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Differential Regulation of Human and Mouse Myometrial Contractile Activity by FSH as a Function of FSH Receptor Density[1]

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ABSTRACT

Previous studies from our laboratory revealed that the follicle-stimulating hormone receptor (FSHR) is expressed at low levels in nonpregnant human myometrium and that it is up-regulated in pregnant term nonlaboring myometrium; however, the physiological relevance of these findings was unknown. Herein, we examined signaling pathways stimulated by FSH in immortalized uterine myocytes expressing recombinant FSHR at different densities and showed that cAMP accumulation is stimulated in all cases but that inositol phosphate accumulation is stimulated only at high FSHR densities. Because an increase in cAMP quiets myometrial contractile activity but an increase in 1,4,5-triphosphoinositol stimulates contractile activity, we hypothesized that FSHR density dictates whether FSH quiets or stimulates myometrial contractility. Indeed, in human and mouse nonpregnant myometrium, which express low levels of FSHR, application of FSH resulted in a quieting of contractile activity. In contrast, in pregnant term nonlaboring myometrium, which expresses higher levels of FSHR, application of FSH resulted in increased contractile activity. Examination of pregnant mouse myometrium from different stages of gestation revealed that FSHR levels remained low throughout most of pregnancy. Accordingly, through mid-gestation, the application of FSH resulted in a quieting of contractile activity. At Pregnancy Day (PD) 16.5, FSHR was up-regulated, although not yet sufficiently to mediate stimulation of contractility in response to FSH. This outcome was not observed until PD 19.5, when FSHR was further up-regulated. Our studies describe a novel FSHR signaling pathway that regulates myometrial contractility, and suggest that myometrial FSHR levels dictate the quieting vs. stimulation of uterine contractility in response to FSH.

contractility, FSH, FSH receptor, myometrium, parturition

INTRODUCTION

The follicle-stimulating hormone receptor (FSHR) is essential for female fertility by virtue of its expression and actions in the ovary. There it mediates follicular growth and estrogen synthesis in response to FSH released by the pituitary. The FSHR is a member of the Family A of G protein-coupled receptors and is most closely related to the luteinizing hormone (LH) and thyroid-stimulating hormone (TSH) receptors. Collectively termed the glycoprotein hormone receptors, the FSH, LH, and TSH receptors have a large extracellular domain that confers high affinity binding and a prototypical 7-transmembrane domain that interacts with G proteins. The glycoprotein hormones FSH, LH, TSH, and human chorionic gonadotropin (the placental homolog of LH) are composed of a common α subunit that is noncovalently associated with a hormone-specific β subunit. Upon binding to hormone, the glycoprotein hormone receptors stimulate G_s, resulting in the activation of adenylyl cyclase and increased synthesis of cAMP [1]. At high receptor densities, however, the glycoprotein hormone receptors also activate $G_{q/11}$, resulting in stimulation of the inositol phosphate signaling cascade [2–5].

Traditionally, FSHR expression in women was thought to be limited to the ovary. However, this receptor is increasingly appreciated to be expressed in additional tissues [6–14], and in several instances, such expression has been demonstrated to have unique physiological roles. For example, FSHR is expressed in osteoclasts, where FSH directly regulates bone mass [11, 15]. FSHR is also expressed in vascular endothelial cells, which are stimulated by FSH to undergo angiogenic processes as well as to synthesize nitric oxide [14]. Of particular relevance to female reproduction are findings that FSHR is expressed in several tissues of the extragonadal reproductive tract in both nonpregnant and pregnant women [13] and that FSHR in the fetal placental vasculature is essential for normal placental and fetal growth [13]. Notably, the extraovarian reproductive tract is also a site of FSH synthesis, with both *CGA* mRNA (encoding the common α subunit) and *FSHB* mRNA (encoding the FSH-specific β

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subunit) detected in placental, decidual, and myometrial tissues of pregnant women [13].

Our recent studies revealed FSHR expression in the muscle fibers and stroma of human myometrium and demonstrated an up-regulation of FSHR in pregnant term nonlaboring myometrium relative to that in nonpregnant myometrium [13]. Using PCR conditions that differentiate among the full-length FSHR mRNA and its many splice variants, we further determined that human pregnant term nonlaboring myometrium expresses only full-length FSHR mRNA [13]. These findings were particularly intriguing in light of recent studies that had identified *FSHR* as a gene associated with the timing of birth in women [16, 17]. The current studies were undertaken to more thoroughly examine the regulation of myometrial FSHR expression during pregnancy and to determine the functional ramifications of such regulation with respect to FSH-mediated changes in contractile activity. Our studies implicate FSH-provoked FSHR signaling in the myometrium as a novel mechanism controlling uterine contractile activity and suggest that the regulation of myometrial FSHR expression in the myometrium determines the balance between the quieting and the activating of contractile activity in response to FSH.

MATERIALS AND METHODS

Sources of Human Myometrial Tissues

Samples of pregnant myometrial tissue were excised from the lower uterine segment from women 18–50 yr of age undergoing cesarean section under spinal anesthesia at term pregnancy (38–40 wk gestation) in the absence of spontaneous or induced labor contractions. All study participants provided consent to the Maternal Fetal Tissue Bank of the University of Iowa Hospital and Clinics Department of Obstetrics and Gynecology (IRB no. 200910784), which provided clinical information, and all patients signed Institutional Review Board-approved written consent forms for the collection of tissue samples (IRB no. 201307720). Samples of nonpregnant myometrial tissue were excised from the lower uterine segment of uteri removed from women 18–50 yr of age; these women were free of cancer and were undergoing elective hysterectomy. The nonpregnant samples were obtained through the University of Iowa Carver College of Medicine Tissue Procurement Core and the Department of Pathology, and all patients signed written consent forms for the University of Iowa Biobank protocol, which was approved by the Institutional Review Board (IRB no. 201103721). The samples were coded or de-identified before they were provided to us. One portion of each sample was prepared for histology, a second portion was stored at −80°C in RNAlater solution (Qiagen) for isolation of RNA, and a third portion was stored in Hanks balanced salt solution (Life Technologies) overnight at 4°C for measurement of myometrial contractile activity.

