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Endothelin-1 promotes migration and induces elevation of $[Ca^{2+}]_i$ and phosphorylation of MAP kinase of a human extravillous trophoblast cell line☆

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Abstract

A highly proliferative, migratory and invasive subpopulation of human placental trophoblasts, known as extravillous trophoblasts (EVT), invades the uterus and its vasculature, to establish an adequate exchange of key molecules between the maternal and fetal circulation. Our earlier studies provided evidence for a positive regulation of migration/invasion of EVT by an autocrine factor IGFII and a paracrine, decidua-derived factor IGFBP1. The present study examined the role played by endothelin (ET)-1, also produced at the fetal-maternal interface, and its receptor subtypes ET_A and ET_B in the regulation of human EVT cell functions. We utilized an in vitro propagated EVT cell line (HTR-8/SVneo) which exhibits the phenotypic and functional characteristics of EVT in situ. Reverse transcription-PCR with primers specific for prepro-ET-1, ET_A and ET_B cDNAs demonstrated the expression of all these genes in HTR-8/SVneo cells. While proliferation was not influenced, migration of these cells through porous Transwell membranes was stimulated by exogenous ET-1. ET-1 also induced biphasic elevation of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) consisting of an initial transient followed by a sustained plateau, as measured by spectrofluorimetry. The dependence of the Ca^{2+} response on phospholipase C (PLC) was demonstrated by its abrogation in the presence of PLC inhibitor U73122. Furthermore, ET-1 treatment of EVT cells rapidly stimulated phosphorylation of MAP kinase (ERK1/2). By using ET receptor antagonists and agonists, it was shown that both ET_A and ET_B receptors were responsible for the effects of ET-1 on migration, $[Ca^{2+}]_i$ and MAPK phosphorylation. Thus, ET-1 may represent an autocrine/paracrine mediator of invasive trophoblast function.

Keywords: Extravillous trophoblast; Endothelin; Migration; Intracellular calcium; Extracellular signal-regulated kinase

1. Introduction

Placenta in many species, including the human, is an invasive structure in which a subpopulation of trophoblast cells invade the uterus and its vasculature to establish an adequate exchange of nutrients and other molecules between the mother and the fetus. Following the establishment of chorionic villi during placental development, cytotrophoblast stem cells within the villi proliferate and differentiate into two distinct pathways: villous syncytiotrophoblast primarily providing absorptive and endocrine functions; and extravillous cytotrophoblast providing the migratory and invasive functions (Chakraborty et al., 2002; Kurman 1992; Kaufmann and Castelluci, 1997). In certain chorionic villi designated as anchoring villi, extravillous trophoblast (EVT) cells arise as migratory cell columns which ultimately penetrate the decidua and modify utero-placental vessels (Chakraborty et al., 2002; Kurman 1992).

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During normal pregnancy, the proliferative, migratory and invasive functions of EVT cells remain under tight regulatory influence of a variety of factors in the microenvironment, inclusive of numerous growth factors, growth factor-binding proteins, proteoglycans and extracellular matrix (ECM) proteins (Chakraborty et al., 2002). These regulatory mechanisms maintaining a normal uteroplacental homeostasis may break down in trophoblast hypoinvasive disorders such as preeclampsia and hyperinvasive disorders such as invasive mole and choriocarcinomas. Pure human EVT cell cultures from chorionic villous explants have been established (Yagel et al., 1989) and characterized (Graham et al., 1992; Irving et al., 1995) in order to study the regulatory influence of different molecules on EVT cell functions. Using these cells, it was shown that a negative regulation of proliferation, migration and invasiveness was provided by transforming growth factor (TGF)-β (Irving et al., 1995; Irving and Lala, 1995) and a TGF-β-binding proteoglycan decorin (Xu et al., 2002), both produced predominantly by the decidual cells. Epidermal growth factor (EGF) ligands (EGF, TGF-α, amphiregulin), colony stimulating factor (CSF)-1, vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) have been found to stimulate proliferation of these cells (Lysiak et al., 1994, 1995; Hamilton et al., 1998a; Athanassiades et al., 1998; Athanassiades and Lala, 1998). Trophoblast-derived insulin like growth factor (IGF)-II and decidua-derived IGF-binding protein (IGFBP)-1 have been shown to stimulate migration/invasion of EVT cells without exerting any effect on proliferation (Irving and Lala, 1995; Hamilton et al., 1998b; McKinnon et al., 2001; Gleeson et al., 2001). A vasoactive peptide endothelin (ET)-1 has been demonstrated to be produced by most of the cell types at the fetal-maternal interface, viz. placental fibroblasts, endothelial and vascular smooth muscle cells, syncytiotrophoblast and cytotrophoblast cells, and decidual cells (Malassine et al., 1993; Kubota et al., 1992; Fant et al., 1992). ET-binding sites have also been demonstrated in most of these cells (Fant et al., 1992; Mondon et al., 1993; Kohnen et al., 1997; Cervar et al., 2000; Kohnen et al., 1998). The ETs therefore, may also act as paracrine or autocrine factors within these cell systems.

The ETs are a family of 21-amino acid iso-peptides called ET-1, ET-2 and ET-3. Originally discovered as an endothelial cell product having the strongest vasoconstrictor function, ET-1 has been found to be produced and secreted by diverse types of cells and to elicit a wide spectrum of biological effects (Rubanyi and Polokoff, 1994). Circulating concentrations of ETs are lower than those causing biological activities in most cases, suggesting that ET-1 mainly acts as a locally-active autocrine/paracrine factor rather than as a circulating hormone (Giannessi et al., 2001). Two main receptor subtypes, ET_A and ET_B mediate biological actions of ETs (Rubanyi and Polokoff, 1994; Giannessi et al., 2001). Both are seven transmembrane domain classical G protein-coupled receptors (Rubanyi and Polokoff, 1994; Giannessi et al., 2001). ET_A receptors are preferentially activated by ET-1 and ET_B receptors can be activated equally by all the isoforms of ET (Rubanyi and Polokoff, 1994; Giannessi et al., 2001).

ET-1 has been demonstrated to modulate proliferation of many cell types, including fibroblasts, endothelial cells, and many neoplastic cells (Takuwa et al., 1989; Mallat et al., 1995; Morbidelli et al., 1995; Bagnato and Catt, 1998). ET-1 has also been shown to stimulate migration of many cell types which include melanocytes (Scott et al., 1997) and endothelial cells (Noiri et al., 1997). The roles played by ET-1 in pure EVT cell functions, however, have never been investigated before, even though both ET_A and ET_B receptors have been documented in EVT cells in situ (Kohnen et al., 1997).

The present study was undertaken to determine whether human EVT cells in culture express ET-1, ET_A and ET_B genes, and to evaluate the effects of exogenous ET-1 on proliferation/survival and migration of these cells. We also sought to examine two signal transduction pathways namely, cytosolic Ca^{2+} ($[Ca^{2+}]_i$) and mitogen activated protein kinase (MAPK/ERK) which might underlie these putative effects.

2. Materials and methods

2.1. Reagents and antibodies

RPMI 1640 and FBS were was purchased from GIBCO (Burlington, ON). Bovine serum albumin (BSA), MTT [3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide], U73122 and U73343 were obtained from Sigma (Oakville, ON). ET-1, BQ788, BQ610, IRL1038 and IRL1620 were purchased from American Peptide Company (Sunnyvale, California). Indo-1-penta-(acetoxymethyl) ester (indo-1 AM), BAPTA-AM and thapsigargin were obtained from Molecular Probes (Eugene, OR). The specific MEK inhibitor PD 98059 was purchased from Calbiochem (San Diego, CA). Monoclonal *anti*-human phosphorylated-ERK antibody and polyclonal goat *anti*-human ERK-1 (cross-reactive with ERK-2) were obtained from Santa Cruz (Santa Cruz, CA). Horseradish peroxidase-conjugated goat *anti*-mouse IgG and swine *anti*-goat IgG were purchased from Cedarlane (Hornby, ON). IGFBP-1 from Upstate Biotechnology, Inc. (Lake Placid, NY).

