Metalloproteinase expression by control and telomerase immortalized human endometrial endothelial cells

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Summary. Vascular endothelial cells play a critical role in the maintenance of endometrial homeostasis. Indeed many pathological conditions causing abnormal endometrial bleeding including progestin only contraception, hormone replacement therapy, endometrial polyps, myomas, hyperplasia and cancer are associated with aberrant angiogenesis. Critical to the process of angiogenesis is the breakdown of the surrounding tissues by matrix metalloproteases (MMPs). In addition to the cells surrounding the endometrial endothelial cells, the endothelial cells themselves produce their own panel of MMPs. We now characterize the specific MMPs that are expressed by endothelial cells derived from human endometrium. These include MMP-1, MMP-2 and MMP-10 but not MMP-3. In addition, in order to successfully carry out consistent, homogeneous and sufficient numbers of studies we investigated the *in vitro* expression of the MMPs with both freshly isolated, early passaged endometrial endothelial cells (HEECs) as well as with newly telomerase immortalized HEECs (T-HEECs). The latter were karyotypically normal and expressed classic endothelial cell endpoints such as tubulogenesis on matrigel and expression of the endothelial cell markers CD-31 (PECAM), von Willebrand's factor, and the Tie-2 receptors. The levels of MMP expression as well as that of the metalloprotease inhibitors TIMP-1 and TIMP-2 were similar in parent and immortalized endothelial cells.

Key words: Endometrium, Endothelial cells, Immortalization, Telomerase

Introduction

The process of angiogenesis (i.e. the generation of new capillary blood vessels from pre-existing ones) represents the concerted effort of many players creating a very specific coordinated balance. Generally, physiological angiogenesis in the adult is limited to the female reproductive tissue, namely in the ovary and endometrium. Hence, endometrial angiogenesis plays a key role in endometrial remodeling throughout the menstrual cycle as well as throughout pregnancy (Rogers and Gargett, 1998; Smith, 1998; Lockwood et al., 2004). So who are some of the major players that allow physiologic angiogenesis to occur?

As in an orchestra there are many diverse instruments, all of which have to come in at the right time and with exquisite pitch. Generation of proteolytic activity is thought to be pivotal in the regulation of cell migration and capillary tube formation (Pepper, 2001). Potentiation of angiogenesis is then exquisitively sensitive to hypoxia, nitric oxide (NO), VEGF, the angiopoietins and a long line of cytokines (Shifren et al., 1996; Davis and Yancopoulos, 1999; John and Tuszynski, 2001; Liekens et al, 2001). All together, this results in an increase in vascular permeability which is in turn aided by the receptivity of the endothelial cells via the expression of high affinity receptors for VEGF, Flt-1 and KDR, and the angiopoietin receptor (Tie-2) (Ferrara et al, 2001; Yancopoulos et al., 2000) In this study, we focus on the proteolytic aspect of angiogenesis by analyzing the expression of the specific MMPs expressed by the endothelial cells themselves utilizing parent and a newly immortalized HEEC line of cells that are karyotypically and phenotypically normal.

Materials and methods

Human endometrial cell isolation and cell culture

The isolation and culturing of primary human endometrial endothelial cells (HEECs) were carried out as previously described (Schatz et al., 2000). The cells were cultured and grown to confluence on flasks coated with 2% gelatin in EGM-2 MV Singlequot Medium with 5% stripped fetal calf serum (Cambrex Bio Science, Inc., Walkersville, MD). The isolation and culture of human endometrial stromal cells was carried out as previously described by our laboratory (Lockwood et al., 2004))

Immortalization Protocol

Immortalization of early passaged HEECs was achieved by transfection of telomerase (hTERT) using a retroviral system utilizing pA317 hTERT expressing cell

line (Geron Corporation, Menlo Park, CA). Transfected cells were selected by puromycin resistance as previously described (Krikun et al., 2004).

