

# Placental lactogen-I gene activation in differentiating trophoblast cells: extrinsic and intrinsic regulation involving mitogen-activated protein kinase signaling pathways

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## Abstract

Trophoblast giant cells are one of the primary endocrine cell types of the rodent placenta. Placental lactogen-I (PL-I) is the initial prolactin (PRL) family member expressed as trophoblast giant cells differentiate. In this report, we use the Rcho-1 trophoblast cell line as a model for studying the regulation of PL-I gene expression during trophoblast giant cell differentiation. Evidence is provided for trophoblast cell expression of epidermal growth factor receptor (EGFR), ErbB2, fibroblast growth factor receptor 1 (FGFR1), transforming growth factor- $\alpha$ , and heparin-binding EGF. EGF and FGF-2 stimulated PL-I mRNA and protein accumulation and PL-I promoter activity in a concentration-dependent manner. These latter growth factor actions on PL-I promoter activities were specifically

inhibited by cotransfection with dominant negative constructs for EGFR and FGFRs respectively. Utilization of the mitogen-activated protein kinase (MAPK) pathway by EGF and FGF-2 in trophoblast cells was demonstrated by growth factor stimulation of a Gal4 DNA binding/Elk1 transactivational domain fusion construct, and more specifically by activation of extracellular signal regulated kinase and p38 MAPK. PL-I gene activation was also sensitive to disruption of MAPK and activation protein-1 (AP-1) signaling pathways. In conclusion, autocrine/paracrine pathways involving EGFR and FGFR1, MAPK and AP-1 are shown to participate in the regulation of the PL-I gene in differentiating trophoblast cells.

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## Introduction

Development of the trophoblast cell lineage is essential for the establishment of an effective exchange and pathway of communication between maternal and fetal tissues. The rodent placenta contains four distinct trophoblast cell types including syncytial, spongiotrophoblast, glycogen and trophoblast giant cells that arise from a common precursor stem cell and serve important biological functions (Soares *et al.* 1995, 1996). Trophoblast giant cells are one of the first cells of the trophoblast lineage to differentiate (Gardner & Beddington 1988, Soares *et al.* 1993, 1996, Rossant 1995) and undergo a morphological transformation arising from continued DNA synthesis without karyokinesis and cytokinesis, called endoreduplication (Ilgren 1983). Trophoblast giant cells are located at the maternal-placental interface and express members of the placental prolactin (PRL) gene family (Soares *et al.* 1991, 1996, 1998), including placental lactogen-I (PL-I) and PL-II, in a differentiation-dependent manner.

PLs serve a number of important biological functions in rodents (Soares *et al.* 1998), including the maintenance of

pregnancy through actions on the corpus luteum (Galosy & Talamantes 1995) and preparation for lactation through actions on the mammary glands (Thordarson *et al.* 1986, Colosi *et al.* 1988). In the rodent, *in vivo* PL-I expression begins shortly after implantation and terminates by mid-gestation (Faria *et al.* 1990). PL-II expression begins after PL-I and continues throughout gestation (Campbell *et al.* 1989, Duckworth *et al.* 1990, Faria *et al.* 1990). PL-I expression serves as an early endocrine marker of trophoblast giant cell differentiation, while PL-II expression serves as an intermediate-to-late endocrine marker of trophoblast giant cell differentiation.

The precise factors and signaling pathways regulating the expression of PL-I have yet to be fully characterized. The anterior pituitary is a possible source of regulators that are associated with decreased serum PL-I levels (Lopez *et al.* 1992, 1993). This may be through the production of factors that inhibit PL-I production or modulate PL-I clearance. A number of growth factors/cytokines/hormones potentially modulating PL-I production in mixed mouse placental cell primary cultures through direct or indirect mechanisms have been reported

(Yamaguchi *et al.* 1992a,b, 1995b,c). The nature of the *in vitro* models used in these experiments makes mechanistic interpretations difficult.

Rcho-1 trophoblast cells represent an *in vitro* rat trophoblast model that can be maintained in a proliferative state or induced to differentiate along the trophoblast giant cell lineage (Faria & Soares 1991, Hamlin *et al.* 1994, Peters *et al.* 1999b). As Rcho-1 trophoblast cells differentiate they express PL-I and other members of the PRL gene family in a stage-specific manner that mimics their ontogeny *in vivo* (Faria *et al.* 1990, Faria & Soares 1991, Hamlin *et al.* 1994). The Rcho-1 trophoblast culture model has been used to demonstrate the involvement of activation protein-1 (AP-1) and GATA cis-regulatory elements in the regulation of PL-I gene transcription (Shida *et al.* 1993, Ng *et al.* 1994). Rcho-1 trophoblast cells have also been utilized to investigate modulatory roles of growth factors and cytokines on trophoblast cell proliferation (Verstuyf *et al.* 1993, Hamlin & Soares 1995). Attempts to identify extracellular signals controlling trophoblast cell differentiation with the Rcho-1 trophoblast cell model have been limited (Yamaguchi *et al.* 1995a, Yamamoto *et al.* 1997).

Based on *in vivo* and some limited *in vitro* analyses, evidence exists for the utilization of epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) signaling pathways in the regulation of trophoblast cell development. Trophoblast giant cells express EGFR and the epidermal growth factor (EGF)-related receptor, ErbB2 (Adamson & Meek 1984, Lim *et al.* 1997). EGF stimulates PL-I protein secretion in mixed placental cell cultures (Yamaguchi *et al.* 1992a) and PL-I gene expression in a rat choriocarcinoma cell line (Sun *et al.* 1998). Furthermore, disruption of either EGFR or FGFR signaling pathways results in aberrations in placental development (Sibilia & Wagner 1995, Threadgill *et al.* 1995, Arman *et al.* 1998, Nichols *et al.* 1998, Tanaka *et al.* 1998, Xu *et al.* 1998).

In this report, we investigate the actions of EGF and fibroblast growth factor (FGF)-2 on differentiating trophoblast cells using the Rcho-1 trophoblast cell model. We demonstrate the existence of autocrine/paracrine pathways involving EGFR and FGFR1 and the participation of components of the mitogen-activated protein kinase (MAPK) and AP-1 pathways in regulating the activation of the PL-I gene in differentiating trophoblast cells.

## Materials and Methods

### Reagents

Horse serum (HS) was obtained from JRH Scientific (Lenexa, KS, USA). Reagents for SDS-PAGE, protein assays, and protein standards were purchased from Bio-Rad (Hercules, CA, USA). Nitrocellulose and nylon membranes were acquired from Schleicher and Schuell (Keene, NH, USA). X-Omat AR X-ray film was

obtained from Eastman Kodak (Rochester, NY, USA). DNA extraction kits were purchased from Qiagen (Chatsworth, CA, USA). Prime-it random primed DNA labeling kits and Pfu polymerase were purchased from Stratagene (La Jolla, CA, USA). [ $\alpha$ - $^{32}$ P]deoxyadenosine triphosphate was purchased from DuPont-New England Nuclear (Boston, MA, USA). All restriction enzymes, DNA ligases, and phosphoPlus extracellular signal regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK antibody kits were obtained from New England Biolabs (Beverly, MA, USA). Lipofectamine, Opti-MEM, subcloning competent DH5 $\alpha$  cells, Superscript preamplification kits, Taq polymerase, oligonucleotide primers, and the MAPK kinase (MEK) inhibitor, PD98059, were obtained from GibcoBRL (Gaithersburg, MD, USA). The p38 MAPK inhibitor, SB203580, was acquired from CalBiochem (San Diego, CA, USA). Luciferase assay kits, the pGL2 vector, anti-active MAPK antibodies and donkey anti-rabbit IgG horseradish peroxidase conjugate were acquired from Promega (Madison, WI, USA). A Rous sarcoma virus promoter- $\beta$ -galactosidase (RSV- $\beta$ GAL) reporter plasmid was obtained from the American Type Culture Collection (Rockville, MD, USA). Mouse receptor grade EGF was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Recombinant human FGF-2 was purchased from Austral Biologicals (San Ramon, CA, USA). The enhanced chemiluminescence (ECL) Western blot detection kit was purchased from Amersham Life Science (Arlington Heights, IL, USA). Unless indicated otherwise all other reagents and chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

### Cell culture

Rcho-1 trophoblast cells were cultured as previously indicated (Faria & Soares 1991, Hamlin *et al.* 1994, Peters *et al.* 2000). Briefly, Rcho-1 trophoblast cells were maintained in a proliferative subconfluent state in NCTC-135 culture media supplemented with 10 mM HEPES, 50  $\mu$ M  $\beta$ -mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml of penicillin and 100  $\mu$ g/ml of streptomycin, and 20% fetal bovine serum (FBS) in a 37 °C incubator under 95% air/5% CO $_2$ . After 3 days of culture in supplemented NCTC-135 with 20% FBS, confluent monolayers of Rcho-1 trophoblast cells were switched to supplemented NCTC-135 culture medium containing 1% HS, 10% HS or to serum-free medium to allow differentiation. Each of these culture conditions promotes trophoblast giant cell differentiation (Hamlin *et al.* 1994, Hamlin & Soares 1995).