2.2. EVT cell line and culture

The present study utilized an EVT cell line HTR8/svneo (Graham et al., 1993), produced in our laboratory

by SV40 Tag immortalization of a short lived, first trimester EVT cell line HTR8 (Graham et al., 1992; Irving et al., 1995). These cells share all the phenotypic and functional characteristics of the parental HTR8 cells produced by propagation of EVT cells migrating out of a first trimester chorionic villus explant, as detailed previously (Irving et al., 1995). They express all the markers of EVT cells in situ, including cytokeratin 7, 8 and 18, placental type alkaline phosphatase, high affinity urokinase type plasminogen activator (uPA) receptor (uPAR), HLA framework antigen w6/32, IGF-II mRNA and protein, and a selective integrin repertoire (e.g. α1, α3, α5, αv, β1 and the vitronectin receptor, αvβ3/β5) (Irving and Lala, 1995). These cells have also been shown to express HLA-G mRNA and protein when grown on Matrigel™ or laminin (Zdravkovic et al., 1999). HTR8/svneo cells, like HTR8 cells respond to migration/invasion-inhibitory signals of TGFβ (Irving and Lala, 1995; Gleeson et al., 2001; Xu et al. 2002), migration/invasion-stimulating signals of IGFII (Irving and Lala, 1995; Hamilton et al., 1998a; McKinnon et al., 2001) and IGFBP-1 (Irving and Lala, 1995; Hamilton et al., 1998b; Gleeson et al., 2001) and exhibit phenotypic behavior of freshly isolated cytotrophoblast cells during the process of Matrigel invasion (Kilburn et al., 2000). They were utilized at 70–80th passage during the present study and grown in RPMI 1640 supplemented with 10% FBS and 2% penicillin/streptomycin, unless specified otherwise.

2.3. RT-PCR

Total RNA was isolated by TRIZOL method. First strand cDNA synthesis was performed using Superscript-II™ system (Gibco). The amplifications were carried out according to the method used before (Chen et al., 2000), using the following primer sequences: ET-1(sense), 5′-GGA CAT CAT TTG GGT CAA CAC TCC-3′; ET-1(antisense), 5′-CCA AGC TTG GAA CAG TCT TTT CCT-3′; ET_A(sense), 5′-TGG CCT TTT GAT CAC AAT GAC TTT-3′; ET_A (antisense), 5′-TTT GAT GTG GCA TTG AGC ATA CAG GTT-3′; ET_B(sense), 5′-ACT GGC CAT TTG GAG CTG AGA TGT-3′; ET_B(antisense), 5′-CTG CAT GCC ACT TTT CTT TCT CAA-3′. The amplification was carried out as 1 min at 94 °C (denaturation), 1 min at 60 °C (annealing) and 3 min at 72 °C (extension) for 35 cycles. For the receptor genes 54 °C was used as annealing temperature.

2.4. MTT cell proliferation/survival assay

Cellular proliferation/survival was quantitatively determined utilizing MTT colorimetric assay as described earlier (Gleeson et al., 2001), where MTT is cleaved to formazan by mitochondrial succinate-tetrazolium reductase system in metabolically active cells. In brief, cells in complete medium were placed in 96-well microtiter plates (1×10^4 cells/100 μl per well) and cultured overnight (37 °C, 5% CO_2). Complete medium was replaced with serum-free medium including 0.1% BSA in place of 10% FBS, to serum-starve the cells for 48 h. Proliferation assays were initiated during serum-starved conditions by addition of ET-1 (0.01–100 nmol/l), or serum-free medium, or FBS to the wells in triplicate. To study the effects of ET-receptor antagonists assays were carried out in serum-reduced (1% FBS and 2% BSA) medium. The plates were incubated for 24 or 48 h at 37 °C prior to the addition of MTT solution, followed by 2 h incubation, extraction and spectrophotometric quantification at 540 nm.

2.5. Transwell migration assay

Migratory ability of EVT cells was determined by their ability to cross the 8 μm pores of migration chambers consists of Transwells fitted with millipore membranes (6.5 mm filters, 8 μm pore size; Costar Corp, Toronto, ON) as described earlier (McKinnon et al., 2001; Gleeson et al., 2001). In brief, 2×10^4 HTR8/SVneo cells per 100 μl serum-reduced medium (SRM) (RPMI 1640 with 1% FBS) were plated in upper wells of transwell chambers containing either 200 μl SRM or SRM with various concentrations of ET-1, or ET-receptor antagonists, or both, or only SRM. The lower wells of the apparatus contained 800 μl SRM into which the transwells were immersed. The migration apparatus was assembled and incubated in a humidified environment (5% CO_2) at 37 °C. The number of migratory cells appearing at 48 h on the undersurface of the polycarbonate membranes was scored visually following staining of cells with Hemacolor Stain Set (EM Science, Gibbstown, NJ) in five random non-overlapping fields at 400× magnification using light microscope. Each condition was repeated 6–11 times.

2.6. Measurement of $[Ca^{2+}]_i$

HTR-8/SVneo cells were loaded with the Ca^{2+}-sensitive fluorescent dye indo-1 AM (2 μM) for 30 min at 37 °C, washed and trypsinized. After inactivating trypsin, the cells were sedimented and resuspended in HEPES-buffered MEM. Aliquots of cell suspensions were sedimented and resuspended in 2 ml of continuously stirred Na^+–HEPES buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, pH 7.3, 290 mOsmol/l) in a fluorimetric cuvette maintained at 37 °C. $[Ca^{2+}]_i$ was monitored using a dual wavelength fluorimeter (model RF-M 2004, Photon Technology International, London, ON, Canada) with excitation wavelength of 355 nm and emission wavelengths of 405 and 485 nm (Chen et

al., 1998). The system software was used to subtract background fluorescence and calculate the ratio R, which is the fluorescence intensity at 405 nm divided by the intensity at 485 nm. $[Ca^{2+}]_i$ was determined from the relationship $[Ca^{2+}]_i = K_d[(R-R_{min})/(R_{max}-R)]\beta$, where K_d (the dissociation constant for the indo-1-Ca_i^{2+} complex) was 250 nmol/l, R_{min} and R_{max} were the values of R at low and saturating concentrations of Ca_i^{2+}, respectively, and β was the ratio of the fluorescence at 485 nm measured at the low and saturating Ca_i^{2+} concentrations (Grynkiewich et al., 1985).

