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Smoothelin-Like 1 Protein is a Bifunctional Regulator of the Progesterone Receptor During Pregnancy

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Abstract

During pregnancy uterine smooth muscle (USM) co-ordinately adapts its contractile phenotype in order to accommodate the developing foetus and then prepare for delivery. Herein we show that SMTNL1 plays a major role in pregnancy to promote adaptive responses in USM and that this process is specifically mediated through interactions of SMTNL1 with the steroid hormone receptor PR-B. *In vitro* and *in vivo* SMTNL1 selectively binds PR and not other steroid hormone receptors. The physiological relationship between the two proteins was also established in global gene expression and transcriptional reporter studies in pregnant *smtnl1-/-* mice and by RNA interference in progesterone sensitive cell lines. We show that the contraction-associated and progestin-sensitive genes (oxytocin receptor, connexin 43 and cyclooxygenase-2) and prolactins are down-regulated in pregnant *smtnl1-/-* mice. We suggest SMTNL1 is a bifunctional co-regulator of PR-B signaling and thus provides a molecular mechanism whereby PR-B is targeted to alter gene expression patterns within USM cells to co-ordinately promote alterations in USM function during pregnancy.

Introduction

Uterine smooth muscle cells (USM) show a high degree of plasticity and are capable of transforming their phenotype in response to a variety of physiological stresses such as pregnancy. In pregnancy, USM cells go through a number of extensive phenotypic changes characterized by the expression of a distinct set of proteins (1,2). In early pregnancy, USM cells proliferate rapidly under the influence of endocrine-stimulated growth factors (3). A significant increase in the expression of anti-apoptotic factors is a key feature of this proliferative phase (4). In mid pregnancy, USM cells switch into a synthetic phase characterized by cellular hypertrophy, extracellular matrix remodelling and activation of apoptotic pathways (3). The changes that occur during this phase are under the influence of both mechanical stimuli and endocrine hormones such as progesterone (2). Towards parturition, the cells adopt a more contractile phenotype, characterized by an up-regulation in the expression of contraction-associated proteins (CAPs) (2). The phenotypic changes in this phase are modulated by mechanical stimuli and sex-related hormones such as progesterone and estrogen. The molecular mechanisms regulating the transition of the USM through these different stages are limited and investigation of these mechanisms is of great importance given the fact that preterm labour is still the leading cause of neonatal morbidity and mortality (5,6).

USM contraction is mainly regulated by phosphorylation of the regulatory light chain of myosin (MLC20) by Ca^{2+} /calmodulin-dependent myosin light chain kinase (MLCK), whereas relaxation is promoted by dephosphorylation of MLC20 by myosin phosphatase (PP1M) (7,8). Both MLCK and PP1M are regulated by several accessory proteins and one such protein is smoothelin-like protein 1 (SMTNL1) (9). Mouse SMTNL1 is divided into two separate domains: a unique N-terminal domain (amino acids 1-346) and a C-terminal single, type-2 calponin-homology domain (CH) domain (amino acids 346-459) that is helix-rich and globular in structure essential for binding tropomysin (9,10). Physiological studies in *smtnl1*-/- mice indicate that the protein plays a key role in modulating the contractile activity of smooth and striated muscle and is involved in adaptation in response to exercise and pregnancy (11,12). We recently showed that during sexual development and pregnancy MYPT1 (the myosin targeting subunit of PP1M) is a signalling target of SMTNL1. Deletion of SMTNL1 results in more than 30-fold increase in the expression of MYPT1 in neonates, gradually declining during sexual development. A significant increase in the levels of MYPT1 was also seen in pregnant *smtnl1-/-* mice (11). Our findings suggested that SMTNL1 may function as a transcriptional regulator of MYPT1 expression in smooth and striated muscle altering their contractile properties. This putative role is supported by nuclear localization of SMTNL1 upon phosphorylation at S301 in response to cAMP/cGMP and by the presence of a number of regulatory transcription factor binding sites in the promoter region of *Smtnl1* (11,13). SMTNL1 also shows sex related differences in expression and function, in that male mice have a two-fold increase in levels compared with females and exercise-adapted muscle tissues from *smtnl1-/-* female mice respond differently to adrenergic agonists (11,12).

Progesterone acting through its receptor PR plays a key role in the establishment and maintenance of pregnancy, coordinating many of the adaptive responses observed in USM (14-16). PR is a member of the steroid hormone family of nuclear receptors and functions as ligand-activated transcriptional factor to regulate the expression of genes through binding to the corresponding hormone response elements within the promoter regions of target genes (17,18). PR is expressed in target cells as two distinct isoforms: PR-A and PR-B, generated from a single gene through the use of two alternative promoters (19,20). In USM, although the precise molecular mechanisms are unknown, PR is thought to promote relaxation by speficially inhibiting expression of the oxytocin receptor (OXTr) and FP receptor, targeted by the contractile agonists oxytocin and PGF2α (21-25). In the case of PGF2α, PR is also involved in inhibiting the expression of key enzymes involved in the metabolism of PGF2α such as cyclooxygense-2 (COX-2) and 15-hydroxy-PG-dehydrogenase (PGDH) (26-29). Additionally, PR reduces the contractility and excitability of USM cells by inhibiting the expression of key gap junction proteins such as connexin 43 (Cnx43) (30). Parturition in humans does not involve a drop in progesterone concentrations and thus, "functional progesterone withdrawal" in USM is achieved via an increase in the PR-A:PR-B ratio (31,32). Concomitantly, this is associated with changes in the expression of both co-activators and repressors of PR transcriptional activity such as the co-activators SRC-2 and -3 and the co-repressors PSF (polypyrimidine tract-binding protein-associated splicing factor) and p54nrb (non-POU-domain-containing, octamer binding protein) (33-36). Additionally, the miR-200 family and their targets, ZEB1 and ZEB2 are identified as unique progesterone/PR-mediated regulators of USM contractility during pregnancy (37).

In this study, we identify SMTNL1 as a previously unrecognized co-regulator of PR-B and in this capacity functions to regulate USM plasticity during pregnancy. We show that SMTNL1 directly and specifically binds PR-B and suppresses its transcriptional activity both *in vivo* and *in vitro*. We suggest that regulation of PR-B by SMTNL1 provides a molecular mechanism whereby PR-B is targeted to alter gene expression patterns within USM cells to

co-ordinately promote alterations in smooth muscle function during pregnancy.

Experimental Procedures

Biochemicals

SV40-hPR-B plasmid was provided by Ligand Pharmaceuticals. Flag-hPR-B was a gift from S Nordeen (University of Colorado health Sciences Center). Pgr 1294 antibody was a kind gift from Dean Edwards (Baylor College of Medicine).

Mouse colony maintenance and pregnancy studies

The *smtnl1-/-* mouse was created using standard protocols as described (Wooldridge et al, 2008). The transgenic mice were maintained by backcross breeding over 9 generations to a 129 SvEv background. All mice were fed standard mouse chow (PMI5058 Picolab Mouse Diet 20, LabDiet) and water ad libitum. Care of the mice used in the experiments met the standard set forth by the National Institutes of Health (NIH) guidelines for the care and use experimental animals. All procedures involving mice were approved by Duke University Animal Care and Use Committee. In order to avoid shifts in the circadian rhythm and hence the estrous cycle, all animals were housed in identical polycarbonate cages and kept in environmentally controlled room (23+/-2 ^{o}C, 50+/-10 % relative humidity, frequent ventillation, and 12-12 hr light-dark cycle). For pregnancy studies 6-8 weeks matured female mice in estrus were selected by the apperanace of their vagina and also with lavage method of removing by gentle mechanical disruption following vaginal washes, stained with hematoxilin/eosin solution and analyzed by light microscopy. Female littermates caged together presented synchronized estrus in 98 % of cases. Females in estrus were mated with adult males overnight and then examined the following morning(s) for the presence of a vaginal plug. This was designated as day 0 of pregnancy. Mice were euthanized on selected day of pregnancy (day 7, 13, and 17), the day of giving birth (birth) and the second day of lactation.

Study of reproductive fitness

Smtnl1-/- colony breeding data was collected over a period of 1 ½ year from age-matched crosses generated between *smtnl1*+/+ x *smtnl1*+/+ and *smtnl1-/-*x *smtnl1-/-* mice (n=1651) given data on the litter size and the proportion of sexes. To gain data on embryonic lethality, time to pregnancy and the interval between pregnancy as the hallmarks of reproductive phenotype and the frequency of pregnancy *smtnl1*+/+ x *smtnl1*+/+ and *smtnl1-/-*x *smtnl1-/-* experimental breeder pairs (n=40 each) were used. The already proved fertile males and virgin females were in the age 6-8 w-old. Data are expressed as means and SEM.

Cell culture

HeLa, T47D and 293T cells were maintained in DMEM and HepG2 cells were maintained in MEM. When cells were treated with hormones, cells were cultured in phenol red-free medium containing 10% charcoal-stripped FBS plus the appropriate hormones for the indicated time periods.

