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Modulation of trophoblast stem cell and giant cell phenotypes: analyses using the Rcho-1 cell model

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Abstract Trophoblast giant cells are located at the maternal–embryonic interface and have fundamental roles in the invasive and endocrine phenotypes of the rodent placenta. In this report, we describe the experimental modulation of trophoblast stem cell and trophoblast giant cell phenotypes using the Rcho-1 trophoblast cell model. Rcho-1 trophoblast cells can be manipulated to proliferate or differentiate into trophoblast giant cells. Differentiated Rcho-1 trophoblast cells are invasive and possess an endocrine phenotype, including the production of members of the prolactin (PRL) family. Dimethyl sulfoxide (DMSO), a known differentiation-inducing agent, was found to possess profound effects on the *in vitro* development of trophoblast cells. Exposure to DMSO, at non-toxic concentrations, inhibited trophoblast giant cell differentiation in a dose-dependent manner. These concentrations of DMSO did not significantly affect trophoblast cell proliferation or survival. Trophoblast cells exposed to DMSO exhibited an altered morphology; they were clustered in tightly packed colonies. Trophoblast giant cell formation was disrupted, as was the expression of members of the PRL gene family. The effects of DMSO were reversible. Removal of DMSO resulted in the formation of trophoblast giant cells and expression of the PRL gene family. The phenotype of the

DMSO-treated cells was further determined by examining the expression of a battery of genes characteristic of trophoblast stem cells and differentiated trophoblast cell lineages. DMSO treatment had a striking stimulatory effect on eomesodermin expression and a reciprocal inhibitory effect on Hand1 expression. In summary, DMSO reversibly inhibits trophoblast differentiation and induces a quiescent state, which mimics some but not all aspects of the trophoblast stem cell phenotype.

Key words trophoblast stem cells · trophoblast differentiation · placenta · prolactin · dimethyl sulfoxide · eomesodermin

Introduction

Trophoblast cells are the earliest cell lineage to differentiate during mammalian development, arising from trophoblast of the blastocyst (Rossant and Cross, 2002). These stem cells can continue to proliferate or go on to differentiate along a multilineage pathway (Gardner and Beddington, 1988), which in rodents leads to five phenotypically distinct cell types: trophoblast giant cells, spongiotrophoblast cells, invasive extravillous trophoblast cells, glycogen cells, and syncytial trophoblast cells. Each of these individual cell types is identified on the basis of morphology, uteroplacental location, and pattern of gene expression (Soares et al., 1996; Georgiades et al., 2002; Rossant and Cross, 2002; Ain et al., 2003). Expression of the placental prolactin (PRL) family of cytokines provides cell-specific and temporal-specific measures of trophoblast cell differentiation (Soares and Linzer, 2001; Soares, 2004). Of the differentiated trophoblast cell types, trophoblast giant cells are the earliest to develop. Trophoblast giant cells possess endocrine properties and are situated at the maternal–fetal interface.

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Factors controlling decisions for continued stem cell proliferation or for differentiation toward each of the differentiated phenotypes are yet to be fully elucidated. Some of the factors implicated in early decisions regulating the trophoblast lineage, include, Cdx2 (Beck et al., 1995; Chawengsaksophak et al., 1997, 2004; Strumpf et al., 2005), eomesodermin (Eomes et al., 2000; Strumpf et al., 2005), estrogen-receptor-related receptor- β (ERR- β , Luo et al., 1997; Tremblay et al., 2001), suppressor of cytokine signaling 3 (SOCS3; Roberts et al., 2001; Takahashi et al., 2003), cyclin E (Geng et al., 2003; Parisi et al., 2003), fibroblast growth factor-4 (FGF4)/FGF receptor-2 (FGFR2; Orr-Uretgeger et al., 1993; Feldman et al., 1995; Arman et al., 1998; Nichols et al., 1998; Tanaka et al., 1998); and activator protein-2 γ (AP-2 γ ; Auman et al., 2002; Werling and Schorle, 2002). Other factors like mammalian achaete-scute homologue-2 (Mash2), Hand1, and glial cell missing 1 (Gcm1) are essential regulators of differentiation of spongiotrophoblast cells, trophoblast giant cells, and labyrinthine syncytial trophoblast cells of the mature chorioallantoic placenta (Guillemot et al., 1994; Cross et al., 1995; Tanaka et al., 1997; Firulli et al., 1998; Kraut et al., 1998; Riley et al., 1998; Anson-Cartwright et al., 2000; Schreiber et al., 2000; Scott et al., 2000). The hierarchical relationship of these critical regulators of trophoblast cell development and placental morphogenesis are still under investigation.

Identification of strategies to manipulate the entry of trophoblast stem cells into a specific pathway of development are critical to understanding mechanisms involved in normal placental morphogenesis. Dysregulation of these processes will likely impact pregnancy outcomes. In this respect, the Rcho-1 cell line provides an effective *in vitro* model system for dissecting the trophoblast giant cell differentiation pathway.

The Rcho-1 cell line was established from a rat transplantable choriocarcinoma (Teshima et al., 1983; Faria and Soares, 1991). The cells exhibit many characteristics of trophoblast stem cells (Faria and Soares, 1991; Cross et al., 1995; Peters et al., 2000; Takahashi et al., 2003). These Rcho-1 trophoblast stem cells can be manipulated to proliferate or differentiate by altering their culture conditions. Proliferation of Rcho-1 trophoblast stem cells can be stimulated by factors present in fetal bovine serum (FBS) and is facilitated by maintaining the cells at low densities. Increasing cell density and removal of mitogenic stimuli lead to spontaneous differentiation into a phenotype resembling trophoblast giant cells (Peters et al., 2000). Rcho-1 trophoblast stem cell differentiation recapitulates *in vivo* trophoblast giant cell development, including endoreplication (Hamlin and Soares, 1995) and sequential expression of members of the PRL family (Faria et al., 1990; Hamlin et al., 1994; Dai et al., 2002). These features of Rcho-1 trophoblast stem cells make them a valuable *in vitro* tool for studying the process of

trophoblast cell differentiation (Kamei et al., 1997, 2002).

Dimethyl sulfoxide (DMSO) is a reagent frequently used as a cryoprotectant for the storage of cells and as a solvent for a variety of drugs. DMSO has also been shown to influence differentiation in a variety of embryonic, extraembryonic, and hematopoietic cell systems (Preisler and Giladi, 1975; Collins et al., 1978; Lever, 1979; McBurney et al., 1982; Omary et al., 1992; Wang and Scott, 1993; Yu and Quinn, 1994; Angello et al., 1997; Thirkill and Douglas, 1997; Skerjanc, 1999).

In this report, the effects of DMSO on the differentiation of trophoblast cells were evaluated. DMSO reversibly inhibited trophoblast giant cell differentiation and activated the expression of Eomes, a gene associated with the stem cell stage of trophoblast development.

Materials and methods

Reagents

FBS was obtained from Atlanta Biologicals (Norcross, GA), while donor horse serum (HS) was purchased from JRH Biosciences (Lenexa, KS). RPMI 1640 culture medium was obtained from Cellgro (Herndon, VA). Trizol reagent for RNA extraction, expressed sequence tags (ESTs), and TOPO TA cloning kits were obtained from Invitrogen (Carlsbad, CA). Nitrocellulose and nylon membranes for Northern and Western blotting were purchased from Schleicher and Schuell (Keene, NH). [³²P]-labeled ATP was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA). Pfu polymerase and Primer-It Random Primer Labeling Kits were purchased from Stratagene (La Jolla, CA). Kits for DNA extraction were obtained from Qiagen Inc. (Valencia, CA). Reagents for SDS-PAGE gels were obtained from Bio-Rad Laboratories Inc. (Richmond, CA). Kodak Bio-Max film was obtained from Eastman Kodak Co. (Rochester, NY). Reagents for the detection of immune complexes by enhanced chemiluminescence (ECL) were obtained from Amersham Corp. (Arlington Heights, IL). The Live/Dead Viability/Cytotoxicity kit was obtained from Molecular Probes (Eugene, OR). Antibodies to rat placental lactogen-II (PL-II, Cat. No. AB1289) and PRL-like protein-A (PLP-A, Cat. No. AB1290) were acquired from Chemicon International (Temecula, CA). Unless otherwise stated, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Holtzman Sprague-Dawley rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Timed pregnancies were established by housing females overnight with fertile males. The presence of sperm in the vaginal lavage was designated as day 0 of pregnancy. Placental tissues were dissected from day 18 of gestation, frozen in liquid nitrogen, and stored at -80°C until analyzed. The University of Kansas Medical Center Animal Care and Use Committee approved all procedures for handling and experimentation with rodents.