2.7. Adenylyl cyclase (AC) activity assay

Membrane fractions isolated from HTR-8/SVneo cells were resuspended in ice-cold 75 mM Tris, 10 mM $MgCl_2$ plus protease inhibitors. AC enzyme activity was measured utilizing a protocol outlined by Johnson et al. (1994)). AC activity assay was started with the addition of 10 μl of purified cell membranes (0–10 μg membrane protein) to 30 μl AC mix ([^{32}P]ATP (5×10^5 cpm), 500 μm ATP, 200 μM cAMP, 5 mM PEP, 50 μM GTP, 0.5 I.U. pyruvate kinase, 2 I.U. myokinase, 1 mM FSK) plus 20 μl ligand solution (0–100 nM of ET-1 or IRL-1620 in PBS). The mixture was then incubated with shaking at 30 °C for 10 min. The assay was stopped with the addition of 1 ml AC stop solution ([^{3}H]cAMP (1×10^4 cpm), 1 mM ATP, 200 μM cAMP, 0.25% sodium dodecyl sulfate (SDS) (w/v)) and stored on ice. To purify cyclic nucleotides, sequential column chromatography was utilized with Dowex 50 columns followed by elution onto alumina columns. Following appropriate adjustment for efficiency, [^{32}P] counts were used to determine the rate of cAMP formation (i.e. pmol of cAMP produced/min/μg of membrane protein added to the assay).

2.8. Immunoblotting MAPK proteins

HTR8/svneo cells were grown on poly-L-lysine coated dishes then serum-starved overnight. Following treatment with 100 nmol/l ET-1 for 0, 5, 10, 30, or 60 min the cells were rinsed twice with cold PBS and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing phosphatase inhibitors 50 mM NaF and 1 mM Na_3VO_4, including a complete mini tablet protease inhibitor cocktail (Roche, Indianapolis, IN) for 0.5 h at 4 °C. The cell lysates were sedimented at 15 000 rpm for 15 min to remove the insoluble material. Total protein concentration was determined using the BCA protein assay reagent (Pierce, Brockville, ON). The protein samples were adjusted to equal amounts before the direct immunoblotting of total cell proteins. The proteins were diluted in reducing sample buffer, the samples heated to 100 °C for 5 min and then analyzed by SDS-PAGE before being transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk prior to immunoblot analysis using anti-phosphorylated ERK or anti-ERK-1 antibodies (cross-reactive with ERK-2) and HRP-conjugated goat *anti*-mouse secondary antibody. The blots were visualized using the enhanced chemiluminescence system (ECL Plus Western Blotting Detection System, Amersham, Oakville, ON).

2.9. Statistical analysis

Migration scores were analyzed using one-way ANOVA, followed by the Tukey test to determine the level of significance of differences of various treatment groups (different doses of ET-1) and then the values were normalized as a percent of the control (migration indices). For analyzing the difference of migration between BQ-610 or BQ-788 and ET-1 treated groups with 100 nmol/l ET-1 treated group, Student's t-test was employed. Differences of $P < 0.05$ were considered significant.

3. Results

3.1. HTR-8/SVneo cells express ET-1 and its receptors

ET-1 immunoreactivity has earlier been demonstrated in situ in endothelium of placental blood vessels, syncytiotrophoblast, villous and EVT cells (Kubota et al., 1992). Using radio-receptor assay on tissue sections. ET_A and ET_B receptors were also documented in EVT cells in situ (Kohnen et al., 1997). To confirm their expression in EVT cells propagated in vitro, we examined their mRNA expressions in HTR8/SVneo cells. Prepro-ET-1, ET_A and ET_B mRNAs were detected after 35 cycles of amplification as bands of the expected sizes of 269, 302 and 428 bp, respectively (Fig. 1).

3.2. ET-1 does not affect HTR-8/SVneo cell proliferation

ET receptors were reported to be high affinity receptors (K_d values in the range of pmol/l to low nmol/l) (Mondon et al., 1993; Rubanyi and Polokoff, 1994). Accordingly, both ET_A and ET_B agonists at 10 pmol/l–10 nmol/l doses were found to have biological effects in most cell types (Scott et al., 1997; Noiri et al., 1997). Therefore, we have chosen concentrations of ET-1 ranging from 10 pmol/l to 100 nmol/l to study their effects on EVT cell functions. None of these concentrations of ET-1 affected proliferation/survival of HTR-8/SVneo cells in our MTT assay conducted for a period of 24 or 48 h (data not shown). Furthermore, neither an ET_A antagonist BQ-610 (Ihara et al., 1992) nor an ET_B

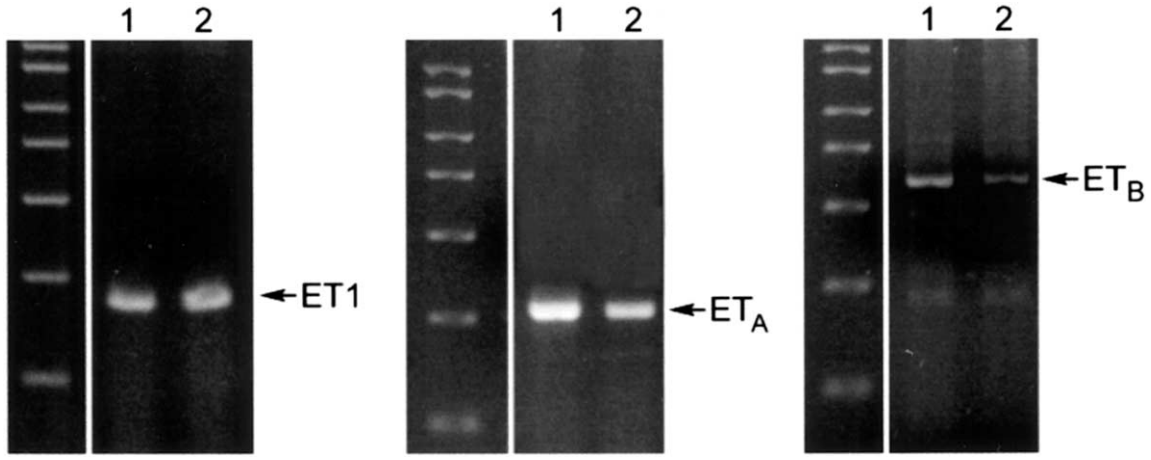

Fig. 1. ET-1, ET_A and ET_B mRNA expressions in HTR-8/SVneo cells. Total RNA (2 μg) isolated from these cells were subjected to RT-PCR using specific primers for respective genes, as described in Section 2. PCR products were separated by gel electrophoresis. Representative gel pictures with results of only two samples (lanes 1 and 2) are shown for ET-1 (left), ET_A (middle) and ET_B (right). Experiments were performed using RNA samples isolated from nine different cultures of HTR-8/SVneo cells.

antagonist BQ-788 (Ishikawa et al., 1994) each at 1 μmol/l affected basal proliferation/survival of these cells (data not shown), suggesting that endogenous ET-1 is not mitogenic to EVT cells. In contrast, both 1 and 10% FBS significantly stimulated proliferation of HTR-8/SVneo cells (data not shown).

3.3. ET-1 stimulates HTR-8/SVneo cell migration

To test whether ET-1 could affect EVT cell motility, HTR-8/SVneo cells were incubated in transwell chambers with different concentrations of ET-1 for 48 h in our chemokinesis assay. ET-1 induced a concentration-dependent (up to 10 nmol/l) increase of HTR-8/SVneo cell migration (Fig. 2). IGFBP1 (1 nmol/l) used as a positive control (Gleeson et al., 2001) also significantly stimulated migration. To elucidate the type of ET receptor involved in the stimulation of migration, experiments were performed in presence of either ET_A receptor antagonist BQ-610 (Ihara et al., 1992) or an ET_B receptor antagonist BQ788 (Ishikawa et al., 1994). Both ET_A selective as well as ET_B selective antagonists strongly inhibited the actions of ET-1. Therefore, ET-1-induced EVT cell migration appears to be mediated both by ET_A as well as by ET_B receptor subtypes.