### DNA plasmid constructs

Expression vectors containing dominant negative EGFR (dn EGFR; Wu & Adamson 1993), dominant negative

FGFRs (dn FGFR1/pCEP4 and dn FGFR2/pCEP4; Li *et al.* 1994), dominant negative c-jun (Tam67/pCMV; Chen *et al.* 1996), and dominant negative c-fos (A-Fos/pRCCMV500; Olive *et al.* 1997) were obtained from Drs Eileen D Adamson of the Burnham Institute, Alka Mansukhani of New York University Medical Center, Michael J Birrer of the National Cancer Institute and Charles Vinson of the National Cancer Institute respectively. Gal4 DNA binding domain/Elk1 transactivational chimeric expression construct and 5 × Gal4/luciferase reporter construct were provided by Dr Mark Roberson of Cornell University (Roberson *et al.* 1995).

PL-I promoter/luciferase constructs were generated from the previously characterized mouse PL-I promoter provided by Dr Daniel I H Linzer of Northwestern University (Shida *et al.* 1993) using Pfu polymerase. Briefly, the 274 bp mouse PL-I promoter served as a template for PCR using an upstream primer with an NdeI restriction site DNA overhang (sense, -274 to -260) and a downstream primer with a BglII restriction site DNA overhang (antisense, -16 to +2). PCR was performed as previously described (Rasmussen *et al.* 1997) using a Perkin-Elmer thermocycler (Model 480; Perkin-Elmer, Norwalk, CT, USA). Briefly, 30 cycles of PCR were with the denaturing temperature at 94 °C (1 min), annealing temperature at 50 °C (1 min), and extension temperature at 72 °C (1 min). The end product was digested with NdeI and BglII and ligated into the pGL2 luciferase reporter plasmid. The accuracy of the PCR-generated PL-I promoter-luciferase reporter construct was verified by DNA sequencing by using an Applied Biosystems Model 310 sequencer and an Applied Biosystems Dye Terminator Cycle Sequencing kits (Applied Biosystems, Foster City, CA, USA).

Gelatinase B promoter/luciferase reporter constructs were generated from a previously characterized minimal mouse gelatinase B promoter (Sato *et al.* 1993) as previously described (Peters *et al.* 1999). Briefly, the mouse gelatinase B-569/chloramphenicol acetyl transferase (CAT) served as a template for PCR using an upstream primer with an NdeI restriction site DNA overhang (sense, -569 to -550) and a downstream primer with a BglII restriction site DNA overhang (antisense -1 to +17).

#### Reverse-transcriptase-polymerase chain reaction analysis

Total RNA was extracted from trophoblast cells using TRIzol (Chomczynski & Sacchi 1987). RNA was reverse transcribed with 5 µg total RNA and 0.5 µg oligo(deoxythymidine) primers. Following reverse transcription, PCR was performed with a Perkin-Elmer Model 2400 thermocycler for 30 cycles with the denaturing temperature at 94 °C (1 min), annealing temperature at 65 °C (2 min), and extension temperature at 72 °C (2 min).

**EGFR and ErbB2** Primers corresponding to rat EGFR were used to assess the expression of EGFR in Rcho-1

trophoblast cells (upstream primer: sense, 760–781; downstream primer: antisense, 1040–1060). PCR conditions were followed as indicated above for creation of the PL-I/luciferase construct with the exception that the elongation temperature was 65 °C. ErbB2 transcripts were detected with minor modifications of a previously described protocol (Lim *et al.* 1997, upstream primer: sense, 2391–2412; downstream primer: antisense, 2694–2713). The amplified products (EGFR, 281 bp; ErbB2, 320 bp) for each RT-PCR reaction were then subjected to 2% agarose gel electrophoresis and processed for Southern blot hybridization using <sup>32</sup>P-end labeled oligonucleotides corresponding to sequences located within the amplified products (EGFR, 5' GTACAACCCACC ACGTACC 3'; ErbB2, 5'ATGCAGATGGGGGCAAG GTG3').

**FGFR1** Primers corresponding to the extracellular domain of rat FGFR1 (Yazaki *et al.* 1993) were used to evaluate the expression of FGFR1 in Rcho-1 trophoblast cells (upstream primer: sense, 23–43; downstream primer: antisense, 1108–1128). PCR was performed as described above with the exception that the elongation temperature was 68 °C and the inclusion of primers for the amplification of β-actin were included as an internal control as previously described (Orwig *et al.* 1997).

#### Northern blot analyses

Analysis of trophoblast cell expression of PL-I and PL-II mRNAs following treatment with growth factors was performed by Northern blotting. Trophoblast cells were initially incubated for 60 h in FBS-containing medium followed by an 18 h exposure to various concentrations of growth factors in serum-free NCTC-135 culture medium. Total RNA was isolated using TRIzol (Chomczynski & Sacchi 1987), separated on formaldehyde agarose gels (15 µg/lane), and blotted onto nylon membranes as previously described (Faria & Soares 1991). Prehybridization was carried out at 42 °C for 16 h in prehybridization buffer (5 × SSPE, 50% formamide, 5 × Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA). Linearized PL-I and PL-II cDNA probes (Dai *et al.* 1996) were radiolabeled by random priming and heat denatured. Hybridization was carried out for 16 h at 42 °C in prehybridization buffer containing the radiolabeled probes. Blots were washed twice for 15 min at 42 °C in 4 × SSC, 0.1% SDS and once at 42 °C in 1 × SSC, 0.1% SDS for 30 min. Blots were exposed to X-ray film at -80 °C. Ribosomal RNA was assessed for equal loading and general integrity of isolated RNA. Hybridizations with radiolabeled cDNAs to β-tubulin (Valenzuela *et al.* 1981) were used as controls.

Transforming growth factor-α (TGF-α) and heparin-binding-EGF (HB-EGF) mRNAs were assessed from poly(A)+-enriched RNA samples as previously described

(Tamada *et al.* 1991, Das *et al.* 1994). Mouse day 8 decidua poly(A)<sup>+</sup>-enriched RNA served as a positive control for TGF- $\alpha$  and HB-EGF.

#### Western blot analyses

PL-I protein was monitored in conditioned culture medium by Western blot analysis. Conditioned culture medium was prepared by washing cells for 60 h after initial plating twice in Hanks' balanced salt solution (HBSS) and then incubating cells for 40–44 h in NCTC-135 culture medium supplemented with 1% HS. Conditioned medium was harvested, clarified by centrifugation, and aliquots stored at  $-80^{\circ}\text{C}$  until analyzed. Proteins present in conditioned medium were concentrated 10-fold by acetone precipitation. Protein pellets were collected by centrifugation, air dried, resuspended in water, and then mixed in non-reducing SDS-PAGE buffer. Samples were then separated in 12.5% polyacrylamide gels according to the procedure of Laemmli (1970) and electrophoretically transferred to nitrocellulose as previously described (Towbin *et al.* 1979). Western blots were then blocked overnight with shaking at  $4^{\circ}\text{C}$  in a blocking solution consisting of 5% non-fat dry milk in Tris-buffered saline (TBS, 0.2 M Tris, pH 7.2 and 1.5 M NaCl). Blots were then incubated with primary antibodies for PL-I (1:500 dilution; Hamlin *et al.* 1994) in fresh blocking solution for 16 h at  $4^{\circ}\text{C}$ , followed by washing three times for 15 min at room temperature in TBST (TBS with 0.5% Triton X-100). The blots were blocked at room temperature in blocking solution for 1 h and incubated with blocking solution containing secondary antibody for 2 h at room temperature, both with shaking. Western blots were then washed three times with shaking in TBST for 15 min each at room temperature. Immune complexes were detected by ECL and exposed to X-ray film.

