 transcription factors (109, 111) such as c-Fos by a rapid and transient phosphorylation of MAPK through three different signaling pathways, extracellular signal-regulated protein kinase (ERK) pathway, c-Jun NH2-terminal kinase (JNK) pathway, and p38 pathway in uterine smooth muscle cells (110). Studies in the human myometrium demonstrate that stretch-regulated uterine contractility might also be induced by the activation of focal adhesion signaling assisted by the increase of ERK activator, smooth muscle Archvillin (SmAV) (104).

Data suggest that the mechanical stretch pathway interacts with inflammation response and steroid hormone signaling. First of all, intrauterine balloon insertion in women elevates amniotic prostaglandin levels (151). In addition, static stretch of human uterine myocytes increases IL-8 mRNA expression with activated promoter activity (112). Moreover, there is a proven association between uterine occupancy and increased expression of the pro-inflammatory chemokine, monocyte chemotactic protein-1 (MCP-1) (113), which is also inhibited by P4 signaling. Last but not least, stretch-induced up-regulation of CAP genes can be abolished by P4 treatment (107).

1.5 Inflammatory Pathway

An inflammatory cytokine-mediated response is also involved in parturition. Various biomarkers of intra-amniotic inflammation, including C-reactive protein, tumor necrosis factor-alpha (TNF- α), MCP-1, matrix metalloproteinase-8 (MMP-8), and angiogenin have been associated with pregnancy loss (23). Cytokines such as interleukin-1 β (IL-1 β), TNF- α , interleukin-8 (IL-8) and IL-6 have been shown to play

an important role in preterm labor, as evidenced by both in vitro and in vivo studies (22, 24, 25). Even in normal term labor without signs of inflammation, levels of IL-1 β and IL-8 in the amnion increase (114). IL-1 β , IL-6 and TNF- α can trigger an inflammation cascade by translocating NF κ B to the nucleus and promoting the transcription of other inflammation mediators (118). There is also evidence showing CAP genes, including Cx43, are up-regulated by cytokines (124).

Prostaglandins (PGs) as key inflammatory mediators for the initiation of labor can promote myometrial contractility (26). Studies in both humans and animal models suggest that prostaglandin concentrations increase prior to labor initiation (27, 28, 29). Indomethacin, an inhibitor of PG endoperoxide H synthase (PGHS), postpones the onset of term labor (115). It has been shown that IL-1 β and TNF- α stimulate PGHS-2 expression and PG synthesis mediated by NF κ B (119, 120). In contrast, PG 15-hydroxy dehydrogenase (PGDH) which catalyzes PGs into inactive metabolites, decreases in human term and preterm labor (121), while IL-1 β and TNF- α down-regulate PGDH expression (122). Two types of PGs mediate myometrial contraction: PGE₂ via EP₃ receptor, and PGF₂ α via FP receptor. During human pregnancy, myometrial EP₃ and FP gene expression is reduced by more than 40 percent (116); however, FP increases significantly at term labor (117). Pro-inflammatory cytokines also up-regulate PG receptor expression (123) mediated through binding to the NF κ B response element.

In human myometrial cells, PGF₂ α increases the relative PR-A to PR-B ratio, and IL-1 β up-regulates the putative truncated PR isoform, PR-C, through an action on

NFκB. This might be one mechanism by which a ‘functional P4 withdrawal’ is induced (126,127).

The increase of matrix metalloproteinases, MMP-2 and -9, at term might enhance leukocyte migration into the decidua and modify IL-1β activity (125).

PGs activate another pathway of non-genomic regulation of myometrial contractility. PGF_{2α} directly induces uterine contractile activity by stimulating the influx of Ca²⁺. In contrast, PGI₂ inhibits myometrial contraction by raising intracellular cAMP levels. Oxytocin and relaxin partially follow the same pathways with stimulatory or inhibitory PGs, respectively (163).

1.6 Hypothesis and Specific Aims

Hypothesis:

Androgen signaling plays a role similar to that of P4 signalling in the maintenance of pregnancy; hormonal and mechanical stimuli facilitate the onset of labor by down-regulating androgen signalling and its co-repressors (PSF and p54nrb) at late pregnancy.

Specific Aims:

1. To examine myometrial AR expression:
 - a) Throughout gestation using normal pregnant rat model
 - b) By the regulation of steroid hormones using non-pregnant ovariectomized rat model
 - c) By the regulation of physiological mechanical stretch using unilaterally pregnant rat model

- d) By the regulation of artificial mechanical stretch using Laminaria-inserted unilaterally pregnant rat model
2. To examine myometrial PSF and p54nrb expression:
- a) By the regulation of steroid hormones and hormone receptor blockers using non-pregnant ovariectomized rat model
 - b) By the regulation of physiological mechanical stretch using unilaterally pregnant rat model
 - c) By the regulation of artificial mechanical stretch using Laminaria-inserted unilaterally pregnant rat model

CHAPTER 2:

THE EXPRESSION OF ANDROGEN RECEPTOR IN RAT MYOMETRIUM

DURING GESTATION AND AT TERM LABOR IS REGULATED BY

HORMONAL AND MECHANICAL STIMULI

2.1 Introduction

The association between androgen signaling and reduced myometrial contractility/CAP gene expression suggests a potential role of androgen signaling in the maintenance of pregnancy and labor initiation (94). However, the mechanisms that regulate androgen signaling in the myometrium remain unclear. Steroid hormone signaling and mechanical stretch are known to be two determining factors influencing both term and PTB. Thus, we hypothesize that in concert with P4 signaling, androgen signaling may inhibit myometrial contraction as well as the onset of labor. Furthermore, we suggest that AR expression may be regulated by steroid hormones and/or mechanical stretch. The goal of the present study was to investigate protein and mRNA expression of AR in rat myometrium throughout normal gestation, during labor and in postpartum, as well as to investigate the effect of hormonal and mechanical signals on AR expression. In this study, we used rat models of spontaneous term labor and hormone-treated non-pregnant, ovariectomized (OVX) model. We also aimed to determine the impact on AR expression of mechanical stretch exerted 1) physiologically as a result of embryo growth by using unilaterally pregnant rat model, and 2) artificially through implantation of the expandable material (Laminaria) within the non-gravid horn of unilaterally pregnant rats. Cx43 protein expression was previously known to be up-regulated in rat myometrium during term labor and by E2 treatment, but reduced by P4 treatment (143). We measured Cx43 expression in rat myometrium to validate our AR data in these *in vivo* rat models.

2.2. Methods

2.2.1. Animals:

Wistar rats (Charles River Co., St. Constance, Canada) were housed individually under standard environmental conditions (12 h light, 12 h dark cycle) and fed Purina Rat Chow (Ralston Purina, St. Louis, MO) and water *ad libitum*. Female virgin rats were mated with male Wistar rats. Day one of gestation was designated as the day when vaginal plug was observed. The average time of delivery was the morning of day 23. The Samuel Lunenfeld Research Institute Animal Care Committee approved all animal experiments.

2.2.2. Experimental design:

Normal rat pregnancy and term labor:

Animals were euthanized by carbon dioxide inhalation and myometrial tissue samples were collected on gestational days 0 (NP), 8, 10, 14, 15, 19, 21, 23 and 1 day post partum (1PP) (n=4). Myometrial tissue was collected at 10 a.m. on all days except for samples from laboring animals (d23), where tissues were collected once the animals had delivered at least one pup.

Hormone-treated OVX rat model:

Ovariectomy was performed on virgin Wistar rats under general anesthesia via isoflurane inhalation. Bilateral flank incisions were made from both sides of the animal. A hemostat was clamped between the ovary and the tip of the uterine horn. A surgical silk suture was tied to the area between the oviduct and the ovary, and then the ovaries were removed using fine scissors. After the surgery, female rats were

allowed to recover for seven days to ensure that the endogenous levels of estrogens were reduced. The following hormone treatments were designed to modulate the action of steroid hormones on the expression of AR and Cx43 in the myometrium.

The OVX animals were split into four groups (n =5, Figure 2.1):

Group 1 (control): treated with subcutaneous injections of vehicle (10% ethanol and 90% corn oil) at time 0 and time 12h.

Group 2 (estrogen): treated with subcutaneous injections of E2 only (4 µg/kg per injection per rat) (130) at time 0 and time 12h.

Group 3 (P4): primed with subcutaneous injections of E2 (4 µg/kg per injection per rat) at time 0 and subcutaneous injections of both E2 (4 µg/kg per injection per rat) and MPA (medroxyprogesterone acetate, 16 mg/kg per injection per rat) (131) at time 12h.

Group 4 (androgen): primed with subcutaneous injections of E2 (4 µg/kg per injection per rat) at time 0 and subcutaneous injections of E2 (4 µg/kg per injection per rat) plus intraperitoneal injections of DHT (dihydrotestosterone, 1mg/kg per injection per rat) (132) at time 12h.

Myometrial samples were collected at 24h for biochemical analysis.

Unilaterally pregnant rat model:

Under general anesthesia, virgin female rats underwent tubal ligation through a flank incision to ensure that they subsequently became pregnant in only one horn (133). Animals were allowed to recover from surgery for at least 7 days before mating. Pregnant rat myometrial samples from non-gravid and gravid horns were

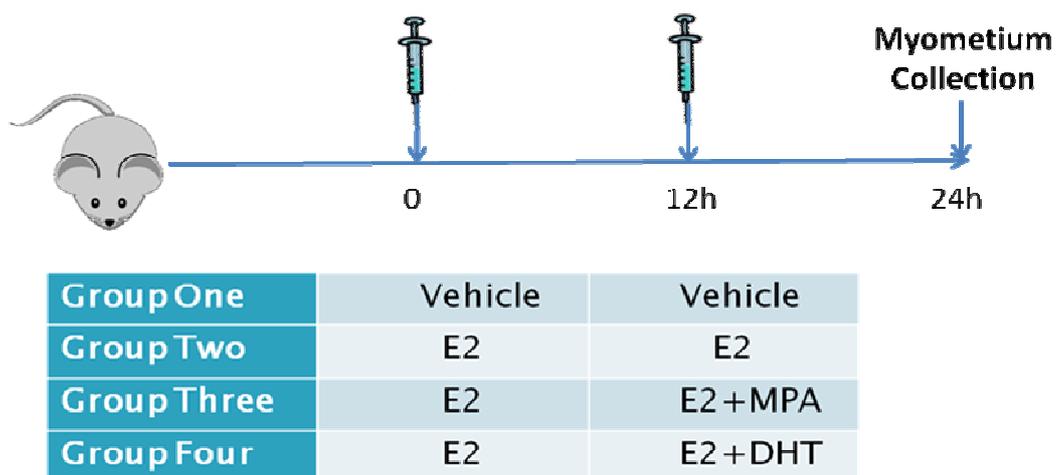


Figure 2.1 Scheme of injections and tissue collection for hormone-treated OVX rats.

Four different groups of OVX animals were treated with (1) vehicle (10% ethanol and 90% corn oil), (2) E2 (estradiol, 4 $\mu\text{g}/\text{kg}$ per injection per rat), (3) E2 + MPA (medroxyprogesterone acetate, 16 mg/kg per injection per rat), or (4) E2 + DHT (dihydrotestosterone, 1 mg/kg per injection per rat). Myometrial tissue was collected 24 hour after the first injection from all four groups.

collected on gestational days 17, 21 and 23 (Labor) or day 1 postpartum (n = 3 animals at each time point). Labor samples (d23L) were collected during active labor once the animals had delivered at least one pup.

Laminaria-inserted unilaterally pregnant rat model:

After pregnancy was confirmed, unilaterally ligated rats were randomized into 3 groups (Figure 2.2).

Group 1, Sham: On day 19, rats underwent sham surgery (n = 2).

Group 2, Control Tube: On day 19, rats underwent insertion of a non-stretchable polyvinyl tube, 1 mm in diameter and 3 cm in length, into the non-gravid uterine horn through a midline abdominal incision (n = 3). This tube is a control for insertion of a foreign object into the uterine lumen and does not impart stretch to the uterine wall.

Group 3, Laminaria: On day 19, rats underwent insertion of an expandable Laminaria tube, 2 mm in diameter and 3 cm in length, into the non-gravid uterine horn through midline abdominal incision. Laminaria, dried seaweed stem of 2 mm width which swells as it absorbs moisture (*in situ* for 12 hours achieving 6-8 mm dilation and becoming comparable in size to the gravid horn) (n = 3).

Myometrial samples were collected on day 23 during spontaneous term labor once the animals had delivered at least one pup.

Casodex-treated pregnant rat model:

Androgen levels have been reported to be high during late gestation in rats and may inhibit the synthesis of the "contraction associated proteins". To investigate the impact of AR signaling on the initiation of labour we treated animals subcutaneously

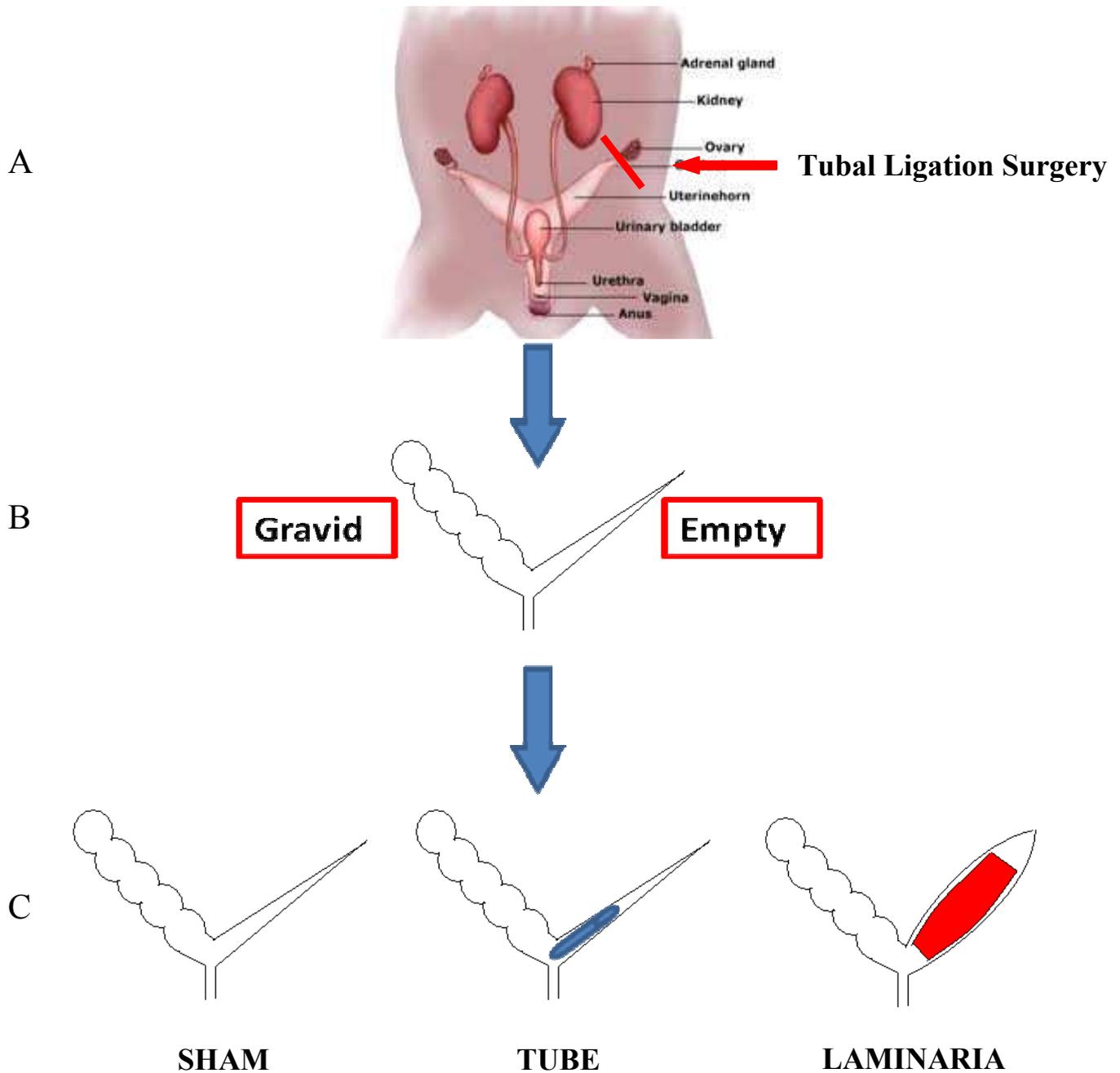


Figure 2.2 Scheme of Laminaria-inserted unilaterally pregnant rat model.

(A) Tubal ligation surgery was performed on one of the two uterine horns on virgin female rat. (B) After 7-day recovery, the rats were mated and subsequently became pregnant in only one horn. (C) On day 19 of gestation, the rats were randomized into 3 groups: 1) sham group (sham surgery), 2) control tube insertion group (insertion of a non-stretchable polyvinyl tube into the non-gravid uterine horn as control for intrauterine device) and 3) Laminaria group (insertion of an expandable Laminaria tube into the non-gravid uterine horn, which swells as it absorbs moisture and achieved comparable size to the gravid horn after 12 hours). Myometrial tissues were collected 24 hours later (n=5/group).

with the AR antagonist Casodex (bicalutamide, 100mg/kg) or vehicle (dimethyl sulfoxide, DMSO) on day 17 of gestation. Myometrial samples were collected after 48 hours (n=5/group).

