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RESEARCH PAPER

Endothelin (ET)-1 and ET-3 promote expression of *c-fos* and *c-jun* in human choriocarcinoma via ET_B receptor-mediated G_i- and G_q-pathways and MAP kinase activation

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Background and purpose: Endothelins (ETs) and their G protein-coupled receptors exert key physiological functions during normal and aberrant placental development. Trophoblast cells mediate the contact between the embryo and the mother, by establishing a transient organ, the placenta. Choriocarcinoma cells display many of the biochemical and morphological characteristics of *in utero* invasive trophoblast cells and may therefore be used as a suitable model to study epithelial tumour progression of foetal-derived cells.
Experimental approach: The present study aimed at investigating ET receptor-mediated activation of the mitogen-activated protein kinase (MAPK) pathway in human choriocarcinoma.
Key results: Both JAR and Jeg-3 choriocarcinoma cell lines expressed *ET receptor subtype B* (*ET_B*) but not *ET_A* receptor transcripts. ET_B receptor engagement by ET-1 and ET-3 resulted in a similar time- and concentration-dependent phosphorylation of p42/44 MAPK, also known as extracellular regulated kinase 1/2. Using specific pharmacological antagonists/inhibitors, we showed that ET-1/-3-mediated signal transduction by the ET_B receptor is transmitted via G_i- and G_q-dependent pathways through activation of the Src (G_i) and protein kinase C (G_q) axis that converge at Ras/Raf, leading to downstream activation of p42/44. On a functional level, ET_B engagement and subsequent phosphorylation of p42/44 resulted in enhanced transcription of the immediate early response genes *c-fos* and *c-jun*, a process commonly assumed to be mediated by the ET_A receptor, and increased cell growth and relative cell area.
Conclusions and implications: As human choriocarcinoma cells secrete ETs, pharmacological antagonism of ETs and/or ET_B receptor-mediated signal transduction could represent a likely target therapy for choriocarcinoma.
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Keywords: trophoblast; intracellular signal transmission; tumour cell growth; G-protein coupled receptor

Abbreviations: EGF, epidermal growth factor; ET, endothelin; ET_A, endothelin A; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PTX, *Pertussis* toxin; RT-PCR, reverse transcription-PCR

Introduction

Endothelins (ETs), a family of small vasoactive peptides, have key physiological functions in normal tissue, acting as modulators of the vascular tone, tissue differentiation, development, cell proliferation and hormone production (see Nelson *et al*., 2003). In mammals, the ETs comprise a family of three peptides: ET-1, ET-2 and ET-3. Whereas ET-1 and ET-2 have similar structures, ET-3 differs in structure at 6 of 21 amino acids.

Endothelins exert their effects by binding to the ET A (ET_A) and ET_B receptor, two cell-surface proteins that belong to the G-protein-coupled receptor system and share about 59% similarity in the primary structure (Nelson *et al*., 2003). The ET_A receptor selectively binds ET-1 and ET-2, whereas the ET_B receptor binds all three ET forms with similar affinity. After ligand binding, ET_A receptors become internalized and

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undergo receptor recycling followed by relocation to the cell membrane, whereas ET_B receptors can be translocated to the lysosomal compartment for degradation (Foster *et al*., 2003) or translocated to nuclear membranes for further signalling events (Bkaily *et al*., 2006). While ET_A receptor primarily mediates vaso-/bronchoconstriction, mitogenesis, antiapoptosis, matrix formation, acute and neuropathic pain, the ET_B receptor mediates inflammatory pain and vasodilatation, and has further been proposed to contribute to clearance of ET as well as to autoinduction of ET-1 (Nelson *et al*., 2003).

Endothelin-1 acts most commonly in a paracrine and/or autocrine manner and is abundantly present in the placenta where it plays a potential role in early trophoblast proliferation and invasion, which is an essential process in normal placentation (Chakraborty *et al*., 2003). Placental fibroblasts, endothelial and vascular smooth muscle cells, decidual cells, and syncytiotrophoblast and cytotrophoblast cells synthesize ET-1. In most of the cells of the feto-maternal interface, ET receptors have been characterized and identified by radioligand saturation analysis, ligand competition and reverse transcription-PCR (RT-PCR) (Wilkes *et al*., 1990). The high ET_A receptor concentration observed in first-trimester placenta has been found to decrease progressively towards term (Kilpatrick *et al*., 1993) and the preponderance of ET_B receptors throughout gestation is probably important in dilating the placental vascular bed and maintaining feto-maternal blood flow.

During placental development, trophoblast cells develop along a cell lineage, forming the villous cytotrophoblast with the overlaying syncytiotrophoblast (Benirschke *et al*., 2006). Imbalances in the intrauterine milieu around the time of implantation and invasion may lead to abnormal trophoblast development associated with early pregnancy failure as seen in gestational trophoblastic disease (Bowen *et al*., 2002). Choriocarcinoma cell lines, replicating trophoblastic cells, derived from the malignant tumour or produced by viral transformation of normal first-trimester trophoblast cells, are suitable systems to study trophoblast behaviour *in vitro* (White *et al*., 1988; Babalola *et al*., 1990; Grümmer *et al*., 1994). As placental trophoblasts may act as *in vitro* models for tumour progression (Lala *et al*., 2002), two choriocarcinoma cell lines were used. JAR cells share many of the characteristics of early placental trophoblast cells (White *et al*., 1988) and the ability to differentiate into syncytiotrophoblast-like cells *in vitro* (Grümmer *et al*., 1994). Jeg-3 cells form large, multinucleated syncytia in culture (Babalola *et al*., 1990), which resemble that of syncytiotrophoblasts *in vivo*.

As choriocarcinoma cell lines secrete ET-1 (Bilban *et al*., 2000), and both ETs and their receptors have an emerging role in tumour cell invasion, the present study aimed at investigating ET receptor expression and G-protein coupled downstream signalling cascades in JAR and Jeg-3 cells. Mitogen-activated protein kinase (MAPK) cascades are key signalling pathways involved in the regulation of normal cell proliferation and differentiation, and aberrant regulation of MAPK cascades contributes to induction of early responsive genes in cancer and other pathological disorders. The MAPKs include three different pathways where the extracellular signal regulated protein kinases (ERK1/2, also known as p44 MAPK and p42 MAPK) has been the subject of intense research scrutiny leading to the development of pharmacological inhibitors for the treatment of cancer (see Dorsam and Gutkind, 2007; Roberts and Der, 2007).

Therefore, the present study was aimed to focus on ET-receptor expression in choriocarcinoma cell lines, and the corresponding G-protein coupled-dependent and -independent pathways leading to activation/phosphorylation of p42/44 MAPK. We then tried to assess whether activation/phosphorylation of p42/44 MAPK was tightly coupled with expression of early-response genes known to be involved in cell growth. Using ET-1 and ET-3 as suitable agonists and different pharmacological inhibitors, we show that only the ET_B receptor is operative in both choriocarcinoma cell lines to activate intracellular signalling and that transactivation of the epidermal growth factor (EGF) receptor is not involved.

Materials and methods

Cell culture

Human choriocarcinoma cell lines (JAR and Jeg-3, kindly supplied by Drs G Desoye, Graz; and R Fuchs, Vienna) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (JAR) or 5% (Jeg-3) fetal calf serum and 1% (v:v) gentamycin at 37 °C in a humidified atmosphere of 5% CO_2 (Wadsack *et al*., 2003; Kovacevic *et al*., 2006). Cells were used between the fifteenth and the thirtieth passage (JAR) or between the eighteenth and the twenty-fifth passage (Jeg-3), counted from the day of recipient. The cells were cultured in 75 cm^2 tissue culture flasks and, prior to treatment, seeded in six-well plates (Corning Incorporated, New York, NY, USA), at a concentration of 5×10^5 cells per well.

Human umbilical cord vein endothelial cells (kindly supplied by Dr R Heller, Erfurt) were prepared with 0.05% collagenase and maintained in M199 containing 15% (v:v) fetal calf serum, 5% (v:v) human serum and 7.5 $\mu g\,ml^{-1}$ endothelial cell growth supplement (Marsche *et al*., 2004).

Cell culture experiments

For time-dependent activation of the MAPK pathway and expression of early genes, cells were incubated in medium containing 20 nM ET-1 or ET-3 for up to 90 min. For agonist-concentration-dependent activation of MAPK, cells were incubated in medium containing various concentrations (0.1–100 nM) of ET-1 or ET-3 for 5 min. For investigation of signal transduction pathways, cells were incubated for 30 min in medium containing ET-receptor antagonists and/or various signal transduction inhibitors, BQ123 (250 and 500 nM), BQ788 (250 and 500 nM), manumycin A (10 μM), GÖ6983 (250 nM), PD98059 (25 μM), PP2 (10 μM), and U73122 (10 and 30 μM), propranolol (100 μM), SQ 22536 (25 μM), Rp-cAMPS (25 μM), LY294002 (25 μM), Akt inhibitor (20 μM), tyrphostin AG1478 (10 μM); using *Pertussis* toxin (PTX; 100 $ng\,ml^{-1}$) as an inhibitor of signal transduction, cells were preincubated overnight. The signal transduction inhibitors were dissolved as recommended by the manufacturer; the highest concentration of dimethyl sulphoxide in cell culture dishes was 0.1% (v:v).

