Trophoblast giant-cell differentiation involves changes in cytoskeleton and cell motility

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Trophoblast giant-cell differentiation is well-characterized at the molecular level, yet very little is known about how molecular changes affect the cellular functions of trophoblast in embryo implantation. We have found, using both explanted E7.5 mouse embryo ectoplacental cone and the rat choriocarcinoma (Rcho-1) cell line, that trophoblast differentiation is distinguished by dramatic changes in cytoarchitecture and cell behavior. Undifferentiated trophoblast cells contain little organized actin and few small, peripheral focal complexes and exhibit high membrane protrusive activity, while differentiated trophoblast giant cells contain prominent stress fibers, large internal as well as peripheral focal adhesions, and become immotile. The dramatic changes in cell behavior and cytoskeletal organization of giant cells correlate with changes in the activities of the Rho family of small GTPases and a decrease in tyrosine phosphorylation of focal adhesion kinase. Together, these data provide detailed insight into the cellular properties of trophoblast giant cells and suggest that giant-cell differentiation is characterized by a transition from a motile to a specialized epithelial phenotype. Furthermore, our data support a phagocytic erosion, rather than a migratory infiltration, mechanism for trophoblast giant-cell invasion of the uterine stroma.

Key Words: trophoblast; implantation; invasion; cytoskeleton; focal adhesion; mouse embryo.

INTRODUCTION

In this paper we characterize changes in the cytoskeleton, cell motility, and other cell behaviors involved in the differentiation of the trophoblast giant cells of the mouse embryo. These cells are the first to differentiate during mouse development and mediate the implantation of the embryo into the uterus at the blastocyst stage of development (4.5 days of gestation). Trophoblast giant cells, which arise from the epithelial trophectoderm cells of the blastocyst, transform at the time of implantation into invasive cells that displace and phagocytose the uterine epithelial cells, penetrate the uterine stroma, and make vascular connections with the maternal blood supply. This is a striking example of a naturally occurring invasive and vasculogenic process, yet almost nothing is known about the cellular mechanisms involved.

The trophectoderm (TE) layer of the blastocyst can be divided into two regions, mural and polar, on the basis of position of the trophectoderm cells relative to the inner cell mass, or ICM (Fig. 1). Each group of TE cells has a different fate in the postimplantation embryo but both will produce trophoblast giant cells. Mural TE initiate implantation at E4.5 and subsequently differentiate to form the primary trophoblast giant cells (Bevilacqua and Abrahamsohn, 1988; Copp, 1978; Dickson, 1963; Dickson and Araujo, 1966), which form an anastomosing network of blood sinuses at the periphery of the embryo (Alden, 1947; Bevilacqua and Abrahamsohn, 1988; Enders and Schlafke, 1967; Potts, 1968; Tachi et al., 1970). These blood sinuses are lined on the side facing Reichert’s membrane by a layer of trophoblast giant cells that are highly flattened and fenestrated, facilitating diffusion of nutrients and oxygen to the embryonic region to allow growth of the embryo prior to formation of the definitive chorioallantoic placenta.

In contrast, the polar TE is maintained in a proliferative state by the action of FGF-4 produced by the underlying ICM and thus differentiates later than the mural TE (Chai et al., 1998; Copp, 1979; Gardner et al., 1973). Prior to implantation the polar TE cells feed asymmetrically into, and expand, the mural population (Copp, 1979; Gardner, 2000), and after implantation they give rise to two extraembryonic structures, the extraembryonic ectoderm and the ectoplacental cone.
The EPC gives rise to secondary trophoblast giant cells and later to the spongiotrophoblast of the chorioallantoic placenta (Cross, 2000; Rossant, 1986). Trophoblast giant-cell differentiation is fairly well understood at the morphological and molecular level (reviewed in Cross, 2000). Giant cells were first identified in rats and mice by their size and high DNA content (Barlow and Sherman, 1972; Hoffman and Wooding, 1993), and subsequent studies showed that giant-cell differentiation begins with exit from the cell cycle and DNA endo-reduplication (Barlow and Sherman, 1972; Snow and Ansell, 1974; Varmuza et al., 1988), followed by expression of hormones and growth factors such as the placental lactogens, proliferins, and proliferin-related proteins (Colosi et al., 1988; Deb et al., 1991; Faria et al., 1991; Hall and Talamantes, 1984; Hamlin et al., 1994; Lee et al., 1988; Lin et al., 1997a,b; Linzer et al., 1985; Linzer and Nathans, 1985; Ogren and Talamantes, 1988; Southard and Talamantes, 1991; Toft and Linzer, 1999; Wilder and Linzer, 1986; Yamaguchi et al., 1992, 1994), required for maintenance of the pregnancy and for the proper formation and vascularization of the chorioallantoic placenta. Trophoblast giant cells also mediate interactions with the decidual cells (Bevilacqua and Abrahamsohn, 1989; Bevilacqua et al., 1985), secrete metalloproteinases (Rinkenberger et al., 1997), and intercalate into maternal capillaries to redirect maternal blood flow into the trabecular spaces that they have formed (Bevilacqua and Abrahamsohn, 1988; Welsh and Enders, 1987).

