Characterization of endoglin on mouse uterine stromal cells

M.-J. Ouellette^{1,2}, L. Hamel³, N. Tremblay³, M. Lamothe¹ and S. St-Jacques^{2,4*}

¹Unité de Recherche en Ontogénie et Reproduction, Centre de Recherche du Centre Hospitalier, Universitaire de Québec, Pavillon CHUL, Ste-Foy, Québec, Canada G1V 4G2; ²Centre de Recherche en Biologie de la Reproduction, Université Laval, Ste-Foy, Québec, Canada;

³Centre de Recherche Guy-Bernier, Hôpital Maisonneuve-Rosemont, Montréal, Québec, Canada H1T 2M4; and ⁴Département d'Obstétrique et Gynécologie, Université Laval, Ste-Foy, Québec, Canada

During the oestrous cycle and early pregnancy, the uterus undergoes a variety of morphological and physiological modifications involving uterine cell proliferation and differentiation as well as extensive tissue remodelling. Transforming growth factor β (TGF- β) has powerful effects on these events and thus is thought to have a critical role in uterine physiology. Endoglin is a transmembrane glycoprotein that binds TGF- β 1 and - β 3 and interacts with TGF- β signalling receptors to modulate many effects of this growth factor in different types of cell. Studies in mice revealed the highest concentrations of endoglin in the reproductive tract, notably on stromal cells of cyclic and pregnant uteri. The objective of the present study was to investigate the role of endoglin expressed on uterine stromal cells in binding TGF- β and in the cellular responses induced by this growth factor. Highly purified populations of uterine stromal cells were isolated by cell affinity to the monoclonal antibody MJ7/18, which is specific to mouse endoglin. Affinity labelling of these cells with ¹²⁵I-labelled TGF- β followed by immunoprecipitation with endoglin-specific polyclonal 1256:4b antiserum indicated that endoglin expressed at the surface of uterine stromal cells binds TGF- β 1 and interacts with TGF- β signalling receptors. Treatment of uterine stromal cells with different concentrations of TGF- β 1 induced a biphasic proliferative response and addition of MJ7/18 as well as neutralizing TGF- β antibodies showed endoglin to be a modulator of TGF- β -induced stromal cell proliferation. Given the importance of TGF- β in the regulation of uterine physiology, these results indicate a role for endoglin during uterine tissue remodelling and decidualization.

Introduction

The endometrium is a dynamic tissue that responds to steroids, cytokines and growth factors to accommodate, regulate and sustain developing embryos. Among these cytokines and growth factors, transforming growth factor β (TGF- β) appears to be critical to endometrial functions (for review see Godkin and Doré, 1998). TGF- β and its receptors are present in both cyclic and pregnant uteri in different species (Tamada *et al.*, 1990; Manova *et al.*, 1992; Chegini *et al.*, 1994; Roelen *et al.*, 1994; Tang *et al.*, 1994; Das *et al.*, 1997; Ando *et al.*, 1998). Cyclic growth and tissue remodelling of the uterus occur throughout reproductive life during the oestrous cycle and trophoblastic invasion (Iwahashi *et al.*, 1996). TGF- β stimulates DNA synthesis in endometrial stromal cells, whereas it inhibits uterine epithelial cell proliferation (Rotello *et al.*, 1991; Hammond *et al.*, 1993; Marshburn *et al.*, 1994; Tang *et al.*, 1994; Dumont *et al.*, 1995). Tissue remodelling involves controlled enzymic degradation of extracellular matrix (ECM) (Bischof *et al.*, 1993; Rodgers *et al.*, 1994) as well as dynamic

temporal and spatial patterns of integrin expression, mirroring the changes in ECM proteins (Tabibzadeh, 1992; Lessey *et al.*, 1994). TGF- β stimulates the synthesis of laminin and collagen IV by endometrial stromal cells (Ueda *et al.*, 1992) and induces the expression of the α 1 β 1 collagen-laminin receptor at the surface of these cells (Grosskinsky *et al.*, 1996). TGF- β produced in the uterine stroma under the influence of progesterone mediates the suppression of epithelial metalloproteinases (Bruner *et al.*, 1995). Similarly, TGF- β 1 significantly influences the plasminogen-activating system in endometrial stromal cells (Ueda *et al.*, 1992; Sandberg *et al.*, 1997; Casslén *et al.*, 1998). During implantation, endometrial TGF- β 1 regulates the invasion of trophoblastic cells by modulation of their proteolytic activity and by promoting differentiation of invasive trophoblast into non-invasive multinucleated giant cells (Graham and Lala, 1992). Furthermore, TGF- β is a key molecule for the local suppression of immune effector cells in the uterus and thus for protection of the fetoplacental unit from rejection (Clark *et al.*, 1990).

TGF- β exerts its biological effects by binding to highly specific cell surface receptors including type I and type II receptors (T β RI and T β RII), as well as the type III receptor betaglycan (for review see Miyazono, 1997). T β RI and T β RII are related transmembrane serine-threonine kinases that

*Correspondence address: Ontogénie et Reproduction, Centre de Recherche du Centre Hospitalier, Universitaire de Québec, Pavillon CHUL, 2705 boul. Laurier, Ste-Foy, Québec, Canada G1V 4G2.

Received 20 November 1998.

associate in the presence of ligand to mediate TGF- β signals (Wrana *et al.*, 1992). Betaglycan, a membrane-anchored proteoglycan without an intracellular kinase domain, presents the ligand to T β RII, thus enhancing cell responsiveness (Lopez-Casillas *et al.*, 1991, 1993). TGF- β first binds to T β RII, which is constitutively autophosphorylated. T β RI is then recruited into the complex and phosphorylated by T β RII to initiate downstream signalling cascades (Ventura *et al.*, 1994; Wrana *et al.*, 1994; Chen *et al.*, 1995). Activated T β RI phosphorylates Smads (*Drosophila*, mothers against dpp homologues) proteins, a family of downstream targets, which translocate to the nucleus in which they may induce gene transcription (Massagué, 1996; Heldin *et al.*, 1997).