Trophoblast cell *in vitro* model

Elucidation of regulatory networks controlling trophoblast giant cells has been facilitated by the availability of the Rcho-1 trophoblast stem cell line (Faria and Soares, 1991; Peters et al., 2000). Rcho-1 trophoblast stem cells can be manipulated to proliferate or

to differentiate along the trophoblast giant cell lineage (Faria and Soares, 1991; Peters et al., 2000). The Rcho-1 trophoblast stem cell line was routinely maintained in sub-confluent conditions with RPMI 1640 culture medium supplemented with 20% FBS as previously reported (Peters et al., 2000). Differentiation was induced by growing cells to near confluence in FBS-supplemented culture medium and then replacing the culture medium with NCTC 135 supplemented with 1% HS (Peters et al., 2000). High cell density and the absence of sufficient proliferation stimulatory factors (removal of FBS) facilitate trophoblast giant cell formation (Peters et al., 2000). DMSO was added to the culture medium during trophoblast cell proliferation and differentiation at a range of concentrations. The differentiation protocol involved an 8-day exposure to differentiating culture conditions. *Chronic DMSO treatment*: Cells were exposed to DMSO (0.5%–1.5%) for 8 days during the differentiation phase. *Acute DMSO treatment*: differentiated trophoblast cells were exposed to DMSO for 48 hr (days 7–8 of differentiation). In some experiments, the reversibility of DMSO actions was evaluated. Rcho-1 trophoblast cells were exposed to differentiating conditions in the presence of DMSO for 8 days followed by a continuation of differentiating conditions in the absence of DMSO for an additional 6 days.

Trophoblast cell proliferation/survival assay

The effects of DMSO on trophoblast cell proliferation/survival were assessed using a crystal violet staining-elution assay. Cultures were established by plating 5,000 cells/well in RPMI 1640 culture medium supplemented with 20% FBS in 24-well plates. Following cell attachment the medium was changed to NCTC 135 with 1% HS. DMSO treatments were added, and medium was changed daily. Cultures were maintained for 8 days, followed by assessment of relative cell density as previously described (Hamlin et al., 1994; Hamlin and Soares, 1995). Briefly, wells were rinsed with PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, 1.8 mM potassium phosphate, pH 7.4), and stained with crystal violet (300 µl/well; 5% formalin, 50% ethanol, 150 mM NaCl, and 0.5% crystal violet) for 10 min with agitation. Cell cultures were then washed repeatedly in tap water, and allowed to dry. Crystal violet dye was then eluted with ethylene glycol. Relative cell

density was quantified by measuring absorbance of each eluant at 600 nm with a microplate reader (Bio-Rad Laboratories Inc.). In this assay, cell density is directly correlated with absorbance of the cellular eluates (Hamlin et al., 1994; Hamlin and Soares, 1995).

Trophoblast giant cell viability assay

The effect of DMSO on trophoblast giant cell viability was assessed by staining cells with components of the Live/Dead Viability-Cytotoxicity kit: SYTO 10 green fluorescent nucleic acid stain detects all cells, whereas DEAD Red nucleic acid stain detects cells with damaged plasma membranes. Rcho-1 trophoblast stem cells were first differentiated into trophoblast giant cells (exposure to 8 days of differentiating conditions). Trophoblast giant cells were then treated with DMSO (1.5%) for 48 hr followed by staining with the Live/Dead Viability-Cytotoxicity kit according to the manufacturers instructions. Ten random fields per control and DMSO-treated flasks were examined for trophoblast giant cells using a Leica MZFL III microscope (Wetzlar, Germany). All cells are detected with a green fluGFP filter (wavelength 510 nm), while dead cells are detected with a rhodamine filter (wavelength 630 nm).

PRL family mini-array analysis

The PRL family mini-array assay is a hybridization-based tool for simultaneously monitoring expression of each known member of the PRL family (Dai et al., 2002). It has been effectively used to monitor the phenotypes of the placenta and trophoblast cells (Table 1). The PRL family mini-array assay was performed, as previously described (Dai et al., 2002). Twenty nanograms of PCR-amplified cDNA for each of the members of the rat PRL family was spotted, in duplicate, onto nylon membranes. Membranes were cross-linked and stored at 4°C until used. Total RNA was extracted from tissues using TRIzol reagent (Chomczynski and Sacchi, 1987). [α P³²]dCTP-labeled cDNA probes were generated by reverse transcription using 10 µg of total RNA. Probes were purified using micro-bio-spin columns. Membrane filters were briefly rinsed with water and pre-hybridized for 2 hr at 42°C with buffer containing 6 × SSPE (1 × SSPE: 0.145 M NaCl, 0.01 M NaH₂PO₄, 1 mM

Table 1 Expression patterns for members of the PRL family in the pregnant rat

PRL family member	Source	Reference
PRL	Lactotrophs, anterior pituitary	Freeman et al. (2000)
PL-I	TGC, CV and CA placentas	Faria et al. (1990)
PL-Iv	TGC and spongiotrophoblast of the JZ, CA placenta	Deb et al. (1991a)
PL-II	TGC of JZ and LZ, CV and CA placentas	Campbell et al. (1989), Faria et al. (1990)
PLF-RP	Trophoblast, labyrinth zone, CA placenta	Sahgal et al. (2000)
PLP-F	TGC, junctional zone, CA placenta	Sahgal et al. (2000)
PLP-B	Spongiotrophoblast cells, CA placenta	Cohick et al. (1997)
PLP-J	Uterine decidua	Dai et al. (2000)
D/tPRP	Uterine decidua	Roby et al. (1993)
PLP-A	TGC & spongiotrophoblast of the JZ, CA placenta; invasive trophoblast, metrial gland	Campbell et al. (1989), Ain et al. (2003)
PLP-I	JZ, CA placenta; specific cell type not determined	Wiemers et al. (2003b), Dai et al. (2002)
PLP-K	Trophoblast, labyrinth zone, CA placenta	Dai et al. 2000
PLP-L	Invasive trophoblast, metrial gland	Ain et al. (2003)
PLP-M	Invasive trophoblast, metrial gland	Ain et al. (2003)
PLP-C	TGC and spongiotrophoblast of the JZ, CA placenta	Deb et al. (1991b)
PLP-Cv	TGC and spongiotrophoblast of the JZ, CA placenta	Dai et al. (1996)
PLP-D	TGC and spongiotrophoblast of the JZ, CA placenta	Iwatsuki et al. (1996)
PLP-H	TGC and spongiotrophoblast of the JZ, CA placenta	Iwatsuki et al. (1998)
PLP-N	Invasive trophoblast, metrial gland	Wiemers et al. (2003a)

TGC, trophoblast giant cell; CV, choriovitelline placenta; CA, chorioallantoic placenta; JZ, junctional zone; LZ, labyrinth zone; PRL, prolactin; PLP, PRL-like protein.

Table 2 cDNAs for trophoblast cell lineage analysis obtained as expressed sequence tags (ESTs)

Description	Abbreviation	GenBank accession no.	Functional group	Probe GenBank accession no.
Cyclin D3	CCND3	NM_012766	Cell cycle regulator	BI291261
Cyclin E	CCNE	D14015	Cell cycle regulator	BE112508
Hand1	Hand1	NM_021592	Transcription regulator	AI070613
Id-1	Id1	NM_012797	Transcription regulator	BI292304

EDTA, pH 7.4), 5 × Denhardt's reagent (1 × Denhardt's: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, dissolved in water), 50% deionized formamide, 0.5% SDS, and salmon sperm DNA (100 µg/ml). Hybridizations were performed overnight with the labeled probes at 42°C. Membranes were washed once with 2 × SSPE and 0.1% SDS for 30 min at 42°C and once with 1 × SSPE and 0.1% SDS at 68°C for 60 min. Membranes were then wrapped with plastic wrap and exposed to Kodak Bio-Max film for 1–4 hr and developed. For signal quantification, membranes were placed in PhosphoImager cassettes, for 24 hr and then quantified using ImageQuant software (version 4.2A, Molecular Dynamics Inc., Sunnyvale, CA).