Sources of Mouse Myometrial Tissues

C57Bl/6 mice were obtained from Harlan Laboratories and housed under standard conditions with a 12L cycle and access to water and food ad libitum. Animal care procedures were approved by the Institutional Animal Care and Use Committee for the University of Iowa and performed in accordance with the standards set by the National Institutes of Health. Postpubertal females were synchronized in the estrous cycle as described by Whitten [18]. Females thus treated were caged overnight with adult males, and pregnant dams were euthanized on Pregnancy Days (PD) 7.5, 11.5, 16.5, and 19.5 to obtain uteri. Nonpregnant, cycling females were also euthanized, and their uteri were collected.

Uterine Myography

Mouse uteri were excised en masse and placed into ice-cold Hanks balanced salt solution. Uteri were bisected longitudinally, and in pregnant uteri, the fetoplacental units were removed. Uterine tissue was isolated, and sites of implantation sites were avoided if necessary. The tissue was cut into 4 × 8 mm strips in ice-cold Krebs buffer (118.3 nM NaCl, 4.7 KCl, 1.2 nM $MgSO_4$, 1.2 nM KH_2PO_4, 25 nM $NaHCO_3$, 2.5 nM $CaCl_2$, and 11 nM glucose). The strips were then mounted to force transducers in organ baths filled with oxygenated Krebs buffer (20% O_2, 5% CO_2, balanced N_2) solution at 37C, and tension was recorded with a Powerlab data acquisition system (AD Instruments). Basal tension (1 g) was applied to the strips, and the tissues were equilibrated to this condition for 1 h prior to study. First vehicle only and then FSH at sequentially increasing concentrations were applied to the organ bath at 12.5-min intervals. Time controls were treated with vehicle only at all time points. Contractile activity during the latter 10 min of each time interval was quantified as a function of the integral under the contraction curve as described by Arthur et al. [19] and is expressed relative to the contractile activity during the final 10 min of the equilibration period.

For the analysis of nonpregnant and pregnant human myometria, myometrial muscle stroma with longitudinal muscle fibers was isolated and cut into 4 × 8 mm strips in ice-cold Krebs buffer. Strips were mounted to force transducers as described above for mouse uteri and were treated sequentially with 2.5 mM KCl (2 min) and 1 nM oxytocin (5 min) and then rinsed three times with fresh Krebs buffer. Basal tension (1.5 g) was applied to the strips, and the tissues were equilibrated to this condition for 2 h prior to study. Vehicle only and then sequentially increasing concentrations of FSH were applied to the organ baths at 12.5-min intervals. Time controls were treated with vehicle only at all time points. Contractile activity was quantified as described above for mouse myometrial tissues.

Antibodies

FSHR-323 hybridoma cells, which express an immunoglobulin G2a (IgG2a) that recognizes the extracellular domain of human FSHR (hFSHR) [20], were obtained from American Type Culture Collection. Ascites fluid was prepared, from which IgG2a was then purified using the NAb protein G Spin Kit (Thermo Fisher Scientific Inc.). Purified nonimmune mouse IgG2a was obtained from R&D Systems and used as the negative control.

Rabbit anti-rat FSHR was generously provided by Dr. Mario Ascoli (University of Iowa). Its characterization and specificity have previously been described [13, 21]. This antibody, unlike FSHR-323, cross-reacts with mouse FSHR [13].

Immunohistochemical Localization of FSHR in Human Myometrium

Sections of paraffin-embedded human myometrium were deparaffinized in xylene and rehydrated in an ethanol series. Immunolocalization of FSHR in human myometrium was performed as previously described [13]. FSHR-323 IgG2a and nonimmune IgG2a were each used at 5 μg/ml and were applied in blocking buffer overnight at 4°C. After samples were washed, biotinylated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.) was added at 42 μg/ml for 1 h at room temperature. The ABC Standard Kit (Vector Laboratories, Inc.) was used according to manufacturer's instructions, and immunoreactivity was visualized with 3,3-diaminobenzidine (DAB; Dako North America, Inc.). Samples were counterstained with 10% Harris hematoxylin (Leica Microsystems Inc.). Images were captured using a BX61 model light microscope (Olympus). Staining was quantified using Image J software (U.S. National Institutes of Health; http://imagej.nih.gov/ij.) with the following modifications. The analysis was limited to FSHR from myometrial smooth muscle by focusing on rectangles of uniform size at the top, right, bottom, left, and center of each image, shifting these as necessary to avoid blood vessels. For each rectangle, the percentage of pixels exceeding a threshold value of 190 was determined, and then the percentages for all rectangles on a given slide were averaged.

Immunohistochemical Localization of FSHR in Mouse Myometrium

The protocol was essentially the same as that described above for human tissues except that rabbit anti-rat FSHR (1:5000 dilution) or preimmune rabbit serum at the same dilution (negative control) was used as the primary antibody, and biotinylated goat anti-rabbit IgG Fab (1:500 dilution; Jackson Immuno-Research Laboratories) was used as the secondary antibody. Morphometric analyses were performed as described above for human myometrium, except that a threshold value of 160 was determined.

Quantitative Real-Time PCR Measurements of FSHR mRNA

Myometrial tissue stored in RNAlater solution was minced and homogenized. Total RNA was isolated using the PureLink RNA mini kit (Ambion) according to manufacturer's instructions, with the addition of on-column treatment with PureLink DNase treatment and postextraction purification with the Turbo DNA-free kit (Life Technologies). RNA was quantified using spectrophotometry (model 1000 spectrophotometer; NanoDrop).

Quantitative measurements of the relative expression of *FSHR* mRNA were made in duplicate, using 100 ng of RNA with iTaq Universal Probes One-Step quantitative PCR (qPCR) kit (Bio-Rad Laboratories). Primer-probe sets were purchased from Integrated DNA Technologies, Inc. To avoid splice variants and complications with extension, we designed the following primer-probe set for amplification of a sequence within exon 10: forward primer was CTATCCACACTGACGCATTACA; reverse primer was CCCAGA GAATTTCCCAGAGAA; and the 6-fluorescein amidite-labeled probe was AATTGAACACCCATCTGAAGCCTT. Negative controls were run with each sample to ensure the absence of genomic DNA contamination. Expression levels of the genes of interest were normalized to that of 18S RNA (Integrated DNA Technologies) and quantitated using the delta delta quantification cycle (Cq) method [22].

hTERT-HM Cells

The hTERT-HM cells, an immortalized line of human uterine myocytes derived from premenopausal nonpregnant myometrium [23], were generously provided by Dr. Jennifer C. Condon (Wayne State University). Cells were cultured in growth medium consisting of Dulbecco modified Eagle medium/F12 medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) and antibiotic/antimycotic agent (10 000 U/ml; Invitrogen) in a humidified incubator at 37°C in 95% air with 5% CO_2.