Total and activated ERK, JNK and p38 MAPK were determined from trophoblast cell lysates by Western blot analysis with phosphoPlus ERK, JNK and p38 MAPK antibody kits according to the manufacturer's instructions (New England BioLabs). Cells were plated at  $1 \times 10^5$  per 35 cm<sup>2</sup> dish in NCTC-135+20% FBS. After 48 h of culture, the cells were washed twice in HBSS and changed to serum-free NCTC-135, and incubated for 5 h, followed by the addition of fresh serum-free NCTC-135 medium with or without growth factors (EGF or FGF-2 at 20 ng/ml). Cells were harvested at 0, 5, and 30 min after addition of the growth factors and lysates prepared and analyzed for phosphorylated and total ERK, JNK and p38 MAPK.

#### Transient transfection assays

Trophoblast cells were transiently transfected with various DNA constructs using a liposome-mediated procedure (Peters *et al.* 1999a). Initially, cells were plated and

incubated for 60 h in FBS-containing culture medium. Cells were then exposed to the transfection reagents (lipofectamine (10  $\mu\text{l}$ ), DNA (1–5  $\mu\text{g}$ ) and Opti-MEM (200  $\mu\text{l}$ )) for 7 h. Culture medium was then replaced and the cells exposed to serum-free conditions in the presence or absence of growth factors for 12–14 h. Cellular lysates were prepared via three consecutive cycles of freezing and thawing and luciferase activity determined with a Promega kit according to the manufacturer's instructions. RSV- $\beta\text{GAL}$  was routinely cotransfected with the constructs and used to correct for transfection efficiency. Results from the experimentation did not differ whether data were normalized to either  $\beta\text{-GAL}$  activity and protein concentration or only protein concentration. For simplicity the transfection analyses are expressed per protein concentration. pGL-2 (promoterless control) or a gelatinase B promoter/luciferase reporter construct were also utilized in some experiments as a control for specificity.

#### Statistical analysis

The data were analyzed by ANOVA. The source of variation from significant *F* ratios was determined with the Newman–Keuls multiple comparison test (Keppel 1973). In some experiments, observations were quantified by digital image processing using Optimus Bioscan (Optimus Corporation, Edmonds, WA, USA) and then subjected to statistical analysis.

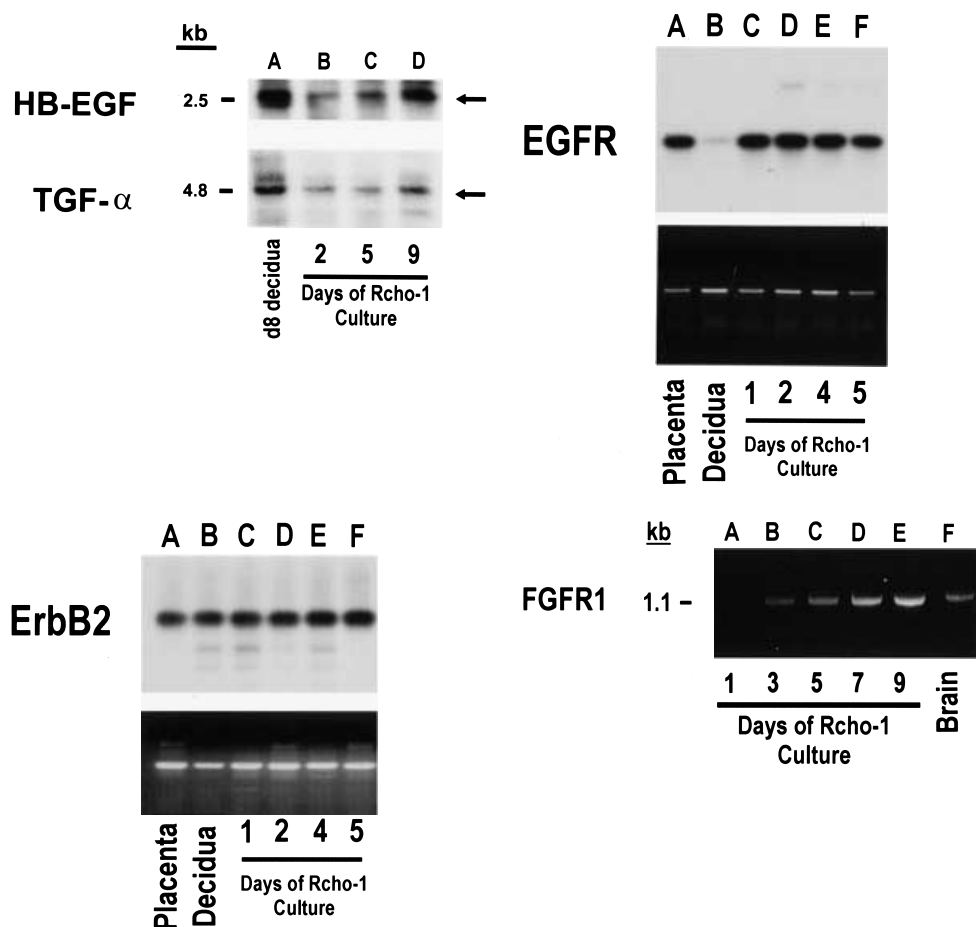
## Results

#### *Trophoblast cells express EGFR, ErbB2, TGF- $\alpha$ , and HB-EGF*

A potential role for EGFR in placental development has been suggested by its *in vivo* trophoblast expression patterns (Adamson & Meek 1984, Lim *et al.* 1997), *in vitro* actions on trophoblast hormone secretion (Yamaguchi *et al.* 1992b, 1995b), and from gene-targeting experimentation (Sibilia & Wagner 1995, Threadgill *et al.* 1995). In the present study, two members of the EGFR family, EGFR and ErbB2, were found by RT-PCR analysis to be expressed in differentiating Rcho-1 trophoblast cells (Fig. 1). Two ligands for EGFR, TGF- $\alpha$  and HB-EGF, have been previously shown to be expressed in trophoblast giant cells developing *in situ* (Tamada *et al.* 1991, Das *et al.* 1994) and were shown here to be expressed in differentiating trophoblast cells by Northern blotting (Fig. 1). Thus, the required participants for an autocrine pathway of EGFR signaling exist within the Rcho-1 trophoblast cell model as they exist in trophoblast giant cells developing *in situ*.