Protein isolation and western blot analysis

Confluent cells were treated as indicated in the corresponding figure legends by addition of compounds directly into the medium. For protein isolation, the cells were washed with chilled phosphate-buffered saline (pH 7.4) and lysed on ice for 10 min in 60 μl of lysis buffer (HEPES, 50 mM; NaCl, 150 mM; ethylene diamine tetraacetic acid, 1 mM; $Na_4P_2O_7$, 10 mM; Na_3VO_4, 2 mM; NaF, 10 mM; Triton X-100, 1% (v:v); glycerol, 10% (v:v); protease inhibitor cocktail tablets; pH 7.4). The cell lysates were scraped using a rubber scraper and cell debris were removed by centrifugation at 11 000 *g* (4 °C, 10 min). Protein content was determined using BCA protein assay, according to the manufacturer's instructions. Protein lysates (50 μg) were diluted in NuPAGE LDS sample buffer and NuPAGE sample reducing agent to a final volume of 20 μl. The samples were boiled for 10 min at 70 °C, and then subjected to electrophoresis on 10% NuPAGE Bis-Tris Gels in NuPAGE MES sodium dodecyl sulphate (SDS) running buffer (30 min at 50 V, followed by 50 min at 150 V). Proteins were transferred to nitrocellulose membranes (2 h, 80 V). After blocking the membranes (5% (w:v) non-fat milk powder in Tris-buffered saline Tween-20 (TBS-T)) for 30 min at room temperature, the blots were incubated with mouse monoclonal anti-pp42/44 antibody (dilution 1:1000 in 3% (w:v) bovine serum albumin) for 2 h at room temperature. The membranes were washed 3 × 10 min in TBS-T buffer and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (dilution 1:1000 000) for 1 h at room temperature. After another 3 × 10 min washes with TBS-T, immunoreactive signals were detected with SuperSignal West Pico chemiluminescent substrate and by exposure to Hyperfilm MP. For loading controls, membranes were stripped and re-probed with a primary antibody recognizing total cellular p42/44 MAPK (Marsche *et al*., 2007); horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution 1:500 000) was used as a secondary antibody.

RNA isolation and RT-PCR

Total cellular RNA from JAR and Jeg-3 cells was isolated using TRIzol Reagent and then treated with DNaseI as recommended by the manufacturer. RT-PCR was performed with the QIAGEN OneStep RT-PCR kit (according to the manufacturer's instructions) in 50-μl reactions containing 100 ng of template RNA, 400 μM of each dNTP, 10 μl of 5 × QIAGEN OneStep RT-PCR buffer, 2 μl of QIAGEN OneStep RT-PCR enzyme mix and gene-specific primers in a final concentration of 0.6 μM. The reverse transcription reaction was conducted for 1 h at 42 °C, followed by an initial PCR activation step for 15 min at 95 °C, 30 or 35 amplification cycles, each of 10 s at 94 °C, 30 s at annealing temperature, 30 s at 72 °C, followed by a final 7 min elongation at 72 °C. Primer sequences, annealing temperatures, number of amplification cycles and amplicon size for each PCR are listed in Tables 1 and 2. The RT-PCR products were run on 1.5% agarose gels supplemented with 0.1% (v:v) ethidium bromide, in 1 × TBE buffer for 1 h at 100 V and visualised on UV transilluminator (Herolab, Heidelberg, Germany). To

Table 1 Description of primers, the expected amplified fragment size, the annealing temperature and the number of amplification cycles

Gene accession no.	*Primers (start position on + strand)*	*Fragment size (bp)*	*Annealing temperature (°C)*	*Cycle no.*	*Reference*
ET_A receptor	F: 5′ AGCTTCCTGGTTACCACTCATCAA 3′ (620)	714	55	35	Harneit *et al*. (1997)
NM_001957	R: 5′ TCAACATCTCACAAGTCATGAG 3′ (1334)				
ET_B receptor	F: 5′ CGAGCTGTTGCTTCTTGGAGTAG 3′ (838)	701	55	35	Harneit *et al*. (1997)
NM_000115	R: 5′ ACGGAAGTTGTCATATCCGTGATC 3′ (1539)				
GAPDH	F 5′: ACAGTCCATGCCATCACTGCC 3′(562)	265	58	30	Iochmann *et al*. (1999)
M17851	R: 5′ GCCTGCTTCACCACCTTCTTG 3′ (827)				
c-fos	F: 5′ AGCTCTGCTTCACAGCGC 3′ (87)	423	54	30	Doneda *et al*. (1997)
NM_005252	R: 5′ GGCCTCCTGTCATGGTCTT 3′ (510)				
c-jun	F: 5′ GGAAACGACCTTCTATGACGATGCCCTCAA 3′ (1275)	315	63	30	Doneda *et al*. (1997)
J04111	R: 5′ GAACCCCTCCTGCTCATCTGCACGTTCTT 3′ (1590)				
c-myc	F: 5′ TGGTCTTCCCCTACCCTCTCAAC 3′ (1136)	264	55	35	Zhang *et al*. (2006)
V00568	R: 5′ GATCCAGACTCTGACCTTTTGCC 3′ (1400)				

Abbreviations: ET, endothelin; ET_B, endothelin B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase.

Table 2 Description of primers, the expected amplified fragment size, the annealing temperature and the number of amplification cycles of ET_B receptor variants

Gene accession no.	*Primers (start position on + strand)*	*Fragment size (bp)*	*Annealing temperature (°C)*	*Cycle no.*	*Reference*
ET_B	F: 5′ CTGTGCTGAGTCTATGTGCT 3′ (793)	450	50	30	Shyamala *et al*. (1994)
S44866	R: 5′ GGAAGCCAGCAGAGGGCAAA 3′ (1243)				
ET_{B1}	F: 5′ CAGCTTAAAATACAATTCTATTTTTATCTT 3′ (165)	480	50	30	Shyamala *et al*. (1994)
F: S75586	R: 5′ GGAAGCCAGCAGAGGGCAAA 3′ (1243)				
ET_{B-276}	F: 5′ ATCCCTATAGTTTTACAAGACAGC 3′ (75 448)	169	50	30	Puffenberger *et al*. (1994)
AY547312	R: 5′ ATTTTCTTACCTGCTTTAGGTG 3′ (75 617)				

Abbreviation: ET_B, endothelin B.

ensure equal RNA loading, RT-PCR for glyceraldehyde-3-phosphate dehydrogenase was performed for each experiment.

Cell counts, relative cell area and motility studies
Cell counts were assessed by manual counting. JAR and Jeg-3 cells were seeded in six-well plates (5×10^4 cells per well) in Dulbecco's modified Eagle's medium supplemented with 2.5% (v:v) heat (56 °C for 45 min) inactivated fetal calf serum. Following overnight attachment, cells were treated with 20 nM ET-1 or ET-3 for 24 and 48 h. At the end of the incubation period, the cells were washed with ice-cold phosphate-buffered saline, trypsinized and cell numbers (six aliquots of each duplicate) were counted using a Bürker-Turk chamber. Cell counts were further statistically evaluated using boxplots and analysis of variance tests in SPSS. In the boxplots, the median for each data set is indicated by the black centre line. The first quartile of a group of values is the value such that 25% of the values fall at or below this value. The third quartile of a group of values is the value such that 75% of the values fall at or below this value, and the first and third quartiles are the upper and lower edges of the boxed area, which is known as the interquartile range. The maximum length of each whisker is 1.5 times the interquartile range.

Measurement of relative cell area. JAR cells (2×10^4 cells per well) were cultured as described above. Following overnight attachment, cells were stimulated in the presence of ET-1 up to 50 h. The 24-h values are boxplots of area values grouped from 22 to 26 h, whereas the 48 h values are boxplots of area values grouped from 46 to 50 h. Cell images were recorded every 20 min using a Chipman technologies Cell IQ microscope (objective $\times 10$; phase contrast; field size 1016×759 μm; pixel image size 1392×1040; pixel resolution 0.73 μm). For evaluation of the data, the software was used ImageJ (Rasband, 1997–2007). The area occupied by the cells in each image was calculated using the cell area macro as written for ImageJ, which makes use of a background subtraction and a statistical variance filter and thresholding to remove interference and enable the area calculation. The occupied cell area, thus calculated, compared with the area in the first image of the respective sequence of images represents the relative value, that is, '1' means the same area as in the first image '2' means double the area of the first image, and so on.