Northern blot analysis

Northern blot analysis was performed as described previously (Faria et al., 1990). Total RNA was isolated using Trizol (Chomczynski and Sacchi, 1987), resolved in 1% formaldehyde-agarose gels, and transferred to nylon membranes and cross-linked. Blots were probed with [³²P]-labeled cDNAs. cDNAs for rat spongiotrophoblast specific protein (SSP) and rat Gcm1 were obtained from Dr. Kunio Shiota (University of Tokyo, Japan; Iwatsuki et al., 2000) and Dr. David Anderson (California Institute of Technology, Pasadena, CA; Kim et al., 1998), respectively. Rat cDNAs for cyclin D3, cyclin E1, Hand1, and inhibitor of DNA binding-1 (Id1) were identified by searching the EST database (National Center for Biotechnology Information, Altschul et al., 1990) and obtained from Invitrogen (Table 2). The identities of the ESTs were verified by DNA sequencing. Rat cDNAs for Eomes, SOCS3, and Cdx2 were isolated by reverse transcription polymerase chain reaction (RT-PCR) from total RNA extracted with the TRIzol reagent (Chomczynski and Sacchi, 1987) from proliferative Rcho-1 trophoblast cells. Five micrograms of total RNA and 0.5 µg of oligo dt (deoxythymidine) were used for reverse transcription reactions. PCR reactions were performed using *Pfu* polymerase with sets of primers based on the cDNA sequences (Table 3). PCR was performed for 30 cycles (denature, 95°C for 1 min; anneal, 55°C for 1 min; extension, 72°C for 1.5 min). Amplified products were subcloned into pCRII-TOPO vector flanked by SP6 and T7 promoters with the TOPO TA Cloning kit. cDNAs were sequenced by the Biotechnology Support Facility of the University of Kansas Medical Center. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a control.

Table 3 Primer sets used for isolating trophoblast cell lineage cDNAs

CDNA	GenBank accession no.	Forward primer	Reverse primer	Amplified product (bp)
Mash 2	X53724	5'-GAGGACGCATTAAGCTAAGCA-3'	5'-ACGCAGCTTAGGGGTGTG-3'	830
Cdx2	AJ278466	5'-TGCGCCTCCGGATTGCTGCAG-3'	5'-GCTGGCCCCAGAACCCCAGG-3'	508
Eomes	AY457971	5'-GGCTCAAATTCCACCGGCACC-3'	5'-GGTCCCTGTTAGACTACCCTA-3'	600
SOCS3	AF075383	5'-TCACACTGAGCGTCGAGA-3'	5'-GTGGAGCATCATACTGGT-3'	434

Eomes, eomesodermin; SOCS3, suppressor of cytokine signaling 3.

RT-PCR

Five micrograms of total RNA isolated from Rcho-1 trophoblast cells and 0.5 µg of oligo dt were used for reverse transcription reactions. RNA isolated from day 18 junctional zone tissue was used as a positive control. Three microliters of each RT product was used in the 50 µl PCR amplification reaction. PCR was performed for Mash2 using primers shown in Table 3. PCR was done using *Taq* DNA polymerase under the following conditions: 30 cycles; denature, 94°C for 1 min; anneal, 60°C for 1 min; extension, 72°C for 1 min. Primers for the amplification of G3PDH were included as controls. The amplified products for each RT-PCR reaction were then separated by electrophoresis in 1% agarose gels.

Western blot analysis

Concentrations of PL-II and PLP-A in Rcho-1 trophoblast cell conditioned medium were monitored by immunoblotting (Deb et al., 1989, 1993; Hamlin et al., 1994). Samples were resolved by SDS-PAGE. Separated proteins were electrophoretically transferred to nitrocellulose and filters were probed with polyclonal antibodies to PL-II (Deb et al., 1989) or PLP-A (Deb et al., 1993). Blots were incubated with horseradish peroxidase-conjugated antibodies to rabbit IgG for 45 min at room temperature. Reaction products were visualized by incubation with ECL substrate and developed with X-Omat AR X-ray film (Rochester, NY) (Kamei et al., 1997).

Statistical analysis

The data were analyzed by analysis of variance. The source of variation from significant *F* ratios was determined with the Newman-Keuls multiple comparison test (Keppel, 1973).

Results

Effects of DMSO on trophoblast cell proliferation, survival, and trophoblast cell morphology

Initially, we examined the effect of DMSO on trophoblast cell proliferation following DMSO exposure to Rcho-1 trophoblast stem cells cultured under proliferating (RPMI 1640/20% FBS) or differentiating (NCTC 135/1% HS) conditions. DMSO did not impair

proliferation in cells cultured under proliferating conditions (data not shown) and did not affect viability in cells exposed to differentiating conditions (Fig. 1A). DMSO at concentrations of 1.5% or less did not significantly affect cell density (Fig. 1A); however, concentrations at or above 3% were toxic. The potential selective toxicity of trophoblast giant cells to DMSO was also examined. DMSO at a concentration of 1.5% did not significantly affect the viability of trophoblast giant cells differentiated *in vitro* (Fig. 1B). The morphology of Rcho-1 trophoblast stem cells cultured in proliferation-promoting medium (RPMI

1640/20% FBS) for 3 days and then transferred to differentiation-promoting medium (NCTC 135/1% HS) with or without DMSO for 8 days was assessed by phase contrast microscopy. Cultures from DMSO-treated cultures (1.5%) had fewer numbers of trophoblast giant cells than did control cultures (Figs. 1C, 1D).

Effects of DMSO on expression of PRL family cytokines

Treatment with DMSO during the 8-day differentiation period significantly disrupted acquisition of the tropho-

