The expression of transforming growth factor β in pregnant rat myometrium is hormone and stretch dependent

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Abstract

From a quiescent state in early pregnancy to a highly contractile state in labor, the myometrium displays tremendous growth and remodeling. We hypothesize that the transforming growth factor β (TGF β) system is involved in the differentiation of pregnant myometrium throughout gestation and labor. Furthermore, we propose that during pregnancy the mechanical and hormonal stimuli play a role in regulating myometrial TGF β s. The expression of TGF β 1-3 mRNAs and proteins was examined by real-time PCR, Western immunoblot, and localized with immunohistochemistry in the rat uterus throughout pregnancy and labor. *Tgf β 1-3* genes were expressed differentially in pregnant myometrium. *Tgf β 2* gene was not affected by pregnancy, whereas the *Tgf β 1* gene showed a threefold increase during the second half of gestation. In contrast, we observed a dramatic bimodal change in *Tgf β 3* gene expression throughout pregnancy. *Tgf β 3* mRNA levels first transiently increased at mid-gestation (11-fold on day 14) and later at term (45-fold at labor, day 23). Protein expression levels paralleled the changes in mRNA. Treatment of pregnant rats with the progesterone (P4) receptor antagonist RU486 induced premature labor on day 19 and increased *Tgf β 3* mRNA, whereas artificial maintenance of elevated P4 levels at late gestation (days 20–23) caused a significant decrease in the expression of *Tgf β 3* gene. In addition, *Tgf β 3* was up-regulated specifically in the gravid horn of unilaterally pregnant rats subjected to a passive biological stretch imposed by the growing fetuses, but not in the empty horn. Collectively, these data indicate that the TGF β family contributes in the regulation of myometrial activation at term integrating mechanical and endocrine signals for successful labor contraction.

Reproduction (2007) **134** 503–511

Introduction

The transforming growth factor β (TGF β) superfamily is composed of five related peptides, three of which are found in mammalian tissues, namely TGF β 1, TGF β 2, and TGF β 3 (Massague *et al.* 1994). They exert their actions via three receptors: type I, type II, and type III (Massague *et al.* 1994). The intracellular signaling cascade linking TGF β family ligands and TGF β receptors to a cellular response is extremely complex and includes a large group of SMAD proteins carrying signals from the cell surface directly to the nucleus (Attisano & Wrana 2002). TGF β isoforms have been studied extensively in the reproductive system. They are multifunctional growth factors expressed by fetoplacental, cervical, and uterine tissues, where they regulate (1) cellular proliferation, (2) tissue remodeling, and (3) inflammatory response (Barnard *et al.* 1990, Lawrence 1996). The

mRNA of all three TGF β isoforms is present in human term placenta in the syncytiotrophoblastic layer, chorionic plate, and in cells of the extravillous trophoblast (Schilling & Yeh 2000). TGF β s were amongst the first identified regulators of invasive trophoblast differentiation since they inhibited the proliferation of first trimester cytotrophoblasts (Graham *et al.* 1992). Furthermore, TGF β s exert anti-invasive effects on trophoblasts by increasing tissue inhibitor of matrix metalloproteinase (TIMP) expression and blocking matrix metalloproteinase (MMP) activity (Graham & Lala 1991, Ma & Chegini 1999). In placental trophoblasts and myometrial smooth muscle cells (SMCs), TGF β has been shown to up-regulate the expression of fibronectin, a marker of preterm labor (Goldenberg *et al.* 1997, Chegini *et al.* 1999). TGF β s mRNA and proteins are expressed in the human myometrium during menstrual cycle (Chegini *et al.* 1994) and pregnancy (Chegini *et al.* 1999, Kuscu

et al. 2001), and have been suggested to play a central role regulating excitability and contractility in the human myometrium at term (Hatthachote & Gillespie 1999).