Recombinant hFSHR was expressed in hTERT-HM cells by using recombinant adenoviral particles encoding hFSHR (Ad-FSHR). Ad-FSHR was previously described [4]. It and control recombinant adenoviral particles encoding β-galactosidase (β-gal) were prepared by the University of Iowa Carver College of Medicine Viral Vector Core Facility. hTERT-HM cells were plated into 6-well plates (100 000 cells/well). On the following day, the cells were washed with serum-free growth medium and incubated 20–21 h at 37°C in the same medium but with recombinant adenovirus particles added at various multiplicities of infection (range, 2–300 multiplicity of infection). The incubation was terminated on the following day by replacing the medium with serum-containing growth medium, and the cells were used for experiments 1 day later.

FSH Binding Assays

Iodine-125-labeled FSH (^{125}I-FSH) binding assays were performed using hTERT-HM cells as we described previously for other cells [24]. Briefly, intact cells were incubated with a saturating concentration of ^{125}I-hFSH with respect to binding assays (300 ng/ml, final) in the absence or presence of pregnant mare serum gonadotropin (220 IU/ml final concentration) for 1 h at room temperature.

Second Messenger Assays

Basal and FSH-stimulated intracellular cAMP in hTERT-HM cells was measured as we described previously for other cells [24]. Briefly, cells were incubated with FSH at a saturating concentration with respect to cAMP accumulation (300 ng/ml, final) or vehicle only for 1 h at 37°C, at which point the intracellular concentration of cAMP was extracted and quantified by radioimmunoassay.

Basal and FSH-stimulated inositol phosphates in hTERT-HM cells were measured as previously described for other cells [4, 25]. hTERT-HM cells were incubated in serum-free medium containing Ad-FSHR or Ad-β-gal as described above, after which the medium was removed and replaced with serum-containing growth medium supplemented with 4 μCi/ml [2-^{3}H]*myo*-inositol (Perkin-Elmer). After incubation for 19 h, the cells were washed and incubated for 1 h at 37°C with 20 mM LiCl plus either vehicle or FSH at a saturating concentration with respect to accumulation of inositol phosphates (500 ng/ml, final). Inositol phosphates were extracted and quantified as described previously [4, 25].

Statistical Analysis

Data were analyzed statistically using Prism software (GraphPad Software, Inc.) by using Student *t*-test or one-way ANOVA and Tukey test for post hoc pairwise multiple comparisons where appropriate, unless otherwise noted. Statistically significant differences were defined as those with a P value of <0.05 and are denoted by an asterisk.

RESULTS

Our previous immunohistochemical study demonstrated that FSHR is expressed in human nonpregnant myometrium and that it is up-regulated in pregnant term myometrium [13]. Because the pregnant term myometrium specimens used previously were pathological, we first confirmed these findings by using normal samples of both nonpregnant and pregnant term nonlaboring myometrium (Fig. 1). Consistent with our earlier findings, in both nonpregnant and pregnant term nonlaboring myometrium, the vascular endothelium expresses FSHR at similar levels. However, whereas the staining for FSHR in myometrial muscle fibers and stroma appeared faint in nonpregnant myometrium, it was much stronger in pregnant term nonlaboring myometrium. Quantitative morphometric analyses of the staining for FSHR specifically in muscle fibers and stroma (i.e., excluding vessels) in several experiments revealed a ~9-fold increase in FSHR protein expression in pregnant term nonlaboring myometrium relative to that in nonpregnant myometrium (Fig. 2A). To determine whether the up-regulation of FSHR protein in human myometrium observed at term pregnancy was due, at least in part, to an increase in expression of the *FSHR* mRNA, we measured relative *FSHR* gene expression by using real-time qPCR. This analysis revealed a ~5-fold increase in the *FSHR* mRNA in pregnant term human myometrium compared to that in nonpregnant myometrium (Fig. 2B).

To determine the signaling pathways stimulated by FSH in myometrial cells and specifically whether they differed as a function of low versus high levels of FSHR expression, we used hTERT-HM cells into which we introduced increasing densities of recombinant hFSHR. hTERT-HM cells, an immortalized line of human nonpregnant myometrial cells, retain many of the characteristics of primary cultures of human nonpregnant myometrial cells [23]. We determined that these cells do not, however, express endogenous FSHR at low levels, as is the case in nonpregnant myometrium; neither specific ^{125}I-hFSH binding nor FSH-stimulated cAMP accumulation was detected (data not shown). Consequently, these cells are an excellent experimental system for expressing recombinant FSHR in myometrial cells at defined levels and quantitatively evaluating the signaling pathways stimulated by FSH as a function of FSHR density. Specifically, we transduced hTERT-HM cells with increasing amounts of adenovirus encoding recombinant hFSHR and then measured cAMP and 1,4,5-triphosphoinositol (IP3) accumulation in response to a saturating concentration of FSH (Fig. 3). Whereas FSH stimulated cAMP accumulation at low and high FSHR densities, FSH stimulated IP3 accumulation only at high FSHR densities. These data suggest that myocytes behave similarly to other cells that express either the FSHR or other glycoprotein hormone receptors, in that hormone-stimulated IP3 occurs only at relatively high receptor densities [2–5].