3.4. ET-1 elevates $[Ca^{2+}]_i$ in HTR-8/SVneo cells

Alteration of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) regulates a variety of cellular functions including cell migration (Komuro and Rakic, 1996). Therefore, we examined and characterized the Ca_i^{2+} signaling in HTR-8/SVneo cells in response to ET-1. We first examined the effects of three different concentrations (0.1, 1.0 and 10 nmol/l) of ET-1 on $[Ca^{2+}]_i$. ET-1 evoked a concentration dependent biphasic elevation of $[Ca^{2+}]_i$

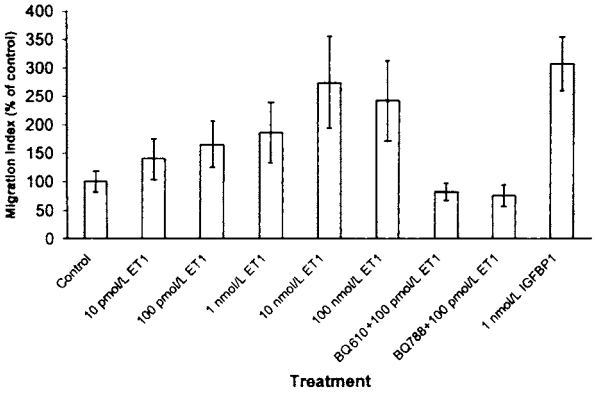

Fig. 2. ET-1 stimulates HTR-8/SVneo cell migration in a concentration-dependent manner up to 10 nmol/l ($P < 0.05$; $n = 11$); and both ET_A antagonist (BQ-610) and ET_B antagonist (BQ-788) at 1 μmol/l concentration inhibit ET-1-induced stimulation of migration ($P < 0.001$ for both the antagonists; $n = 6$ for both treatments). IGFBP1 has been used as positive control. Values are means ± SD. Each 'n' represents sum of total number of cells in five different fields on each membrane. Data were normalized to 100% for control (absolute number of migrated cells in control group was 208 ± 17).

in HTR-8/SVneo cells (Fig. 3). These results were reproduced in four separate experiments. No change in fluorescence was detected when the cells were treated with vehicle alone (data not shown). ET-1 induced an increase in fluorescence intensity at 405 nm concurrently with a decrease in fluorescence intensity at 485 nm, indicating true elevation of $[Ca^{2+}]_i$ (data not shown). In all of our experiments, 0.1 and 1.0 nmol/l ET-1 exhibited distinct dose response; however, in most experiments, 100 nmol/l ET-1elicited almost equal response as 1.0 or 10 nmol/l. Treatment of cells with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (Tg) (which blocks the re-uptake of Ca^{2+} into intracellular stores), evoked a large transient increase followed by a

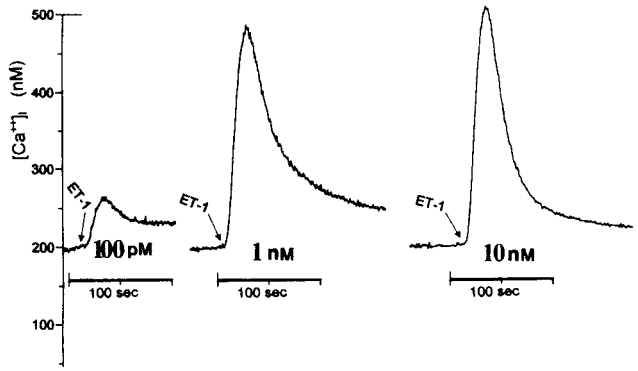

Fig. 3. ET-1 induces a concentration-dependent increase in $[Ca^{2+}]_i$ in suspended HTR-8/SVneo cells. Cells were loaded with Ca^{2+} sensitive dye indo-1 and susupended in Na^+ buffer (with 1 mM Ca^{2+}) in a fluorimetric curvette at 37 °C with continuous stirring. $[Ca^{2+}]_i$ was monitored by fluorescence spectrophotometry. At the time indicated by the *arrows*, 100 pmol/l, or 1 or 10 nmol/l of ET-1 were added to the cuvette. Traces are representative of responses from four separate cell preparations.

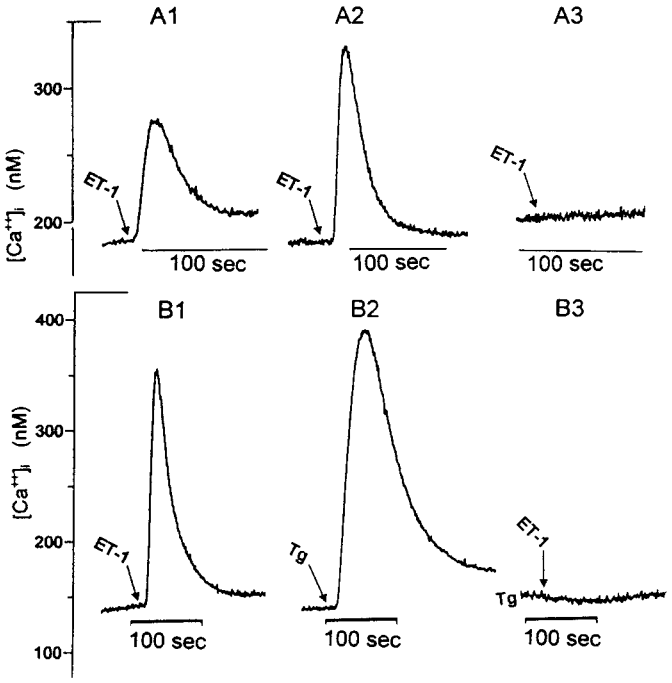

Fig. 4. Effects of EGTA, BAPTA-AM and thapsigargin on the $[Ca^{2+}]_i$ response to ET-1 in HTR-8/SVneo cells. Cells were loaded with indo-1 and suspended in Na^+ buffer in a fluorimetric cuvette at 37 °C, $[Ca^{2+}]_i$ was monitored by fluorescence spectrophotometry. *Upper panel*: $[Ca^{2+}]_i$ responses to ET-1 in the presence of EGTA (extracellular Ca^{2+} chelator) or BAPTA-AM (intracellular Ca^{2+} chelator). A1, control cells were susupended in Na^+ buffer (with 1 mM Ca^{2+}) and challenged with 10 nmol/l ET-1; A2, cells were suspended in Na^+ buffer (with 1 mM Ca^{2+}) plus 5 mM EGTA for 2 min and then challenged with 10 nmol/l ET-1; A3, cells were loaded with 5 μM BAPTA-AM for 30 min concomitantly with indo-1 AM, and later suspended in Na^+ buffer (with 1 mM Ca^{2+}) and challenged with 10 nmol/l ET-1. Note the difference in time scale bars for each of the tracings. Time scale in B_2 and B_3 is same as that of B_1. Note, with EGTA pretreatment ET-1 yielded a transient elevation of $[Ca^{2+}]_i$ but the plateau response was abolished. With BAPTA-AM pretreatment, the initial Ca^{2+} transient was totally abolished. *Lower panel*: $[Ca^{2+}]_i$ responses to thapsigargin in EVT cells. B_1, control (10 nmol/l ET-1) B_2, 1 μM thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATP ase. B_3, cells were treated with Tg for 30 min and then challenged with 10 nmol/l ET-1. The time scale in both tracings is the same. Traces are representative of responses from three separate cell preparations.

smaller sustained elevation of $[Ca^{2+}]_i$ (Fig. 4). Pretreatment of the cells with Tg for 30 min completely inhibited the Ca^{2+} response to ET-1 (Fig. 4); whereas their pretreatment with 5 mM EGTA for 2 min to chelate extracellular Ca^{2+} reduced the sustained plateau phase of Ca^{2+} response to ET-1 (Fig. 4). Furthermore, in cells loaded with intracellular Ca^{2+} chelator BAPTA-AM, the initial Ca^{2+} transient was totally abolished (Fig. 4). The aminosteroid U-73122 is an inhibitor of phospholipase C (PLC), whereas U-73343 is a close but inactive analog of U-73122 (Bleasdale et al., 1989). While pretreatment of cells for 2 min with 1 μM of U73122 totally abolished the Ca^{2+} response to ET-1, the same concentration of U-73343 had no effect on the response to ET-1 (Fig. 5). U-73122 itself triggered a transient increase in $[Ca^{2+}]_i$ (data not shown).