#### *Trophoblast cells express FGFR1*

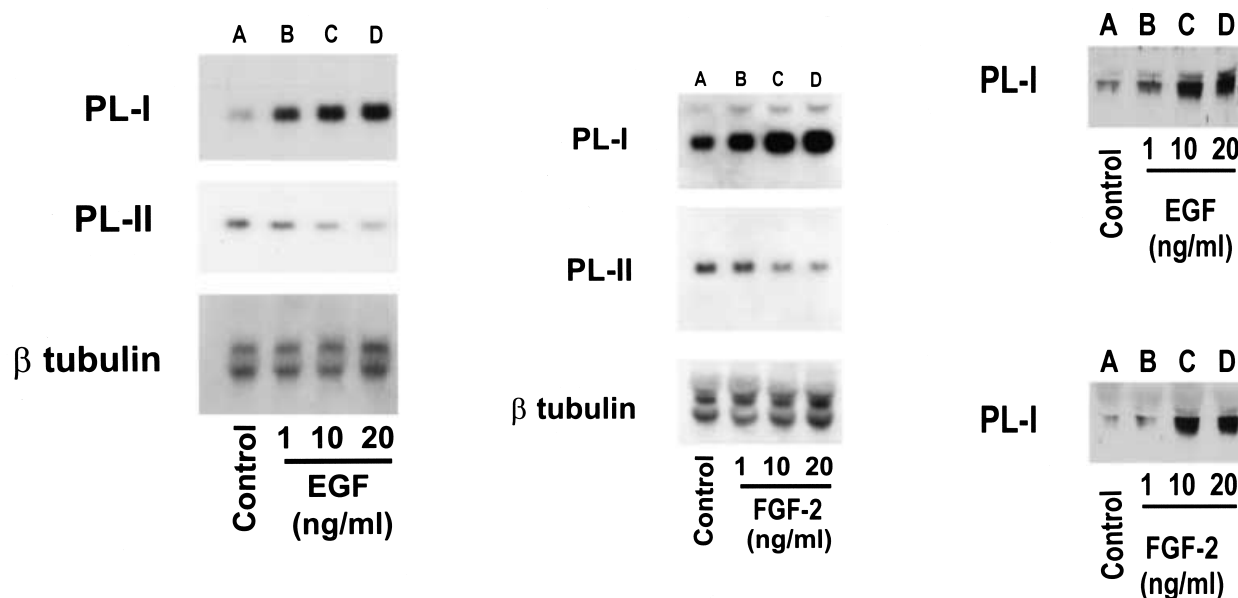
We originally obtained evidence for the expression of FGFR1 in trophoblast cells through a screen for receptor



**Figure 1** Rcho-1 trophoblast cell-specific expression of growth factor receptors and ligands. Top left panel. Northern blot analysis of poly(A)<sup>+</sup>-enriched RNA (2 µg/lane) from differentiating Rcho-1 trophoblast cells probed for HB-EGF (upper) or TGF- $\alpha$  (lower). Lane A, day 8 mouse decidua; lanes B–D, days 2, 5, and 9 of Rcho-1 trophoblast cell culture respectively. Top right panel. RT-PCR and Southern blot analysis of EGFR expression in Rcho-1 trophoblast cells. Lane A, rat placenta; lane B, day 8 mouse decidua/embryo; lanes C–F, days 1, 2, 5, and 9 of Rcho-1 trophoblast cell culture respectively. The upper portion of the panel represents RT-PCR followed by Southern blot analysis. The bottom portion of the panel represents the ethidium bromide-stained gel. Bottom left panel. RT-PCR and Southern blot analysis of ErbB2 expression in Rcho-1 trophoblast cells. Lanes contain samples as described for the EGFR analysis. Bottom right panel. RT-PCR analysis of FGFR1 expression in Rcho-1 trophoblast cells. Lanes A–E, days 1, 3, 5, 7 and 9 of Rcho-1 trophoblast cell culture respectively. Lane F, brain. The panel represents an ethidium bromide-stained gel and contains amplified products for FGFR1 and  $\beta$ -actin. Please note that days 1 and 2 of culture represent proliferative Rcho-1 trophoblast cells, while days 4–9 represent a continuum of differentiating trophoblast giant cells. The blots presented in this figure are representative. Each experiment was replicated a minimum of three times.

tyrosine kinases in proliferating and differentiating trophoblast cells (B M Chapman & M J Soares, unpublished data). Here we show by RT-PCR that FGFR1 is expressed in differentiating trophoblast cells (Fig. 1). Amplified FGFR1 products were isolated and sequenced in order to verify their identity. FGFR2 was identified in proliferating trophoblast cells but not in differentiating trophoblast cells (B M Chapman & M J Soares, unpublished data). Unlike the autocrine pathway described above for the EGFR signaling pathway, we were not able

to detect the expression of FGFR ligands in differentiating trophoblast cells (data not shown) using published procedures for the detection of FGF-2 and FGF-4 (Rappolee *et al.* 1988, 1994). Although we were not exhaustive in our search for possible FGF family members in trophoblast cells, our present observations and those presented below are consistent with a paracrine signaling pathway involving the elaboration of FGFR ligands by inner cell mass derivatives (Rappolee *et al.* 1994, Nichols *et al.* 1998, Tanaka *et al.* 1998) or by decidual cells surrounding the



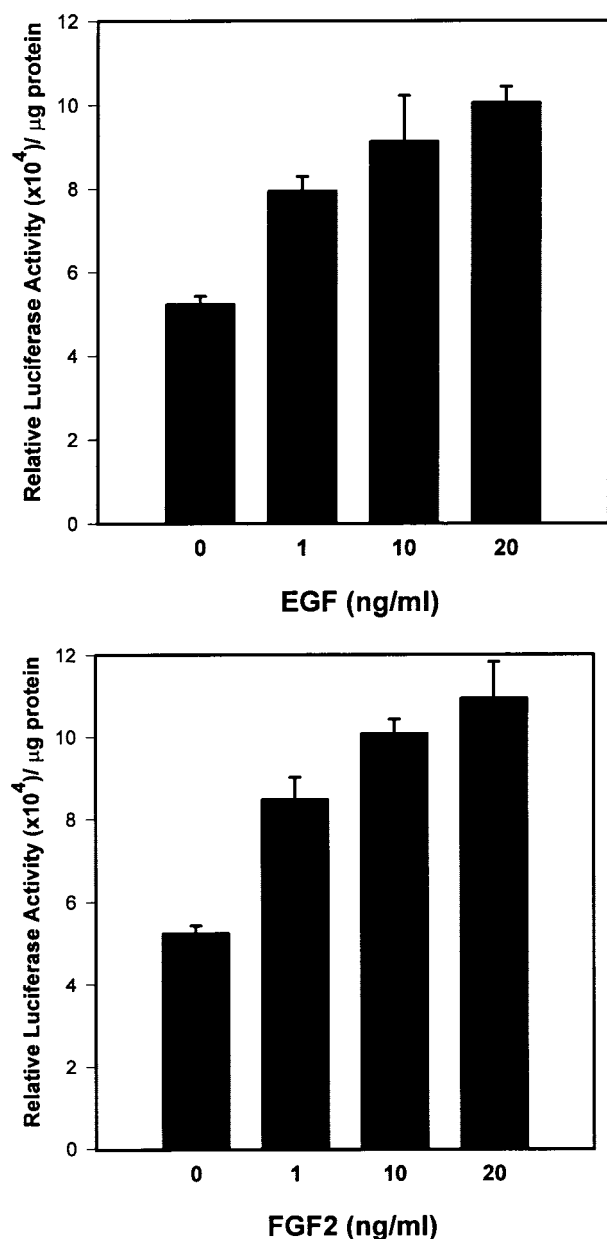
**Figure 2** Effects of EGF and FGF-2 on PL expression in differentiating Rcho-1 trophoblast cells. Left panel. Assessment of the effects of EGF on Rcho-1 trophoblast expression of PL-I, PL-II and  $\beta$ -tubulin mRNAs by Northern blot analysis. Trophoblast cells were treated for 18 h in serum-free culture medium with various concentrations of EGF and total RNA was harvested. Total RNA (15  $\mu$ g/lane) was separated by electrophoresis, blotted, and probed with radiolabeled cDNAs for PL-I, PL-II, or  $\beta$ -tubulin. Lanes A–D, contain samples treated with 0, 1, 10 and 20 ng/ml EGF respectively. Middle panel. Assessment of the effects of FGF-2 on Rcho-1 trophoblast expression of PL-I, PL-II, and  $\beta$ -tubulin mRNAs by Northern blot analysis. Lanes contain samples as described for the EGF treatment. Right panel. Western blot analyses of EGF (upper panel) and FGF-2 (lower panel) effects on PL-I protein secretion by Rcho-1 trophoblast cells. Conditioned medium was collected from Rcho-1 trophoblast cells exposed for 40 h in NCTC-135 culture medium supplemented with 1% HS and various concentrations of growth factors. Samples were concentrated  $\times 10$ , immunoblotted, and probed with polyclonal antibodies for PL-I. Lanes A–D, 0, 1, 10 and 20 ng/ml of growth factor respectively. The blots presented in this figure are representative. Each experiment was replicated a minimum of three times. Observations were quantified by digital image processing and then subjected to statistical analysis. Growth factor-treated cells expressed significantly more PL-I mRNA and protein than did untreated controls ( $P < 0.05$ ).

developing trophoblast giant cells (Carlone & Rider 1993).