The above-described data show that muscle fibers and stroma from nonpregnant human myometrium express relatively low levels of FSHR and that, in human pregnant term nonlaboring myometrium, the levels are higher, suggesting that nonpregnant tissue responds to FSH with increased cAMP, but term nonlaboring myometrium responds to FSH with the synthesis of IP3 as well as cAMP. Because the G_s/adenylyl cyclase signaling system is down-regulated in myometrium from term pregnancy [26–29], we presume that the inositol phosphate cascade is likely the predominant pathway stimulated by FSHR at term. In light of the known roles of intracellular cAMP in quieting and IP3 in stimulating myometrial contractile activity [30–34], we hypothesized that nonpregnant myometrium would respond to FSH with a quieting of contractile activity, whereas pregnant term myometrium would respond to FSH with a stimulation of contractile activity. To test whether the observed changes in

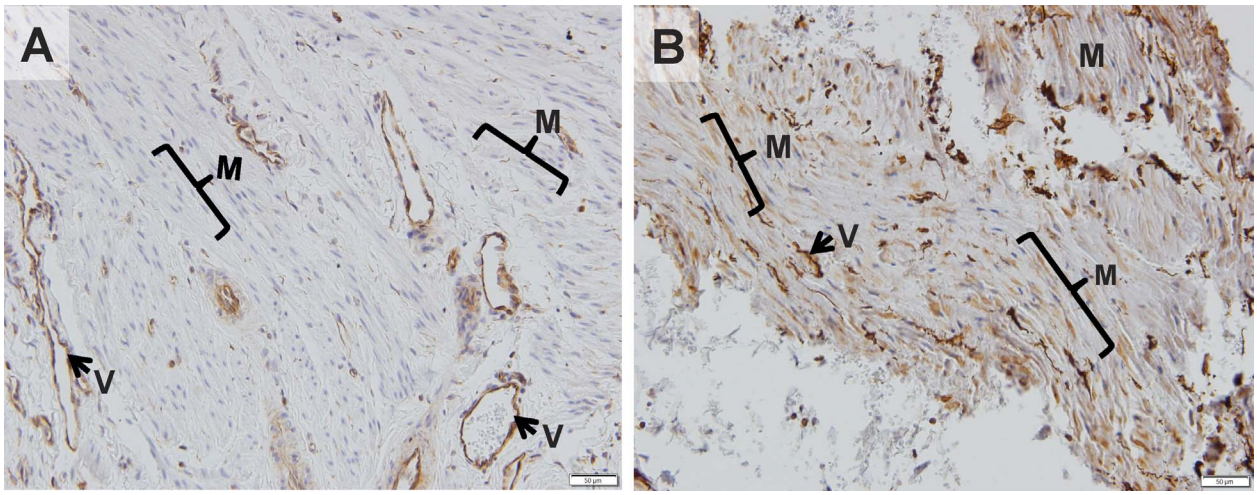

FIG. 1. FSHR protein expression is higher in muscle fibers and stroma of human pregnant term myometrium than in nonpregnant myometrium. Human nonpregnant myometrium (**A**) and nonpathological pregnant term myometrium (**B**) are shown stained with antibody FSHR-323 IgG2a for FSHR (brown) and counterstained with hematoxylin (blue). Myometrial muscle fibers ([M]) and endothelium of blood vessels ([V]) are labeled. **A** and **B**) Five representative samples each. Original magnification ×200; bar = 50 μm. Samples stained with nonimmune IgG2a did not exhibit staining (not shown).

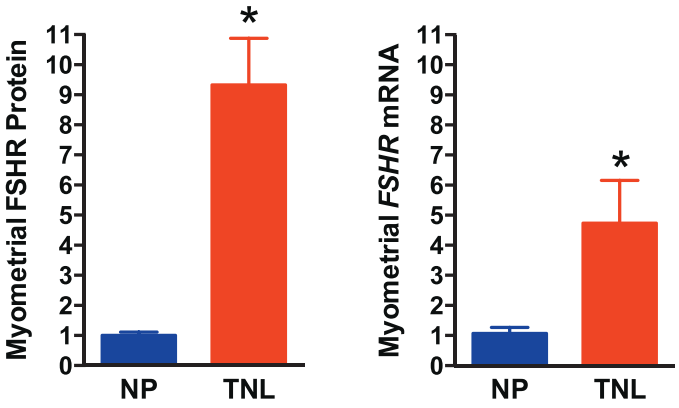

FIG. 2. FSHR protein and *FSHR* mRNA are each up-regulated in human pregnant term nonlaboring myometrium relative to that in nonpregnant myometrium. **A**) FSHR protein immunostaining of muscle fibers and stroma in five samples each of nonpregnant (NP) and pregnant term nonlaboring (TNL) human myometrium was quantified as described in *Materials and Methods*. **B**) *FSHR* mRNA from four samples each of nonpregnant (NP) and pregnant TNL human myometrium was quantified by real-time PCR. Protein and mRNA data are mean ± SEM of the fold increase relative to those in nonpregnant myometrium. *Difference of $P < 0.05$ from nonpregnant myometrium.

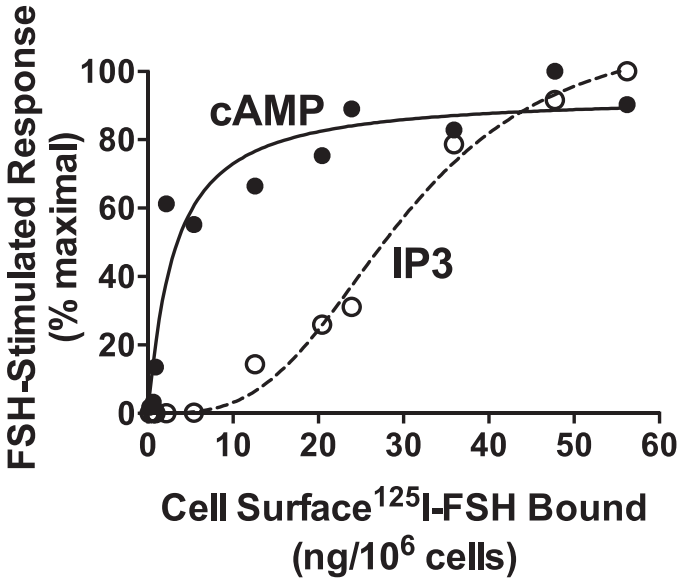

FIG. 3. In myometrial hTERT-HM cells, FSH stimulates cAMP production at all densities of FSHR but stimulates IP3 synthesis only at high FSHR densities. hTERT-HM cells were transduced with adenovirus encoding FSHR to achieve different densities of cell surface recombinant FSHR as determined by cell surface binding of ^{125}I-labeled FSH. cAMP and IP3 accumulation were each measured in response to a saturating concentration of FSH as described in *Materials and Methods*. Control cells transduced with adenovirus encoding β-galactosidase did not reveal detectable FSH-stimulated cAMP or IP3 accumulation. Data are percentages of maximally FSH-stimulated response of each signaling pathway as a function of FSHR density.