Endoglin, also known as CD105 (Letarte *et al.*, 1994), is a homodimeric transmembrane glycoprotein that is structurally related to betaglycan and binds TGF- β 1 and - β 3 with high affinity (Gougos and Letarte, 1990; Lopez-Casillas *et al.*, 1991; Cheifetz *et al.*, 1992). In the presence of ligand, endoglin forms heteromeric complexes with T β RI and T β RII and, despite the absence of signalling motif, modulates cellular responses to TGF- β in different types of cell (Yamashita *et al.*, 1994; Lastres *et al.*, 1996; Caniggia *et al.*, 1997). Transfection of endoglin cDNA into the U-937 monocytic line totally abrogated the inhibition of cellular proliferation and the associated increase in *c-myc* transcript induced by TGF- β 1 (Lastres *et al.*, 1996). Exposure of human villous explants from first trimester pregnancy to endoglin monoclonal antibody 44G4, neutralizing TGF- β antibodies, or endoglin antisense oligonucleotides, stimulated trophoblast cell outgrowth and migration (Caniggia *et al.*, 1997). The discovery that endoglin is the gene for hereditary haemorrhagic telangiectasia type 1, an autosomal dominant disorder characterized by multisystemic vascular dysplasia and recurrent haemorrhage (McAllister *et al.*, 1994), confirms the importance of this molecule in the mediation of TGF- β effects *in vivo*. These findings identify endoglin as an important accessory protein of the TGF- β receptor complex.

Although endoglin is characteristically expressed on vascular endothelial cells (Gougos and Letarte, 1988; O'Connell *et al.*, 1992; Letarte *et al.*, 1994), estimation of its relative concentration in mouse tissues by semi-quantitative RT-PCR revealed highest expression in tissues of the reproductive tract, notably in the uterus in which endoglin co-localizes with TGF- β secreted in the stroma (St-Jacques *et al.*, 1994). Given the importance of TGF- β in uterine physiology (Flanders *et al.*, 1991; Godkin and Doré, 1998) and the regulatory functions attributed to endoglin in the mediation of cell responses induced by TGF- β (Lastres *et al.*, 1996; Caniggia *et al.*, 1997), the purpose of this study was to determine whether endoglin expressed by mouse uterine stromal cells interacts with TGF- β receptors and participates in cell proliferation induced by TGF- β .

Materials and Methods

Animals

Randomly bred female CD1 mice at 5–10 weeks of age (Charles River Laboratories, St Constant, Québec) were used

to provide uterine cells. Experiments were carried out in accordance with the procedures described in the guidelines for the care and use of laboratory animals as approved by the institution's animal care committee.

Isolation and culture of uterine cells

Cells were isolated from mouse uterine horns by a combination of enzymic and mechanical dispersion techniques adapted from protocols described in the literature (Chegini *et al.*, 1992; Hammond *et al.*, 1993; Zhang *et al.*, 1995). Mice were killed by CO₂ inhalation, and uterine horns were excised and opened into Dulbecco's modified Eagle medium: nutrient mixture F12 (D-MEM/F-12) (Gibco BRL, Burlington, Ontario). Epithelial cells were recovered by enzymic digestion (60 min at 37°C) with 1 mg collagenase type I ml⁻¹ (Sigma, St Louis, MO) in D-MEM/F12. A second enzymic digestion was performed by adding fresh collagenase solution to the tissues and shaking (140 rpm) for 30 min at 37°C. The resulting suspension consisted of epithelial and stromal cells. Stromal cells were recovered by crushing the remaining tissues on a metal sieve (200 μ m). Cells were resuspended in D-MEM/F12 supplemented with 10% fetal bovine serum (FBS) (PDI Bioscience, Aurora, Ontario) and 1% (v/v) penicillin (5000 U)–streptomycin (5 mg ml⁻¹) solution (Sigma), and cultured at 37°C in a humidified atmosphere of 5% CO₂.

Characterization of uterine cells

Epithelial and stromal cells were seeded at 1.5 and 2.0 $\times$ 10⁴ cells per well, respectively, in tissue culture chamber slides (Nunc, Napierville, Québec) and allowed to attach for 48 h. Slides were washed with Dulbecco's PBS (DPBS) and cell membranes were permeabilized by a 5 min incubation in a solution of acetone:methanol (1:1). After washes with DPBS, slides were incubated for 2 h at 37°C with polyclonal rabbit anti-mouse vimentin or rabbit anti-human panCytokeratin (ICN, Montréal, Québec) diluted 1:25 in PBS. Normal rabbit serum was used as a control. The cells were washed in PBS and incubated for 60 min at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) diluted 1:25 in PBS. Slides were washed with PBS and immunostaining was observed using a fluorescence microscope.

Preparation of endoglin-positive uterine stromal cell populations

Stromal cells isolated by enzymic digestion were cultured for 3 days in D-MEM/F12 supplemented with 10% FBS. Near-confluent monolayers were detached with trypsin–EDTA (Gibco BRL), washed twice and resuspended in PBS (5 $\times$ 10⁶ cells ml⁻¹). Cells were then incubated for 30 min at 4°C with MJ7/18 hybridoma supernatant containing mouse endoglin monoclonal antibody (mAb) (Stanford University School of Medicine, Stanford, CA; Ge and Butcher, 1994).

The cells were washed in PBS and incubated for 20 min at 4°C with FITC-conjugated goat anti-rat IgG (8 µg ml⁻¹) (Sigma). After incubation, the cells were washed, resuspended in PBS (5 × 10⁶ cells ml⁻¹), and sorted by flow cytometry. Endoglin-positive stromal cells were recovered and maintained in D-MEM/F12 supplemented with 10% FBS. These cells were used for affinity labelling and proliferation assays after a first passage.

TGF-β affinity labelling and immunoprecipitation

Affinity labelling was performed as described by Mitchell *et al.* (1992). Briefly, endoglin-positive cells were seeded at 2 × 10⁵ cells per well in a 12-well plate. Near-confluent cell monolayers were washed for 30 min with ice-cold binding buffer (DPBS with Mg²⁺ and Ca²⁺, pH 7.4, containing 0.1% BSA; Sigma) and incubated on ice for 4 h with 100 pmol [¹²⁵I]-labelled TGF-β1 I⁻¹ (Mandel, Guelph, Ontario) with or without 4 nmol competing unlabelled TGF-β1 I⁻¹. The receptor-ligand complexes were crosslinked with 2 mmol bis(sulfosuccinimidyl)suberate I⁻¹ (Pierce, Rockford, IL). Cells were washed with DPBS and solubilized in a buffer containing 1% (v/v) Triton-X100, 10% glycerol, 1 mmol EDTA I⁻¹, 20 mmol Tris-HCl I⁻¹, pH 7.4, and a mixture of proteolytic inhibitors containing 1 mmol phenylmethylsulfonylfluoride I⁻¹, 20 µg aprotinin ml⁻¹, 20 µg soybean trypsin inhibitor ml⁻¹ and 25 mmol benzamidine I⁻¹ (Boehringer Mannheim, Laval, Québec). Cell lysates were either analysed directly by SDS-PAGE or were immunoprecipitated overnight with 40 µg rabbit polyclonal antibody 1256:4b raised against human endoglin (Hospital for Sick Children, Toronto, Ontario) (Gougos and Letarte, 1990) or preimmune serum. Immune complexes were precipitated for 2 h at 4°C with protein G-Sepharose (Pharmacia, Baie d'Urfé, Québec) slurry consisting of 50% packed beads in DPBS and 1% (v/v) Triton-X100. Extracts and radiolabelled endoglin complexes were fractionated on 4–12% SDS-PAGE under non-reducing and reducing conditions and visualized by autoradiography.