DHT-treated pregnant rat model:

Plasma androgen levels normally fall prior to the onset of labor in rats. To prevent the fall in androgen at term and to check whether maintaining androgen levels postpone the onset of term labor, rats were treated with DHT (dehydrotestosterone, non-aromatized androgen, intraperitoneally, 1mg/kg per day) or vehicle (10% ethanol and 90% corn oil) starting from day 18 of gestation. Myometrial samples were collected once the animals had delivered at least one pup (n=5/group).

2.2.3. Tissue collection:

For protein extraction, the uterine horns were placed into ice-cold PBS, bisected longitudinally and dissected away from both pups and placentas. The endometrium was carefully removed from the myometrial tissue by mechanical scraping on ice which eliminates the entire luminal epithelium and the majority of the uterine stroma. The myometrial tissue was flash-frozen in liquid nitrogen and stored at -80°C . For immunohistochemical studies the uterine horns were placed in formalin or 4% paraformaldehyde (PFA) for fixation and then cross-sectioned or sectioned longitudinally.

2.2.4. Western immunoblot analysis:

Frozen myometrial tissue was crushed under liquid nitrogen using a mortar and pestle. Crushed tissue was homogenized for 1 minute in RIPA lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1% (vol/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, supplemented with 100 μ M sodium orthovanadate and protease inhibitor cocktail tablets (CompleteTM Mini, Roche, Quebec, Canada). Samples were spun at 12 000g for 15 min at 4°C and the supernatant was transferred to a fresh tube to obtain a crude protein lysate. Protein concentrations were determined using Pierce® BCA Protein Assay Kit (Pierce Biotechnology, Thermo Scientific, USA). Protein samples (40–50 μ g) were resolved by electrophoresis on 8 or 12% SDS-polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) in 25 mM Tris-HCl, 250 mM glycine, 0.1% (wt/vol) SDS, pH 8.3, for 1 h at 100 volts at room temperature. Protein expression levels of AR and Cx43 were measured by immunoblot analysis using primary and secondary antibodies summarized in Table 2.1. Probed membranes were developed using LuminataTM Crescendo Western HRP detection system (Millipore Corporation, USA) and analyzed using densitometry. To confirm that equal amount of total protein was examined in each sample, the blot was stripped and reprobed with alpha-Tubulin primary antibody using the condition described above. The expression of AR and Cx43 was normalized to alpha-Tubulin expression.

Table 2.1. Summary of Antibodies Used in Immunoblot Analysis.

Antibody	Primary or secondary	Mono or polyclonal	Dilution	Source
AR (N-20)	Primary	Polyclonal	1:2000	(Rabbit) Santa Cruz
Cx43	Primary	Polyclonal	1:2000	(Rabbit) Millipore
Anti-mouse IgG	Secondary	HRP-linked	1:2000	GE Healthcare
Anti-rabbit IgG	Secondary	HRP-linked	1:2000	(Donkey) GE Healthcare
Alpha Tubulin	Primary	Monoclonal	1:2000	(Mouse) Sigma

2.2.5. Real-time PCR analysis:

Total RNA was extracted from the frozen tissues using TRIZOL (Gibco BRL, Burlington, ON, Canada) according to manufacturer's instructions. RNA samples were column purified using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada), and treated with 2.5 μ l DNase I (2.73 Kunitz units/ μ l; Qiagen) to remove genomic DNA contamination. Total RNA (2 μ g) was primed with random hexamers to synthesize single-strand cDNAs in a total reaction volume of 100 μ l using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The thermal cycling parameters of RT were modified according to the Applied Biosystems manual. Hexamer incubation at 25°C for 10 min and RT at 42°C for 30 min were followed by reverse transcriptase inactivation at 95°C for 5 min. Complementary DNA (20 ng)

from the previous step was subjected to real-time PCR using specific sets of primers (Table 2.2) in a total reaction volume of 5 μ l (Sigma). Primer specificity was confirmed by blast analysis. Real-time PCR was performed in an optical 384-well plate with a BIO-RAD CFX384TM Real-Time System (BIO-RAD) using SYBR Green detection chemistry. The run protocol was as follows: initial denaturation stage at 95°C for 20 sec, and 40 cycles of amplification at 95°C for 5 sec and 60°C for 20 sec. After PCR, a dissociation curve was constructed by increasing temperature from 65°C to 95°C for detection of PCR product specificity. In addition, a no-template control (H₂O control) was analyzed for possible contamination in the master mix. A cycle threshold (Ct) value was recorded for each sample. PCR reactions were set up in triplicates, and the mean of the three Cts was calculated. A comparative Ct method ($\Delta\Delta$ Ct method) was applied to the raw Ct values to find a relative gene expression across normal gestation. PAPPA, IGF1R and PDGFA were chosen as candidate reference genes based on their gestational profiles of myometrial expression (Figure 2.3). In Figure 2.4, we showed standard curves and melting curves for all our target genes and reference gene. We checked consistency of their expression in all rat models and PDGFA appears to have the most consistent expression among all experiment groups (Figure 2.5). Therefore, mRNA level from each rat myometrial sample was normalized to PDGFA mRNA. Validation experiments were performed to ensure the PCR efficiencies between the target genes and PDGFA were approximately equal.

Table 2.2. Real-time PCR primer sequences of target genes and housekeeping gene.

Genes		Primer Sequences	Accession #
AR	Forward	5' - CCTGGATTCTGTGCAGCCTATTGCA-3'	NM_012502
	Reverse	5' - AGGATCTTGGGCACTTGCACAGA-3'	
Cx43	Forward	5' - TGGGGGAAAGGCGTGAGGAAAGT -3'	NM_012567
	Reverse	5' - ACCCATGTCTGGGCACCTCTCTT -3'	
PDGFA	Forward	5' - AGCGACTGGCTCGAAGTC -3'	NM_012801
	Reverse	5' - CTCCAAGGCATCCTCAGC -3'	

2.2.6. Immunohistochemistry:

The formalin fixed myometrial tissues were gradually dehydrated in ethanol and embedded in paraffin. Sections of 5 µm thickness were collected on Superfrost Plus slides (Fisher scientific ltd., Nepean, ON, Canada). Paraffin sections were deparaffinized and rehydrated. After immersion in 3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) the antigens were unmasked using a microwave heating retrieval treatment in 10 mM Sodium Citrate solution (pH6) in 2 steps for 5 and 3 min with 20 min cooling down, blocked for 1 hour with DAKO Protein Serum-Free Blocking solution (DAKO Corporation, Caspinteria, CA) and incubated with primary AR polyclonal rabbit antibody (1:100, Santa Cruz, USA) overnight. For the negative controls, ChromPure non-specific sheep IgGs (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used at the same concentration and sections were also incubated with secondary antibodies in the absence of primary antibodies. Secondary antibodies used for detection were biotinylated goat anti-rabbit (1:300,

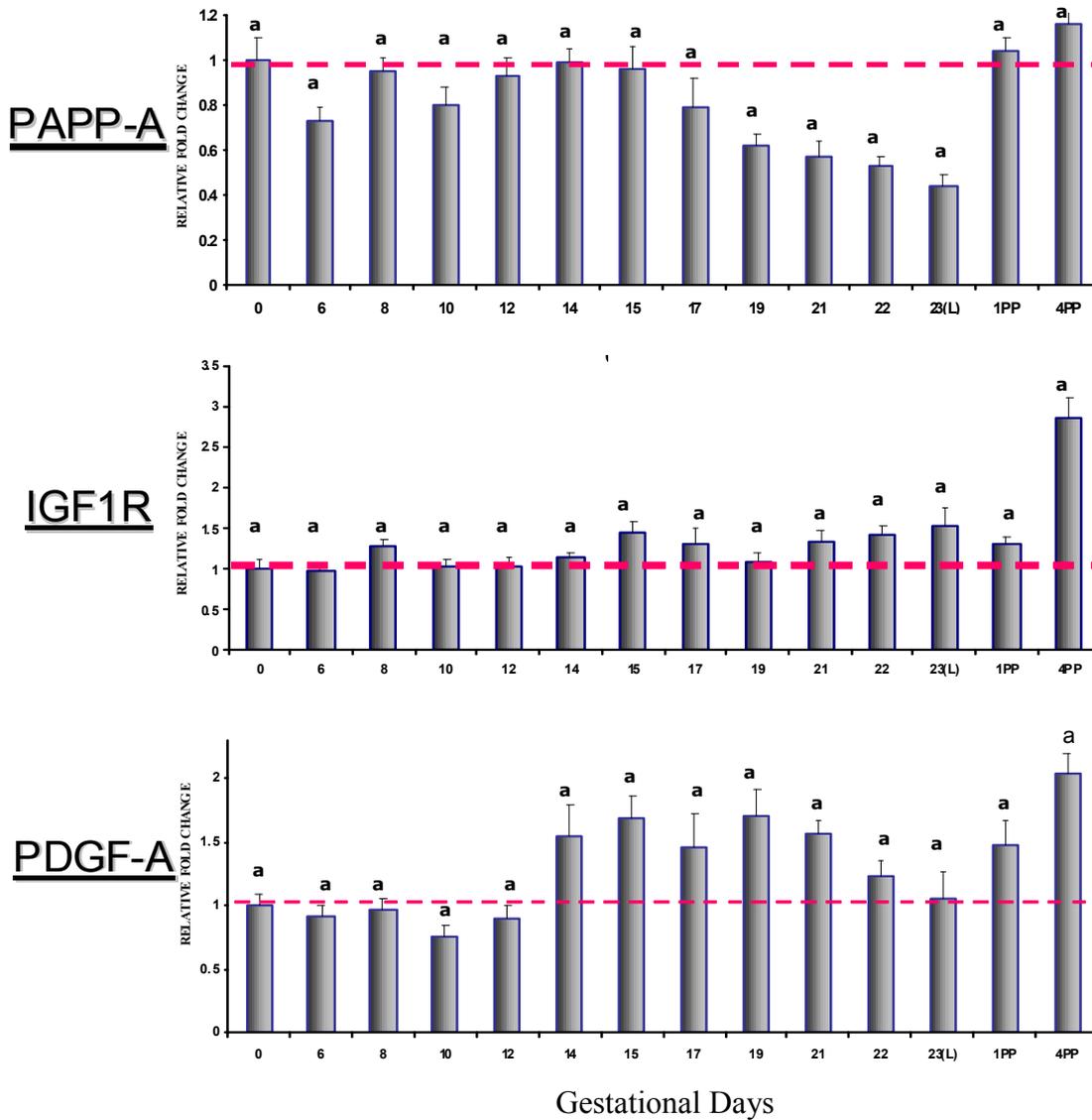
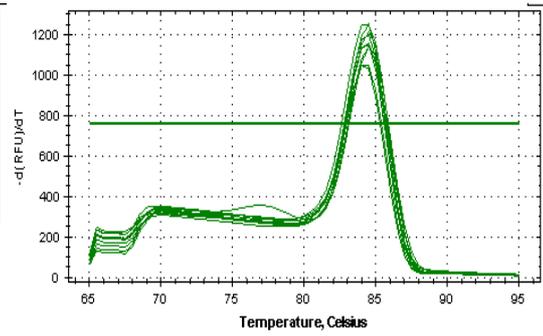
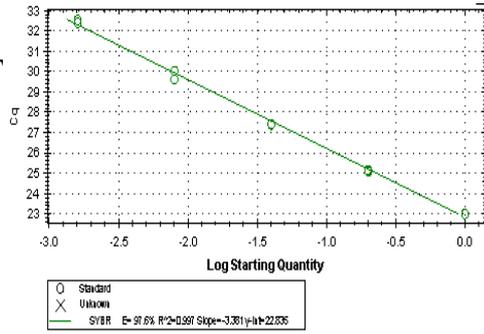


Figure 2.3 Gene expression profiles of PAPP-A, IGF1R and PDGFA across gestation in the rat myometrium. Statistical analysis was performed with one-way ANOVA followed by Student-Newman-Keuls test. Results are expressed as mean \pm SEM (n=4). Data labeled with same letters are not significantly different from each other (P>0.05). Adapted from Master thesis of Prudence Pui-Hing Tsui.

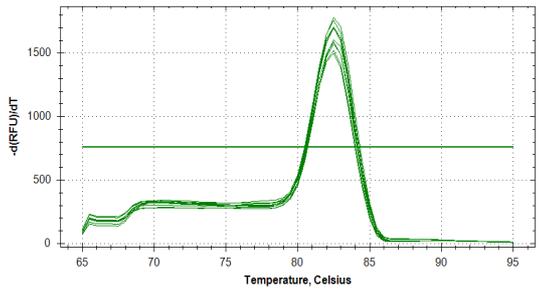
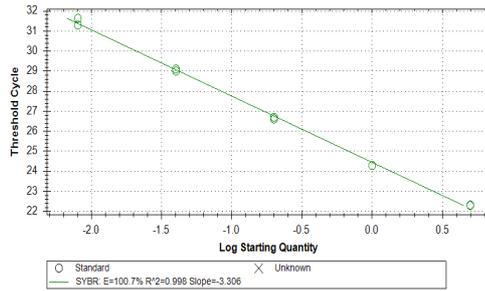
Standard Curves

Melting Curves

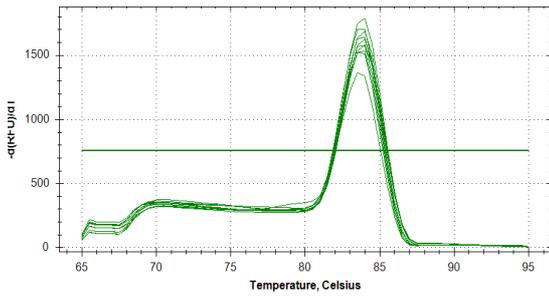
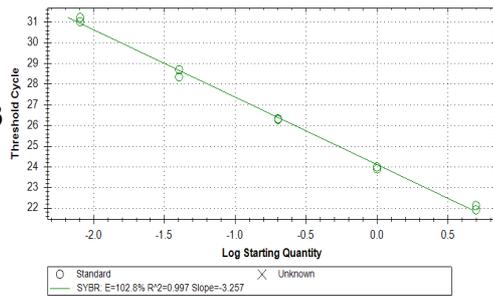
PDGF



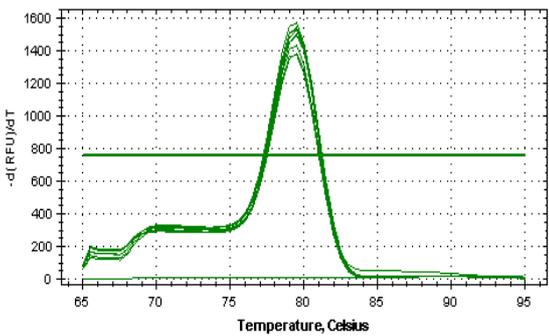
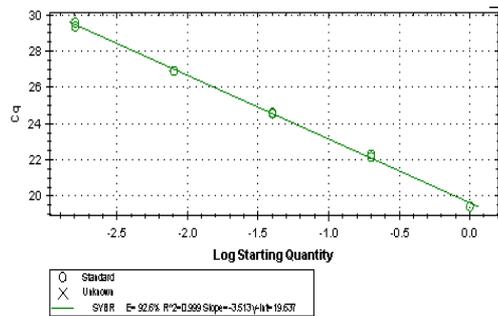
AR



Cx43



PSF



P54

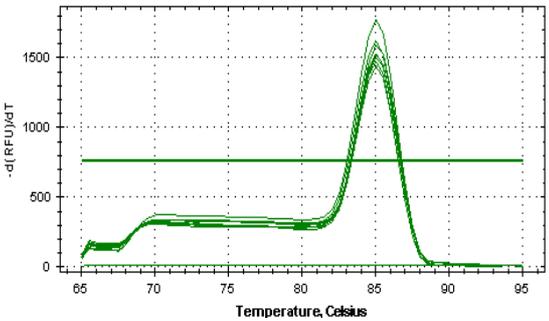
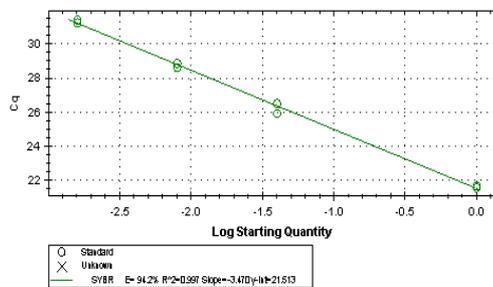


Figure 2.4 Standard curves and melting curves of primers for real-time PCR.