Measurement of cell motility. JAR cells were cultured as described above. Images, recorded using the Cell IQ microscope as described above, were used to estimate cell motility by making use of ImageJ and the manual Tracking macro (Fabrice Cordelières, Institut Curie, Orsay France). The 24-h values are boxplots of velocity values grouped from 22 and 26 h, whereas the 48-h values are boxplots of velocity values grouped from of 46 and 50 h. The cell motility statistics were evaluated using SPSS.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay
Cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. JAR and Jeg-3 cells (3×10^4 cells per well) were seeded in 12-well plates as described above. Following overnight attachment, cells were stimulated in the presence of ET-1 or ET-3 for 24 and 48 h. At the end of the incubation period, the cells were washed with warm phosphate-buffered saline (10 mM) and 500 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution (0.5 mg ml^{-1} in Dulbecco's modified Eagle's medium) was added to each well. After incubation for 2 h (37 °C), the medium was removed and acidic isopropanol (0.04 M HCl) was added. Measurements were performed spectrophotometrically at 570 nm, with background subtraction at 630 nm.

Reagents
Fetal calf serum and trypsin/ethylene diamine tetraacetic acid were purchased from PAA (Linz, Austria). Dulbecco's modified Eagle's medium, gentamycin and nitrocellulose membranes (0.45 μm pore size) were obtained from Gibco Invitrogen (Lofer, Austria). Collagenase, M199 and human serum were from BioWhittaker Europe (Verviers, Belgium). ET-1 and ET-3 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). BQ123 was from Bachem (San Carlos, CA, USA); BQ788 and manumycin A were from Sigma-Aldrich. PTX, GÖ6983, PD98059, propranolol, SQ 22536, LY294002, the Akt inhibitor, tyrphostin AG1478 and PP2 were obtained from Calbiochem (La Jolla, CA, USA). Rp-cAMPS and U73122 were from BIOMOL (Hamburg, Germany). Mouse monoclonal anti-phosphorylated p44/42 (pp42/44) MAPK was obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal anti-p42/44 MAPK (clone K-23) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-rabbit IgG was from Pierce Biotechnology Inc. (Rockford, IL, USA) and peroxidase-conjugated goat anti-mouse IgG was from Rockland (Gilbertsville, PA, USA). Antibody stripping buffer was from Gene Bio Application Ltd. (Kfar-Hanagid, Israel). Complete Mini protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany). NuPAGE 10% Bis-Tris Gels, NuPAGE MES SDS running buffer, NuPAGE LDS sample buffer, NuPAGE sample reducing agent, SeeBlue Plus prestained standard, TRIzol Reagent and DNaseI were purchased from Invitrogen (Carlsbad, CA, USA). BCA Protein Assay and SuperSignal West Pico Chemiluminescent Substrate were from Pierce Biotechnology, and Hyperfilm MP was obtained from Amersham Biosciences (Buckinghampshire, UK). QIAGEN OneStep RT-PCR kit was from QIAGEN (Hilden, Germany). All primer pairs (Tables 1 and 2) were purchased from TIB MOLBIOL (Berlin, Germany).

Results

Expression of ET receptors in human choriocarcinoma cells
Placenta-derived cells express ETs and *ET* receptor transcripts. To clarify ET-receptor expression in foetus-derived choriocarcinoma cell lines, RT-PCR using specific forward and reverse primer pairs (Table 1) was performed. No expression of ET_A receptor transcripts was observed in non-stimulated and stimulated JAR choriocarcinoma (Figure 1) that are regarded as poorly differentiated cells. However, also

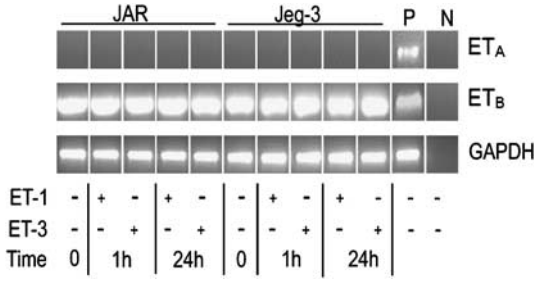

Figure 1 RT-PCR of ET receptors in human choriocarcinoma cells. JAR and Jeg-3 cells were cultured in medium containing 20 nM of ET-1 or ET-3 for 1 and 24 h. RNA was isolated and ET_A and ET_B receptor fragments were amplified by RT-PCR using specific forward and reverse oligonucleotide primer pairs (Table 1). The PCR products were separated on 1.5% agarose gels. P (positive control: human umbilical venous endothelial cells); N (negative control: RNA free water). To ensure equal gel loading, control glyceraldehyde-3-phosphate dehydrogenase RT-PCR (Table 1) was performed. One representative experiment out of three is shown. ET, endothelin; ET_A, endothelin A; ET_B, endothelin B; RT-PCR, reverse transcription-PCR.

Jeg-3 cells, which have been reported to proliferate slowly, but have a higher degree of differentiation, that is, they contain several syncytial elements locked ET_A receptor transcripts (Figure 1). Also, use of different passages of both choriocarcinoma cell lines revealed no ET_A-receptor expression on RNA level. However, expression of ET_B-receptor transcripts could be confirmed under non-stimulated conditions, as well as under short- and long-term stimulated conditions using ET-1 or ET-3 as an agonist (Figure 1).

Recently, a splice variant of the ET_B receptor (named ET_{B1}), with similar ligand affinity as reported for ET_B, has been identified in human uterus and placenta (Shyamala *et al*., 1994). However, using specific primer pairs for both ET receptors (Table 2) and sequencing the corresponding PCR product, no ET_{B1} could be identified in JAR and Jeg-3 cell lines (data not shown).

Another ET_B-receptor variant ($ET_{B\text{-}276}$) that carries a single missense mutation (G→T) in exon 4 has been reported (Puffenberger *et al*., 1994). This mutation (Trp276Cys) results in impaired signal transduction due to the lack of G_q-coupling (Imamura *et al*., 2000). However, using specific primer pairs for both ET receptors (Table 2) and sequencing the corresponding PCR product, no $ET_{B\text{-}276}$ receptor could be identified in JAR and Jeg-3 cells (data not shown).

Activation of the MAPK pathway in human choriocarcinoma cells
The ET_A receptor is considered to be the primary receptor for ET-1-mediated activation of the MAPK pathway. We show here that in both choriocarcinoma cell lines, ET-1 and ET-3, induced comparable time-dependent phosphorylation of p42/44 (reaching maximum activation at approximately 2.5 min after stimulation) (Figure 2). The concentration-dependent activation of the MAPK pathway in JAR and Jeg-3 cells by ET-1 and ET-3 was paralleled by an increasing extent of phosphorylation of p42/44 (Figure 3).

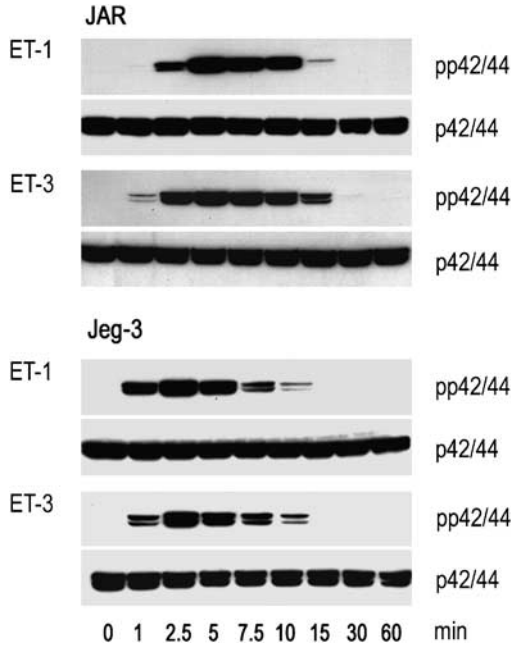

Figure 2 Western blot of ET-induced time-dependent p42/44 activation in human choriocarcinoma cells. JAR and Jeg-3 cells were incubated in medium containing 20 nM ET-1 or ET-3 at indicated time periods (1–60 min). The cells were lysed, aliquots of protein lysates were subjected to SDS-PAGE and transferred to nitrocellulose as described under Materials and methods. Phosphospecific mouse monoclonal anti-p42/44 and rabbit anti-p42/44 were used as primary antibodies. Immunoreactive bands were visualized with peroxidase-conjugated secondary antibodies using the Super Signal system. One representative experiment out of three is shown. ET, endothelin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Inhibition of the MAPK pathway in human choriocarcinoma cells
To clarify whether ET-receptor blockade in JAR and Jeg-3 cells is paralleled by lack of phosphorylation of p42/44, both choriocarcinoma cell lines were preincubated (either alone or in combination) with two well-defined ET-receptor antagonists, prior to stimulation with ET-1 or ET-3. BQ123 (an ET_A-receptor antagonist) had no effect on the extent of ET-mediated phosphorylation pattern of p42/44 (Figure 4); data that confirm results shown in Figure 1. When JAR and Jeg-3 cells were preincubated with BQ788 (an ET_B-receptor blocker), either alone or in combination with BQ123, no phosphorylation of p42/44 was observed. In a next set of experiments, we investigated effects of a specific inhibitor of the MAPK pathway on phosphorylation of p42/44; Figure 5 shows that the specific MAPK kinase inhibitor, PD98059, was effective in preventing phosphorylation of p42/44.