Karyotyping

Actively dividing HEECs which reached 40-70% confluence were harvested as follows: Colcemid was added to a final concentration of 0.04 mg/ml at least two hours prior to harvesting. Cells were trypsinized and harvested to produce G-banded metaphase preparations as described (Krikun et al., 2004). Karyotype analysis and documentation was performed as previously described (Baker et al., 1998) by a computerized acquisition and analysis system (Applied Imaging). A minimum of 20 cells were analyzed from each immortalized and parent primary cell culture for chromosomal clonal structural or numerical abnormalities.

Telomerase detection

Telomerase activity was assayed using the TRAPeze ELISA Detection Kit per manufacturer's instructions (Chemicon International, Inc., Temecula, CA) as previously described (Krikun et al., 2004). Absorbance readings were determined at 450 and 595 nM and telomerase activity was measured using the equation Abs450-Abs595. As positive controls for the assay, lysate from a telomerase-positive cell pellet and TSR8 template provided in the kit were used. The lysis buffer alone as well as heat-inactivated cell lysates (10 minutes at 85°C) were used as negative controls.

Tube formation

Cold, unpolymerized Matrigel (BD Biosciences, Bedford, Mass) was placed in a pre-chilled 24-well cell culture plate and then incubated at 37°C for 30 min. Either control or immortalized HEECs (4×10^4 cells/500ul of media) were seeded onto the Matrigel and allowed to grow overnight in a 37°C incubator. Tube formation was examined and photographed (200x) with an inverted contrast microscope (Olympus, Mellville, NY).

ELISAs

HEECs and THEECs were seeded and grown to confluence in basal medium as previously described (Krikun et al., 2005). The medium was then changed to a defined medium (Krikun et al., 2005) and the experimental conditions were carried out for 6 hours. The conditioned media were tested in multiplex sandwich ELISAs and analyzed as described by the manufacturer (Pierce Biotechnology, Inc. Rockford, IL). All results were adjusted for total cell protein.

mRNA microarray analysis

Cells grown on T-25 flasks were harvested with QIAzol™ lysis reagent (Qiagen, Valencia, CA) and used to prepare total RNA. One hundred µg of total RNA was cleaned and precipitated using RNeasy Mini Kit (Qiagen, Valencia, CA) to prepare the template for cDNA synthesis. A T7-(dT)24 oligo-primer was used to synthesize double-stranded cDNA by the Superscript Choice System (Gibco-Invitrogen, CA), which was subsequently cleaned up by Phase Lock Gels (PLG)-Phenol/Chloroform extraction and ethanol precipitation. Biotinylated cRNA was prepared with the ENZO BioArray High Yield RNA Transcript Labeling Kit (T7) (ENZO, Farmingdale, NY). Additional cRNA clean up was carried out by RNeasy Mini Kit prior to the fragmentation of biotinylated cRNAs with 5X fragmentation buffer (Tris 200 mM, pH 8.1, KOAc 500 mM, MgOAc 150 mM). The chemically fragmented cRNAs were then hybridized to Affymetrix HG_U133A 2.0 human chips, screening for 18,400 human genes and ESTs, followed by fluorescence labeling and optical scanning.

Raw data without normalization generated from Affymetrix GeneChip® Operating Software Version 1.1.1 (GCOS 1.1.1) (Affymetrix, Santa Clara, CA) were analyzed by GeneSpring software 6.1 (Silicon Genetics, Redwood City, CA)

The gene readouts were then normalized to the fiftieth percentile of the distribution of all measurements in each chip. Per gene normalization was performed using the median value of each gene throughout different chips in the same experimental condition.

