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Transforming growth factor β mediates the progesterone suppression of an epithelial metalloproteinase by adjacent stroma in the human endometrium

(cell–cell interactions/stromelysin/matrilysin)

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ABSTRACT **Unlike most normal adult tissues, cyclic growth and tissue remodeling occur within the uterine endometrium throughout the reproductive years. The matrix metalloproteinases (MMPs), a family of structurally related enzymes that degrade specific components of the extracellular matrix are thought to be the physiologically relevant mediators of extracellular matrix composition and turnover. Our laboratory has identified MMPs of the stromelysin family in the cycling human endometrium, implicating these enzymes in mediating the extensive remodeling that occurs in this tissue. While the stromelysins are expressed *in vivo* during proliferation-associated remodeling and menstruation-associated endometrial breakdown, none of the stromelysins are expressed during the progesterone-dominated secretory phase of the cycle. Our *in vitro* studies of isolated cell types have confirmed progesterone suppression of stromal MMPs, but a stromal-derived paracrine factor was found necessary for suppression of the epithelial-specific MMP matrilysin. In this report, we demonstrate that transforming growth factor β (TGF-β) is produced by endometrial stroma in response to progesterone and can suppress expression of epithelial matrilysin independent of progesterone. Additionally, we find that an antibody directed against the mammalian isoforms of TGF-β abolishes progesterone suppression of matrilysin in stromal–epithelial cocultures, implicating TGF-β as the principal mediator of matrilysin suppression in the human endometrium.**

The matrix metalloproteinases (MMPs) make up a complex highly regulated family of enzymes that are able to degrade most components of the extracellular matrix (ECM) at a neutral pH (1). The regulation of ECM composition is an extremely important consideration; it promotes the structural integrity of tissues and provides bioactive signals that can independently affect cellular behavior. For example, the matrix directs the development and morphogenesis of vertebrate embryos and influences basic cellular processes such as proliferation, migration, and differentiation (2). In the adult, although the MMPs participate in the normal process of wound healing, overexpression or altered expression of these enzymes is associated with certain pathological conditions such as arthritis (3) and metastatic tumor invasion (4). Other examples of normal MMP expression in the adult occur in the female reproductive tract and appear to coincide with events that require tissue remodeling or repair such as ovulation (5, 6), implantation (7, 8), and postpartum uterine and mammary gland involution (9–11).

Among the reproductive tissues, the primate endometrium is perhaps the most dynamic, undergoing regular periods of extensive tissue growth, cellular differentiation, and breakdown (12). Not unexpectedly, several laboratories have identified MMP expression during the menstrual cycle (13–17). MMPs of the stromelysin family are focally expressed in areas of active tissue growth during the proliferative stage of the cycle, are absent during the early-to-mid secretory stage, and are broadly reexpressed during the extensive tissue breakdown that occurs during menstruation (15–17). In addition to cycle-dependent stromelysin expression, the endometrial surface and glandular epithelium and supporting stroma also demonstrated a cell-specific pattern of expression. Matrilysin (EC 3.4.24.23, MMP-7) is localized only to glandular or luminal epithelium, and other stromelysins, stromelysin 1 (EC 3.4.24.17, MMP-3), stromelysin 2 (EC 3.4.24.22, MMP-10), and stromelysin 3 (MMP-12), are localized only to the stromal compartment. *In vitro* studies have confirmed that progesterone can suppress the expression of endometrial stromelysins although estradiol does not appear to directly activate their expression (17, 18).