Activation of the MAPK pathway in human choriocarcinoma cells via G_i and/or G_q
A further set of experiments was aimed to identify G-protein-mediated coupling of the ET_B receptor to the MAPK pathway. Inhibition of G_i signalling with PTX led to nearly complete inhibition of ET-1- and ET-3-stimulated phosphorylation of p42/44 in JAR cells and clearly attenuated response in Jeg-3 cells (Figure 6a). These observations demonstrate that

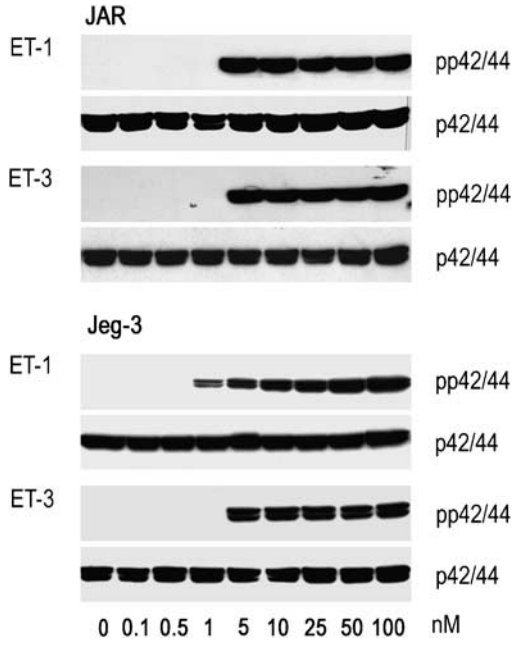

Figure 3 Western blot of ET-induced concentration-dependent p42/44 activation in human choriocarcinoma cells. JAR and Jeg-3 cells were incubated in medium containing ET-1 or ET-3 at the indicated concentrations (0.1–100 nM) for 5 min. The cells were lysed, aliquots of protein lysates were subjected to SDS-PAGE and transferred to nitrocellulose as described under Materials and methods. Phosphospecific mouse monoclonal anti-p42/44 and rabbit anti-p42/44 were used as primary antibodies. Immunoreactive bands were visualized with peroxidase-conjugated secondary antibodies using the Super Signal system. One representative experiment out of three is shown. ET, endothelin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

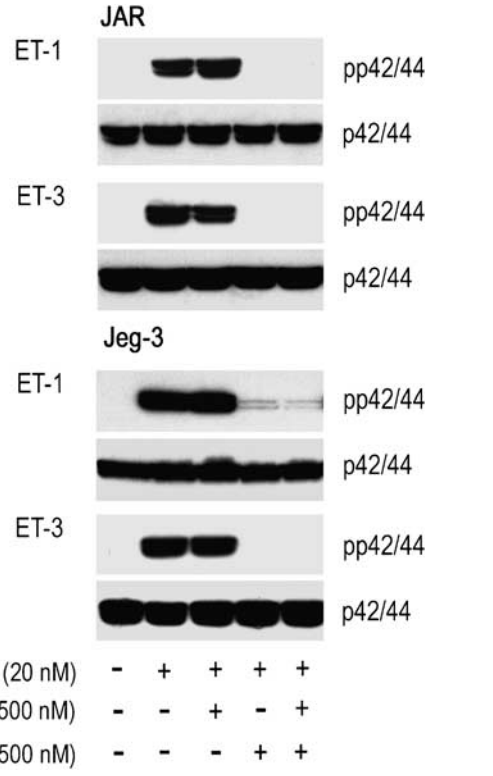

Figure 4 Western blot of ET-induced p42/44 activation in human choriocarcinoma cells in the presence of ET receptor antagonists. JAR and Jeg-3 cells were preincubated for 30 min in medium with a ET_A (BQ123) or ET_B receptor antagonist (BQ788) at the indicated concentration. Pre-incubated and non pre-incubated cells were stimulated with ET-1 or ET-3 (added at the indicated concentration to the medium) for 5 min. The cells were lysed, aliquots of protein lysates were subjected to SDS-PAGE and transferred to nitrocellulose as described under Materials and methods. Phosphospecific mouse monoclonal anti-p42/44 and rabbit anti-p42/44 were used as primary antibodies. Immunoreactive bands were visualized with peroxidase-conjugated secondary antibodies using the Super Signal system. One representative experiment out of three is shown. ET, endothelin; ET_A, endothelin A; ET_B, endothelin B; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

coupling of G_i type of heterotrimeric G-proteins to the ET_B-receptor population partially mediates ET-induced activation of p42/44 MAPK. As Src complexes and Src kinase activation are early events in Ras-dependent activation of MAPK via PTX-sensitive G-protein-coupled receptors (Luttrell *et al*., 1996), choriocarcinoma cells were pretreated with a selective inhibitor of the Src family of protein tyrosine kinases, PP2, as well as the Ras inhibitor, manumycin. Inhibition of p42/44 phosphorylation by PP2 and manumycin (Figure 6b) clearly indicates a G_i-Src-Ras-mediated signalling pathway. As expected, no phosphorylation of p42/44 was observed when cells stimulated with ET-1 and ET-3 were preincubated with manumycin and BQ123 (an ET_A receptor antagonist) or BQ788 (an ET_B receptor antagonist) (Figure 6b).

Next, we investigated whether the G_q-subtype of G-proteins also contributes to ET-mediated activation of the MAPK pathway in choriocarcinoma cell lines. Indeed, ET-1- and ET-3-induced activation of phospholipase C and protein kinase C (PKC) was blocked using specific inhibitors U73122 and GÖ6983 (Figure 6a). Similarly as shown with PTX, both inhibitors attenuated p42/44 phosphorylation. As the combination of PTX with U73122 and GÖ6983 completely blocked phosphorylation of p42/44 (Figure 6a), G-protein coupling of the ET_B receptor population to the G_i- and G_q-subtypes of G-protein-α subunits is likely to occur in both choriocarcinoma cell lines. These observations parallel our findings (mentioned above) that the Tyr276Cys mutation (reported to impair G_q coupling; Imamura *et al*., 2000) is not present in either choriocarcinoma cell line.

Next we tested, whether additional pathways (also known to be coupled via G_i/G_q-mediated signalling) are also involved in ET-1- and ET-3-mediated phosphorylation of p42/44 in both choriocarcinoma cell lines. However, use of SQ22536 (an adenylate cyclase inhibitor) and Rp-cAMPS (a specific protein kinase A inhibitor) are indirect and direct evidence, respectively, that protein kinase A activation was not contributing to p42/44 phosphorylation (Figure 6c). Neither propranolol (a PLD inhibitor and thus indirect inhibitor of the phosphatidyl inositol 3-kinase/Akt pathway) nor LY294002 (a specific phosphatidyl inositol 3-kinase inhibitor), nor a specific Akt inhibitor altered p42/44 phosphorylation (Figure 6c). Also AG1478, a highly specific and selective inhibitor of the EGF-receptor kinase, did not affect phosphorylation of p42/44 (Figure 6c).

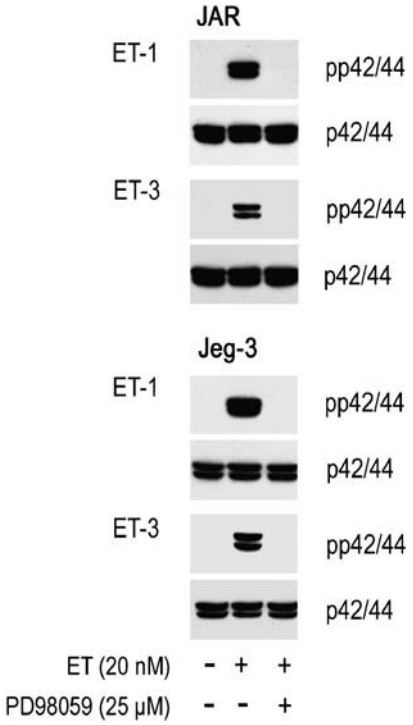

Figure 5 Western blot of ET-induced p42/44 activation in human choriocarcinoma cells in the presence of MAPK inhibitors. JAR and Jeg-3 cells were preincubated for 30 min in medium with the MAPK kinase inhibitor PD98059 at the indicated concentration. Preincubated and non-preincubated cells were stimulated by ET-1 or ET-3 (added at the indicated concentration to the medium) for 5 min. The cells were lysed and aliquots of protein lysates were subjected to SDS-PAGE and transferred to nitrocellulose. Phosphospecific mouse monoclonal anti-p42/44 and rabbit anti-p42/44 were used as primary antibodies. Immunoreactive bands were visualized with peroxidase-conjugated secondary antibodies using the Super Signal system. One representative experiment out of three is shown. ET, endothelin; MAPK, mitogen-activated protein kinase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Expression of early-response genes in the absence and presence of ET_B blockade and MAPK kinase inhibition

Stimulation of the Ras–MAPK signal transduction pathway results in a multitude of events, including expression of immediate-early genes such as *c-fos*, *c-jun* and *c-myc* (Dunn *et al*., 2005). In ovarian cancer, ET-1-mediated activation of the ET_A receptor promotes *c-fos* expression via phosphorylation of p42/44 and the EGF-receptor kinase (Bagnato *et al*., 1997; Vacca *et al*., 2000). We here show that ET_B receptor engagement by ET-1 and ET-3, respectively, led to time-dependent induction of *c-fos* and *c-jun* in both choriocarcinoma cell lines (Figure 7). In general, expression of both genes returned to baseline levels after 90 min. Only the proto-oncogene, *c-myc*, a major inducer of proliferation and differentiation in cancer cells, is constitutively expressed at the RNA level in JAR and Jeg-3 cells.