[³H]thymidine incorporation assay

Proliferation of endoglin-positive uterine stromal cells in response to TGF-β was studied by evaluating [³H]thymidine incorporation into trichloroacetic acid-precipitable material using a method adapted from protocols for human endometrial stromal cells (Tang *et al.*, 1994; Dumont *et al.*, 1995). Cells were seeded at 2.5 × 10⁴ cells per well in 96-well microtitre culture plates (Falcon, Toronto, Ontario) in D-MEM/F12 supplemented with 10% FBS. At 24 h after plating, cells were washed with Mg²⁺- and Ca²⁺-free PBS and incubated in 2% FBS-supplemented D-MEM/F12 medium in the presence of different concentrations of TGF-β1 (0–10 ng ml⁻¹) with or without 15 µg per well of mAb MJ7/18, Pan specific TGF-β neutralizing antibody (R&D Systems, Minneapolis, MN), or non-immune rat IgG (Sigma). After 48 h, 0.2 µCi [³H]thymidine (NEN, Mississauga, Ontario) was added to each well and the incubation was continued for a further 18–24 h. Cells were then detached with

trypsin-EDTA, harvested onto filter paper discs with a multiple cell harvester (Fisher Scientific Ltd, Neapan, Ontario), and the incorporated radioactivity was measured by liquid scintillation counting. Two assays were performed in triplicate using three different cell preparations.

Statistical analysis

Increases in stromal cell proliferation induced by TGF-β1 were analysed individually using *t* tests. A modified *t* test (in which variances are pooled) was used to compare unilaterally, at each concentration of TGF-β1 tested, differences in the increase in cell proliferation between groups with and without antibodies. The same test was also performed to confirm the stimulatory effect of MJ7/18 antibody at a TGF-β1 concentration of 2 ng ml⁻¹.

Results

Isolation of uterine cells expressing endoglin

A protocol derived from established procedures (Chegini *et al.*, 1992; Hammond *et al.*, 1993; Zhang *et al.*, 1995) was used to prepare primary cultures of uterine cells from mature randomly cyclic mice. Uterine epithelial cells were recovered after a first digestion step with collagenase. Cells attached over a period of 72 h after plating and showed a typical cuboidal morphology (Fig. 1a). They were specifically stained with antibody directed against cytokeratin (Fig. 1c), which is a marker for epithelial cells (Chegini *et al.*, 1992; Zhang *et al.*, 1995). Stromal cells were released after additional enzymic digestion followed by grinding of the remaining uterine tissue. Live cells attached to the culture dishes within 18 h, whereas other contaminating cells, such as dead cells and epithelial cells, were eliminated by replacement of the culture medium. These cells showed a typical fibroblast-like morphology (Fig. 1b) and expressed high concentrations of vimentin (Fig. 1d), a marker for fibroblastic cells (Chegini *et al.*, 1992; Mani *et al.*, 1992; Mulholland *et al.*, 1992). They did not express cytokeratin. At the time of plating, both types of cell showed a cell viability > 95%.

Flow cytometric analysis of viable stromal cell populations (Fig. 2a) revealed that approximately 60% of these cells express endoglin at their surface, as determined by labelling with mAb MJ7/18, which is specific to mouse endoglin (Fig. 2b). Highly purified populations of endoglin-positive uterine stromal cells were then isolated by cell sorting (Fig. 2c) to perform functional studies. Antibodies bound to the cell surface were released after a few hours in culture and endoglin expression was stable for up to 10 days, as determined by flow cytometry using mAb MJ7/18 (data not shown). The doubling time of these cells was 48 h when seeded at 1 × 10⁴ cells cm⁻². The populations of endoglin-positive cells showed a typical fibroblast-like morphology, expressed high concentrations of vimentin, and did not contain any macrophages or endothelial cells, which are both endoglin-expressing cells, as confirmed by immunostaining

Fig. 1. Isolation and culture of mouse uterine cells. Epithelial cells were recovered after enzymic digestion of mouse uterine horns with collagenase type I. Stromal cells were isolated after an additional enzymic digestion followed by grinding and sieving of the remaining uterine tissues. After a few days in culture, epithelial cells showed a typical cuboidal morphology (a), while stromal cells showed a fibroblast-like morphology (b). Characterization of both types of cell by immunocytochemistry with antibodies specific to cytokeratin and vimentin revealed the expression of cytokeratin by epithelial cells (c) and vimentin by stromal cells (d). Scale bars represent 200 μm (a, b) and 50 μm (c, d).

with antibodies specific for F4/80 antigen and von Willebrand factor, respectively (data not shown).

Expression of endoglin at the surface of uterine stromal cells and binding of TGF- β 1

Endoglin-positive uterine stromal cells were affinity labelled with ^{125}I -labelled TGF- β 1 and TGF- β -receptor complexes were analysed electrophoretically after chemical crosslinking and cell membrane solubilization. Autoradiograms of labelled complexes analysed by SDS-PAGE under reducing conditions (Fig. 3a, lane T) revealed four major bands of apparent relative molecular masses of 49, 67, 80–90 and > 150 kDa. Competition experiments using unlabelled TGF- β 1 confirmed the specificity of the labelling of the 67, 80–90 and > 150 kDa complexes (Fig. 3b, lane T). The apparent molecular mass, binding specificity, and migration pattern of these binding complexes are characteristic of T β RI, T β RII and betaglycan (Massagué and Like, 1985; Cheifetz *et al.*, 1986; Cheifetz *et al.*, 1988). The similar intensity of the 49 kDa band in the presence of an excess of unlabelled ligand indicated that

this radiolabelled complex was not specific. Immunoprecipitation of affinity labelled complexes with the polyclonal anti-human endoglin serum 1256:4b, previously shown to react with mouse endoglin (St-Jacques *et al.*, 1994), was performed after affinity crosslinking to determine whether TGF- β complexes contained endoglin. The antiserum precipitated a major radiolabelled band of 167 kDa in non-reducing conditions (Fig. 3a, line NR), which migrated as an affinity labelled band of 96 kDa after reduction (Fig. 3a, line R). Faint bands, likely to correspond to T β RII and T β RI, co-immunoprecipitated with endoglin in the presence of ligand (Fig. 3a, lines NR and R). These complexes are not detected in the presence of an excess of unlabelled ligand (see Fig. 3b, lines R and NR). Free ^{125}I -labelled TGF- β 1 migrated at 26 kDa and non-specific labelling was observed at the top of the gels.

