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Id-2 regulates critical aspects of human cytotrophoblast differentiation, invasion and migration

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SUMMARY

During early human placental development, the conceptus attaches itself to the uterus through cytotrophoblast invasion. Invasive cytotrophoblast cells differentiate from precursor villous cytotrophoblasts, but the essential regulating factors in this process are unknown. Basic helix-loop-helix (bHLH) transcription factor dimers are essential regulators of mouse trophoblast development. We therefore examined the importance of this family of factors in the human placenta. In many cell lineages, bHLH factors are sequestered by members of the Id family, HLH proteins that lack the basic DNA binding domain (Inhibitor of DNA binding proteins (Id-1 to Id-4)). During differentiation of some tissues, Id expression declines, allowing bHLH factors to dimerize, bind DNA and *trans*-activate lineage-specific genes. To begin to study the role of bHLH transcription factors in human placental development, we first characterized Id expression in cytotrophoblast cells. The cells expressed Id-3 constitutively; Id-2 was downregulated, at the mRNA and protein levels, as the cells differentiated in culture and in situ, respectively. In cases when cytotrophoblast differentiation was compromised (in placentas from women with preeclampsia, or in cells grown under hypoxic conditions in culture), Id-2 expression was maintained. To assess the functional relevance of these correlations, we used an adenovirus vector to maintain Id-2 protein expression in cultured cytotrophoblasts. Compared to control (*lacZ*-expressing) cells, cytotrophoblasts transduced to constitutively express Id-2 retained characteristics of undifferentiated cells: α1 integrin expression was low and cyclin B expression was retained. Furthermore, invasion through Matrigel was partially inhibited and migration was strikingly enhanced in Id-2-expressing cells. These results suggest that Id-2 and the bHLH factors that it partners play important roles in human cytotrophoblast development.

Key words: Id, Cytotrophoblast, Placenta, Differentiation, Integrin, Cyclin, Invasion, Migration, Human

INTRODUCTION

Development of Eutherian embryos depends on the placenta (reviewed in Cross et al., 1994; Rinkenberger et al., 1997). Formation of this transient but vital organ is the first full-fledged test of the embryo's differentiative capacity – a point that is graphically illustrated by the number of genetically engineered mice with phenotypes that are first evident as placental abnormalities. In humans, development of the placenta also presents an interesting opportunity to study seemingly unique processes. For example, uterine attachment requires the organ's specialized epithelial cells, termed cytotrophoblasts, to aggressively invade the uterine lining (Fisher et al., 1989; Librach et al., 1991). Other than the fact that these cells stop proliferating and invasion is limited to a circumscribed area (the decidualized endometrium and the inner third of the myometrium), this process is more akin to tumorigenesis than to organogenesis. The method whereby cytotrophoblasts initiate maternal blood flow to the placenta is equally unusual. During invasion, a subpopulation of cells opens the termini of uterine blood vessels. Subsequently, these fetal cells replace the resident maternal endothelium and portions of the smooth muscle wall, thereby creating a hybrid vasculature that is particularly evident on the arterial side of the circulation (Zhou et al., 1997b).

Viewed from a developmental perspective, these unusual attributes are the end result of cytotrophoblast differentiation along the invasive pathway. Key morphological aspects of this process are diagrammed in Fig. 2. The cytotrophoblast stem cells form a polarized epithelium that is attached to the basement membrane that surrounds the stromal cores of chorionic villi. During differentiation/invasion, cytotrophoblasts leave this basement membrane to form columns of unpolarized cells that attach to, then penetrate, the

uterine wall. The ends of the columns terminate within the superficial endometrium, where they give rise to invasive cytotrophoblasts. During interstitial invasion, a subset of these cells, either individually or in small clusters, commingles with resident decidual, myometrial and immune cells. During endovascular invasion, masses of cytotrophoblasts migrate into the vessels (likened to dripping candle wax; Ramsey et al., 1976) before the lumina eventually recanalize. Together, the two components of cytotrophoblast invasion anchor the placenta to the uterus and permit a steady increase in the supply of maternal blood that is delivered to the developing fetus.

Some of the key molecular aspects of human cytotrophoblast differentiation and invasion are also known. In this regard the cells' expression of several classes of downstream regulators is precisely modulated as they invade either in situ (the uterine wall) or in vitro (extracellular matrix, ECM). For example, proliferating cytotrophoblast stem cells express the $\alpha_6\beta_4$ integrin cell-ECM adhesion molecule (Damsky et al., 1992, 1994). As the cells stop dividing and differentiate/invade the uterine wall, expression of this molecule is decreased coincidentally with increases in $\alpha_1\beta_1$ integrin, which promotes cytotrophoblast invasion. Successful cytotrophoblast invasion also depends on the regulated expression and activation of the matrix metalloproteinase MMP-9 (Librach et al., 1991). The expression of other molecules appears to be more closely related to the cells' ability to function as a pseudo-endothelium (Zhou et al., 1997b). Finally, invading cytotrophoblasts upregulate the expression of both surface and secreted proteins that likely enable them to escape maternal immune rejection (Ellis et al., 1990; Kovats et al., 1990; Roth et al., 1996). The regulated expression of these molecules provides insights into how cytotrophoblasts achieve their remarkable functions, as well as stage-specific antigens that precisely mark important transitions in their differentiation/invasion.