Despite the depth of understanding of the molecular aspects of giant-cell differentiation, however, the temporal and causal relationships between trophoblast giant-cell differentiation and morphogenesis are only poorly defined and the related cellular changes are uncharacterized (Gard-
ner and Davies, 1993). Changes in protrusive activity occur in the transition from trophectoderm to invasive trophoblast (Gonzales et al., 1996a,b; Sutherland et al., 1988), and changes in adhesive behavior (Cross et al., 1995) and in cadherin and connexin expression (Grummer et al., 1996; Reuss et al., 1996; Winterhager et al., 2000) are seen in the transition from proliferative trophoblast to trophoblast giant cell, yet nothing is known about how these changes contribute to the functional behavior of the cells nor to the morphogenesis of the trophoblast blood spaces. In order to understand trophoblast giant cell differentiation and the process of implantation in the broadest sense, it is important to know what cellular changes are occurring.

We have examined the changes in cell behavior and cytoskeletal organization in differentiating trophoblast giant cells in two systems: the rat choriocarcinoma (Rcho-1) cell line and freshly explanted E7.5 mouse embryo EPC and Reichert’s membrane-associated trophoblast giant cells. We show that there is a dramatic decrease in cell motility and protrusive activity upon giant-cell differentiation, which is accompanied by reorganization of the actin cytoskeleton, changes in focal adhesion formation and distribution, and an increase in organization and stability of cell–cell interactions. The changes in cytoskeletal organization correlate with changes in the activity of members of the Rho family of GTPases and a decrease in the phosphorylation of focal adhesion kinase (FAK). The phenotypic changes that occur with trophoblast giant-cell differentiation appear incompatible with the interpretation that they are a migratory invasive cell type and point instead to phagocytosis as the primary mechanism of trophoblast invasion in the mouse.

**MATERIALS AND METHODS**

**Antibodies**

The vinculin (Hvin-1) and talin (8d4) antibodies were from Sigma Chemicals Inc.; the β-catenin antibody was from Zymed Labora-
Cultures and Immunofluorescence of Rcho-1 Cells and Ectoplacental Cone Explants

Rat choriocarcinoma (Rcho-1) cells were the gift of Dr. Michael Soares and were routinely maintained in growth medium: NCTC-135 (Sigma) supplemented with 20% fetal bovine serum (FBS; Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), 2 mM l-glutamine (Gibco BRL), 50 µM β-mercaptoethanol, and 100 units/ml penicillin G, and 100 µg/ml streptomycin. The cells were passed every other day and fed on the intervening day. Differentiation was induced in cells on the second day after passage by first performing a 1-min cold trypsinization and then culturing the remaining cells (trypsin resistant) in differentiation medium, which is identical to the growth medium except that it contains 10% horse serum instead of 20% FBS. In other experiments, Rcho-1 cells were cultured in TS medium or in Eagle Plus medium as described below for EPC explants. The results obtained were identical under all culture conditions.

Ectoplacental cones were dissected from E7.5 day mouse embryos as described previously (Sutherland et al., 1993). In some cases explants were cultured for 24 h under conditions described for trophoblast stem (TS) cells; in 70% mouse embryo fibroblast (MEF) -conditioned RPMI-1640 medium supplemented with 1 ng/ml FGF-2 and 1 U/ml heparin (Tanaka et al., 1998). The conditioned medium was prepared as previously described (Tanaka et al., 1998). MEF cells were isolated from E13.5 mouse embryos using standard techniques (Hogan et al., 1993) and used without freezing between passages 2 and 6. For conditioned medium, the cells were treated with mitomycin C, washed and trypsinized, and then replated at confluent density and incubated for 72 h in RPMI-1640 supplemented with 20% FBS, sodium pyruvate, β-mercaptoethanol, glutamine, and pen-strep. The medium was then removed, filtered, and frozen at −20°C until use. In other experiments, EPC explants were cultured in Eagle Plus medium with 10% FBS (Stephens et al., 1995), which resulted in giant-cell differentiation by 5 days of culture. The behavior and cytoskeleton of freshly isolated EPC cells after 12–15 h in culture was identical in either TS medium or Eagle Plus medium. To examine trophoblast giant cells that had differentiated in vivo, Reichert’s membrane was dissected away from E7.5 embryos and trypsinized at 37°C for 5 min, then the isolated cells were spun down and plated in Eagle Plus medium and cultured for 1–5 days. The resulting cell populations were mixed, containing smaller, rounded parietal endoderm cells and large flattened trophoblast giant cells, which were easily identified on the basis of morphology and expression of integrin α7 (AES, unpublished observations).

Immunofluorescence staining was performed on Rcho-1, EPC, and trophoblast giant cells fixed in 3.7% formaldehyde and permeabilized in 0.2% Triton X-100. Cells were blocked for 30 min with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) incubated with primary antibody for 1 h at room temperature, washed in PBS, incubated with secondary antibody, then washed again in PBS, and mounted. Images were obtained using a Zeiss fluorescence microscope and Openlab 2.0 software on a Macintosh G3 computer.

Time-Lapse Microscopy of Rcho-1 Cells, EPC Explants, and Isolated Trophoblast Giant Cells

Cells in 35-mm dishes were maintained at 37°C under 5% CO₂ throughout the duration of the time-lapse session using a PDM-12 stage incubator (Harvard Instruments) on an Olympus IX-70 microscope outfitted with Hoffman optics. Digital images were collected every 20 s over a period of 3 h using a Hamamatsu Orca camera, and image processing and analysis were performed using Openlab 2.0 software on a Macintosh G3 computer.

