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Smoothelin-like 1 Protein Is a Bifunctional Regulator of the Progesterone Receptor during Pregnancy*[S]

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During pregnancy, uterine smooth muscle (USM) coordinately adapts its contractile phenotype in order to accommodate the developing fetus 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 that 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 coordinately promote alterations in USM function during pregnancy.

Uterine smooth muscle (USM)[5] cells 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 midpregnancy, USM cells switch into a synthetic phase characterized by cellular hypertrophy, extracellular matrix remodeling, 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). Toward 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 labor 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, whereas relaxation is promoted by dephosphorylation of MLC20 by myosin phosphatase (7, 8). Both myosin light chain kinase and myosin phosphatase 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 (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 myosin phosphatase) is a signaling target of SMTNL1. Deletion of SMTNL1 results in a 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 Ser-301 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

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[5] The abbreviations used are: USM, uterine smooth muscle; CAP, contraction-associated protein; PR, progesterone receptor; ER, estrogen receptor; AR, androgen receptor; GR, glucocorticoid receptor; MMTV, murine mammary tumor virus; RMA, robust multichip analysis; IP, immunoprecipitation.

mice have a 2-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 a 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 specifically inhibiting expression of the oxytocin receptor and prostaglandin F 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 and 15-hydroxy-PG-dehydrogenase (26–29). Additionally, prostaglandins 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 it 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 coordinately 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 (12). 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 guidelines for the care and use experimental animals. All procedures involving mice were approved by the 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 an environmentally controlled room (23 $\pm$ 2 °C, 50 $\pm$ 10% relative humidity, frequent ventillation, and 12/12-h light/dark cycle). For pregnancy studies, 6–8 weeks mature female mice in estrus were selected by the appearance of their vagina and also with the 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 a 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 were collected over a period of 1.5 years from age-matched crosses generated between *smtnl1*$^{+/+}$ $\times$ *smtnl1*$^{+/+}$ and *smtnl1*$^{-/-}$ $\times$ *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*$^{+/+}$ $\times$ *smtnl1*$^{+/+}$ and *smtnl1*$^{-/-}$ $\times$ *smtnl1*$^{-/-}$ experimental breeder pairs (n = 40 each) were used. The already proved fertile males and virgin females were in the age range of 6–8 weeks old. Data are expressed as means $\pm$ S.E.

Cell Culture—HeLa, T47D, and 293T cells were maintained in DMEM and HepG2 cells were maintained in minimum Eagle's medium. 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 Applied Science) 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), estrogen receptors α and β (ERα and ERβ), androgen receptor (AR), and glucocorticoid receptor (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 3 μg of DNA per triplicate. Following a 24-h transfection, cells were treated with vehicle (ethanol) or with the corresponding receptors ligands as follows: 10 nM R5020 for PR-A and -B, 100 nM 17β-estradiol for ERα and -β, 100 nM dexamethasone for GR, 100 nM R1881 for AR, or 100 nM 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 3

μg 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 to 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 3 μg of DNA per triplicate. The following day, cells were incubated with vehicle or 10 nM R5020 in the presence or absence of 100 μM 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 with or without R5020 and 8-Br-cAMP were 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 I*A*.

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 I*C*). 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—The 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 (1 μg) 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 a 0.2 mM concentration of each primer (see supplemental Table I*B* 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 multichip analysis (RMA) normalization was done on the entire data set. Multiway analysis of variance and -fold change were performed to select target genes that were differentially expressed between the different comparisons. Top differentially expressed genes were selected with a p value of 0.05 based on an analysis of variance test and a -fold change cut-off of ≥2. Gene ontology enrichment analysis was performed with a χ^2 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 Web site.

Immunoprecipitation, Immunoblotting, and Mass Spectrometry—Uterus 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, medium was replaced with fresh medium with or without 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.

Enzyme Immunoassays—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, or 17 of pregnancy or at birth and is reported as the mean number of dead embryos. The 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 (for >2 groups). Parametric statistical tests were used if the assumptions of such tests were met. We also log-transformed data for analyses. In general linear models, 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 general linear models, we applied Tukey's honestly significant difference procedure to test for pairwise 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 SMTNL1 in the regulation of smooth and striated muscle plasticity during pregnancy. In both vascular and USM cells,