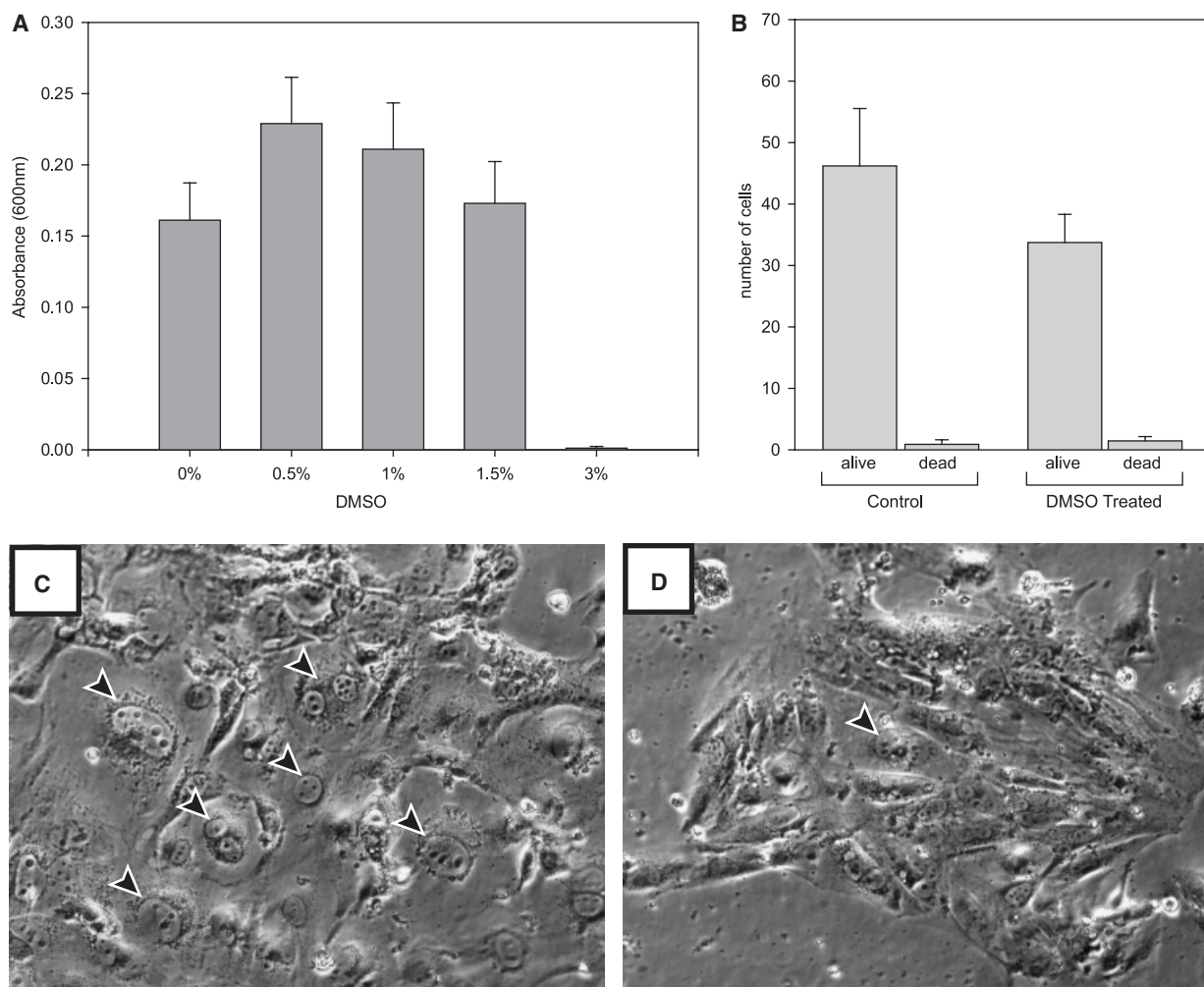


Fig. 1 Examination of the effects of dimethyl sulfoxide (DMSO) on trophoblast cell proliferation/survival/viability and morphology. (A) Effects of DMSO on trophoblast cell proliferation/survival. Rcho-1 cells maintained in NCTC 135 culture medium supplemented with 1% HS (differentiating medium) were exposed to varying concentrations of DMSO for 8 days. Relative changes in cell density were quantified by crystal violet staining followed by elution and measurements of absorbance at 600nm. Concentrations of DMSO greater than 1.5% were found to be toxic. (B) Effects of DMSO on trophoblast giant cell viability. Rcho-1 trophoblast stem cells were first differentiated into trophoblast giant cells for 8 days and then treated with DMSO for 48 h. The effect of DMSO (1.5%) on trophoblast giant cell viability was

assessed with the Live/Dead Viability-Cytotoxicity kit. No significant differences were observed in the numbers of live and dead trophoblast giant cells exposed to DMSO (1.5%) or control conditions. (C, D) Effects of DMSO on trophoblast cell morphology. (C) Morphology of Rcho-1 trophoblast cells after 8 days of differentiation. (D) Morphology of Rcho-1 trophoblast cells exposed to 1.5% DMSO for 8 days during differentiation. Images shown in (C) and (D) were obtained using phase contrast microscopy. Arrowheads depict the location of trophoblast giant cells in the cultures. Please note the decreased number of trophoblast giant cells in the DMSO-treated cultures (D). All experimental results presented in this figure were replicated at least three times.

blast giant cell phenotype, as monitored by the PRL family miniarray assay. Expression of PRL family members was significantly decreased following chronic exposure to DMSO (days 1–8 of differentiation, Fig. 2). The effects of DMSO were dose dependent with a maximally effective concentration of 1.5% (Figs. 2A, 2B). Acute DMSO treatment also effectively suppressed PRL family gene expression in differentiated trophoblast cells (48 hr of DMSO treatment on days 7 and 8 of differentiation, Figs. 2A, 2B). Furthermore, the accumulation of PL-II and PLP-A proteins in medium conditioned by differentiating trophoblast cells treated chronically or acutely with DMSO was inhibited (Figs. 2C). Finally, the inhibitory actions of DMSO were reversible (Fig. 3). Following an 8-day treatment with 1.5% DMSO during differentiation, removal of DMSO for 6 days led to a dramatic induction of PRL family gene expression and PL-II and PLP-A protein production (Figs. 3A, 3B). Removal of DMSO also resulted in an increase number of trophoblast giant cells in the

cultures (data not shown) and an increased accumulation of PL-II and PLP-A proteins (Figs. 3C). In summary, DMSO is a reversible inhibitor of trophoblast giant cell differentiation.

In these experiments, PLP-B expression was detected in the differentiated Rcho-1 trophoblast cells (Figs. 2 and 3). This observation contrasts with our earlier results (Faria and Soares, 1991). *In vivo*, PLP-B expression is restricted to the spongiotrophoblast cell lineage (Duckworth et al., 1990; Cohick et al., 1997). The discrepancy may reflect differences in the sensitivities of the assays used in the two studies or a gradual selection of Rcho-1 trophoblast cells with the potential to differentiate along the spongiotrophoblast lineage.

Effects of DMSO on trophoblast lineages

As DMSO was not selectively toxic to trophoblast giant cells (Fig. 1B) but did inhibit aspects of trophoblast

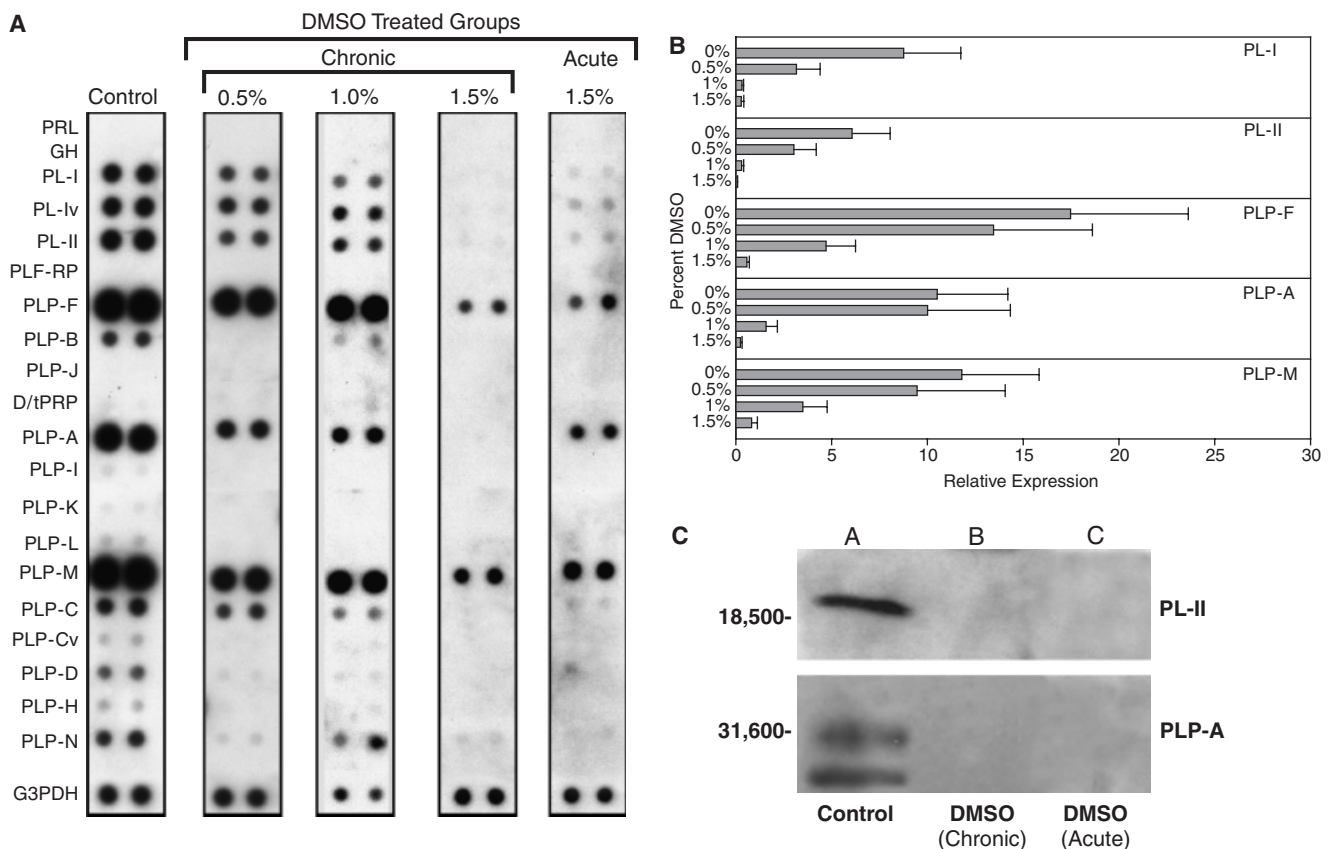


Fig. 2 Evaluation of the effects of dimethyl sulfoxide (DMSO) on the Rcho-1 trophoblast giant cell differentiated phenotype. (A) Representative prolactin (PRL) family mini-array assay of Rcho-1 trophoblast cells treated chronically or acutely with DMSO. *Chronic DMSO treatment*: cells were exposed to DMSO (0.5%–1.5%) for 8 days during the differentiation phase. *Acute DMSO treatment*: differentiated trophoblast cells were exposed to DMSO for 48 hr (days 7–8 of differentiation). At the termination of treatment, cells were harvested, total RNA isolated, and PRL family gene expression

assessed with the PRL family mini-array assay (A). (B): Graphic representation of five replicate PRL family mini-arrays. Please note that data is presented for a subset of PRL family members exhibiting the highest levels of expression (PL-I, PL-II, PRL-like protein (PLP)-F, PLP-A, and PLP-M). (C) Western blot analysis of PL-II and PLP-A in conditioned medium generated from control cultures (Lane A) and following chronic (Lane B) and acute (Lane C) treatment with DMSO (1.5%). The Western blotting experiment was repeated three times on different sets of samples.