FSHR density and FSH-stimulated signaling pathways affected contractility, we recorded isometric tension in isolated strips of tissue from human nonpregnant myometrium and from pregnant term nonlaboring myometrium, and assessed the cumulative FSH dose response in each case (Fig. 4). The data, which reflect a combination of contractile force, duration, and frequency [19], demonstrate that indeed human nonpregnant myometrium responds to FSH with an attenuation (12%–25%) of contractile activity, and pregnant term nonlaboring myometrium responds to FSH with a stimulation (14%–30%) of contractile activity.

To determine the relative expression levels of myometrial FSHR and the responsiveness of myometrial tissue to FSH throughout the course of pregnancy, we examined myometrial tissues from mice obtained at defined stages of gestation. Similar to the patterns observed in human tissues, myometrial fibers and stroma from the nonpregnant, cycling mouse exhibited weak FSHR staining (Fig. 5A), and those from pregnant term nonlaboring myometrium at PD 19.5 displayed relatively strong staining (Fig. 5E). In early (PD 7.5) and mid (PD 11.5) gestation myometrium, FSHR staining appeared to be slightly increased relative to that in nonpregnant myometrium, but the increase was more noticeable at PD 16.5 (Fig. 5). Quantitative morphometric analyses from several experiments and comparison to mouse nonpregnant myometrium revealed

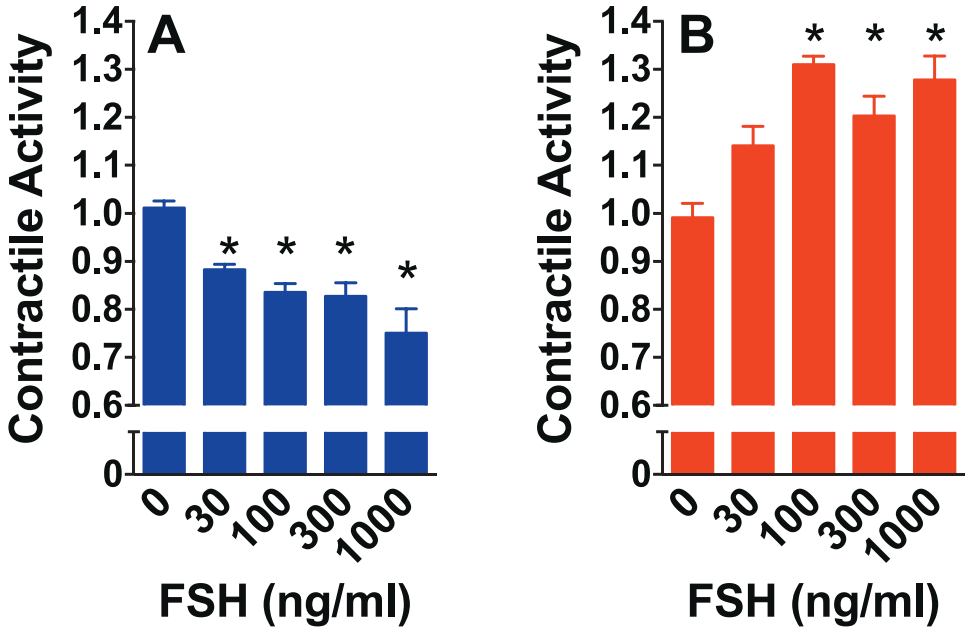

FIG. 4. FSH quiets contractile activity in human nonpregnant myometrium and stimulates contractile activity in pregnant term nonlaboring myometrium. Relative contractile activities of strips of human nonpregnant myometrium (**A**) or human pregnant term nonlaboring myometrium (**B**) in response to increasing concentrations FSH were determined using isometric tension recordings as described in *Materials and Methods*. **A** and **B**) Data are mean ± SEM from four independent experiments. *Difference of $P < 0.05$ from no FSH addition.

that a significant increase in myometrial FSHR protein expression does not occur until PD 16.5, and that a further ~2-fold increase occurs between PD 16.5 and 19.5 (Fig. 6, top). These data suggest that FSHR expression remains relatively low through most of gestation, and increases substantially only closer to term.

Contractile activity of mouse myometrium in response to FSH at various stages of gestation was measured by isometric tension recordings in myometrial strips (Fig. 6). In nonpregnant mouse myometrium, contractile activity quieted (17%–36% reduction) in response to FSH, even at the lowest concentration tested. At PD 7.5 and 11.5, contractile activity also quieted in an FSH-dependent manner but to a lesser extent. At PD 16.5, when FSHR expression was ~3-fold increased, FSH no longer quieted contractile activity at a statistically significant level. It was only at PD 19.5, which in this mouse strain represents term but prior to the onset of labor and at which point FSHR expression was ~6-fold increased, that the mouse myometrium displayed a significant FSH-dependent increase (10%–35%) in contractile activity. These data demonstrate that, in the nonpregnant state and to a lesser extent in early through at least mid-pregnancy, FSH quiets contractile activity. For most of the remainder of pregnancy, FSH has no net effect on contractile activity. At term, when FSHR is significantly up-regulated, FSH stimulates contractile activity.

DISCUSSION

Although much has been learned regarding the mechanisms underlying the regulation of uterine contractility (for reviews see [34–38]), determining how its timing and that of parturition is controlled remains a challenge. Generally, uterine contractility during pregnancy can be divided into three phases. A quiescent phase is maintained throughout most of pregnancy to ensure successful implantation and maintenance of the pregnancy. An active phase is initiated a few days prior to the clinical onset of labor, whereupon the expression of contraction-associated proteins increases, allowing the uterus to subsequently respond to endogenous agonists and achieve strong, coordinated contractions. The final stimulation phase occurs at the onset of clinical labor, and at this time the myometrium is capable of responding to endogenous agonists such as oxytocin and PGF2α with increased contractility. The relative balance between uterine quiescence and stimulation is ultimately regulated by dephosphorylation (inactivation) versus phosphorylation (activation) of myosin regulatory light chains within uterine myocytes. Although particular agonists are known to mediate either uterine quiescence or contraction due to their stimulation of particular signaling pathways, until now, no endogenous agonists capable of mediating both quiescence and contraction have been identified. However, the results reported herein identify a novel hormone signaling pathway, FSH signaling through myometrial FSHR, which indeed mediates both uterine quiescence and uterine stimulation depending upon the relative expression of myometrial FSHR.