A principal role of the stroma in mediating steroid action on adjacent epithelium has been recognized during growth and differentiation in numerous adult tissues (19), including the endometrium (20, 21). Interestingly, we found that the ability of progesterone to suppress epithelial promatrilysin, the secreted form of matrilysin, required a soluble factor of stromal origin (17). Potential candidates for this stromal-derived factor(s) capable of regulating MMP expression in adjacent epithelium are numerous, including a variety of cytokines (22) and growth factor families (21, 23). However, transforming growth factor β (TGF-β) has been shown to suppress the stromelysin 1 gene in rat fibroblasts through a TGF-β inhibitory element in the stromelysin promoter (24, 25). Sequences within the human matrilysin gene are highly homologous to the rat stromelysin TGF-β inhibitory element (26), and Marti *et al.* (27) have recently shown inhibition of matrilysin by TGF-β in cultures of kidney mesangial cells. In this report, we provide evidence that in the human endometrium, progesterone enhances expression of TGF-β mRNA and protein by the endometrial stroma and that this growth factor serves as the principal stromal-derived factor leading to the suppression of epithelial promatrilysin expression.

MATERIALS AND METHODS

Acquisition of Human Tissue. Endometrial tissue was obtained during the proliferative and secretory interval of the

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Abbreviations: MMP, matrix metalloproteinase; ECM, extracellular matrix; TGF-β, transforming growth factor β.

menstrual cycle from diagnostic or donor biopsies removed with a Pipelle suction instrument from the fundus region. Additional samples were obtained from uteri removed by hysterectomies performed for benign conditions. The use of human tissue was approved by Vanderbilt University's Institutional Review Board and Committee for the Protection of Human Subjects.

Explant Cultures of Endometrium. Sections of endometrial tissue were dissected into uniform 1×2 mm^2 explants, 8–10 of which were suspended within tissue culture inserts (Millicell; Millipore) near the surface of the air–culture medium interface as described (17). Explant cultures were maintained in phenol red-free Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) (Sigma) supplemented with 1% ITS$^+$ (Collaborative Biomedical Products; Bedford, MA) and 0.1% Excyte (Miles Scientific) and incubated at 37°C in a humidified chamber with 95% air/5% CO_2. Cultures were maintained for 48 hr followed by collection of conditioned medium containing secreted proteins, which were stored at −80°C for Western blot analysis. Explanted tissues were quick-frozen in liquid N_2 for Northern blot analysis.

Endometrial Cell Isolation and Culture Procedures. Isolated endometrial epithelial and stromal cells were obtained by sequential enzymatic dissociation as initially described (28) and modified (17). Briefly, endometrial tissue was dissected into small (1 cm^3) fragments and incubated in medium containing 0.4% collagenase, 0.02% DNase, and 2% (vol/vol) chicken serum for 1 hr at 37°C. Stromal cells were isolated from epithelial fragments by filtration. Epithelial fragments were further digested in medium containing 0.4% collagenase, 0.1% hyaluronidase, and 0.1% Pronase. A short digestion of 0.05% trypsin was performed to obtain single cells. Cell purity was assessed by immunolocalization of cytokeratin and vimentin; <5% contamination by epithelial or stromal cells was routine within either purified cell population (data not shown). Epithelial cells were cultured on Matrigel (Collaborative Biomedical)-coated nitrocellulose tissue culture inserts with a 0.4-μm pore size (Millicell; Millipore). Stromal cells were cultured on a coating of type I rat tail collagen (Becton Dickinson) on the bottom surface of tissue culture wells. Epithelial or stromal cells (3×10^5 cells per well, 24-well plate) were allowed to attach to the culture matrix for 24 hr in DMEM/F-12 with 5% (vol/vol) charcoal-stripped heat-inactivated calf serum before initiating serum-free conditions. After the cell attachment period, cultures were maintained in DMEM/F-12 medium supplemented with 1% ITS$^+$ and 0.1% Excyte; medium was changed every second day. For analysis of promatrilysin secretion, cultures were labeled with [^{35}S]methionine during the last 18 hr of culture.

Steroid and Growth Factor Treatments. Steroid treatments of explant cultures or cultures of isolated cell types included estradiol alone (10 nM) or estradiol (1 nM) plus progesterone (500 nM). Some cultures were treated with TGF-β1 (1–2 ng/ml), TGF-β2 (0.5–1 ng/ml), or pan-specific blocking antibody to TGF-β (R & D Systems) (10 μg/ml). For standardization, experimental treatments were begun with initiation of serum-free conditions, considered day 0.