To confirm that the ET_B-receptor–MAPK axis is operative in inducing *c-fos* and *c-jun*, choriocarcinoma cell lines were pretreated with the ET_B receptor antagonist BQ788 or the MAPK kinase inhibitor PD98059 prior to stimulation with ET-1 or ET-3. RT-PCR analysis revealed marked reduction of *c-fos* and *c-jun* in both choriocarcinoma cell lines, whereas expression of *c-myc* was unaffected (Figure 8).

Cell counting, relative cell area and motility of choriocarcinoma cells

To investigate whether ET treatment of human choriocarcinoma alters the cell number, cells were incubated with ET-1 or ET-3 for 24 and 48 h. Whereas after 24 h both ETs had no effect, a significant ($P<0.001$) increase in cell number was found after 48 h in both the cell lines in response to ET treatment (Figure 9a). As cell viability studies, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium test, revealed no significant differences between non-stimulated and ET-stimulated JAR and Jeg-3 cells (data not shown), the next series of experiments aimed at investigating the relative cell area of non-stimulated and ET-stimulated JAR cells by video time-lapse microscopy. Both, ET-1 and ET-3 significantly increased the relative cell area after 24 and 48 h (Figure 9b). Next, cell motility was estimated using the same image sequences. Data shown in Figure 9c reveal significant ($P<0.001$) differences in cell motility in ET-1-treated JAR cells after 24 and 48 h compared with non-stimulated cells.

Discussion

Endothelins are potent vasoactive peptides that mediate their effects via heptahelical G-protein-coupled receptors of the ET_A and ET_B type to transmit receptor-mediated signalling depending on cell or tissue type. In addition to their role as vasoactive peptides, ETs are also recognized as growth factors, as they are potent mitogens for a variety of cell types. Expression of ET-1 and ET receptors has been shown in various cancer cell lines and tumours such as cervical, ovarian, prostate, colon, glioma, lung and renal (Nelson *et al*., 2003; Bagnato *et al*., 2005). Therefore, ETs have been proposed as progression factors in many tumour types and to promote tumour growth. In addition, ETs suppress apoptosis of cancer cells. These proliferation and survival functions of ETs are mediated via both ET_A and ET_B receptor populations, depending on the cell type (Nelson *et al*., 1996; Bagnato and Catt, 1998; Shichiri *et al*., 1998; Dong *et al*., 2005). Moreover, ET_A receptor blockade completely inhibits growth and neoangiogenesis of cervical carcinoma cell xenografts (Bagnato *et al*., 2002).

Endothelins and ET receptors are ubiquitously expressed and regulate a wide range of physiological and pathophysiological functions, such as cardiovascular, mitogenic and neuroregulatory events and placental development. Expression levels of ET_A and ET_B receptors have further been reported to significantly change during normal pregnancy (Wilkes *et al*., 1990; Germain *et al*., 1997; Kohnen *et al*., 1997; Sand *et al*., 1998). ET_B receptor-binding sites in the placenta increased from the first ($70 \pm 6\%$) to the third trimester ($97 \pm 3\%$) (Kilpatrick *et al*., 1993). ET_A and ET_B receptor transcripts are present in first- and second-trimester placental tissue, whereas some third-trimester preparations are lacking ET_A receptor transcripts (Kilpatrick *et al*., 1993). Also, in isolated trophoblast preparations, a decrease in ET_A receptor transcripts from the first to the third trimester has been reported (Cervar *et al*., 2000).

Contradictory findings on ET_A receptor expression in choriocarcinoma cells have been reported. Fiore *et al*.

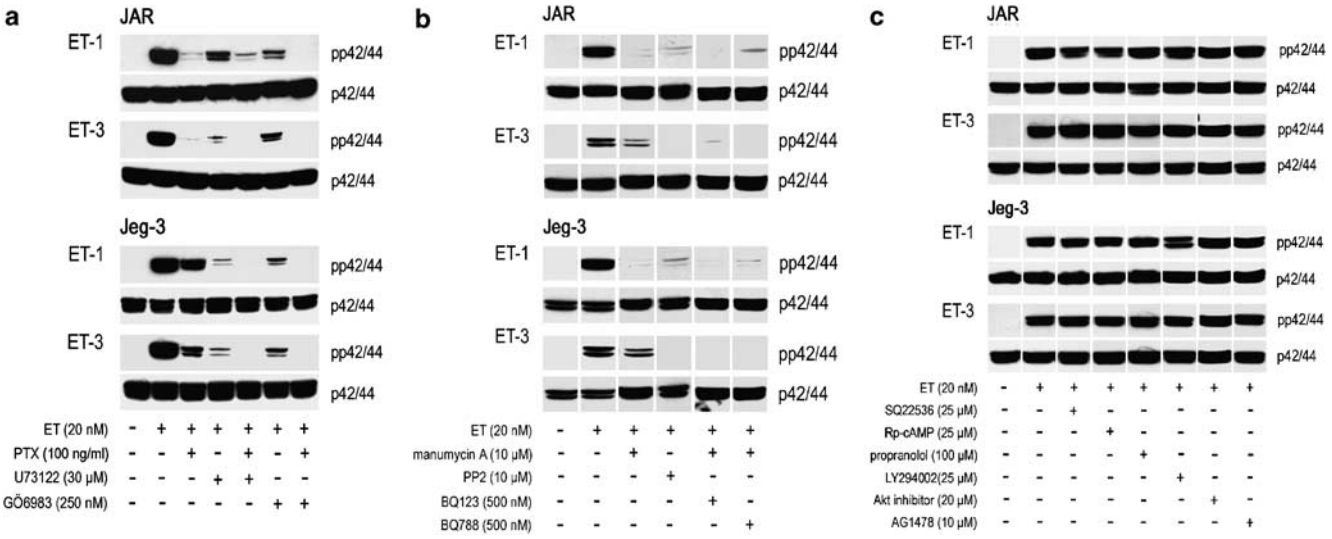

Figure 6 Western blot of ET-induced p42/44 activation in human choriocarcinoma cells in the presence of pharmacological inhibitors. JAR and Jeg-3 cells were preincubated (**a**) for 12 h with PTX and for 30 min in medium with phospholipase C inhibitor (U73122) and PKC inhibitor (GÖ6983), alone or in combinations; (**b**) for 30 min in medium with an inhibitor of the Src family of tyrosine kinases (PP2), the Ras inhibitor (manumycin A) or combinations of manumycin with ET_A (BQ123) or ET_B receptor antagonist (BQ788); (**c**) with various inhibitors of receptor-mediated signal transduction at indicated concentration for 30 min. Preincubated and non-preincubated cells were stimulated with ET-1 or ET-3 (at the indicated concentrations) for 5 min. The cells were lysed and aliquots of protein lysates were subjected to SDS-PAGE and transferred to nitrocellulose as described under Materials and methods. Phosphospecific mouse monoclonal anti-p42/44 and rabbit anti-p42/44 were used as primary antibodies. Immunoreactive bands were visualized with peroxidase-conjugated secondary antibodies using the Super Signal system. One representative experiment out of three is shown. ET, endothelin; ET_A, endothelin A; ET_B, endothelin B; PTX, *Pertussis* toxin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