We proposed earlier that the ability of the myometrium to contract at term can be defined biochemically as an increase in expression of a cassette of genes encoding 'contraction-associated proteins' (CAPs), which control the contractile activity and responsiveness of the myometrium during labor (Challis 1994). We have shown that the timely expression of putative CAPs (Cx43, oxytocin receptor, prostaglandin receptor) is regulated by the integration of fetal endocrine and growth signals underlying myometrial activation (Ou *et al.* 1997, 1998). In addition, we have reported that the activation of CAP genes at term correlated with the increase in extracellular matrix (ECM) protein expression (Shynlova *et al.* 2004). It has been reported by others that the CAP and ECM expression could be regulated by cytokines, such as TGF β 1, which may play a role in preparing the myometrium for parturition (Hatthachote *et al.* 1998). There is an evidence for the involvement of other cytokines in the modulation of myometrial function. Specifically, pro-inflammatory cytokines increase in human myometrium at term, suggesting that a cascade of cytokine interactions might prepare the myometrium for spontaneous preterm or term labor (Romero *et al.* 1991, 2006).

While several studies have reported the immunolocalization of TGF β s and their receptors in pregnant term and preterm human myometrium (Hatthachote *et al.* 1998, Chegini *et al.* 1999, Kuscu *et al.* 2001), very limited information is available on the expression of TGF β ligands and their function throughout pregnancy. We hypothesized that cytokines, specifically TGF β s, may assist in the myometrial activation at labor by mediating changes in pregnant uterine smooth muscle during consecutive phases of myometrial differentiation (Shynlova *et al.* 2006). We further proposed that mechanical and hormonal stimuli might regulate the expression of TGF β s in pregnant myometrium. In this study, we defined the expression profile of TGF β 1-3 in the rat myometrium during normal pregnancy, spontaneous term labor, and *post partum* using real-time PCR, immunoblotting, and immunohistology techniques. We also investigated the effect of progesterone (P4) on the expression of *Tgf β 3* gene using a P4-delayed labor and RU486-induced preterm labor models. In addition, the effect of gravidity on the expression and localization of TGF β 3 was investigated using a unilateral tubal-ligation rat model.

Materials and Methods

Animals

Wistar rats (Charles River Co., St Constance, Canada) were housed individually under standard environmental

conditions (12 h light:12 h darkness cycle) and fed Purina Rat Chow (Ralston Purina, St Louis, MO, USA) and water *ad libitum*. Female virgin rats were mated with male rats. Day 1 of gestation was designated as the day a vaginal plug was observed. The average time of delivery under these conditions was during the morning of day 23. Our criteria for labor were based on delivery of at least one pup. The Samuel Lunenfeld Research Institute Animal Care Committee approved all animal experiments.

Experimental design

Normal pregnancy and term labor

Animals were killed by carbon dioxide inhalation and myometrial samples were collected on gestational days 0 (non-pregnant, NP), 6, 8, 10, 12, 14, 15, 17, 19, 21, 22, 23 (labor), or 1 and 4 day *post partum* (PP). Tissue was collected at 1200 h on all days with the exceptions of the labor sample (day 23L) that was collected once the animals had delivered at least one pup ($n=4$).

P4-delayed labor

To determine whether high plasma levels of P4 might modulate the expression of TGF β family genes, pregnant rats were randomized to receive daily s.c. injections of either P4 (medroxyprogesterone acetate, 16 mg/kg in 0.4 ml sterile saline, Pharmacia Canada Inc.) or vehicle starting on day 20 of gestation. Animals ($n=4$ at each time point for each treatment) were killed on days 21, 22, or 23L in the vehicle-treated group or days 21, 22, 23, or 24 in the P4-treated group.

RU486-induced preterm labor

On day 19 of gestation two groups of rats were treated with either RU486 (10 mg/kg, s.c., at 1000 h, in 0.5 ml corn oil containing 10% EtOH, Mifepristone;17 β -hydroxy-11 β -[4-dimethylaminophenyl]-17-[1-propynyl]-estra-4,10-dien-3-one; Biomol International, Plymouth Meeting, PA, USA) or vehicle. Myometrial samples were collected from both groups of animals on day 20 when the RU486-treated animals had delivered at least one pup ($n=4$).