Western Blot Analysis. Secreted proteins from explant cultures were quantitated by bovine serum albumin/colorimetric assay (Pierce), separated by SDS/PAGE, and transferred to Immobilon-P (Millipore). The membrane was then incubated in 5% (vol/vol) milk containing a 1:1000 dilution of antibody against human matrilysin (as described in the ^{35}S-labeling experiments) overnight at 4°C followed by horseradish peroxidase-coupled secondary antibody incubation for 1 hr at room temperature. Proteins were visualized by a 10-min exposure to Kodak XAR film after a 1-min incubation in Amersham's enhanced chemiluminescence (ECL) reagents for Western blot analysis.

Northern Blot Analysis. RNA was extracted from explant cultures of endometrium by cell disruption in guanidine isothiocyanate with a tissue homogenizer followed by phenol/chloroform purification. RNA was separated electrophoretically and transferred to a nitrocellulose membrane by standard procedure (29). [^{32}P]UTP-labeled complementary RNA probes were made to specific regions of human matrilysin (849 bp encoding the entire cDNA; ref. 30) and TGF-β1 and TGF-β2 (nt 997–1277 and 253–853, respectively; ref. 31). Each blot was stripped and hybridized to a probe for cyclophilin, a constitutively expressed mRNA (32).

Analysis of ^{35}S-Labeled Promatrilysin Secretion. Secretion of promatrilysin was analyzed after [^{35}S]methionine labeling (100 μCi; 1 Ci = 37 GBq) for 18 hr at 37°C in methionine-free medium as described (17). Prior to labeling, cultures were incubated for 2 hr in methionine-free medium without ^{35}S. Secreted proteins were quantitated by trichloroacetic acid precipitation and equivalent trichloroacetic acid-precipitable counts (1×10^5 cpm) were selectively immunoprecipitated with a rabbit polyclonal antibody raised against a glutathione *S*-transferase–matrilysin fusion protein containing the last 100 amino acids of matrilysin (30, 33). The resulting complexes were removed with protein A-Sepharose and identified by SDS/PAGE and autoradiography.

Immunohistochemical Analysis of TGF-β Expression. Immunohistochemistry was performed as described by Gold *et al.* (31). Briefly, deparaffinized formalin-fixed tissue sections were analyzed for TGF-β by using polyclonal antibodies raised in rabbits against a synthetic peptide corresponding to amino acids 4–19 (TGF-β1 and TGF-β2) and 9–20 (TGF-β3) of the mature polypeptide and shown to be isoform specific by Western blot analysis (34). Primary antibody was detected by using a biotinylated goat anti-rabbit secondary antibody and the Vectastain Elite streptavidin–biotin complex kit (Vector Laboratories). Secondary antibody was visualized by using a 3,3′-diaminobenzidene tetrahydrochloride solution resulting in a brown precipitate. Slides were counterstained with Gill's hematoxylin (Fisher). Normal rabbit serum was used as a negative control.

RESULTS

In our previous *in vivo* studies (15, 16), growth-related expression of mRNA for endometrial stromelysins could be appreciated in focal areas of active tissue reorganization during the estrogen-directed proliferative phase but not during the progesterone-dominated secretory menstrual interval. Our initial *in vitro* study confirmed the ability of progesterone to suppress the secretion of promatrilysin protein in intact endometrial tissue explants (17), but suppression of matrilysin mRNA was not determined. Northern and Western blot analysis reveal that specific matrilysin mRNA (Fig. 1*A*) and secretion of the promatrilysin protein (Fig. 1*B*) can be suppressed *in vitro* by progesterone treatment in endometrial explants. In contrast to