Role of endoglin in the proliferation of uterine stromal cells in response to TGF- β 1

Uterine stromal cell proliferation was measured using a [^3H]thymidine incorporation assay. Results were expressed

Fig. 2. Flow cytometric analysis of mouse uterine stromal cell population with monoclonal antibody (mAb) MJ7/18. A viable stromal cell population isolated by collagenase digestion (a) was immunolabelled with mAb MJ7/18 specific to mouse endoglin. R1 represents the selected cell population. Approximately 60% of these cells expressed endoglin on the surface (b) as determined by incubation with rat anti-mouse endoglin followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG. Sorting of these cells allowed the isolation of a highly purified population (> 99%) of endoglin-positive uterine stromal cells (c). Cells that did not express endoglin are in the lower left-hand box (b,c). PE: phycoerythrin.

as a percentage increase compared with cell proliferation in the absence of TGF- β 1 and statistical analyses were performed on these values to take into consideration variations in basal cell proliferation rate among the different cell populations studied. The results are summarized (Fig. 4). A biphasic curve of cell proliferation was observed in response to TGF- β 1 (Fig. 4a). [3 H]thymidine incorporation into DNA was significantly increased by approximately 20% after treatment with 0.02 ng TGF- β 1 ml $^{-1}$ ($P < 0.025$). Although TGF- β 1 significantly stimulated cell proliferation at 0.2 ng ml $^{-1}$ ($P < 0.05$), great variability was observed between responses in the three different cell preparations. At a concentration of 2 ng ml $^{-1}$, TGF- β 1 had no significant effect on cell proliferation. Thymidine incorporation was stimulated by approximately 20% at a concentration of 5 ng ml $^{-1}$ ($P < 0.025$) and by approximately 60% at 10 ng ml $^{-1}$ ($P < 0.005$).

mAb MJ7/18 was added to endoglin-positive cells in the presence of the ligand to determine whether endoglin had a role in the proliferation of uterine stromal cells in response to TGF- β 1 (Fig. 4b). In the presence of mAb MJ7/18, the increase in DNA synthesis induced by TGF- β 1 at concentrations of 0.02 ($P < 0.005$) and 10.0 ng ml $^{-1}$ ($P < 0.025$) was significantly inhibited. Conversely, at 2 ng ml $^{-1}$, TGF- β 1 had no effect, whereas the addition of anti-endoglin significantly increased the rate of thymidine incorporation ($P < 0.025$). The specificity of the action of TGF- β on [3 H]thymidine incorporation was confirmed by using TGF- β neutralizing antibodies ($P < 0.01$ and 0.05). Since the expression of both T β RII and endoglin shows cyclic variation in the uterus (Takahashi *et al.*, 1994; M-J. Bergeron, C. Brown, M-J. Ouellette and S. St-Jacques, unpublished), high standard deviations result from the comparison of cell populations isolated from randomly cyclic mice.

Effect of TGF- β 1 on the expression of endoglin at the surface of uterine stromal cells

Since the expression of endoglin is stimulated by TGF- β 1 on activated monocytes (Lastres *et al.*, 1996) and bone marrow stromal cells (Robledo *et al.*, 1998), its expression at the surface of uterine stromal cells treated with different concentrations of TGF- β 1 was verified by flow cytometric analysis. Cells were incubated with TGF- β 1 for 48 h and the expression of endoglin was studied using mAb MJ7/18 and FITC-conjugated anti-rat IgG, as described for the preparation of endoglin-positive uterine stromal cells. Flow cytometric analysis showed that incubation with 0–5 ng TGF- β 1 ml $^{-1}$, concentrations known to stimulate the expression of endoglin in other types of cell (Lastres *et al.*, 1996; Robledo *et al.*, 1998), for 48 h did not affect either the percentage of endoglin-positive cells or the expression of endoglin (Fig. 5).

Discussion

Unlike most normal adult tissues, cyclic growth and remodelling occur within the uterus. Luminal and glandular epithelial cells, stromal cells and endothelial cells undergo

Fig. 3. Analysis of ^{125}I -labelled transforming growth factor β (TGF- β) affinity labelled membrane proteins of mouse uterine stromal cells. Endoglin-positive uterine stromal cells were affinity labelled with ^{125}I -labelled TGF- β 1 and TGF- β -receptor complexes were analysed electrophoretically after chemical crosslinking. Endoglin- ^{125}I -labelled TGF- β 1 complexes were identified after immunoprecipitation with polyclonal anti-human endoglin serum. Autoradiograms of Triton-X100 extracts of affinity labelled cells analysed by SDS-PAGE under reducing conditions (a, lane T) revealed four major bands of apparent relative molecular masses of 49, 67, 80–90 and > 150 kDa. In the presence of unlabelled TGF- β 1, radiolabelled complexes of 67, 80–90 and > 150 kDa have been competed for by the specific ligand (b, lane T), and are likely to correspond to type I transforming growth factor β receptor (T β RI), T β RII and betaglycan (B). The 49 kDa radiolabelled band that has not been competed for is not specific for the ligand. Non-specific labelling was observed at the top of the gels. Polyclonal endoglin antiserum precipitated a major radiolabelled band of 167 kDa under non-reducing conditions (a, lane NR) and of 96 kDa after reduction (a, lane R). Faint bands, probably corresponding to T β RII and T β RI, co-immunoprecipitated with endoglin in the presence of ligand (a, lanes NR and R). These complexes are not detected in the presence of excess unlabelled ligand (b, lanes R and NR) confirming their specificity for TGF- β 1. Free ^{125}I -labelled TGF- β 1 migrated at 26 kDa.

periods of proliferation, migration, differentiation and apoptosis. TGF- β has potent effects on these processes and is thus thought to play a pivotal role in uterine physiology (Flanders *et al.*, 1991; Shull and Doetschman, 1994; Godkin and Doré, 1998). Endoglin, a TGF- β -binding membrane glycoprotein, is highly expressed in mouse uterine stroma (Gougos and Letarte, 1990; Cheifetz *et al.*, 1992; St-Jacques *et al.*, 1994). The present study demonstrated that endoglin is expressed at the surface of uterine stromal cells, binds TGF- β 1, interacts with signalling TGF- β receptors and participates in the proliferation of stromal cells induced by this growth factor.