Mammalian two hybrid and luciferase assays

Mammalian two hybrid assays were carried out in HepG2 cells. Cells were transfected Fugene 6 reagent (Roche)with 1500 ng of 5xGal4-luciferase reporter plasmid, 500 ng of VP16 or 500 ng of nuclear receptor-VP16 fusion constructs (PR-A, PR-B (or PR-B deletion/mutant constructs), ERα, ERβ, AR, and GR), 500 ng of pM-Gal4DBD or pM-Gal4DBD-SMTNL1 construct (or pM-SMTNL1 deletion constructs), 200 ng of cytomegalovirus β-galactosidase (β-gal), and 300 ng of pBSIIKS plasmid for a total of 3µg of DNA per triplicate. Following a 24 h transfection, cells were treated with vehicle (ethanol) or with the corresponding receptors ligands as follows: 10nM of R5020 for PR-A and-B, 100 nM of 17ß-Estradiol for ERα and β, 100 nM of Dexamethasone for GR, 100nM of R1881 for AR, or 100 nM of RU486 and ZK98299. After an additional 24 h of incubation, the cells were harvested for luciferase and β-galactosidase assays. For analyzing the effects of 8-br-cAMP on PR-B transcriptional activity, HeLa cells were transfected with 10 ng of SV40-PR-B, 1500 ng

of MMTV-luciferase reporter plasmid, 200 ng of β-gal, and 1290 ng of pBSIIKS plasmid for a total of 3µg of DNA per well. After transfection, the cells were incubated with vehicle or 10 nM R5020 in the presence of 8-br-cAMP (10 nM-1 mM) for 24 h and subsequently harvested for luciferase and β-gal assays. For analyzing the effects of SMTNL1 on PR-B transcriptional activity, reporter assays were carried out in HeLa cells. Cells were transiently transfected with 10 ng of SV40-PR-B, without (0 ng) or with increasing concentrations (75, 150 and 300 ng) of pcDNA 3.1-SMTNL1 together with 1500 ng of the MMTV-luciferase reporter plasmid and varying amounts of pBSIIKS plasmid for a total of 3µg of DNA per triplicate . The following day, cells were incubated with vehicle or 10 nM R5020 in the presence and/or of 100 µ 8-br-cAMP for 24 h and subsequently harvested for luciferase and β-gal assays. Assays for endogenous PR-B transcription activity were carried out in T47D cells. pcDNA3.1-SMTNL1 was transfected into T47D and the effects of increasing amounts of SMTNL1 (15 and 80 ng) on PR-B transcriptional activity ± R5020 and 8-br-cAMP was measured. Data are presented as a normalized response, representing the absolute luciferase activity corrected for transfection efficiency by normalizing against the β-gal activity.

Site-directed Mutagenesis

All SMTNL1 mutants were created with the QuikChange mutagenesis kit (Stratagene) and completely sequenced for confirmation. Primers used in the mutagenesis experiments are listed in supplemental table IA.

SMTNL1 silencing in T47D cells

Double-stranded siRNA to knock down endogenous SMTNL1 protein levels and a scrambled-sequence were synthesized by Dharmacon Inc. (listed in supplemental table IC). The siRNAs were transfected into T47D cells using Dharmafect 1 transfection reagent (Dharmacon) according to the manufacturer's protocol. After 48 h, the cells were treated with 1 nM R5020 for another 12 h. Subsequently, cells were lysed and analyzed by Western blot analysis using anti-SMTNL1 and anti-PR antibodies.

RNA isolation and RT-PCR

RNeasy® Lipid Tissue Mini kit (Qiagen) was used to isolate total RNA from the uterus (n = 3) of WT and/or *smtnl1-/-* of pregnant and/or non-pregnant mice. RNA (1µg) was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad). PCR amplification was performed with iQ SYBR Green Supermix (Bio-Rad) with 0.2 mM of each primer (see supplemental table IB for primer sequences). The 36B4 gene was used for the normalization of amplification.

Microarray

RNA was isolated from the uterus (n = 3) of WT and/or *smtnl1-/-* of pregnant (day 17) and/or non-pregnant mice. For microarray hybridizations, 100 ng of total RNA was amplified and labeled using the MessageAmp Premier Kit (Ambion). Equal amounts of labeled cRNA were hybridized to the Affymetrix Mouse Genome 430 2.0 microarray (Affymetrix) according to the manufacturer's protocol. Partek Genomics Suite 6.4 (Partek Inc., St. Louis, MO) was used to perform data analysis. Robust multi-chip analysis (RMA) normalization was done on the entire data set. Multi-way ANOVA and fold change were performed to select target genes that were differentially expressed between the different comparisons. Top differentially expressed genes were selected with p value of 0.05 based on ANOVA test and fold change cut-off of ≥ 2. Gene Ontology Enrichment analysis was performed with chi-square test and limited to functional groups with more than two genes. Hierarchical Clustering was performed based on Average Linkage with Pearson's Dissimilarity. GeneGo software was used for obtaining pathway maps and biological networks. All microarray experimental results are available at the Duke Microarray facility website (https://discovery.genome.duke.edu/express/resources/2419).

Immunoprecipitation, immunoblotting and mass spectrometry

Uterus (UT) samples of WT and *smtnl1-/-* mice in day 0 and 14 of pregnancy were homogenized as described previously (9). Samples were processed for immunoprecipitation (IP) and immunoblotting with specific antibodies against SMTNL1, PR and ERα. For IP experiments with 293T cells, cells were transfected with Flag-Smtnl1, Flag-PR-B, SV40-PR-B and pcDNA-Smtnl1 as indicated. 48 h after transfection, media was replaced with fresh media ± 10 nm R5020 and incubated for 2 h. Subsequently, cells were processed as described previously (67). IPs were boiled for 10 min in SDS sample buffer, and proteins were resolved by 10% SDS-PAGE and visualized with silver staining. For immunoblotting experiments, cells were lysed and processed for Western blotting with the corresponding antibodies as described previously (11). For mass spectrometry experiments, bands of interest from IP studies were in gel-digested with 0.6 µg of trypsin, and the tryptic peptides were subjected to MALDI TOF-TOF mass spectrometry.

EIA assays

Circulating progesterone and estradiol concentrations were determined by using ACE™ competitive enzyme immunoassays (Cayman Chemicals). Hormone concentrations were calculated in pg/ml.

Variables and statistical analysis

Litter size was the number of pups counted in each litter. Embryonic lethality was measured by searching uteri for dead embryos at the time of dissection either on day 7, 13, 17 of pregnancy or at birth and is reported as the mean number of dead embryos. Proportion of males was the percentage of male pups within each litter. Time to pregnancy was the number of days between the set-up of the breeding pair (with female in estrus) and the first observation of the plug (considered as day 0). Interval between pregnancies was the number of days between the first observation of the plug in the first pregnancy and the first observation of the plug in the second pregnancy.

Normalized data were analyzed by t-tests (for two groups) or by general linear models (GLM, for >2 groups). Parametric statistical tests were used if the assumptions of such tests were met. We also log-transformed data for analyses. In GLMs, we tested all possible interaction terms and report here the final models obtained by excluding non-significant ($p > 0.05$) interactions. When any covariate or factor was significant in GLMs, we applied Tukey's HSD procedure to test for pair wise differences in group means. Tests were conducted using the R statistical environment (R Development Core Team 2008) or SPSS 17.0 for Windows.

Results

Deletion of SMTNL1 results in a poor reproductive phenotype

Previous findings suggested an important role for SMTNL1in the regulation of smooth and striated muscle plasticity during pregnancy. In both vascular and USM SMTNL1 expression increased by more than 10 fold by day 13-17 of pregnancy compared to non-pregnant mice then declines steadily through parturition and the onset of lactation (11). To determine if loss of SMTNL1 function effects normal pregnancy we examined our *smtnl1-/-* mouse breeding database. After several thousand breedings we observed that litter sizes were smaller and embryonic lethality was higher in *smtnl1-/-* mice (table I). There was also a statistically significant increase in the proportion of male mice born relative to females (table I). Increased proportion of males to females within a population has been shown to be related to reduce overall fitness. Additionally, time to pregnancy was longer in *smtnl1-/-* mice and the intervals between pregnancies increased by 3-4 days (table I) although the latter difference was not statistically significant. These findings reveal that loss of SMTNL1 results in a phenotype of lower overall reproductive fitness as a physiological effect.

Global gene expression profiles of uterine samples from *smtnl1-/-* and wild-type mice during pregnancy

To more fully define SMTNL1 role in pregnancy, we performed microarray analysis using Affymetrix Mouse Genome 430 2.0 Array. Over 39,000 transcripts per single array were examined essentially encompassing the entire expressed mouse genome. Total uterine RNA was isolated from 3 different mice for each group; non-pregnant wild type (WN), wild type pregnant (WP), knock-out non-pregnant (KN), and knock-out pregnant (KP). Differentially expressed genes between the four comparisons are shown in a Venn diagram in figure 1A. Comparison 1 consisted of WT mice vs. *smtnl1-/-* non-pregnant mice. This comparison identified 31 genes differentially regulated between the two groups. Comparison 2 consisted of WT non-pregnant mice and WT pregnant mice. This comparison identified 3276 that were differentially expressed. Comparison 3 consisted of knock out non-pregnant mice vs. knock out pregnant mice. This comparison identified 3766 genes that were differentially expressed. Finally, comparison 4 consisted of analyzing WT pregnant mice vs. knock out pregnant. This comparison identified 128 genes (tables II and III) that were differentially expressed (Fig. 1B). Complete lists of genes for all four comparisons are available at the Duke Microarray facility website (https://discovery.genome.duke.edu/express/resources/2419). Table II show a list of 81 genes which were up-regulated by more than 2-50 fold (n=3) in the WT pregnant mice vs. the *smtnl1-/-* pregnant ones and table III show a list of 47 genes which were down-regulated by more than 2-18 fold (n=3) in the WT pregnant mice when compared to the *smtnl1-/-* pregnant mice. Bioinformatic analysis of genes differentially expressed in this comparison identified the most significant pathways involved including: immune response (i.e., oncostatin M signaling via MAPK and histamine H1 receptor signalling), cell adhesion, cytoskeleton remodelling, proteolysis, histidine and proline metabolism, atherosclerosis, cAMP biosynthetic process and activation of protein kinase A, transport, plasminogen activation, steroid metabolic process and glucocorticoid receptor signalling, regulation of growth hormone receptor signaling pathway, and negative regulation of protein import into nucleus. Additionally, analysis of networks of transcriptional regulation identified sp1, c-myc, NF-kappaB1, stat3, stat5a, smad-2, -3, and -4, estrogen receptor α, and progesterone receptor as the major transcription factors involved in modulating gene expression in this comparison. Taken together, microarray analysis identified a number of signalling pathways involved in mediating the actions of SMTNL1 and strongly points to a role for the sex-related hormones estrogen and progesterone and their cognate receptors in SMTNL1 functions during pregnancy.