To examine which of the ET receptor subtypes could be involved in eliciting $[Ca^{2+}]_i$ elevations, two ET_B selective antagonists IRL-1038 (Gomez-Garre et al., 2001) and BQ-788 (Ishikawa et al., 1994), one ET_A selective antagonist BQ-610 (Ihara et al., 1992) and one ET_B agonist IRL-1620 (Mazzoni et al., 1999) were used. Pretreatment with 1 μM of each of the antagonists resulted in at least 50% inhibition of peak Ca_i^{2+} (Fig. 6). Furthermore, IRL-1620 elicited a dose-dependent increase in $[Ca^{2+}]_i$ (Fig. 7). Maximum stimulatory dose (100 pmol/l) of IRL-1620 in eliciting $[Ca^{2+}]_i$ response (Fig. 7) was found to be much lower than that of ET-1 (Fig. 3). This may indicate that ET_B receptor of HTR-8/SVneo cells are higher affinity receptors than ET_A receptors.

3.5. ET-1 does not modulate AC activity in EVT cell membrane

Most studies suggest that the ET receptors are coupled to G_q (Rubanyi and Polokoff, 1994). However, G_i and G_s have also been documented to mediate ET response in many cell types (Fujitani and Bertrand, 1997; Aramori and Nakanishi, 1992; Takigawa et al., 1995). While G_q-coupled receptors, upon binding with their ligands, cause elevation of $[Ca^{2+}]_i$, and stimulation of PLC and PI-3 kinase activities, G_i and G_s coupled receptors, upon ligand binding cause inhibition and stimulation of AC activity, respectively. Therefore, we examined the role of G_i and/or G_s in ET-1 signaling in

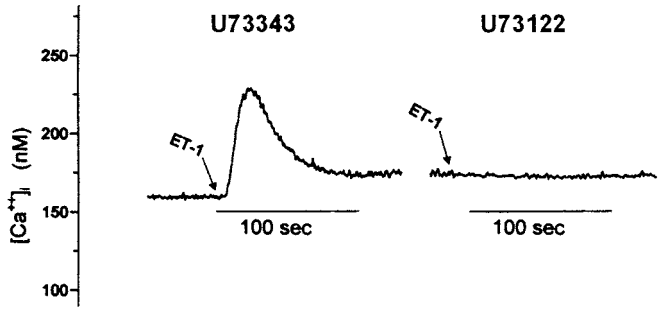

Fig. 5. Effect of PLC inhibitor on $[Ca^{2+}]_i$ in response to ET-1 in HTR-8/SVneo cells. Cells were loaded with indo-1 and suspended in Na^+ buffer in a fluorimetric cuvette at 37 °C, $[Ca^{2+}]_i$ was monitored by fluorescence spectrophotometry. Cells were incubated for 2 min with 1 μM of U-733122 (PLC inhibitor) or U-73343 (an inactive analogue of U-73122) before challenging 10 nmol/l of ET-1. U-733122 pretreatment abolished ET-1-induced Ca^{2+} respoose in EVT cells. Time scale in both tracings is same. Traces are representative of responses from three separate cell preparations.

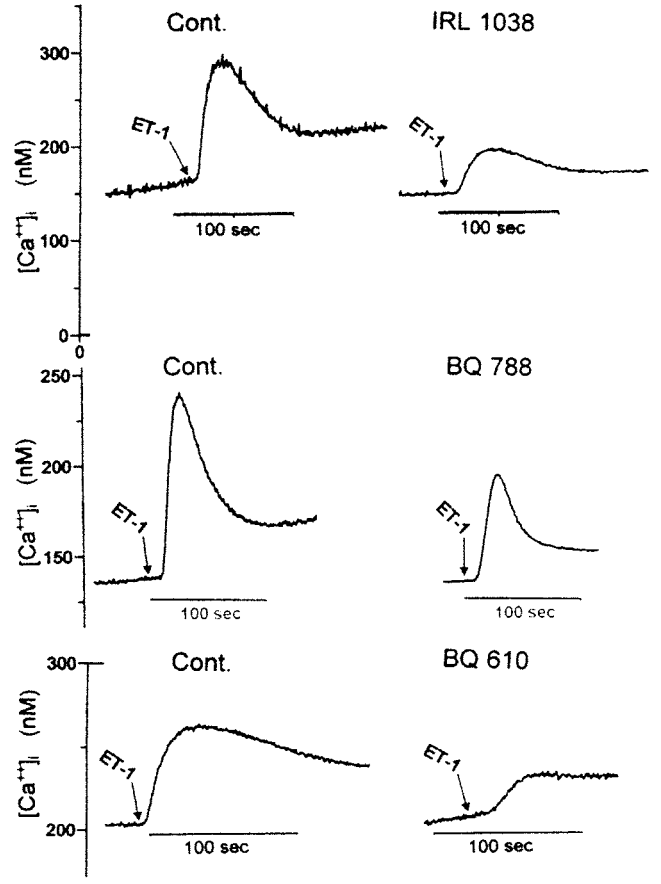

Fig. 6. Inhibition of ET1-induced $[Ca^{2+}]_i$ by ET receptor antagonists. HTR-8/SVneo cells were loaded with indo-1 and suspended in Na^+ buffer in a fluorimetric cuvette at 37 °C, $[Ca^{2+}]_i$ was monitored by fluorescence spectrophotometry. Suspended cells after 30 min pretreatment with ET_B antagonists (IRL1038, or BQ788, 1 μM each) or ET_A antagonist (BQ610, 1 μM) were challenged with 10 nmol/l ET-1. Note the difference in $[Ca^{2+}]_i$ time scale bars for tracing among the panels. But the time scale of both the tracings in each panel is the same. Traces are representative of responses from three separate cell preparations.

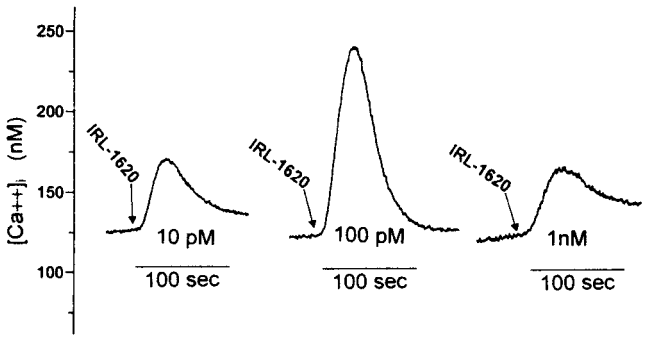

Fig. 7. An ET_B agonist IRL 1620 induces concentration-dependent increase in $[Ca^{2+}]_i$; in susupended HTR-8/SVneo cells which were preloaded with indo-1, and monitored fluorescence spectrophotometry. At the time indicated by the *arrows*, 10 or 100 pmol/l or 1 nmol/l of IRL 1620 were added to the cuvette. The time scale in all three tracings is the same. Traces are representative of responses from four separate cell preparations.