Our data show that changes in FSHR density in myometrial muscle fibers and stroma strongly correlate with the quieting versus stimulation of myometrial contractile activity. Thus, in the nonpregnant state and during the initial and middle stages of pregnancy, myometrial FSHR expression is relatively low, and the myometrium responds to FSH with a quieting of contractile activity. At term pregnancy, myometrial FSHR protein and *FSHR* mRNA are significantly up-regulated, and this change correlates with a switch in responsiveness of the myometrial tissue to FSH such that contractile activity is stimulated. Therefore, the functional outcome of FSH addition to myometrium is dependent upon the relative expression levels of myometrial FSHR, with low FSHR levels mediating a quieting action and high FSHR levels a stimulatory action. Our data suggest that the up-regulation of FSHR at term, but prior to the onset of labor, is a component of the activation phase.

How differences in hFSHR levels within the myometrium differentially regulate uterine contractile activity has not yet been fully elucidated, but our data and those of others suggest that it involves the activation of distinct signaling pathways by different densities of receptor. Cells that express endogenous or

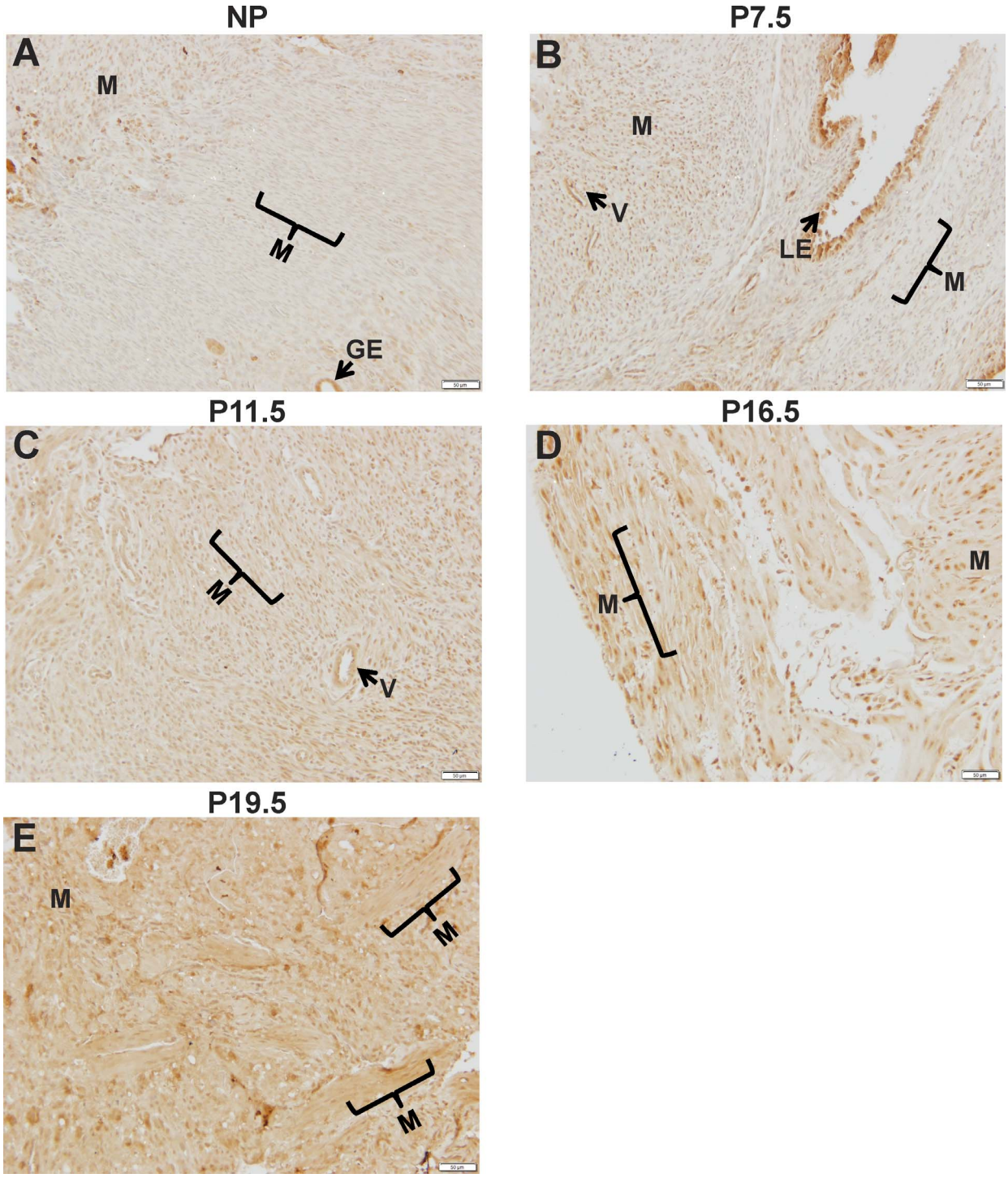

FIG. 5. In the mouse, myometrial FSHR protein expression remains relatively low until late in pregnancy. Representative samples of mouse myometrium are shown from nonpregnancy (NP [**A**]) or at different stages of gestation (PD 7.5–19.5 [**B–E**]) stained with anti-rat FSHR (brown) and counterstained with hematoxylin (blue). Myometrial muscle fibers ([M]), endothelium of blood vessels ([V]), luminal epithelium ([LE]), and glandular epithelium ([GE]) are labeled. Images are representative of 6 NP, 3 PD-7.5, 3 PD-11.5, 4 PD-16.5, and 3 PD-19.5 samples. Original magnification ×200; bar = 50 μm. Samples stained with preimmune IgG did not exhibit staining (not shown).

recombinant FSHR or those that express the structurally related LH and TSH receptors respond to their cognate hormones with increased cAMP production and, when receptor density is high, a parallel increase in the synthesis of IP3 [2–5]. Similarly, we show herein that hTERT-HM cells, immortalized human nonpregnant uterine myocytes that have not retained expression of endogenous FSHR, stimulate cAMP accumulation in response to FSH regardless of the levels of expression of recombinant FSHR, but only stimulate IP3 synthesis when the density of recombinant FSHR is high. We therefore expect that myocytes expressing relatively low levels of FSHR (i.e., from nonpregnant tissue or from early through mid-gestation tissues) respond to FSH with increased cAMP and little or no IP3, but that myocytes expressing high FSHR (i.e., from tissue at term