Most notable in this comparison is the deregulation of the prolactin (PRL) family members in the *smtnl1-/-* pregnant mice. All members of this family with an observed expression on the array were found to be significantly down-regulated (3-18 fold) in the *smtnl1-/-* pregnant mice. The deregulation of prolactins in *smtnl1-/-* pregnant mice was confirmed by RT-PCR analysis. Accordingly, the mRNA for Prl3b1, Prl2a1, Prl7a1 and Prl2c2 were all decreased markedly in knock out (KO-P) pregnant mice when compared to their wild type counterparts (WT-P) (Fig. 2A). The significance of this finding will be elaborated on later in the context of the function of prolactins as mediators of pregnancy adaptations. Additionally, consistent with microarray results, RT-PCR analysis showed that the mRNA for Cyp26a1 and Slc13a1 were down-regulated in the KO-P mice (Fig. 2A). Uterine cyp26a1 down-regulation in KO-P mice is intriguing given its role in the maintenance of pregnancy during the process of blastocycst implantation and the finding that cyp26a1 expression in the uterus is induced by steroid hormones (38-40)**.** Additionally, we confirmed that the mRNA of a number of proteases (i.e., matrix metallopeptidases 13 and 3, arylsulfatase K, and ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin-like motifs)) which play an essential role in the ovulation process, through extracellular matrix remodelling, were all found to be down-regulated in the KO-P mice (41,42). We also confirmed that mRNA of Hal (histidine-ammonia lyase), the histidine-degrading rate-limiting enzyme is up-regulated in KO-P mice when compared to their WT-P counterparts (Fig. 2B). The increase in Hal mRNA in *smtnl1-/-* pregnant mice is intriguing given the findings that the Hal gene is activated by glucocorticoids and glucagon via the PKA signalling pathway (43).

SMTNL1 regulates the expression of ERα and PR-B in the primary genital tissues

Microarray analysis support a role for SMTNL1 is a key player in USM plasticity mediated through the sex-related hormones. To investigate this hypothesis further we examined the expression of SMTNL1 in the primary genital tissues selectively targeted by estrogen and progesterone. In non-pregnant uterus, SMTNL1 is expressed within myometrial cells of the uterus as well as in the endometrial layer, and by day 13 of pregnancy expression is increased >10 fold in both cell types (Fig. 3A and B). Non-pregnant mammary gland shows a low level of SMTNL1 expression, with staining being confined to alveolus cells lining the lumens of intralobular duct systems, but by day 13 of pregnancy, the protein is induced 8-14 fold (Fig. 3A and B). As observed previously the deletion of SMTNL1 does not appear to alter the ultra-structure of any tissues, either non-pregnant or pregnant including uterus or mammary gland (12). Additionally, we observed a low level of SMTNL1 expression in the adrenal gland, but by day 13 of pregnancy, the protein is induced by 3-4 fold (Fig. 3B). The adrenal cortex is devoted to the synthesis of glucocorticoids, mineralocorticoids and androgens and specific cell types within the cortex are associated with the synthesis of each of these steroid hormones. Examination of circulating progesterone (P) and estradiol (E) levels in *smtnl1-/-* mice showed SMTNL1 deletion produced a marginal 10-15% reduction in circulating E and P levels, suggesting a potential role in regulating steroid biosynthesis (Fig. 3C and D).

The findings that SMTNL1 is discretely expressed within reproductive tissues, coupled with the microarray analysis, supports links between the protein and steroid hormone action. To more fully understand this relationship, we first investigated the expression of both ER and PR in the uterus. Pregnancy induced expression of both ERα and PR-B in USM (Fig. 4A). The expression of both proteins increased 3-4 fold in non-pregnant USM from *smtnl1-/-* mice and 8-9 fold in USM from pregnant *smtnl1-/-* mice (Fig. 4A). These data suggest a direct relationship between SMTNL1 expression levels and ER and PR expression. Next, we performed IP experiments from USM isolated from pregnant WT and *smtnl1-/-* mice. Figure 4B show that in both tissues SMTNL1 co-IP with PR-B but not ERα. As observed in Western blot experiments, IP of PR-B from USM extracts prepared from

smtnl1-/- mice showed a large induction of the protein. Similarly, although IP with ERα antibody confirmed the presence of ERα and PR-B in the IP as expected, Western analysis with anti-SMTNL1 antibodies indicated that SMTNL1 does not bind directly to ERα *in vivo*. Although our data do not support direct interactions of SMTNL1 with ERα, its increased expression observed after IP and Western analysis from *smtnl1-/-* mice may reflect either disruption of the ability of PR to suppress expression of ERα at the transcriptional level or that the increased expression of PR-B, by binding to ERα, stabilizes the protein. This notion is supported in studies by Beato *et al* demonstrating an interaction of PR-B with ERα in T47D cells (44,45). Although SMTNL1 does not bind ERα directly, any effects on ER expression are likely to indirectly affect SMTNL1 expression through the feedback mechanisms between PR and ER. Next, we performed quantitative RT-PCR experiments from USM isolated from both pregnant and non-pregnant WT and *smtnl1-/-* mice to determine if the observed changes in PR-B could be seen at the transcriptional level. We chose a primer set directed at the sequence specific for PR-B. Figure 4C show that the mRNA levels of PR-B are up-regulated by more than 2 fold in pregnant *smtnl1-/-* mice when compared with WT pregnant mice. A slight, non-significant, increase in PR-B transcript was also observed in non-pregnant *smtnl1-/-* mice relative to the wild type non-pregnant mice (Fig. 4C). To further investigate the effect of SMTNL1 on the expression of PR-B *in vivo*, we developed several small interfering RNA (siRNA) constructs to knock down endogenous SMTNL1 expression. For these experiments we chose the progestin-sensitive breast cancer cell line T47D due to the lack of a well-characterized USM cell lines expressing both proteins. Figure 4D shows R5020 induced the expression of both SMTNL1 and PR-B by 5-10-fold. Treatment of the cells with SMTNL1 siRNAs effectively suppresses the expression of SMTNL1 regardless of R5020 treatment. However, Western analysis of PR-B shows SMTNL1 siRNAs dramatically induce the expression of the receptor ~10 fold above that induced by R5020 alone (Fig. 4D).

Collectively, these data provide direct evidence that SMTNL1 directly regulates expression of PR-B *in vivo* and that the effects of SMTNL1 deletion on PR expression in *smtnl1-/-* mice are not the result of a non-specific adaptive response to chronic deletion of the gene in these animals.

SMTNL1 binds PR *in vivo* and *in vitro*

To determine the selectivity of SMTNL1 towards the major steroid hormone receptors; progesterone receptor-A and -B, estrogen receptor-α and -β, androgen receptor (AR) and glucocorticoid receptor (GR) we carried out mammalian two-hybrid analysis. SMTNL1 was fused to the C-terminus of the GAL4 DNA binding domain in the pM vector and each of the steroid receptors was fused downstream of the Gal4 activation domain in the VP16 vector. Interaction between SMTNL1 and the steroid receptors was assessed by measuring the ability of the plasmids to activate transcription from a luciferase reporter in the presence or absence of the corresponding hormones. Figure 5A shows the remarkable degree of selectivity of SMTNL1 for PR-B and -A over AR, GR, ERα and ERβ. Significantly, the interaction between SMTNL1 and both isoforms of PR is hormone dependent. In the presence of R5020, reporter activity showed that SMTNL1 has much higher binding preference to the PR-B isoform when compared to PR-A. In contrast, SMTNL1 did not show any significant interaction with ERα, ERβ in the presence or GR. A small, non-hormone dependent interaction was observed between SMTNL and AR. In comparative experiments, binding of SMTNL1 to PR-B and PR-A compares favourably with the well defined co-activator steroid receptor co-activator-1 (SRC-1) NR box peptide (supplemental Fig. 1). However, unlike SMTNL1, SRC1-NR box does not discriminate between the steroid hormone receptors and binds to ERα as shown previously (46,47). Three proteins (SRC-1,-2, -3) have been identified in the p160 steroid hormone co-activator family and each contains a short conserved NR interaction motif (NR box), which has the core sequence LXXLL (L = leucine and X = any amino acid) (48-50). SMTNL1 is therefore discriminated from all other known

steroid receptor-binding proteins in that it shows an exquisite degree of selectivity for PR A and B only. Additionally, SMTNL1 does not contain LXXLL motifs, suggesting that the protein interacts with PR at other sites and may have other functions. The finding that SMTNL1 only interacts with PR-B and A and not with either isoform of ER in 2-hybrid and Co-IP experiments suggests that the mechanisms of altered ER expression observed *in vivo* is likely to be mediated through PR via a feed back mechanism. Both estrogen and progesterone are known to regulate the expression of their corresponding receptors at the transcriptional level.