#### *EGF and FGF-2 stimulate PL-I expression in trophoblast cells*

Based on the observation that EGFR and FGFR1 were present in Rcho-1 trophoblast cells, we evaluated whether prototypical ligands for these receptors, EGF and FGF-2, were capable of regulating trophoblast-specific gene expression. We examined PL-I and PL-II expression since they represent markers of early and later trophoblast-specific endocrine differentiation and since previous reports had suggested that EGF could regulate PL-I and PL-II protein secretion (Yamaguchi *et al.* 1992b, Sun *et al.* 1998). Treatment of Rcho-1 trophoblast cells at early stages of differentiation (day 2.5 of culture) with either EGF or FGF-2 stimulated the accumulation of PL-I mRNA with variable effects on PL-II mRNA, and without significantly affecting  $\beta$ -tubulin (Fig. 2). These growth factor treatments similarly stimulated trophoblast cell accumulation of PL-I protein (Fig. 2).

In order to better determine mechanisms underlying the actions of EGF and FGF-2 on PL-I expression, we examined the effects of the growth factors on PL-I promoter activity. A 274 bp promoter previously characterized by Shida and coworkers (Shida *et al.* 1993) was subcloned into a luciferase reporter vector, pGL2. The 274 bp PL-I promoter/luciferase reporter construct behaved similarly to the previously reported 274 bp PL-I promoter/CAT reporter construct (Shida *et al.* 1993) with regard to cell-specific and differentiation-dependent expression (data not shown). PL-I promoter activity in Rcho-1 trophoblast cells was significantly stimulated by either EGF or FGF-2 (Fig. 3). Maximal growth factor activation of the PL-I promoter was in the range 2.5- to 3.5-fold. The involvement of EGFR- and FGFR-signaling pathways in the growth factor responses was further assessed by cotransfection of EGFR or FGFR dominant negative constructs with the PL-I promoter/reporter constructs. Dominant negative EGFR was shown to significantly decrease both intrinsic and EGF-stimulated PL-I promoter activity, whereas the dominant negative FGFR inhibitory actions were restricted to FGF-2-stimulated



**Figure 3** EGF and FGF-2 stimulation of PL-I promoter activity in Rcho-1 trophoblast cells. Rcho-1 trophoblast cells were transiently transfected with the PL-I promoter/luciferase reporter construct, incubated in serum-free media with various concentrations of EGF (upper panel) or FGF-2 (lower panel) for 12–14 h, lysed, and luciferase activity measured. Please note that treatment with either EGF or FGF-2 led to a stimulation of PL-I promoter activity. All experiments were performed in triplicate and repeated at least three times. Data are presented as the mean  $\pm$  S.E.M. Growth factor-treated cultures showed significantly greater PL-I promoter activity than did control cultures ( $P < 0.05$ ).

PL-I promoter activity (Fig. 4). The inhibitory effects of dominant negative EGFR on intrinsic PL-I promoter activity provide further support for the involvement of an

autocrine EGFR ligand–EGFR signaling pathway in trophoblast cells. Growth factor treatment or exposure to dominant negative receptor constructs did not significantly influence pGL2 (promoterless control) activity (data not shown). Collectively, these experiments demonstrated that EGF and FGF-2, operating through their respective receptors in differentiating trophoblast cells, specifically activate the PL-I promoter which culminates in the stimulation of PL-I mRNA and protein formation.

#### *Growth factors activate MAPK in differentiating trophoblast cells*

Since stimulation of either EGFR or FGFRs resulted in the transcriptional activation of the PL-I gene, we next investigated potential site(s) for convergence of the growth factor receptor signaling pathways. Previous studies have reported that EGFR and FGFRs can both activate MAPK signaling cascades (Friesel & Maciag 1995, Riese & Stern 1998). Consequently, we evaluated the effects of EGF and FGF-2 on trophoblast cell MAPK activities. We first evaluated the influence of EGF and FGF-2 on a MAPK-responsive gene reporter assay that employs a Gal4 DNA binding domain/Elk1 transactivational chimeric (Gal4/Elk1) expression plasmid with a  $5 \times$  Gal4 binding site/luciferase reporter plasmid (Roberson *et al.* 1995). Both growth factors significantly stimulated the MAPK-responsive gene reporter system introduced into trophoblast cells (Fig. 5). Since each of the three MAPK modules, ERK, JNK and p38 MAPK can participate in the activation of Elk1 (Brunet & Pouyssegur 1998, Lewis *et al.* 1998), we next examined the effects of EGF and FGF-2 on activation of ERK, JNK and p38 MAPK. EGF and FGF-2 significantly stimulated the accumulation of the phosphorylated (activated) forms of ERK and p38 MAPK (Fig. 6). Immunoreactive JNK was below the limits of detection in the intrinsic or growth factor-stimulated trophoblast cell lysates (Fig. 6). We next examined the effects of inhibitors of the ERK (PD98059) and p38 MAPK (SB203580) pathways on growth factor-stimulated PL-I promoter activities. Antagonism of individual MAPK signaling modules significantly inhibited both intrinsic and growth factor PL-I gene promoter activities, but did not prevent growth factor activation (Figs 7 and 8). Similar results were obtained with a dominant negative MAPK construct (data not shown). Exposure to MAPK inhibitors did not significantly affect pGL2 (promoterless control) activity (data not shown). Our cumulative evidence indicates that EGFR- and FGFR-signaling pathways activate the PL-I promoter via MAPK signaling cascades.

#### *Involvement of AP-1 in activation of the PL-I promoter*

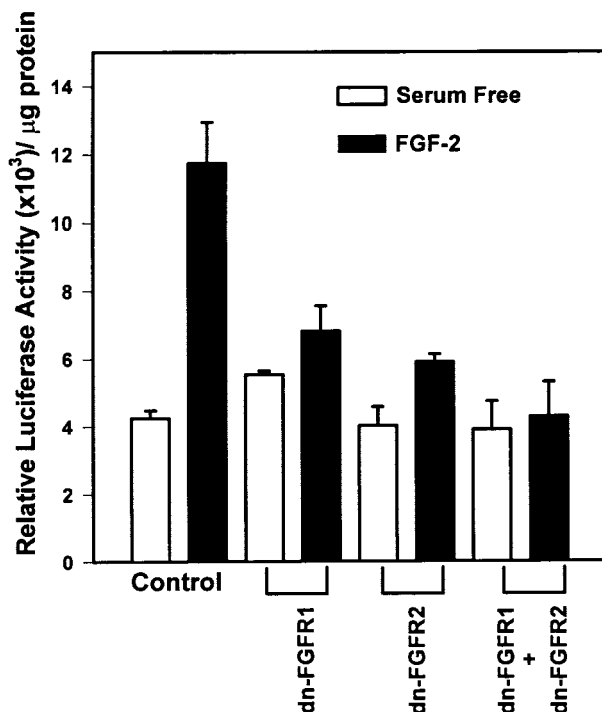
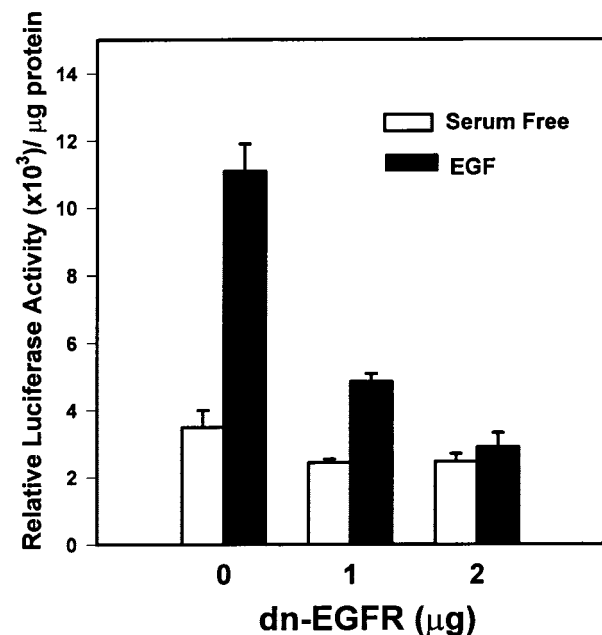
Previous studies on transcriptional regulation of the PL-I gene identified two AP-1 sites that were critical for promoter activity (Shida *et al.* 1993). Since in other

systems, growth factor and MAPK signaling pathways impinge upon the formation of active AP-1 complexes (Angel & Karin 1991, Karin 1995), we evaluated the involvement of AP-1 in PL-I gene activation in differentiating trophoblast cells. The effects of dominant negative c-jun and c-fos DNA constructs on PL-I promoter activity in differentiating trophoblast cells were evaluated. The