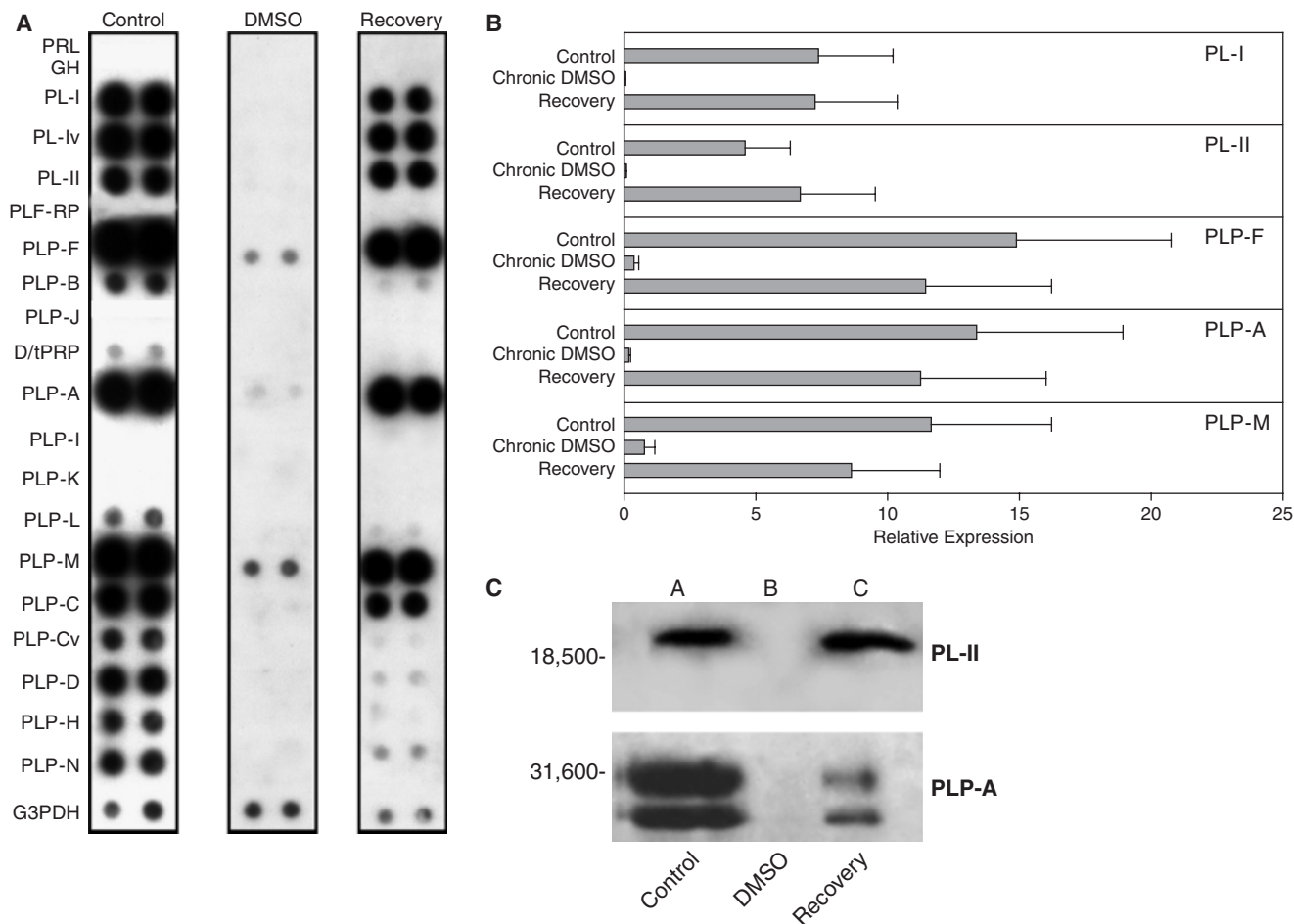


Fig. 3 Reversible effects of dimethyl sulfoxide (DMSO) on trophoblast cell differentiation. (A) Representative PRL family mini-array analysis of control Rcho-1 trophoblast cells (following 14 days of differentiation, *Control*), Rcho-1 trophoblast cells exposed to DMSO (1.5%) for 14 days (*DMSO*), or Rcho-1 trophoblast cells exposed to DMSO (1.5%) for 8 days, followed by culture without DMSO for an additional 6 days (*Recovery*). (B) Graphic representation of five replicate prolactin (PRL) family mini-arrays. Please note that data are presented for a subset of PRL family members

exhibiting the highest levels of expression (PL-I, PL-II, PLP-F, PLP-A, and PLP-M). (C) Representative Western blot analysis of PL-II and PRL-like protein (PLP-A) protein production in control Rcho-1 trophoblast cells (following 14 days of differentiation, *Control*, Lane A), Rcho-1 trophoblast cells exposed to DMSO (1.5%) for 14 days (*DMSO*, Lane B), or Rcho-1 trophoblast cells exposed to DMSO (1.5%) for 8 days, followed by culture without DMSO for an additional 6 days (*Recovery*, Lane C). The Western blotting experiment was repeated three times on different sets of samples.

giant cell differentiation (Figs. 1 and 2), we determined whether DMSO was arresting trophoblast cell development or directing differentiation along a trophoblast cell lineage other than trophoblast giant cells (e.g., spongiotrophoblast cells, invasive extraplacental trophoblast cells, or syncytial trophoblast cells). Specific genetic markers have been identified for trophoblast stem cells and each of the five differentiated trophoblast cell lineages (Tanaka et al., 1998; Anson-Cartwright et al., 2000; Iwatsuki et al., 2000; Takahashi et al., 2003; L. Canham, N. Sahgal, M.J. Soares, unpublished results). PLP-B and PLP-N are specific markers for spongiotrophoblast and invasive trophoblast populations, respectively (Duckworth et al., 1990; Cohick et al., 1997; Wiemers et al. 2003a). Neither PLP-B nor PLP-N mRNAs were induced by DMSO treatment (Fig. 2), suggesting that DMSO was not capable of

promoting differentiation toward spongiotrophoblast or invasive trophoblast cells. We extended this analysis by using Northern blot and RT-PCR analyses of Rcho-1 trophoblast cells chronically treated with 1.5% DMSO (days 1–8 of differentiation) for expression of Id1, cyclin D3, cyclin E1, Cdx2, Eomes, SOCS3, Hand1, SSP, Gcm1, and Mash2 (Figs. 4A,4B). DMSO treatment did not activate the expression of additional spongiotrophoblast (SSP, Mash2) or syncytial trophoblast cell lineage markers (Gcm1), or a number of genes known to be up-regulated in trophoblast stem cells (Cdx2, cyclin D3, cyclin E1, Id1, SOCS3, and Mash2). However, chronic DMSO treatment (days 1–8 of differentiation) resulted in the enhanced expression of the trophoblast stem cell regulator, Eomes, and decreased expression of the trophoblast giant cell regulator, Hand1 (Fig. 4A). The actions of DMSO on the