Understanding the differentiation of any cell type ultimately requires knowledge of the transcriptional regulatory mechanisms that are involved. In this regard, very little is known about the mechanisms that normally regulate human placental development in general, and cytotrophoblast differentiation/invasion specifically. This information is vital to understanding how these cells fulfill their many unique functions. In addition, the findings could help explain the etiology of preeclampsia, a life-threatening pregnancy complication that is associated with abnormal cytotrophoblast differentiation, evidenced by the cells' inability to upregulate several stage-specific antigens that are normally expressed coincidentally with uterine invasion (Zhou et al., 1993).

In devising an approach for gaining entry into the transcriptional networks that regulate human cytotrophoblast differentiation/invasion, we took into consideration the fact that helix-loop-helix (HLH) family members are critical to the differentiation of many cell types, including myocytes (reviewed by Ludolph and Konieczny, 1995; Molkentin and Olson, 1996), neurons (reviewed by Kageyama et al., 1995), hematopoietic cells (reviewed by Orkin, 1995) and, notably, mouse placental trophoblasts (Cross et al., 1995; Guillemot et al., 1994; Nakayama et al., 1997; Riley et al., 1998). HLH transcription factors are classified on the basis of their expression patterns and structure/function. For example, numerous basic HLHs (bHLH) are tissue specific. In contrast, their dimerization partners – E-factors and Ids (Inhibitors of DNA binding) – are more widely expressed. During differentiation, transcription is activated when the HLH domains of tissue-specific proteins dimerize with the homologous regions of E-factors, an interaction that allows the basic regions of both molecules to bind DNA cooperatively. Transcription and, consequently, differentiation are inhibited when one of the four Ids, which lack the basic domains that mediate interactions with DNA (reviewed by Murre et al., 1994), binds E-factors to form inactive heterodimers (Benezra et al., 1990; Sun et al., 1991). In addition, Id and E-factor expression alone can regulate differentiation (hematopoiesis; Orkin, 1996). Here we exploited the ability of Id family members to function as dominant negative inhibitors to test the hypothesis that bHLH family members play a critical role in human placental development.

MATERIALS AND METHODS

cDNAs and antibodies

The Id-1, Id-3 and Id-4 constructs were the kind gift of Dr Maria-Clementia Hernandez (University of California San Francisco). Generation of a human Id-2 cDNA by PCR was described previously (Biggs et al., 1992). A polyclonal antibody raised against the entire Id-2 protein was used for immunolocalization experiments (Iavarone et al., 1994). A commercially available antibody that recognizes Id-2 was used for immunoblotting (sc-489; Santa Cruz Biotechnology, Inc., Santa Cruz, California). The anti-cytokeratin rat mAb (7D3), which reacts with all human trophoblasts, was raised in this laboratory (Damsky et al., 1994). An antibody that recognizes human cyclin B was obtained from Pharmingen (14551A; San Diego, California). Production of an anti-HLA-G mAb, as well as an HLA-G cDNA, was described previously (McMaster et al., 1995, 1998). An antibody (IA1007) that recognizes human α1 integrin (VLA-1) was purchased from Endogen (Woburn, MA).

Cell isolation and culture

Cytotrophoblasts were isolated as previously described from first and second trimester human placentas (Fisher et al., 1989; Librach et al., 1991). The cells were cultured under standard tissue culture conditions (5% CO_2/95% air) except when they were maintained in an hypoxic atmosphere. In the latter experiments, cytotrophoblasts were placed in a Bactron anaerobic incubator (Sheldon Manufacturing Inc., Cornelius, OR) where they were maintained in a 2% O_2/93% N_2/5% CO_2 environment, as described (Genbacev et al., 1996).