SMTNL1 expression increased by more than 10-fold by day 13–17 of pregnancy compared with non-pregnant mice and then declines steadily through parturition and the onset of lactation (11). To determine if loss of SMTNL1 function affects normal pregnancy, we examined our *smtnl1*$^{-/-}$ mouse breeding data base. After several thousand breedings, we observed that litter sizes were smaller and embryonic lethality was higher in *smtnl1*$^{-/-}$ mice (Table 1). There was also a statistically significant increase in the proportion of male mice born relative to females (Table 1). An increased proportion of males to females within a population has been shown to be related to a reduction in overall fitness. Additionally, time to pregnancy was longer in *smtnl1*$^{-/-}$ mice and the intervals between pregnancies increased by 3–4 days (Table 1), 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 the role of SMTNL1 in pregnancy, we performed microarray analysis using the Affymetrix Mouse Genome 430 2.0 Array. Over 39,000 transcripts/single array were examined, essentially encompassing the entire expressed mouse genome. Total uterine RNA was isolated from three different mice for each group: non-pregnant wild type (*WN*), wild type pregnant (*WP*), knock-out non-pregnant (*KN*), and knock-out pregnant (*KP*) (see Fig. 1). Differentially expressed genes between the four comparisons are shown in a Venn diagram in Fig. 1*A*. Comparison 1 consisted of WT mice *versus 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 *versus* knock-out pregnant mice. This comparison identified 3766 genes that were differentially expressed. Finally, comparison 4 consisted of analyzing WT pregnant mice *versus* knock-out pregnant. This comparison identified 128 genes (Tables 2 and 3) that were differentially expressed (Fig. 1*B*). Complete lists of genes for all four comparisons are available at the Duke Microarray facility Web site. Table 2 shows a list of 81 genes that were up-regulated by more than 2–50-fold ($n = 3$) in the WT pregnant mice *versus* the *smtnl1*$^{-/-}$ pregnant ones, and Table 3 shows a list of 47 genes that were down-regulated by more than 2–18-fold ($n = 3$) in the WT pregnant mice when compared with 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 signaling), cell adhesion, cytoskeleton remodeling, proteolysis, histidine and proline metabolism, atherosclerosis, cAMP biosynthetic process and activation of pro-

TABLE 1

***Smtnl1* deletion results in a poor reproductive phenotype**

Smtnl1$^{-/-}$ colony breeding data were collected over a period of 1.5 years from age-matched crosses generated between *smtnl1*$^{+/+}$ × *smtnl1*$^{+/+}$ and *smtnl*$^{-/-}$ × *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 ± S.E.

	smtnl1$^{+/+}$	*smtnl1*$^{-/-}$	Statistics
Litter size	7.14 ± 1.47	4.23 ± 1.25	$t_{30} = 5.961$, $p < 0.001$
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%	$t_{30} = 1.768$, $p = 0.087$
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	$t_{16} = 1.827$, $p = 0.086$

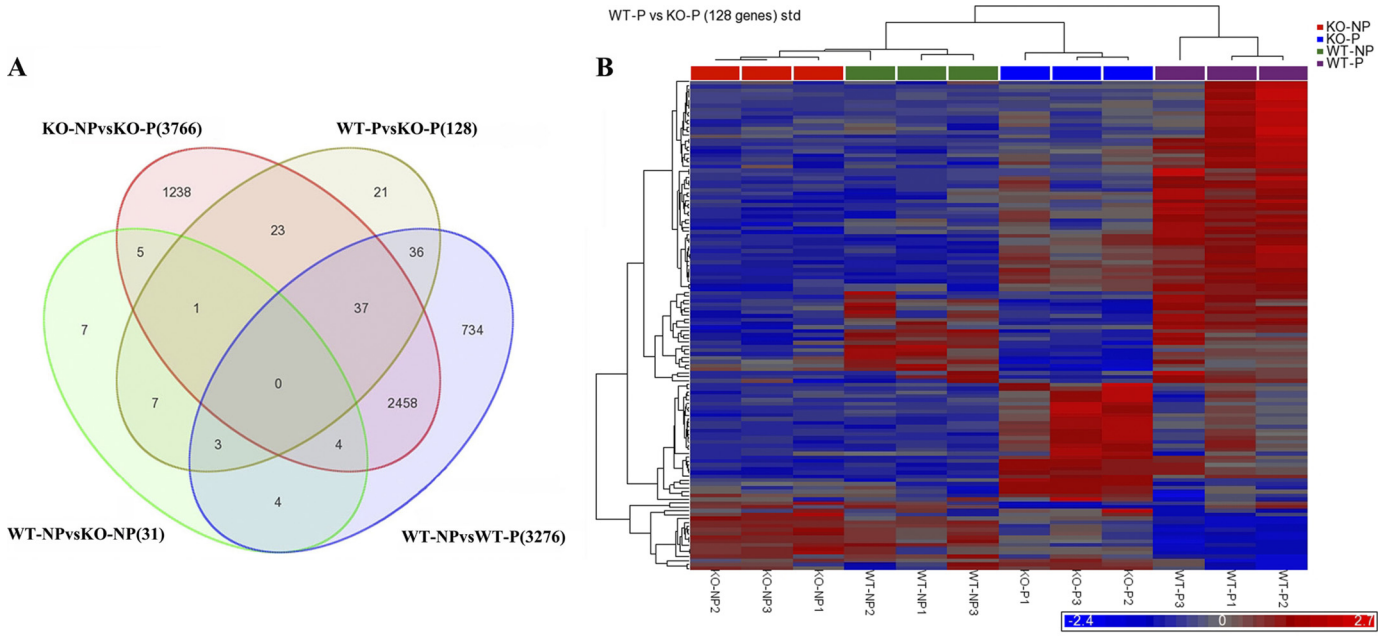


FIGURE 1. **Venn diagram demonstrating the relationship between genes differentially regulated in the different comparisons and hierarchical clustering analysis of the wild type pregnant (*WP*) *versus* knock-out pregnant (*KP*) mice.** *A*, Venn diagram; the *numbers* within the *intersections* of the *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 wild type pregnant *versus* knock-out pregnant 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*. *WN*, non-pregnant wild type; *KN*, knock-out non-pregnant.