The specific localization of endoglin enabled the development of a highly reproducible and straightforward technique for the isolation and culture of endoglin-positive uterine stromal cells suitable for functional studies. In the mouse uterus, endoglin is expressed in the stroma, whereas epithelial and muscular cells are totally negative (St-Jacques *et al.*, 1994). Freshly isolated uterine epithelial and stromal cells showed characteristic morphology and immunocytochemical staining for intermediate filaments (Chegini *et al.*, 1992; Zhang *et al.*, 1995). However, after 3 days

in culture, immunocytochemical analysis of stromal cell populations revealed that only 60% of cells expressed endoglin at the surface. Contaminating epithelial cells, usually estimated as approximately 5% (Bigsby *et al.*, 1986; Jacobs and Carson, 1991; Chegini *et al.*, 1992), are unlikely to account for this relatively high proportion of endoglin-negative cells. Indeed, immunohistochemical studies revealed that the expression of endoglin varies during the oestrous cycle (M.-J. Bergeron, C. Brown, M.-J. Ouellette and S. St-Jacques, unpublished). Since randomly cyclic mice were used for the isolation of uterine cells, it is likely that endoglin-low or endoglin-negative stromal cells isolated from mice in dioestrus, proestrus or metoestrus account for the lower than expected proportion of endoglin-positive cells observed after a few days in culture. It is critical that homogeneous populations of endoglin-positive uterine stromal cells are used in functional studies. Therefore, highly purified populations of stromal cells expressing endoglin were prepared by affinity to the monoclonal antibody MJ7/18, which is specific for mouse endoglin (Ge and Butcher, 1994).

Affinity labelling of endoglin-expressing stromal cells

Fig. 4. [3H]thymidine incorporation into the DNA of endoglin-positive mouse uterine stromal cells after treatment with transforming growth factor β1 (TGF-β1) in the presence or absence of antibodies specific to mouse endoglin or TGF-β. (a) A biphasic curve of cell proliferation was observed in response to TGF-β1. Thymidine incorporation was significantly increased by approximately 20% after incubation with TGF-β1 at concentrations of 0.02 ($P < 0.025$) and 0.2 ng ml⁻¹ ($P < 0.05$). No stimulation was observed at 2.0 ng ml⁻¹. Thymidine incorporation was stimulated by approximately 20% at a concentration of 5 ng TGF-β1 ml⁻¹ ($P < 0.025$) and by approximately 60% at 10 ng TGF-β1 ml⁻¹ ($P < 0.005$). (b) Increases in DNA synthesis induced by TGF-β1 at concentrations of 0.02 and 10.0 ng ml⁻¹ were significantly inhibited by mAb MJ7/18 ($P < 0.005$ and 0.025) and TGF-β neutralizing antibodies ($P < 0.01$ and 0.05). At 2 ng ml⁻¹, TGF-β1 had no effect, while the addition of anti-endoglin significantly increased the rate of thymidine incorporation ($P < 0.025$). Values represent means ± SEM; * $P \leq 0.05$, ** $P \leq 0.01$. □, TGF-β1; ■, TGF-β1 + mAb MJ7/18; ▨, TGF-β1 + anti-TGF-β.

using ¹²⁵I-labelled TGF-β1 revealed the presence of TβRI, TβRII and betaglycan. Immunoprecipitation of total cell lysates with polyclonal anti-endoglin serum indicated that endoglin expressed by uterine stromal cells binds TGF-β1 and interacts with TβRI and TβRII. Endoglin-¹²⁵I-labelled

TGF-β1 complexes migrated to endoglin monomer and dimer immunoprecipitated from TGF-β1 affinity labelled mouse fibroblast NCTC-2071 cells analysed under reducing and non-reducing conditions, respectively (St-Jacques *et al.*, 1994). Monomeric endoglin migrated slightly above TβRII and

Fig. 5. Flow cytometric analysis of endoglin expression at the surface of mouse uterine stromal cells after treatment with different concentrations of transforming growth factor β 1 (TGF- β 1). Freshly isolated stromal cells were incubated with increasing concentrations of TGF- β 1 for 48 h and the expression of endoglin was evaluated by flow cytometry using mAb MJ7/18. Although no staining is observed with control non-immune rat IgG (a), approximately 60% of uterine stromal cells expressed endoglin on their surface (b). Treatment of uterine stromal cells with (c) 0.0, (d) 1.0, (e) 2.0 and (f) 5.0 ng TGF- β 1 ml $^{-1}$ did not affect the expression of endoglin. M1 represents the area of relative fluorescence intensity corresponding to cells expressing endoglin, as determined by comparing the pattern of non-specific fluorescence after incubation of cells with negative control (rat IgG) with that after incubation with anti-endoglin (MJ7/18).

was visualized best by comparing the specific non-reduced and reduced immunoprecipitates. Furthermore, co-immunoprecipitation of faint bands corresponding to T β RI and T β RII indicated that endoglin and the TGF- β signalling receptors interact with each other on the surface of uterine stromal cells in the presence of ligand, as reported for pig endothelial cells (Yamashita *et al.*, 1994), human bone marrow stromal cells (Robledo *et al.*, 1996) and pre-B leukaemic cells (Zhang *et al.*, 1996). These results indicate that endoglin on uterine stromal cells may function as a modulator of the effects of TGF- β , as demonstrated in U-937 monocytic cells (Lastres *et al.*, 1996) and invading cytotrophoblast (Caniggia *et al.*, 1997).