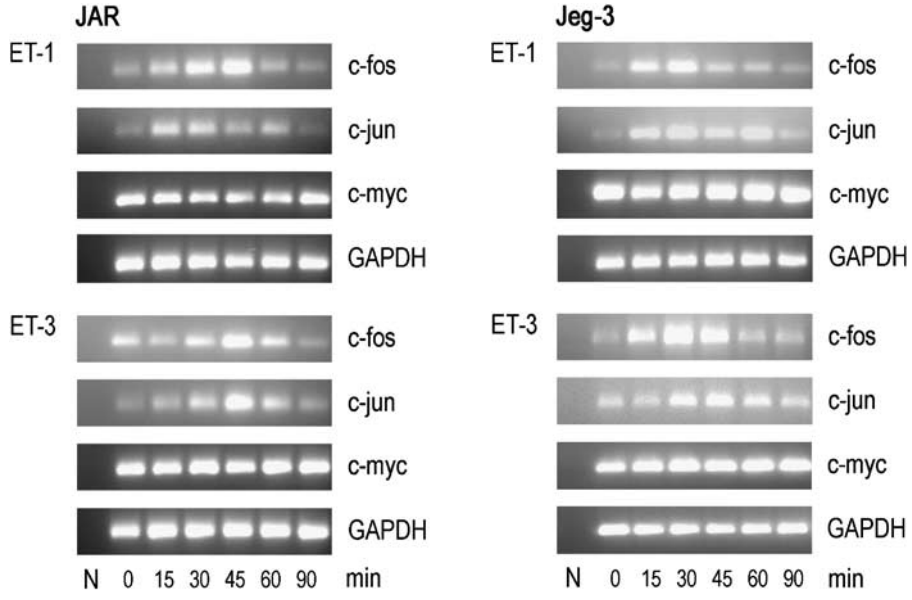

Figure 7 RT-PCR of ET-mediated, time-dependent induction of early-response genes in human choriocarcinoma cells. JAR and Jeg-3 cells were cultured in medium containing ET-1 or ET-3 (20 nM) for the indicated time periods (15–90 min). RNA was isolated and *c-fos*, *c-jun* and *c-myc* fragments were amplified by RT-PCR using specific forward and reverse oligonucleotide primer pairs (Table 1). The PCR products were separated on 1.5% agarose gels; N (negative control, RNA-free water). To ensure equal gel loading, control glyceraldehyde-3-phosphate dehydrogenase RT-PCR (Table 1) was performed. One representative experiment out of three is shown. ET, endothelin; RT-PCR, reverse transcription-PCR.

(2005) observed low ET_A receptor expression (compared with ET_B receptor) in Jeg-3 cells. Other authors reported pronounced expression of ET_A receptor proteins in JAR and Jeg-3 (Mauschitz *et al*., 2000) despite absence of ET_A receptor mRNA (Bilban *et al*., 2000). Our data using RNA treated with DNAse and published primer pairs for RT-PCR (Table 1) clearly demonstrate expression of ET_B but no ET_A receptors at the mRNA level in JAR and Jeg-3 choriocarcinoma cells (Figure 1). These results are confirmed by observations that ET-3 promotes phosphorylation of p42/44 via ET_B receptor,

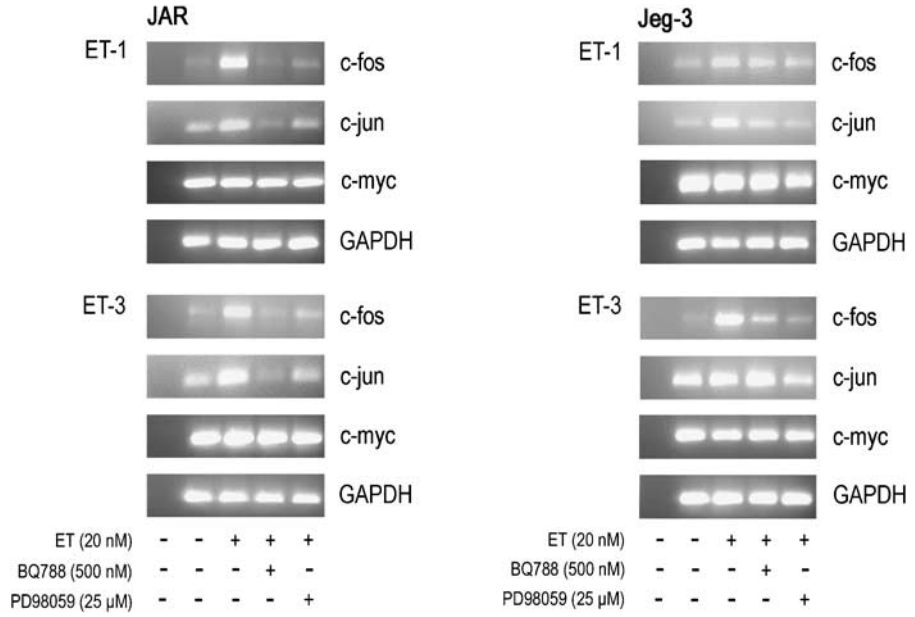

Figure 8 RT-PCR of ET-dependent induction of early response genes in human choriocarcinoma cells in the presence of specific inhibitors. JAR and Jeg-3 cells were preincubated for 30 min in medium with ET_B receptor antagonist (BQ788) or MAPK kinase inhibitor (PD98059) at the indicated concentrations. Preincubated and non-preincubated cells were stimulated by adding ET-1 or ET-3 at the indicated concentrations for 45 min. RNA was isolated and *c-fos*, *c-jun* and *c-myc* fragments were amplified by RT-PCR using specific forward and reverse oligonucleotide primer pairs (Table 1) as described under Materials and methods. The PCR products were separated on 1.5% agarose gels; N (negative control, RNA-free water). To ensure equal gel loading, control glyceraldehyde-3-phosphate dehydrogenase RT-PCR (Table 1) was performed. One representative experiment out of three is shown. ET, endothelin; ET_B, endothelin B; MAPK, mitogen-activated protein kinase; RT-PCR, reverse transcription-PCR.

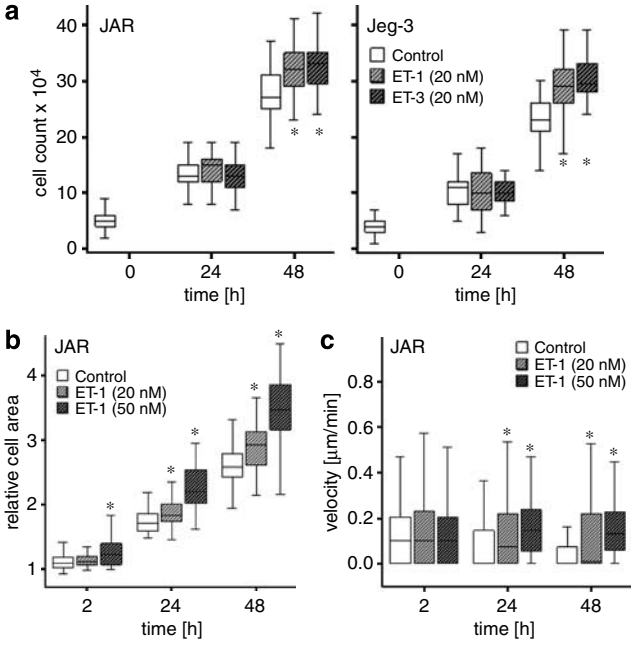

Figure 9 Effect of ET-1 and ET-3 on cell counts, relative cell area and cell motility of human choriocarcinoma cell lines. JAR and Jeg-3 cells (**a**) were cultured in heat-inactivated medium for the indicated times in the absence or presence of ET-1 or ET-3 concentrations for the indicated time intervals. (**a**) Manual cell counting using the Bürker-Turk chamber. (**b** and **c**) JAR cells were cultured as described under Materials and methods, and relative cell area (**b**) and cell motility (shown here as velocity; (**c**)) were recorded using images from the Cell IQ microscope. Statistical evaluations were performed using SPSS. Values are expressed as boxplots and indicate significant differences (*$P<0.001$; analysis of variance) between ET-1 and ET-3-treated cells versus non-stimulated cells. ET, endothelin.

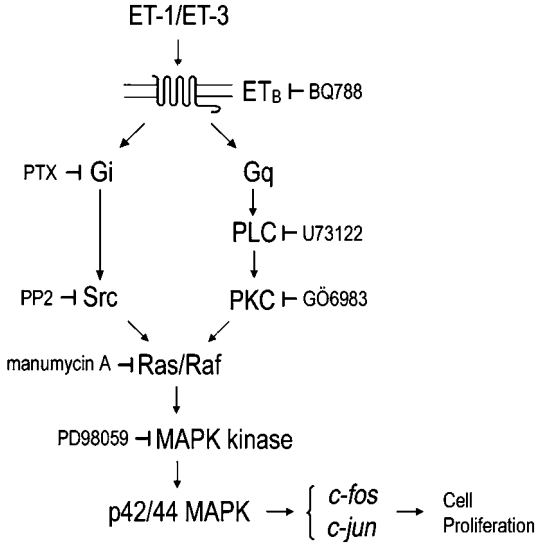

Figure 10 The proposed activation cascade of ET-mediated signalling via the ET_B receptor. Upon binding to ET_B, ET (ET-1 or ET-3) initiates signalling cascades leading to p42/44 MAPK activation. The possible cooperation of G-protein ($G_i/_0$ and G_q-PKC)-dependent pathways leads to Ras activation. Activated Ras recruits MAPK kinase Raf and activated Raf signals through its substrate, MAPK kinase, to p42/44 MAPK. Activated p42/44 MAPK subsequently induces the expression of early-response genes such as *c-jun* and *c-fos* and promotes cell proliferation. ET, endothelin; ET_B, endothelin B; MAPK, mitogen-activated protein kinase.

as does ET-1; the ET_B receptor has been reported to bind ET-1 and ET-3 with similar affinity (Nelson *et al.*, 2003). Our observations are further confirmed on a pharmacological level, as BQ788 (an ET_B receptor antagonist), but not BQ123