Northern analyses

Total RNA was extracted from purified first and second trimester cytotrophoblasts either immediately upon isolation or after 12, 24, 36 or 48 hours in culture. Total RNA was isolated according to published methods (Chomczynski and Sacchi, 1987) and blots were prepared as previously described (McMaster et al., 1995). Before transfer, gels were stained with Acridine orange to ensure integrity of the RNA samples and confirm equal loading. The probes were generated by random priming of the entire Id-1 to Id-4 cDNAs, or the 450 bp *Pvu*II fragment from the 3′ untranslated region of HLA-G, by using [^{32}P]CTP (Amersham Life Science, Inc., Piscataway, NJ) and the Klenow fragment of DNA polymerase I according to standard methods (Tabor et al., 1993). Probes had a specific activity of 2×10^9 disints/minute/μg. The final post-hybridization washes were conducted in 0.3× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.4) and 0.1% sodium dodecyl sulfate (SDS) at 65°C. Blots were stripped in 0.1× SSC with 0.5% SDS at 95°C. One set of blots was probed to assess Id-1 and Id-4 RNA expression; Id-2 and Id-3 RNA expression patterns were confirmed in three separate experiments.

Immunohistochemistry (Id-2)

First and second trimester tissue samples (n=4) were obtained by biopsying the basal plate of the placenta or the uterine site of placental attachment (e.g., placental bed biopsy). The biopsies were performed as previously described (Damsky et al., 1992; Zhou et al., 1993). Control, third trimester samples (n=3) were obtained either from women who delivered at term after uncomplicated pregnancies, or from women who delivered before term for reasons other than preeclampsia (e.g., incompetent cervix). Experimental samples (n=3) were obtained from women with preeclampsia, diagnosed as described previously (Lim et al., 1997; Zhou et al., 1993, 1997a).

Double indirect immunofluorescence analysis of frozen sections prepared from these tissue samples and biopsies was performed as follows. Nonspecific reactivity was blocked by incubating the sections in PBS containing 0.7% fish skin gelatin (G-7765; Sigma, St. Louis, MO) and 0.1% Triton X-100 (Sigma) for 30 minutes at room temperature. Then the sections were exposed (1 hour, room temperature) to antibodies against Id-2 (Iavarone et al., 1994) and cytokeratin (7D3), diluted 1:50 (v/v) in blocking buffer. After washing in PBS at 4°C, they were incubated (1 hour) in fluorescein-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch; West Grove, PA) and rhodamine-conjugated goat anti-rat IgG (Jackson ImmunoResearch) diluted 1:100 (v/v) in blocking solution. Sections were washed again in cold PBS and mounted. Antibody binding was detected by using filters that permitted visualization of either fluorescein or rhodamine with no overlap. Control experiments included incubation of tissue sections with either primary or secondary antibodies alone.

Adenovirus construction and transduction

The adenovirus constructs were made and transduction was accomplished as previously described (Janatpour et al., 1999). Control constructs (AdclacZ, AdcGFP) and AdcId-2 were used at a multiplicity of infection (m.o.i.) of 100/cytotrophoblast. The cells were preincubated (30 minutes) with virus before plating, and virus was present, at the same titer, throughout the entire culture period.

Detection of β-galactosidase activity

Cytotrophoblasts transduced with AdclacZ for 36 hours were washed once with PBS before they were fixed for 10 minutes (room temperature) in PBS containing 0.2% glutaraldehyde and 2% formaldehyde. After three additional washes in PBS, the fixed cells were incubated overnight at 37°C in 30 mM potassium ferricyanide and 1 mg/ml X-gal (Promega) to develop the color reaction. The product was visualized at a magnification of 200× by using a light microscope (Lim and Chae, 1989).

Immunocytochemistry (integrin α1)

Cytotrophoblasts that had been cultured for 66 hours in medium containing either AdclacZ or AdcId-2 were washed in PBS and fixed (10 minutes, room temperature) in 3% paraformaldehyde/PBS. Nonspecific reactivity was blocked by incubating the samples in PBS/0.2% bovine serum albumin (30 minutes, room temperature). Double indirect immunofluorescence analysis was then carried out as described above for Id-2 localization, using anti-VLA-1 (IA1007) together with anti-cytokeratin (7D3). Similar results were obtained in three separate experiments.

Immunoblotting

Cytotrophoblasts (10^6) were isolated, cultured and transduced to express cDNAs encoding either *lacZ* or Id-2 as described above. After 40 hours, the cells were washed three times with PBS before the addition of 200 μl lysis buffer (150 mM NaCl/1.0% NP-40/50 mM Tris, pH 8.0). A portion of the lysate (20 μl) was subjected to 10% SDS-PAGE under reducing conditions, then electroblotted to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Nonspecific reactivity was blocked by incubating the membranes (4 hours, room temperature) in Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 8.0) that contained 0.1% Tween-20 and 5% Carnation non-fat dry milk. Then the membranes were reacted (overnight, 4°C) with antibodies that specifically recognized either Id-2 (sc489; 50 ng/ml), cyclin B1 (14551A;1 mg/ml), or HLA-G (1:100; McMaster et al., 1998). Each antibody was diluted in blocking solution. After washing once in TBS/0.1% Tween-20, the membranes were incubated (2 hours, room temperature) in the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody; goat anti-mouse IgG was used to detect binding of all primary antibodies except anti-Id-2, which was identified by using goat anti-rabbit IgG (Jackson ImmunoResearch). Membranes were then processed for chemiluminescence according to the manufacturer's instructions (ECL; Amersham, Buckinghamshire, UK) and exposed to autoradiography film (Hyperfilm; Amersham). The NIH Image 1.60 program was used for densitometry. Before re-probing, membranes were stripped by incubation (50°C, 30 minutes) in 62.5 mM Tris HCl, pH 6.7, containing 100 mM 2-mercaptoethanol. The Id-2 experiment was repeated three times with similar results; the cyclin B experiment was repeated six times with similar results.