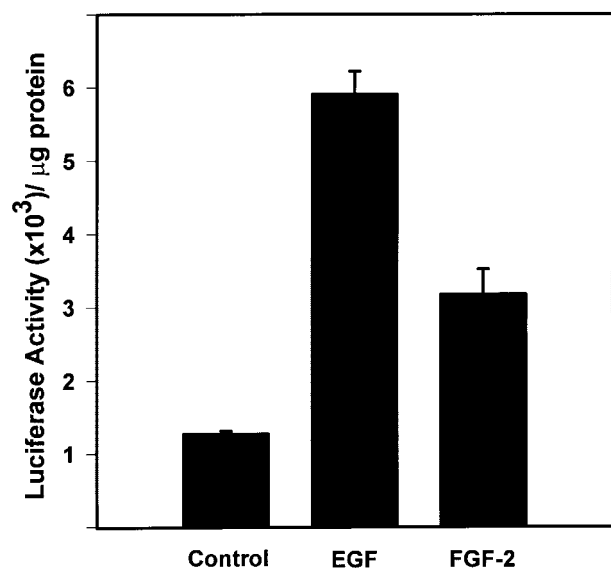
dominant negative c-jun DNA construct inhibited intrinsic PL-I promoter activities by more than 95% (Fig. 9). Surprisingly, the dominant negative c-fos construct stimulated intrinsic PL-I promoter activity (Fig. 9). Neither AP-1 dominant negative construct entirely prevented growth factor activated PL-I promoter activities, nor did they affect the intrinsic activities of two constructs used as controls: pGL2 and the gelatinase B promoter (data not shown). The dominant negative c-jun acts by binding to DNA and preventing transactivation (Chen *et al.* 1996), whereas, the dominant negative c-fos is known to act by squelching, thus by sequestering factors from binding the AP-1 element (Olive *et al.* 1997). The data support a role for AP-1 (proteins and DNA response element) in the transcriptional regulation of the PL-I gene. The nature of AP-1 activation of the PL-I gene may be complex, potentially involving both negative and positive AP-1 regulators.

## Discussion

The differentiation of trophoblast giant cells is accompanied by the stage-specific expression of endocrine-related genes. One of the first endocrine genes activated by developing trophoblast giant cells is PL-I (Soares *et al.* 1996). *In vivo* PL-I synthesis is initiated immediately postimplantation; it can reach levels in the



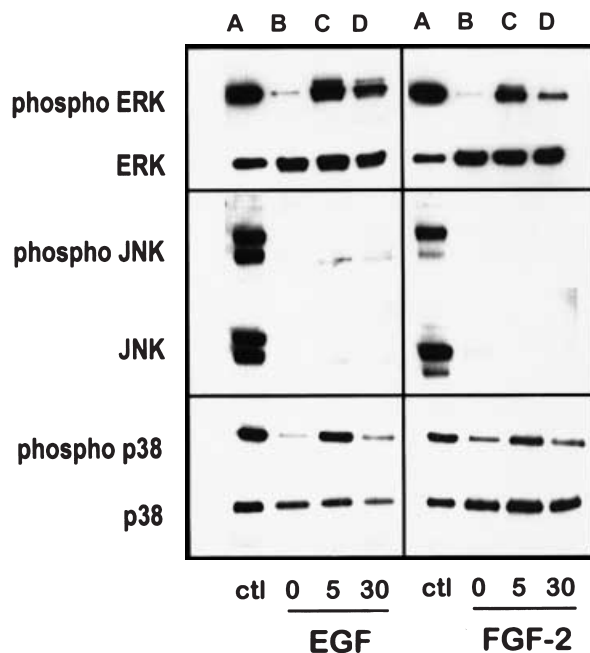
**Figure 4** Inhibition of EGF- and FGF-2-stimulated PL-I promoter activity in Rcho-1 trophoblast cells by cotransfection of dominant negative EGFR or FGFR1 and FGFR2 expression constructs respectively. Rcho-1 trophoblast cells were transiently cotransfected with PL-I promoter/luciferase reporter constructs and dominant negative growth factor receptor constructs in the presence or absence of growth factors. Cells were lysed following a 12–14 h incubation and luciferase activity measured. The concentration of DNA per transfection was the same for all treatments. All experiments were performed in triplicate and repeated at least three times. Data are presented as the mean  $\pm$  S.E.M. Statistical comparisons were made between control (0) cultures (serum-free or growth factor-treated) and dominant negative-treated cultures. Top panel. Transient cotransfection of Rcho-1 trophoblast cells with a PL-I promoter/luciferase reporter construct in the absence or presence of EGF (20 ng/ml) and an EGFR dominant negative construct (dn-EGFR) or a control expression vector. Bottom panel. Transient cotransfection of Rcho-1 trophoblast cells with a PL-I promoter/luciferase reporter construct in the absence or presence of FGF-2 (20 ng/ml) and an FGFR1 dominant negative construct (dn-FGFR1), an FGFR2 dominant negative construct (dn-FGFR2), or a control expression vector. Dn-EGFR-treated cultures showed significantly less PL-I promoter activity than did serum-free or EGF-treated control cultures ( $P < 0.05$ ). Dn-FGFR-treated cultures showed significantly less PL-I promoter activity than did FGF-2-treated control cultures ( $P < 0.05$ ). Please note the inhibitory effects of the dn-EGFR construct in the absence of EGF, and the lack of effect of the dn-FGFRs in the absence of FGF-2. These observations further demonstrate the autocrine activation of the EGFR pathway in the trophoblast cells.



**Figure 5** EGF- and FGF-2 mediated activation of Gal4 DNA binding/Elk1 transactivation domain fusion gene constructs in trophoblast cells. Rcho-1 trophoblast cells were cotransfected with the Gal4/Elk1 construct (1 µg) and 5 × Gal4/luciferase (1 µg) and then maintained in serum-free medium with or without EGF (20 ng/ml) or FGF-2 (20 ng/ml). Cells were lysed following a 12–14 h incubation and luciferase activity measured. All experiments were performed in triplicate and repeated at least three times. Data are presented as the mean ± S.E.M. Growth factor-treated cultures showed significantly greater Elk1 activity than did control cultures ( $P < 0.05$ ).

