# The Change in Tenascin Expression in Mouse Uterus During Early Pregnancy

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***Purpose:*** *Our aim was to examine the changes in spatiotemporal tenascin (TN) expression in mouse uterus during early pregnancy, when the uterine tissue undergoes a tremendous restructuring.*
***Methods:*** *Using immunohistochemistry and in situ hybridization, the changes in distribution of TN protein in mouse uterine tissues in pregnancy Day 0 through Day 5 were analyzed.*
***Results:*** *Immunoreactive TN and TN mRNA were expressed in the basement membrane of the epithelium as well as in the smooth muscle layer, and their distribution shifted from the subbasement region on Day 0–3 to the smooth muscle layer on Days 4 and 5.*
***Conclusions:*** *These results indicate that TN expression in the uterus during early pregnancy is spatiotemporally different and may be regulated by a different mechanism.*

**KEY WORDS:** early pregnancy; in situ hybridization; tenascin; uterus.

## INTRODUCTION

Tenascin (TN) is an extracellular matrix (ECM) glycoprotein composed of disulfide-bonded six subunits (1,2). The primary structure of TN has been predicted from the nucleotide sequences. It is a multidomain macromolecule containing signal peptides, epidermal growth factor-like repeats, and fibronectin type III-like repeats and concluding with a fibrinogen homologous sequence. The tissue distribution of TN is characteristic because it is present transiently in the dense mesenchyme surrounding the growing epithelium during embryogenesis and in the stroma during tumorigenesis and tissue repair. Thus, the pattern of TN expression in tissues is highly restricted compared with that of other ECM proteins such as fibronectin, laminin, and collagens, suggesting that TN expression is confined to special conditions probably regulated by physiological and pathological microenvironments.

A possible hint for the induction of de novo TN synthesis came from observations of female endocrine target organs. In human breast and uterus, TN was detected by immunohistochemistry in the basement membrane and subbasement membrane zones beneath the ductal, glandular, and luminal epithelia in a cyclic manner during menstruation. TN expression was found in the stroma of normal breast more or less throughout the cycle but reached its peak in the fourth week of the cycle (3). TN in uteri, in contrast, was demonstrated around the endometrial glands during the proliferative phase but not during the secretory phase (4). Our preliminary study suggested, by immunohistochemistry and in situ hybridization, that in mouse uterus, immunoreactive TN and TN mRNA were found in the stroma and muscle layer at metestrus and diestrus but not at proestrus and estrus and that no TN was found in ovariectomized mice. Therefore, ovarian hormones are likely to be involved in TN induction in the uterus. Further, stromal TN in ovariectomized mouse uterus was restored by progesterone but not estrogen injection, whereas muscle layer TN was restored by either estrogen or progesterone. These observations suggest that TN expression is differently regulated between stroma and smooth muscle. We assume that TN expression in the uterus may exert the most dynamic changes during early pregnancy because tremendous restructuring occurs in this tissue during implantation and subse-

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quent uterine and fetal growth. However, there have been few studies concerning the change in the localization of TN expression during early pregnancy. The present study was undertaken to elucidate the biological importance of TN in the uterus during early pregnancy by analyzing the change in TN expression in the periimplantation period.

## MATERIALS AND METHODS

### Animals

BALB/c mice, purchased from CLEA Japan Inc. (Tokyo), were used in this study. The date of pregnancy was determined by vaginal plug, and the day the vaginal plug was recognized was defined as Day 0.

### Antibody

Monoclonal rat anti-human TN antibody, 3–6, diluted 1/100, was used for TN immunohistochemistry. This monoclonal antibody, which was developed in our laboratory by immunizing purified human TN (5), can specifically react with both human and mouse TN. This antibody recognizes the EGF domain so that both isoforms are detectable. As a positive control for TN staining, we used several rat and human tissues which were previously reported to be positive by Oike *et al.* (5). We confirmed that this antibody reacted positively to mouse TN under the experimental conditions we used for immunohistochemistry. We also confirmed negative TN staining, with rat IgG used as the negative control.

### Immunohistochemistry

After five mouse uteri from each stage of early pregnancy were removed, they were fixed with 4% paraformaldehyde in PBS(−), embedded in polyester wax, sectioned at 4 μm, and placed on egg albumin-coated glass slides. Sections were immunohistochemically stained with a rat anti-human TN antibody by an indirect immunofluorescence method according to the procedures described by Kusakabe *et al.* (6). Briefly, sections were first incubated for 30 min with a blocking solution of PBS(−) containing 1% BSA and 5% normal goat serum and subsequently incubated with the monoclonal rat anti-human TN antibody overnight at room temperature. After being washed with PBS(−) in 0.1% Tween 20 (Sigma), they were again incubated with FITC-labeled anti-rat IgG (Zymed, San Fancisco, CA) for 2 hr. All samples were observed under a laser microscope (Zeiss). Immunohistochemical observation was also carried out using the same anti-TN antibody by the method of immunoperoxidase staining.