T β RI, T β RII and betaglycan are synthesized and expressed by uterine stromal and epithelial cells in cyclic uterus and decidua of different species (Chegini *et al.*, 1994; Roelen *et al.*, 1994; Tang *et al.*, 1994; Das *et al.*, 1997; Ando *et al.*, 1998). Five

TGF- β binding proteins have been identified on human endometrial stromal cells (Dumont *et al.*, 1995). However, although the presence of T β RI, T β RII, and betaglycan as well as two glycosyl-phosphatidylinositol (GPI)-anchored proteins were reported by Dumont *et al.* (1995), endoglin- 125 I-labelled TGF- β complexes were not detected. Since endoglin is expressed in the stroma of human endometrium (S. St-Jacques, P. DesRosiers, C. Nielly, M. Lamothe and D. Provencher, unpublished), the absence of TGF- β -labelled endoglin complexes might be explained by the fact that endoglin could not be readily detected in total cell lysates without immunoprecipitation (Cheifetz *et al.*, 1992). After chemical crosslinking, endoglin-TGF- β 1 complexes co-migrated with T β RII under reducing conditions and with the 180 kDa GPI-anchored protein under non-reducing conditions (Cheifetz *et al.*, 1992; Dumont *et al.*, 1995; Robledo *et al.*, 1996). Since endoglin, T β RI and T β RII were co-

precipitated by antibodies to either endoglin or T β R II in the presence of ligand in other types of cell (Yamashita *et al.*, 1994; Robledo *et al.*, 1996; Zhang *et al.*, 1996), detection of endoglin-TGF- β 1 complexes would be expected after immunoprecipitation of affinity labelled human endometrial cell lysates with anti-T β R II . However, immunoprecipitated complexes were analysed after electrophoresis under reducing conditions (Dumont *et al.*, 1995) which do not allow T β R II -TGF- β 1 and endoglin-TGF- β 1 complexes to be distinguished. Further studies are required to verify that endoglin expressed on human endometrial cells binds to TGF- β 1 and interacts with signalling receptors.

Overall, the results of the present study demonstrate that treatment of endoglin-positive mouse uterine stromal cells with TGF- β 1 significantly stimulates DNA synthesis, as reported for human endometrial stromal cells (Hammond *et al.*, 1993; Marshburn *et al.*, 1994; Tang *et al.*, 1994; Dumont *et al.*, 1995). Furthermore, a biphasic curve of proliferation in response to the growth factor was observed: there was a slight increase in thymidine incorporation at the lower concentrations investigated, followed by an absence of response at 2 ng ml $^{-1}$, and a stimulation at higher concentrations of TGF- β 1. Similarly, Tang *et al.* (1994) observed that TGF- β 1 did not have an effect on the proliferation of human endometrial cells at 1 ng ml $^{-1}$, whereas lower concentrations ($\leq$ 0.5 ng ml $^{-1}$) exerted a stimulatory effect. However, the increase in thymidine incorporation into mouse uterine stromal cells in response to concentrations of TGF- β 1 < 0.5 ng ml $^{-1}$ was smaller than that reported for human cells (Hammond *et al.*, 1993; Tang *et al.*, 1994). This difference might be explained by species variation and the phase of the cycle at which uterine tissues were isolated. A bimodal proliferative response to TGF- β 1 was observed in human smooth muscle cells, chondrocytes and fibroblasts (Battegay *et al.*, 1990).

Significant inhibition of [^3H]thymidine incorporation was observed in the presence of mAb MJ7/18 after treatment of stromal cells with 0.02 and 10.0 ng TGF- β 1 ml $^{-1}$. Interestingly, at 2 ng TGF- β 1 ml $^{-1}$, anti-mouse endoglin significantly stimulated stromal cell proliferation, whereas the growth factor alone had no effect. Since endoglin modulates cellular responses to TGF- β 1 (Lastres *et al.*, 1996; Caniggia *et al.*, 1997), the obstruction of endoglin molecules by specific antibodies might alter the stromal cell proliferative response to the growth factor. Thus, the present study indicates that endoglin is involved in the regulation of uterine stromal cell proliferation in response to TGF- β 1.

The expression of endoglin is upregulated by TGF- β 1 on activated monocytes (Lastres *et al.*, 1996) and bone marrow stromal cells (Robledo *et al.*, 1998). Since TGF- β 1 is synthesized and secreted by uterine stromal and epithelial cells in the mouse uterus (Tamada *et al.*, 1990; Manova *et al.*, 1992; Roelen *et al.*, 1994; Das *et al.*, 1997), the effect of this growth factor on the expression of endoglin on mouse uterine stromal cells was investigated. The results demonstrate that treatment of freshly isolated cells with TGF- β 1 has no effect on the expression of endoglin at the surface of uterine stromal cells.

In preparation for implantation, a wave of fibroblast-like cell proliferation is observed in the uterine stroma to increase the

fibroblast population capable of undergoing decidualization (Moulton and Koenig, 1984; Abrahamsohn and Zorn, 1993). It has been proposed that TGF- β 1 may act to stimulate stromal cell proliferation in preparation for decidualization (Marshburn *et al.*, 1994). On the basis of the critical functions attributed to TGF- β in the uterus (Godkin and Doré, 1998), the high expression of endoglin in the endometrial stroma (St-Jacques *et al.*, 1994), and the demonstration that endoglin is involved in the mediation of cellular effects of this growth factor on uterine stromal cells, it is likely that endoglin is an important regulator of the actions of TGF- β in the uterine stroma.

The authors wish to thank Michelle Letarte from the Hospital for Sick Children of Toronto, Canada, for providing the rabbit polyclonal antibody 1256:4b raised against human endoglin, and Eugene Butcher of Stanford University School of Medicine, Stanford, CA, USA, for providing the rat monoclonal anti-mouse endoglin MJ7/18. They also thank Marie-Josée Bergeron for helpful discussion and review of the manuscript. This work was supported by grant MT-12703 from the Medical Research Council of Canada. S. St-Jacques is a Junior Scholar from Fonds de la Recherche en Santé du Québec (960141-103).