Real time quantitative RT-PCR

Quantitative real time RT-PCR was conducted with the Roche Light Cycler (Roche, IN) as previously described (Krikun G, et al., in press). A quantitative standard curve was created utilizing a range of 500 pg to 250 ng total-RNA. The curve was created with the Roche Light Cycler (Roche, Indianapolis, IN) by monitoring the increasing fluoresence of PCR products during amplification. Values for the unknowns were adjusted to the quantitative expression of ß-actin from these same unknowns. Melting curve analysis was conducted to determine the specificity of the amplified products and the absence of primer-dimer formation. All products obtained yielded the correct melting temperature. Primers for MMP-1 were as follows: Forward: ACTACGATTCGGGGAG Reverse: CTTGGGGTATCCGTGT and yielded a 225 bp band. The primers for ß-actin were previously described (Krikun et al., 2005). All primers were synthesized and gel purified at the Yale DNA Synthesis Laboratory, Critical Technologies. All bands were sequenced to assure correct product formation.

Immunocytochemistry and immunohistochemistry

HEECs or THEECs were grown to between 40-70% confluence and immunocytochemistry was carried out as previously described (Guller et al., 1993). As a

comparison, endometrial stromal cells were also grown to sub-confluence and stained. Immunohistochemistry was conducted as previously described (Krikun et al., 2005). A monoclonal antibody to CD31 was obtained from DAKO-Cytomation (Carpinteria, CA).

## Results

HEEC immortalization and characterization

Parent and immortalized HEECs were assayed before and after heat inactivation. Figure 1 shows that immortalized HEECs and positive controls (telomerase-positive cell pellet or TSR8 template) displayed similar levels of hTert expression. By contrast, primary parent cells or heat-inactivated cells displayed low or background levels of hTert expression. Chromosome analysis of the immortalized cells showed a normal female karyotype (46,XX)
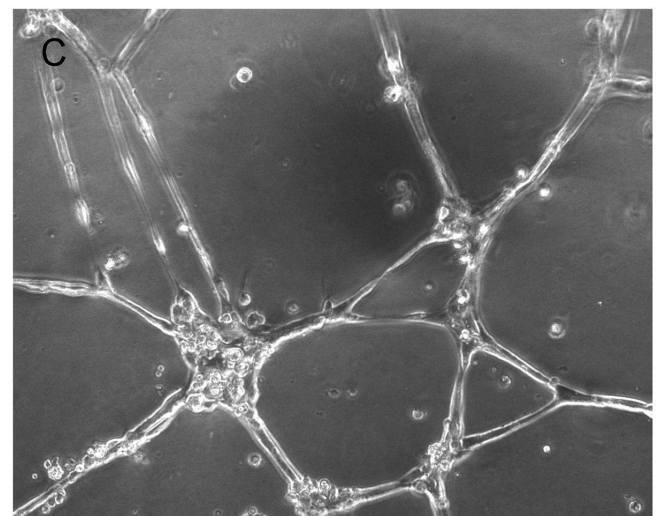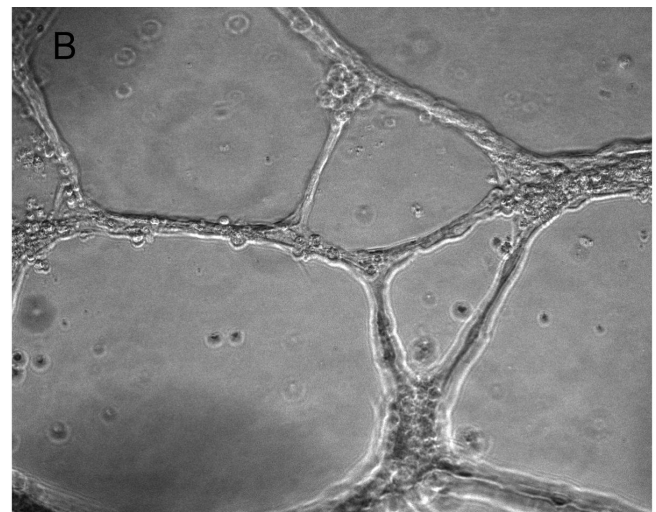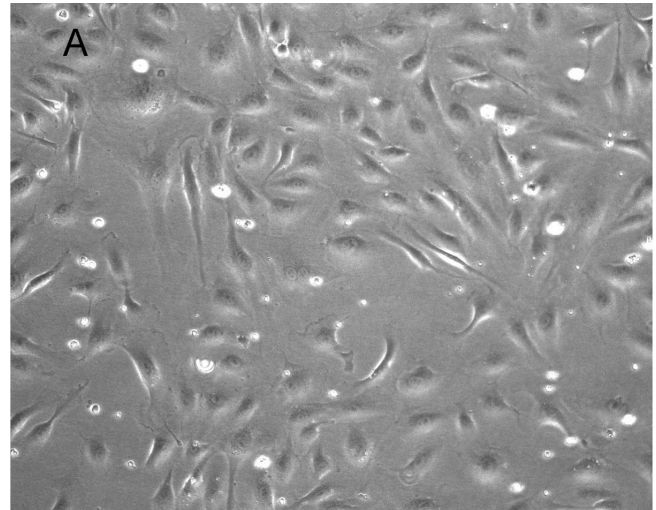
Tube Formation