### Hybridization Probe

A mouse TN complementary DNA (cDNA) clone was made in our laboratory in Riken (7). This cDNA clone, a fragment of about 2.5 kilobases (kb), was inserted into the *Eco*RI site of the pBluescript-II SK(+) vector.

### Northern Blot Hybridization

Northern blot hybridization of early pregnant uterus was performed according to the method of Maniatis *et al.* (8). Total RNA extracted from five mice uteri of each stage of early pregnancy or from 2H6GR cells by the AGPC method. Twenty micrograms of total RNA per each lane was resolved by electrophoresis through 0.8% agarose gels containing formaldehyde, followed by transfer to a nylon membrane. Blots were irradiated in UV cross-linker for 30 sec and hybridized with $2 \times 10^5$ cpm/ml mouse TN cDNA (2.5 kb) multiprimed by the random primer method. Hybridization was performed at 42°C overnight in 50% formamide, 4× SSC (1× SSC = 0.15 *M* NaCl and 0.015 *M* sodium citrate), 20 m*M* Tris–HCl (pH 7.0), 0.1× Denhart's reagent (100× Denhart's reagent = 2% each BSA, Ficoll, and polyvinylpyrolidone), 10% sodium dodecyl sulfate (SDS), and 100 μg/ml salmon sperm DNA. Washes were performed in 2× SSC and 0.1% SDS at 50°C for 15 min four times. Blots were exposed on an imaging plate at room temperature overnight and analyzed using an imaging analyzer (BAS2000, Fuji Film Co., Tokyo). 2H6GR cells, a mouse breast carcinoma cell line, from which mouse TN cDNA was cloned, served as the positive control for mouse TN mRNA.

### In Situ Hybridization

Five mice uteri from each stage of early pregnancy were dissected and irradiated in a microwave oven (BioRad, Tokyo) for 20 min at 35°C in 0.1 *M* Na–phosphate buffer. The tissues were placed in a cold fixative solution containing 4% paraformaldehyde in PBS(−) and incubated overnight at 4°C. Then after washed in PBS(−), they were dehydrated and embedded in polyester wax. Serial sections of 5-μm thickness were mounted on Biobond (Biocell Inc.)-coated glass

slides. After dewaxing and air-drying, they were incubated for 15 min in 0.5 *M* HCl at room temperature (RT), then with 50% formamide in 2× SSC (1× SSC: 0.15 *M* NaCl and 0.015 *M* sodium citrate) for 30 min at 60°C. Sections were digested with 4 mg/ml proteinase K (Boehringer, Mannheim) for 20 min at 37°C. After rinsing in PBS, sections were treated with glycine (2 mg/ml) in PBS and then fixed with 4% paraformaldehyde in PBS for 5 min at RT. They were again treated with glycine solution and incubated with 1 m*M* levamisole for 5 min at RT. Sections were acetylated with 0.1 *M* triethanolamine solution, pH 8.0, containing 0.25% acetic anhydrate and 1 m*M* EDTA for 10 min at RT twice. After rinsed in PBS, sections were dehydrated by immersion in an up-graded ethanol series and dried. A cRNA probe for in situ hybridization was made by dividing the full length into six fragments of approximately 1 kb each, subcloned to pBluescript-II(+) vector, and mixing them. A digoxigenin (DIG)-labeled cRNA probe for mouse TN was made according to the method described in the manual of DIG systems. Hybridization was carried out according to the protocol of DIG systems (Boehringer, Mannheim) with only minor modifications. Briefly, the hybridization buffer, composed of 50% formamide, 10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 600 m*M* NaCl, 10 m*M* DTT, 1× Denhart's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidine, 0.02% BSA), 0.25% SDS, 10% dextran sulfate, and 200 mg/ml *E.coli* tRNA, was heated at 85°C for 10 min. Then it was heated again at 85°C for 2 min after the DIG-labeled cRNA probe (2 ng/ml) was added. This heated probe buffer was finally applied to the sections. Hybridization was carried out overnight at 45°C in a 50% formamide moistened chamber. To remove the probe solution, the sections were washed in 50% formamide with 2× SSC at 42°C for 15 min and subsequently washed in 2× SSC at 42°C to remove further nonspecifically attached cRNA probes. Positive signals in situ hybridization were analyzed according to the protocol attached to the DIG detection kit (Boehringer Mannheim) with some minor modifications using anti-digoxigenin–alkaline phosphatase conjugate and NBT/BCIP, then the sections were mounted with coverslips. The control study for TN mRNA localization by in situ hybridization was done using a sense probe.