TABLE 2
Genes up-regulated in wild type pregnant *versus* *smtnl1*$^{-/-}$ pregnant mice

Probe set ID	Gene symbol	Change	*p* value
		-fold	
1452426_x_at		50.3404	0.0118484
1415835_at	*Prl3b1*	17.5723	0.000565294
1427760_s_at	*Prl2c2///Prl2c3///Prl2c4*	12.0257	7.87×10^{-5}
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	2.88×10^{-6}
1417256_at	*Mmp13*	4.82501	3.77×10^{-5}
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	4.67×10^{-7}
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	6.58×10^{-6}
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	2.72×10^{-5}
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 3
Genes down-regulated in wild type pregnant *versus* *smtnl1*$^{-/-}$ pregnant mice

Probe set ID	Gene symbol	Change	*p* value
		-fold	
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	5.53×10^{-5}
1418645_at	*Hal*	−18.0734	7.10×10^{-6}

tein kinase A, transport, plasminogen activation, steroid metabolic process and glucocorticoid receptor signaling, 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-κB1, 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 signaling 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 with their wild type

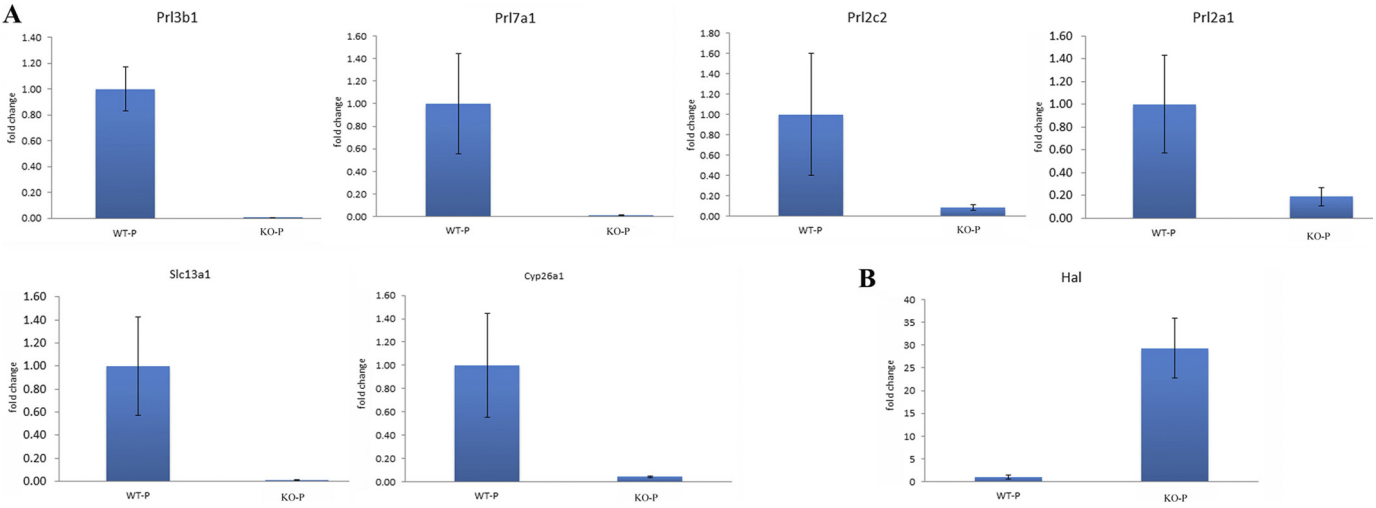


FIGURE 2. **Quantitative RT-PCR validation of microarray data.** Quantitative RT-PCR analysis of Prl3b1, Prl2a1, Prl7a1, Prl2c2, Cyp26a1, and Slc13a1 (*A*) and Hal (*B*) in wild type pregnant (*WT-P*) and *smtnl1*$^{-/-}$ pregnant (*KO-P*) mouse uteri. The results represent the mean ± S.E. (*error bars*), $n = 3$ RNA sets.

counterparts (WT-P) (Fig. 2*A*). The significance of this finding will be elaborated upon below in the context of the function of prolactins as mediators of pregnancy adaptations. Additionally, consistent with microarray results, RT-PCR analysis showed that the mRNAs for *Cyp26a1* and Slc13a1 were down-regulated in the KO-P mice (Fig. 2*A*). 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)) that play an essential role in the ovulation process, through extracellular matrix remodeling, 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 with their WT-P counterparts (Fig. 2*B*). 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 signaling pathway (43).

SMTNL1 Regulates the Expression of ERα and PR-B in the Primary Genital Tissues—Microarray analysis supports a role for SMTNL1 as 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. 3, *A* 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. 3, *A* and *B*). As observed previously, the deletion of SMTNL1 does not appear to alter the ultrastructure of any tissues, either non-pregnant or pregnant, including uterus and 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. 3*B*). 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 that SMTNL1 deletion produced a marginal 10–15% reduction in circulating estradiol and progesterone levels, suggesting a potential role in regulating steroid biosynthesis (Fig. 3, *C* and *D*).