Unilaterally pregnant rats

Under general anesthesia virgin female rats underwent tubal ligation through a flank incision to ensure that they subsequently became pregnant in only one horn (Ou *et al.* 1998). Animals were allowed to recover from surgery for at least 7 days before mating. Pregnant myometrial samples from empty and gravid horns were collected on days 6, 12, 14, 15, 17, 19, 21, 22, 23, or 1PP ($n=4$).

Tissue collection

Animals were killed by carbon dioxide inhalation. For RNA and protein extraction the uterine horns were placed into ice-cold PBS, bisected longitudinally, and dissected away from both pups and placentas. The endometrium was carefully removed from the myometrial tissue by mechanical scraping on ice, which we have previously shown removes the entire luminal epithelium and the majority of the uterine stroma (Piersanti & Lye 1995). The myometrial tissue and decidua were flash-frozen in liquid nitrogen and stored at -70°C . For immunohistochemical studies the intact uterine horns were placed in ice-cold PBS and fixed immediately in 4% paraformaldehyde solution at 4°C for 48 hours. For each day of gestation, tissue was collected from four different animals.

Real-time-PCR analysis

Total RNA was extracted from the frozen tissues using TRIzol (Gibco BRL) according to manufacturer's instructions. RNA samples were column purified using RNeasy Mini Kit (Qiagen), and treated with $2.5\ \mu\text{l}$ DNase I ($2.73\text{Kunitz unit}/\mu\text{l}$, Qiagen) to remove genomic DNA contamination. RT and real-time PCR (RT-PCR) was performed to detect the mRNA expression of TGF β s and TGF β -related genes in rat myometrium. Total RNA ($2\ \mu\text{g}$) was primed with random hexamers to synthesize single-strand cDNAs in a total reaction volume of $100\ \mu\text{l}$ using the TaqMan RT Kit (Applied Biosystems, Foster City, CA, USA) as described earlier (Shynlova *et al.* 2005). cDNA ($20\ \text{ng}$) from the previous step was subjected to real-time PCR using specific sets of primers (see legend to Fig. 1) in a total reaction volume of $25\ \mu\text{l}$ (Applied Biosystems). RT-PCR was performed in an optical 96-well plate with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), using the SYBR Green detection chemistry. The run protocol was as follows: initial denaturation stage at 95°C for 10 min, 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. After PCR, a dissociation curve was constructed by increasing temperature from 65 to 95°C for detection of PCR product specificity. In addition, a no-template control (H_2O control) was analyzed for possible contamination in the master-mix. A cycle threshold (Ct) value was recorded for each sample. PCRs were set up in triplicates and the mean of the three Ct s was calculated. A comparative Ct method ($\Delta\Delta\text{Ct}$ method) was applied to the raw Ct values to find a relative gene expression across normal gestation. To obtain experimental results, the expression of individual gene at every gestational day (1) was normalized to ribosomal 18S mRNA and (2) a fold change was calculated relative to the expression of the same gene in corresponding NP sample using an arithmetic formula (see ABI User Bulletin #2). For unilaterally pregnant animals, the gene expression was

Figure 1 The effects of gestational age on the expression of *TGF β 1-3* genes in the pregnant rat myometrium. Total RNAs were extracted from frozen myometrial tissues, single-strand cDNAs were synthesized as described under 'Materials and Methods' and mRNA levels were analyzed on the indicated days of gestation by real-time PCR. Specific forward and reverse primers were designed using Primer Express software, version 2.0.0 (Applied Biosystems) as follows: TGF β 1 mRNA, 5'-AGAAGTCACCCGCGTGCTAAT-3' (sense primer) and 5'-CACTGCTTCCGAATGTCTGA-3' (antisense primer; GenBank accession #: NM_021578); TGF β 2 mRNA, 5'-TTCA-GAATCGTCCGCTTCGAT-3' (sense primer) and 5'-TTGTTACAGC-CACTCTGGCCTT-3' (antisense primer; GenBank accession #: NM_031131); TGF β 3 mRNA, 5'-ATACAACACCCTGAACCCGGA-3' (sense primer) and 5'-CGACTTCACCACCATGTTGGA-3' (antisense primer; GenBank accession #: NM_013174); 18S, 5'-GCGAAAG-CATTGCCAAGAA-3' (sense primer) and 5'-GGCATCGTT-TATGGTCGGAAC-3' (antisense primer; GenBank accession #: X01117). TGF β 1-3 mRNA levels were normalized to 18S mRNAs and expressed in fold changes relative to a corresponding non-pregnant sample. The bars represent mean $\pm$ s.d. ($n=4$ at each time point). Data labeled with different letters are significantly different from each other ($P<0.05$).