## RESULTS

The immunohistochemical analysis using a monoclonal anti-human TN antibody summarizes the localization of TN in early pregnant mouse uterus as illustrated in Fig. 1. In a control study using rat IgG, no specific staining of TN was found (data not shown). TN was expressed in the basement membrane beneath the epithelial cells of the luminal epithelium on Day 0 through Day 3, especially markedly on Day 0 and Day 3. TN expression was also positive in the basement membrane of the glandular epithelium on Day 0 and Day 1, although it was weaker than that in the luminal epithelium. Immunoreactive TN was present in the inner smooth muscle layer on Day 1 through Day 5, most prominently on Day 4 and Day 5. The intensity of TN expression in the muscular layer increased on Day 4 and Day 5 when the pregnant uterus grew in volume.

In Northern blot analysis, 2 bands, of 7 and 5.5 kb, in agreement with the sizes of TN mRNA, were detected in all samples from early pregnant mouse uterine tissues on Day 0 through Day 5 (Fig. 2). As illustrated in Fig. 2, the TN mRNA signal was most prominent on Day 0 and Day 1.

In situ hybridization using the cRNA probe for TN mRNA and digoxigenin revealed that the TN mRNA was localized in the cells near the basement membrane beneath the luminal epithelial cells on Days 0, 1, and 2, which coincided with the findings for TN protein localization. TN mRNA was also detected in the muscle layer on Days 4 and 5, when immunoreactive TN was most predominant. Figure 3 illustrates the distribution of TN mRNA in the midsagittal plate of the pregnant uterus on Day 5. TN mRNA expressed in the extended muscle layer corresponded to the presence of the fetus. Figure 4 shows the localization of TN mRNA in the basement membrane beneath the luminal epithelium on Day 3 (Fig. 4A) and in the muscle layer on Day 4 (Fig. 4B). No TN mRNA was detected in these sections when we used a sense probe as the negative control (data not shown).

## DISCUSSION

It has been reported that one of the compartments dramatically affected by the changes during early pregnancy is the extracellular matrix (ECM); for instance, fibrillar collagen synthesis by the nonpregnant stroma is replaced by production of the basal lamina-associated proteins collagen IV, laminin, and heparan sulfate proteoglycan. One possible contributor to such regulation is the decidual ECM. Therefore, analysis of the ECM molecules which modulate these interactions is crucial for a better understanding of the process of