The finding 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. 4*A*). 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. 4*A*). 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. Fig. 4*B* shows that in both tissues SMTNL1 co-immunoprecipitated 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 and co-workers (44, 45) demonstrating an interaction of PR-B with ERα in T47D cells. Although SMTNL1 does not bind ERα

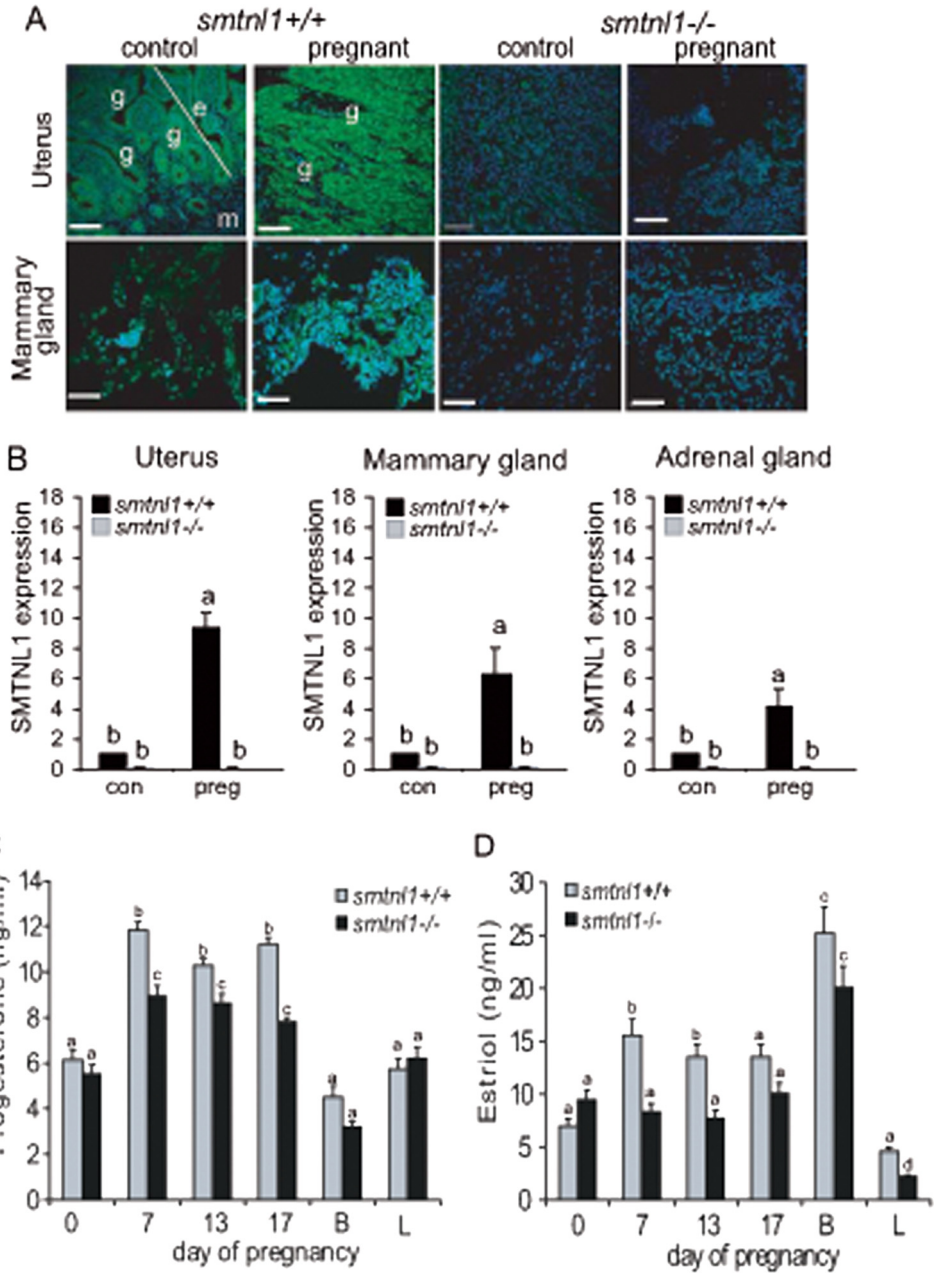


FIGURE 3. **Pregnancy regulates SMTNL1 expression in the primary genital tissues.** *A*, immunohistochemical images of SMTNL1 localization in uterus and mammary gland from control and pregnant WT and *smtnl1*$^{-/-}$ mice. *Scale bars*, 20 μm. *m*, myometrium; *e*, endometrium; *g*, endometrial glands 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. *Insets below* show representative Western blots. Data shown are from day 0 and day 14 of pregnancy. *C* and *D*, circulating hormone concentrations of *smtnl1*$^{+/+}$ and *smtnl1*$^{-/-}$ mice during pregnancy. The circulating progesterone (*C*) and estriol (*D*) concentration is lower in *smtnl11*$^{-/-}$ mice in pregnancy. Results shown are means $\pm$ S.E. (*error bars*), $n = 4-6$ animals, general linear model with Tukey's test. *Different letters* indicate significant differences ($p < 0.05$).