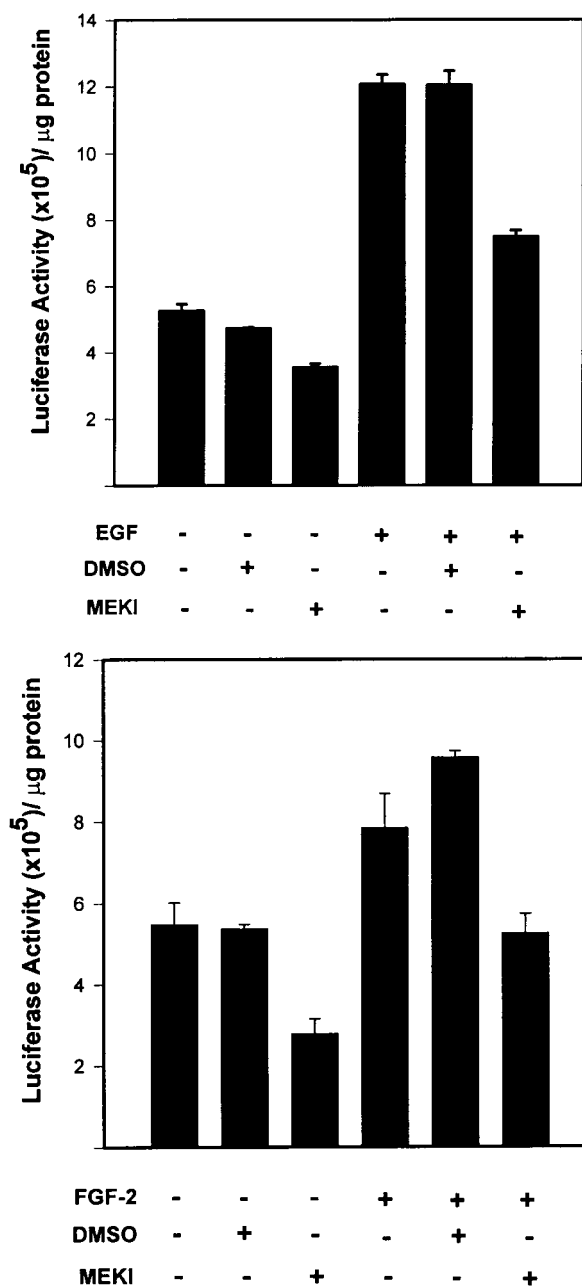
circulation of 10 µg/ml, and its synthesis subsequently terminates at mid-gestation (Ogren *et al.* 1989, Faria *et al.* 1990). PL-I provides a pivotal early signal to the maternal environment, most importantly to the corpus luteum, necessary for the establishment of pregnancy (Galosy & Talamantes 1995, Soares *et al.* 1998). In this report, we present evidence supporting a framework for both autocrine and paracrine signaling pathways controlling the expression of the PL-I gene. These pathways utilize, at least in part, members of the EGFR and FGFR families converging on the activation of the MAPK cascade.

EGFR and FGFR signaling pathways modulate the activity of the PL-I gene in trophoblast cells. These observations are consistent with their actions in regulating gene expression by various lineages of human trophoblast cells (Morrish *et al.* 1987, 1999, Pestell *et al.* 1995). We have shown that ligands and receptors for the EGFR system are present in rodent trophoblast cells differentiating *in vitro*, similar to that previously reported for rodent trophoblast cells developing *in vivo* (Adamson & Meek 1984, Tamada *et al.* 1991, Das *et al.* 1994, Lim *et al.* 1997). Addition of a prototypical ligand for the EGFR, EGF, to trophoblast cell cultures effectively stimulated the expression of PL-I. The actions of EGF were best observed under serum-free culture conditions and during early stages of

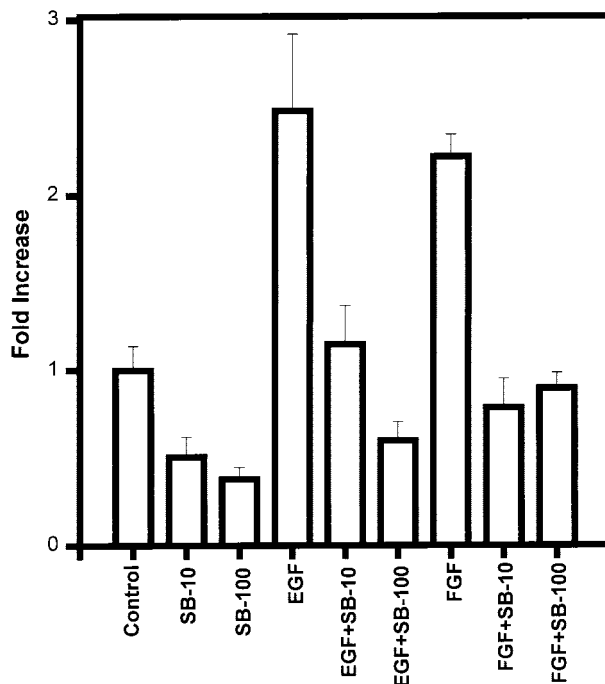


**Figure 6** Growth factor-induced activation of MAP kinases in differentiating trophoblast cells. Trophoblast cells were plated, expanded for 48 h, then exposed to serum-free conditions for 5 h followed by a 0, 5 or 30 min treatment with EGF (20 ng/ml) or FGF-2 (20 ng/ml). Cellular lysates were harvested and processed for Western blot analysis with antibodies to: top panel – active phospho ERK (upper) or total ERK (lower); middle panel – active phospho JNK (upper) or total JNK (lower); bottom panel – active phospho p38 MAPK (upper) or total p38 MAPK (lower). Lane A, positive control; lane B, at the time of growth factor addition; lane C, 5 min after growth factor addition; lane D, 30 min after growth factor addition. The blots presented in this figure are representative. Each experiment was replicated a minimum of three times. Observations were quantified by digital image processing and then subjected to statistical analysis. Phosphorylated ERK and p38 MAPK proteins were significantly increased 5 min following exposure to either EGF or FGF-2 ( $P < 0.05$ ). Please note that EGF and FGF-2 activate ERK and p38 MAPK without affecting their expression.

trophoblast differentiation, suggesting the involvement of autologous controls. Autocrine mechanisms have previously been implicated in the control of trophoblast giant cell differentiation (Hamlin & Soares 1995). Furthermore the inhibitory actions of dominant negative EGFR constructs in the absence of exogenous EGFR ligands reinforced the notion of an autocrine EGFR pathway in developing trophoblast giant cells. In contrast, although FGFR1 was expressed in trophoblast cells and exogenous FGF-2 stimulated trophoblast PL-I expression, ligands for FGFRs were not identified in trophoblast cells, nor were dominant negative FGFR constructs effective in influencing intrinsic trophoblast cell PL-I gene activation. Paracrine signals are likely operatives for the activation of trophoblast cell FGFR1. FGFR ligands may arise *in vivo* from maternal or other extraembryonic sources situated



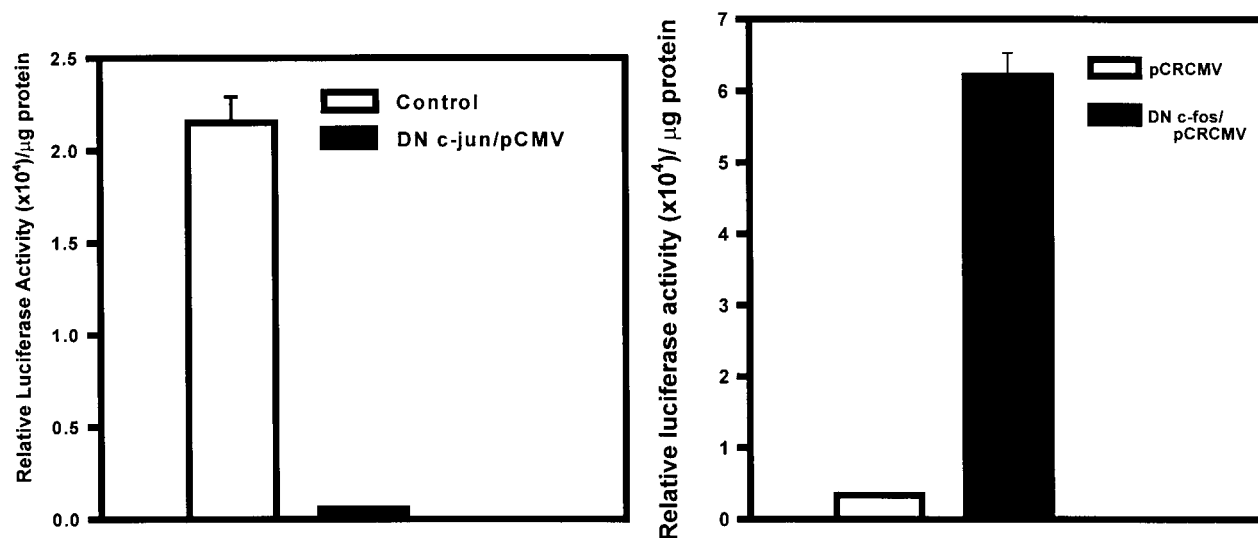
**Figure 7** MEK inhibitor (PD98059) inhibits intrinsic PL-I promoter activity. Rcho-1 trophoblast cells were transiently transfected with a PL-I promoter/luciferase reporter construct in the absence or presence of EGF or FGF-2 for 10 h and then incubated with EGF or FGF-2 in the presence of MEK inhibitor (MEKI; 60  $\mu\text{M}$ ) or the dimethylsulfoxide (DMSO) vehicle control for another 3 h. Growth factors were used at a concentration of 20 ng/ml. Top panel. Analysis of MEKI on EGF stimulated PL-I promoter activity. Bottom panel. Analysis of MEKI on FGF-2 stimulated PL-I promoter activity. MEKI significantly inhibited intrinsic PL-I promoter activity ( $P < 0.05$ ) but did not entirely prevent growth factor stimulated PL-I promoter activity. All experiments were performed in triplicate and repeated at least three times. Data are presented as the mean  $\pm$  S.E.M. of the mean.