Immunoprecipitation

Cytotrophoblasts were isolated, cultured and transduced to express cDNAs encoding either *lacZ* or Id-2 as described above. After 66 hours, the cells were washed twice with PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, then incubated for 90 minutes on ice with the biotin reagent (1 mg/ml NHS-LC-Biotin in PBS; Pierce, Rockford, IL). The reaction was stopped by the addition of 50 mM glycine in PBS. Cells were lysed in PBS containing 2% NP-40/0.1M Tris, pH 7.5 (1 ml/4×10^6 cells). Components that adhered nonspecifically were pre-cleared by adding 100 μl Protein A Sepharose CL-4B (Pharmacia Biotech Products, Piscataway, NJ). The resulting supernatant was isolated by centrifugation (14,000 revs/minute; 10 minutes) before the addition of anti-α1 integrin at a dilution of 1:50 (v/v). The samples were mixed end-over-end at 4°C overnight, then added to 35 μl of packed Protein A Sepharose CL-4B. This mixture was incubated for an additional 1 hour. A series of sequential washing steps was then performed, isolating the beads by centrifugation after they were resuspended in 1 ml of each reagent. The order was as follows: wash buffer 1 (12.5 mM potassium phosphate/0.6 M NaCl, pH 7.4),wash buffer 2 (0.1% SDS/0.05% NP-40/0.3 M NaCl/10 mM Tris, pH 8.3), wash buffer 1, wash buffer 3 (12.5 mM potassium phosphate/0.3 M NaCl, pH 7.4), and 10 mM Tris, pH 8.3. The bound proteins were eluted into sample buffer, separated by SDS-PAGE (7.5% acrylamide), then transferred to nitrocellulose as described above. Nonspecific reactivity was blocked by incubating the membranes (3 hours, room temperature) in PBS/0.1% Tween-20/5% Carnation non-fat dry milk. Proteins in the immunoprecipitates were detected by exposing the membranes to HRP-conjugated streptavidin (Pierce) and visualized by using the ECL method described above.

Invasion assay

Cytotrophoblasts were isolated, cultured and transduced to express cDNAs encoding either green fluorescent protein (GFP) or Id-2 as described above. The cells were plated on Transwell inserts (6.5 mm; Corning, Inc., Corning, NY) containing polycarbonate filters (8 μm pores) coated with a 100 μm layer of Matrigel (Librach et al., 1991). After 60 hours, the filters were rinsed with PBS and processed as described previously (Genbacev et al., 1996). Briefly, the filter was stained with an antibody that recognizes cytokeratin (7D3) and mounted on slides with the underside facing up. Cytokeratin-positive cell processes that had penetrated the Matrigel and appeared on the underside of the filter were counted. Each variable was tested in duplicate or triplicate, and the entire experiment was repeated three times.

Migration assay

Cytotrophoblasts were isolated, transduced to express cDNAs encoding either *lacZ* or Id-2 as described above, and cultured on Transwell inserts containing polycarbonate filters (5 μm pores). The underside of the filter was coated with fibronectin (30 μg/ml; F-1141, Sigma). After 48 hours, cells remaining on the top surface were removed with a cotton swab. Cells that had migrated to the underside of the filter were stained with anti-cytokeratin as in the invasion assay. The filters were inverted onto slides and the remaining cells counted. One additional filter from each experiment was used to examine the morphology of the cells on the top surface. Each variable was tested in triplicate and the entire experiment was repeated three times.

RESULTS

Cytotrophoblasts downregulate Id-2 mRNA expression as they differentiate/invade in vitro

We examined cytotrophoblast expression of Id family members, at the RNA level, as a function of differentiation/invasion in vitro. Before culture (0 hours), the population consists of progenitors and cells that are in the initial stages of differentiation along the invasive pathway. After 36 hours in culture, first (I) and second (II) trimester cytotrophoblasts have fully differentiated, as shown by their expression of stage-specific antigens (Damsky et al., 1994; Fisher et al., 1989). Thus RNA was extracted either from freshly isolated cells or at 12-hour intervals for up to 48 hours.