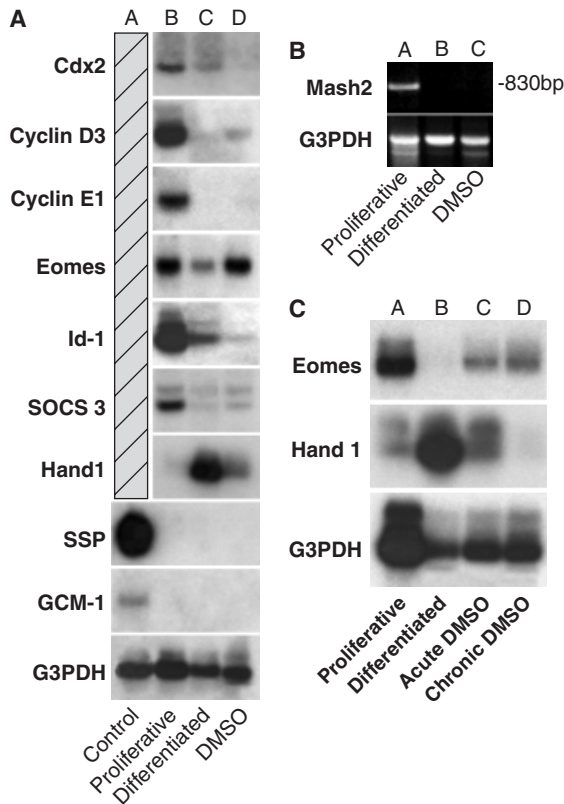


Fig. 4 Lineage analysis of control and dimethyl sulfoxide (DMSO)-treated trophoblast cells. (A) Northern blot analysis was performed on: Lane A, control samples (*Control*); Lane B, Rcho-1 trophoblast cells grown under proliferating conditions (*Proliferative*); Lane C, differentiating Rcho-1 trophoblast cells, 8 days (*Differentiation*); Lane D, Rcho-1 trophoblast cells treated with DMSO (1.5%) for 8 days during the differentiation phase (*DMSO*). Cells were harvested, total RNA was isolated, and the expression of various genes implicated in trophoblast lineage determination was assessed. Trophoblast stem cell-associated genes: Cdx2, cyclin D3, cyclin E1, Eomes, Id1, SOCS3; trophoblast differentiation-associated genes: Hand1, SSP, and Gcm1. G3PDH was used as a control for assessing RNA loading and integrity. Day 18 rat placental junctional and labyrinth zone RNAs were used as controls for the SSP and Gcm1 northern blots, respectively. (B): Reverse transcription polymerase chain reaction (RT-PCR) analysis of Mash2 expression. Total RNA was isolated from each of the representative cell cultures after 8 days and analyzed by RT-PCR using primers based on the Mash2 cDNA sequence. Lane A, Rcho-1 trophoblast cells grown under proliferating conditions (*Proliferative*); Lane B, differentiating Rcho-1 trophoblast cells (*Differentiated*); Lane C, Rcho-1 trophoblast cells treated with DMSO (1.5%) for 8 days during the differentiation phase (*DMSO*). (C) Effects of acute and chronic DMSO treatment of trophoblast cells on the expression of Eomes and Hand1. Northern blot analysis was performed on: Lane A, Rcho-1 trophoblast cells grown under proliferating conditions (*Proliferative*), Lane B, differentiating Rcho-1 trophoblast cells (following 8 days of differentiation; *Differentiated*), and Lane C, differentiated Rcho-1 trophoblast cells were treated for 48 hr (days 7–8 of differentiation, *Acute DMSO*); Lane D, Rcho-1 trophoblast cells treated with DMSO (1.5%) for 8 days during the differentiation phase (*Chronic DMSO*). Cells were harvested, total RNA isolated, and the expression of Eomes and Hand1 was assessed. Blots were probed with cDNAs to Eomes, and Hand1. G3PDH was used as a control for assessing RNA loading and integrity. All experimental results presented in this figure were replicated at least three times.

expression of Eomes and Hand1 were further examined following acute and chronic exposure of differentiated trophoblast cells (days 7–8 of differentiation). DMSO effectively stimulated Eomes expression and inhibited Hand1 expression (Fig. 4C). In summary, DMSO treatment effectively reactivated a gene associated with trophoblast stem cells and inhibited the expression of a key regulator of trophoblast giant cell development.

Discussion

Trophoblast cells are the first cell lineage to arise from the early embryo. Trophoblast stem cells have been isolated from the mouse blastocyst and extraembryonic ectoderm and have the potential to differentiate into all of the trophoblast lineages of the mature placenta (Tanaka et al., 1998). These include: trophoblast giant cells, spongiotrophoblast cells, glycogen cells, invasive extraplacental trophoblast cells, and syncytial trophoblast cells. A population of cells resembling trophoblast stem cells has been isolated from rat blastocysts (Buehr et al., 2003). However, the developmental potential of these putative rat trophoblast stem cells has not been determined. Rcho-1 trophoblast stem cells were derived from a transplantable rat choriocarcinoma and exhibit many characteristics of trophoblast stem cells (Faria and Soares, 1991; Cross et al., 1995; Peters et al., 2000; Takahashi et al., 2003). Collectively mouse trophoblast stem cells and Rcho-1 trophoblast stem cells have been effectively used to investigate mechanisms controlling trophoblast cell differentiation. In this report, we have utilized Rcho-1 trophoblast stem cells and DMSO as tools for studying trophoblast cell differentiation. DMSO-inhibited trophoblast giant cell development in a concentration-dependent manner and was associated with the reactivation of the trophoblast stem cell-associated gene, Eomes, and the inhibition of a key trophoblast giant cell regulator, Hand1. The effects of DMSO were also reversible.

DMSO has been shown to be an effective modulator of cell differentiation. It promotes the differentiation of embryonic and hematopoietic stem cells (Preisler and Giladi, 1975; Collins et al., 1978; Lever, 1979; McBurney et al., 1982; Omary et al., 1992; Angello et al., 1997) and inhibits the differentiation of trophoblast cell and adipocyte lineages (Wang and Scott, 1993; Thirkill and Douglas, 1997; present study). The actions of DMSO on human and rat trophoblast cells have remarkable parallels. DMSO inhibits the fusion of human cytotrophoblast cells into syncytial trophoblast cells and their endocrine differentiation, including inhibition of chorionic gonadotropin production (Thirkill and Douglas, 1997). DMSO also disrupted the formation and endocrine differentiation of rat trophoblast giant cells (present study). The effects of DMSO in both

human and rat trophoblast cells were concentration dependent, reversible, and restricted to differentiation (Thirkill and Douglas, 1997; present study).

Insights into the actions of DMSO were obtained by characterizing the profiles of gene expression associated with trophoblast stem and differentiated cell lineages. DMSO impacted the expression of two key genes involved in the regulation of trophoblast cells, *Eomes*, and *Hand1*, but not other known genes implicated in the control of trophoblast development (*Cdx2*, *cyclin E1*, *Id1*, *SOCS3*, *Gcm1*, and *Mash2*). DMSO treatment resulted in the elevation of *Eomes* expression and the inhibition of *Hand1* expression. These observations provide information about the hierarchy of regulatory factors controlling trophoblast development and suggest that *Eomes* and *Hand1* are central to the differentiation state of Rcho-1 trophoblast cells. Increased *Eomes* mRNA was associated with an inhibition of trophoblast cell differentiation but not an enhancement of trophoblast cell proliferation. Consequently, DMSO treatment did not return the trophoblast cells to a true stem cell state. The DMSO-treated cells were growth arrested but poised to differentiate once DMSO was removed from the cultures. Other genes whose expression is elevated in the trophoblast stem cell state may be critical for activating proliferation (e.g. *Cdx2*, *cyclin E1*, *Id1*, *SOCS3*, or *Mash2*), whereas *Eomes* may play an essential role in maintaining the undifferentiated state.

Eomes is a member of the T-box family of transcription factors, and is first expressed in trophoblast during early mouse development (Russ et al., 2000). *Eomes* null mutant embryos do not develop past the blastocyst stage exhibiting a lack of differentiation of normal trophoblast cell derivatives (Russ et al., 2000; Strumpf et al., 2005). Although T-box DNA binding motifs have been identified in the promoter regions of several genes (Papaioannou, 2001), target genes for *Eomes* action in trophoblast cells have not been identified.

Hand1 encodes a member of the basic helix-loop-helix (bHLH) transcription factor family, is expressed in the trophoblast cell lineage, and is pivotal to the differentiation of trophoblast giant cells (Cross et al., 1995; Firulli et al., 1998, 2003; Riley et al., 1998). The *Hand1* null mutation results in prenatal lethality because of a placental defect associated with failure in the development of trophoblast giant cells (Firulli et al., 1998; Riley et al., 1998). Overexpression of *Hand1* in Rcho-1 trophoblast cells also promotes trophoblast giant cell differentiation (Cross et al., 1995). Diminished *Hand1* expression associated with DMSO exposure may have been the cause or the result of decreased trophoblast giant cell formation. The regulation of *Hand1* expression in trophoblast cells and its downstream targets are not well understood. Furthermore a potential hierarchical relationship between *Eomes* and *Hand1* is yet to be resolved.

In conclusion, DMSO represents a useful tool in manipulating the differentiation state of trophoblast cells. DMSO selectively impacts trophoblast giant cell differentiation by affecting the expression of at least two key regulatory genes, *Eomes* and *Hand1*.