Both control and immortalized HEECs (Fig. 2 panels B and C) displayed classic endothelial cell tube formation when seeded on matrigel-coated plates. By contrast, no tube formation was observed when the cells were seeded on gelatin-coated plates (Fig. 2 panel A).

Microarrays

A microarray testing 18,400 transcripts, demonstrated 93% identity between the parent and the immortalized HEECs. Of interest the 70kD heat shock protein (HSP) was greatly induced following immortalization of the HEECs (not shown). This is likely as the HSPs play a critical role in stress responses (Kregel, 2002) such as that which would be encountered during the process of immortalization. (Kregel, 2002). Significantly, both control and immortalized HEECs
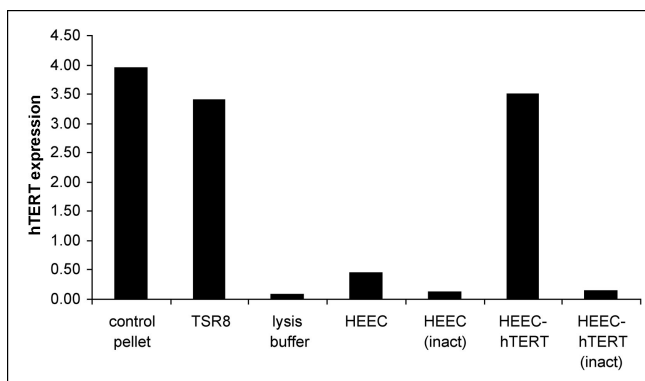
Fig. 1. Telomerase activity. The activity of telomerase was assessed as described in Methods. Positive controls consisted of a telomerase-positive cell pellet or TSR8 template provided by the manufacturer. Negative control consisted of buffer. Parent and immortalized HEECs (HEECs-hTERT) were assayed before and after heat inactivation.

Fig. 2. Tube formation. Representative phase-contrast microscopic images after overnight incubation are shown for control cells grown without matrigel **(A)** as well as control **(B)** and immortalized HEECs **(C)** grown on Matrigel coated wells.

expressed the mRNAs for the classic endothelial cell markers CD-31 (PECAM), von Willebrand's factor, nitric oxide synthase 3 (endothelial cell) (NOS3) as well as the VEGF and angiopoietin receptors FLT-1, KDR, Tie-1 and Tie-2 (Table 1).

Immunocytochemistry and immunohistochemistry

At the protein level, the expression of PECAM was observed both in *in vivo* and *in vitro* endometrial endothelial cells but not in the stroma. Figure 3 demonstrates that cultured parent (HEECs) and immortalized T-HEECs displayed immunostaining for PECAM unlike the cultured endometrial stromal cells. *In vivo* immunohistochemistry of the endometrium also demonstrates PECAM staining that is specifically limited to the endothelial cells.

MMP and MMP inhibitor expression

Microarray chip analysis on RNA derived from either parent or immortalized T-HEECs demonstrated

Table 1. Microarray analysis. Expression of genes which are classically expressed by endothelial cells are demonstrated in control (HEECS) or immortalized endometrial endothelial cells (T-HEECs). The expression levels depicted are normalized to ß-actin.