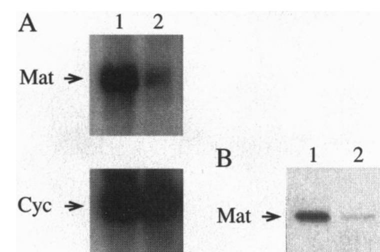

FIG. 1. Detection and steroidal regulation of matrilysin mRNA (Mat) (*A*) and the 30-kDa promatrilysin protein (Mat) (*B*) in proliferative-phase explants of human endometrium. Tissue was cultured for 48 hr under serum-free conditions with 10 nM estradiol (lanes 1) or 1 nM estradiol/500 nM progesterone (lanes 2). (*A*) The same blot was stripped and reprobed for cyclophilin (Cyc), a constitutively expressed mRNA. Results are representative of three experiments in *A* and five experiments in *B*.

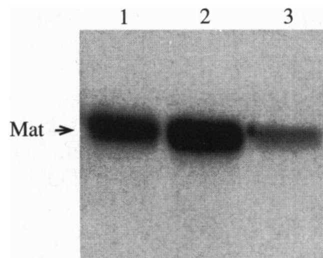

FIG. 2. Detection and steroidal regulation of promatrilysin protein (Mat; 30 kDa) expression in isolated epithelial cells maintained on a Matrigel-coated insert. Cells were cultured for 6–8 days under serum-free conditions with 10 nM estradiol (lane 1), 1 nM estradiol/500 nM progesterone (lane 2), or 1 ng of TGF-β1 (lane 3). Results are representative of five experiments.

the steroid sensitivity of intact endometrial tissues, however, progesterone does not independently suppress promatrilysin secretion by isolated epithelial cells. We found epithelial cells require a progesterone-induced stromal-derived soluble factor(s) for suppression of this enzyme (17). In this study, we have investigated the possibility that a member of the TGF-β family may be the stromal factor necessary for progesterone-induced inhibition of promatrilysin.

As shown in Fig. 2, while treatment with progesterone had no effect, treatment of isolated epithelial cells with human TGF-β1 completely suppressed promatrilysin secretion. These results indicate that TGF-β can suppress promatrilysin secretion *in vitro* but do not determine whether TGF-β is the stromal-derived signal that mediates progesterone-directed promatrilysin suppression. To address this question, cocultures of isolated epithelial and stromal cells in bicameral chambers were established under serum-free conditions and treated with progesterone in the presence of a pan-specific antibody directed against each of the known forms of mammalian TGF-β. This antibody would block the action of progesterone if progesterone acts by induction of TGF-β. As shown in Fig. 3, either TGF-β1 or progesterone treatment can independently suppress promatrilysin secretion in a coculture environment. However, addition of the pan-specific TGF-β antibody completely blocked the ability of progesterone treatment to inhibit the secretion of promatrilysin in stromal/epithelial cocultures. These data strongly suggest that TGF-β is the progesterone-induced factor of stromal origin that mediates suppression of epithelial promatrilysin.

To confirm stromal-derived TGF-β expression, we conducted an immunohistochemical analysis by using polyclonal antibodies directed against TGF-β1, TGF-β2, and TGF-β3. Each of these isoforms has been reported to be produced by the human endometrium, and investigators have found the levels of expression of this growth factor family to increase during the secretory phase of the menstrual cycle (23). Confirming the recent work of Gold *et al.* (31), our analysis of tissues acquired during the proliferative, periovulatory, and secretory phases of the menstrual cycle revealed that TGF-β2 is the principal isotype of TGF-β associated with the endometrial stroma and that TGF-β2 increases in the secretory menstrual interval relative to the proliferative interval (Fig. 4). While increases in the intensity of TGF-β1 and TGF-β3 expression were also apparent during the secretory interval, these isotypes of TGF-β were less specific to stromal cells (data not shown) being primarily associated with epithelial glands. To further confirm the role of progesterone in the induction of TGF-β2 secretion by endometrial stroma, we cultured