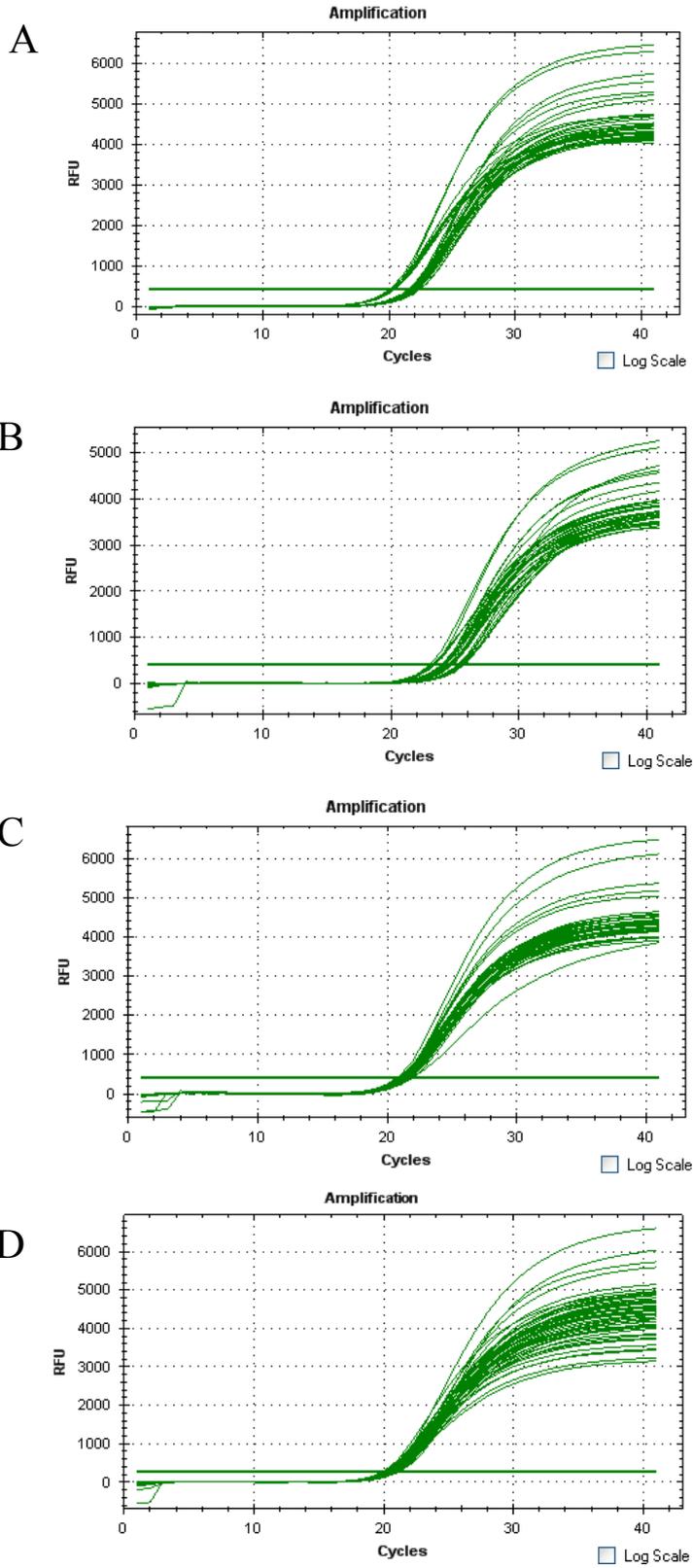


Figure 2.5 Gene expression profiles of candidate reference genes. IGF1R (A), PAPPA (B) and PDGFA (C) in OVX + E2 model. PDGFA (D) in OVX treated with E2, E2+DHT, E2+MPA.

DAKO Corporation, Caspinteria, CA) in combination with Streptavidin HRP (DAKO Corporation, Caspinteria, CA). Final visualization was achieved using Dako Liquid DAB+ Substrate Chromogen System (DAKO Corporation, Caspinteria, CA). Counterstaining with Harris` Hematoxylin (Sigma diagnostics, St. Louis, MO) was carried out before slides were mounted with Cytoseal XYL (Ricard-Allan Scientific, Kalamazoo, MI). For the assessment of staining intensity, myometrial cells from each set of tissues were observed on a Leica DMRXE microscope (Leica Microsystems, Richmond Hill, ON, Canada). A minimum of five fields were examined for each uterine horn for each set of tissue, and representative tissue sections were photographed with a Sony DXC-970 MD (Sony Ltd., Toronto, ON, Canada) 3CCD color video camera.

2.2.7. Statistical analysis:

To determine differences between groups, data from real-time PCR and immunoblot analysis of AR and Cx43 expression in gestational profiles and hormone-treated model were subjected to a one-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Tukey Post-Hoc Test). Data from unilaterally pregnant model and Laminaria-inserted unilaterally pregnant model were subjected to a two-way ANOVA followed by Bonferroni post-tests. Results are transformed by logarithmic (log) transformation if necessary. Statistical analysis was carried out using GraphPad Prism 5 for Windows (Graph Pad Software; San Diego, CA, USA) with the level of significance for comparison set at $P < 0.05$.

2.3. Results

2.3.1. AR gestational profile in rat myometrium

Myometrial tissue was collected throughout rat gestation and protein expression of AR and Cx43 was examined by immunoblot. As was reported previously (144), there was a significant up-regulation of Cx43 protein expression during active labor (D23L vs. NP, $p < 0.001$, Figure 2.6). We found that AR protein expression during early gestation was not significantly different from non-pregnant stage (Figure 2.7). AR expression in the non-pregnant rat myometrium was quite variable, possibly due to variance in hormone levels during estrous cycles since we did not control for stage of estrous cycle when tissue was collected. Starting from gestational day 14, the expression of AR decreased gradually until term labor and was low on day 1 postpartum. There was a significant 3 fold decrease in AR expression during term labor as compared to early gestation (day 8) (D8 vs. D21 & D23L & D1pp, $p < 0.01$). To localize AR protein expression in rat uterine tissue, uterine samples from pregnant rats were immunostained with anti-AR antibody. As shown in Figure 2.8, in both longitudinal and circular myometrium layers, anti-AR immunostaining was detected in the nuclei of uterine myocytes at early gestation (day 8). In mid gestation (day 15), however, anti-AR immunostaining was decreased in both layers and on day 23 during labor, only little trace of anti-AR immunostaining was detected. In summary, immunostaining of AR in both longitudinal layer and circular layer showed a similar trend with immunoblot (Figure 2.8).

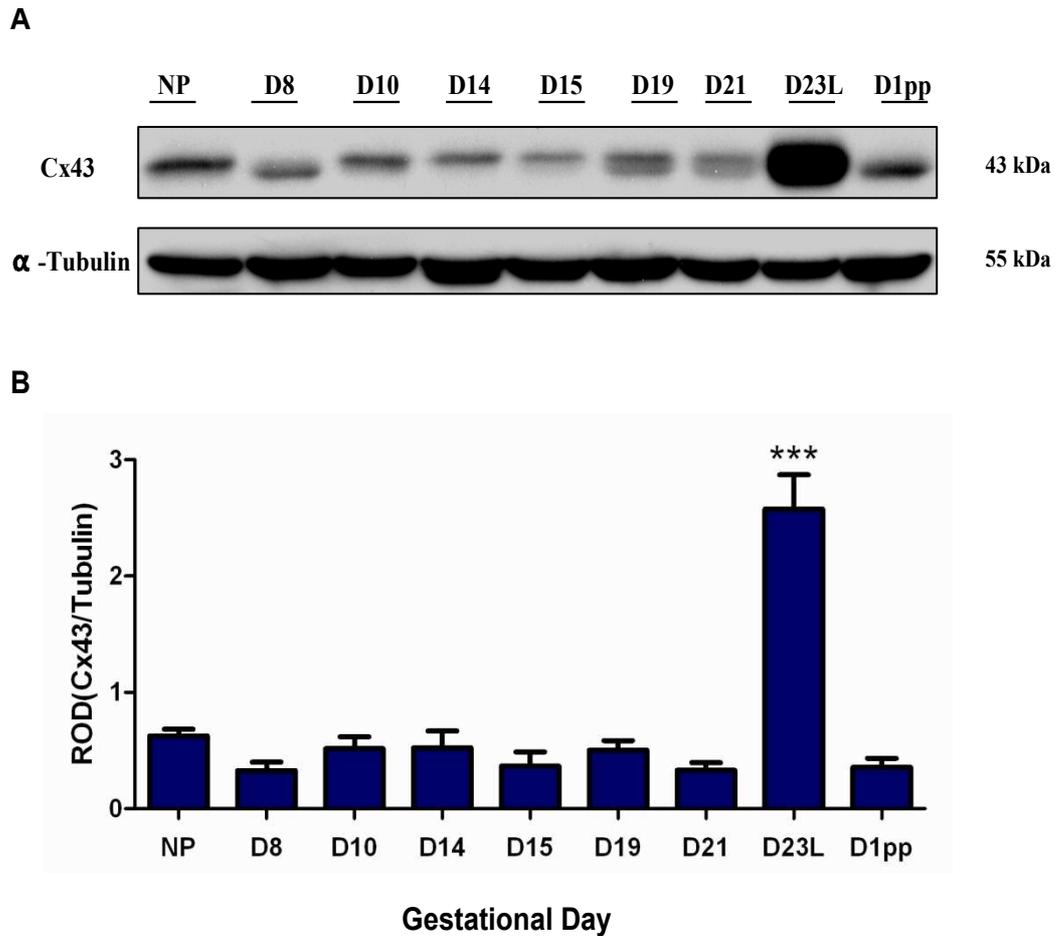


Figure 2.6 Immunoblot analysis of Cx43 protein expression in rat myometrium throughout gestation, during term labor and postpartum. (A) Representative immunoblots of Cx43 and α -Tubulin protein expression. (B) Densitometric analysis illustrating Cx43 protein expression, normalized against α -Tubulin. Results are expressed as mean \pm SEM at each time point in relative optical density (ROD) value (n=4). Statistical analysis was performed using one-way ANOVA followed by Tukey's Multiple Comparison test. A significant difference between NP and D23L is indicated by *** ($p < 0.001$).

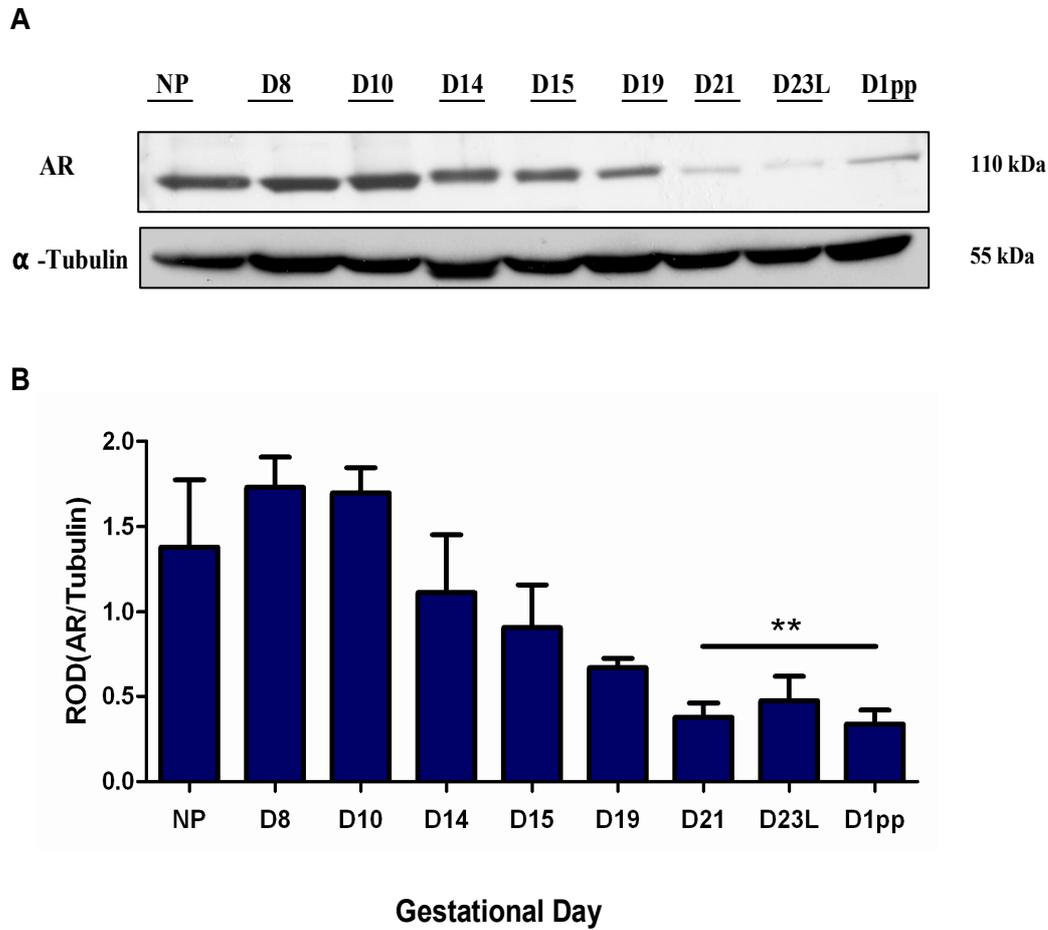


Figure 2.7 Immunoblot analysis of AR protein expression in rat myometrium throughout gestation, during term labor and postpartum. (A) Representative immunoblots of AR and α -tubulin protein expression. (B) Densitometric analysis illustrating AR protein expression, normalized against α -Tubulin. Results are expressed as mean \pm SEM at each time point in relative optical density (ROD) value (n=4). Statistical analysis was performed using one-way ANOVA followed by Tukey's Multiple Comparison test. A significant difference between D8 and D21 & D23L & D1pp is indicated by ** (p < 0.01).

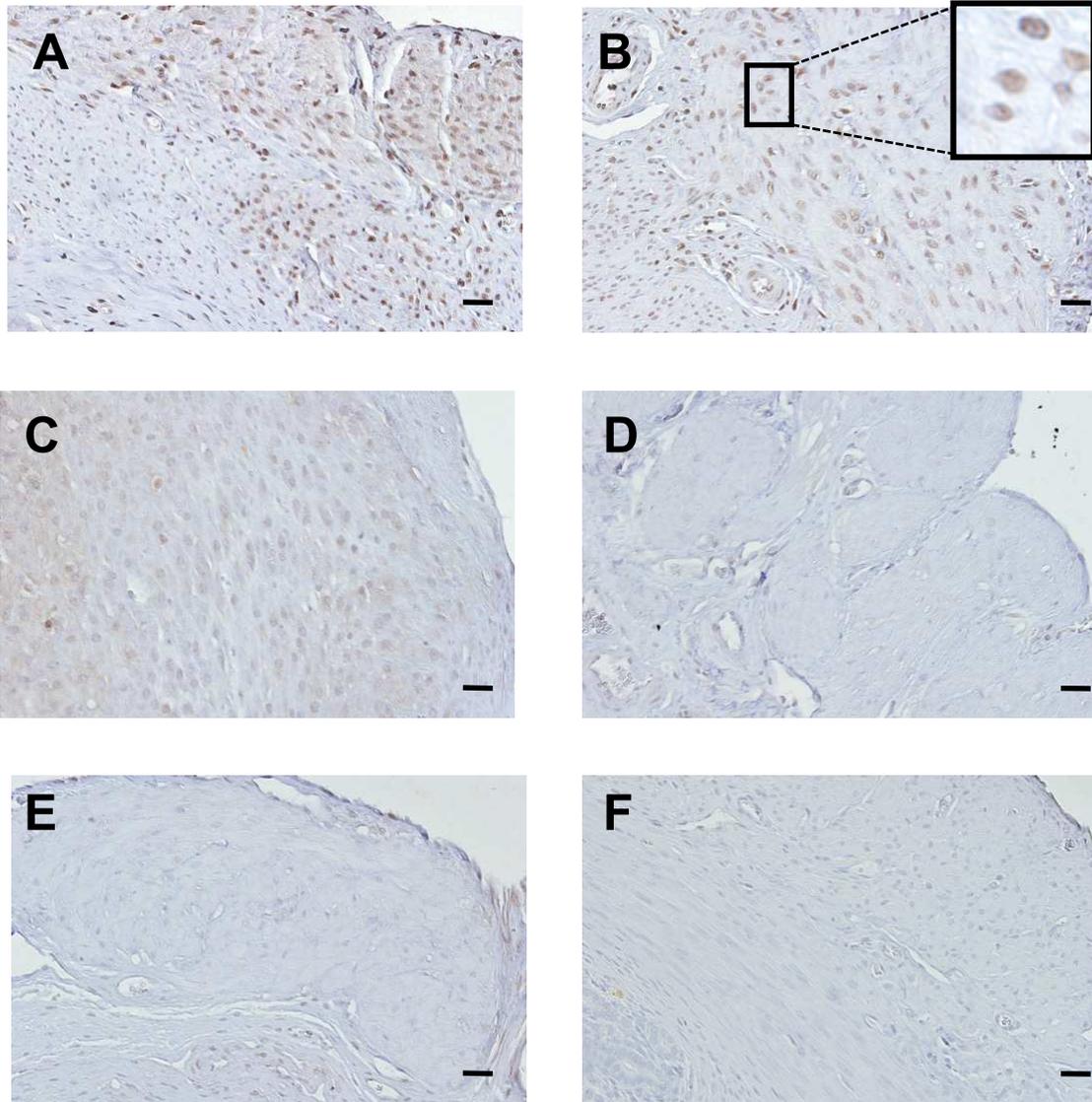
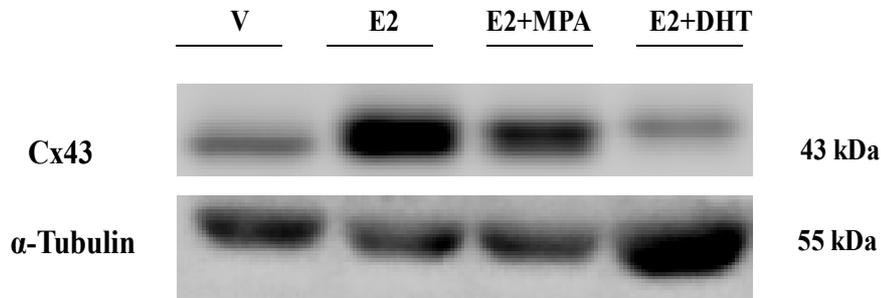


Figure 2.8 Immunohistochemical staining of AR protein expression in rat myometrium throughout gestation. For each day of gestation, tissues were collected from 3 different animals. Images were taken under 400X magnification. Immunohistochemical AR staining of rat myometrium on non-pregnant (A), day 8 (B), day 15 (C), day 23 during labor (D), one day postpartum (E) of gestation are shown above. Note the lack of staining after incubation of myometrial tissue with non-specific rabbit IgG (F).