shown as fold change relative to day 6 gravid horn mRNA level, whereas that of P4- and RU486-treated animals was shown as a fold change relative to the vehicle sample. Validation experiments were performed to ensure that the PCR efficiencies between the target genes and 18S were approximately equal.

Western immunoblot analysis

Total protein was extracted from the frozen tissues using RIPA lysis buffer as described earlier (Shynlova

et al. 2005). Protein samples (40–50 µg) were resolved by electrophoresis on a 12–15% SDS-polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) in 25 mM Tris-HCl (pH 8.3), 250 mM glycine, 0.1% (wt/vol) SDS, for 18 h at 30 V and 4 °C. The protein expression levels of TGFβ1 and TGFβ3 were measured by western analysis using primary antibody (TGFβ1: mouse monoclonal, 1:2000; TGFβ3: rabbit polyclonal, 1:2000; Abcam International, Cambridge, CA, USA). PVDF membranes were stripped and reprobed with anti-tubulin (1:3000, clone DM 1A; Sigma-Aldrich) and anti-calponin (1:3000, clone hCP; Sigma) mouse primary antibodies to control the loading variations. Probed membranes were exposed to X-ray film (Kodak XAR, Eastman Kodak) and analyzed by densitometry (ImageJ software program; National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>). Calponin is constitutively expressed in non-pregnant and pregnant rat tissue under the same protein extraction conditions (Williams *et al.* 2005). Tubulin is present in almost all eukaryotic cells and is commonly used as a housekeeping protein (Vemuganti *et al.* 2004).

Immunohistochemistry

The fixed myometrial tissues were sectioned into 10 µm thickness and collected on Superfrost Plus slides (Fisher Scientific Ltd., Nepean, ON, Canada). The frozen sections were immersed in 0.3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ, USA). Antigen retrieval was performed by cooking the tissues at 90 °C after 5 min, followed by blocking with 5% normal goat serum and incubation with primary antibodies overnight at 4 °C. Primary antibody was rabbit anti-TGFβ3 (1:100, Abcam International). For the negative controls, ChromPure non-specific rabbit IgGs (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) were used at the same concentration and sections were also incubated with secondary antibodies in the absence of primary antibodies. Secondary antibodies used for detection were biotinylated goat anti-rabbit (1:200; Vector, Burlingame, CA, USA). Final visualization was achieved using Vectastain Elite ABC Kit (Vector). Counterstaining with Harris Hematoxylin (Sigma diagnostics) was carried out before slides were mounted with Cytoseal XYL (Ricard-Allan Scientific, Kalamazoo, MI, USA). Myometrial cells from each of the three tissue sets were observed on a Leica DMRXE microscope (Leica Microsystems, Richmond Hill, ON, Canada). A minimum of five fields were examined for each gestational day and uterine horn for each set of tissue, and representative tissue sections were photographed with Sony DXC-970 MD (Sony Ltd., Toronto, ON, Canada) 3CCD color video camera.

Statistical analysis

Gestational profiles were subjected to a one-way ANOVA followed by pairwise multiple comparison procedures (Student–Newman–Keuls method) to determine differences between groups. P4 (days 21, 22, and 23) and tubal-ligation data were analyzed by two-way ANOVA followed by pairwise multiple comparison procedures as described above. The day 24 P4 treated group was compared with the day 23 vehicle group using a *t*-test. RU486 results were compared with vehicle using a one-way ANOVA, where required the data were transformed by the appropriate method to obtain a normal distribution. Statistical analysis was carried out using SigmaStat version 2.01 (Jandel Corp., San Rafael, CA, USA) with the level of significance for comparison set at $P < 0.05$.

