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ORIGINAL ARTICLE

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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

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Introduction

Trophoblast cells are the earliest cell lineage to differentiate during mammalian development, arising from trophectoderm 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 extraplacental 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-Ureteger et al., 1993; Feldman et al., 1995; Arman et al., 1998; Nichols et al., 1998; Tanaka et al., 1998); and activator protein-2γ (AP-2γ; 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 endoreduplication (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). [^{32}P]-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

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