References

- Abrahamsohn PA and Zorn TMT (1993) Implantation and decidualization in rodents *Journal of Experimental Zoology* **266** 603–628
- Ando N, Hirahara E, Fukushima J, Kawamoto S, Okuda K, Funabashi T, Gorai I and Minaguchi H (1998) Differential gene expression of TGF- β isoforms and TGF- β receptors during the first trimester of pregnancy at the human maternal-fetal interface *American Journal of Reproductive Immunology* **40** 48–56
- Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF and Ross R (1990) TGF- β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop *Cell* **63** 515–524
- Bigsby RM, Cooke PS and Cunha GR (1986) A simple efficient method for separating murine uterine epithelial and mesenchymal cells *American Journal of Physiology* **251** E630–E636
- Bischof P, Redar M, Gindre P, Vassilakos P and Campana A (1993) Localization of alpha 2, alpha 5 and alpha 6 integrin subunits in human endometrium, decidua and trophoblast *European Journal of Obstetrics and Gynaecology and Reproductive Biology* **51** 217–226
- Bruner KL, Rodgers WH, Gold LI, Korc M, Hargrove JT, Matrisian LM and Osteen KG (1995) Transforming growth factor beta mediates the progesterone suppression of an epithelial metalloproteinase by adjacent stroma in the human endometrium *Proceedings National Academy of Sciences USA* **92** 7362–7366
- Caniggia I, Taylor CV, Knox JW, Lye SJ and Letarte M (1997) Endoglin regulates trophoblast differentiation along the invasive pathway in human placental villous explants *Endocrinology* **138** 4977–4988
- Casslén B, Sandberg T, Gustavsson B, Willén R and Nilbert M (1998) Transforming growth factor β 1 in the human endometrium. Cyclic variation, increased expression by estradiol and progesterone, and regulation of plasminogen activators and plasminogen activator inhibitor-1 *Biology of Reproduction* **58** 1343–1350
- Chegini N, Rossi MJ and Masterson BJ (1992) Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and EGF and PDGF β -receptors in human endometrial tissue: localization and *in vitro* action *Endocrinology* **130** 2373–2385
- Chegini N, Zhao Y, Williams RS and Flanders KC (1994) Human uterine tissue throughout the menstrual cycle expresses transforming growth factor- β 1 (TGF- β 1), TGF- β 2, TGF- β 3, and type II receptor messenger ribonucleic acid and protein and contains [^{125}I]TGF- β 1 binding sites *Endocrinology* **135** 439–449
- Cheifetz S, Like B and Massagué J (1986) Cellular distribution of type I and type II receptors for transforming growth factor- β *Journal of Biological Chemistry* **261** 9972–9978
- Cheifetz S, Andres JL and Massagué J (1988) The transforming growth factor-