2.3.2. Hormonal regulation of myometrial AR protein expression using a non-pregnant OVX rat model

Circulating levels of P4 in rat maternal serum peak at day 15-19 and dramatically decrease by day 23, the day of delivery. To examine the effect of steroid hormones on myometrial AR expression, we treated OVX rats with different combinations of steroid hormones (E2, E2 + MPA, E2 + androgen. Our results presented in Figure 2.9 indicated that, similar to the findings in human myometrium, Cx43 protein expression in our OVX rat model was also significantly up-regulated by E2 treatment (vehicle vs. E2, $p < 0.05$) and down-regulated by MPA treatment. Importantly Cx43 was significantly inhibited by androgen treatment after priming with E2 (E2 vs. E2 + DHT, $p < 0.05$) (Figure 2.9). In contrast to Cx43, AR expression was not significantly changed by E2 treatment of non-pregnant OVX, however, levels were significantly higher in rats treated with MPA after priming with E2, compared to E2 only treated animals (E2 + MPA vs. E2, $p < 0.05$). Androgen (DHT) treatment did not change AR expression in E2-primed myometrium (Figure 2.10).

A



B

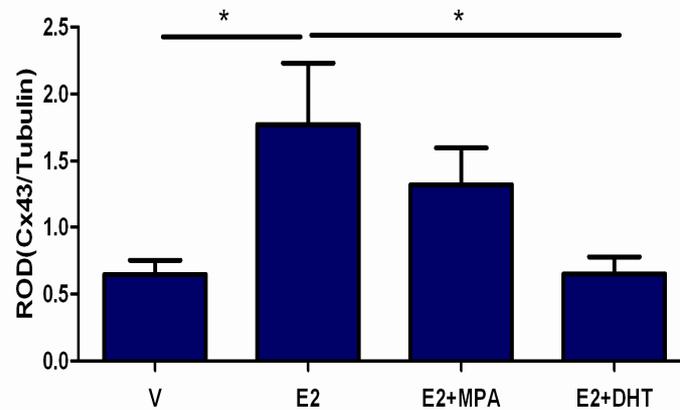


Figure 2.9 Immunoblot analysis of myometrial Cx43 protein expression in hormone-treated OVX rat model. (A) Representative immunoblots of Cx43 and α -Tubulin protein expression. (B) Densitometric analysis illustrating AR protein expression, normalized against α -Tubulin. Results were transformed by logarithmic (log) transformation and expressed as mean \pm SEM for each treatment group in relative optical density (ROD) value (n=4). Statistical analysis was performed using one-way ANOVA followed by Tukey's Multiple Comparison test. A significant difference between V and E2, and between E2 and E2 + DHT is indicated by * (p < 0.05).

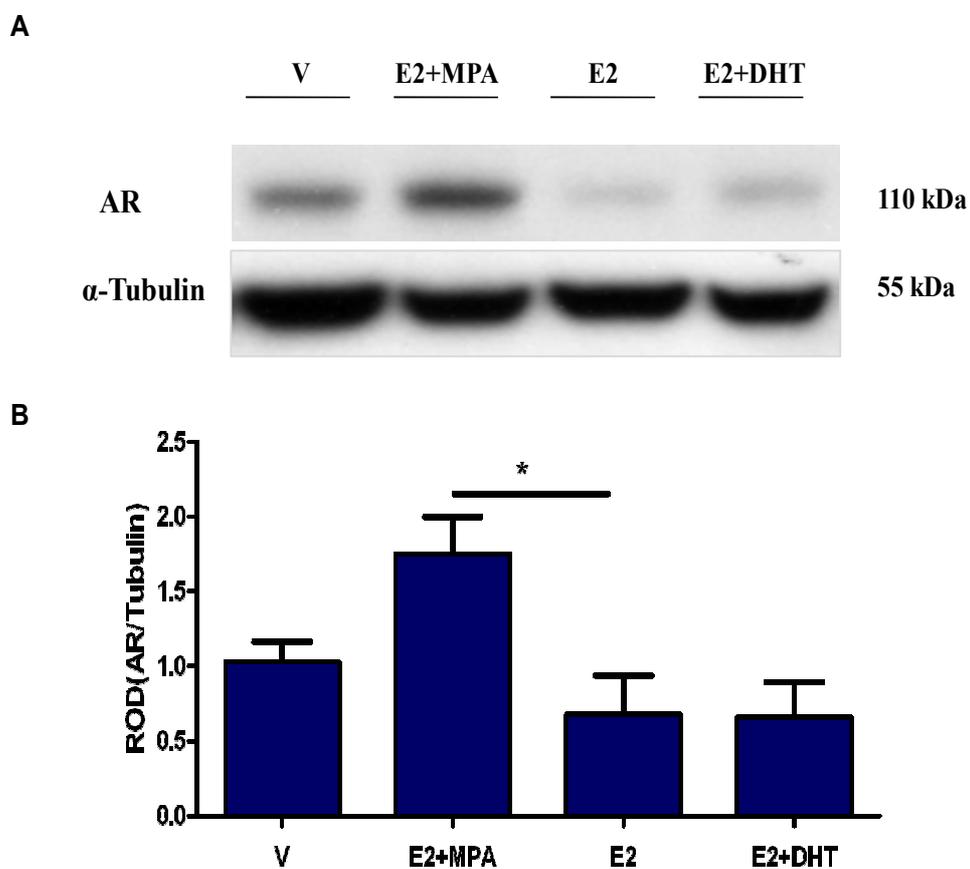


Figure 2.10 Immunoblot analysis of myometrial AR protein expression in hormone-treated OVX rat model. (A) Representative immunoblots of AR and α -Tubulin protein expression. (B) Densitometric analysis illustrating AR protein expression, normalized against α -Tubulin. Results are expressed as mean \pm SEM for each treatment group in relative optical density (ROD) value (n=5). Statistical analysis was performed using one-way ANOVA followed by Tukey's Multiple Comparison test. A significant difference between E2 and E2 + MPA is indicated by * (p < 0.05).

2.3.3. Physiological mechanical stretch inhibits myometrial AR protein expression

Since immunoblot analysis in bilaterally pregnant rats demonstrated an induction of Cx43 expression and a decrease of AR expression in the myometrium during labor, experiments were conducted to investigate the mechanisms involved. We collected myometrial tissue from both non-gravid and gravid uterine horns of tubal-ligated rats during late gestation (day 17 and day 21), during spontaneous labor, as well as 1-day post-partum. Similar to bilaterally pregnant model, our data revealed a significant increase of Cx43 protein expression in labor as compared to other gestational days ($p < 0.01$). There was a significant difference in Cx43 expression between non-gravid and gravid horns on gestational day 17 ($p < 0.05$), day 21 ($p < 0.05$), and 1 day postpartum ($p < 0.001$) (Figure 2.11). AR expression decreased throughout gestation ($p < 0.001$) in both non-gravid and gravid uterine horns of unilaterally pregnant rats, similar to our finding in bilaterally pregnant rats (Figure 2.12). On all gestational days we studied, levels of AR were significantly lower in gravid horns than non-gravid horns (gestational day 17, $p < 0.01$; day 21, $p < 0.01$; day 23 during labor, $p < 0.01$ and 1 day postpartum, $p < 0.05$) indicating that physiological stretch exerted by growing embryo down-regulated myometrial AR expression.

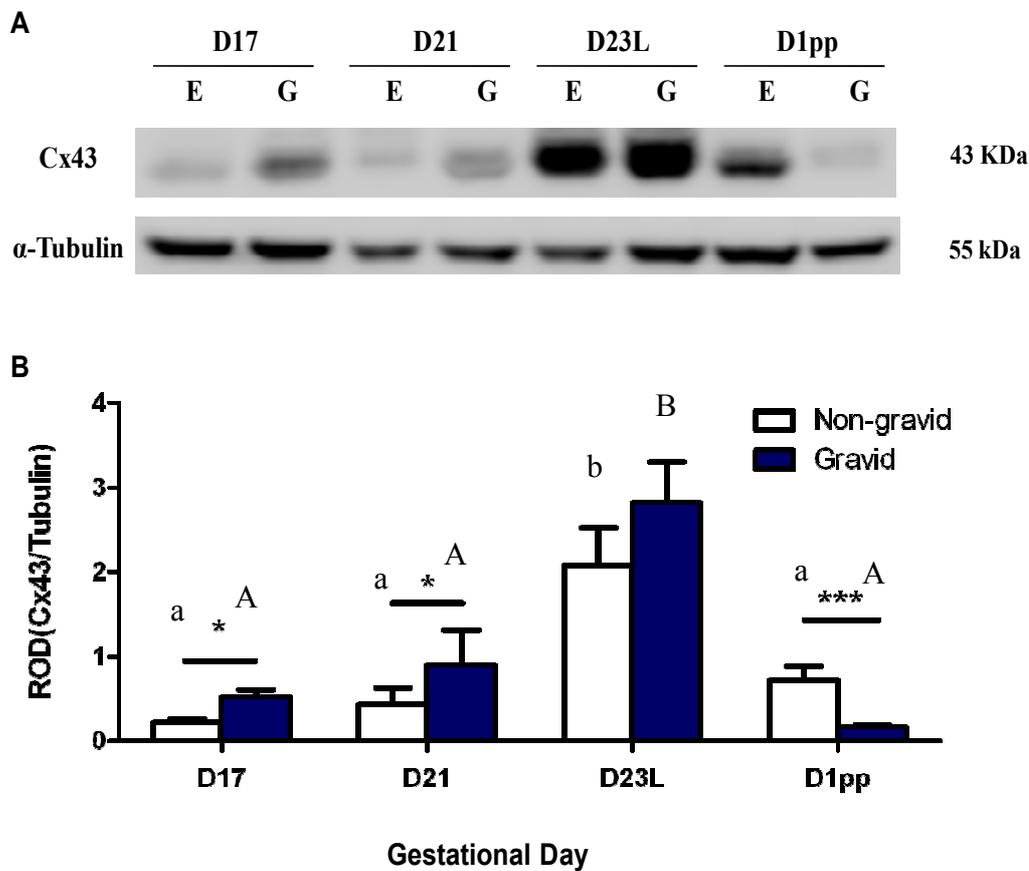


Figure 2.11 Immunoblot analysis of Cx43 protein expression in unilaterally pregnant rat model during late gestation. (A) Representative immunoblots of Cx43 and α -Tubulin protein expression. (B) Densitometric analysis illustrating Cx43 protein expression, normalized against α -Tubulin. Results were transformed by log transformation and expressed as mean \pm SEM at each time point in relative optical density (ROD) value (n=3). Empty bar represents non-gravid horn, and solid bar represents gravid horn. A significant difference between matching non-gravid and gravid horns is indicated by * ($p < 0.05$) or *** ($p < 0.001$). Data labeled with different letter indicates significant changes in Cx43 expression in non-gravid horns (small letters) or gravid horns (capital letters) during gestation ($p < 0.01$).

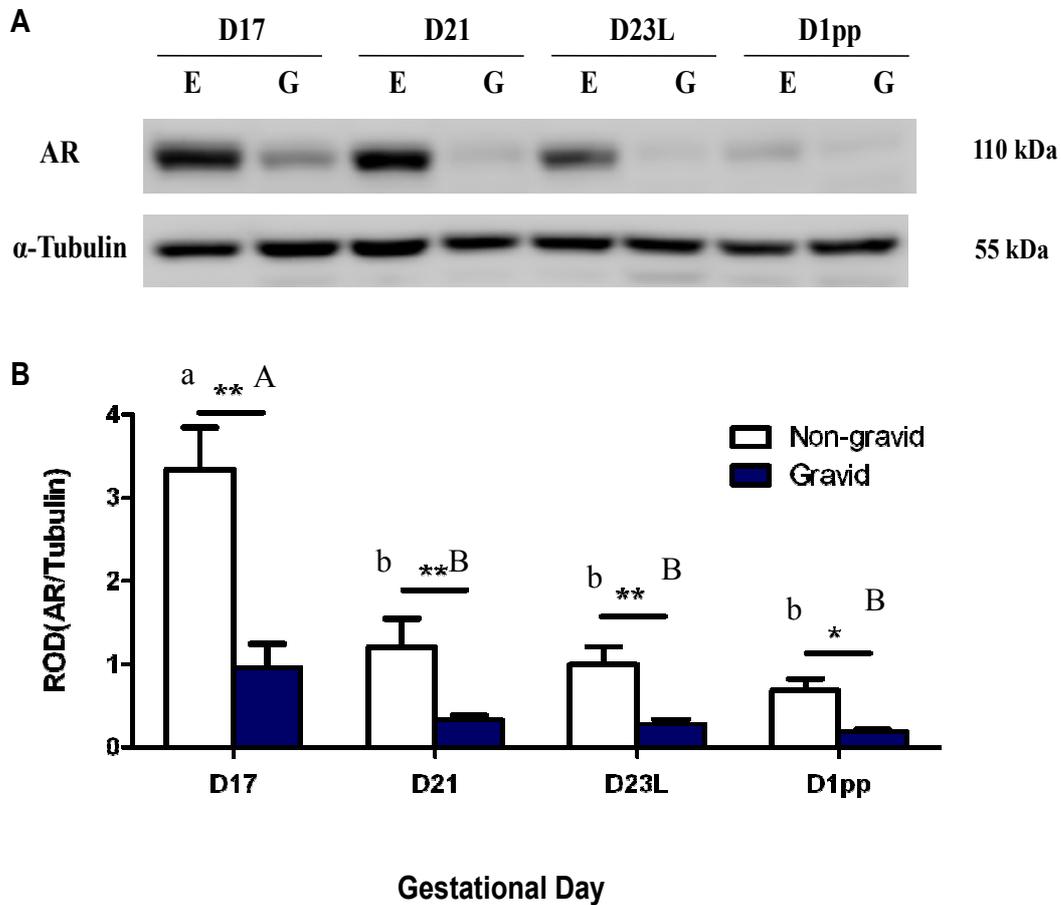


Figure 2.12 Immunoblot analysis of AR protein expression in unilaterally pregnant rat model during late gestation. (A) Representative immunoblots of AR and α -Tubulin protein expression. (B) Densitometric analysis illustrating AR protein expression, normalized against α -Tubulin. Results were transformed by log transformation and expressed as mean \pm SEM at each time point in relative optical density (ROD) value (n=3). Empty bar represents non-gravid horn, and solid bar represents gravid horn. A significant difference between matching non-gravid and gravid horns is indicated by * ($p < 0.05$) or ** ($p < 0.01$). Data labeled with different letter indicates significant changes in AR expression in non-gravid horn (small letters) or gravid horn (capital letters) during gestation ($p < 0.001$).

2.3.4. Artificial mechanical stretch inhibits myometrial AR gene expression

Since a further decrease in AR expression occurred in the gravid uterine horn compared to the non-gravid horns, we speculated that the presence of the fetal placental unit might be responsible for the decrease in AR expression. Therefore, we investigated whether artificial mechanical stretch of the uterine wall by an expendable material that mimics the effect of gravity might inhibit AR gene expression. Using the unilaterally pregnant rat model, we inserted Laminaria or a non-expendable control tube into the non-gravid uterine horns or performed sham surgery on day 19 of gestation and collected myometrial tissue from both non-gravid and gravid uterine horns on day 23 during labor. AR and Cx43 mRNA levels were analyzed by real-time PCR.

As shown in Figure 2.13, artificial mechanical stretch imposed by Laminaria insertion did not influence Cx43 expression. AR expression, however, was significantly down-regulated by Laminaria insertion into the non-gravid horn, to a level similar to that of the matching gravid horn. Sham surgery or insertion of a non-expandable plastic tube, did not reduce AR expression and levels remained significantly higher ($p < 0.01$) than that of the gravid or Laminaria-inserted horns (Figure 2.13).