To further validate the observed *in vivo* interactions between SMTNL1 and PR-B, 293T cells were transfected with expression vectors for Flag-SMTNL1 and SV40-PR-B, and cell lysates were precipitated with flag beads. The Flag-SMTNL1 was able to co-IP SMTNL1 and PR-B (Fig. 5B). Likewise, when cells were transfected with expression vectors for Flag-PR-B and pCDNA-SMTNL1, the Flag beads were able to IP both Flag-PR-B and SMTNL1 (Fig. 5C) and the presence of SMTNL1 was confirmed by Western blotting (Fig. 5C). Mass spectrometry studies confirmed the identity of the bands seen in the IP experiments (Fig. 5D). Additionally, endogenous SMTNL1 was found to co-IP with endogenous PR-B in T47D cell lysates as shown in figure 4E. Finally, co-localization of SMTNL1 and PR-B was observed by immunofluorescent staining in T47D cells showing discrete nuclear localization for both proteins (Fig. 5F).

Mapping the interaction sites between SMTNL1 and PR-B.

Mammalian two-hybrid experiments were carried out to determine the regions(s) involved in interaction between SMTNL1 and PR-B. In the case of SMTNL1, aside from the CH domain, the protein has no defined domains and is quite unique with no sequence similarities to known proteins in the database. Our approach was to create small blocks of the protein and test them for their ability to bind PR-B. Accordingly, we created several truncated pieces of SMTNL1 namely, amino acids 1-50, 1-60, 1-90, 1-150, 1-200, 1-230, and 1-345. All constructs were fused to the C-terminus of the GAL4 DNA binding domain in the pM vector, sequenced and expression-validated. Figure 6A show that the first 60 amino acids of SMTNL1 are dispensable for PR-B binding whereas amino acids between 60 and 90 are capable of restoring full binding capacity when compared to the full length protein. No significant increase in binding was observed when additional amino acids were used however, a significant increase in binding was observed with the 1-230 construct indicating that amino acids 200-230 of SMTNL1 are capable of interacting with PR-B (Fig. 6A). Taken together, the results indicate that amino acids 60 to 90 and amino acids 200 to 230 are required for SMTNL1 interaction with PR-B, with the later exhibiting a robust binding capacity when compared to the full length protein. Furthermore, the results indicate the CH domain, as expected, does not play a role in mediating the interaction between the two proteins.

Next, we carried out mammalian two-hybrid experiments to determine regions of PR-B necessary for its interaction with SMTNL1. The PR-B constructs employed in the assay include: the N-terminal construct (amino acids 1-555), amino acids 306-456, amino acids 456-687, amino acids 680-933, the C587A transcriptional incompetent mutant, the mutant Pro construct, the G722C mutant, the AF2 mutant (E907A/E911A), and the 1-922 construct (i.e., marks the end of the hormone binding domain). All constructs were fused downstream of the Gal4 activation domain in the VP16 vector, sequenced and expression-validated. Figure 6B show that the C587A mutant abolished the interaction with SMTNL1 completely. This construct of PR-B has a point mutation in the first zinc finger of the DNA binding domain and it disrupts the ability of PR to function as a transcription factor. When the AF2 mutant was tested, the level of the reporter activity generated was significantly reduced compared with that of the wild-type PR-B confirming the hormone dependence of the interaction between the two proteins (Fig. 6B). The 1-922 construct was still capable of binding

to SMTNL1 but the reporter level activity was reduced compared with that of the full length PR-B (Fig. 6B). The PR-B mPro mutant showed reduced binding when compared with that of the wild type protein (Fig. 6B). This construct has three essential prolines (P422, P423 and P426) changed to alanines which results in the loss of binding to the SH3 domains of signalling proteins. The G722C mutant (the 722 construct) was found to bind SMTNL1 with levels comparable to those of the wild type protein (Fig. 6B). This mutant generates a receptor that has lost detectable RU486 binding, but retains wild-type binding affinity for progestin agonists (51,52). The fact that SMTNL1 and PR-B is hormone-dependent prompted us to examine if the hormone binding domain of PR-B by itself is sufficient to bind SMTNL1. Figure 6B show that this is not the case, pointing to additional amino acids upstream that are required for mediating the interaction between the two proteins. Finally, as expected from the hormone-dependence of the interaction, constructs missing the ligand-binding domain did not show any reporter activity (Fig. 6B). Comparative experiments with the co-activator SRC1-NR box are shown in supplemental figure 2. Next, we used the mammalian two-hybrid system to examine the effects of anti-progestins (RU486 and ZK98299) on the interaction between SMTNL1 and PR-B. Treating the cells with anti-progestins resulted in significant reduction in the luciferase activity when compared to R5020 as shown in figure 6C. The significant reduction in the interaction of SMTNL1 with PR-B in the presence of anti-progestins suggested that binding between the two proteins is a function of the conformational changes in PR-B and further validates the hormone-dependence of the interaction.

SMTNL1 is a transcriptional co-regulator of PR-B function

The significance of the interaction between SMTNL1 and PR-B was next investigated in the context of progesterone-responsive promoters and PKA mediated signalling. SMTNL1 is phosphorylated at S301in smooth muscle cells in response to 8Br-cAMP resulting in translocation of the protein to the nucleus, an effect blocked by S301A substitution (11). Activators of PKA are also known to affect the transcriptional activity of PR-B (53,54). Figure 7A shows treatment of HeLa cells, transfected with an expression vector for PR-B and MMTV-luciferase reporter plasmid, with increasing amounts of 8Br-cAMP plus R5020 increases the transcriptional activity of PR-B in a dose dependent manner. This assay helped us determine the conditions for analyzing the effect of SMTNL1 on PR-B transcriptional activity at any given [8Br-cAMP]. Thus, HeLa cells were transfected with PR-B expression vector alone or with increasing amounts of SMTNL1 expression vector together with the MMTV-luciferase reporter in the presence and/or absence of R5020 and 8Br-cAMP. Figure 7B show that SMTNL1 inhibits PR-B tranasctivation in a dose-dependent manner (i.e. 300 ng of SMTNL1 causes more than 50% reduction in transcriptional activity). This effect correlates precisely with 8Br-cAMP concentrations (10μM) required to promote nuclear translocation of SMTNL1through S301 phosphorylation (11). Increasing amounts of transfected SMTNL1 was also found to progressively repress endogenous PR-B activity by 20-30% in T47D cells (Fig. 7C). Taken together, these findings suggest that SMTNL1 functions as a repressor of the transcriptional activity of PR *in vivo*.

As discussed earlier, progesterone promotes USM relaxation during pregnancy by repressing the expression of specific genes encoding CAPs (55). Specifically, PR inhibits the expression of key CAPs including OXTR, Cnx-43, and COX-2 (21-29) Therefore, if SMTNL1 represses PR, one might predict loss of this function in smtnl1-/- mice would lead to altered expression of PR regulated genes. Figure 7D shows this is indeed the case. Quantitative RT-PCR studies show that mRNA for OXTR, Cnx-43 and COX-2 were all decreased markedly in the KO-P mice compared to their WT-P counterparts (Fig. 7D). These findings support SMTNL1 as a novel co-regulator of PR-B transcriptional activity in USM.

Discussion

In the present study we show that SMTNL1 plays a key role in pregnancy to promote adaptive responses in USM and that this process is mediated through interactions of SMTNL1 with the progesterone receptor PR-B. Both *in vitro* and *in vivo* SMTNL1 specifically binds to the progesterone receptor and not other steroid hormone receptors, discriminating the protein from all other known co-regulators of this class of nuclear receptors. Both SMTNL1 and PR exhibit co-regulation of expression during normal pregnancy and there is a significant increase in PR expression at the protein level in *smtnl1-/-* mice in both reproductive and non-reproductive tissues. Additionally PR expression is significantly induced in response to RNA interference of SMTNL1 in progesterone sensitive cell lines. These observations therefore show a direct role for SMTNL1 in regulating the expression of PR itself. Additionally, transcriptional reporter assays and global gene expression analysis suggest a mechanism of action in which SMTNL1 functions as a co-regulator of PR-B transcriptional activity to regulate gene expression in reproductive tissues underling the physiological relationship between the two proteins. Consistent with this role, quantitative RT PCR studies of several known contractile associated proteins (CAPs) regulated by PR-B were greatly suppressed in USM from *smtnl1-/-* mice. Global gene analysis also showed that a primary target of SMTNL1 are prolactins, a well defined group of genes governing both pregnancy and lactation that are also involved in progesterone production in the late stages of pregnancy. All prolactin genes were greatly suppressed in global gene expression of tissues from *smtnl1-/-* mice and these findings were also confirmed in RT-PCR studies. Clearly our findings define SMTNL1 as a homeostatic regulator of PR function during pregnancy. Figure 8 outlines three potential modes of action that are currently under investigation.