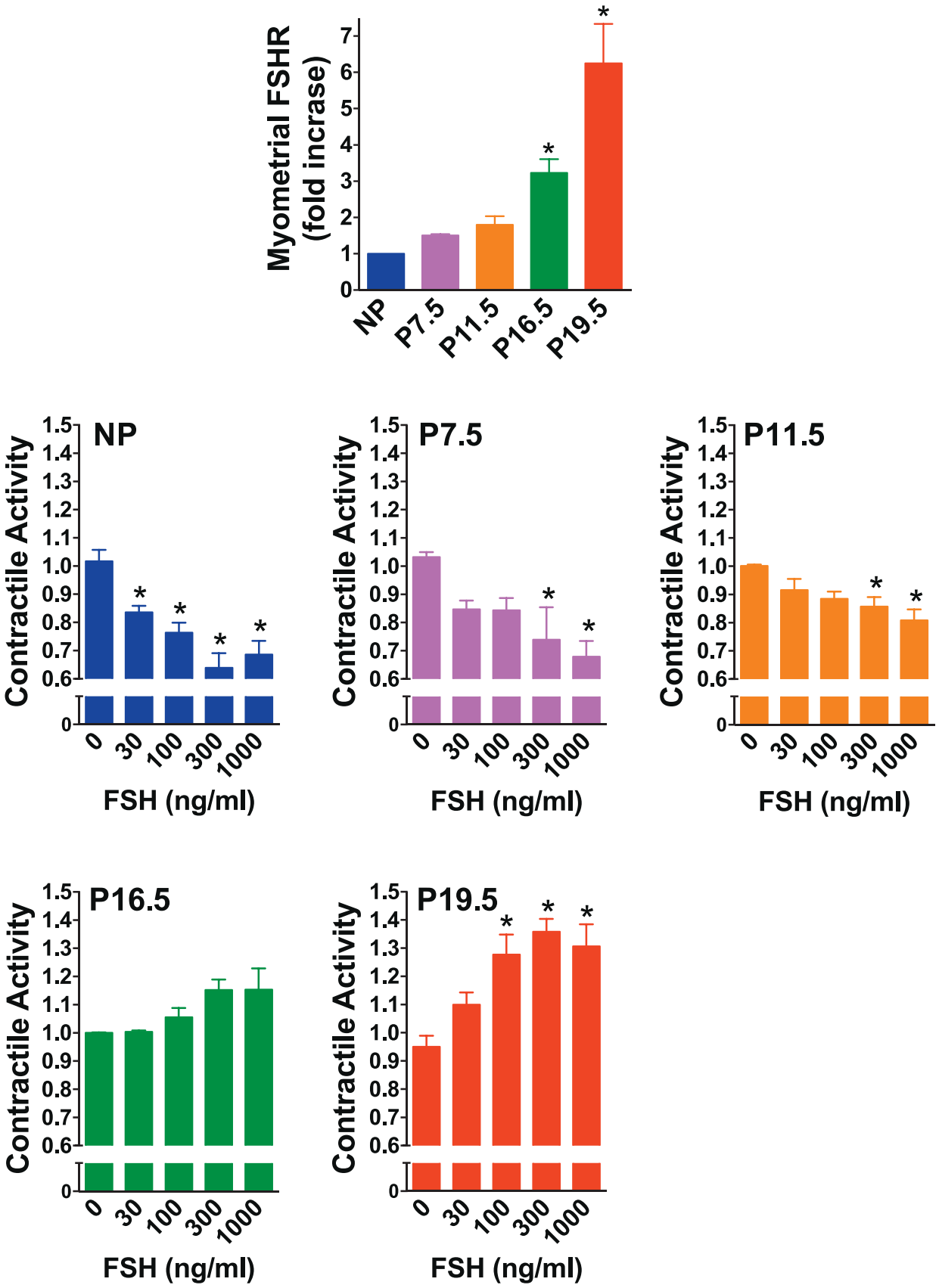

FIG. 6. In the mouse, FSH quiets myometrial contractile activity through mid-gestation, when FSHR remains low, and stimulates contractile activity at term, when FSHR is up-regulated. **Top**) Staining of FSHR in mouse myometrium from 6 NP, 3 PD-7.5, 3 PD-11.5, 4 PD-16.5, and 3 PD-19.5 samples was quantified as described in *Materials and Methods* and shown as mean ± SEM of the fold increase relative to nonpregnant myometrium. **Middle and Bottom Rows**) Relative contractile activities of strips of mouse nonpregnant myometrium (NP) or mouse myometrium from different stages of gestation in response to increasing concentrations FSH were determined using isometric tension recordings as described in *Materials and Methods*. Data are mean ± SEM of 3 NP, 3 PD-7.5, 5 PD-11.5, 3 PD-16.5, and 5 PD-19.5 samples. *Difference of $P < 0.05$ from no FSH addition.

pregnancy) are capable of responding to FSH with increased IP3 synthesis. Theoretically myocytes with high FSHR levels should also respond to FSH with increased cAMP. However, it has been shown that myocytes from term pregnancy are less responsive to agonists that stimulate the Gs/adenylyl cyclase signaling system because components of this pathway are down-regulated [26–29]. Therefore, myocytes from term pregnancy, at which time FSHR levels are high, would be predicted to respond to FSH with increased IP3 in the context of an attenuated cAMP response.

The relative cAMP versus IP3 responses to FSH in myocytes expressing different densities of FSHR is expected to account for the FSHR density-dependent actions of FSH on myometrial contractility because elevated intracellular cAMP promotes quiescence whereas elevated intracellular IP3 stimulates contractile activity [30–34]. Consistent with this, our data show that nonpregnant human and mouse myometrium, as well as pregnant mouse myometrium through mid-gestation, respond to FSH with a quieting of contractile activity. Also, earlier studies showed that addition of FSH to nonpregnant rat myometrium suppressed myoelectrical activity [8, 9]. Myometrium from late pregnancy that is not yet term (i.e., PD 16.5 in the mouse) exhibits an up-regulation of FSHR that is not quite as high as that seen in term nonlaboring tissue (PD 19.5). At PD 16.5, FSH had no effect on contractile activity, consistent with the potential balancing of cAMP-mediated quieting versus IP3-mediated stimulation of activity when FSHR levels are moderately increased. However, in term nonlaboring tissues from PD 19.5 mice, when FSHR levels are further increased, the FSH-stimulated IP3 response is expected to be stronger and the G_s/adenylyl cyclase pathway is expected to be down-regulated, causing the FSH-stimulated IP3 pathway to predominate. Consistent with this, FSH stimulates contractility in pregnant term nonlaboring myometrium from human and mouse. We hypothesize that pregnant term laboring myometrium would exhibit even higher FSHR and a greater stimulation of contractile activity in response to FSH. However, validation of this prediction awaits further experimentation.