Immunoprecipitations and Western Blots for FAK

Whole-cell lysates were prepared by scraping cultured Rcho-1 cells into lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, in TBS, pH 7.5, with 10 mM EDTA, pH 8.0, 0.7 µg/ml pepstatin, 4 mM peflaboc, 5 µg/ml leupeptin, 2 µg/ml aprotonin, and 7 mM sodium ortho-venadate; all protease inhibitors were from Boehringer Mannheim). Cell debris was removed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was passed through a 26-gauge needle to shear the DNA. Cooamassie Reagent (Pierce Chemicals Inc.) was used to determine the protein concentration of the cell and tissue lysates. All lysates were prepared in the identical manner. For Western blots, equal amounts of protein (25 µg) from lysates of each stage were run on an SDS-PAGE gel and transferred to PVDF membrane and FAK was detected by probing the blot with the 2A7 antibody.

For immunoprecipitation and phosphorylation studies, equal amounts (500 µg) of total protein from lysates of each stage were incubated for 1 h with the 2A7 anti-FAK mAb and Gamma-Blut Plus Sepharose beads (Pharmacia). The beads were washed five times with 1:10 diluted lysis buffer, eluted by boiling in Laemmli sample buffer, run on a SDS-PAGE gel, and transferred to PVDF membrane. The proportion of FAK that was tyrosine phosphorylated was examined first, by probing the blot with the PY20 anti-phosphotyrosine antibody, then the total amount of FAK immunoprecipitated was determined by stripping the blot and reprobing with the 2A7 anti-FAK antibody.

Rho, rac, and cdc42 Activity Assays

These assays were modified from previously published protocols for Rho (Ren et al., 1999) and Rac/Cdc42 (Bagrodia et al., 1995), which use GST-fusion proteins of either Rho- (GST-RBD) or Rac/Cdc42-binding sites (GST-PBD) from their respective downstream effectors (rhotekin for Rho and PAK for Rac/Cdc42). These fusion proteins are bound to glutathione sepharose beads and used to precipitate active GTPases from cell lysates. The precipitated proteins are then resolved on 15% SDS-PAGE gels and detected by Western blot. The constructs for GST-RBD and GST-PBD were kind gifts of Bill Arthur and Dr. Keith Burridge (University of North Carolina-Chapel Hill). Both constructs were used to transform BL21 E. coli and expression was induced with IPTG. The bacteria were then lysed by sonication in the following lysis buffers: for
GST-RBD, the lysis buffer contained 50 mM Tris, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% Triton X-100, and 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin as protease inhibitors; for GST-PBD, the lysis buffer was 20 mM Hapes, pH 7.5, 120 mM NaCl, 10% glycerol, 2 mM EDTA, 0.5% NP-40, and protease inhibitors as above. After sonication in lysis buffer, the crude lysate was centrifuged at 12,000 rpm for 10 min at 4°C to remove debris. The supernatant was then incubated with glutathione-sepharose beads (Pharmacia) for 1 h at 4°C, then washed with their respective lysis buffers first, then without, detergent. The protein concentration of the beads was measured by comparison to BSA standards on an SDS–PAGE gel. The beads were used within 1 week of preparation.

For precipitating the activated GTPases, cells were lysed by scraping in 50 mM Tris, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM MgCl₂, and protease inhibitors as above. Cell debris was removed by centrifugation at 14,000 rpm for 10 min at 4°C. Protein concentration was measured using the Coomassie Reagent (Pierce Chemicals, Inc.) and 500 μg total protein was incubated with 30 μg of either GST-RBD or GST-PBD beads for 30 min with rotation at 4°C. The beads were then washed 5 times with 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM MgCl₂, and protease inhibitors as above. Beads were eluted by boiling in Laemmli sample buffer and loaded on 15% SDS-PAGE gels, transferred to PVDF, and subjected to Western blot with antibodies to the appropriate GTPase. These assays were repeated 3 times for Rac and Cdc42, and twice for Rho. For each assay, gels containing 25 μg per lane of total protein from whole-cell lysates of cells of each stage were also blotted with these antibodies for comparison.

RESULTS

Trophoblast Cells Organize Their Actin Cytoskeleton as They Differentiate into Giant Cells

Rcho-1 stem cells are a morphologically heterogeneous population consisting of either round cells, with circularly distributed peripheral actin, or slightly spread cells with no actin stress fibers but with patches of actin at the periphery consistent with the formation of lamellar protrusions (Fig. 2). We found by time-lapse analysis (see below) that the morphological heterogeneity is due to cell-cycle asynchrony, with the round cells being peri-mitotic. When induced to differentiate over a 5-day period, Rcho-1 cells not only grow in size and become more flat and spread, but also dramatically rearrange their actin cytoskeleton into thick bundles of stress fibers (Fig. 2).

To quantitate the change in stress fiber formation, 150–200 cells from each stage were examined and those containing stress fibers were counted and measured as a percentage of the total (Fig. 2, numbers beside images). The onset of stress fiber formation was very fast, as almost three-fourths (69%) of the cells examined on day 1 of differentiation contained prominent stress fibers, as compared to only 5% of those in the stem-cell population. We further examined the time course of stress fiber formation during the first 24 h of differentiation and found that the increase in the number of cells exhibiting stress fibers began between 9 and 12 h of differentiation, implying that this is an early, but not an immediate aspect of differentiation. The number of cells containing stress fibers continued to increase with differentiation, reaching 88% by day 3 and 98% by day 5.