Our hypothesis that SMTNL1 functions as a co-regulator of PR-B stems from several lines of evidence. First, IP and Western assays of PR-B from *smtnl1-/-* pregnant mice USM extracts showed a significant increase in the induction of the protein in comparison to the WT extracts. This increase is also evident at the transcript level; quantitative RT-PCR data showed a 2-3-fold up-regulation of PR-B mRNA levels in pregnant *smtnl1-/-* mice when compared with their WT pregnant litter mates. Second, in reporter assays, SMTNL1 was found to inhibit PR-B transcriptional activation in a dose-dependent manner and the effect was shown to be independent of cell and promoter context, ruling out the involvement of transcriptional interference. Finally, the necessity for a functional DBD and AF2 for SMTNL1 binding puts the protein at the core of PR transactivation given the fact the primary role of the DBD is to bind to specific DNA target sequences within the promoter of responsive genes, and the established functions of AF2 as a mediator of hormone-dependent transcriptional activation. It is intriguing to think that the reduction of PR-B transcription by SMTNL1 is due to the formation of an inhibitory complex that is either unable to bind DNA or is incapable of binding to the transcription machinery. Future studies will test this hypothesis by electrophoretic mobility shift and CHIP assays. Additionally, SMTNL1 phosphorylation could play a role in mediating its effects on PR-B since the S301A SMTNL1to alanine blocks entry into the nucleus (11). Furthermore, reduction of PR-B transcriptional activity was only detected when cells were treated with PKA activators (53,54). If SMTNL1 acts as a transcriptional co-regulator of PR *in vivo* inhibiting its function, phosphorylation of one or more sites may be similar to deleting the protein as observed in *smtnl1-/-* female mice. Such a mechanism of action may imply that the default function for SMTNL1 in non-pregnant animals is to repress PR function from promoting pregnancy-like adaptive responses in USM. The observed repression of key CAPs in *smtnl1-/-* pregnant mice is consistent with such role. The molecular mechanism(s) by which SMTNL1 deletion promotes an increase in the transcriptional activity of PR-B *in vivo* is currently under further investigation.

In specific pathway analysis related to progesterone and pregnancy, most notable was the effect of SMTNL1 deletion on the PRL family members. All members of this family with observed expression on the array were found to be down-regulated in the *smtnl1-/-* pregnant mice. In rodents, PRLs are a large family of proteins that share extensive structural and functional homology and are synthesized in all reproductive tissues in a temporal manner (56,57). The PRL family are associated with an array of functions, however, their involvement in reproduction has received the most attention (58). Studies of *PRL-/-* and *PLR-R-/-* mice results in complete infertility in females due to failure of implantation (59,60). These mice show a lack of functional corpus luteum (CL) and thus, were unable to produce progesterone. The CL plays a central role in the maintenance of pregnancy through regulating the production of progesterone (61). In rodents, the functions of the CL are sustained throughout pregnancy by coordinating the secretion of PRLs (56,62). The deregulation of the PRLs in *smtnl1-/-* pregnant mice is very interesting given our observations that deletion of SMTNL1 is associated with a slight reduction in the levels of progesterone in late pregnancy. Taken together, our results highlight a previously unrecognized role for SMTNL1 as an activator of the expression and/or release of a subset of the PRL family members during late pregnancy. Future studies will focus on elucidating the mechanism(s) of SMTNL1 activation of PRLs and their effect on progesterone production using a culture system of luteal cells from WT and *smtnl1-/-* pregnant mice.

Finally, analysis of *smtnl1-/-* mouse breeding data base showed that the loss of SMTNL1 results in smaller litter sizes and higher embryonic lethality. Additionally, we observed that time to pregnancy was longer in *smtnl1-/-* mice. Collectively, our data indicate that loss of SMTNL1 results in a phenotype of lower overall reproductive fitness as a physiological effect. These phenotypic effects are distinct from those observed in PR null mice. PR-A null mice developed signs of uterine dysplasia and abnormal ovaries resulting in female infertility, whereas PR-B null mice did not show any uterine abnormalities but exhibited signs of mammary glands malformations (63-66). The differences between *smtnl1-/-* mice and the severity of the PR null phenotype further support the hypothesis that SMTNL1 functions to affect a subset of PR genes.

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Figure legends

Table 1. *Smtnl1* deletion results in a poor reproductive phenotype. *Smtnl1-/-* colony breeding data was collected over a period of 1 ½ year from age-matched crosses generated between *smtnl1+/+* x *smtnl1+/+* and *smtnl-/-* x *smtnl1-/-* mice (n=1651). Data on embryonic lethality, time to pregnancy and the interval between pregnancy as the hallmarks of reproductive phenotype and the frequency of pregnancy were collected. Data are expressed as means and ± SEM.

Table II. Genes up-regulated in wild type pregnant (WP) vs. *smtnl1-/-* pregnant (KP) mice.

Table III. Genes down-regulated in wild type pregnant (WP) vs. *smtnl1-/-* pregnant (KP) mice.

Figure 1. Venn diagram demonstrating the relationship between genes differentially regulated in the different comparisons and hierarchical clustering analysis of the wild type pregnant (WP) vs. knock-out pregnant (KP) mice. (A) Venn diagram; the numbers within the intersections of the circles indicate the common genes between the different groups. The numbers outside the circles indicate the number of genes differentially-regulated between the two groups as indicated. (B) Hierarchical clustering analysis of the differentially expressed genes in the WP vs. KP comparison. The color code for the signal strength is shown in the box at the bottom; induced genes are indicated by red and repressed genes are indicated by blue.

Figure 2. Quantitative RT-PCR validation of microarray data. QRT-PCR analysis of Prl3b1, Prl2a1, Prl7a1, Prl2c2, Cyp26a1, Slc13a1 and (D) Hal in wild type pregnant (WT-P) and *smtnl1-/-* pregnant (KO-P) mouse uteri. The results represent the mean ± SEM, n= 3 RNA sets.

Figure 3. Pregnancy regulates SMTNL1 expression in the primary genital tissues. (A) IH images of SMTNL1 localization in uterus and mammary gland from control and pregnant WT and *smtnl1-/-* mice. Scale bars represent 20 μm. m: myometrium, e: endometrium, g: endometrial glands in tunica propria of uterine mucosa (B) Pregnancy induces significant expression of SMTNL1 relative to control (non-pregnant) in USM, mammary gland and adrenal gland. Inserts below show representative Western blots. Data shown is from day 0 and day 14 of pregnancy. (C and D) Circulating hormone concentrations of *smtnl1+/+* and *smtnl1-/-* mice during pregnancy**.** The circulating progesterone (E) and estriol (F) concentration is lower in *smtnl11-/-* mice in pregnancy. Means ± SEM, n = 4-6 animals, GLM with Tukey-test, different letters indicate significant differences ($p < 0.05$).

Figure 4. SMTNL1 regulates the expression of ERα and PR-B in USM. (A) Pregnancy and SMTNL1 deletion promotes a higher expression of PR-B and ERα in USM. Insert below show representative Western blots. Data shown is from day 0 (con.) and day 14 of pregnancy (preg.). (B) SMTNL1, PR-B and ERα co-IP studies from uterine extracts of WT-P and *smtnl1-/-* P. (C) PR-B mRNA levels are up-regulated in the pregnant uteri of *smtnl1-/-* compared to WT mice. RT-PCR results of PR-B mRNA levels in USM from aged matched pregnant (preg.) and non pregnant (con.) WT and *smtnl-/-* mice, n = 3 ± SEM. (D) T47D cells were transfected with either siRNA control or siRNA directed against SMTNL1. Subsequently, cells were treated with the synthetic progestin R5020 (+) (1 nM) or ethanol (-) for 12 hours, lysed and analyzed by Western blot analysis using antibodies against SMTNL1 and PR-B.

Figure 5. SMTNL1 binds PR *in vivo* and *in vitro*. (A) HepG2 cells were transfected with the indicated constructs. After 24 h, cells were treated with different receptors ligands as indicated and subsequently harvested for luciferase and β-gal assays. (B) 293T cells were transfected with the indicated expression vectors. After 48 h, the culture media was replaced ± 10 nM R5020 for 2 hours. Lysates were IP using Flag beads and processed for SDS-PAGE and silver stain. (C) IPs were subjected to the same treatment as in (B) and then probed with an antibody against SMTNL1**.** (D) Designated bands (SMTNL1 and PR-B) were excised and processed for mass spectrometry. Peptide sequences and their position in each protein are shown. (E) T47D cells were lysed and lysates were incubated with SMTNL1 or PR antibodies, and IP with protein A-Sepharose. IPs were Western blotted with SMTNL1 and PR antibodies. (F) Nuclear co-localization (merge, white) of endogenous SMTNL1 (green) and PR (red) in T47D cells. Scale bars = 10μm.

Figure 6. Identification of interaction sites within SMTNL1 and PR-B. (A) HepG2 cells were transfected with the indicated SMTNL1 deletion constructs together with PR-B. (B) HepG2 cells were transfected with the indicated PR-B deletion constructs and mutants together with SMTNL1. In both (A) and (B), 24 h after, the cells were incubated with vehicle or 10 nM R5020 for 24 h then harvested for luciferase and β-gal assays. (C) Effects of anti-progestins on SMTNL1-PR-B interaction. After transfection, the cells were incubated with (vehicle) or 10 nM R5020, 100nM RU486 and 100nM ZK98299 for 24 h and harvested for luciferase and β-gal assays.