(an ET_A receptor antagonist), was able to inhibit cellular responses after stimulation with ET-1 or ET-3 (Figure 4). Functional coupling of the ET_B receptor population to receptor-mediated signal transduction results in phosphorylation, and thereby activation, of p42/44 MAPK. ET_B receptors are assumed to play an important role in clearance of circulating ET-1 (Ozaki *et al*., 1995) and to couple to $G_{\alpha q/11}$ (Takigawa *et al*., 1995), $G_{\alpha i}$ (Eguchi *et al*., 1993) and $G_{\alpha 13}$ (Kitamura *et al*., 1999) subtypes of G_α-subunits, thus stimulating different intracellular signal transduction pathways. In the present study, neither pretreatment of the cells with PTX nor inhibition of $G_{\alpha q/11}$-mediated activation of phospholipase C and PKC completely blocked p42/44 MAPK activity. In contrast, combination of PTX with inhibitors of phospholipase C/PKC prevented p42/44 phosphorylation, suggesting coupling of $G_{\alpha q/11}$ and $G_{\alpha i}$ to ET_B receptors in JAR and Jeg-3 cells. This is in line with findings in astrocytes, where ET-1-mediated activation of MAPK signalling occurs via combination of PTX-sensitive and PTX-insensitive (PKC-dependent pathways) (Kasuya *et al*., 1994). Our findings on ET-1- and ET-3-mediated G-protein coupling in JAR and Jeg-3 cells are supported by the observation that both choriocarcinoma cell lines lack the G→T missense mutation (Trp276Cys) within the human ET_B receptor, and thus no impaired $G_{\alpha q}$ signalling has been observed (Figure 4). Inhibition of p42/44 MAPK after treatment of cells with PP2 or manumycin A demonstrates involvement of the Src-Ras/Raf pathway as previously shown for adrenal glomerulosa cells (Shah *et al*., 2006). Therefore, we propose that phosphorylation of p42/44 MAPK and subsequent expression of early responsive genes occurs along the signalling cascade, as shown in Figure 10. Transactivation of the EGF receptor could be excluded, as tyrphostin AG1478, which selectively inhibits autophosphorylation of the EGF receptor, did not affect p42/44 activation. Jeg-3 cells express functional EGF receptors (Cao *et al*., 1994) and treatment of these cells with EGF has been reported to phosphorylate p38 MAPK (Roberson *et al*., 2000).

Mitogen-activated protein kinase signalling is activated by a multitude of stimuli and plays a central role during cancer development. The p42/44 pathway is the best studied of mammalian MAPK pathways and is deregulated in about one-third of all human cancers (Dhillon *et al*., 2007). G-protein-mediated loading of Ras GTPase leads to recruitment of the Raf kinase, followed by activation of MAPK kinase, which results in phosphorylation of p42/44 MAPK. Most cancer-associated lesions leading to sustained activation of p42/44 occur at early steps of the pathway, for example, overexpression of tyrosine kinases, such as those of the EGF receptor, Ras and Raf mutations (Downward, 2003), and/or sustained autocrine or paracrine production of activating ligands like ET-1 (Wulfing *et al*., 2004). As a consequence of sustained p42/44 activation, deregulation and/or amplification of nuclear transcription factors, most notably c-myc and AP-1, can be observed. Our own data clearly demonstrate that the p42/44 MAPK pathway in human choriocarcinoma cell lines is not constitutively active, as ET-1/3 stimulation is required for p42/44 activation. Also p42/44 dependent induction of the proto-oncogenes *c-jun* and *c-fos*, which is accompanied by significant increase of cell number, relative cell area and motility depends on ET-1 or ET-3. In contrast, ET-1 and ET-3 were without effect on *c-myc* expression levels. Different panels of general and tissue-specific transcription factors operate together to guarantee a constant transcription of *c-myc* (Chung and Levens, 2005). Most importantly, *c-myc*-mutant murine embryos are small and retarded in development compared with their littermates (Davis *et al*., 1993), providing direct evidence that *c-myc* is necessary for normal embryonic development.

The ET_B receptor shares several intracellular signalling pathways with the ET_A receptor; however, unlike ET_A receptor-dependent effects, ET_B receptor-dependent phenomena are shorter lasting and reversible. We here show that ET_B receptor-mediated coupling in choriocarcinoma cell lines leads to activation of the Ras/Raf-MAPK pathway. This pathway is a key downstream effector of the Ras small GTPase, the most frequent mutated oncogene in human cancers. Ras is a key downstream effector of the EGF receptor that is mutationally activated and even overexpressed in a variety of human cancers, which in turn may lead to enhanced cell proliferation as observed in human choriocarcinoma. To date, the Ras/Raf-MAPK pathway is considered a major pathway for therapeutic intervention in human diseases such as cancer. Indeed, ET_B receptor blockade has been reported to inhibit the dynamics of cell interactions and communications in melanoma cell progression. From that point, pharmacological interruption of ET_B receptor signalling may represent a novel therapeutic strategy in the treatment of this malignancy (Bagnato *et al*., 2005; Lahav, 2005). From our findings, we conclude that ET_B receptor-mediated activation of the Ras/Raf-MAPK pathway is operative in human choriocarcinoma, although particular upstream activation via the EGF receptor may be excluded. Whether effects observed in the present study will promote invasiveness, apoptosis or angiogenesis, or whether blockade of the ET_B receptor might represent an alternative approach during abnormal placental development, remains to be elucidated (Bagnato *et al*., 2005).

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Conflict of interest

The authors state no conflict of interest.

References

Babalola GO, Coutifaris C, Soto EA, Kliman HJ, Shuman H, Strauss III JF (1990). Aggregation of dispersed human cytotrophoblastic cells: lessons relevant to the morphogenesis of the placenta. *Dev Biol* **137**: 100–108.

Bagnato A, Catt KJ (1998). Endothelins as autocrine regulators of tumor cell growth. *Trends Endocrinol Metab* **9**: 378–383.

Bagnato A, Cirilli A, Salani D, Simeone P, Muller A, Nicotra MR *et al.* (2002). Growth inhibition of cervix carcinoma cells *in vivo* by endothelin A receptor blockade. *Cancer Res* **62**: 6381–6384.

Bagnato A, Spinella F, Rosano L (2005). Emerging role of the endothelin axis in ovarian tumor progression. *Endocr Relat Cancer* **12**: 761–772.

Bagnato A, Tecce R, Di Castro V, Catt KJ (1997). Activation of mitogenic signaling by endothelin 1 in ovarian carcinoma cells. *Cancer Res* **57**: 1306–1311.

Benirschke K, Kaufmann P, Baergen RN (2006). *Pathology of the Human Placenta*. Springer Verlag: New York.

Bilban M, Barth S, Cervar M, Mauschitz R, Schaur RJ, Zivkovic F *et al.* (2000). Differential regulation of endothelin secretion and endothelin receptor mRNA levels in JAR, JEG-3, and BeWo choriocarcinoma cell lines and in human trophoblasts, their nonmalignant counterpart. *Arch Biochem Biophys* **382**: 245–252.

Bkaily G, Nader M, Avedanian L, Choufani S, Jacques D, D'Orleans-Juste P *et al.* (2006). G-protein-coupled receptors, channels, and Na^+–H^+ exchanger in nuclear membranes of heart, hepatic, vascular endothelial, and smooth muscle cells. *Can J Physiol Pharmacol* **84**: 431–441.

Bowen JM, Chamley L, Mitchell MD, Keelan JA (2002). Cytokines of the placenta and extra-placental membranes: biosynthesis, secretion and roles in establishment of pregnancy in women. *Placenta* **23**: 239–256.

Cao H, Lei ZM, Rao CV (1994). Transcriptional and posttranscriptional mechanisms in epidermal growth factor regulation of human chorionic gonadotropin (hCG) subunits and hCG receptor gene expression in human choriocarcinoma cells. *Endocrinology* **135**: 962–970.

Cervar M, Huppertz B, Barth S, Hahn T, Weiss U, Kaufmann P *et al.* (2000). Endothelin A and B receptors change their expression levels during development of human placental villi. *Placenta* **21**: 536–546.

Chakraborty C, Barbin YP, Chakrabarti S, Chidiac P, Dixon SJ, Lala PK (2003). Endothelin-1 promotes migration and induces elevation of $[Ca^{2+}]_i$ and phosphorylation of MAP kinase of a human extravillous trophoblast cell line. *Mol Cell Endocrinol* **201**: 63–73.

Chung HJ, Levens D (2005). c-myc expression: keep the noise down!. *Mol Cell* **20**: 157–166.

Davis AC, Wims M, Spotts GD, Hann SR, Bradley A (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev* **7**: 671–682.

Dhillon AS, Hagan S, Rath O, Kolch W (2007). MAP kinase signalling pathways in cancer. *Oncogene* **26**: 3279–3290.

Doneda L, Bulfamante G, Grimoldi MG, Volpi L, Larizza L (1997). Localization of fos, jun, kit and SCF mRNA in human placenta throughout gestation using *in situ* RT-PCR. *Early Pregnancy* **3**: 265–271.