Rcho-1 cells have been found in several studies to be an appropriate model for the molecular aspects of giant-cell differentiation (Cross et al., 1995; Dai et al., 1996; Faria and Soares, 1991; Hamlin et al., 1994; MacAuley et al., 1998; Nakayama et al., 1998; Ng et al., 1994; Shida et al., 1993). To examine how closely their cellular behavior resembles EPC and trophoblast giant cells, we made explants of EPC from E7.5 embryos and looked at the cytoskeletal changes during their differentiation in vitro, as well as the differences between EPC cells and trophoblast giant cells isolated directly from Reichert’s membrane (RM-TGC).

The changes in actin organization seen in differentiating Rcho-1 cells also occurred in cells from EPC explants differentiating in vitro (Fig. 3). Cells at the periphery of freshly explanted (≤24 h) E7.5 EPC cultured either in TS medium or in Eagle’s Plus medium (see Materials and Methods) contained no stress fibers, but did exhibit centrally located small round dots of actin (Fig. 3A). These central round dots of actin were a unique and consistent feature of the peripheral cells of these EPC cultures and were never seen in Rcho-1 cells. When EPC cells were differentiated by culturing them in vitro for 3–5 days, they organized their actin into stress fibers, similar to the differentiated Rcho-1 cells (Fig. 3A). In addition, freshly explanted RM-TGC (which had differentiated in vivo prior to explantation) also contained abundant, well-organized stress fibers (Fig. 3B). Organization of the actin cytoskeleton into stress fibers is therefore a specific, early characteristic of trophoblast giant-cell differentiation.

Trophoblast Giant Cells Contain Organized Cell-Matrix and Cell–Cell Contacts

To examine whether there are concomitant changes in focal adhesions, the localization of the focal adhesion protein vinculin was compared with actin organization in stem and differentiated Rcho-1 cells, and in EPC explants and RM-TGC (Fig. 4). Changes in focal adhesion size, number, and distribution correlated directly with changes in actin stress fiber formation during differentiation. Rcho-1 stem cells and peripheral EPC cells had few, if any, focal adhesions, and these were all at the periphery of the cell where they colocalized with areas of actin concentration in lamellipodia (Fig. 4). Interestingly, the small round dots of actin seen in peripheral EPC cells colocalized with circular vinculin structures, such that the dot of actin was at the center of the doughnut-shaped vinculin structure (Fig. 4). These resemble podosomes, cytoskeletal structures identified in macrophages, osteoclasts, and lymphocytes (Marchisio et al., 1988; Nitsch et al., 1989; Tarone et al., 1985; Zambonin-Zallone et al., 1988).

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large central, as well as peripheral, focal adhesions were noted in those cells (Fig. 4), while changes in focal adhesions were not seen in those cells that did not contain stress fibers. By day 3 of differentiation, almost 90% of the cells had made stress fibers, and all of these had numerous, large, central, and peripheral focal adhesions (Fig. 4). Similarly, after 5 days in culture, differentiated EPC cells had numerous organized stress fibers and large, internal, and peripheral focal adhesions (Fig. 4), as did freshly explanted RM-TGCs (data not shown). In differentiated EPC explants, punctate dots of actin were noted at the ends of many stress fibers, where they colocalized with vinculin in stress fibers. It is possible that these are similar to the podosome-like structures seen in the stem EPC cells.

Other than vinculin, both the peripheral focal complexes in the Rcho-1 stem cells and the peripheral and central focal adhesions in the differentiated Rcho-1 cells contained VASP, paxillin, and FAK (Fig. 5). Tensin was not detected in focal adhesions at any stage (data not shown). Talin was not detectable in the peripheral focal complexes of the Rcho-1 stem cells, but was clearly localized to all focal adhesions in the Rcho-1 differentiated cells (Fig. 5A). FAK was not highly concentrated in the focal adhesions of the differentiating Rcho-1 cells, as compared to talin (Figs. 5A and 5B). These observations show heterogeneity in the molecular composition of focal adhesions between stem and differentiating cells. Since only limited co-localization studies were possible, there may actually be more heterogeneity between individual focal adhesions at each stage.

In addition to organization of cell–matrix contact sites, the giant cells also developed elaborate cell–cell junctions, as determined by staining for β-catenin (Fig. 6). The stem trophoblast cells in both cultured Rcho-1 and the EPC explants contained only patches of peripheral β-catenin staining. However, the differentiated cells in both cell populations showed an elaborate pattern of staining for both proteins; the membranes of these giant cells interdigitate as they contact one another, resulting in a complex, web-like junctional pattern (Fig. 6).

It has been previously shown that changes in stress fiber and focal adhesion formation in cultured cell lines can depend on the serum content of the medium in which they are cultured (Ridley, 1994; Ridley and Hall, 1992b; Ridley et al., 1992). We thus examined whether the changes described above are simply a response to the changes in the culture medium. When Rcho-1 stem cells are cultured to confluence in growth media, which contains 20% FBS, a small proportion spontaneously undergoes giant-cell differentiation (Faria and Soares, 1991). When the stem cells are removed from the confluent culture by light trypsinization and the differentiated cells remaining on the plate are maintained in growth medium, no reversion to the stem-cell phenotype is seen (Fig. 7). Furthermore, cells differentiated for 2 days in differentiation medium do not revert to a stem-cell phenotype when switched back to growth medium (Fig. 7). Therefore, the changes in cell morphology and adhesive contacts that we describe are not a transient environmental effect.
Trophoblast Cells Show Differentiation-Dependent Changes in Protrusive Activity and Motility