Membranes with these RNA samples were hybridized with ^{32}P-labeled cDNA probes to Id-1 to Id-4. The results showed that cytotrophoblasts expressed very low levels of Id-1 and undetectable levels of Id-4 at the time points studied (data not shown). In contrast, Id-2 mRNA was abundant in the progenitor population, but rapidly declined (~ 6-fold) once the cells started to differentiate in culture (Fig. 1A). Furthermore, the maximum decrease in Id-2 mRNA expression was evident for both first trimester (data not shown) and second trimester samples after only 12 hours, i.e., before the cells had fully differentiated. Thereafter, cytotrophoblasts isolated from both first and second trimester placentas continued to express low levels of Id-2 mRNA. This is in contrast to Id-3 mRNA (Fig. 1B), which was constitutively expressed by both first and second trimester cells during the same time period. Thus, of the four known Ids, only the pattern of Id-2 mRNA expression was consistent with a potential role in cytotrophoblast differentiation.

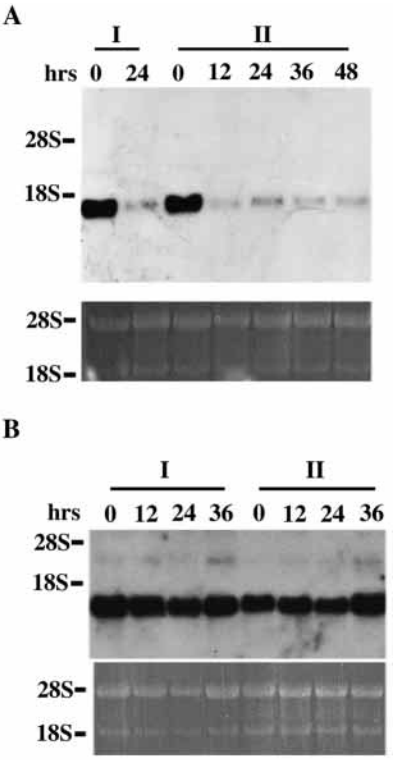


Fig. 1. Cytotrophoblasts downregulate Id-2 mRNA expression as they differentiate/invade in culture, while Id-3 mRNA is constitutively expressed. Total RNA from freshly isolated (0 hours) and cultured first (I) and second (II) trimester cytotrophoblasts was separated by formaldehyde-agarose gel electrophoresis and transferred to Nytran membranes that were hybridized with ^{32}P-labeled cDNA probes to Id-2 and Id-3. Id-2 mRNA was abundant in the progenitor population (A, 0 hours), but expression rapidly declined once the cells started to differentiate in culture (e.g., from 12 hours onward). In contrast, Id-3 was constitutively expressed during the same time period (B). Acridine orange staining of the 28S and 18S ribosomal RNAs demonstrated sample loading.

Cytotrophoblasts transiently downregulate Id-2 protein expression as they differentiate/invade in situ

To determine whether the downregulation of Id-2 mRNA expression that occurred in vitro replicated the in vivo situation, we used an Id-2-specific antibody to stain tissue sections of the maternal-fetal interface. We performed double indirect immunofluorescence to simultaneously localize cytokeratin expression, thereby identifying trophoblasts amid other fetal and maternal cells in the sections. Fig. 2 shows a diagram of the tissue histology, together with an arrow whose segments are labeled according to the regions shown in Fig. 2A-F. Fig. 2A,B contain the portion of an anchoring villus that gives rise to a cell column. Id-2 was expressed in approximately half the cytotrophoblast stem cells. Staining, which was particularly bright at sites of column initiation and in the proximal part of the column, was downregulated in the distal column region (Fig. 2A,B). Cytotrophoblasts near the uterine surface did not react with anti-Id-2, but as the cells invaded the decidua (Fig. 2C,D) and replaced the lining of maternal spiral arterioles (Fig. 2E,F), staining was detected once again. Together, these results suggested that Id-2 expression is downregulated, both in vitro and in situ, during the early phases of cytotrophoblast differentiation/invasion. However, the upregulation of Id-2 expression by cytotrophoblasts during later stages of differentiation, when they enter the uterine wall, was not observed in the culture model.