Dong F, Zhang X, Wold LE, Ren Q, Zhang Z, Ren J (2005). Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial cells: role of ETB receptor, NADPH oxidase and caveolin-1. *Br J Pharmacol* **145**: 323–333.

Dorsam RT, Gutkind JS (2007). G-protein-coupled receptors and cancer. *Nat Rev Cancer* **7**: 79–94.

Downward J (2003). Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer* **3**: 11–22.

Dunn KL, Espino PS, Drobic B, He S, Davie JR (2005). The Ras–MAPK signal transduction pathway, cancer and chromatin remodeling. *Biochem Cell Biol* **83**: 1–14.

Eguchi S, Hirata Y, Marumo F (1993). Endothelin subtype B receptors are coupled to adenylate cyclase via inhibitory G protein in cultured bovine endothelial cells. *J Cardiovasc Pharmacol* **22** (Suppl 8): S161–S163.

Fiore G, Florio P, Micheli L, Nencini C, Rossi M, Cerretani D *et al.* (2005). Endothelin-1 triggers placental oxidative stress pathways: putative role in preeclampsia. *J Clin Endocrinol Metab* **90**: 4205–4210.

Foster N, Loi TH, Owe-Young R, Stanley KK (2003). Lysosomal traffic of liganded endothelin B receptor. *Biochim Biophys Acta* **1642**: 45–52.

Germain AM, MacDonald PC, Casey ML (1997). Endothelin receptor mRNAs in human fetal membranes, chorionic vessels, and decidua parietalis. *Mol Cell Endocrinol* **132**: 161–168.

Grümmer R, Hohn HP, Mareel MM, Denker HW (1994). Adhesion and invasion of three human choriocarcinoma cell lines into human endometrium in a three-dimensional organ culture system. *Placenta* **15**: 411–429.

Harneit S, Paust HJ, Mukhopadhyay AK, Ergun S (1997). Localization of endothelin-1 and endothelin-receptors A and B in human epididymis. *Mol Hum Reprod* **3**: 579–584.

Imamura F, Arimoto I, Fujiyoshi Y, Doi T (2000). W276 mutation in the endothelin receptor subtype B impairs Gq coupling but not Gi or Go coupling. *Biochemistry* **39**: 686–692.

Iochmann S, Reverdiau-Moalic P, Beaujean S, Rideau E, Lebranchu Y, Bardos P *et al.* (1999). Fast detection of tissue factor and tissue factor pathway inhibitor messenger RNA in endothelial cells and monocytes by sensitive reverse transcription-polymerase chain reaction. *Thromb Res* **94**: 165–173.

Kasuya Y, Abe Y, Hama H, Sakurai T, Asada S, Masaki T *et al.* (1994). Endothelin-1 activates mitogen-activated protein kinases through two independent signalling pathways in rat astrocytes. *Biochem Biophys Res Commun* **204**: 1325–1333.

Kilpatrick SJ, Roberts JM, Lykins DL, Taylor RN (1993). Characterization and ontogeny of endothelin receptors in human placenta. *Am J Physiol* **264**: E367–E372.

Kitamura K, Shiraishi N, Singer WD, Handlogten ME, Tomita K, Miller RT (1999). Endothelin-B receptors activate $G_{\alpha 13}$. *Am J Physiol* **276**: C930–C937.

Kohnen G, Mackenzie F, Collett GP, Campbell S, Davenport AP, Cameron AD *et al.* (1997). Differential distribution of endothelin receptor subtypes in placentae from normal and growth-restricted pregnancies. *Placenta* **18**: 173–180.

Kovacevic A, Hammer A, Sundl M, Pfister B, Hrzenjak A, Ray A *et al.* (2006). Expression of serum amyloid A transcripts in human trophoblast and fetal-derived trophoblast-like choriocarcinoma cells. *FEBS Lett* **580**: 161–167.

Lahav R (2005). Endothelin receptor B is required for the expansion of melanocyte precursors and malignant melanoma. *Int J Dev Biol* **49**: 173–180.

Lala PK, Lee BP, Xu G, Chakraborty C (2002). Human placental trophoblast as an *in vitro* model for tumor progression. *Can J Physiol Pharmacol* **80**: 142–149.

Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ (1996). Role of c-Src tyrosine kinase in G protein-coupled receptor- and $G^{\beta\gamma}$ subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* **271**: 19443–19450.

Marsche G, Heller R, Fauler G, Kovacevic A, Nuszkowski A, Graier W *et al.* (2004). 2-Chlorohexadecanal derived from hypochlorite-modified high-density lipoprotein-associated plasmalogen is a natural inhibitor of endothelial nitric oxide biosynthesis. *Arterioscler Thromb Vasc Biol* **24**: 2302–2306.

Marsche G, Semlitsch M, Hammer A, Frank S, Weigle B, Demling N *et al.* (2007). Hypochlorite-modified albumin colocalizes with RAGE in the artery wall and promotes MCP-1 expression via the RAGE-Erk1/2 MAP-kinase pathway. *FASEB J* **21**: 1145–1152.

Mauschitz R, Cervar M, Hahn T, Purstner P, Desoye G (2000). Self-regulation of the endothelin receptor system in choriocarcinoma cells. *Biochim Biophys Acta* **1502**: 224–234.

Nelson J, Bagnato A, Battistini B, Nisen P (2003). The endothelin axis: emerging role in cancer. *Nat Rev Cancer* **3**: 110–116.

Nelson JB, Chan-Tack K, Hedican SP, Magnuson SR, Opgenorth TJ, Bova GS *et al.* (1996). Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. *Cancer Res* **56**: 663–668.

Ozaki S, Ohwaki K, Ihara M, Fukuroda T, Ishikawa K, Yano M (1995). ETB-mediated regulation of extracellular levels of endothelin-1 in cultured human endothelial cells. *Biochem Biophys Res Commun* **209**: 483–489.

Puffenberger EG, Hosoda K, Washington SS, Nakao K, de Wit D, Yanagisawa M *et al.* (1994). A missense mutation of the

endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell* **79**: 1257–1266.

Rasband WS (1997–2007). *ImageJ*. US National Institutes of Health: Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/.

Roberson MS, Ban M, Zhang T, Mulvaney JM (2000). Role of the cyclic AMP response element binding complex and activation of mitogen-activated protein kinases in synergistic activation of the glycoprotein hormone alpha subunit gene by epidermal growth factor and forskolin. *Mol Cell Biol* **20**: 3331–3344.

Roberts PJ, Der CJ (2007). Targeting the Raf–MEK–ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26**: 3291–3310.

Sand AE, Ostlund E, Andersson E, Fried G (1998). Endothelin-induced contractions in placental arteries is mediated by both ETA- and ETB-receptors. *Acta Physiol Scand* **163**: 227–234.

Shah BH, Baukal AJ, Chen HD, Shah AB, Catt KJ (2006). Mechanisms of endothelin-1-induced MAP kinase activation in adrenal glomerulosa cells. *J Steroid Biochem Mol Biol* **102**: 79–88.

Shichiri M, Marumo F, Hirata Y (1998). Endothelin-B receptor-mediated suppression of endothelial apoptosis. *J Cardiovasc Pharmacol* **31** (Suppl 1): S138–S141.

Shyamala V, Moulthrop TH, Stratton-Thomas J, Tekamp-Olson P (1994). Two distinct human endothelin B receptors generated by alternative splicing from a single gene. *Cell Mol Biol Res* **40**: 285–296.

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.

Vacca F, Bagnato A, Catt KJ, Tecce R (2000). Transactivation of the epidermal growth factor receptor in endothelin-1-induced mitogenic signaling in human ovarian carcinoma cells. *Cancer Res* **60**: 5310–5317.

Wadsack C, Hrzenjak A, Hammer A, Hirschmugl B, Levak-Frank S, Desoye G *et al*. (2003). Trophoblast-like human choriocarcinoma cells serve as a suitable *in vitro* model for selective cholesteryl ester uptake from high density lipoproteins. *Eur J Biochem* **270**: 451–462.

White TE, Saltzman RA, Di Sant'Agnese PA, Keng PC, Sutherland RM, Miller RK (1988). Human choriocarcinoma (JAR) cells grown as multicellular spheroids. *Placenta* **9**: 583–598.

Wilkes BM, Mento PF, Hollander AM, Maita ME, Sung S, Girardi EP (1990). Endothelin receptors in human placenta: relationship to vascular resistance and thromboxane release. *Am J Physiol* **258**: E864–E870.

Wulfing P, Kersting C, Tio J, Fischer RJ, Wulfing C, Poremba C *et al*. (2004). Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. *Clin Cancer Res* **10**: 2393–2400.

Zhang P, Li H, Wu ML, Chen XY, Kong QY, Wang XW *et al*. (2006). c-Myc downregulation: a critical molecular event in resveratrol-induced cell cycle arrest and apoptosis of human medulloblastoma cells. *J Neurooncol* **80**: 123–131.