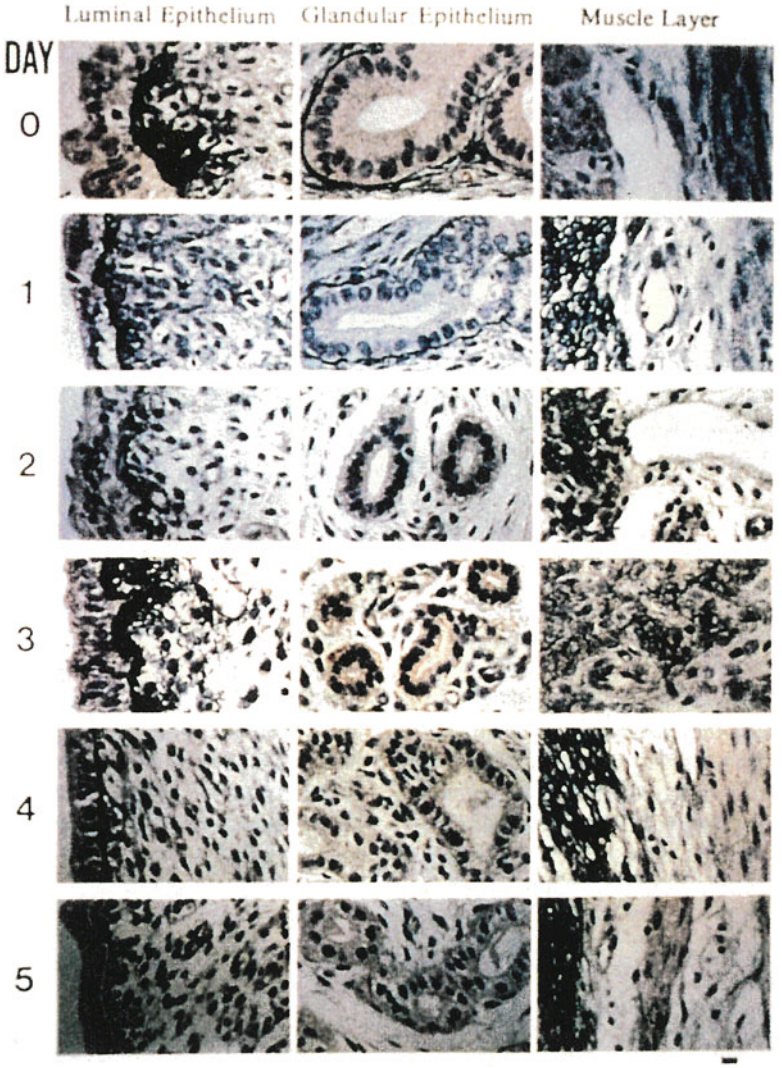


**Fig. 1.** Summary of the daily changes in the localization of immunoreactive TN in the luminal epithelium, glandular epithelium, and muscle layer of the mouse uterus during early pregnancy.

early pregnancy. TN is one of a group of ECM proteins and has been reported to be expressed within the mesenchyme responding to the signals from adjacent epithelia, especially in such remodeling processes as wound healing and tumorigenesis. We reported previously that epithelial cells can induce TN expression within the surrounding stroma in association with extensive tissue restructuring (9). The uterine tissue also undergoes remodeling not only during the sexual cycle but also in early pregnancy. In the human endometrium, we reported that TN is expressed in the mesenchyme beneath the epithelial cells and controlled by sex steroid hormone (10). Julian *et al.* (11) reported recently that TN is induced at the implantation site in mouse uterus. Michie and Head (12) also indicate a unique spatiotemporal TN expression during decidualization in the mouse.

In the present experiment we clearly demonstrated a characteristic change in the distribution of TN expression in early pregnant mouse uterine tissue at both protein and mRNA levels. The gene and protein expressions of TN seem to respond to the morphological changes in the pregnant uterus, suggesting that TN plays an important role in the uterus in early pregnancy.

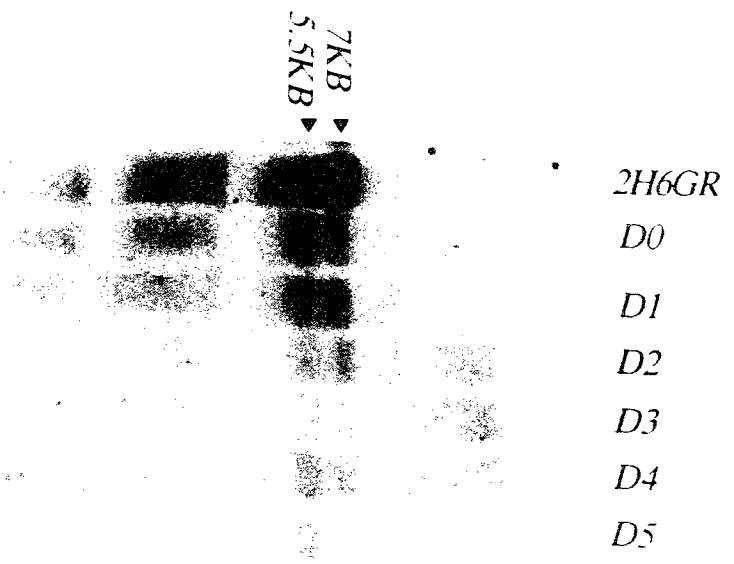


**Fig. 2.** Northern blot analysis of TN mRNA in the mouse uterus during early pregnancy. Twenty micrograms of total RNA was hybridized in each lane using a TN cDNA probe. Total RNA from 2H6GR cells was used as the positive control.

During decidualization, there is a striking redistribution of ECM in the decidualized endometrium. We demonstrated dramatic regionalization of TN expression spatially and temporally within the uterus during early pregnancy. Our finding that there was a small amount of TN in the endometrium immediately below the glandular epithelium is in agreement with the report by Michie and Head (12), which showed that the prominent region of TN expression that exhibited variation during the cycle was within the endometrial stroma immediately adjacent to the uterine epithelium. According to our results, TN first appeared in the

**Fig. 3.** Localization of TN mRNA in the midsagittal plate of a mouse pregnant uterus on Day 5 analyzed by in situ hybridization using a TN cRNA probe.