HEECs NORMALIZED	T-HEECs NORMALIZED	ABBREVIATION	GENBANK	DESCRIPTION
0.9686591	0.83652806	CD31: PECAM1	M37780	Human leukocyte surface protein: platelets/endothelial cell adhesion molecule
0.93500084	0.33672413	VWF, F8VWF	NM_0005552	Coagulation factor VIII: von Willebrands factor
0.9347415	0.6184612	NOS3; eNOS	N_000603	Nitrix oxide synthase 3 (endothelial cell)
1.1041067	0.5387055	FLT1	AA058828	FMS-related tyrosine kinase 1; vascular endotelial growth factor receptor
0.88271236	1.0450658	KDR; FLK1; VEGFR2	NM_002253	Vascular endothelial growth factor receptor-2: kinase insert domain receptor
0.8951956	0.5811104	TIE1; JTK14	NM_005424	Tyrosine kinase with immunoglobulin & epidermal growth factor domains
0.8236676	1.004876	TEK; TIE2	NM_000459	Transmembrane receptor protein tyrosine kinase activity (endothelial)

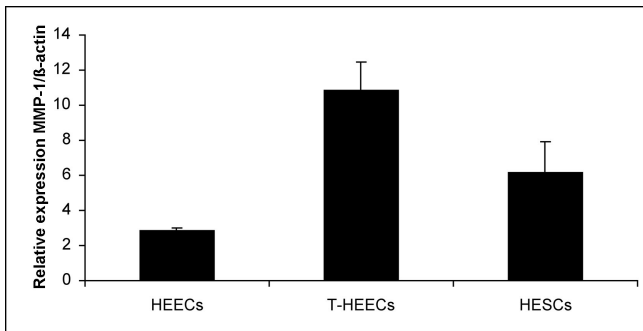## HEECs   THEECs
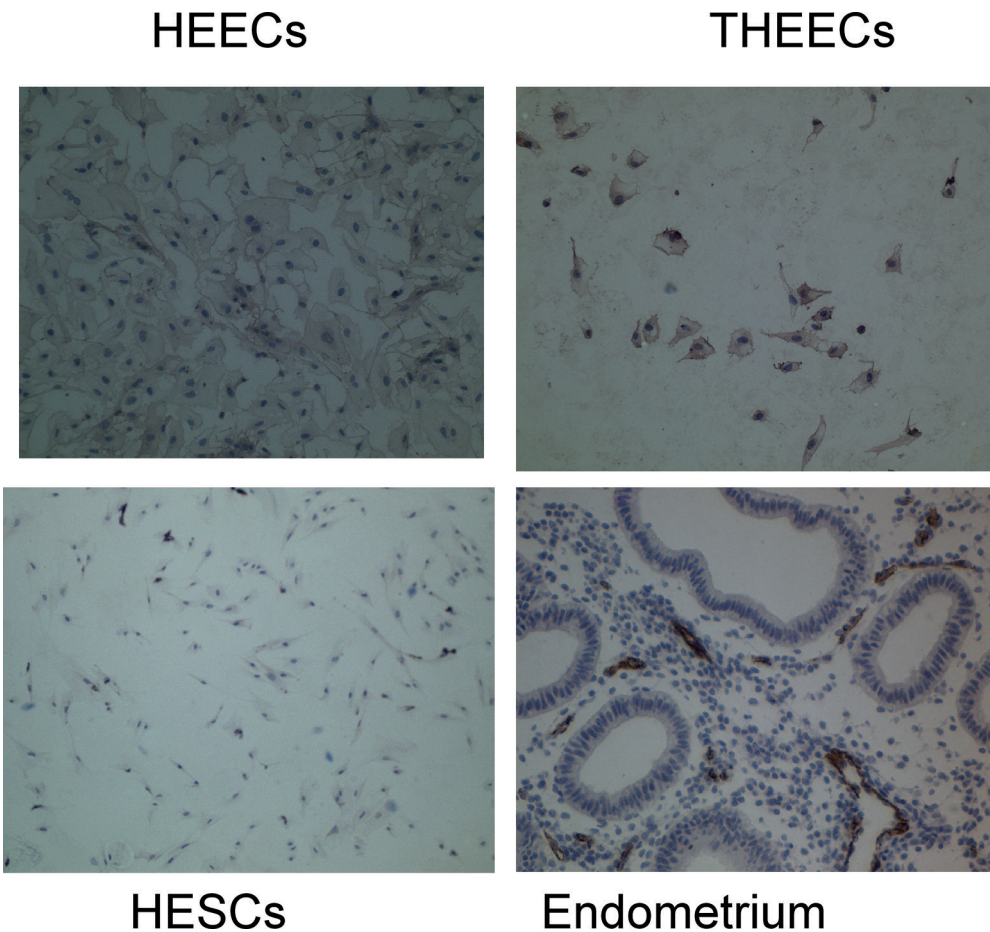
HESCs   Endometrium