EVT cell by measuring the AC activity in cell membrane preparations in the presence and absence of different doses of ET-1 or IRL-1620 (0.01–100 nM). While forskolin (Fsk) stimulated AC activity in untreated cell membranes, neither ET-1 nor IRL 1620 exhibited inhibition or stimulation in either Fsk-stimulated or unstimulated cell membrane preparations (data not shown).

3.6. ET-1 stimulates rapid phosphorylation of ERK in EVT cells

Serine/threonine-specific protein kinases ERK-1 & 2 which belong to MAPK family can be activated by various G protein-coupled receptors and receptor tyrosine kinases. MAPK signaling has been demonstrated in HTR-8/SVneo cells both for IGFII (McKinnon et al., 2001) and IGFBP1 (Gleeson et al., 2001). MAPK has been implicated in cell migration possibly through phosphorylation of myosin light chain kinase (Klemke et al., 1997). Therefore, MAPK (ERK-1 & 2) signaling was investigated in HTR-8/SVneo cells after addition of exogenous ET-1. As depicted in Fig. 8, addition of an ET-1 dose of 10 nmol/l caused a rapid (within 5 min) increase in the levels of phosphorylation of ERK-1 & 2. Addition of vehicle did not have any effect on ERK phosphorylation (data not shown). Like migration and Ca^{2+} responses, MAPK phosphorylation by ET-1 in HTR-8/SVneo cells was also shown to be mediated through both ETA and ETB receptors, because preincubation of these cells either with BQ610 or BQ 788 abrogated the ET-1 effects on ERK phosphorylation (Fig. 8).

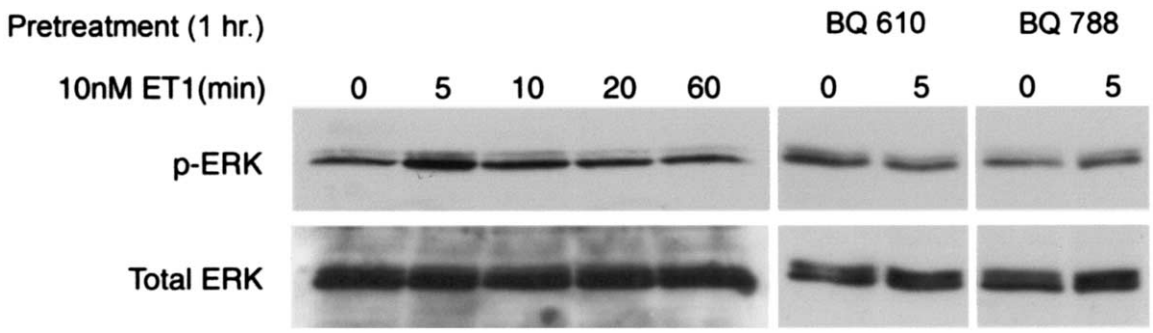

Fig. 8. ET-1 stimulates phosphorylation of MAPK (ERK-1 and ERK-2) at 5 min after treatment of HTR-8/SVneo cells, whereas 1 h pretreatment of cells with ET_A or ET_B antagonists (BQ 610 or BQ 788) reduced the ET-1 effect. The figure shows representative Western blots for phosphorylated ERK-1 and ERK-2 (p-ERK; *top*) and total ERK (*bottom*; visualized with an ERK-1 antibody cross-reactive with ERK-2 after stripping the same blot).

4. Discussion

While invading cytotrophoblast or EVT cells of the human placenta in situ were shown before to express both ET-1 (Malassine et al., 1993; Kubota et al., 1992; Fant et al., 1992) and its receptors ET_A and ET_B (Fant et al., 1992; Mondon et al., 1993; Kohnen et al., 1997; Cervar et al., 2000), functional significance of these expressions has never been investigated before. Using a well characterized human first trimester EVT cell line, the present study showed that these cells expressed ET-1 and its receptors ET_A and ET_B, and ET-1 can signal in these cells through both receptors to stimulate migration, $[Ca^{2+}]_i$ and phosphorylation of MAPK (ERK-1 & 2). This is the first evidence of functional significance of ET receptors in human EVT cells. Whether distinct subpopulations of EVT cells possessing either ET_A or ET_B or both receptors are present is, however, not clear from our study.

Signaling through ET receptors has previously been demonstrated to start very early in embryonic life (Shin et al., 1999). These signaling events are essential for normal embryonic development of subsets of neural crest cell derivatives. The present findings suggest one more role of these signaling events in the maintenance of successful pregnancy. Stimulation of EVT cell migration by ET-1 as shown in the present study is likely a common feature of embryonic/fetal cells. Shin et al. (1999) have determined that ET_B signaling is required during embryonic day (*E*) 10.5–12.5 period in mice for migration of both melanoblasts and enteric neuroblasts from neural tube towards their final destination. A number of adult type cells have also been shown to exhibit ET_B-mediated stimulation of migration by ET-1, e.g. human umbilical vein endothelial cells (HUVEC) (Morbidelli et al., 1995; Noiri et al., 1997). ET_A-mediated migration has also been documented in some carcinoma cell lines (Rosano et al., 2001). ET-1 has been reported to be a potent mitogen for many cell types including vascular smooth muscle cells (Zdravkovic et al., 1999), endothelial cells (Morbidelli et al., 1995), fibroblasts (Takuwa et al., 1989) and some tumor cells (Bagnato and Catt, 1998). It also could be growth inhibitory in some other cell types like hepatic myofibroblastic cells (Mallat et al., 1995). However, ET-1 at migration-stimulatory concentrations had no effect on proliferation of HTR-8/SVneo cells. For some cell types, ET-1 on its own was not mitogenic but was found to act synergistically with other growth factors to stimulate proliferation (Scott et al., 1997). Whether ET-1 could serve as a co-mitogen with other growth factors for EVT cells remains to be examined.

One of the key signaling events of ET action is activation of Ca_i^{2+} release from intracellular stores (Fant et al., 1992; Cervar et al., 2000). ET-1 has been shown in numerous cell lines to stimulate PLC causing breakdown of phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 then releases Ca^{2+} through binding with IP3 receptors on intracellular Ca^{2+} stores (endoplasmic and sarcoplasmic reticulum). This kind of signaling typifies classical G_q-coupled receptors. However, ET receptors have also been shown to be associated with inhibition or stimulation of AC, making these receptors G_i- or G_s-coupled receptors, respectively (Fujitani and Bertrand, 1997; Aramori and Nakanishi, 1992). Sometimes, single class of ET_A receptors can couple to both G_i and G_q. Again, cells overexpressing only ET_A or only ET_B were found to display stimulation or inhibition of AC, respectively (Aramori and Nakanishi, 1992). However, in our study, neither ET_A nor ET_B agonist had any effect on the activity of EVT cell membrane bound AC. Although in the present study we did not measure PLC or PI3-kinase activities after ET-1 treatment of EVT cells, elevation of $[Ca^{2+}]_i$ by ET-1 and ET_B agonist and abrogation ET-1-mediated Ca^{2+} response after pretreatment with PLC inhibitor indicate that both ET_A and ET_B receptors in these cells are coupled to G_q proteins. Present data suggest that ET-1-induced initial transient Ca_i^{2+} response in EVT cells is

due to Ca^{2+} mobilization and the sustained response is due to Ca^{2+} influx. The Ca^{2+} response to ET-1 in these cells however, arise primarily by release of Ca^{2+} from intracellular stores. ET-1, in some studies, have been shown to release Ca^{2+} by IP_3 independent mechanisms (Neylon, 1999), which includes caffeine-sensitive stores mediated through ryanodine receptor. We have not explored involvement of ryanodine-sensitive intracellular Ca^{2+} stores in EVT cells.