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References

- Ain, R., Canham, L.N. and Soares, M.J. (2003) Gestational stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. *Dev Biol* 260:176–190.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410.
- Angello, J.C., Stern, H.M. and Hauschka, S.D. (1997) P19 embryonal carcinoma cells: a model system for studying neural tube induction of skeletal myogenesis. *Dev Biol* 192:93–98.
- Anson-Cartwright, L., Dawson, K., Holmyard, D., Fisher, S.J., Lazzarini, R.A. and Cross, J.C. (2000) The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. *Nat Genet* 25:311–314.
- Arman, E., Haffner-Krausz, R., Chen, Y., Heath, J.K. and Lonai, P. (1998) Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. *Proc Natl Acad Sci USA* 95:5082–5087.
- Auman, H.J., Nottoli, T., Lakiza, O., Winger, Q., Donaldson, S. and Williams, T. (2002) Transcription factor AP-2 γ is essential in the extra-embryonic lineages for early postimplantation development. *Development* 129:2733–2747.
- Beck, F., Erler, T., Russell, A. and James, R. (1995) Expression of *Cdx-2* in the mouse embryo and placenta: possible role in the patterning of the extra-embryonic membranes. *Dev Dyn* 204:219–227.
- Buehr, M., Nichols, J., Stenhouse, F., Mountford, P., Greenhalgh, C.J., Kantachuvesiri, S., Brooker, G., Mullins, J. and Smith, A.G. (2003) Rapid loss of Oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. *Biol Reprod* 68: 222–229.
- Campbell, W.J., Deb, S., Kwok, S.C.M., Joslin, J. and Soares, M.J. (1989) Differential expression of placental lactogen-II and prolactin-like protein-A in the rat chorioallantoic placenta. *Endocrinology* 125:1565–1574.
- Chawengsaksophak, K., de Graaff, W., Rossant, J., Deschamps, J. and Beck, F. (2004) *Cdx2* is essential for axial elongation in mouse development. *Proc Natl Acad Sci USA* 101:7641–7645.
- Chawengsaksophak, K., James, R., Hammond, V.E., Köntgen, F. and Beck, F. (1997) Homeosis and intestinal tumours in *Cdx2* mutant mice. *Nature* 386:84–87.
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Cohick, C.B., Xu, L. and Soares, M.J. (1997) Placental prolactin-like protein-B: heterologous expression and characterization of placental and decidual species. *J Endocrinol* 152:291–302.
- Collins, S.J., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C. (1978) Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc Natl Acad Sci USA* 75:2458–2462.

- Cross, J.C., Flannery, M.L., Blanas, M.A., Steingrimsson, E., Jenkins, N.A., Copeland, N.G., Rutter, W.J. and Werb, Z. (1995) Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development. *Development* 121:2513–2523.
- Dai, G., Liu, B., Levan, G., Szpirer, C., Kwok, S.C.M. and Soares, M.J. (1996) Prolactin-like protein-C variant: complementary DNA, unique six exon gene structure, and trophoblast cell-specific expression. *Endocrinology* 137:5009–5019.
- Dai, G., Lu, L., Tang, S., Pea, I.M.J. and Soares, M.J. (2002) Prolactin family miniarray: a tool for evaluating uteroplacental-trophoblast endocrine cell phenotypes. *Reproduction* 124:755–765.
- Dai, G., Wang, D., Liu, B., Kasik, J.W., Müller, H., White, R.A., Hummel, G.S. and Soares, M.J. (2000) Three novel paralogs of the rodent prolactin gene family. *J Endocrinol* 166:63–75.
- Deb, S., Faria, T.N., Roby, K.F., Larsen, D., Kwok, S.C.M., Talamantes, F. and Soares, M.J. (1991a) Identification and characterization of a new member of the placental prolactin family: placental lactogen-I variant. *J Biol Chem* 266:1605–1610.
- Deb, S., Hamlin, G.P., Roby, K.F., Kwok, S.C.M. and Soares, M.J. (1993) Heterologous expression and characterization of prolactin-like protein A. *J Biol Chem* 268:3298–3305.
- Deb, S., Hashizume, K., Boone, K., Southard, J.N., Talamantes, F., Rawitch, A. and Soares, M.J. (1989) Antipeptide antibodies reveal structural and functional characteristics of rat placental lactogen-II. *Mol Cell Endocrinol* 63:45–56.
- Deb, S., Roby, K.F., Faria, T.N., Szpirer, C., Levan, G., Kwok, S.C.M. and Soares, M.J. (1991b) Molecular cloning and characterization of prolactin-like protein-C complementary deoxyribonucleic acid. *J Biol Chem* 266:23027–23032.
- Duckworth, M.L., Schroedter, I.C. and Friesen, H.G. (1990) Cellular localization of rat placental lactogen II and rat prolactin-like proteins A and B by *in situ* hybridization. *Placenta* 11:143–155.
- Faria, T.N., Deb, S.C.M., Talamantes, F. and Soares, M.J. (1990) Ontogeny of placental lactogen-I and placental lactogen-II expression in the developing rat placenta. *Dev Biol* 141:279–291.
- Faria, T.N. and Soares, M.J. (1991) Trophoblast cell differentiation: establishment, characterization, and modulation of a rat trophoblast cell line expressing members of the placental prolactin family. *Endocrinology* 129:2895–2906.
- Feldman, B., Poueymirou, W., Papaioannou, V.E., DeChiara, T.M. and Goldfarb, M. (1995) Requirement of FGF-4 for post-implantation mouse development. *Science* 267:246–249.
- Firulli, A.B., McFadden, D.G., Lin, Q., Srivastava, D. and Olson, E.N. (1998) Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat Genet* 18:266–270.
- Firulli, B.A., Howard, M.J., McDavid, J.R., McIlreavey, L., Dionne, K.M., Centonze, V.E., Cserjesi, P., Virshup, D.M. and Firulli, A.B. (2003) PKA, PKC, and the protein phosphatase 2A influence HAND factor function: a mechanism for tissue-specific transcriptional regulation. *Mol Cell* 12:1225–1237.
- Freeman, M.E., Kanyicska, B., Lerant, A. and Nagy, G. (2000) Prolactin: structure, function, and regulation of secretion. *Physiol Rev* 80:1523–1631.
- Gardner, R.L. and Beddington, R.S. (1988) Multi-lineage 'stem' cells in the mammalian embryo. *J Cell Sci* 10(suppl.): 11–27.
- Geng, Y.u.Q., Sicinska, E., Das, M., Schneider, J.E., Bhattacharya, S., Rideout, W.M., Bronson, R.T., Gardner, H. and Schinski, P. (2003) Cyclin E ablation in the mouse. *Cell* 114:31–443.
- Georgiades, P., Ferguson-Smith, A.C. and Burton, G.J. (2002) Comparative developmental anatomy of the murine and human definitive placentae. *Placenta* 23:3–19.
- Guillemot, F., Nagy, A., Auerbach, A., Rossant, J. and Joyner, A.L. (1994) Essential role of Mash-2 in extraembryonic development. *Nature* 371:333–336.
- Hamlin, G.P., Lu, X.-J., Roby, K.F. and Soares, M.J. (1994) Recapitulation of the pathway for trophoblast giant cell differentiation *in vitro*: stage-specific expression of members of the prolactin gene family. *Endocrinology* 134:2391–2396.
- Hamlin, G.P. and Soares, M.J. (1995) Regulation of deoxyribonucleic acid synthesis in proliferating and differentiating trophoblast cells: involvement of transferrin, transforming growth factor- β , and tyrosine kinases. *Endocrinology* 136:322–331.
- Iwatsuki, K., Oda, M., Sun, W., Tanaka, S., Ogawa, T. and Shiota, K. (1998) Molecular cloning and characterization of a new member of the rat placental prolactin (PRL) family, PRL-like protein H. *Endocrinology* 139:4976–4983.
- Iwatsuki, K., Shinozaki, M., Hattori, N., Hirasawa, K., Itagaki, S., Shiota, K. and Ogawa, T. (1996) Molecular cloning and characterization of a new member of the rat placental prolactin (PRL) family, PRL-like protein D (PLP-D). *Endocrinology* 137:3849–3855.
- Iwatsuki, K., Shinozaki, M., Sun, W., Yagi, S., Tanaka, S. and Shiota, K. (2000) A novel secretory protein produced by rat spongiotrophoblast. *Biol Reprod* 62:1352–1359.
- Kamei, T., Hamlin, G.P., Chapman, B.M., Burkhardt, A.L., Bolen, J.B. and Soares, M.J. (1997) Signaling pathways controlling trophoblast cell differentiation: src-family protein tyrosine kinases. *Biol Reprod* 57:1302–1311.
- Kamei, T., Jones, S.R., Chapman, B.M., McGonigle, K.L., Dai, G. and Soares, M.J. (2002) The phosphatidylinositol 3-kinase/Akt signaling pathway modulates the endocrine differentiation of trophoblast cells. *Mol Endocrinol* 16:1469–1481.
- Keppel, G. (1973) Design and analysis. Prentice-Hall, Englewood Cliffs, NJ.
- Kim, J., Jones, B.W., Zock, C., Chen, Z., Wang, H., Goodman, C.S. and Anderson, D.J. (1998) Isolation and characterization of mammalian homologs of the *Drosophila* gene *glial cells missing*. *Proc Natl Acad Sci USA* 95:12364–12369.
- Kraut, N., Snider, L., Chen, C.-M.A., Tapscott, S.J. and Groudine, M. (1998) Requirement of the mouse *I-mfa* gene for placental development and skeletal patterning. *EMBO J* 17:6276–6288.
- Lever, J.E. (1979) Inducers of mammalian cell differentiation stimulate dome formation in a differentiated kidney epithelial cell line (MDCK). *Proc Natl Acad Sci USA* 76:1323–1327.
- Luo, J., Sladek, R., Bader, J.A., Matthyssen, A., Rossant, J. and Giguere, V. (1997) Placental abnormalities in mouse embryos lacking the orphan nuclear receptor *ERR β* . *Nature* 388:778–782.
- McBurney, M.W., Jones-Villeneuve, E.M., Edwards, M.K. and Anderson, P.J. (1982) Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* 299:165–167.
- Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Kiewe-Nebenius, D., Chambers, I., Scholer, H. and Smith, A. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor *Oct4*. *Cell* 95:379–391.
- Omary, M.B., de Grandpre, L., McCaffrey, M. and Kagnoff, M.F. (1992) Biochemical and morphological differentiation of the human colonic epithelial cell line SW620 in the presence of dimethylsulfoxide. *J Cell Biochem* 48:316–323.
- Orr-Urtreger, A., Bedford, M.T., Burakova, T., Arman, E., Zimmer, Y., Yayon, A., Givol, D. and Lonai, P. (1993) Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). *Dev Biol* 158:475–486.
- Papaioannou, V.E. (2001) T-box genes in development: from hydra to humans. *Int Rev Cytol* 207:1–70.
- Parisi, T., Beck, A.R., Rougier, N., McNeil, T., Lucian, L., Werb, Z. and Amati, B. (2003) Cyclins E1 and E2 are required for endoreduplication in placental trophoblast giant cells. *EMBO J* 22:4794–4803.
- Peters, T.J., Chapman, B.M. and Soares, M.J. (2000) Trophoblast differentiation: an *in vitro* model for trophoblast giant cell development. In: Tuan, R.S. and Lo, C.W. (eds.) *Methods in*