Results

Figure 1 illustrates the expression of *Tgfb1-3* gene throughout pregnancy and *post partum*. Relative abundance of the *Tgfb1* mRNA was significantly increased starting at mid-gestation (3.01 ± 0.21 -fold increase on day 12 vs NP, $P < 0.05$) and was maintained elevated until labor and *post partum* (5.22 ± 0.80 -fold increase on day 1PP versus NP, $P < 0.05$). No significant changes were found for *Tgfb2* myometrial transcript levels across gestation ($P > 0.05$). In contrast, a dramatic bimodal change in *Tgfb3* mRNA abundance was observed throughout pregnancy. We detected a transient activation of myometrial *Tgfb3* gene expression on gestational days 14–15 (11.30 ± 1.30 - and 12.36 ± 1.91 -fold increase versus NP, $P < 0.05$), a subsequent decrease at late gestation (6.92 ± 0.96 -fold increase on day 19 versus NP, $P < 0.05$), and a second dramatic increase at labor (45.31 ± 8.73 -fold increase on day 23 versus NP, $P < 0.05$). During *post partum* involution *Tgfb3* transcript levels quickly decreased (8.37 ± 1.84 -fold increase on 1PP versus NP, $P < 0.05$). We applied immunoblotting technique to analyze if protein expression of TGFβ1 and TGFβ3 reflects their gene expression. As expected from the mRNA data, the expression of TGFβ1 and TGFβ3 proteins started increasing from mid-gestation ($P < 0.05$ for day 17 versus NP), was further up-regulated at late gestation and during labor (three- to fourfold increase on days 21–23 versus NP for TGFβ1 and TGFβ3 proteins, $P < 0.05$; Fig. 2) and decreased thereafter. From these observations, it appears that the expression of TGFβ1 and TGFβ3 in rat myometrium was clearly dependent on gestational age.

We also studied the temporal and spatial distribution of TGFβ3 protein in the myometrium across gestation (Fig. 3). According to our observation, the TGFβ3 staining in the rat myometrium was significantly altered during pregnancy. Immunostaining of TGFβ3 in uterine smooth muscle of non-pregnant and early pregnant (day 6) animals was extremely weak. However, starting from day 15 the immunoreactivity of the rat myometrium

Figure 2 The effect of gestational age on the expression of TGF β 1 (A) and TGF β 3 (B) proteins in the pregnant rat myometrium. Representative western blots and densitometric analysis of TGF β 1 and TGF β 3 protein levels throughout normal gestation and *post partum*. TGF β 1 and TGF β 3 protein expression levels were normalized to α -tubulin (A) or calponin (B). Bar graphs showing the mean $\pm$ S.E.M. ROD ($n=3-4$ at each time point). Data labeled with different letters are significantly different from each other ($P<0.05$).

increased dramatically (Fig. 3). Consistent with our gene and protein expression results the most intense staining was found in laboring samples. In addition, we detected more intense TGF β 3 immunostaining at late gestation in the circular myometrial layer when compared with the longitudinal myometrial layer (Fig. 3). TGF β 3 protein was always detected in the cytoplasm of myometrial SMCs and this spatial distribution was similar in both uterine muscle layers.

P4 is the major hormone of pregnancy in the rat. In our study, artificial maintenance of elevated P4 levels at late gestation (from day 20 to day 23) by daily injections of hormone caused a failure to initiate labor. We showed before that this treatment prevented the increase in the expression of CAPs, AP-1, and ECM genes in the rat myometrium (Piersanti & Lye 1995, Shynlova *et al.* 2004). We have now shown that the administration of P4 also prevented the increase of *Tgf β 3* mRNA levels at term when compared with vehicle-treated animals (Fig. 4A). *Tgf β 3* gene remained low in myometrium of P4-treated rats on gestational day 22, day 23 (labor), and day 24 (1 day after normal delivery) when compared with controls ($P<0.05$). On the contrary, treatment of pregnant rats with the P4 receptor antagonist RU486 on day 19 caused the onset of preterm labor within 24 h and