Figure 7. SMTNL1 regulates PR-B expression at the transcriptional level. (A) Effect of cAMP on the transcriptional activity of PR-B**.** HeLa cells were transfected with an expression vector for PR-B and an MMTV-luciferase reporter plasmid. After transfection, cells were incubated with vehicle or 10 nM R5020 in the presence of 8Br-cAMP (10 nM-1mM) for 24 h and harvested for luciferase and β-gal assays. (B) Effect of SMTNL1 on PR-B transcriptional activity. HeLa cells were transfected with PR-B expression vector without (0 ng) or with increasing concentrations (75, 150 and 300 ng) of SMTNL1 expression vector together with the MMTV-luciferase reporter plasmid. The following day, cells were incubated with vehicle or 10 nM R5020 for 24 hours and subsequently harvested for luciferase and β-gal assays. (C) SMTNL1 was transfected into T47D and the effects of increasing amounts of SMTNL1 (15 and 80 ng) on PR-B transcriptional activity ± R5020 and ± 10 μm 8Br cAMP was measured. (D) RT-PCR analysis of OXTR, Cnx-43 and Cox-2 in wild type pregnant (WT-P) and *smtnl1-/-* pregnant (KO-P) mouse uteri, n= 3 RNA sets ± SEM.

Figure 8. Mechanisms by which SMTNL1 regulates PR-B to co-ordinately promote USM adaptations in pregnancy. Schematics A and B show regulation of expression of SMTNL1, PR and ER are regulated via two possible homeostatic feedback mechanisms. In (A) the synthesis of PR is controlled by estrogen acting through ER. Progesterone (P4) abrogates estrogen induction by down-regulating ER protein concentration, decreasing circulating estrogen levels and antagonizing ER action at the molecular level. ER may also have a direct effect on SMTNL1 transcription, a hypothesis supported by the presence of ER binding sites in the promoter region of *smtnl1*. In (B) SMTNL1 directly and specifically binds PR-B, in a hormone-dependant manner, and suppresses its transcriptional activity. A likely means by which SMTNL1 regulates PR-B is via PKA-mediated phosphorylation. Both proteins have multiple *in vivo* phosphorylation sites and, at least one of these sites, S301 has the potential to affect SMTNL1 entry into the nucleus. Therefore, by phosphorylating SMTNL1, it may be possible to activate or inhibit its effects on PR function. In (C) SMTNL1 also functions as an activator of the expression and/or release of a subset of the PRL family members (i.e., placental lactogens) which are involved in progesterone production in late pregnancy and thus regulating PR transcriptional activity. Taken together, we suggest that regulation of PR by SMTNL1 provides a molecular mechanism whereby PR is directed to alter gene expression patterns within USM (i.e. CAPs) to co-ordinately promote alterations in USM function during pregnancy. ⊥ (inhibition) → (gene transcription).

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Table 1.

	smtnl1+/+	*smtnl1-/-*	
Litter size	7.14±1.47	4.23±1.25***	t = 5.961,n=70; df = 30, p < 0.001
Embryonic lethality	0.05 ± 0.22	0.4 ± 0.68*	t_{23}=2.185, p=0.039
Proportion of males	44% ± 19.4%	57% ± 21.8%	t_{394} = 1.768, df = 30, p = 0.087
Time to pregnancy	2.8 ± 1.37	4.6 ± 2.23*	t_{38} = 3.156, p = 0.003
Interval between pregnancies	23.6 ± 2.65	27.5 ± 6.22*	t_{16} = 1.827, p = 0.086

Table II.

Probeset ID	Gene Symbol	Fold-Change	p-value
1452426_x_at	---	50.3404	0.0118484
1415835_at	Prl3b1	17.5723	0.000565294
1427760_s_at	Prl2c2 /// Prl2c3 /// Prl2c4	12.0257	7.87E-05
1436717_x_at	Hbb-y	10.5434	0.0402453
1448532_at	Prl8a9	9.53893	0.0063006
1436823_x_at	Hbb-y	9.23758	0.0497143
1457446_at	Opcml	6.89362	0.00252789
1419430_at	Cyp26a1	5.71716	0.0325249
1431379_a_at	Slc13a1	5.55238	0.0271946
1435330_at	Pyhin1	5.16049	2.88E-06
1417256_at	Mmp13	4.82501	3.77E-05
1460480_at	Erv3	4.79078	0.0198751
1416444_at	Elovl2	4.56532	0.0109421
1424959_at	Anxa13	4.54885	0.0185272
1422640_at	Pcdhb9	4.50123	0.00292992
1429835_at	2310033E01Rik	4.46294	0.00207603
1456211_at	Nlrp10	4.26845	0.00389898
1449529_s_at	Prl7a1	4.24167	4.67E-07
1419764_at	Chi3l3	3.95692	0.00726454
1450621_a_at	Hbb-y	3.94281	0.0248618
1449227_at	Ch25h	3.91637	0.00256177
1448608_at	Prl8a2	3.90766	0.0202838
1419700_a_at	Prom1	3.74858	0.0467971
1435212_at	P2rx2	3.52183	0.0191051
1453109_at	Arsk	3.46635	0.00739499
1422240_s_at	Sprr2h	3.34212	0.048739
1460670_at	Riok3	3.32078	0.00880283
1455965_at	Adamts4	3.30621	0.000183407
1416523_at	Rnase1	3.2957	0.0272699
1443109_at	---	3.20581	0.000563566
1443122_at	---	3.11984	0.0268743
1444198_at	---	3.11473	0.000703871
1459183_at	---	3.01381	0.00259112
1455963_at	6332401O19Rik	3.0044	0.00312948
1449211_at	Bpnt1	3.00332	9.99E-05
1437667_a_at	Bach2	2.99958	0.000357257
1434171_at	C330011K17Rik	2.96357	0.0282958
1446130_at	---	2.94778	6.58E-06
1435532_at	LOC100048362	2.90419	0.0176285
1420282_s_at	Prss29	2.88675	0.000879869
1439475_at	Zfp429	2.87303	0.00605254
1450364_a_at	Havcr1	2.85818	0.0352559
1450802_at	Prss28	2.84496	2.72E-05
1435612_at	Opcml	2.76545	0.00503496

1449992_at	Prss29	2.7388	0.00216172
1444262_at	1110017F19Rik	2.72515	0.0069375
1447096_at	---	2.70101	0.0150339
1449032_at	Prl2a1	2.63682	0.0171069
1448650_a_at	Pole	2.59374	0.0247163
1429053_at	1110012J17Rik	2.57509	0.00376077
1420566_at	---	2.48943	0.029121
1429691_at	Ptprg	2.48249	0.00238895
1448025_at	Sirpb1	2.46555	0.00830697
1456688_at	---	2.41953	0.00133759
1418945_at	Mmp3	2.41295	0.0426652
1429982_at	4933426K21Rik	2.4034	0.00943209
1425951_a_at	Clec4n	2.38158	0.00402976
1435533_s_at	4933426K21Rik	2.37328	0.0255649
1458659_at	Plac9	2.37271	0.0287112
1447231_at	---	2.36516	0.0234413
1425451_s_at	Chi3l3 /// Chi3l4	2.34073	0.0443562
1455208_at	Pex19	2.30199	0.00568377
1424502_at	Oit1	2.28795	0.0242847
1418429_at	Kif5b	2.2702	0.000310519
1424843_a_at	Gas5	2.26929	0.0011463
1449475_at	Atp12a	2.26072	0.0445511
1418764_a_at	Bpnt1	2.25707	0.00255535
1459661_at	Dcdc2a	2.24712	0.0225015
1418156_at	Kcne4	2.23922	0.001954
1443939_at	OTTMUSG00000008561	2.22234	0.0276062
1417280_at	Slc17a1	2.21315	0.00660674
1419627_s_at	Clec4n	2.16441	0.00979708
1419405_at	Nmb	2.16288	0.00802374
1447202_at	1200009F10Rik	2.13809	0.000141127
1450291_s_at	Ms4a4c	2.12471	0.0362122
1420604_at	Hesx1	2.1143	0.000794586
1452590_a_at	Plac9	2.10211	0.0306398
1446141_at	---	2.09829	0.0202272
1449388_at	Thbs4	2.09591	0.0033629
1437636_at	LOC623121	2.04626	0.000233143
1436222_at	Gas5	2.01476	0.00126799

Table III.