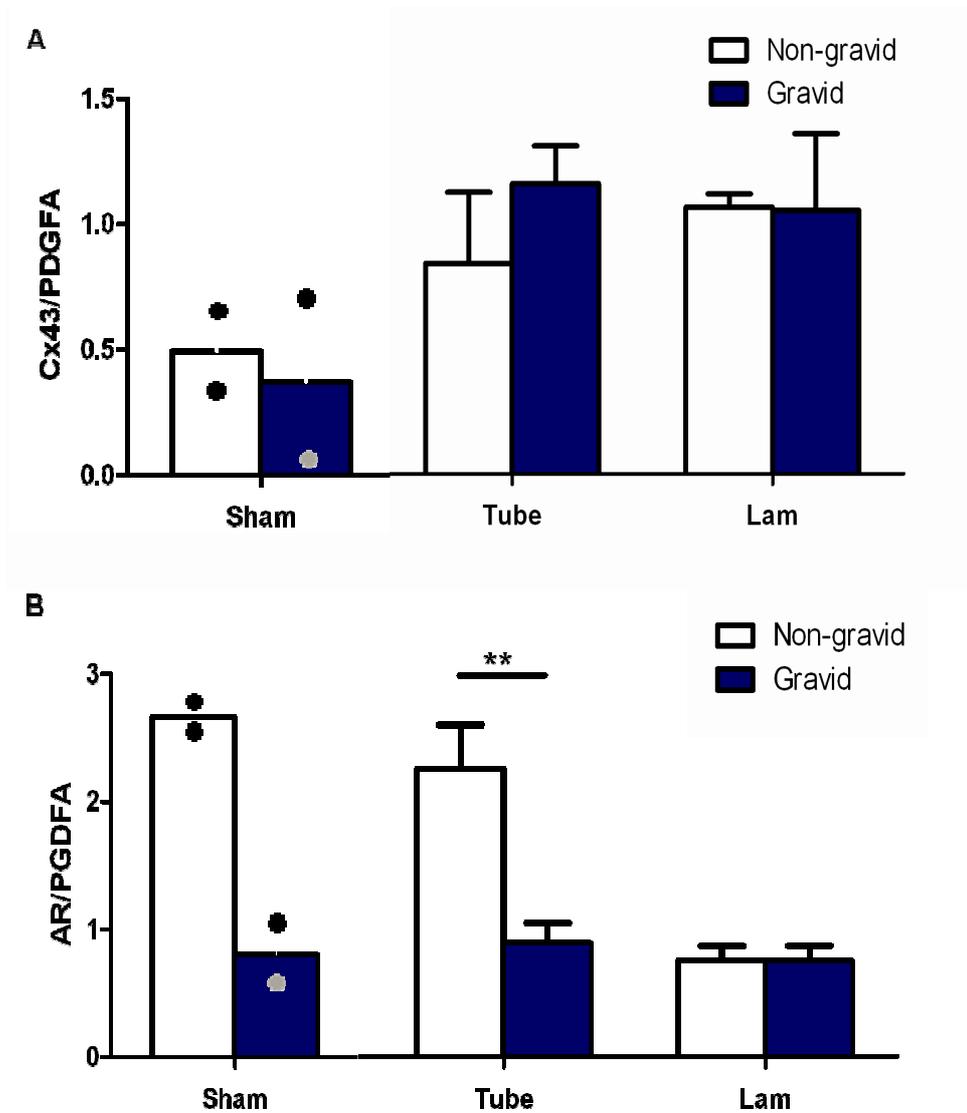


Figure 2.13 AR gene expression on Laminaria-inserted unilaterally pregnant rat model. Cx43 (A) and AR (B) mRNA levels in the myometrium of OVX rats were analyzed by Real-Time PCR using specific forward and reverse primers (see Table 2.2), normalized to PDGFA mRNAs. The bars represent mean \pm SEM (n=3 for Laminaria-inserted and tube-inserted groups, n=2 for sham surgery group). A significant difference between non-gravid and gravid horns in both tube-inserted and sham surgery group is indicated by ** (p < 0.01).

2.3.5. Modulation of androgen signaling in late pregnancy

Our data suggest that androgen signaling may have a similar effect to that of P4 signaling in maintaining pregnancy and the quiescence of the myometrium. Plasma P4 and androgen levels normally fall prior to the onset of labor. It was reported earlier that daily injection of P4 starting from day 20 to maintain plasma P4 levels to that during early stage of pregnancy can delay the onset of spontaneous labor up to 24 hours (P4-delayed labor rat model). Based on androgen serum levels during pregnancy, we performed daily injection of DHT starting from day 18 of gestation until the day of delivery. However, in contrast to that of P4 treatment, there was no change regarding the timing of parturition between DHT and vehicle treated group (data not shown).

High levels of P4 during pregnancy inhibit the synthesis of CAP genes. When PR antagonist RU486 is administered, preterm labor is induced. To test our hypothesis that androgen signaling has a similar effect as P4 signaling in maintaining pregnancy, we treated rats with Casodex (bicalutamide, AR inhibitor) to determine whether blocking androgen signaling could induce preterm labor similar to RU486. In contrast to RU486 treatment, Casodex did not induce preterm labor in pregnant rats (data not shown).

These experiments suggest that while AR expression falls at term, modulation of AR signaling does not impact the timing of delivery.

2.4 Discussion

To our knowledge, this is the first study in which myometrial androgen signaling was investigated throughout pregnancy, during labor and postpartum, and in which the expression of AR by steroid hormones and mechanical stretch in myometrium was systemically studied using rat models. Data from our study show that (1) myometrial AR protein expression throughout gestation demonstrated a similar trend to that of PR protein expression, most notably a significant decrease in expression at term prior to the onset of labor; (2) AR expression in myometrium was up-regulated by P4 treatment, but down-regulated by both physiological and artificial mechanical stretch.

During pregnancy the uterine myocytes undergo a program of differentiation, including hyperplasia during early pregnancy (proliferative phase) and hypertrophy in mid gestation (synthetic phase) (145-148). Our data suggest that these phases are mediated by changes in the endocrine and mechanical environment (the latter as a result of tension placed on the uterine wall by the growing fetus). We found that during early gestation, myometrial AR protein was highly expressed. We speculate therefore that androgen signaling might be directly involved in the process of myometrial differentiation during early pregnancy. It was reported that androgen induces skeletal muscle hypertrophy in human (138, 139) and promotes myogenic differentiation through activating follistatin, known as activin-binding protein (140). It is plausible therefore that similar to the skeletal muscle, androgen signaling is also

involved in the induction of cellular hypertrophy in uterine smooth muscle, myometrium.

It is well-known that P4 signaling plays a predominant role in maintaining the quiescence of myometrium during pregnancy. Solid evidence from previous studies and our own research indicate that androgen signaling might assist P4 signaling in maintaining pregnancy, and that the reduction of androgen signaling at term might facilitate the onset of labor. Evidence to support this notion includes: (1) a similar trend in serum levels of androgen and P4 throughout gestation in both human and rat; (2) similarity in the structure of PR and AR; (3) a similar trend of myometrial PR and AR expression throughout gestation in both human and rat; (4) a similar relaxing effect of P4 and androgen signaling on spontaneous contractile activity in human myometrium; (5) similar down-regulation of CAP gene Cx43 expression by P4 and androgen signaling.

To investigate hormonal regulation of myometrial AR expression, we used a non-pregnant OVX rat model. Our data demonstrated that after surgical removal of endogenous hormones by ovariectomy, MPA treatment boosts the expression of myometrial AR protein in rat, which indicates that AR protein expression in myometrium is positively regulated by P4 signaling, and might also be a possible explanation for the similar trend in rat AR and PR expression profiles during pregnancy. However, we could not detect any significant effect of E2 alone or E2 plus DHT treatment on the regulation of AR protein levels in rat myometrium. Similar experiments have been conducted in other species. In OVX mice, Pelletier G et al.

found E2 administration induces myometrial AR gene expression in 12 hours (149), while in OVX rhesus monkeys, it was shown that E2 treatment alone did not increase myometrial AR mRNA levels significantly, but both E2 plus P4 and E2 plus testosterone treatments increased myometrial AR mRNA levels to approximately the same level (152). It was also reported that the concentration of bovine cytosolic AR is significantly correlated with cytosolic PR and ER concentrations (153). Thus there seems to be no clear consensus relating to the steroid regulation of AR expression. This could in part be due to species differences, further confounded due to the use of different combinations of hormones (or forms of androgen) or the experimental design. It is clear that further investigations need to be conducted to more fully define the role of steroid hormone in the regulation of AR expression.

To investigate the effect of mechanical stretch on myometrial AR expression, we used both a physiological mechanical stretch model as well as an artificial mechanical stretch model. Our data show that mechanical stretch imposed by fetal growth exerts a consistent inhibitory effect on myometrial AR expression at both the mRNA and protein level. Our results of artificial mechanical stretch model confirm that the decrease in AR expression in the gravid horns is not due to the presence of the fetal/placental unit or factors produced by deciduas by showing that mechanical stretch imposed by expandable material Laminaria insertion alone could also reduce myometrial AR expression to the same level as in gravid horns. Our data clearly indicate that mechanical stretch participates in the down-regulation of myometrial AR expression during late pregnancy.

Our data suggest that androgen and P4 signaling have similar effects on the regulation of CAP gene expression and that androgen signaling might assist P4 with maintaining pregnancy. We therefore speculate therefore that (1) similar to P4-delayed labor, the administration of DHT might postpone labor in rats and (2) similar to RU486-induced preterm labor, administration of the antiandrogen (Casodex) would induce preterm labor. We chose the dosage and time of administration based on established animal models and also on the pharmacodynamics of both drugs. However, modulation of androgen signaling in late pregnancy by these treatments had no effect on the timing of labor. This failure to induce preterm labor or to delay term labor could be explained by the fact that (1) androgen signaling might not be a determinant factor in maintaining pregnancy; (2) the role of androgen signaling in maintaining myometrium quiescence might also depend on other intracellular players, such as nuclear co-activators and co-repressors. The role of myometrial AR during pregnancy and the onset of labor needs to be uncovered by further research.

CHAPTER 3:

THE EXPRESSION OF PSF AND P54 IN RAT MYOMETRIUM BY

HORMONAL AND MECHANICAL REGULATION

3.1. Introduction

PSF and p54nrb have been identified as nuclear co-repressors for both PR and AR. They contribute to the transactivation properties of PR and AR through the recruitment of proteins comprising the general transcriptional machinery to AR / PR target promoters. Previous studies have demonstrated that myometrial PSF and p54nrb protein expression decreases significantly near term and during labor (102). The molecular function of PSF and p54nrb, together with their gestational profiles implies that both proteins may play an important role in labor initiation through regulating myometrial contractility and CAP gene expression. However, the exact mechanism remains unclear. Steroid hormone signaling and mechanical stretch are well-accepted as two determining factors regulating both term and PTB. Thus, we hypothesized that the expression of PSF and p54nrb may be regulated by steroid hormones and/or mechanical stretch. Protein and/or mRNA expression of PSF and p54nrb was analyzed by immunoblot analysis, immunohistochemical staining, or real-time PCR analysis in rat myometrium of hormone-treated non-pregnant, ovariectomized (OVX) model, unilaterally pregnant model and Laminaria-inserted unilaterally pregnant model. Cx43 expression was measured by immunoblot to validate our *in vivo* rat models.

3.2. Methods

3.2.1. Animals:

Animal housing information is described in detail under section 2.2.1.

3.2.2. Experimental Design:

Hormone-treated OVX rat model:

All information about model design is described in detail under section 2.2.2.

Hormone/receptor blocker-treated OVX rat model:

Ovariectomy was performed on virgin Wistar rats as described in 2.2.2. The following hormone or hormone receptor treatments were designed to modulate the action of steroid hormones on the expression of PSF and p54nrb in the myometrium.

The OVX animals were split into seven groups (Figure 3.1):

Group 1 (control): treated with subcutaneous injections of vehicle (10% ethanol and 90% corn oil) at time 0 and time 12h (n=5).

Group 2 (estrogen): treated with subcutaneous injections of E2 only (10 µg/kg) (130) at time 0 to test estrogen regulation on PSF and p54nrb expression and as control for E2-primed MPA treated group (n=4).

Group 3 (P4): treated with subcutaneous injections of E2 (10 µg/kg) at time 0 and subcutaneous injections of MPA (medroxyprogesterone acetate, 16 mg/kg) (131) at time 12h to test MPA regulation after E2-priming (to boost PR expression in OVX myometrium) on PSF and p54nrb expression (n=5).

Group 4 (blockade of P4 signaling): treated with subcutaneous injections of E2 (10 µg/kg) and RU486 (mifepristone: 17β-hydroxy-11β-[4-di-methylaminophenyl]-17-[1-propynyl]-estra-4,10-dien-3-one (Biomol), 10 mg/kg) at time 0 and subcutaneous

injections of MPA (medroxyprogesterone acetate, 16 mg/kg) (131) at time 12h to test the effect of blocking PR on PSF and p54nrb expression (n=5).

Group 5 (androgen): treated with intraperitoneal injections of DHT (dihydrotestosterone, 1mg/kg) (132) at time 0 and 12h to test androgen regulation on PSF and p54nrb expression (n=5).

Group 6 (blockade of androgen signaling 1): treated with subcutaneous injections of anti-androgen Casodex (ICI 176,334: (2RS)-4-cyano-3-(4-fluorophenylsulphonyl)-2-anilide, 100mg/kg) (135) at time -1h and 12h, and intraperitoneal injections of DHT (dihydrotestosterone, 1mg/kg) (132) at time 0 and 12h to test the effect of blocking AR by Casodex on PSF and p54nrb expression (n=5).

Group 7 (blockade of androgen signaling 2): treated with subcutaneous injections of a potent anti-androgen Hydroxyflutamide (5 mg/rat) (136) at time -1h and 12h, and intraperitoneal injections of DHT (dihydrotestosterone, 1mg/kg) (132) at time 0 and 12h to test the effect of blocking AR by Hydroxyflutamide on PSF and p54nrb expression (n=5).

Myometrium was collected at 24h for biochemical analysis.

E2-treated OVX rat model:

Ovariectomy was performed on virgin Wistar rats under general anesthesia via isoflurane inhalation. Bilateral flank incisions were made from both sides of the animal, and the ovaries were removed using fine scissors. Rats were allowed to

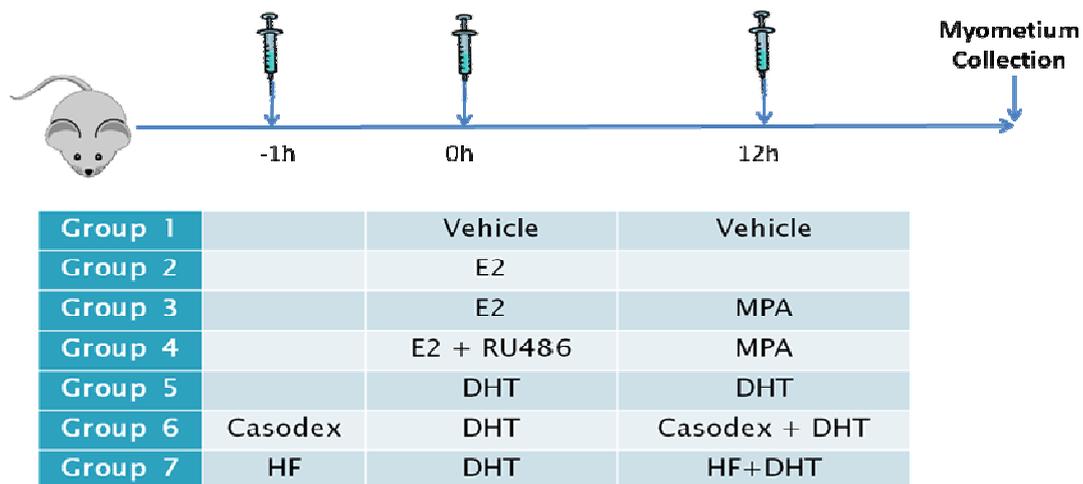


Figure 3.1 Scheme of injections and tissue collection for hormone/receptor blocker-treated OVX rat model.

Seven different groups of OVX animals were treated with steroid hormones and/or steroid hormone receptor blockers as described in the table above with the dosages of E2 (estrodial 10 $\mu\text{g}/\text{kg}$ per rat), MPA (Medroxyprogesterone acetate, 16 mg/kg per rat), DHT (Dihydrotestosterone, 1 mg/kg per injection per rat), Casodex (100 mg/kg per injection per rat), and Hydroxyflutamide: 5 mg per injection per rat. Myometrium samples were collected at 24 hour.

recover for 7 d to ensure that the endogenous levels of estrogens were reduced. The OVX animals were split into four groups. Each group consisted of 4 animals and were treated with sc injections of E2 (4 µg/kg per injection per rat) at time 0, 3h, and 6h. Animals (n=4) were euthanized at time 0, 3h, 6h and 24 h.

Unilaterally pregnant rat model:

All information about model design is described in detail under section 2.2.2.

Laminaria-inserted unilaterally pregnant rat model:

All information about model design is described in detail under section 2.2.2.

3.2.3. Tissue collection:

All information about tissue collection is described in detail under section 2.2.3.

3.2.4. Western immunoblot analysis:

Frozen myometrial tissue was crushed under liquid nitrogen using a mortar and pestle. Total proteins were extracted as described in section 2.2.3 and 2.2.4. Protein expression levels of PSF and p54 were measured by immunoblot analysis using primary and secondary antibodies summarized in Table 3.1.

Table 3.1. Summary of antibodies used in immunoblot analysis.