Figure 3 TGF β 3 immunolocalization in pregnant rat myometria during gestation. Immunohistochemical examination was performed on sections of uterus from non-pregnant (NP) (A), 6 days (B), 15 days (C), 17 days pregnant (D), laboring (day 23) (E), and 1 day *post partum* (F) animals. Expression of TGF β 3 in circular myometrial layers increased significantly during late gestation (C–E, arrows). Relatively weak immunostaining was present in longitudinal myometrial layers (C–E). The lack of immunostaining after incubation of myometrial tissue with non-specific rabbit IgGs on gestational day 14 (G) or with anti-rabbit secondary antibodies in the absence of primary antibody on labor (day 23) is shown in H. Magnification, 200 $\times$; scale bar A–E=50 μ m.

a 3.6-fold increase in *Tgf β 3* gene expression ($P<0.05$, Fig. 4B). These results demonstrated that decreased P4 signaling during late pregnancy caused by RU486 led to *Tgf β 3* gene induction, whereas maintenance of high plasma P4 levels prevented this increase.

Since TGF β 1 and TGF β 3 protein expression increased specifically at the second half of gestation when mechanical stretch of uterine walls imposed by growing fetuses was apparent, we decided to study *Tgf β 3* gene and protein expression using the unilaterally pregnant rat model (Fig. 5). This model enables us to distinguish the effects of endocrine and mechanical stimuli, given that both gravid and empty horns were subjected to the same hormonal environment. *Tgf β 1* mRNA levels were not statistically different between empty and gravid horns (data not shown). We found that *Tgf β 3* gene expression in

Figure 4 The effects of progesterone (A) and RU486 (B) on myometrial TGF β 3 expression. Real-time PCR analysis of TGF β 3 mRNA levels in pregnant rat myometrium during progesterone-delayed (A) and RU486-induced preterm (B) labor normalized versus 18S mRNA and expressed in fold change relative to a vehicle day 21 (A) or day 20 (B) samples. Shown are vehicle (black bars) and P4- or RU486-treated (white bars) samples. Values represent mean $\pm$ s.d. ($n=4$ at each time point). A significant difference is indicated by * $P<0.05$ or *** $P<0.001$.

the empty horn was very low throughout gestation. In contrast, Tgf β 3 transcript levels were dramatically increased in the gravid uterine horns, showing a profile similar to that of normal pregnant animals. Relative quantification indicated a transient induction of Tgf β 3

Figure 5 Expression of TGF β 3 gene in the myometrium of unilaterally pregnant rats during gestation. mRNA levels were analyzed on the indicated days of gestation by real-time PCR using specific primers (see Fig. 1). TGF β 3 gene expression levels in empty (white bars) and gravid (black bars) were normalized to 18S mRNAs and expressed in fold changes relative to a day 6 gravid sample. The bars represent mean $\pm$ s.d. ($n=4$ at each time point). A significant difference between gravid and empty horn of the same gestational day is indicated by * $P<0.05$ or *** $P<0.001$.

gene in the gravid horn at mid-pregnancy (12.4-fold increase on day 14 when compared with day 6) and at term (22.4-fold increase on day 23L when compared with day 6; Fig. 5). This increase in Tgf β 3 mRNA of the gravid horn was statistically different from the corresponding empty horn (two-way ANOVA, $P<0.05$). We also found a dramatic increase in TGF β 3 protein immunoreactivity in the gravid horns of unilaterally pregnant rats on late gestation (Fig. 6A, C and E) when compared with their pairing empty horns (Fig. 6B, D and F).