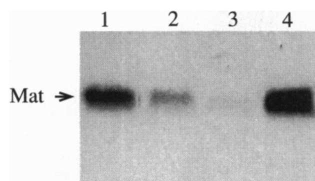

FIG. 3. Detection and steroidal regulation of promatrilysin protein (Mat; 30 kDa) expression in isolated epithelial cells maintained on a Matrigel-coated insert and cocultured with isolated stromal cells plated on collagen type I on the bottom of the culture well. Cells were cultured for 6–8 days under serum-free conditions with 10 nM estradiol (lane 1), 1 nM estradiol/500 nM progesterone (lane 2), TGF-β at 1 ng/ml (lane 3), or 1 nM estradiol/500 nM progesterone/pan-specific TGF-β antibody (10 μg/ml) (lane 4). Results are representative of eight experiments.

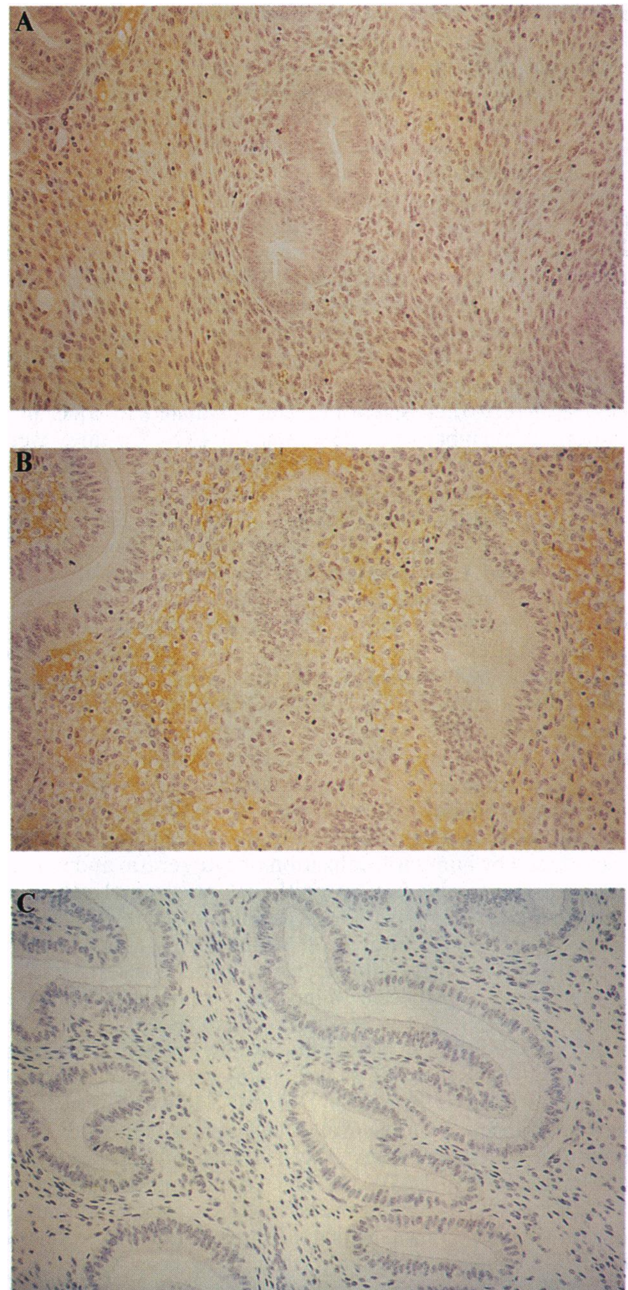

FIG. 4. Detection of TGF-β2 protein in formalin-fixed paraffin-embedded human endometrial tissue. TGF-β2 protein localization in normal proliferative endometrium (*A*) and normal secretory endometrium (*B*). Normal rabbit IgG was used as a negative control in normal secretory endometrium (*C*). All sections were counterstained with Gill's hematoxylin. Results shown are representative of multiple experiments performed on numerous tissue samples (proliferative, n = 8; secretory, n = 12). (×95.)