Fig. 3. Immunocytochemistry: Control (HEECs) or immortalized endometrial endothelial cells (T-HEECs) or cultured human endometrial stromal cells (HESCs) or an endometrial section were immunostained for CD 31 and detected by peroxidase reactivity (brown). The cells were counterstained with hematoxylin (magenta). x 20

Table 2. Microarray analysis. Expression of matrix metalloproteases and inhibitors expressed by control HEECs or immortalized T-HEECs. The expression levels depicted are normalized to ß-actin.

HEECs NORMALIZED	T-HEECs NORMALIZED	ABBREVIATION	GENBANK	DESCRIPTION
1.0259563	1.3115485	MMP-1	NM_002421	*Homo sapiens* matrix metalloproteinase 1 (interstitial collagenase; fibroblast collagenase)
0.88636935	1.151058	NMP-2	NM_004530	*Homo sapiens* matrix metalloproteinase 2 (gelatinase A, 72kDa type IV collagenase)
Absent	Absent	MMP-3	NM_002422	*Homo sapiens* matrix metalloproteinase 3 (stromelysin 1, progelitinase)
0.9516322	3.6750405	MMP-10	NM_002425	*Homo sapiens* matrix metalloproteinase 19 (stromelysin 2, hydrolase activity)
0.9939604	1.0757039	TIMP-1	NM_003254	*Homo sapiens* tissue inhibitor of metalloproteinase 1
0.98578143	0.98087215	TIMP-2	NM_003255	*Homo sapiens* tissue inhibitor of metalloproteinase 2

Fig. 4. mRNA Expression of MMP-1: The expression of MMP-1 was compared for control (HEECs) or immortalized endometrial endothelial cells (T-HEECs) by real time quantitative RT-PCR. The expression of the transcripts is expressed as a ratio to ß-actin used as an internal control (n=3)