The changes observed in the cytoskeletal and focal adhesion organization of the giant cells implied concomitant changes in cell-motile behavior. To characterize these changes, cell behavior of Rcho-1 stem cells and differentiated cells and EPC and TGC explants were recorded by time-lapse digital microscopy. Figure 8 shows panels taken from a time-lapse movie of Rcho-1 stem cells and EPC explants. The Rcho-1 stem cells and peripheral EPC cells display high protrusive activity and are highly motile, but whereas Rcho-1 cells display almost no persistence, peripheral EPC cells appear more polarized, with a stable leading edge and high persistence. Upon contact with one another, Rcho-1 stem cells and peripheral EPC cells do not exhibit contact-inhibition, contact-induced retraction, or contact-induced paralysis (Abercrombie and Heaysman, 1953, 1954; Trinkaus, 1984), but rather continue to actively expand their area of intercellular contact. Despite this contact expansion behavior, the newly formed areas of cell–cell interaction are highly dynamic, and the cells in contact slide relative to each other, making and breaking cell–cell contacts frequently (Fig. 8). The EPC cells were frequently observed to crawl underneath one another (Fig. 8). As noted above, the localization of β-catenin in these cells is patchy (Fig. 6A), consistent with the idea of limited stability in the contacts between adjacent cells. In contrast to the very motile behavior of the peripheral EPC cells, the central cells of the explant remained as a cohesive cell cluster. They showed outward movement as a cell sheet during in vitro differentiation, but showed limited rearrangement within the cell sheet (Fig. 8).

With differentiation, Rcho-1 and EPC-derived giant cells undergo dramatic changes in cell behavior. Figure 9 shows panels from time-lapse movies of day-3 differentiated Rcho-1 cells and freshly isolated RM-TGC. Unlike either Rcho-1 stem cells or peripheral EPC cells, the giant cells of both types show little or no protrusive activity and are completely immotile. Furthermore, they exhibit very stable cell–cell contacts and no exchange of neighbors. These changes in protrusive activity and cell contact stability can be seen to start as early as 1 day postdifferentiation for the Rcho-1 cells and characterize the majority of the Rcho-1 cells by 3 days postdifferentiation. This differentiation-specific change in motility and protrusive activity characterized giant cells that differentiate in vitro from explanted EPC as well, indicating that the behavior changes are characteristic and do not depend on the environment in which differentiation takes place.

We also examined the effect of substrate on the behavior of Rcho-1, EPC, and RM-TGC cells by plating each on a layer of Matrigel and making time-lapse observations. We obtained identical results to those on plastic substrates: the Rcho-1 stem cells and the EPC cells were very motile, and EPC cells could penetrate the matrix, while the differentiated Rcho-1 and RM-TGC were not motile and showed

FIG. 4. Changes in cell–matrix adhesion sites with giant-cell differentiation. Rcho-1 stem and differentiated cells and peripheral EPC and EPC-derived giant cells (TGC) were stained for vinculin, as a focal contact marker, and phalloidin, showing filamentous actin. Rcho-1 stem cells and peripheral EPC cells contain only a few small, peripheral focal complexes (small arrows), while the differentiated cells have numerous large, peripheral as well as internal focal adhesions. Peripheral EPC cells also contain central patches of doughnut-shaped vinculin staining, which form a ring around the internal dots of actin localization (arrowheads). On the other hand, EPC-derived giant cells (TGC) contain bona fide focal adhesions, very similar to differentiated Rcho-1 cells. Bar for Rcho-1 = 10 μm. Bar for EPC = 5 μm.
FIG. 5. Composition of cell–matrix adhesion sites in stem and differentiating Rcho-1 cells. (A) Rcho-1 stem and differentiated cells were stained for VASP, paxillin, or talin. Both the small, peripheral focal complexes in the stem cells and the large, central focal adhesions in the differentiated cells contain VASP and paxillin. In contrast, talin was not detected in the focal complexes of Rcho-1 stem cells, but is localized to the focal adhesions of the differentiated cells. (B) Rcho-1 stem and differentiated cells were double-stained for FAK and actin (stem cells) or FAK and talin (d3 differentiated cells). FAK localizes to both the small focal complexes of Rcho-1 stem cells and to the large focal adhesions of differentiated cells. Bar = 5 μm.

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little if any protrusive activity. Thus the changes in trophoblast cell behavior are not substrate-dependent.

These results show that Rcho-1 stem cells and peripheral EPC cells are highly motile cells that form many protrusions and only small, peripheral focal complexes. As they differentiate, they grow in size and flatten considerably, while decreasing their protrusive activity and organizing large focal adhesions. These changes are reminiscent of the effects of regulatory molecules such as focal adhesion kinase and the Rho family of small GTPases, on cell behavior and the cytoskeleton. We therefore looked at these proteins next.

**Differentiation-Induced Changes in Phosphorylation of Focal Adhesion Kinase in Trophoblast Cells**

Focal adhesion kinase, or FAK, is a protein tyrosine kinase that is activated by tyrosine phosphorylation induced by integrin-mediated cell adhesion to extracellular matrix and by signaling through growth factor receptors (Burridge et al., 1992; Chen et al., 1998; Knight et al., 1995; Lipfert et al., 1992; Schlaepfer et al., 1994). FAK associates with both integrins and growth factor receptors and has been shown to play a role in integrin- and growth factor-
mediated cell migration (Cary et al., 1996; Owen et al., 1999; Schaller et al., 1992; Sieg et al., 2000) and in focal adhesion turnover (Hildebrand et al., 1993; Ilic et al., 1997, 1995; Otey, 1996; Schaller et al., 1992).