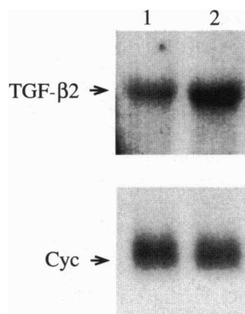

FIG. 5. Detection and steroidal regulation of TGF-β2 mRNA in explants of proliferative human endometrium. Tissue was cultured for 4 days under serum-free conditions with 10 nM estradiol (lanes 1) or 1 nM estradiol/500 nM progesterone (lanes 2). The same blot was stripped and reprobed for cyclophilin (Cyc), a constitutively expressed mRNA. Results are representative of three experiments.

explants of tissue, acquired during the proliferative interval, in the presence of estradiol or estradiol and progesterone. By using a specific RNA probe to human TGF-β2, Northern blot analysis confirmed an increase in TGF-β2 mRNA with progesterone treatment (Fig. 5). Since TGF-β2 appears to be the primary isoform of TGF-β secreted by the endometrial stroma, we cultured isolated endometrial epithelial cells in the presence of TGF-β1 or TGF-β2. As shown in Fig. 6, either isoform is equally capable of suppressing epithelial matrilysin expression whereas progesterone alone cannot, further supporting the specific role of TGF-β2 in mediating the effects of this steroid.

DISCUSSION

The human endometrium is a dynamic complex glandular tissue that is among the most steroid-sensitive of all adult tissues. The cellular mechanisms by which steroids may influence ECM degradation or composition is an extremely important biochemical consideration in normal reproductive and nonreproductive tissues and in the pathophysiology of disease. MMPs of the stromelysin family are expressed in the human endometrium in focal areas of active glandular and stromal remodeling during the proliferative phase of the menstrual cycle but are suppressed during the secretory interval (15, 16). At present, there is only an association of estradiol-stimulated growth and the expression of endometrial stromelysins. However, progesterone suppression of stromelysins (17, 18) and other endometrial MMPs (14) has been documented by *in vitro* studies. Numerous reproductive disorders are associated with inadequate progesterone action (35, 36). We have demonstrated (17) that suppression of matrilysin, the epithelial-specific stromelysin, required a progesterone-induced stroma factor(s).

In the present study, by using cultures of intact endometrial explants, we demonstrated that progesterone treatment can

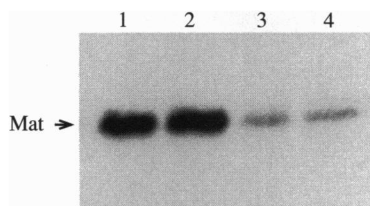

FIG. 6. Detection and steroidal regulation of promatrilysin protein (Mat; 30 kDa) expression in isolated epithelial cells maintained on a Matrigel-coated insert. Cells were cultured for 6–8 days under serum-free conditions with 10 nM estradiol (lane 1), 1 nM estradiol/500 nM progesterone (lane 2), TGF-β1 at 1 ng/ml (lane 3), or TGF-β2 at 0.5 ng/ml (lane 4). Results are representative of three experiments.