When cytotrophoblast differentiation/invasion fails in situ, Id-2 protein expression is not downregulated

Next, we investigated whether the failure of cytotrophoblast differentiation/invasion observed in preeclampsia is associated with ectopic cytotrophoblast Id-2 staining in situ. Three pairs of samples obtained from control patients and patients with preeclampsia, matched for gestational age, were analyzed. Typical results are shown in Fig. 3. Consistent with the results

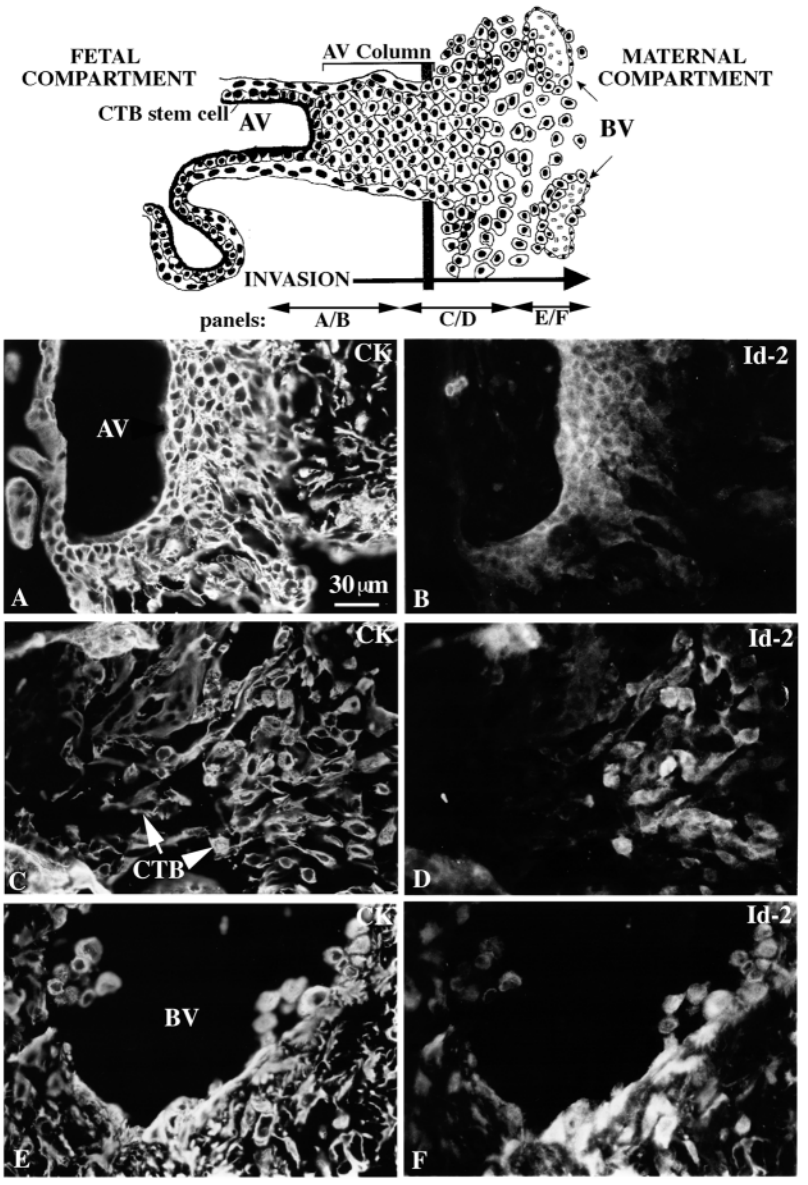


Fig. 2. Cytotrophoblasts transiently downregulate Id-2 protein expression as they differentiate/invade in situ. The diagram shows the histology of the maternal-fetal interface. (A-F) The tissue sections were prepared from biopsies at 18 weeks of gestation of the regions spanned by the coordinately lettered arrows at the bottom of the diagram. The sections were stained with an antibody that specifically reacted with cytokeratin (CK; A,C,E), to identify cytotrophoblasts, and anti-Id-2 (B,D,F). In the fetal compartment, Id-2 staining was detected in association with cytokeratin-positive cytotrophoblasts (A,B). Staining, which was brightest at sites of column initiation, decreased in the distal column region. As cytotrophoblasts invaded the uterus, Id-2 expression was downregulated to the extent that no immunoreactivity was observed. As cytotrophoblasts invaded the deeper decidua (C,D) and replaced the lining of the maternal spiral arterioles (BV; E,F), Id-2 protein expression was again detected. AV, anchoring villus; CTB, cytotrophoblast.

above, staining of tissue sections from a control sample showed that cytotrophoblasts near the uterine surface did not stain with an antibody that recognized Id-2 (Fig. 3A,B). When the pregnancy was complicated by preeclampsia, cytotrophoblasts in a comparable location stained brightly for Id-2 (Fig. 3C,D). Analysis of cytotrophoblasts throughout the entire region, from the site of column initiation to the invasion front, showed that cytotrophoblasts in all areas stained brightly for Id-2 (data not shown). These results suggested that abnormal cytotrophoblast differentiation/invasion in vivo is associated with a failure to downregulate Id-2 expression.