Fig. 5. Protein Expression of MMPs and MMP-inhibitors: HEECs or THEECs were grown to confluence and the media was switched to DM for 6 hours. ELISAs were conducted on the collected medium (n=3).
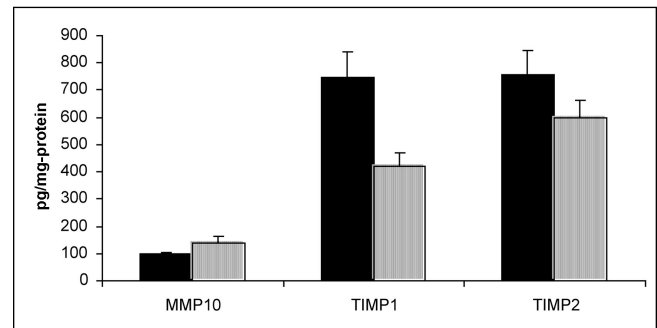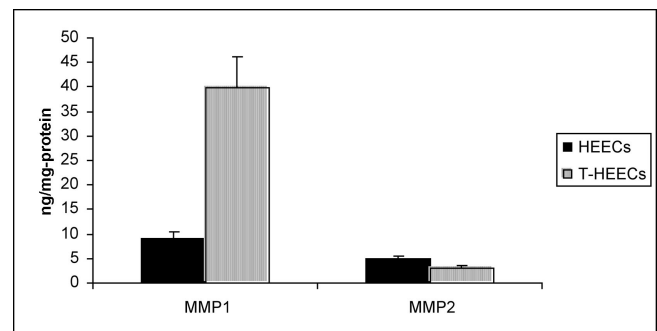
the presence of MMP1 (interstitial collagenase); MMP-2 (gelatinase 72kD type); and MMP-10 (collagen catabolism (stromelysin 2). The expression of the classic MMP inhibitors TIMP-1 and TIMP-2 were also observed in the parent or immortalized HEECs (Table 2). By contrast no expression of MMP3 was demonstrated in either the parent or the immortalized HEECs (Table 2). This is particularly interesting as previous studies demonstrated high levels of both MMP-1 and MMP3 expression in endometrial stromal cells (Schatz et al., 1994; Lockwood et al., 1998).

Q-RT-PCR

Since MMP-1 was commonly expressed by HEECs, T-HEECs and endometrial stromal cells, we compared the mRNA level of expression by these cultured cells utilizing Q-RT-PCR. As can be seen in Figure 4, similar levels of MMP-1 mRNA were observed for all three cell types.

ELISAs

To determine the expression of MMPs and TIMP at the protein level, multiplex sandwich ELISAs were carried out on parent or immortalized HEECs. Figure 5 demonstrates that MMP-1 has the highest level of expression and MMP-10 has the lowest level of expression. While MMP-1 expression is somewhat higher in the THEECs, the overall level of protein expression for the MMPs and for the TIMPs is similar for both parent and immortalized cells with MMP-1 and MMP-2 being expressed in the nanogram range (top panel) and MMP-10 and TIMPs being expressed in the picogram range (bottom panel).

Discussion

The matrix metalloproteases degrade the bulk of the extracellular components during endometrial tissue remodeling. Based on substrate specificity, the MMPs are grouped into several groups including the collagenases, which regulate ECM turnover by degrading interstitial collagens, the gelatinases, which degrade basement membrane collagens and the

stromelysins, which degrade such diverse proteins as proteoglycans, glycoproteins, fibronectin, and laminin, and can cleave the globular domain of interstitial (type III) and basement membrane collagen type IV and V (Woessne Jr, 1991; Johnson et al., 1998). Initial experiments from our laboratory identified the expression and regulation of the MMPs in the stromal/decidual cells. These were believed to be regulated by the hormonal milieu of the endometrium to play an integral role in implantation by modulating migration of the invading trophoblast (Schatz et al., 1994). We now demonstrate that the endometrial endothelial cells also express MMPs and their inhibitors, although they produce a different pattern of expression. Since in humans, the trophoblast needs to ultimately invade the maternal circulation in order to survive, it is possible that part of the control of this invasion occurs at the level of the endothelial cell itself.

In addition, pathologic angiogenesis such as that observed with aberrant endometrial bleeding due to progestin only contraception or hormone replacement therapy may be in part due to a shift in the expression of the MMPs. Further studies will be necessary to ascertain the function of the endothelial MMPs in physiologic and pathologic angiogenesis. To facilitate studies with human endometrial endothelial cells we immortalized these cells by expressing hTert, the catalytic subunit of telomerase. While all endothelial cells display classic markers such as von Willebrand factor (VwF) and PECAM (CD31) studies of endothelial cells from different tissues have shown that they display variations in morphology, intercellular junctions, cell surface and growth properties as well as in production of basal lamina components, both in vivo and in vitro (Sage et al., 1981). The findings that the immortalized HEECs appear to maintain the repertoire of classic endometrial endothelial cell markers, morphology and function suggest that this may be a novel and ideal system for future *in vitro* studies.

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