We compared the expression and level of tyrosine phosphorylation of FAK between Rcho-1 stem and differentiated cells (Fig. 10). The results show that while the overall expression of FAK did not change with differentiation, there was a consistent, differentiation-dependent decrease in the proportion of immunoprecipitated FAK that was tyrosine phosphorylated, suggesting a decrease in FAK activity with differentiation. A decrease in FAK activity would be consistent with the immotile phenotype observed in differentiated Rcho-1 cells, since FAK-null cells display an identical inability to migrate (Ilic et al., 1995).

Changes in the Activity of Rho Family Members during Trophoblast Giant-Cell Differentiation

Members of the Rho family of small GTPases are involved in signal transduction downstream of growth factor and cell adhesion receptors, and their activity causes cy-
toskeletal rearrangements; specifically, Rho induces formation of stress fibers and focal adhesions; Rac causes membrane ruffling and formation of lamellipodia, and Cdc42 induces formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995; Ridley and Hall, 1992a,b; Ridley et al., 1992). To examine whether changes in GTPase activity correlate with the changes in cytoskeletal and focal adhesion organization, we used GTPase activity assays, which take advantage of the high binding affinity of certain downstream effectors for the activated forms of their respective GTPases (Bagrodia et al., 1995; Ren et al., 1999). Rcho-1 stem cells have relatively high Rac and Cdc42 and low Rho activities when compared with differentiated cells (Fig. 11). This is consistent with the protrusive activity, lack of stress fibers, and sparse peripheral focal complexes seen in the stem cells. At 1 day postdifferentiation, there is a dramatic decrease in Rac and Cdc42 activity and a substantial increase in Rho activity (Fig. 11), consistent with the decrease in membrane protrusive activity and increased organization of stress fibers and focal adhesions in the differentiated cells. At days 3 and 5 of differentiation, the Rac and Cdc42 activity remained low, while Rho activity decreased to about the level found in the stem cells. These results were consistently obtained each time the assay was repeated. Interestingly, there appears to be an increase in the absolute level of all three GTPases in whole-cell lysates (Fig. 11). It is not clear why Rac and Cdc42 expression would increase, since there is no concomitant increase in their activity. It is possible that this might reflect an increase in their general extractability in cell lysates.

**DISCUSSION**

Previous studies of Rcho-1 cells have shown that trophoblast giant-cell differentiation is characterized by dramatic changes in cell size, morphology, adhesive, and invasive behavior (Cross et al., 1995; Faria et al., 1990; Faria and Soares, 1991; Kamei et al., 1997; Peters et al., 1999; Reuss et al., 1996), implying that the cytoskeleton and adhesive structures of these cells are undergoing reorganization during the differentiation process. We demonstrate here that there are specific changes in cytoskeletal organization, in focal adhesion formation and localization, and in cell behavior that occur during trophoblast giant-cell differentiation. These changes in cell behavior and cytoarchitecture are integral to the trophoblast giant-cell differentiation program, characterize both primary cultures of mouse trophoblast cells and the Rcho-1 cell line, and correlate with changes in the activity of at least three members of the Rho family of small GTPases. Characterization of the changes in cytoarchitecture and cell behavior that occur during trophoblast giant-cell differentiation gives us the tools with which to further explore the temporal and causal correlates of differentiation and morphogenesis during implantation.

**Correlation between Position and Differentiation in the EPC**

Comparison of the cellular characteristics of EPC explants and Rcho-1 cells allows us to refine our notion of the correlation between cell position and relative degree of differentiation in the EPC. The central cells have been identified as proliferative stem cells and the peripheral cells as trophoblast giant cells by the criteria of expression of specific transcription factors (Cross, 2000; Guillemot et al., 1994; Scott et al., 2000). This has led to the model of proliferating central cells that differentiate into secondary
trophoblast giant cells as they are displaced peripherally (Cross, 2000; Hoffman and Wooding, 1993). Rcho-1 cells exhibit the same pattern of transcription factor expression during giant-cell differentiation (Cross, 2000; Cross et al., 1995; Dal et al., 1996; Hamlin et al., 1994; Nakayama et al., 1998; Scott et al., 2000). However, we find that the peripheral EPC cells are similar in behavior and cytoskeletal organization to Rcho-1 stem cells; they are highly motile with poorly organized actin, few, and always peripheral, matrix adhesion structures, and dynamic cell–cell interactions. By the criteria of cellular characteristics, they are not differentiated trophoblast giant cells. In contrast, the central cells of the EPC are more cohesive, have stable cell–cell interactions, and display little motility (Albieri and Bevilacqua, 1996; Albieri et al., 1999). These cells are not similar to Rcho-1 stem cells, but are more closely modeled by the trophoblast stem cell line (Tanaka et al., 1998), which has similar motile behavior and is more epithelial in character than the Rcho-1 cell line (A. E. Sutherland, unpublished observations). These results suggest that the correlation between molecular and cellular differentiation varies between in vivo differentiated trophoblast and the Rcho-1 cell line. This may be due to effects of uterine factors or decidual cell interactions on EPC cells, or perhaps is a result of the immortalization of the Rcho-1 line.