The potency of ET-1 in elevating peak $[Ca^{2+}]_i$ in HTR-8/SVneo cells corresponded closely to the potency of ET-1 in stimulating the migration of these cells. ET-1 induced elevation in $[Ca^{2+}]_i$, like migration response, were inhibited both by ET_A and ET_B antagonists. Furthermore, a selective ET_B agonist caused increase of $[Ca^{2+}]_i$. However, the extent to which the two receptors may interact with respect to migration stimulation or Ca^{2+} signaling is not apparent from our present data.

G_i-independent but G_q-dependent activation of MAPK (ERK-1 & 2) resulting from treatment of different cell types with ET-1 has been demonstrated (Cramer et al., 2001). Likewise, we have shown here stimulation of phosphorylation of ERK-1 & 2 within 5 min of exposure of HTR-8/SVneo cells to ET-1. It is likely that the rise of $[Ca^{2+}]_i$ is a requirement for ERK phosphorylation by ET-1 as demonstrated in COS cells and CHO cells overexpressing ET_A or ET_B receptor (Cramer et al., 2001).

Present findings suggest that ET-1 is one of the physiological factors produced in situ which promote the migratory function of the EVT. Other migration stimulatory factors identified in this laboratory are IGFII (Irving and Lala, 1995; Hamilton et al., 1998a; McKinnon et al., 2001), produced by trophoblast cells and IGFBP-1 (Irving and Lala, 1995; Hamilton et al., 1998b; Gleeson et al., 2001), produced by the decidua. The role of ET-1 in trophoblast dysfunction remains conjectural at present. Several investigators have shown higher plasma concentrations of ET in women with preeclampsia (a trophoblast hypomigratory/hypoinvasive disorder) than normal pregnant women (Granger et al., 2001). However, plasma concentrations of ET-1, even in preeclampsia is so low that circulating ET-1 is unlikely to elicit its biological actions, rather it is likely to act locally in an autocrine or paracrine manner (Rubanyi and Polokoff, 1994; Giannessi et al., 2001). Placental trophoblast cells isolated from preeclamptic pregnancies express higher levels of ET-1 mRNA than those from normotensive pregnancies (Napolitano et al., 2000). Therefore, while increased ET-1 produced by the vascular tissues might elicit pressure response in vascular smooth muscle cells (Rubanyi and Polokoff, 1994; Giannessi et al., 2001), ET-1 overproduction by the trophoblast in preeclampsia, could be a compensatory mechanism to rescue the fetus. Results of the elegant studies of Shin et al. (1999), establishing the important role of ET receptor signaling in normal embryonic development combined with our present findings on EVT cell migration indicate that administration of ET receptor agonists and antagonists during pregnancy may have adverse effects on embryonic development and placental function.

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References

Aramori, I., Nakanishi, S., 1992. Coupling of two endothelin receptor subtypes to differing signal transduction in transfected Chinese hamster ovary cells. J. Biol. Chem. 267, 12 468–12 474.

Athanassiades, A., Hamilton, G., Lala, P.K., 1998. Vascular endothelial growth factor stimulates proliferation but not migration or invasiveness in human extravillous trophoblast. Biol. Reprod. 59, 643–654.

Athanassiades, A., Lala, P.K., 1998. Role of placenta growth factor (PlGF) in human extravillous trophoblast proliferation, migration and invasiveness. Placenta 19, 465–473.

Bagnato, A., Catt, K.J., 1998. Endothelins as autocrine regulators of tumor cell growth. Trends Endocrinol. Metab. 9, 378–383.

Bleasdale, J.E., Bundy, J.L., Bunting, S., et al., 1989. Inhibition of phospholipase C dependent process by U-73122. Adv. Prostaglandin Thromboxane Leukotriene Res. 19, 590–593.

Cervar, M., Huppertz, B., Barth, B., Hahn, T., Weiss, U., Kaufmann, P., Desoye, G., 2000. Endothelin A and B receptors change their expression levels during development of human placental villi. Placenta 21, 536–546.

Chakraborty, C., Gleeson, L.M., McKinnon, T., Lala, P.K., 2002. Regulation of human trophoblast migration and invasiveness. Can. J. Physiol. Pharmacol. 80 (2), 116–124.

Chen, S., Apostolova, M.D., Cherian, M.G., Chakrabarti, S., 2000. Interaction of endothelin-1 with vasoactive factors in mediating glucose induced increased permeability in the endothelial cells. Lab. Invest. 80, 1311–1321.

Chen, Y., Shyu, J.F., Santhagopal, A., et al., 1998. The calcitonin receptor stimulates Shc tyrosine phosphorylation and Erk1/2 activation: involvement of Gi, protein kinase C and calcium. J. Biol.Chem. 273, 19 809–19 816.

Cramer, H., Schmenger, K., Heinrich, K., Horstmeyer, A., Boning, H., Breit, A., Piiper, A., Lundstrom, A., Muller-Esterl, W., Schroeder, C., 2001. Coupling of endothelin receptors to the ERK/MAP kinase pathway. Roles of palmitoylation and G (alpha)q. Eur. J. Biochem. 268, 5449–5459.

Fant, M.E., Nau, L., Word, R.A., 1992. A potential role for endothelin-1 in human placental growth: interactions with insulin-like growth factor family of peptides. J. Clin. Endocrinol. Metab. 74, 1158–1163.

Fujitani, Y., Bertrand, C., 1997. ET-1 cooperates with EGF to induce mitogenesis via a PTX-sensitive pathway in airway smooth muscle cells. Am. J. Physiol. 272, C1492–C1498.

Giannessi, D., Delry, S., Vitale, R.L., 2001. The role of endothelins and their receptors in heart failure. Pharmacol. Res. 43, 111–126.

Gleeson, L.M., Chakraborty, C., McKinnon, T., Lala, P.K., 2001. Insulin-like growth factor binding protein 1 stimulates human

trophoblast migration by signaling through α5β1 integrin via mitogen-activated protein kinase pathway. J. Clin. Endocrinol. Metab. 86, 2484–2493.

Gomez-Garre, D., Largo, R., Tejera, N., Fortes, J., Manzarbeitia, F., Egido, J., 2001. Activation of NF-κB in tubular epithelial cells of rats with intense proteinuria: role of angiotensin II and endothelin-1. Hypertension 37, 1171–1178.

Graham, C.H., Hawley, T.S., Hawley, T.G., et al., 1993. Establishment and characterization of first trimester human trophoblast cells with extended life span. Exp. Cell Res. 206, 204–211.

Graham, C.H., Lysiak, J.J., McCrae, K.R., Lala, P.K., 1992. Localization of transforming growth factor-β at the human fatal-maternal interface: role in trophoblast growth and differentiation. Biol. Reprod. 46, 561–572.

Granger, J.P., Alexander, B.T., Llinas, M.T., Bennett, W.A., Khalil, W.A., 2001. Pathophysiology of hypertension during preeclampsia linking placental ischemia with endothelial dysfunction. Hypertension 38, 718–722.

Grynkiewich, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Hamilton, G.S., Lysiak, J.J., Watson, A., Lala, P.K., 1998a. Effects of colony stimulating factor-1 on human extravillous trophoblast growth and invasion. J. Endocrinol. 159, 69–77.