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. Fig. 4*C* shows 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. 4*C*). 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 well characterized USM cell lines expressing both proteins. Fig. 4*D* shows that 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

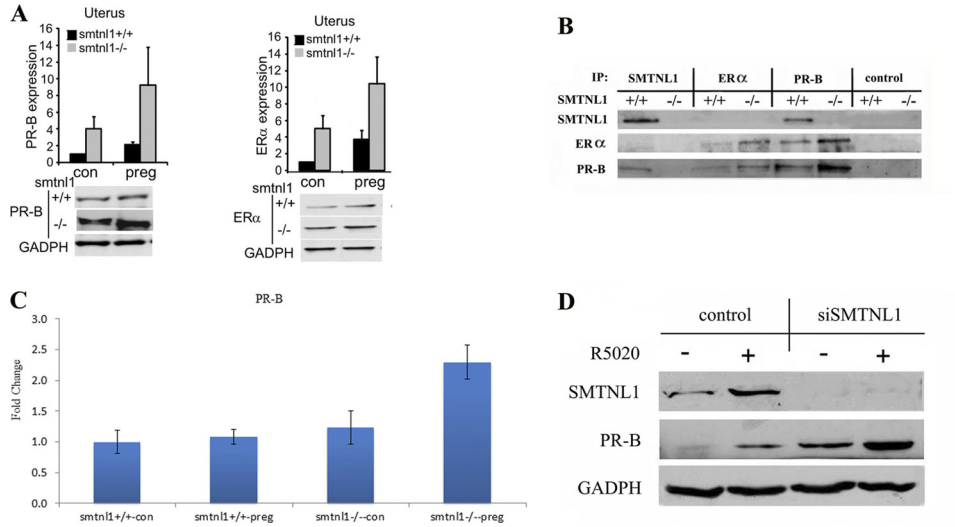


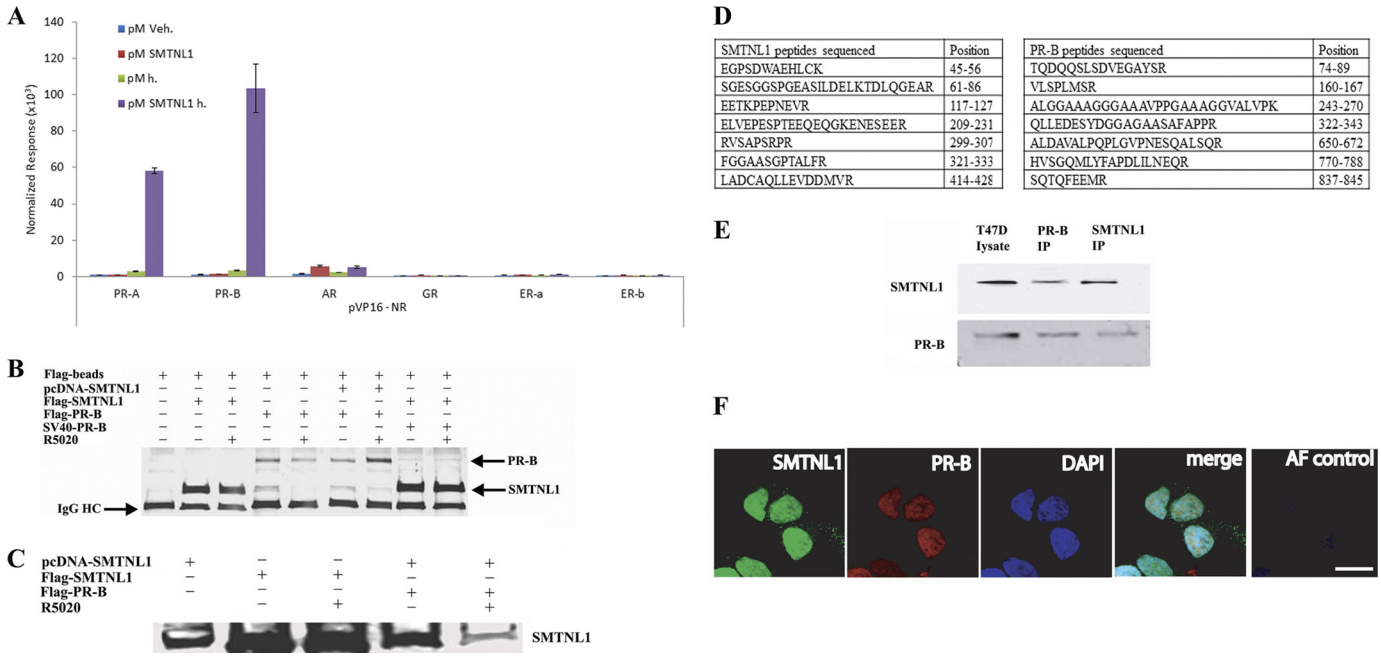
FIGURE 4. **SMTNL1 regulates the expression of ERα and PR-B in USM.** *A*, pregnancy and SMTNL1 deletion promote a higher expression of PR-B and ERα in USM. *Insets below* show representative Western blots. Data shown are from day 0 (*con.*) and day 14 of pregnancy (*preg.*). *B*, SMTNL1, PR-B, and ERα co-IP studies from uterine extracts of WT-P and *smtnl1*$^{-/-}$ pregnant. *C*, PR-B mRNA levels are up-regulated in the pregnant uteri of *smtnl1*$^{-/-}$ compared with WT mice. RT-PCR results of PR-B mRNA levels in USM from aged matched pregnant and non-pregnant WT and *smtnl*$^{-/-}$ mice, $n = 3 \pm$ S.E. (*error bars*). *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 h, lysed, and analyzed by Western blot analysis using antibodies against SMTNL1 and PR-B.