Discussion

We have previously reported that the myometrium undergoes gradual changes in phenotype throughout gestation. These stages are characterized by an early proliferative phase, an intermediate phase of cellular hypertrophy and matrix elaboration, and the final contractile phase (Shynlova *et al.* 2006). We proposed that phenotypic modulation of uterine myocytes is the result of integration of endocrine signals and mechanical stimulation of the uterus by the growing fetus. We have

Figure 6 TGF β 3 localizes within the circular layer SMCs of the gravid myometrium from unilaterally pregnant rats. Uterine tissue was collected from empty and gravid horns of unilaterally pregnant rats on different gestational days, including day 17 (A and B), day 19 (C and D), and day 23 (E and F). Tissues were labeled with anti-TGF β 3 antibody and light microscopy images of cross-sections were collected. TGF β 3 localized mainly within the cytoplasm of circular muscle SMCs of the gravid uterine horn (arrows) and was much less abundant in the empty horn. No gestational changes in localization were observed. Magnification, 200 $\times$; scale bar A–H = 50 μ m.

also demonstrated that these signals are important in regulating the onset of labor (Lye *et al.* 2001). In this report we showed that cytokines, specifically the TGF β s, were (1) differentially expressed in the pregnant rat myometrium during specific phases of gestation and labor and (2) regulated by gravidity and ovarian hormones.

Numerous studies have shown that TGF β s control a remarkable diversity of cellular functions, many of which are directly related to cell growth. We found that TGF β mRNA and protein levels were low during the early phase of gestation when rat myometrial SMCs undergo hyperplasia, but increased during the synthetic phase when the proliferative activity of myometrial SMCs was substantially reduced. Consistent with our results, total TGF β 1 levels (Hatthachote *et al.* 1998) and TGF β 3 immunoreactive proteins (Kuscu *et al.* 2001) have been reported to be elevated in the pregnant human myometrium when compared with non-pregnant tissues. TGF β 1 is well-known for its bimodal and dose-dependent effects on the growth of SMCs. Arici and colleagues demonstrated that low concentrations of TGF β 1 stimulate cell proliferation in leiomyoma (Arici & Sozen 2003) and vascular cells (Battegay *et al.* 1990), while these stimulatory effects disappear at high TGF β 1 concentrations. It has been also shown that increased TGF β 1 gene expression induced by angiotensin II led to *de novo* protein synthesis in cultured vascular SMC (Koibuchi *et al.* 1993). New protein synthesis is a property of hypertrophic cells. We have previously documented myometrial hypertrophic growth during the second part of gestation (Shynlova *et al.* 2006). Our finding that TGF β 1 and TGF β 3 expression was induced during that specific time period raises the possibility that TGF β s may support cellular hypertrophy in late pregnant uterus.

Interestingly, we observed two periods of transient myometrial induction of TGF β 3 gene during rat gestation. The first increase in TGF β 3 gene expression occurred at mid-gestation (around day 14). As was shown before (Reynolds 1949) at that time fetal growth mediates an acute stretch of the uterine walls creating a transient hypoxemia of myometrial SMCs. We have previously shown that at this time there is a transient activation of the stress-induced (intrinsic) apoptotic pathway in the myometrium, a characteristic signal that we believe stops myometrial proliferative activity and promotes smooth muscle differentiation to a synthetic and later to a contractile phenotype (Shynlova *et al.* 2006). Interestingly, we found that the expression of hypoxia-induced transcription factor (HIF-1 α) gene was up-regulated in the rat myometrium around day 14 of gestation and later at term, supporting the occurrence of two periods of hypoxia during gestation (Shynlova, Lye, unpublished). It has been shown in human placental explants that TGF β 3 expression correlates closely with the expression of HIF-1 α (Caniggia *et al.* 1999, 2000). In addition, HIF-1 α has been shown to directly regulate