**Trophoblast Giant-Cell Differentiation Begins with Changes in Cohesion and Motility**

These results are consistent with the notion that there are (at least) three stages in trophoblast giant-cell differentiation (Cross, 2000) and demonstrate the cellular characteristics of each (Fig. 12). Our data suggest that trophoblast giant-cell differentiation begins with a type of epithelial–mesenchymal transition, characterized by stabilization of cell–cell interactions, changes in cytoskeletal architecture, and an increase in protrusive activity, followed by a re-epithelialization characterized by stabilized cell–cell and cell–matrix interactions and little or no motility. Thus the stem cells are proliferative, cohesive, and nonmotile; the intermediate cells are loosely associated and highly motile, and the differentiated giant cells are postmitotic, cohesive, and nonmotile.

The initial transition can be seen in the phenotypes of central and peripheral EPC cells, as well as by changes in the mural trophectoderm as it differentiates to primary trophoblast giant cells (Bevilacqua and Abrahamsohn, 1988, 1989; Reuss et al., 1996). Interestingly, mSna, which is expressed in the EPC and inhibits the onset of endoreduplication (Nakayama et al., 1998; Smith et al., 1992), has recently been shown to regulate epithelial–mesenchymal transitions through transcriptional repression of the E-cadherin promoter (Batlle et al., 2000; Cano et al., 2000). Previous studies have shown progressive switching of cadherin isotype expression in the EPC followed by loss of cadherin expression from peripheral cells (Nose and Takeichi, 1986; Reuss et al., 1996).

**Trophoblast Giant Cells Undergo Re-Epithelialization to Form Vascular Structures**

The cells forming the trophoblast blood spaces of the yolk sac placenta at E7.5 are differentiated trophoblast giant cells (Bevilacqua and Abrahamsohn, 1988, 1989; Welsh and Enders, 1987). We find that these cells are phenotypically similar to differentiated Rcho-1 cells and to EPC cells that have differentiated in vitro, in that they exhibit no protrusive activity and have a highly organized cytoskeleton, well-developed matrix adhesion structures, and stable cell–cell interactions when cultured in vitro. Trophoblast giant cells in situ have also been shown to have well-developed cell–cell junctional structures and organized actin, as well as specialized adhesive structures and adherens-type junctions with decidual cells (Bevilacqua and Abrahamsohn, 1988, 1989; Bevilacqua et al., 1985). The in vitro characteristics that we describe for these cells are thus reflective of their in vivo cell architecture and are likely required for the function of the trophoblast giant cells as vascular cells forming the blood sinuses of the yolk sac placenta. Stable cell–cell contacts allow the giant cells to form the walls of the vessels and to make contacts with decidual cells; organized actin allows them to maintain their cell shape and to resist maternal blood pressure, and organized cell–matrix adhesions allow them to form anchoring contacts with the decidual matrix. We conclude from these observations that the last phase of trophoblast giant-cell differentiation involves a specialized type of re-epithelialization that includes stabilization of cell–cell contacts, reorganization of the actin cytoskeleton, and a decrease in protrusive activity.

**Changes in Cell Behavior during Trophoblast Giant-Cell Differentiation: Role of Focal Adhesion Proteins**

We find that the differentiation-related changes in actin organization, focal adhesion formation, and cell behavior described above correlate exactly with the decreasing level
of FAK tyrosine phosphorylation (Fig. 10). FAK activity is required for cell migration, as cells from FAK-null embryos demonstrate decreased motility (Illic et al., 1995). Interestingly, FAK-null cells also contain an increased number of, and particularly larger, focal adhesions (Illic et al., 1995), leading to the concept that FAK activity is required for focal adhesion turnover which takes place during cell migration (Illic et al., 1997). Based on the decrease in the proportional amount of tyrosine-phosphorylated FAK in differentiated giant cells and their complete immobility (Figs. 8 and 9), we hypothesize that the large focal adhesions in these cells have a very low rate of turnover. Future studies, using GFP-tagged focal adhesion proteins, will directly examine this question.

The adhesive structures of both stem and differentiated Rcho-1 cells contain vinculin, VASP, paxillin, and FAK. However, talin is found in focal adhesions only in giant cells and is diffusely localized in stem cells (see Fig. 5). This is interesting because talin has been associated with activation of integrins and maturation of focal adhesions (Calderwood et al., 1999; Sampath et al., 1998). Furthermore, decreased talin expression and calpain-mediated cleavage of talin have both been associated with rapid disassembly of focal adhesions (Carragher et al., 1999; Priddle et al., 1998).

The changes in cell behavior and cytoarchitecture are correlated with changes in the activity of three members of the Rho family of GTPases, RhoA, Rac1, and Cdc42. The changes in GTPase activity occur very early in differentiation, concomitant with some of the earliest changes seen in molecular differentiation (Cross, 2000; Cross et al., 1995; Rinkenberger et al., 1997). The GTPases or their upstream regulators may thus be early targets of giant-cell-specific transcriptional activity. Alternatively, it is possible that the observed changes in GTPase activity may drive some of the molecular aspects of differentiation, as Rho, Rac, and Cdc42 have all been found to signal to the nucleus and to cause changes in gene expression (Coso et al., 1995; Hill et al., 1995; Minden et al., 1995; Perona et al., 1997). Future experiments will focus on defining the relationship of Rho family activity to other aspects of giant-cell differentiation.