The source of FSHR that is relevant to stimulating myometrial FSHR during pregnancy (and potentially in the nonpregnant state as well) is likely to be locally synthesized FSH. Indeed, maternal pituitary FSH secretion is suppressed during pregnancy in women [39]. However, our previous studies suggest that FSH is synthesized in the placenta, decidua, and myometrium, where it can act as a paracrine and/or autocrine signal to engage the myometrial FSHR [13]. It is not possible experimentally to determine the concentrations of FSH synthesized locally in these tissues. However, as with other locally synthesized hormones and growth factors that act in a paracrine or autocrine manner, it is possible that the local concentrations may exceed those in the systemic circulation. Although we cannot extrapolate our findings to the concentrations of FSH synthesized locally in the placenta and uterus, the concentrations of FSH used in the present study are within the range of concentrations used to examine signaling of endogenous FSHR in different tissues and of recombinant FSHR in heterologous cells [4, 11, 14, 24, 40–42] (and Segaloff et al., unpublished data).

Our data suggest that signaling through the myometrial FSHR may contribute to both the establishment and maintenance of pregnancy, as well as the timing of parturition. By maintaining uterine quiescence early in pregnancy, FSH signaling through low densities of myometrial FSHR may promote embryo implantation and the maintenance of pregnancy. At the end of gestation when myometrial FSHR expression is up-regulated, signaling by this pathway may contribute to increased contractile activity and therefore the timing of parturition. Notably, recent studies identified the *FSHR* as a candidate gene for preterm birth in women (i.e., gestation earlier than 37 wk) [16, 17]. Interestingly, single-nucleotide polymorphisms within introns 1 and 2, rather than variants within the coding sequence of the *FSHR* gene, were associated with preterm birth. Specifically, one haplotype consisting of six SNPs was risk-promoting, and another haplotype consisting of seven SNPs was a protective haplotype with respect to preterm birth. At the time of this discovery, it was difficult to reconcile the mechanistic connection between the FSHR, whose expression was thought to be restricted to the ovary, with the timing of birth. The findings herein demonstrating that, firstly, uterine myometrium expresses FSHR and, secondly, the dynamic expression of this receptor during pregnancy governs the FSH-mediated quieting vs. stimulation of contractile activity, suggest a potential mechanistic basis for the identified contributions of *FSHR* haplotypes to the timing of birth. Given that these *FSHR* SNPs are in introns, they are not expected to alter the FSHR protein structure, but rather FSHR expression, potentially altering tissue specificity and/or the timing of changes in expression levels. Unfortunately, given the complexity of the *FSHR* haplotypes associated with preterm birth and their distribution across an extremely large region (>100 kb) of the gene, it is not yet experimentally feasible to elucidate their effects on FSHR expression.

Extragonadal, nonmyometrial sites of FSHR expression should also be considered and assessed for potential contributions to pregnancy. For example, FSHR is also present in the uterine endometrium, with particularly strong expression in endometrial glands [13]. In pregnant women, FSHR is expressed in decidualized cells surrounding the maternal arteries of the maternal decidua, and in endothelial cells of the maternal arteries before and after they are remodeled [13]. It remains to be determined whether these sites of FSHR may therefore contribute to implantation or spiral artery remodeling or both. Notably, FSHR is not limited to maternal tissue. FSHR is expressed on endothelial cells in fetal portions of the placenta, where it mediates angiogenesis [13, 14]. This site of extragonadal FSHR expression is essential for normal pregnancy, with deletion of the *Fshr* gene from fetal portions of the mouse placenta (i.e., fetal vascular endothelium) resulting in growth restriction of the placenta and the fetus, as well an increase in the rate of fetal demise [13].

The roles of ovarian FSHR in achieving fertility and potential contributions of extragonadal FSHR to pregnancy and parturition should be considered in light of studies suggesting that the risks of failed implantation, spontaneous miscarriage, and adverse perinatal outcomes in infertile women undergoing assisted reproductive technologies (ART) are not necessarily due to ART itself but to the maternal factors underlying infertility [43–51]. For example, in women having spontaneous pregnancies, infants conceived after 12 or more months of attempting conception have been found to exhibit an increased risk of adverse perinatal outcomes relative to those conceived within 12 mo [44–47, 49, 50]. Furthermore, a comparison of pregnancy outcomes in subfertile women who conceived naturally (long time to pregnancy) vs. those of ART pregnancies revealed no differences in adverse outcomes [51]. Notably, in a large population-based cohort study that compared the outcomes of two consecutive singleton pregnancies (in one, conception occurred after ART, and in the other, it was spontaneous conception, i.e., the mothers served as the controls rather than a different population cohort), adverse

perinatal outcomes did not differ between infants of the two pregnancies. These data suggest that some adverse outcomes of pregnancies aided by assisted fertilization can be attributed to the factors that lead to infertility rather than to factors related to reproductive technology [48]. We propose that, in addition to ovarian FSHR being critical for female fertility, extraovarian FSHR, including myometrial FSHR, contributes to the establishment of pregnancy and to the maintenance of a successful pregnancy, potentially including the proper timing of parturition. As such, a general decrease in FSHR expression and/or responsiveness that would dampen the ovarian response to FSH might also dampen the extra-ovarian responses to FSH, and thereby contribute to implantation failure, early pregnancy loss, and/or parturition defects.

In summary, the data presented demonstrate that the relative expression of FSHR in myometrium, low in nonpregnant tissue and through most of pregnancy but up-regulated at term pregnancy, governs a switch between FSH mediating quiescence versus stimulation of uterine contractile activity. It will be important in future studies to elucidate the contributions of the myometrial FSHR to pregnancy and parturition.

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