PR-B shows that SMTNL1 siRNAs dramatically induce the expression of the receptor ~10-fold above that induced by R5020 alone (Fig. 4*D*). 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 nonspecific 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 toward the major steroid hormone receptors (progesterone receptor-A and -B, estrogen receptor-α and -β, AR, and 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. Fig. 5*A* 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 with 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 favorably 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, and -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 L*XX*LL (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 L*XX*LL 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 two-hybrid and co-IP experiments suggests that the mechanisms of altered ER expression observed *in vivo* are likely to be mediated through PR via a feedback 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-immunoprecipitate SMTNL1 and PR-B (Fig. 5*B*). Likewise, when cells were transfected with expression vectors for FLAG-PR-B and pCDNA-SMTNL1, the FLAG beads were able to immunoprecipitate both FLAG-PR-B and SMTNL1 (Fig. 5*C*), and the presence of SMTNL1 was confirmed by Western blotting (Fig. 5*C*). Mass spectrometry studies confirmed the identity of the bands seen in the IP experiments (Fig. 5*D*). Additionally, endogenous SMTNL1 was found to co-IP with endogenous PR-B in T47D cell lysates as shown in Fig. 4*E*. Finally, co-localization of SMTNL1 and PR-B was observed by immunofluorescent staining in T47D cells showing discrete nuclear localization for both proteins (Fig. 5*F*).

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 calponin homology domain, the protein has no defined domains and is quite unique with no sequence similarities to known proteins in



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 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 receptor 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 medium was replaced with or without 10 nM R5020 for 2 h. Lysates were immunoprecipitated 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 immunoprecipitated 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*, 10 μm. *Error bars*, S.E.

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. Fig. 6*A* 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 with 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. 6*A*). Taken together, the results indicate that amino acids 60–90 and amino acids 200–230 are required for SMTNL1 interaction with PR-B, with the latter exhibiting a robust binding capacity when compared with the full-length protein. Furthermore, the results indicate that the calponin homology 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. Fig. 6*B* shows 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. 6*B*). 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. 6*B*). The PR-B mPro mutant showed reduced binding when compared with that of the wild type protein (Fig. 6*B*). This construct has three essential prolines (Pro-422, Pro-423, and Pro-426) changed to alanines, which results in the loss of binding to the Src homology 3 domains of signaling proteins. The G722C mutant (the 722 construct) was found to bind SMTNL1 with levels comparable with those of the wild type protein (Fig. 6*B*). 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 are hormone-dependent prompted us to examine if the hormone binding domain of PR-B by itself is sufficient to bind SMTNL1. Fig. 6*B* shows 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. 6*B*). Comparative experiments with the co-activator SRC1-NR box are shown in supplemental Fig. 2. Next, we used the mammalian two-hybrid system to examine the effects of anti-progestins (RU486 and