**Redefining the Mechanism of Trophoblast Invasion**

The changes that we observe in trophoblast cell behavior with giant-cell differentiation are at odds with the general perception that the giant cells are an invasive cell population and the attending concept that invasive cells are typically highly motile and have decreased cell–cell interactions, fewer focal adhesions, and a less organized cytoskeleton (Keely et al., 1998; Mareel et al., 1990; Strauli and Haemmerli, 1984). The perception of trophoblast giant cells as invasive is based on many observations: the evidence of invasive behavior both in vitro and in vivo (Bevilacqua and Abrahamsohn, 1991, 1994; Bevilacqua et al., 1999; Carr, 1979; Kirby, 1960, 1963a,b; Macpherson et al., 1989); the evidence of membrane activity in trophoblast giant cells in the early phases of implantation (Bevilacqua and Abrahamsohn, 1988, 1989); and the specific expression of the matrix metalloproteinase MMP-9 by giant cells which is required for matrix degradation during implantation (Alexander et al., 1996; Behrendt et al., 1992; Das et al., 1997; Harvey et al., 1995; Peters et al., 1999; Sharkey et al., 1996). How can the well-developed cell–cell interactions, increased cell–matrix adhesion, highly organized actin cytoskeleton, and lack of motility that characterizes trophoblast giant cells be reconciled with these facts? The answer lies in redefining our notion of the mechanism of trophoblast invasion. Invasion of trophoblast cells into ectopic sites invariably results not in diffuse infiltration of the trophoblast cells into the tissue, but rather erosion and displacement of the endogenous cells, formation of vascular channels, and intercalation into adjacent capillaries (Bevilacqua and Abrahamsohn, 1991, 1994; Bevilacqua et al., 1991; Carr, 1979; Kirby, 1960, 1963a,b; Macpherson et al., 1989). Similarly, when trophoblast cells are cultured on monolayers of cultured cells, they induce retraction of the monolayer cells and displace them on the substrate, and then locally degrade the underlying extracellular matrix (Glass et al., 1979, 1983). The degree of invasive behavior in rodent trophoblast cells during implantation has been an issue of some dispute, as many of the events attributed to trophoblast invasion have been found to depend instead on decidual cell activity and endothelial cell migration (Welsh and Enders, 1987, 1991a,b). In fact, the behavior of trophoblast giant cells in situ appears to be characterized primarily by intense phagocytic activity leading to erosion and displacement of the uterine epithelial and decidual cells (Bevilacqua and Abrahamsohn, 1988, 1989). There is no evidence that trophoblast giant cells exhibit the classical behavioral characteristics of invasive, metastatic cells in vivo.

Based on these observations, we envision the invasion of trophoblast cells as a process of directed, progressive phagocytosis of stromal cells and matrix that would create space to accommodate the growth of the embryo and concomi-
tant expansion of the yolk sac placenta. The expression of MMP-9 associated temporally with this phagocytic activity (Alexander et al., 1996; Behrendtson et al., 1992; Das et al., 1997; Harvey et al., 1995; Peters et al., 1999; Sharkey et al., 1996) would, in our view, function to clear the matrix surrounding the decidual cells as they are phagocytosed. We further hypothesize, based on our results, that the peripheral EPC cells are an intermediate population, postproliferative but not yet giant cells, whose function is to migrate distally to undergo morphogenesis and add to the yolk sac placenta, where they will differentiate into giant cells.

Human Cytotrophoblast Show a Similar Pattern of Phenotypic Changes with Differentiation

In the human, the epithelial cytotrophoblast cells of the villus undergo an epithelial–mesenchymal transition to form the columns of the anchoring villi, then become invasive at the tips of the anchoring villi (Damsky et al., 1994). These invasive cells differentiate to become pseudo-endothelial cells which intercalate into the spiral arteries of the uterus, forming large-bore vessels that direct maternal blood to the periphery of the implanting embryo (Zhou et al., 1997). Thus, despite the considerable differences in the structure of the placenta and degree of invasion of the trophoblast, there are fundamental similarities in the cellular aspects of trophoblast differentiation between these two species. We would speculate that this program of sequential epithelial–mesenchymal transition, invasion, and mesenchymal–endothelial transition may be a common mechanism for trophoblast differentiation in other species as well.

In summary, we have shown that there are specific changes in cell behavior, cytoskeletal organization, and adhesion structures that define the differentiation of trophoblast cells into trophoblast giant cells. Understanding how these cells differentiate at the cellular level will allow us to interpret the effects of molecular changes and to uncover the functional characteristics of the giant cells in embryo implantation.

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FIG. 12. Summary model for cellular differentiation of trophoblast giant cells. This figure shows a detail of one-half of the cylindrically symmetrical EPC and yolk sac placenta from an E7.5 embryo. The portion illustrated is from the area boxed in on the small figure of the whole embryo at the upper left. Below, the three major states of trophoblast differentiation and the cells that exemplify these states are illustrated and their major cellular characteristics are listed. The central cells of the EPC are proliferative and cohesive, with strong cell–cell contacts. The peripheral EPC cells have undergone an epithelial–mesenchymal transition and exhibit high motility and dynamic cell–cell contacts. The final stage of differentiation is in the yolk sac placenta, where the trophoblast cells have undergone a re-epithelialization to form the walls of the blood spaces. These trophoblast giant cells (green) are epithelial and exhibit no motility, but are very phagocytic. They engulf the adjacent uterine decidual cells (purple) and their matrix, thus eroding the stromal layer surrounding the embryo and making room for growth of the embryo and concomitant enlargement of the yolk sac placenta.
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