Probeset ID	Gene Symbol	Fold-Change	p-value
1432216_s_at	Mpp7	-2.00481	0.00230603
1428891_at	9130213B05Rik	-2.00806	0.02324
1449394_at	Slco1b2	-2.01627	0.0272746
1427451_a_at	BC018473	-2.07375	0.0153867
1451625_a_at	C8g	-2.0821	0.0437489
1427001_s_at	Hnf4a	-2.08448	0.0224681
1445824_at	Zfp458	-2.11182	0.00204125
1417920_at	Amn	-2.11539	0.0438993
1442255_at	---	-2.11813	0.0241017
1450839_at	D0H4S114	-2.15096	0.0101531
1448837_at	Vil1	-2.16171	0.0234569
1439109_at	Ccdc68	-2.17935	0.0153504
1459733_at	---	-2.18599	0.000581386
1451610_at	Cxcl17	-2.1862	0.0493382
1457435_x_at	Myom2	-2.20231	0.0138509
1419670_at	Ftcd	-2.23909	0.00127039
1439117_at	Clmn	-2.25626	0.0125216
1425749_at	Stxbp6	-2.27681	0.0413114
1417600_at	Slc15a2	-2.29336	0.0182215
1438699_at	Srd5a1	-2.30676	0.0383523
1431101_a_at	Srd5a1	-2.32013	0.0246874
1457025_at	4833413O15Rik	-2.36177	0.01158
1424072_at	2010107G23Rik	-2.38241	0.000154301
1422846_at	Rbp2	-2.4153	0.0449952
1433845_x_at	Dusp9	-2.47057	0.00662899
1456495_s_at	Osbpl6	-2.48547	0.0397768
1456321_at	Npal1	-2.52829	0.0261785
1429166_s_at	Clmn	-2.52865	0.00630892
1453444_at	5730437C12Rik	-2.58443	0.0125215
1416677_at	Apoh	-2.65625	0.0337181
1417462_at	Cap1	-2.7564	0.0142922
1457483_at	---	-2.7689	0.00187144
1417461_at	Cap1	-2.83218	0.0082928
1452707_at	Klhl30	-3.00085	0.027121
1421445_at	Slc26a3	-3.29019	0.008087
1416980_at	Mettl7b	-3.44204	0.00469039
1449088_at	Fbp2	-3.68493	0.0415826
1419063_at	Ugt8a	-3.98828	0.0118296
1451788_at	F11	-4.324	0.00883293
1428301_at	LOC671957	-4.41548	0.00540577

1429467_s_at	Slc26a3	-4.41934	0.0103699
1459253_at	Arrdc3	-4.77537	0.0106758
1452731_x_at	B930046C15Rik	-5.40964	0.00252296
1427798_x_at	---	-5.44483	0.0171833
1427797_s_at	---	-7.61701	0.0375428
1439055_at	OTTMUSG00000001305	-9.19592	5.53E-05
1418645_at	Hal	-18.0734	7.10E-06

Figure 1.

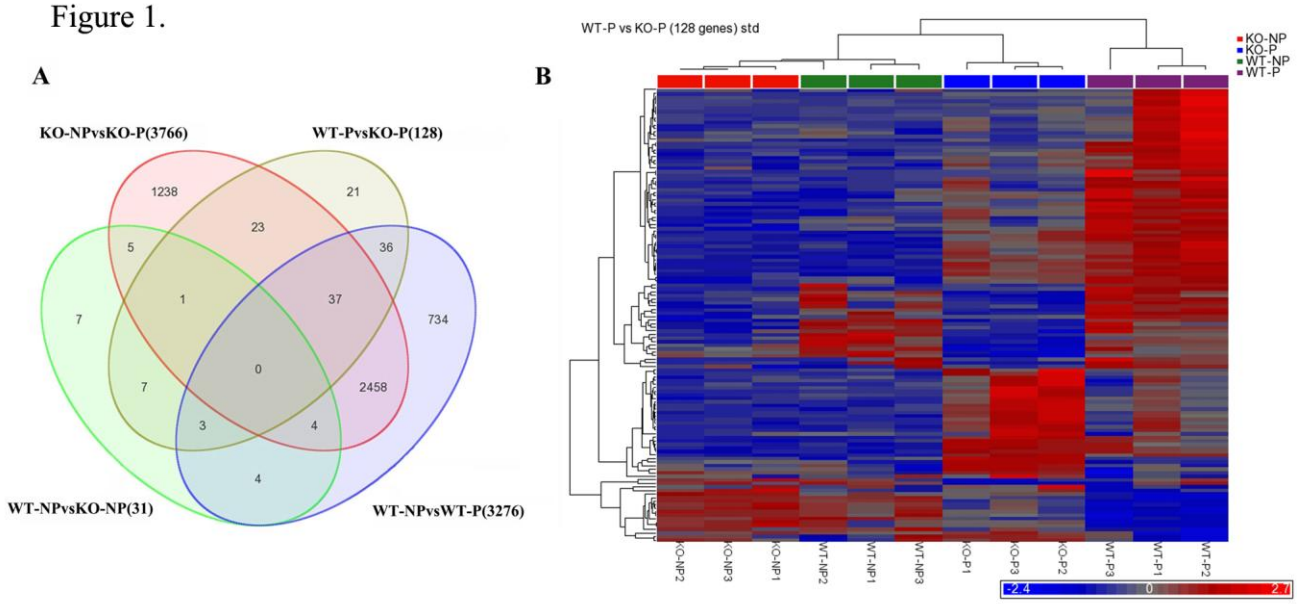


Figure 2

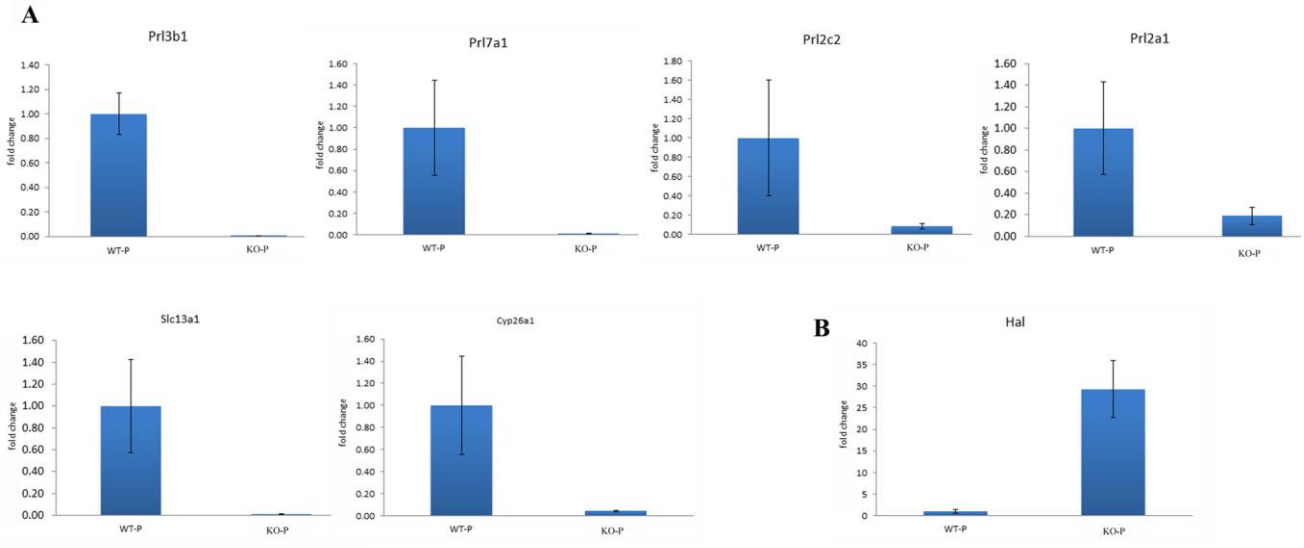
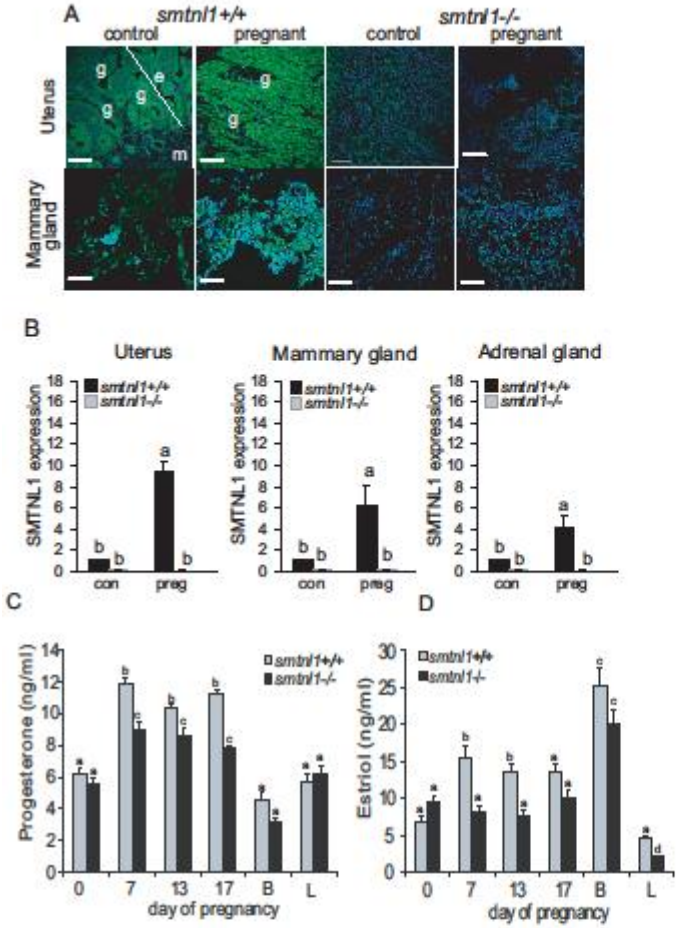


Figure 3.