Inhibition of differentiation/invasion in vitro results in a partial failure to downregulate Id-2 mRNA

Under standard culture conditions (20% oxygen), isolated cytotrophoblasts exit the cell cycle and replicate the differentiation pathway that leads to uterine invasion in vivo. In hypoxia (2% oxygen), cytotrophoblasts upregulate expression of hypoxia-inducible factor (HIF) 2α protein (but not HIF 1α), continue to proliferate, fail to express $\alpha_1\beta_1$ integrin and do not invade (Genbacev et al., 1996, 1997, and unpublished observations). Thus, we tested the effects of hypoxia on Id-2 expression (Fig. 4). After cytotrophoblasts were maintained for 12 hours under standard conditions (Fig. 4, lane S), only low levels of Id-2 mRNA expression were detected. In comparison, cytotrophoblasts that were cultured for 12 hours in a 2% oxygen atmosphere expressed approximately 2-fold higher levels of Id-2 mRNA (Fig. 4, lane H). This was in contrast to HLA-G mRNA, the abundance of which is not regulated by oxygen tension (Genbacev et al., 1996, 1997). Together, the results of the foregoing experiments suggested that in cytotrophoblasts, Id-2 levels correlate with the state of differentiation.

Adenovirus (AdcId2) transduction maintains Id-2 expression in cultured cytotrophoblasts

As a first step toward testing the functional importance of these correlative observations, we investigated several methods for altering the pattern of cytotrophoblast Id-2 expression observed in vitro. As has often been reported for primary cells, conventional methods for transfection resulted in very low (<1%) efficiencies. This technical problem was circumvented by using adenovirus transduction. Control cultures were transduced, at the time of plating on Matrigel, with an adenovirus carrying the *lacZ* gene under control of the CMV promoter. As shown by assaying for β-galactosidase activity at 36 hours (Fig. 5A), transduction efficiency was routinely at least 90%.

In subsequent experiments, cytotrophoblasts were transduced with AdclacZ or the same adenovirus vector carrying an Id-2 cDNA (AdcId-2) rather than a *lacZ* cDNA. After 36 hours, Id-2 mRNA levels were assessed by northern hybridization (Fig. 5B). As expected, cells infected with the control AdclacZ virus expressed very low levels of endogenous Id-2 mRNA. Cells transduced with AdcId-2 contained similar levels of endogenous Id-2 message (1.3 kb). In addition, the

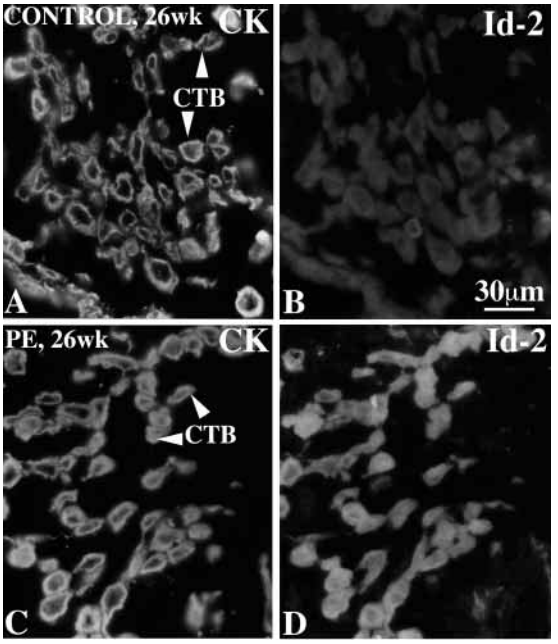


Fig. 3. When cytotrophoblast differentiation/invasion fails in situ, Id-2 protein expression is not downregulated. The Id-2 staining patterns of samples from a control patient and a patient diagnosed with preeclampsia (PE), both at 26 weeks of gestation, were analyzed. The tissue sections were stained with anti-cytokeratin (CK; A,C) and anti-Id-2 (B,D). In the control sample, cytotrophoblasts near the uterine surface did not react with the Id-2 antibody (A,B). In contrast, when pregnancy was complicated by preeclampsia, cytotrophoblasts in a comparable location stained brightly for Id-2 (C,D).