- β receptor type III is a membrane proteoglycan. Domain structure of the receptor *Journal of Biological Chemistry* **263** 16 984–16 991
- Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massagué J and Letarte M (1992) Endoglin is a component of the transforming growth factor- β receptor in human endothelial cells *Journal of Biological Chemistry* **267** 19 027–19 030
- Chen R-H, Moses HL, Maruoka EM, Derynck R and Kawabata M (1995) Phosphorylation-dependent interaction of the cytoplasmic domains of the type I and type II transforming growth factor- β receptors *Journal of Biological Chemistry* **270** 12 235–12 241
- Clark DA, Flanders KC, Banwatt D, Millar-Book W, Manuel J, Stedronska-Clark J and Rowley B (1990) Murine pregnancy decidua produces a unique immunosuppressive molecule related to transforming growth factor- β 2 *Journal of Immunology* **144** 3008–3014
- Das SK, Lim H, Wang J, Paria BC, BazDresch M and Dey SK (1997) Inappropriate expression of human transforming growth factor (TGF)- α in the uterus of transgenic mouse causes downregulation of TGF- β receptors and delays the blastocyst attachment reaction *Journal of Molecular Endocrinology* **18** 243–257
- Dumont N, O'Connor-McCourt MD and Philip A (1995) Transforming growth factor- β receptors on human endometrial cells: identification of the type I, II and III receptors and glycosyl-phosphatidylinositol anchored TGF- β binding proteins *Molecular and Cellular Endocrinology* **111** 57–66
- Flanders KC, Marascalco BA, Roberts AB and Sporn MA (1991) Transforming growth factor β : a multifunctional regulatory peptide with actions in the reproductive system. In *Growth Factors in Reproduction* pp 23–37. Springer-Verlag, New York
- Ge AZ and Butcher EC (1994) Cloning and expression of a cDNA encoding mouse endoglin, an endothelial cell TGF- β ligand *Gene* **138** 201–206
- Godkin JD and Doré JJE (1998) Transforming growth factor β and the endometrium *Reviews of Reproduction* **3** 1–6
- Gougos A and Letarte M (1988) Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line *Journal of Immunology* **141** 1925–1933
- Gougos A and Letarte M (1990) Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells *Journal of Biological Chemistry* **265** 8361–8364
- Graham CH and Lala PK (1992) Mechanisms of placental invasion of the uterus and their control *Biochemistry and Cell Biology* **70** 867–874
- Grosskinsky CM, Yowell CW, Sun J, Parise LV and Lessey BA (1996) Modulation of integrin expression in endometrial stromal cells *in vitro*. *Journal of Clinical Endocrinology and Metabolism* **81** 2047–2054
- Hammond MG, Oh S-T, Anners J, Surrey ES and Halmé J (1993) The effect of growth factors on the proliferation of human endometrial stromal cells in culture *American Journal of Obstetrics and Gynecology* **168** 1131–1138
- Heldin CH, Miyazono K and ten-Dijke P (1997) TGF- β signalling from cell membrane to nucleus through SMAD proteins *Nature* **390** 465–471
- Iwahashi M, Muragaki Y, Ooshima A, Yamoto M and Nakano R (1996) Alterations in distribution and composition of the extracellular matrix during decidualization of the human endometrium *Journal of Reproduction and Fertility* **108** 147–155
- Jacobs AL and Carson DD (1991) Proteoglycan synthesis and metabolism by mouse uterine stroma cultured *in vitro*. *Journal of Biological Chemistry* **266** 15 464–15 473
- Lastres P, Letamendia A, Zhang H et al. (1996) Endoglin modulates cellular responses to TGF- β 1 *Journal of Cell Biology* **133** 1109–1121
- Lessey BA, Castelbaum AJ, Buck CA, Lei Y, Yowell CW and Sun J (1994) Further characterization of endometrial integrins during the menstrual cycle and in pregnancy *Fertility and Sterility* **62** 497–506
- Letarte M, Greaves A and Vera S (1994) CD105 (endoglin) cluster report. In *Leukocyte Typing V: White Cell Differentiation Antigen* pp 1756–1759. Oxford University Press, Oxford
- Lopez-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS and Massagué J (1991) Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF- β receptor system *Cell* **67** 785–795
- Lopez-Casillas F, Wrana JL and Massagué J (1993) Betaglycan presents ligand to the TGF- β signaling receptor *Cell* **73** 1435–1444
- McAllister KA, Grogg KM, Johnson DW et al. (1994) Endoglin, a TGF- β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1 *Nature Genetics* **8** 345–351
- Mani SK, Julian J, Lampelo S and Glasser SR (1992) Initiation and maintenance of *in vitro* decidualization are independent of hormonal sensitization *in vivo*. *Biology of Reproduction* **47** 785–799
- Manova K, Paynton BV and Bachvarova RF (1992) Expression of activins and TGF- β 1 and - β 2 RNAs in early postimplantation mouse embryos and uterine decidua *Mechanisms of Development* **36** 141–152
- Marshburn PB, Arici AM and Casey ML (1994) Expression of transforming growth factor- β 1 messenger ribonucleic acid and the modulation of deoxyribonucleic acid synthesis by transforming growth factor- β 1 in human endometrial cells *American Journal of Obstetrics and Gynecology* **170** 1152–1158
- Massagué J (1996) TGF- β signaling: receptors, transducers and MAD proteins *Cell* **85** 947–950
- Massagué J and Like B (1985) Cellular receptors for type β transforming growth factor. Ligand binding and affinity labelling in human and rodent cell lines *Journal of Biological Chemistry* **260** 2636–2645
- Mitchell EJ, Fitz-Gibbon L and O'Connor-McCourt M (1992) Subtypes of betaglycan and of type I and type II transforming growth factor- β (TGF- β) receptors with different affinities for TGF- β 1 and TGF- β 2 are exhibited by human placental trophoblast cells *Journal of Cellular Physiology* **150** 334–343
- Miyazono K (1997) TGF- β receptors and signal transduction *International Journal of Hematology* **65** 97–104
- Moulton BC and Koenig BB (1984) Uterine deoxyribonucleic acid synthesis during preimplantation in precursors of stromal cell differentiation during decidualization *Endocrinology* **115** 1302–1307
- Mulholland J, Aplin JD, Ayad S, Hong L and Glasser SR (1992) Loss of collagen type VI from rat endometrial stroma during decidualization *Biology of Reproduction* **46** 1136–1143
- O'Connell PJ, McKenzie A, Fiscaro N, Rockman SP, Pearse MJ and D'Apice AJF (1992) Endoglin: a 180-kD endothelial cell and macrophage restricted differentiation molecule *Clinical and Experimental Immunology* **90** 154–159
- Robledo MM, Hidalgo A, Lastres P, Arroyo AG, Bernabéu C, Sanchez-Madrid F and Teixido J (1996) Characterization of TGF- β 1-binding proteins in human bone marrow stromal cells *British Journal of Haematology* **93** 507–514
- Robledo MM, Ursa MA, Sanchez-Madrid F and Teixido J (1998) Associations between TGF- β 1 receptors in human bone marrow stromal cells *British Journal of Haematology* **102** 804–811
- Rodgers WH, Matrisian LM, Giudice LC, Dsupin B, Cannon P, Svitek C, Gorstein F and Osteen KG (1994) Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones *Journal of Clinical Investigations* **94** 946–953
- Roelen BA, Lin HY, Knezevic V, Freund E and Mummery CL (1994) Expression of TGF- β s and their receptors during implantation and organogenesis of the mouse embryo *Developmental Biology* **166** 716–728
- Rotello RJ, Lieberman RC, Furchio AF and Gerschenson LE (1991) Coordinated regulation of apoptosis and cell proliferation by transforming growth factor β 1 in cultured uterine epithelial cells *Proceedings National Academy of Sciences USA* **88** 3412–3415
- Sandberg T, Eriksson P, Gustavsson B and Casslén B (1997) Differential regulation of the plasminogen activator inhibitor-1 (PAI-1) gene expression by growth factors and progesterone in human endometrial stromal cells *Molecular Human Reproduction* **3** 781–787
- Shull MM and Doetschman T (1994) Transforming growth factor- β 1 in reproduction and development *Molecular Reproduction and Development* **39** 239–246
- St-Jacques S, Cymerman U, Pece N and Letarte M (1994) Molecular characterization and *in situ* localization of murine endoglin reveal that it is a transforming growth factor- β binding protein of endothelial and stromal cells *Endocrinology* **134** 2645–2657
- Tabibzadeh S (1992) Patterns of expression of integrin molecules in human endometrium throughout the menstrual cycle *Human Reproduction* **7** 876–882
- Takahashi T, Eitzman B, Bossert NL, Walmer D, Sparrow K, Flanders KC, McLachlan J and Gray Nelson K (1994) Transforming growth factors β 1, β 2 and β 3 messenger RNA and protein expression in mouse uterus and vagina during estrogen-induced growth: a comparison to other estrogen-regulated genes *Cell Growth and Differentiation* **5** 919–935
- Tamada H, McMaster MT, Flanders KC, Andrews GK and Dey SK (1990) Cell type-specific expression of transforming growth factor- β 1 in the mouse uterus during the periimplantation period *Molecular Endocrinology* **4** 965–972
- Tang X-M, Zhao Y, Rossi MJ, Abu-Rustum RS, Ksander GA and Chegini N (1994) Expression of transforming growth factor- β (TGF- β) isoforms and TGF- β type II receptor messenger ribonucleic acid and protein, and the effect of TGF- β s on endometrial stromal cell growth and protein degradation *in vitro*. *Endocrinology* **135** 450–459
- Ueda M, Ueki M, Tsurunaga T, Yamada T, Okamoto Y, Seiki Y, Iwai E and Sugimoto O (1992) *In vitro* study on the effect of sex steroid and growth

- factor on growth and laminin, collagen IV, and tissue plasminogen activator production of normal endometrial cells and endometrial cancer cells in culture *Acta Obstetrica Gynaecologica Japonica* **44** 1219–1226
- Ventura F, Doody J, Liu F, Wrana JL and Massagué J** (1994) Reconstitution and transphosphorylation of TGF- β receptor complexes *EMBO Journal* **13** 5581–5589
- Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang X-F and Massagué J** (1992) TGF β signal through a heteromeric protein kinase receptor complex *Cell* **71** 1003–1014
- Wrana JL, Attisano L, Wieser R, Ventura F and Massagué J** (1994) Mechanism of activation of the TGF- β receptor *Nature* **370** 341–347
- Yamashita H, Ichijo H, Grimsby S, Morén A, ten Dijke P and Miyazono K** (1994) Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor- β *Journal of Biological Chemistry* **269** 1995–2001
- Zhang H, Shaw ARE, Mak A and Letarte M** (1996) Endoglin is a component of the transforming growth factor (TGF)- β receptor complex of human pre-B leukemic cells *Journal of Immunology* **156** 565–573
- Zhang L, Rees MC and Bicknell R** (1995) The isolation and long-term culture of normal human endometrial epithelium and stroma. Expression of mRNAs for angiogenic polypeptides basally and on estrogen and progesterone challenges *Journal of Cell Sciences* **108** 323–331