suppress the matrilysin gene and protein expression, indicating that a progesterone-induced stromal factor(s) likely regulates matrilysin expression by epithelial cells at the genomic level. While the identity of the stromal factor(s) required for matrilysin suppression was not previously identified, TGF-β is known to modulate both epithelial cell growth and differentiated function (37). Additionally, TGF-β has been shown to inhibit secretion of promatrilysin by mesangial cells *in vitro* (27) and recent studies of the human matrilysin gene revealed sequences that are highly homologous to the TGF-β inhibitory element (26) described in rat fibroblasts (24, 25). We show here that TGF-β1 or TGF-β2 treatment *in vitro* acts directly on isolated endometrial epithelial cells, independently suppressing promatrilysin protein expression. Furthermore, blocking the action of endogenously produced TGF-β in cocultures abolished the progesterone-dependent ability of stroma to suppress epithelial promatrilysin secretion. This data indicates that stromal TGF-β secretion may serve the principal role in mediating the suppression of endometrial matrilysin expression during the secretory phase of the normal menstrual cycle. A similar TGF-β suppression of stromelysin 1 expression has been described in cultured rat fibroblasts (24, 25), and we have demonstrated that TGF-β can suppress prostromelysin expression in explant cultures of endometrial tissue (K.G.O., W.H.R., J.T.H., J. Vasquez, F. Gorstein, and L.M.M., unpublished data). Thus, these results indicate that TGF-β may serve both an autocrine and paracrine role in the regulation of endometrial stromelysins. In support of this position, numerous studies have shown the TGF-β gene and protein expression increasing during the secretory stage of the menstrual cycle relative to the proliferative phase (K.G.O., K.L.B., L.I.G., and J.T.H., unpublished data and refs. 34, 38, and 39). Chegini *et al.* (39) found TGF-β mRNA and protein expression to be lowest during the early proliferative phase, increased to their highest levels in the late proliferative and early-to-mid secretory phase, and dramatically decreased during the late secretory phase as serum progesterone levels fall.

While each of the TGF-β isotypes can have similar activities *in vitro* (40), specific TGF-β isotype(s) may serve different and interactive roles in the inhibition of endometrial MMP expression *in vivo*. TGF-β1, TGF-β2, and TGF-β3 have each been localized primarily to either stromal (TGF-β2) or epithelial cells (TGF-β1 and TGF-β3) within the human endometrium (K.G.O. *et al.*, unpublished data and refs. 34, 39, and 41) but the expression patterns of TGF-β isoforms is quite complex. Our study indicates that TGF-β2 may be the primary isoform to be produced by endometrial stroma in response to progesterone. Although stromal TGF-β2 is present in the proliferative phase, staining intensity increases after ovulation. Northern blot analysis revealed that progesterone treatment can increase the expression of TGF-β2 mRNA in serum-free explant cultures of proliferative endometrium while TGF-β1 mRNA levels changed only slightly. In support of our findings, Altman *et al.* (42) found TGF-β2 mRNA at its highest levels during midpregnancy in the mouse uterus whereas TGF-β1 mRNA was undetectable at this time. Other groups (38, 41), however, have reported an increase in TGF-β1 in either secretory phase stroma or decidual cells. This discrepancy may reflect differences in explant cultures vs. monolayer cultures or potential regulatory interactions among isotypes of this growth factor. TGF-β1 has been shown to decrease TGF-β2 and TGF-β3 expression (43) while enhancing its own expression (44, 45). TGF-β2 appears to increase expression of all three mammalian isotypes (43). Such interactions between the TGF-β isoforms likely play a role in their regulation in the human endometrium as well. Indeed, our studies would indicate that either TGF-β1 or TGF-β2 can suppress epithelial matrilysin expression, indicating the potential for interactions of TGF-β isotypes in the regulation of endometrial MMPs.

In summary, we report here an important stromal–epithelial interaction in normal tissue involving stromal-derived TGF-β as a paracrine mediator of MMP regulation in adjacent epithelium. In concert with the ability of TGF-β to block production of matrix-degrading enzymes, this cytokine simultaneously induces secretion of both protease inhibitors and ECM components (46). While virtually all normal cells are responsive to TGF-β, many transformed cells are not (47). There is a growing body of evidence demonstrating aberrant TGF-β expression in several cancers (48–50), including adenocarcinoma of the endometrium (31). Clearly, understanding the mechanisms by which TGF-β regulates MMP expression in the cycling human endometrium will provide important insights into not only normal physiology but also disease states.

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