TGF β 3 gene expression in mouse trophoblast cells *in vitro* (Schaffer *et al.* 2003). Taken together, we suggest that the activation of TGF β 3 gene expression at mid- and late gestation is likely mediated by HIF-1 α . It is also plausible that a similar molecular mechanism (mechanical stretch imposed by growing fetuses on myometrial SMCs) is responsible for the second period of TGF β 3 gene induction in the rat myometrium before and during parturition. A number of *in vitro* studies have shown up-regulation of TGF β by mechanical stretch in a variety of cell types such as vascular SMCs (Li *et al.* 1998), intestinal SMCs (Gutierrez & Perr 1999), pulmonary arterial SMCs (Mata-Greenwood *et al.* 2005), and cardiomyocytes (van Wamel *et al.* 2001). These data correspond well with our *in vivo* studies using the unilaterally pregnant rats where we demonstrated that TGF β 3 gene and protein expression was induced specifically in the gravid horns but not in the empty horns of late pregnant and laboring animals. Our results are further supported by a study using a similar experimental approach in which mRNA and protein expression of components of the TGF β -signaling axis are up-regulated by a stretch in the unilateral ureteric obstruction model in fetal sheep (Yang *et al.* 2001).

Furthermore, we found a difference in spatial distribution of TGF β 3 protein in a gravid horn; the circular myometrial layer showed more intense immunoreactivity than the longitudinal. Our previous studies have reported that the circular layer of the myometrium is more responsive to mechanical stretch than longitudinal based on the fact that the expression of the connexin43 (Doualla-Bell *et al.* 1995; a putative CAP gene) and γ -actin (Shynlova *et al.* 2005; a component of SM contractile apparatus) were increased in uterine circular muscle at late gestation. Others have also reported different responses to stretch, nor-adrenaline, and estrogen stimulation in circular versus longitudinal muscle (Matsumoto 1980, Doualla-Bell *et al.* 1995). This suggests that the two myometrial layers play different roles in labor contractions. The circular muscle primarily contracts rhythmically, while the longitudinal layer shortens the uterus upon expulsion of each fetus. We believe that stretch-induced activation of TGF β signaling in the circular myometrial layer is one of the factors preparing the myometrium for labor. In other independent studies using cultured vascular SMCs, mechanical stretch not only stimulated TGF β mRNA expression in a time- and elongation-dependent manner, but also up-regulated expression of type I and type IV collagen, and fibronectin genes, which was largely inhibited by addition of neutralizing antibody against TGF β (Li *et al.* 1998, Joki *et al.* 2000). TGF β 3 treatment was also found to stimulate fibronectin expression in human cultured leiomyoma cells (Arici & Sozen 2000). We have reported earlier that expression of fibronectin, as well as major components of basement membrane, namely type IV collagen and laminin, are dramatically

up-regulated prior to and during labor in rats (Shynlova *et al.* 2004). Thus, the parallel increase of TGF β 3 and ECM components at late gestation suggests that TGF β 3 may mediate ECM induction, providing the mechanism to anchor hypertrophied uterine myocytes in order to produce coordinated, forceful labor contractions.

It has been shown *in vitro* that the TGF β -signaling system can be regulated by ovarian hormones in human myometrial cells (Chegini *et al.* 1996, Awad *et al.* 1997). In the non-pregnant ovariectomized mouse uterus, the expression of TGF β can be transiently increased by the *in vivo* injection of estrogen (Das *et al.* 1992). In the present study we found a negative correlation between plasma P4 levels and the expression of TGF β 3 at late pregnancy, suggesting additional hormonal regulation of this gene. We speculate that the decrease in P4 levels, followed by an increase in estrogen plasma levels and mechanical stimulation of myometrium are all responsible for the activation of TGF β 3 at term.

To date, the physiological role of TGF β s in preparing the myometrium for labor is not fully understood. Among the three TGF β isoforms we studied, TGF β 1 and TGF β 3 genes show significant changes across gestation. We suggest that at mid-gestation TGF β 3 may influence the transition from proliferative and synthetic myometrial phenotypes. We also suggest that at late gestation both TGF β 1 and TGF β 3 proteins (1) support myometrial cellular hypertrophy and (2) play a role in the preparation of myometrium for labor contractions. Our results support and expand the understanding of myometrial phenotypic modulation during pregnancy and demonstrate a significant role for members of the TGF β family in this process.

Acknowledgements

This study was supported by a grant from the CIHR. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 3 January 2007

First decision 26 January 2007

Revised manuscript received 22 May 2007

Accepted 1 June 2007