Antibody	Primary or secondary	Mono or polyclonal	Dilution	Source
PSF	Primary	Polyclonal	1:1000	(Rabbit) Cedarlane
P54	Primary	Monoclonal	1:2000	(Mouse) BD Biosciences
Anti-mouse IgG	Secondary	HRP-linked	1:2000	GE Healthcare
Anti-rabbit IgG	Secondary	HRP-linked	1:2000	(Donkey) GE Healthcare
Alpha Tubulin	Primary	Monoclonal	1:2000	(Mouse) Sigma

3.2.5. Real-Time PCR Analysis:

Total mRNAs were extracted as described in section 2.2.5. mRNA expression levels of PSF and p54 were measured by real-time PCR analysis using primary and secondary antibodies summarized in Table 3.2.

Table 3.2. Real-time PCR primer sequences of target genes and housekeeping gene.

Genes		Primer Sequences	Accession #
PSF	Forward	5' – CCAGGAAGAATTAAGGCGCA -3'	NM_001025271
	Reverse	5' – TCCTCTTGCCTCAACTGCATT -3'	
P54	Forward	5' – TGGAAACACGAACCCTAGCG -3'	NM_001012356
	Reverse	5' – CACGTACTGAGGAAGGTTGCG -3'	
PDGFA	Forward	5' – AGCGACTGGCTCGAAGTC -3'	NM_012801
	Reverse	5' – CTCCAAGGCATCCTCAGC -3'	

3.2.6. Immunohistochemistry:

Immunohistochemical analysis was performed as described in detail under section 2.2.6. The antigens were unmasked using Trypsin 0.125% 15mins, and incubated with primary PSF monoclonal mouse antibody (1:250, Sigma, USA) overnight. Secondary antibodies used for detection were biotinylated goat anti-mouse (1:300, DAKO Corporation, Caspinteria, CA) in combination with Streptavidin HRP (DAKO Corporation, Caspinteria, CA). Stained and total consecutive cells were counted using 400X magnification, from 5 noncontiguous, randomly selected fields of all layers of myometrium under blinded experimental design. Percentages of PSF-positive nuclei were determined by the number of cells having positively staining nuclei divided by total number of cells in the field (labeled and unlabeled) and multiplied by 100.

3.2.7. Statistical Analysis:

To determine differences between groups, data from hormone-treated OVX model were subjected to a one-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Tukey Post-Hoc Test). Data from unilaterally pregnant model and Laminaria-inserted unilaterally pregnant model were subjected to a two-way ANOVA followed by Bonferroni post-tests. Results were transformed by log transformation if necessary. Statistical analysis was carried out using GraphPad Prism 5 for Windows (Graph Pad Software; San Diego, CA, USA) with the level of significance for comparison set at $P < 0.05$.

3.3. Results

3.3.1. E2 regulation of myometrial PSF expression on OVX rat model

P4 plays a predominant role in maintaining pregnancy. In our laboratory, PSF has been identified as a PR co-repressor and has been considered to play a major role in the putative mechanisms leading to a “functional withdrawal of P4”. Yu et al. reported earlier that myometrial PSF protein level in rats was down-regulated at term and during spontaneous labor. To investigate whether the increase in estrogen concentrations at term contributes to the decrease of PSF expression at labor, we treated OVX rats with E2 and collected uterine tissue at different time points to examine myometrial PSF expression by immunohistochemical staining. Relative PSF protein expression, calculated by PSF positive cells versus total cell number is shown in Figure 3.2 B. In myometrium, PSF expression significantly decreased, starting from 6h (vehicle vs. 6h, $p < 0.05$, vehicle vs. 24h, $p < 0.01$). Our data indicates estrogen signaling down-regulates myometrial PSF expression.

Importantly, consistent with our data from the E2-treated OVX rat model, Cx43 expression was significantly up-regulated by E2 treatment as detected by immunoblot analysis (vehicle vs. 24h, $p < 0.01$) (Figure 3.2 A).

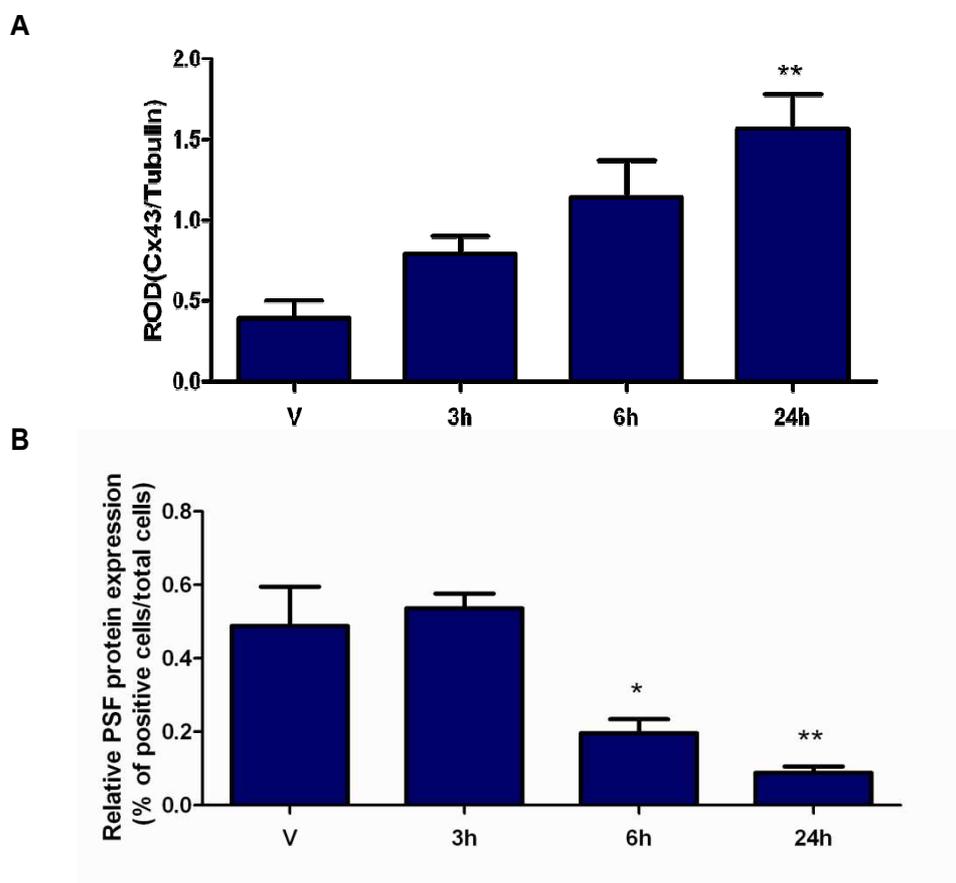


Figure 3.2 Myometrial Cx43 (A) and PSF (B) protein expression in E2-treated OVX rat model. (A) Densitometric analysis illustrating Cx43 protein expression, normalized against α -Tubulin. (B) Relative PSF protein expression was determined by the number of cells having PSF positively staining divided by total number of cells in the field and multiplied by 100. Results are expressed as mean \pm SEM for each treatment group in relative optical density (ROD) value (n=4). Statistical analysis was performed using one-way ANOVA followed by Tukey's Multiple Comparison test. A significant difference in Cx43 expression between V and 24h is indicated by ** (p < 0.01); in PSF expression between V and 6h, and between V and 24h is indicated by * (p < 0.05) and ** (p < 0.01).

3.3.2. Hormonal regulation of myometrial PSF and p54 protein expression on OVX rat model

During rat pregnancy, circulating levels of P4 and androgen levels are elevated in rat maternal serum, but levels falls towards term, when estrogen levels increase. To mimic pregnancy and labor conditions in OVX rats and to compare the effect of androgen with P4 on myometrial PSF and p54nrp expression, we treated OVX rats with different combinations of steroid hormones (E2, E2 + MPA, E2 + DHT) and examined myometrial PSF and p54 expression. In our OVX model, PSF protein expression was significantly up-regulated by DHT treatment (E2 + DHT vs. E2, $p < 0.05$), but not significantly regulated by either E2 or MPA treatment (E2 vs. E2 + MPA, $p > 0.05$) (Figure 3.3). Our data indicate that androgen signaling has a greater effect on regulating myometrial PSF expression than P4 signaling, which might account for the high expression of PSF during early pregnancy. p54nrp expression was not influenced by any of the hormones examined ($p > 0.05$) (Figure 3.4).

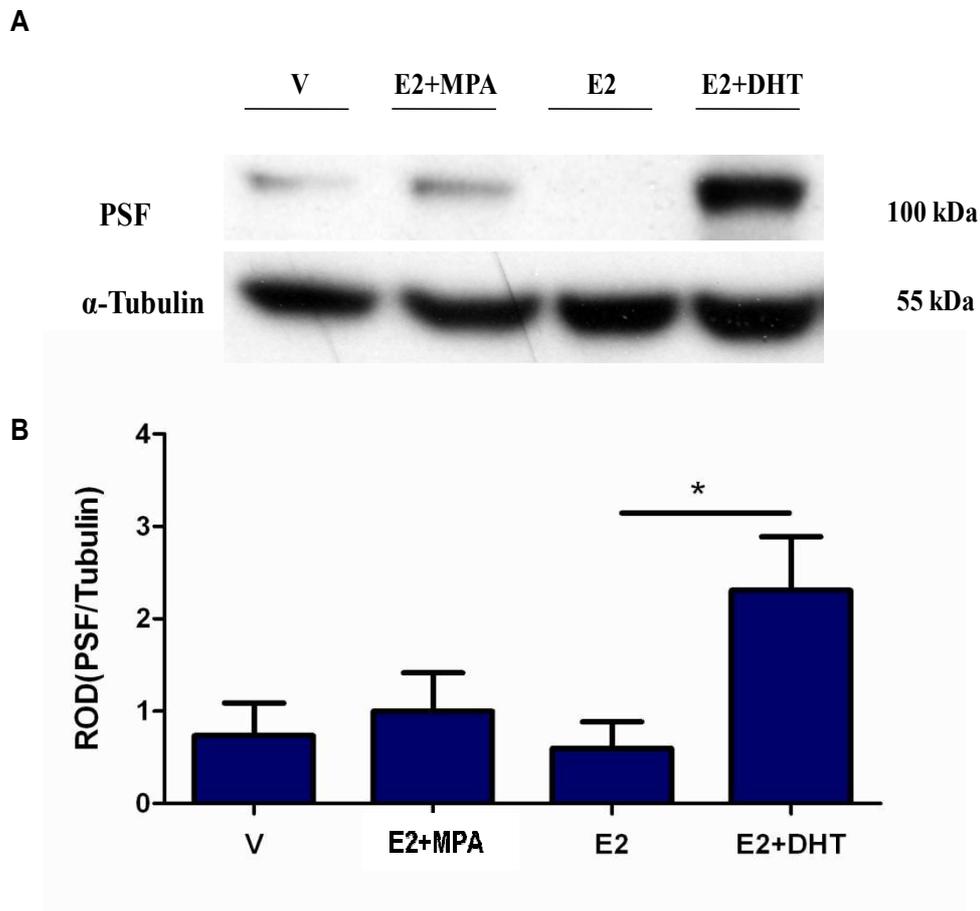
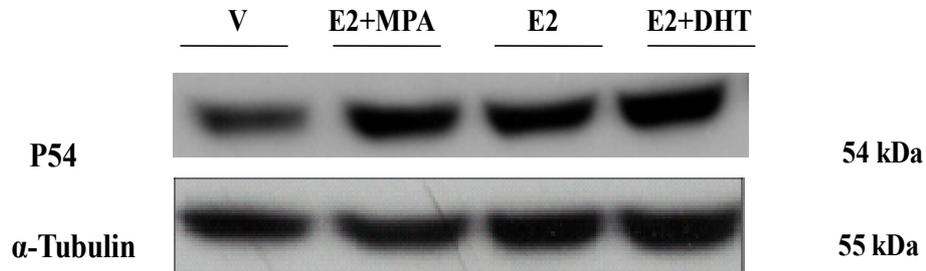


Figure 3.3 Immunoblot analysis of myometrial PSF protein expression in hormone-treated OVX rat model (A) Representative immunoblots of PSF and α -Tubulin protein expression. (B) Densitometric analysis illustrating PSF protein expression, normalized against α -Tubulin. Results are expressed as mean \pm SEM for each treatment group in relative optical density (ROD) value (n=5). Statistical analysis was performed using one-way ANOVA followed by Tukey's Multiple Comparison test. A significant difference between E2 and E2 + DHT is indicated by * (p < 0.05).

A



B

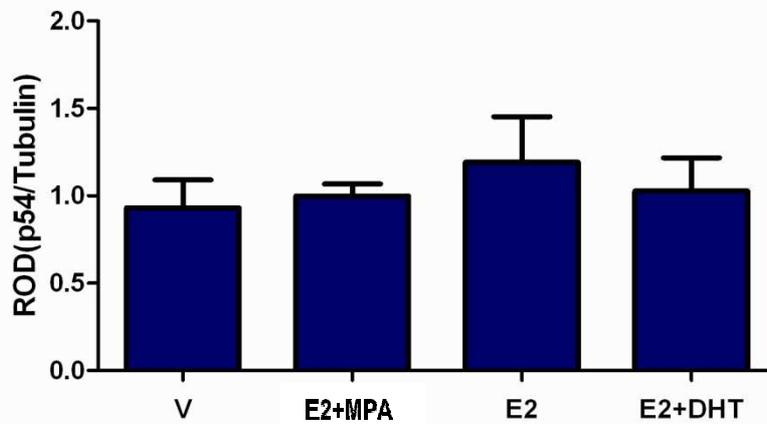


Figure 3.4 Immunoblot analysis of myometrial p54nrp protein expression in hormone-treated OVX rat model (A) Representative immunoblots of p54nrp and α -Tubulin protein expression. (B) Densitometric analysis illustrating p54nrp protein expression, normalized against α -Tubulin. Results are expressed as mean \pm SEM for each treatment group in relative optical density (ROD) value (n=5). Statistical analysis was performed using one-way ANOVA followed by Tukey's Multiple Comparison test, no significance detected.

3.3.3. Regulation of myometrial PSF and p54 mRNA expression by modulation of steroid hormone signaling in OVX rat model

Interestingly, reports suggest that there are differences in the expression of PSF mRNA and protein during rat gestation: up-regulation of PSF gene expression occurred near term and during labor whereas PSF protein was down-regulated. Our data (Figure 3.4) indicate that androgen treatment up-regulates the expression of myometrial PSF protein. To systemically study the regulation of myometrial PSF and p54nrb gene expression by steroid hormone signaling, we treated OVX rats with different combinations of steroid hormones (E2, E2 + MPA, DHT) and/or steroid hormone receptor blockers (PR blocker: RU486, AR blockers: Hydroxyflutamide and Casodex) and examined myometrial PSF and p54 mRNA expression by real-time PCR. To examine the effect of DHT alone, in this set of experiment, DHT treatment was not preceded by E2.

Similar to what was observed in protein levels, Cx43 mRNA expression in OVX rats was significantly up-regulated by E2 treatment (vehicle vs. E2, $p < 0.001$) and significantly down-regulated by MPA treatment (E2 vs. E2 + MPA, $p < 0.001$). Injection of RU486 before MPA treatment blocked the effect of MPA treatment (E2 + MPA + RU486 vs. E2 + MPA, $p < 0.001$). However, there were no changes in Cx43 mRNA expression after modulation of androgen signaling by DHT alone or in combination with AR inhibitor (Hydroxyflutamide or Casodex) (Figure 3.5).

Consistent with the gestational profile of PSF mRNA expression, PSF mRNA expression was significantly up-regulated by E2 treatment (vehicle vs. E2, $p < 0.01$). Although the reduction of PSF mRNA by MPA did not reach significance (E2 vs. E2

+ MPA, $p > 0.05$), RU486 treatment increased PSF mRNA expression significantly (E2 + MPA + RU486 vs. E2+ MPA, $p < 0.01$), whereas neither androgen nor androgen blockers changed PSF mRNA expression (Figure 3.6). Similar to the results of protein study, p54nrb expression was not influenced by any hormone or hormone blocker examined in our OVX model ($p > 0.05$) (Figure 3.7).