**Figure 8** p38 MAPK inhibitor (SB203580) inhibits intrinsic PL-I promoter activity. Rcho-1 trophoblast cells were transiently transfected with PL-I promoter/luciferase reporter construct for 5 h, incubated in the presence of p38 MAPK inhibitor (SB203580, 10  $\mu\text{M}$  (SB-10) or 100  $\mu\text{M}$  (SB-100)) or the dimethylsulfoxide vehicle control for 30 min then treated with growth factors with the p38 MAPK inhibitor or vehicle control for 12–14 h. Growth factors (EGF and FGF-2) were used at a concentration of 20 ng/ml. The p38 MAPK inhibitor significantly inhibited intrinsic PL-I promoter activity ( $P < 0.05$ ) but did not entirely prevent growth factor-stimulated PL-I promoter activity. All experiments were performed in triplicate and repeated at least three times. Data are presented as the mean  $\pm$  S.E.M.

in close proximity to developing trophoblast giant cells (Carlone & Rider 1993, Rappolee *et al.* 1994, Yamaguchi & Rossant 1995, Nichols *et al.* 1998, Tanaka *et al.* 1998). Most interestingly, decidual cells produce FGF-2 and are also rich sources of TGF- $\alpha$  and HB-EGF (Tamada *et al.* 1991, Carlone & Rider 1993, Das *et al.* 1994, Zhang *et al.* 1994). Thus, both autocrine and paracrine signaling systems are likely to participate in growth factor regulation of PL-I gene expression in differentiating trophoblast giant cells.

The MAPK pathway effectively provides a conduit between signals at the cell surface and potential nuclear responses (Brunet & Pouyssegur 1998, Lewis *et al.* 1998). EGFR and FGFR1 are receptor tyrosine kinases known to activate the MAPK cascade in a number of different model cell systems (Friesel & Maciag 1995, Riese & Stern 1998). Differentiating rat trophoblast cells respond to growth factor stimulation with an activation of ERK and p38 MAPK modules (present study). Furthermore, growth factor-stimulated MAPK cascades participate in the



**Figure 9** Dominant negative c-jun and c-fos expression construct modulation of PL-I promoter activity. Rcho-1 trophoblast cells were transiently cotransfected with PL-I promoter/luciferase (1.5 μg) constructs and either dominant negative c-jun (dn c-jun, 2 μg) or dominant negative c-fos (dn c-fos, 2 μg). Controls were performed with the respective empty expression vectors (pCMV and pCRCMV) or with gelatinase B substituted for the PL-I promoter (data not shown). Please note that exposure to the dn c-jun construct resulted in a significant inhibition of PL-I promoter activity (left panel). Surprisingly, the dn c-fos construct stimulated PL-I promoter activity (right panel). All experiments were performed in triplicate and repeated at least three times. Data are presented as the mean ± S.E.M. Statistical comparisons were made between control and dominant negative treated cells and they are significantly different ( $P < 0.01$ ).

regulation of the PL-I gene. EGFR signaling pathways have also been shown to stimulate the human chorionic gonadotropin- $\alpha$  gene promoter via activation of the cAMP response element binding protein (CREB) in heterologously transfected Rcho-1 trophoblast cells (Matsumoto *et al.* 1998). p38 MAPK is also known to activate CREB (Xing *et al.* 1998) and may represent a commonality in the two studies. Pestell *et al.* (1995) have demonstrated that EGF activates MAPK signaling pathways in human JEG-3 choriocarcinoma cells. In their studies with JEG-3 cells, ERK and JNK modules were shown to be growth factor responsive, with the ERK module participating in the regulation of P-450 side-chain cleavage enzyme gene transcription. Specificity of MAPK modules controlling gene expression is likely cell lineage, differentiation stage and gene dependent.

Progress in understanding the regulation of the PL-I promoter in differentiating trophoblast cells (Shida *et al.* 1993, Ng *et al.* 1994) offers potential insight into the actions of EGF and FGF-2 on PL-I gene activation. A trophoblast-specific core promoter (274 bp) has been identified that directs trophoblast giant cell-specific and differentiation-dependent PL-I gene activation (Shida *et al.* 1993). Linzer and his colleagues (Shida *et al.* 1993) further demonstrated a role for AP-1 trans-acting factors and cis-regulatory elements controlling PL-I promoter activation. Pestell *et al.* (1995) further showed that growth factor activation of an ovine P450 side-chain cleavage promoter in human choriocarcinoma cells was mediated

via MAPK modulation of AP-1 activities. Our observations with dominant negative c-jun and c-fos constructs are in agreement with a role for AP-1 in the regulation of the PL-I gene but may not represent the only site for growth factor modulation of PL-I promoter activities. The GATA family of transcription factors may represent an alternative site for growth factor modulation. Rat trophoblast cells express GATA-2 and GATA-3 which bind GATA elements within the PL-I promoter, and which have been proposed to positively regulate PL-I transcription (Ng *et al.* 1994). ERK has also been shown to phosphorylate GATA-2 in hematopoietic cells (Towatari *et al.* 1995). The nature of the effects of phosphorylation on GATA-2 activities and whether p38 MAPK similarly modulates GATA factor transcriptional activities in trophoblast cells remains to be determined. Additional data exist for basic helix-loop-helix transcription factors regulating PL-I promoter activity (Cross *et al.* 1995); however, mechanisms underlying their interface with growth factor signaling pathways in trophoblast cells have not been reported. Finally, MAPK cascades are known to activate members of the ets family of transcription factors (Brunet & Pouyssegur 1998, Lewis *et al.* 1998, Wasyluk *et al.* 1998). Ets-2 has recently been implicated in regulating trophoblast cell development and gene expression (Pestell *et al.* 1995, Ezashi *et al.* 1998, Yamamoto *et al.* 1998). Ets elements have also been shown to be important in the activation of the PL-II and decidual PRL-related protein genes in differentiating trophoblast giant cells (Orwig &

Soares 1999, Sun & Duckworth 1999). Although, a putative ets-binding site is located within the 274 bp PL-I promoter, the presence of ets-2 or any other member of the ets transcription factor family in Rcho-1 trophoblast cells and their role in growth factor-stimulated PL-I gene activation are yet to be resolved.

In conclusion, EGFR and FGFR signaling pathways act through the MAPK cascades to specifically upregulate the expression of PL-I in differentiating trophoblast cells. Specific nuclear targets for ERK and p38 MAPK action within differentiating trophoblast giant cells are unknown and represent the focus of our current research efforts. Growth factor action on developing trophoblast giant cells may positively influence adjustments in PL-I expression required for successfully establishing a pregnancy.

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