Hamilton, G.S., Lysiak, J.J., Han, V.K.M., Lala, P.K., 1998b. Autocrine-paracrine regulation of human trophoblast invasiveness by IGF-II and IFGBP-1. Exp. Cell Res. 217, 147–156.

Ihara, M., Noguchi, K., Saeki, T., et al., 1992. Biological profiles of highly potent novel endothelin antagonists selective for the ETA receptor. Life Sci. 50, 247–255.

Irving, J.A., Lala, P.K., 1995. Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-β, IGF-II and IGFBP-1. Exp. Cell Res. 217, 419–427.

Irving, J.A., Lysiak, J.J., Graham, C.H., Han, V.K.M., Hearn, S., Lala, P.K., 1995. Characteristics of trophoblast cells migrating from first trimester chorionic villus explants and propagated in culture. Placenta 16, 413–433.

Ishikawa, K., Ihara, M., Noguchi, K., et al., 1994. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. Proc. Natl. Acad. Sci. USA 91, 4892–4896.

Johnson, R.A., Alvarez, R., Salomen, Y., 1994. Determination of adenylyl cyclase catalytic activity using single and double column procedures. Methods Enzymol. 238, 31–57.

Kaufmann, P., Castelluci, M., 1997. Extravillous trophoblast in human placenta. Trophoblast Res. 10, 21–65.

Kilburn, B.A., Wang, J., Duniec-Dmuchkowski, J.M., Leach, R.E., Ramero, R., Armant, D.R., 2000. ECM and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. Biol. Reprod. 62, 739–747.

Klemke, R.L., Cai, S., Giannini, A.L., Gallagher, P.J., de Lanerolle, P., Cheresh, D.A., 1997. Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol. 137, 481–492.

Kohnen, G., Campbell, S., Irvine, G.A., Church, H.J., MacLachlan, F., Titterington, M., Cameron, I.T., 1998. Endothelin receptor extression in human decidua. Mol. Human Reprod. 4, 185–193.

Kohnen, G., Mackenzie, F., Collett, G.P., Campbell, S., Davenport, A.P., Cameron, A.D., Cameron, I.T., 1997. Differential distribution of endothelin receptor subtypes in placentae from normal and growth-restricted pregnancies. Placenta 18, 173–180.

Komuro, H., Rakic, P., 1996. Intracellular Ca^{++} fluctuations modulate the rate of neuronal migration. Neuron 17, 275–285.

Kubota, T., Kamada, S., Hirata, Y., Eguchi, S., Imai, T., Mauromo, F., Aso, T., 1992. Synthesis and release of endothelin-1 by human decidual cells. J. Clin. Endocrinol. Metab. 75, 1230–1234.

Kurman, R.J., 1992. The morphology, biology and pathology of intermediate trophoblast. Hum. Pathol. 22, 847–855.

Lysiak, J.J., Connelly, I., Khoo, N., Stetler-Stevenson, W., Lala, P.K., 1994. Role of transforming growth factor-α and epidermal growth factor (EGF) on proliferation and invasion by first trimester human trophoblast. Trophoblast Res. 8, 455–467.

Lysiak, J.J., Johnson, J.R., Lala, P.K., 1995. Localization of amphiregulin in the human placenta and decidua throughout gestation: role in trophoblast growth. Placenta 16, 359–366.

Malassine, I., Cronier, L., Mondon, F., Mignot, T.M., Ferre, F., 1993. Localization and production of immunoreactive endothelin-1 in the trophoblast of human placenta. Cell Tissue Res. 271, 491–497.

Mallat, A., Fouassier, L., Praeaux, A.M., et al., 1995. Growth inhibitory properties of endothelin-1 in human hepatic myofibroblastic Ito cells: an endothelin$_B$ receptor-mediated pathway. J. Clin. Invest. 96, 42–49.

Mazzoni, M.R., Breschi, M.C., Caccarelli, F., Lazzeri, N., Giusti, L., Paola, N.A., 1999. Lucacchini. Suc-[Glu^9, $Ala^{11,15}$]-endothelin-1 (8-21), IRL 1620, identifies two populations of ET_B receptors in guinea-pig bronchus. Br. J. Pharmacol. 127, 1406–1414.

McKinnon, T., Chakraborty, C., Gleeson, L.M., Chidiac, P., Lala, P.K., 2001. Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF type 2 receptor involving inhibitory G protein(s) and phosphorylation of MAPK. J. Clin. Endocrinol. Metab. 86, 3665–3674.

Mondon, F., Malassine, A., Robaut, C., Vial, M., Bandet, J., Tanguy, G., Rostene, W., Cavero, I., Ferre, F., 1993. Biochemical characterization and autoradiographic localization of [125I] endothelin-1 binding sites on trophoblast and blood vessels of human placenta. J. Clin. Endocrinol. Metab. 76, 237–244.

Morbidelli, L., Orlando, C., Maggi, C.A., Ledda, F., Ziche, M., 1995. Proliferation and migration of endothelial cells is promoted by endothelins via activation of ETB receptors. Am. J. Physiol. 269, 4686–4695.

Napolitano, M., Miceli, F., Calce, A., Vacca, A., Gulino, R., Apa, A., 2000. Lanzone. Expression and relationship between endothelin-1 messenger ribonucleic acid (mRNA) and inducible/endothelial nitric oxide synthase mRNA isoforms from normal and preeclamptic placentas. J. Clin. Endocrinol. Metab. 85, 2318–2323.

Neylon, C.B., 1999. Vascular biology of endothelin signal transduction. Clin. Exp. Pharmacol. Physiol. 26, 149–153.

Noiri, E., Hu, Y., Bahou, W.F., et al., 1997. Permissive role of nitric oxide in endothelin-induced migration of endothelial cell migration. J. Biol. Chem. 272, 1747–1752.

Rosano, L., Varmi, M., Salani, D., Di Castro, C., Spinella, F., Natali, P.G., Bagnato, A., 2001. Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. Cancer Res. 61, 8340–8346.

Rubanyi, G.M., Polokoff, M.A., 1994. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol. Rev. 46, 325–415.

Scott, G., Cassidy, L., Abdel-Malek, Z., 1997. Alpha-melanocyte-stimulating hormone and endothelin-1 have opposing effects on melanocyte adhesion, migration, and pp125FAK phosphorylation. Exp. Cell Res. 237, 19–28.

Shin, M.K., Levorse, J.M., Ingram, R.S., Tilghman, S.M., 1999. The temporal requirement for endothelin receptor-B signalling during neural crest development. Nature 402, 496–501.

Takigawa, M., Sakurai, T., Kasuya, Y., Abe, Y., Masaki, T., Goto, K., 1995. Molecular identification of guanine-nucleotide-binding regulatory proteins which couple to endothelin receptors. Eur. J. Biochem. 228, 102–108.

Takuwa, N., Takuwa, Y., Tanagisawa, M., et al., 1989. A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. J. Biol. Chem. 264, 7856–7861.

Xu, G., Guimond, M.J., Chakraborty, C., Lala, P.K., 2002. Control of proliferation, migration and invasiveness of the human extravillous

trophoblast by decorin, a decidual product. Biol. Reprod. 67, 681–689.

Yagel, S., Casper, R.F., Powel, W., Parhar, R.S., Lala, P.K., 1989. Characterization of pure human first-trimester cytotrophoblast cells in long-term culture: growth pattern, markers and hormone production. Am. J. Obstet. Gynecol. 160, 938–945.

Zdravkovic, M., Aboegyne-Matiesen, G., Lala, P.K., Hager, H., Ebbsen, P., 1999. Susceptibility of MHC Class-I expressing EVT cell lines to killing by natural killer cells. Placenta 20, 431–440.