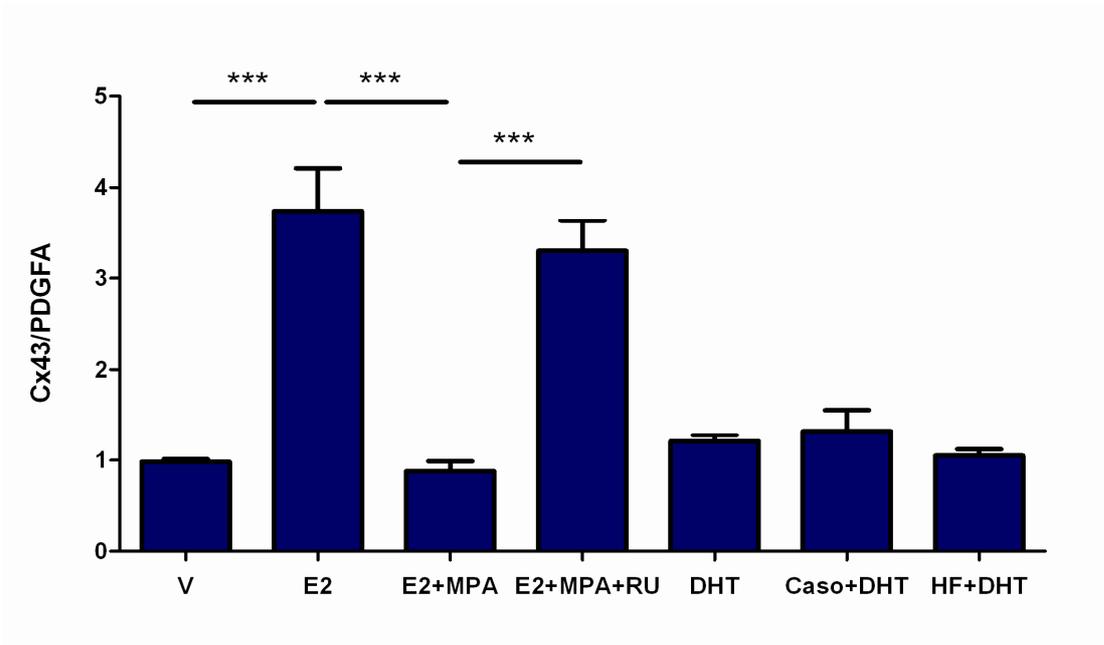


Figure 3.5 Cx43 gene expression on hormone/receptor blocker treated OVX rat model. Cx43 mRNA levels in the myometrium of OVX rats were analyzed by Real-Time PCR using specific forward and reverse primers (see Table 3.2), normalized to PDGFA mRNAs. The bars represent mean \pm SEM (n=5). A significant difference between vehicle and E2, between E2 and E2 + MPA, and between E2 + MPA and E2 + MPA + RU486 is indicated by *** ($p < 0.001$).

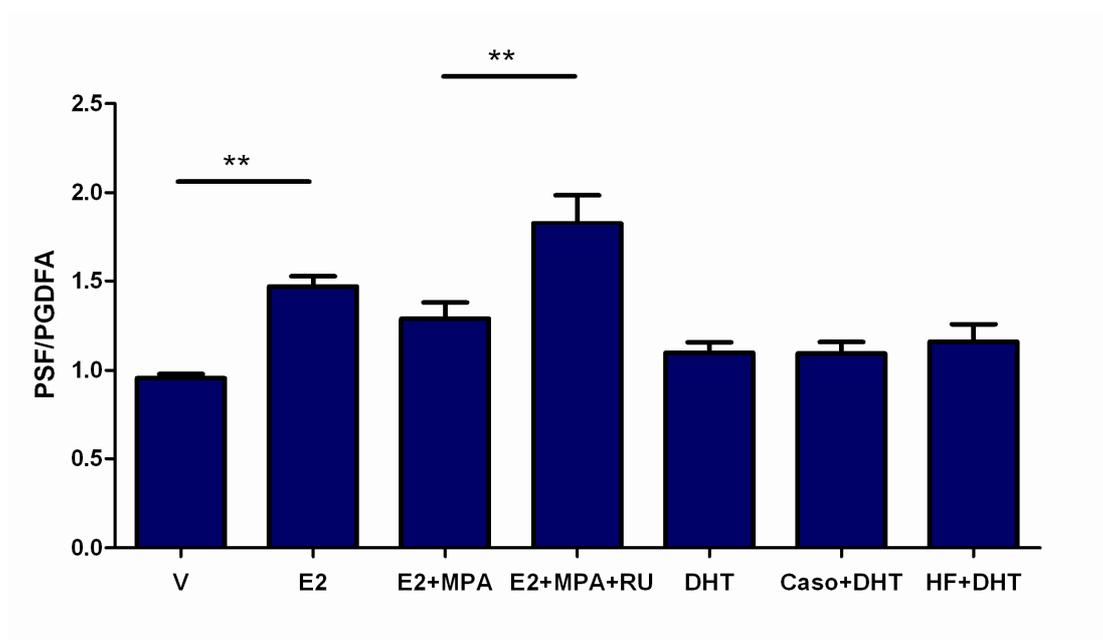


Figure 3.6 PSF gene expression on hormone/receptor blocker treated OVX rat model. PSF mRNA levels in the myometrium of OVX rats were analyzed by Real-Time PCR using specific forward and reverse primers (see Table 3.2), normalized to PDGFA mRNAs. The bars represent mean \pm SEM (n=5). A significant difference between vehicle and E2, and between E2 + MPA and E2 + MPA + RU486 is indicated by ** ($p < 0.01$).

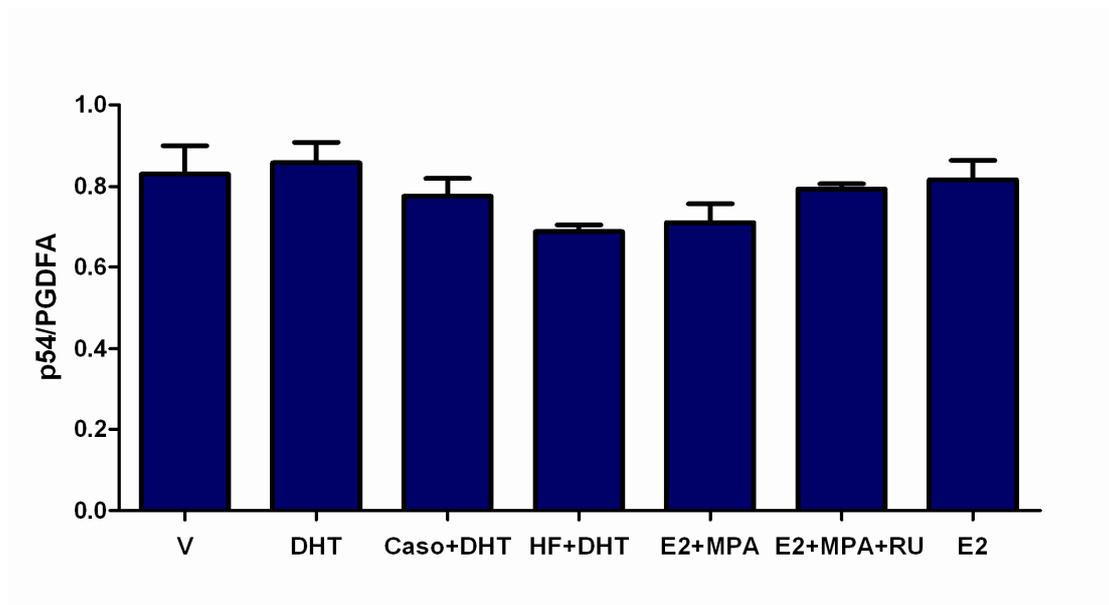


Figure 3.7 p54nrb gene expression on hormone/receptor blocker treated OVX rat model. P54nrb mRNA levels in the myometrium of OVX rats were analyzed by Real-Time PCR using specific forward and reverse primers (see Table 3.2), normalized to PDGFA mRNAs. The bars represent mean \pm SEM (n=5). No significant difference was detected ($p > 0.05$).

3.3.4. Physiological mechanical stretch inhibits myometrial PSF and p54nrb protein expression

Immunoblot analysis in bilaterally pregnant rats demonstrated a decrease of both PSF and p54nrb protein expression in the myometrium during labor. We therefore focused our attention on the impact of mechanical stretch during late gestation and labor. We collected myometrial tissue from both non-gravid and gravid uterine horns of tubal-ligated rats during late gestation including day 17 and day 21, during spontaneous labor, as well as 1- day post-partum. Similar to the bilaterally pregnant model, our data revealed significant decrease of PSF protein expression near term and during labor in gravid uterine horns compared to matching non-gravid uterine horns ($p < 0.001$). There was a significant reduction in PSF mRNA in gravid versus non-gravid horns on gestational day 21 ($p < 0.05$), day 23 during labor ($p < 0.05$), and 1 day postpartum ($p < 0.01$) (Figure 3.8). For p54nrb expression, there was significant reduction in mRNA expression in gravid versus non-gravid horns only on gestational day 23 during labor ($p < 0.05$) (Figure 3.9). Our data indicate that physiological mechanical stretch inhibits myometrial PSF protein expression and may also influence myometrial p54nrb protein expression during late pregnancy and labor.

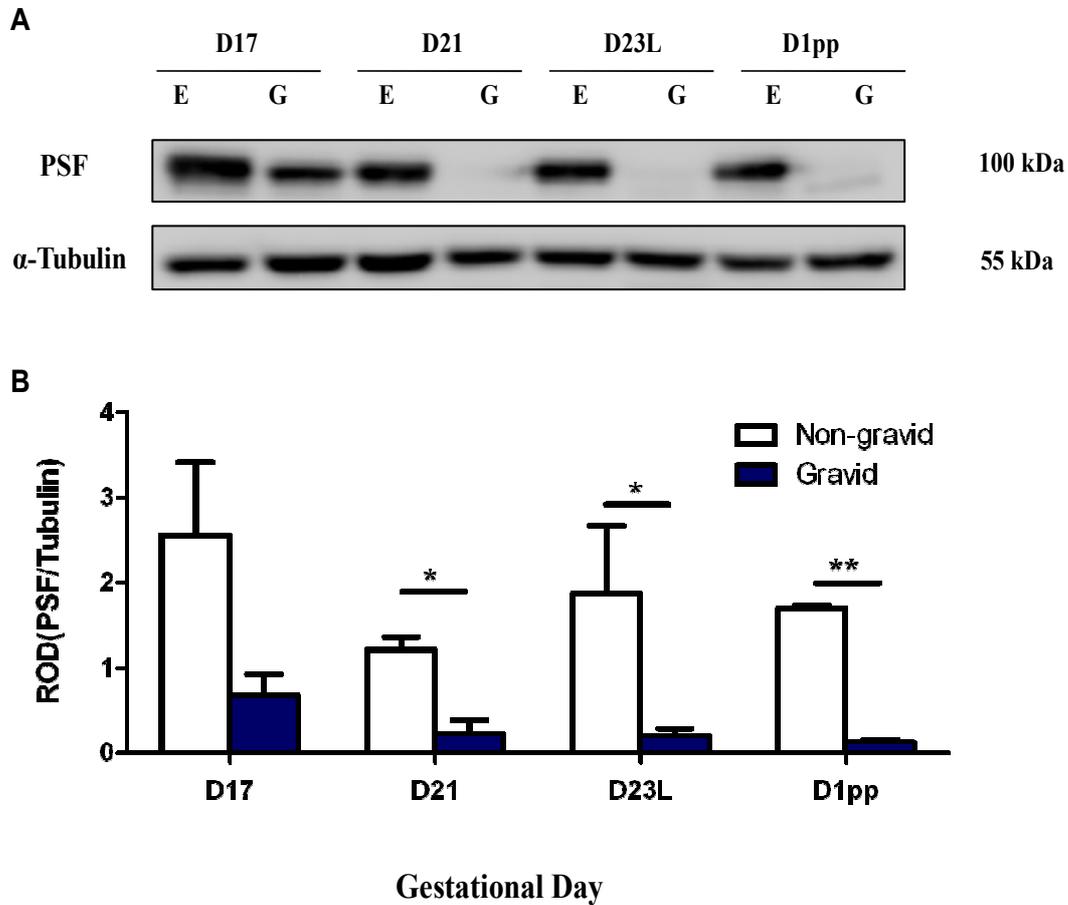


Figure 3.8 Immunoblot analysis of PSF protein expression in unilaterally pregnant rat model during late gestation. (A) Representative immunoblots of PSF and α -Tubulin protein expression. (B) Densitometric analysis illustrating PSF protein expression, normalized against α -Tubulin. Results were transformed by log transformation and expressed as mean \pm SEM at each time point in relative optical density (ROD) value (n=3). Empty bar represents non-gravid horn, and solid bar represents gravid horn. Statistical analysis was performed using two-way ANOVA followed by Bonferroni posttests. A significant difference between non-gravid and gravid horns on D21, D23L and D1pp is indicated by * ($p < 0.05$) and ** ($p < 0.01$).

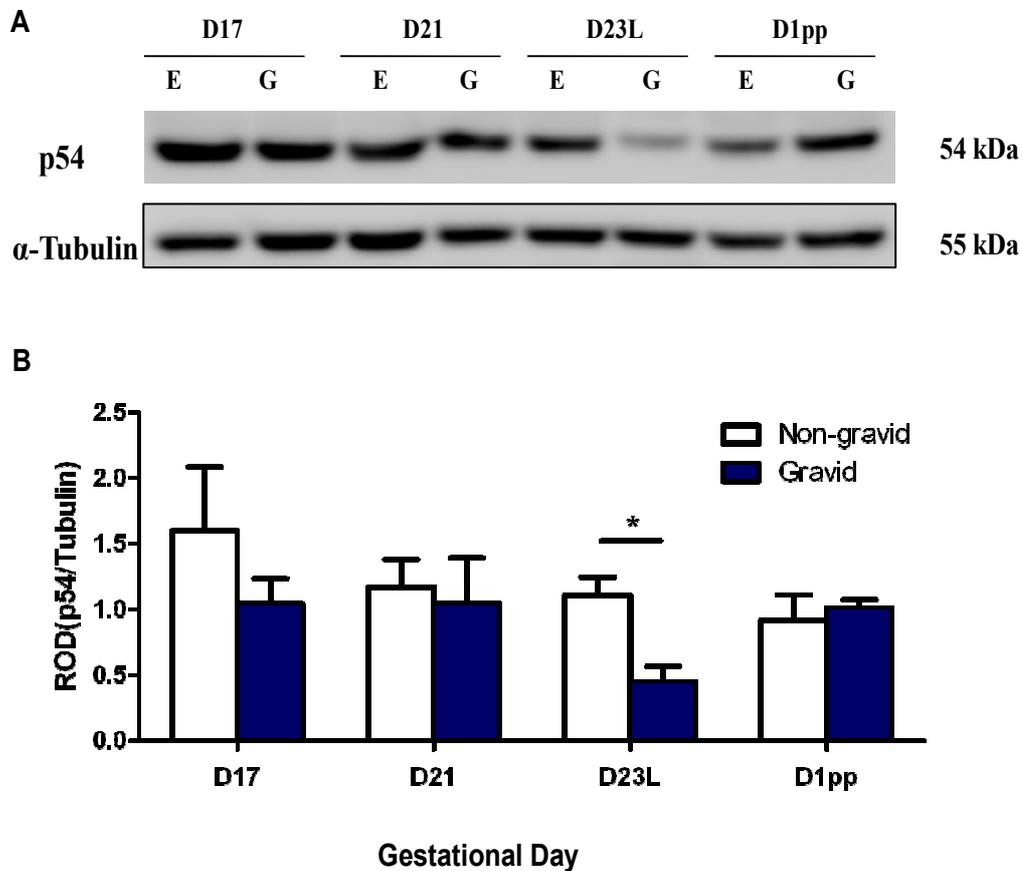


Figure 3.9 Immunoblot analysis of p54nrp protein expression in unilaterally pregnant rat model during late gestation. (A) Representative immunoblots of p54nrp and α -Tubulin protein expression. (B) Densitometric analysis illustrating p54nrp protein expression, normalized against α -Tubulin. Results were transformed by log transformation and expressed as mean \pm SEM at each time point in relative optical density (ROD) value (n=3). Empty bar represents non-gravid horn, and solid bar represents gravid horn. Statistical analysis was performed using two-way ANOVA followed by Bonferroni posttests. A significant difference between D23L non-gravid and D23L gravid is indicated by * ($p < 0.05$)

3.3.5. Artificial mechanical stretch influences myometrial PSF and p54nrb gene expression

Since a decrease in PSF and p54nrb expression occurred only in the gravid uterine horn, we speculated that the presence of the fetal-placental unit might be responsible for the decrease in PSF and p54nrb expression. Therefore we investigated whether artificial mechanical stretch of the uterine wall by an expendable material that mimics the effect of gravity could inhibit PSF and p54nrb mRNA expression. Using the unilaterally pregnant rat model, we either inserted Laminaria or non-expendable control tube into non-gravid uterine horns, or performed sham surgery on day 19 of gestation. Myometrial tissue was collected from both non-gravid and gravid uterine horns on day 23 during labor. PSF and p54nrb mRNA levels were analyzed by real-time PCR.

As shown in Figure 3.10, artificial mechanical stretch imposed by Laminaria insertion down-regulated PSF mRNA expression, to a level similar to that of the matching gravid horn. There was a non-significant trend for higher PSF mRNA level in non-gravid horns compared to gravid horns in the control groups. While the values of PSF mRNA were higher in the non-gravid versus gravid horns of both sham and tube groups these levels did not reach statistical significance. This is likely due to the small sample size (n=3). We found no significant difference in p54nrb mRNA expression between non-gravid and gravid horns in any group.