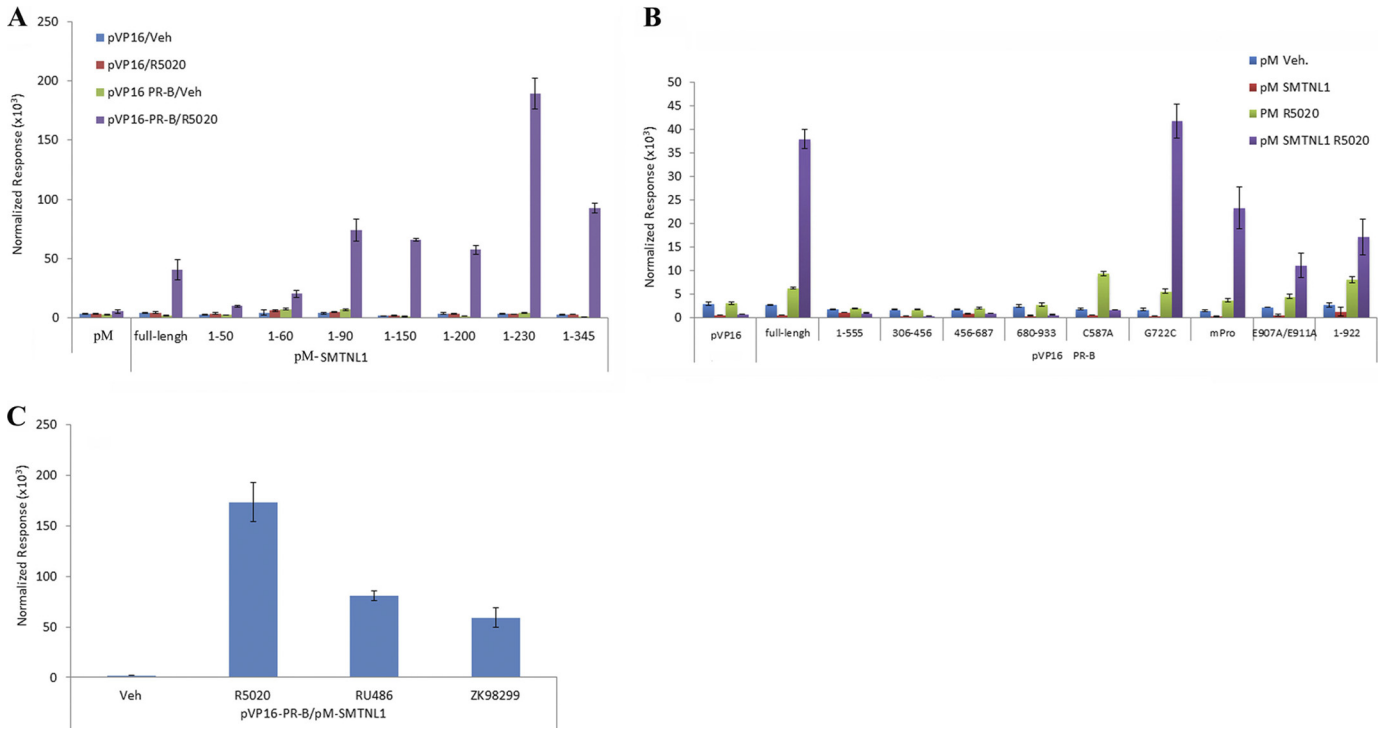


FIGURE 6. **Identification of interaction sites within SMTNL1 and PR-B.** *A*, HepG2 cells were transfected with the indicated SMTNL1 deletion constructs together with PR-B. *B*, HepG2 cells were transfected with the indicated PR-B deletion constructs and mutants together with SMTNL1. In both *A* and *B*, 24 h after, the cells were incubated with vehicle or 10 nM R5020 for 24 h and 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 (*Veh*) or 10 nM R5020, 100 nM RU486, and 100 nM ZK98299 for 24 h and harvested for luciferase and β-gal assays. *Error bars*, S.E.

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 with R5020, as shown in Fig. 6*C*. 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 signaling. SMTNL1 is phosphorylated at Ser-301 in smooth muscle cells in response to 8-Br-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). Fig. 7*A* shows that treatment of HeLa cells, transfected with an expression vector for PR-B and MMTV-luciferase reporter plasmid, with increasing amounts of 8-Br-cAMP plus R5020 increases the transcriptional activity of PR-B in a dose-dependent manner. This assay helped us to determine the conditions for analyzing the effect of SMTNL1 on PR-B transcriptional activity at any given [8-Br-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 8-Br-cAMP. Fig. 7*B* shows that SMTNL1 inhibits PR-B transactivation in a dose-dependent manner (*i.e.* 300 ng of SMTNL1 causes a more than 50% reduction in transcriptional activity). This effect correlates precisely with 8-Br-cAMP concentrations (10 μM) required to promote nuclear translocation of SMTNL1 through Ser-301 phosphorylation (11). Increasing amounts of transfected SMTNL1 were also found to progressively repress endogenous PR-B activity by 20–30% in T47D cells (Fig. 7*C*). 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 oxytocin receptor, Cnx-43, and cyclooxygense-2 (21–29). Therefore, if SMTNL1 represses PR, one might predict that loss of this function in *smtnl1*$^{-/-}$ mice would lead to altered expression of PR-regulated genes. Fig. 7*D* shows that this is indeed the case. Quantitative RT-PCR studies show that mRNAs for oxytocin receptor, Cnx-43, and cyclooxygense-2 were all decreased markedly in the KO-P mice compared with their WT-P counterparts (Fig. 7*D*). 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