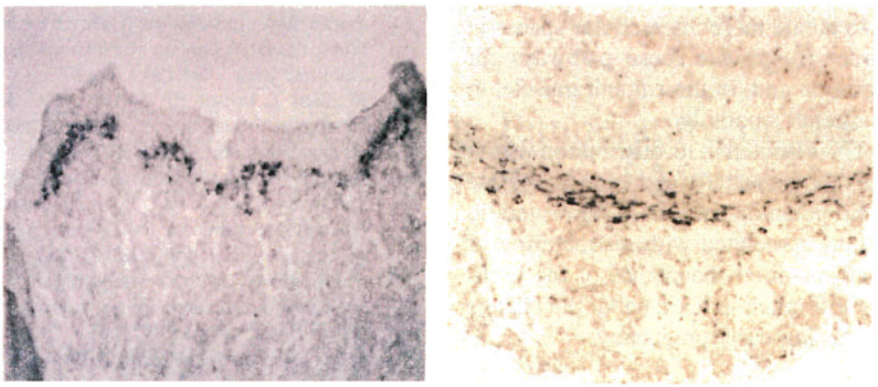


**Fig. 4.** Localizations of TN mRNA in the stromal cells adjacent to the basement membrane beneath the luminal epithelium on Day 3 (A) and in the muscle layer on Day 4 (B) by in situ hybridization using a DIG-labeled TN cRNA probe.

stroma immediately beneath the luminal epithelia and then shifted to the smooth muscle layer as epithelial expression regressed. TN expression in the stromal portion seems to be short-lived because it disappears by Day 4 and is completely negative. Compared with other ECM proteins which are modulated within the uterine tissue during early pregnancy, TN is unique in that its expression and loss are reexpressed within another region of the uterine tissue. This feature shows the intricate nature of TN regulation in this tissue. Another region where TN was constantly expressed was the subepithelial region, along with the basement membrane, as observed on Days 1–4 of gestation. However, it was apparent that, by Day 5, there were interruptions of TN expression along the length of this structure. Compared with TN expression in the subepithelial region, the smooth muscle showed a constant TN expression from Day 1 to Day 5. The prominent expression of TN around the smooth muscle was also reported to be a constant feature during the different stages of the cycle and in ovariectomized rats (12). Therefore, we speculate that the expression of TN in the smooth muscle is not under steroid hormone control but is an important reflection of the dynamics of the expansion of the tissue due to decidua formation in early pregnancy. The expression of TN in the uterine muscle seems to increase, possibly due to remodeling during the dramatic expansion of decidual tissue. The expression of TN in the smooth muscle may restructure the ECM in such a way that the muscle layer can resist the expansion of decidual volume, while it virtually disappears from the epithelial basement membrane by Day 4.

The regulator which controls TN expression is unclear. However, β-type transforming growth factor (TGF-β) and basic fibroblast growth factor (bFGF) are considered as possible factors in the regulation of TN expression because TGF-β has been shown to induce TN at the mRNA level in chick embryo fibroblasts (13) as well as at the protein level in mouse L cells (14). Recently, Rettig *et al.* (15) reported the induction of human TN by cytokines such as interleukin-6. Two recent papers have reported that angiotensin II vasoconstrictor peptide could enhance TN expression in vascular smooth muscle cells in vitro (16,17). In hypertensive rats, focal expression of TN was found in vascular smooth muscles at sites apt to receive a high blood pressure (17). It can be speculated, therefore, that mechanical stress induces TN expression in the smooth muscle, which is perhaps mediated by angiotensin II. The uterine smooth muscles are indeed stressed continuously by the growing embryo, and angiotensin II is known to be rich during pregnancy (17). Taking these points into consideration, we conclude that TN expression in the uterus is probably regulated in a complex way through various factors such as ovarian hormones, TGF-βs, and angiotensin II. Coexamination of TN and TGF-β2 by immunohistochemistry and in situ hybridization demonstrated that stromal TN may be induced by TGF-β2, which is produced in the epithelium at a progesterone-excessive stage, while smooth muscle TN is induced by the mechanical stress of the growing embryo. The function of TN also remains unclear, but it has been suggested that it may have many functions including migration and immunomodulation (16). It can be speculated that a possible function of TN in

pregnant uteri is to create more intercellular spaces in the stroma in order to provide loose pathways for decidual cell migration and, in the smooth muscle, to make these cells stretch smoothly.

In conclusion, TN is limited in its distribution within the pregnant uterus, both spatially and temporally, and this may reflect the potential for this ECM protein to have many functions associated with restructuring of tissues in vivo and maternal acceptance of the fetus. However, there is still much to learn about the change in TN expression during early pregnancy.

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