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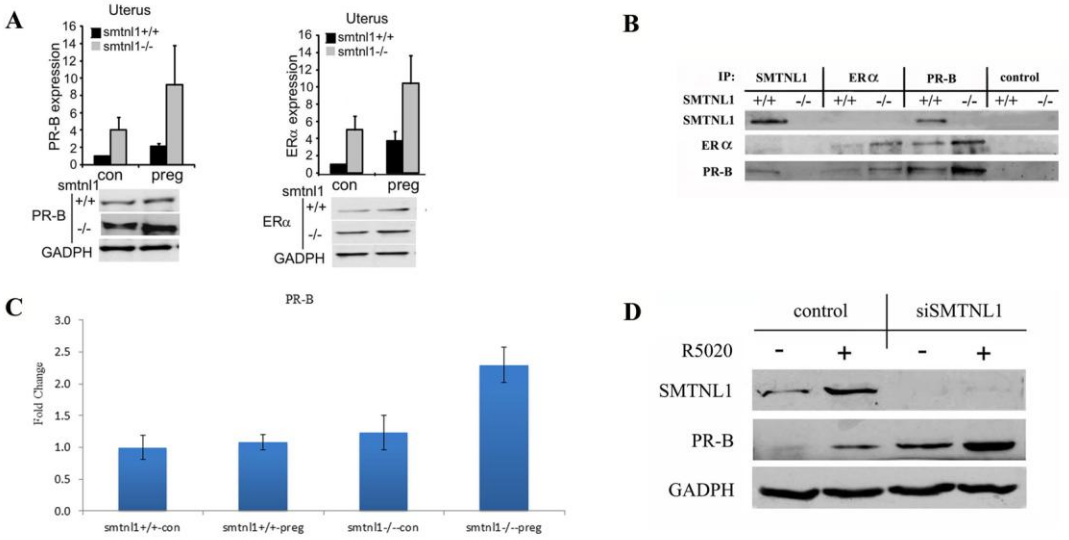
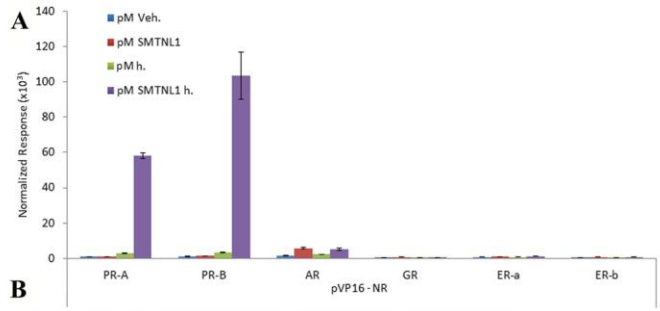
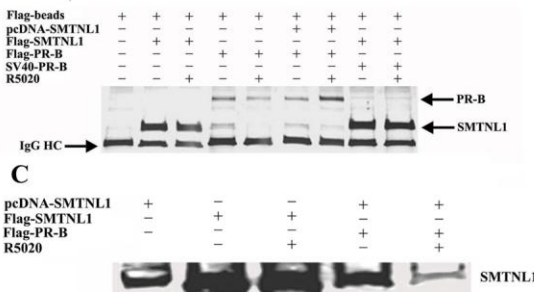


Figure 5.

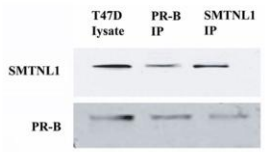




D

SMTNL1 peptides sequenced	Position
EGPSDWAEHLCK	45-56
SGESGGSPGEASILDELKTDLQGEAR	61-86
EETKPEPNEVR	117-127
ELVEPESPTEEQEQGKENESEER	209-231
RVSAPSRPR	299-307
FGGAASGPTALFR	321-333
LADCAQLLEVDDMVR	414-428

PR-B peptides sequenced	Position
TQDQQSLSDVEGAYSR	74-89
VLSPLMSR	160-167
ALGGAAAGGGAAAVPPGAAAGGVALVPK	243-270
QLLEDESYDGGAGAASAFAPPR	322-343
ALDAVALPQPLGVPNESQALSQR	650-672
HVSGQMLYFAPDLILNEQR	770-788
SQTQFEEMR	837-845



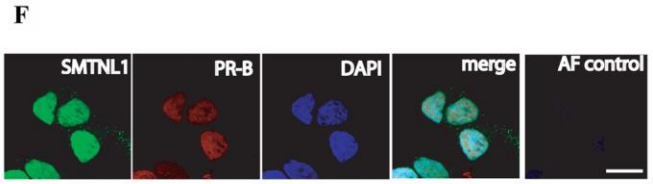
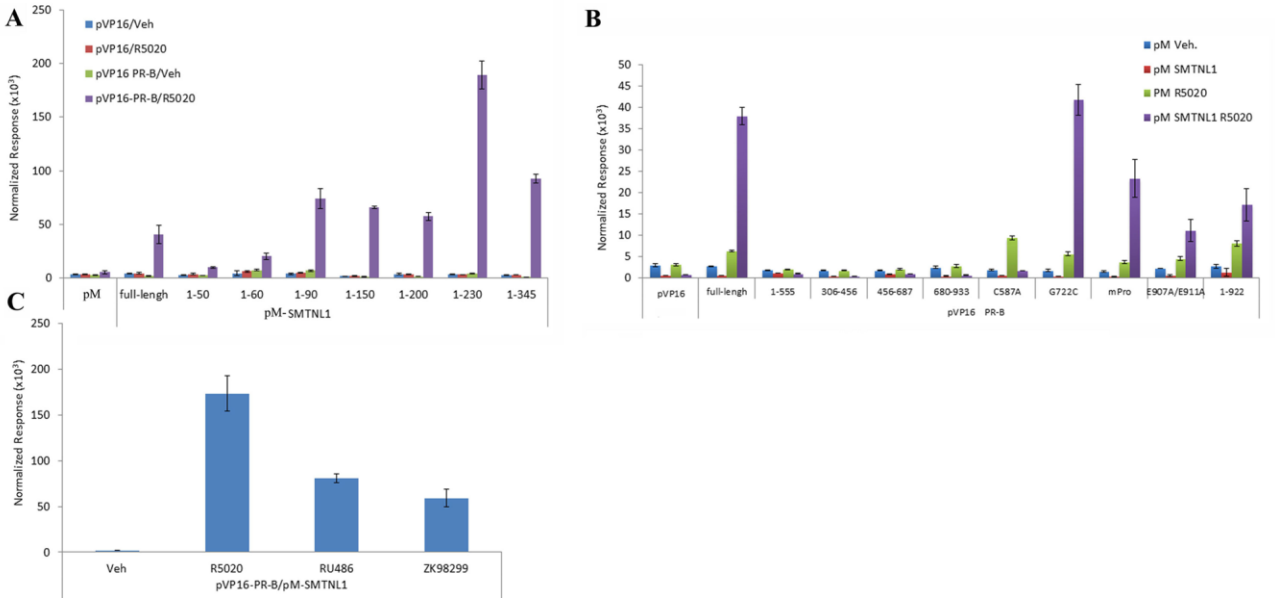


Figure 6.



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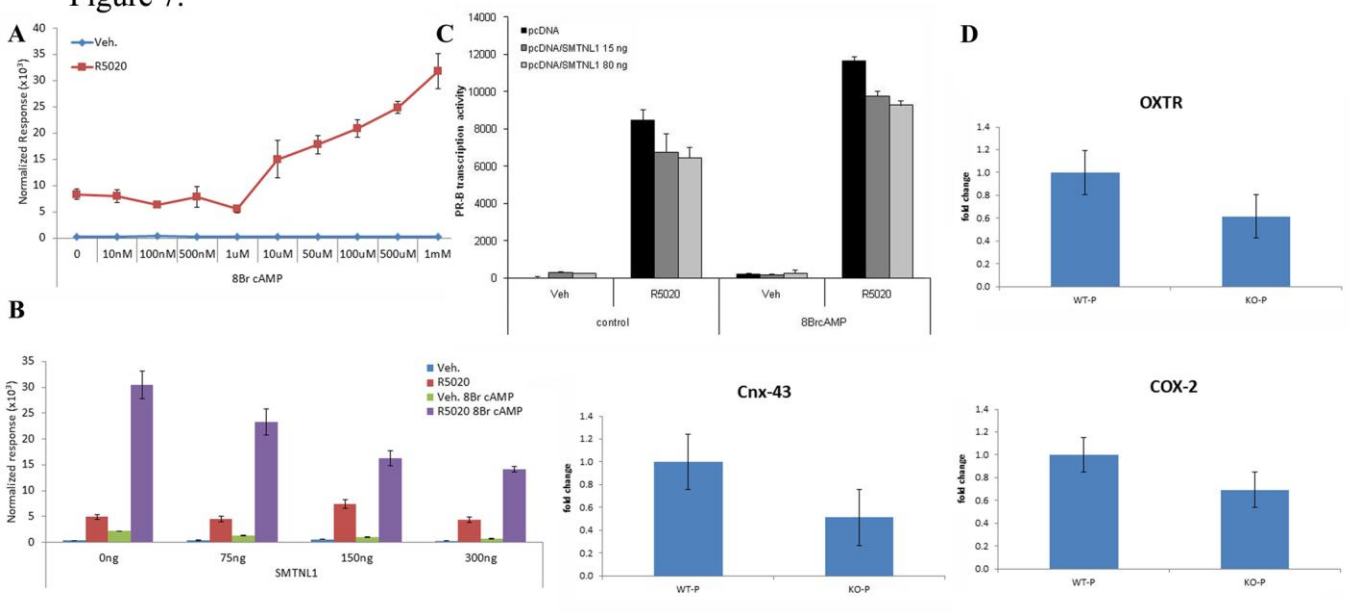

Figure 8.