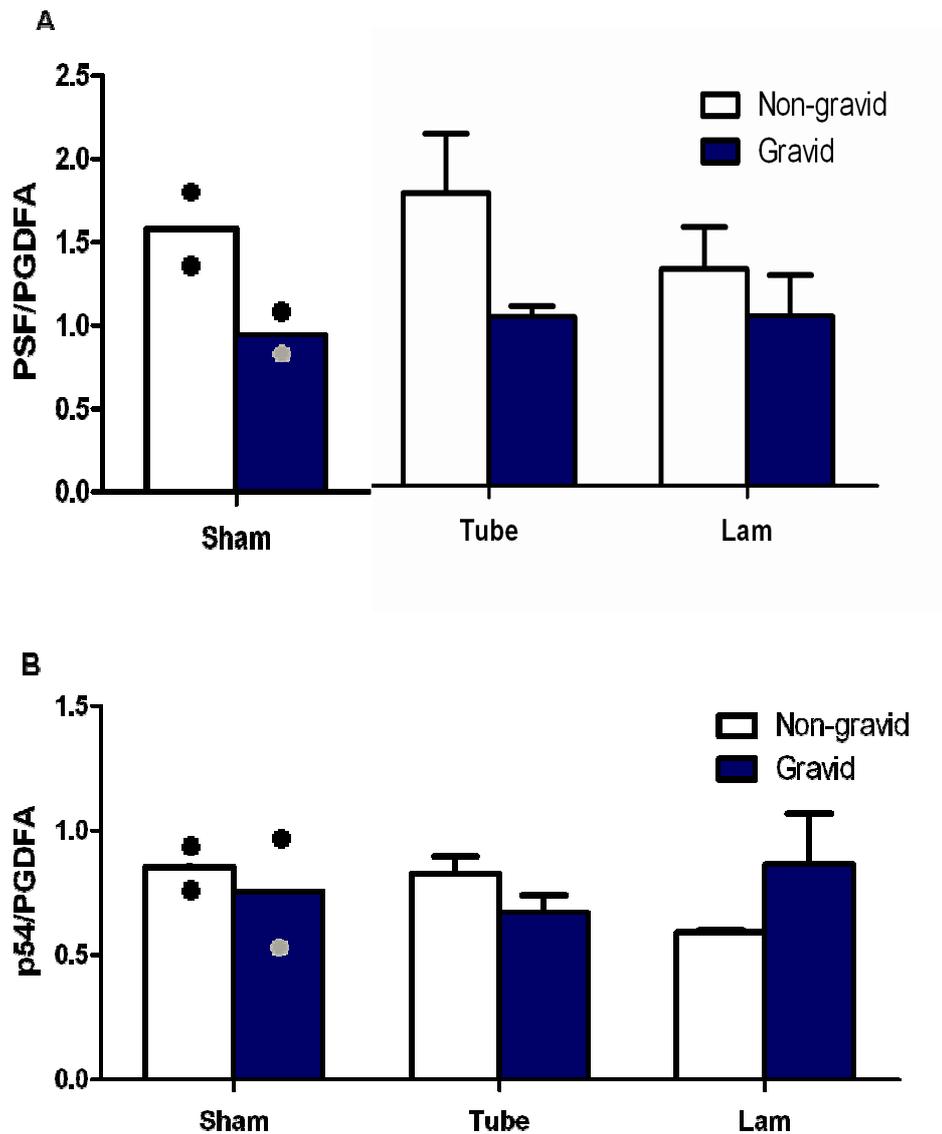


Figure 3.10 PSF and p54 gene expression on Laminaria-inserted unilaterally pregnant rat model. PSF (A) and p54nrB (B) mRNA levels in the myometrium of OVX rats were analyzed by Real-Time PCR using specific forward and reverse primers (see Table 2.2), normalized to PDGFA mRNAs. The bars represent mean \pm SEM (n=3 for Laminaria-inserted and tube-inserted groups, n=2 for sham surgery group). No significant difference was detected ($p > 0.05$).

3.4 Discussion:

The splicing factors, PSF and p54nrb are two highly homologous multifunctional factors that have been identified as nuclear co-repressors for both PR and AR and are considered participating into the putative mechanism of “P4 functional withdrawal”. It has been suggested that PSF and p54nrb might play an important role in pregnancy and during labor by modulating the transcriptional activity of PR and AR. In this study we investigated two potential mechanisms of hormonal and mechanical regulation of nuclear co-repressors PSF and p54nrb expression in myometrium.

Data from our study suggest that (1) myometrial PSF protein expression was down-regulated by estrogen signaling, and up-regulated by androgen signaling; (2) myometrial PSF mRNA expression was up-regulated by estrogen signaling and also by blocking P4 signaling with RU486; (3) myometrial PSF protein and mRNA expression was both down-regulated by physiological and artificial mechanical stretch.

PSF and p54nrb are highly homologous proteins that can form heterodimers which might interact to regulate transcription of target genes. Our data suggest that hormone signaling regulates the expression of myometrial PSF but not p54nrb. It was shown that PSF protein expression was induced by androgen signaling but reduced by estrogen signaling in OVX models, while p54nrb expression was not regulated by steroid hormone treatment. Dieli-Conwright et al. reported differential regulation of ER co-regulators gene expression in human skeletal muscle cells by estrogen and

selective ER modulators (SERMs): (1) E2 and SERMs up-regulate steroid receptor co-activator (SRC) mRNA expression and down-regulate the silencing mediator of retinoid and thyroid hormone receptors (SMRT, tamoxifen (TAM) and raloxifene (RAL)); (2) E2 treatment increases MyoD mRNA expression but SERMs decrease expression; (3) E2 and RAL up-regulate GLUT4 mRNA expression; however, TAM down-regulates it (154). It was also reported that the expression of nuclear co-regulators, SRC-1 and NCoR was differentially regulated in different parts of neonatal rat brain (155). All these evidence indicates that the regulation of nuclear co-regulators is a complex process, which may involve hormone signaling crosstalk and depend on cell type and location. Differential recruitment of nuclear co-regulators, SRC-1 and SMRT to ERE by E2 treatment and ER inhibitor 4-Hydroxy-tamoxifen treatment was also reported (156). We speculate, therefore, that while hormone treatment may not alter the expression of p54nrb it could influence the recruitment of p54nrb to the promoters of target genes.

Mechanical stretch also regulated myometrial PSF and p54nrb expression differentially. Our data showed that mechanical stretch imposed by fetal growth exerts consistent inhibitory effect on myometrial PSF expression at both the gene and protein levels. Our finding that insertion of an expandable material into the non-gravid horn is able to reduce PSF expression to levels similar to that of the gravid horn, supports a specific role for mechanical stretch (rather than the presence of the fetal/placenta unit) in the down-regulation of PSF at term. However, stretch does not appear to regulate p54nrb expression in myometrium.

Our lab previously reported that mechanical stretch in primary rat myometrial smooth muscle cells increased the expression of activator protein-1 (AP-1) family members, including c-fos, fosB, fra-1, c-jun, and junB (157). We speculate that mechanical stretch may regulate the recruitment of p54nrb to target gene promoters instead of directly regulating myometrial p54nrb expression, as p54nrb can be recruited to AP-1 transcriptional factors and regulate target gene expression (102).

The differential regulation of PSF and p54nrb expression in myometrium could possibly be explained as that (1) the regulation of myometrial p54nrb expression is a result of the synergistic effect of steroid hormones and mechanical stretch; (2) the expression of p54nrb is not regulated by either steroid hormones or mechanical stretch, however, hormonal and mechanical stimuli may influence the recruitment of p54nrb to AP-1 binding to the target gene promoters in order to regulate target gene expression. The differential regulation of PSF and p54nrb might also lead to different PSF/p54nrb ratio under diverse hormonal or mechanical stimuli, which might cause different recruitment of other nuclear factors (such as mSin3A) to the transcription machinery to regulate target genes differently.

Our data also indicate that the expression of myometrial PSF mRNA and protein were differentially regulated by hormone signaling. It was shown by immunostaining that E2 treatment decreased PSF protein expression in myometrium, whereas by real-time PCR, E2 treatment up-regulated PSF gene expression. Interestingly, this differential regulation also occurred during normal rat gestation: in early pregnancy while P4 signaling dominated, PSF gene expression was low and PSF protein

expression was high; but in late pregnancy and during labor, PSF gene expression was up-regulated (134), while PSF protein expression was down-regulated instead. It might be related to post-transcription modulation, which involves polyadenylation and RNA editing.

Surprisingly, we did not detect any effect of blocking androgen signaling on myometrial PSF or p54nrb expression, although we found an inductive effect of DHT treatment on myometrial PSF expression in the OVX model. This observation might be due to cross talk between androgen and other steroid hormone signaling. It was reported that anti-androgen Hydroxyflutamide participated into the regulation of PR in rat uterus (141). Cárdenas et al. also reported the association between DHT-attenuated estrogenic effect and the down-regulation of ER (142).

In summary, our data suggest that P4 signaling and mechanical stretch in association with estrogen and androgen signaling regulate the myometrial expression of the nuclear co-repressor PSF, which in turn can modulate PR and AR transactivation. This might act as a feedback mechanism, which can partially explain P4 functional withdraw at the onset of labor. Putative mechanism of labor initiation by hormonal and mechanical stimuli is shown in Figure 3.11.

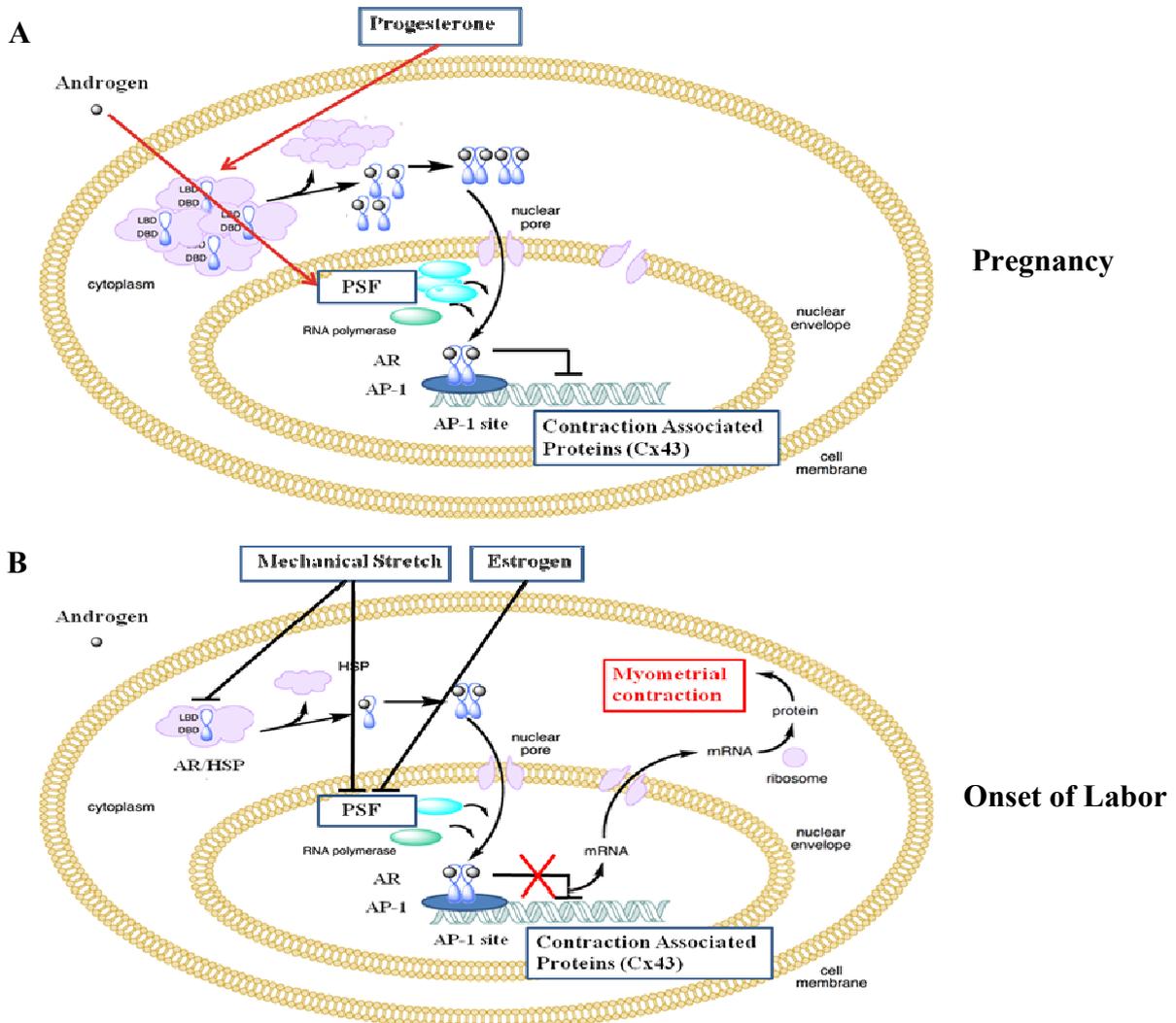


Figure 3.11 Putative mechanism of labor initiation by hormonal and mechanical stimuli.

A. During pregnancy, P4 up-regulates myometrial AR expression. Androgen in myometrium consequently binds with more AR which interacts with AP-1 on the promoters of target genes such as CAP gene Cx43 and further recruits nuclear co-repressors such as PSF. In addition, androgen up-regulates AR co-repressor PSF expression, which assists AR further suppressing CAP expression.

B. At the onset of labor, mechanical stretch inhibits AR myometrial expression. Androgen in myometrium consequently binds with less AR which interacts with AP-1 on promoters of CAP genes. In addition, mechanical stretch and estrogen signaling inhibits PSF expression, which further de-repress CAP expression. As a result, CAP expression is up-regulated followed by myometrial contraction and baby expulsion.

Note that the myometrial PSF expression regulated by hormonal and mechanical stimuli may also modulate CAP expression through interaction with PR.

CHAPTER 4:

SUMMARY OF RESULTS AND FUTURE DIRECTIONS

4.1 Summary of Results

In summary, our data demonstrate that (1) myometrial AR protein expression throughout gestation shows a trend similar to that of PR protein expression: it is highly expressed in early gestation and levels fall significantly towards term before the onset of labor; (2) AR expression in the myometrium is up-regulated by MPA treatment, but down-regulated by both physiological and artificial mechanical stretch; (3) myometrial PSF protein expression is down-regulated by estrogen signaling, and up-regulated by androgen signaling; (4) myometrial PSF mRNA expression is up-regulated by estrogen signaling and also by blocking P4 signaling with RU486; (5) myometrial PSF protein and gene expression are both down-regulated by physiological and artificial mechanical stretch; (6) p54nrb expression in the myometrium is not regulated by either steroid hormone treatment or mechanical stretch.

4.2 Future Directions

It was reported that androgen induces skeletal muscle hypertrophy in human (138, 139) and promotes myogenic differentiation through Wnt signaling cascade or myostatin (158, 159). In our *in vivo* model for pregnancy and term labor, we detected high expression of AR protein in myometrium during early gestation, when uterine myocytes undergo hyperplasia (proliferative phase of myometrial differentiation). To investigate the role of AR in myometrial differentiation, we can use normal pregnancy rat model to check (1) the expression of upstream and downstream molecules of Wnt

signaling cascade, such as beta-catenin, follistatin and T-cell factor-4 (TCF-4); (2) the expression of myostatin gene and/or protein expression throughout gestation. In addition, we can treat OVX animals with testosterone or its non-aromatized analog DHT to check the morphology of the uterus after hormone administration, and the expression of differentiation-related factors. We can also knock-down the key factors of Wnt pathway (beta-catenin, follistatin et al.) either *in vitro* using primary myometrial cells in combination with siRNA or *in vivo* using an animal model to check the effect of blocking Wnt pathway on myometrial tissue differentiation.

We investigated the hormonal and mechanical regulation of myometrial AR expression using *in vivo* rat models. To check whether the same mechanism also applies to human myometrium, we can treat primary human myometrial cells or h-TERT human myometrium cell lines with steroid hormones in combination with static mechanical stretch to mimic gestational changes and measure AR and co-repressors (PSF and p54nrb) expression. We can also knock-down AR in the h-TERT human myometrium cell line and check whether this influences hormonal or mechanical regulation of PSF and p54nrb expression.

One weakness of our study on artificial stretch is the limited sample size, which might explain why we were unable to detect a significant difference in PSF expression between the non-gravid and gravid uterine horns. To confirm the effect of mechanical stretch on myometrial PSF and p54nrb expression, we would need to increase the sample size.

We found no effect on the course of pregnancy by modulating androgen signaling. It might, nevertheless, be possible that modulation of androgen signaling affects proximal components of the labor process, such as CAP gene expression. This could be investigated in future studies. Based on the results of CAP gene expression, we can design dosage curve and time curve for each drug using rats during late gestation.

Our data showed hormonal and mechanical regulation of PSF but not p54nrb. To test whether hormone treatment or mechanical stretch influences recruitment of PSF and p54nrb to HRE or the phosphorylation of PSF and p54nrb proteins, chromatin immunoprecipitation (ChIP) could be used to measure PSF and p54nrb binding to HRE and measure the phosphorylated PSF and p54nrb proteins in OVX model treated with steroid hormones, and following physiological or artificial mechanical stretch.

Steroid hormone regulation, mechanical stretch and inflammatory responses are three determining factors in pregnancy and the onset of labor. Our study focused on hormone signaling and mechanical stretch. However, it is reported that in prostate cancer cells, IL-1 β secreted by macrophages can de-repress steroid hormone receptors (PR, ER and AR) through interaction with MEKK1 and NCoR/TAB2 complex mediated by L/HX7LL motif (160). We can treat OVX rats or primary human myometrial cells with IL-1 β and test the recruitment of MEKK1 and NCoR/TAB2 complex on promoters of steroid hormone receptors. This might integrate hormone signaling, mechanical stretch and inflammatory responses together and add additional insights to the current view of the mechanisms of labor initiation.

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