- molecular biology, Vol. 137. Developmental biology protocols. Vol. III, Humana Press Inc., Totowa, NJ, pp. 301–311.
- Preisler, H.D. and Giladi, M. (1975) Differentiation of erythroleukemic cells *in vitro*: irreversible induction by dimethyl sulfoxide (DMSO). *J Cell Physiol* 85:537–546.
- Riley, P., Anson-Cartwright, L. and Cross, J.C. (1998) The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat Genet* 18:271–275.
- Roberts, A.W., Robb, L., Rakar, S., Hartley, L., Cluse, L., Nicola, N.A., Metcalf, D., Hilton, D.J. and Alexander, W.S. (2001) Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3. *Proc Natl Acad Sci USA* 98: 9324–9329.
- Roby, K.F., Deb, S., Gibori, G., Szpirer, C., Levan, G., Kwok, S.C.M. and Soares, M.J. (1993) Decidual prolactin related protein: identification, molecular cloning and characterization. *J Biol Chem* 268:3136–3142.
- Rossant, J. and Cross, J.C. (2002) Extraembryonic lineages. In: Rossant, J. and Tam, P.P.L. (eds.) *Mouse development*. Academic Press, New York, pp. 155–180.
- Russ, A.P., Wattlers, S., Colledge, W.H., Aparicio, S.A.J.R., Carlton, M.B.L., Pearce, J.J., Barton, S.C., Surani, M.A., Ryan, K., Nehis, M.C., Wilson, V. and Evans, M.J. (2000) Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature* 404:95–99.
- Sahgal, N., Knipp, G.T., Liu, B., Chapman, B.M., Dai, G. and Soares, M.J. (2000) Identification of two new nonclassical members of the rat prolactin family. *J Mol Endocrinol* 24:95–108.
- Schreiber, J., Riethmacher-Sonnenberg, E., Riethmacher, D., Tuerk, E.E., Enderich, J., Bosl, M.R. and Wegner, M. (2000) Placental failure in mice lacking the mammalian homolog of glial cells missing, GCMA. *Mol Cell Biol* 20:2466–2474.
- Scott, I.C., Anson-Cartwright, L., Riley, P., Reda, D. and Cross, J.C. (2000) The Hand1 basic helix–loop–helix transcription factor regulates trophoblast differentiation via multiple mechanisms. *Mol Cell Biol* 20:530–541.
- Skerjanc, I.S. (1999) Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. *Trends Cardiovasc Med* 9:139–143.
- Soares, M.J. (2004) The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal–fetal interface. *Reprod Biol Endocrinol* 2:51.
- Soares, M.J., Chapman, B.M., Rasmussen, C.A., Dai, G., Kamei, T. and Orwig, K.E. (1996) Differentiation of trophoblast endocrine cells. *Placenta* 17:277–289.
- Soares, M.J. and Linzer, D.I.H. (2001) Rodent prolactin family and pregnancy. In: Horseman, N.D. (ed.) *Prolactin*. Kluwer Academic, Norwell, pp. 139–167.
- Strumpf, D., Mao, C.-A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F. and Rossant, J. (2005) Cdx2 is required for correct cell fate specification and differentiation of trophoctoderm in the mouse blastocyst. *Development* 132: 2093–2102.
- Takahashi, Y., Carpino, N., Cross, J.C., Torres, M., Parganas, E. and Ihle, J.N. (2003) Socs3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. *EMBO J* 22: 372–384.
- Tanaka, M., Gertsenstein, M., Rossant, J. and Nagy, A. (1997) Mash2 acts cell autonomously in mouse spongiotrophoblast development. *Dev Biol* 190:55–65.
- Tanaka, S., Kunath, T., Hadjantonakis, A.-K., Nagy, A. and Rossant, J. (1998) Promotion of trophoblast stem cell proliferation by FGF4. *Science* 282:2072–2075.
- Teshima, S., Shimosato, Y., Koide, T., Kuroki, M., Kiruchi, M. and Aizawa, M. (1983) Transplantable choriocarcinoma of rats induced by fetectomy and its biological activities. *Gann* 74: 205–212.
- Thirkill, T.L. and Douglas, G.C. (1997) Differentiation of human trophoblast cells *in vitro* is inhibited by dimethyl sulfoxide. *J Cell Biochem* 15:460–468.
- Tremblay, G.B., Kunath, T., Bergeron, D., Lapointe, L., Champigny, C., Bader, J.-A., Rossant, J. and Giguere, V. (2001) Dihydrostilbesterol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERR β . *Genes Dev* 15: 833–838.
- Wang, H. and Scott, R.E. (1993) Inhibition of distinct steps in the adipocyte differentiation pathway in 3T3T mesenchymal stem cells by dimethyl sulphoxide (DMSO). *Cell Prolif* 26: 55–66.
- Wiemers, D.O., Ain, R., Ohboshi, S. and Soares, M.J. (2003a) Migratory trophoblast cells express a newly identified member of the prolactin gene family. *J Endocrinol* 179:335–346.
- Wiemers, D.O., Shao, L.-J., Ain, R., Dai, G. and Soares, M.J. (2003b) The mouse prolactin gene family locus. *Endocrinology* 144:313–325.
- Werling, U. and Schorle, H. (2002) Transcription factor gene AP-2 γ essential for early murine development. *Mol Cell Biol* 22:3149–3156.
- Yu, Z.W. and Quinn, P.J. (1994) Dimethyl sulphoxide: a review of its applications in cell biology. *Biosci Rep* 14:259–281.