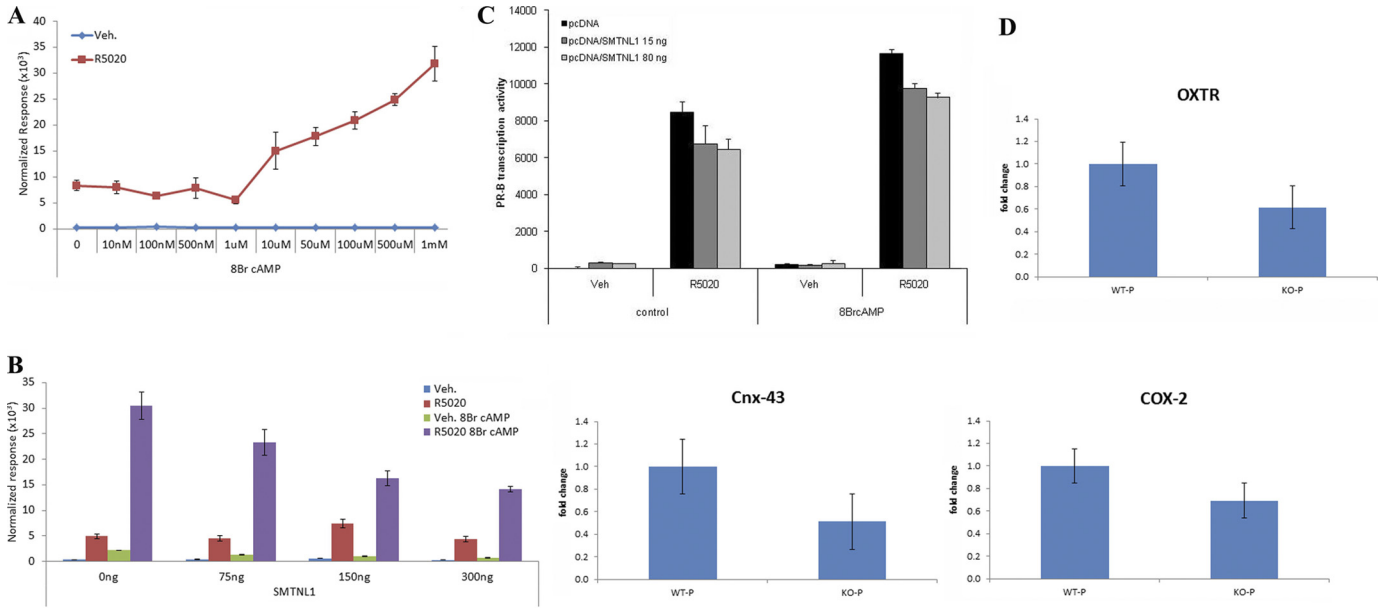


FIGURE 7. **SMTNL1 regulates PR-B expression at the transcriptional level.** *A*, effect of cAMP on the transcriptional activity of PR-B. HeLa cells were transfected with an expression vector for PR-B and an MMTV-luciferase reporter plasmid. After transfection, cells were incubated with vehicle or 10 nM R5020 in the presence of 8-Br-cAMP (10 nM to 1 mM) 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 h 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 with or without R5020 and with or without 10 μM 8-Br-cAMP were measured. *D*, RT-PCR analysis of oxytocin receptor (*OXTR*), Cnx-43, and cyclooxygense-2 in wild type pregnant (*WT-P*) and *smtnl1*$^{-/-}$ pregnant (*KO-P*) mouse uteri, $n = 3$ RNA sets ± S.E. (*error bars*).

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 underlying the physiological relationship between the two proteins. Consistent with this role, quantitative RT-PCR studies of several known 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. Fig. 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 mouse USM extracts showed a significant increase in the induction of the protein in comparison with 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 littermates. 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 because the S301A SMTNL1 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.

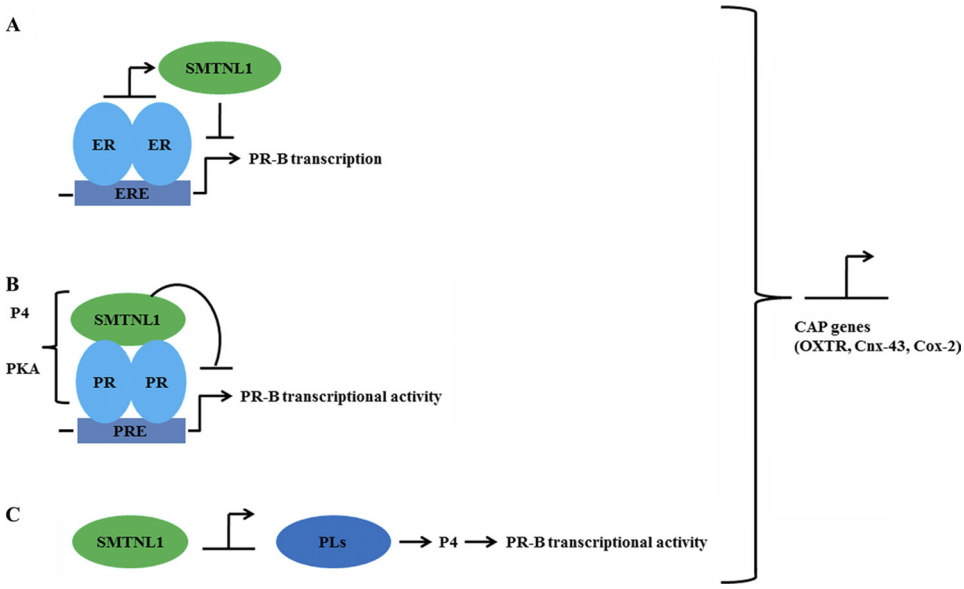


FIGURE 8. **Mechanisms by which SMTNL1 regulates PR-B to coordinately promote USM adaptations in pregnancy.** *Schematics A* and *B* show that regulation of expression of SMTNL1, PR, and ER is via two possible homeostatic feedback mechanisms. In *A*, the synthesis of PR is controlled by estrogen acting 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, Ser-301, 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) that 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 coordinately promote alterations in USM function during pregnancy. ⊥(inhibition) → (gene transcription).

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). Members of 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 result in complete infertility in females due to failure of implantation (59, 60). These mice show a lack of functional corpus luteum and thus were unable to produce progesterone. The corpus luteum plays a central role in the maintenance of pregnancy through regulating the production of progesterone (61). In rodents, the functions of the corpus luteum 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 an *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 gland 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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