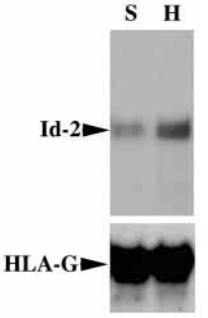


Fig. 4. Inhibition of differentiation/invasion partially rescues cytotrophoblast downregulation of Id-2 mRNA expression in vitro. Second trimester cytotrophoblasts were maintained under either standard (S; 20% O_2) or hypoxic (H; 2% O_2) conditions. After 12 hours, samples of total RNA were prepared from the cells. A membrane with both samples (10 μg of each) was hybridized with a probe for Id-2. Cytotrophoblasts maintained under standard conditions expressed low levels of Id-2, whereas cytotrophoblasts maintained under hypoxic conditions, which inhibit differentiation/invasion, expressed 2-fold higher levels of Id-2 mRNA (top panel). Acridine orange staining of the 28S and 18S ribosomal RNAs demonstrated sample loading (data not shown). As another control for sample loading the blot was stripped and reprobed for HLA-G mRNA expression (bottom panel), the abundance of which is not regulated by oxygen tension (Genbacev et al., 1997).

probe hybridized to a 1.1 kb message encoded by the Id-2 cDNA that was inserted into the viral vector. Densitometry showed that transduction resulted in an 8-fold increase in Id-2 mRNA expression. Consequently, message levels after transduction with AdcId-2 were comparable to those observed in freshly isolated cytotrophoblasts (Fig. 1A, 0 hours). As an additional control for sample loading, the blot was then stripped and reprobed for HLA-G.

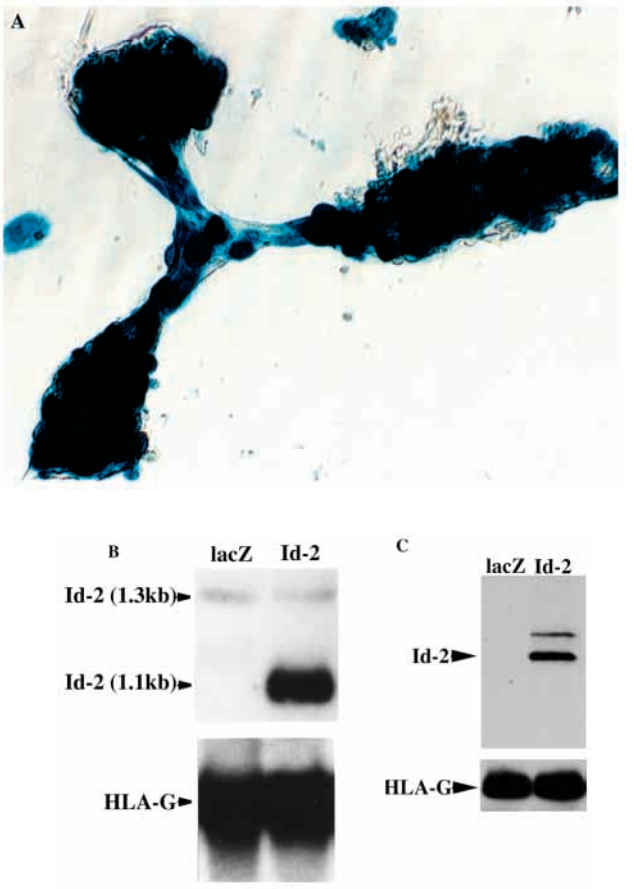


Fig. 5. Adenovirus transduction (AdcId-2) maintains Id-2 expression in cytotrophoblasts differentiating/invading in vitro. (A) Cytotrophoblasts were cultured in the presence of adenovirus carrying the *lacZ* cDNA driven by the CMV promoter. After 36 hours, the cells were fixed and assayed for β-galactosidase activity. As shown by the percentage of blue cells, transduction efficiency was at least 90% in this and all other experiments. (B) Cytotrophoblasts were transduced with adenovirus carrying cDNAs for either *lacZ* or for Id-2, both driven by the CMV promoter. After 36 hours in culture, Id-2 levels were assessed by northern hybridization. Cells transfected with control AdclacZ virus expressed low levels of endogenous Id-2 mRNA (1.3 kb). Cells transduced with AdcId-2 also expressed low levels of endogenous Id-2 mRNA, as well as a ~1.1 kb message encoded by the transduced cDNA. As a control for sample loading the blot was stripped and reprobed to detect HLA-G mRNA expression. (C) Cytotrophoblasts were transduced with AdclacZ or AdcId-2. After 36 hours lysates were prepared and analyzed for Id-2 protein expression by immunoblotting (top panel). No immunoreactive bands were detected in control cells transduced with AdclacZ. In cells transduced with AdcId-2, two bands, M_r 13,000 and 15,800, were detected; the higher molecular weight band is probably the phosphorylated form of the molecule. The blot was then stripped and reprobed with an antibody against HLA-G to confirm equal protein loading (bottom panel).

Finally, we investigated whether enhanced RNA production changed Id-2 expression by cytotrophoblasts at the protein level. Lysates of cells transduced with either AdclacZ or AdcId-2 were analyzed by immunoblotting with an antibody that specifically reacts with Id-2 (Fig. 5C). No immunoreactive bands were detected in the control cells transduced with

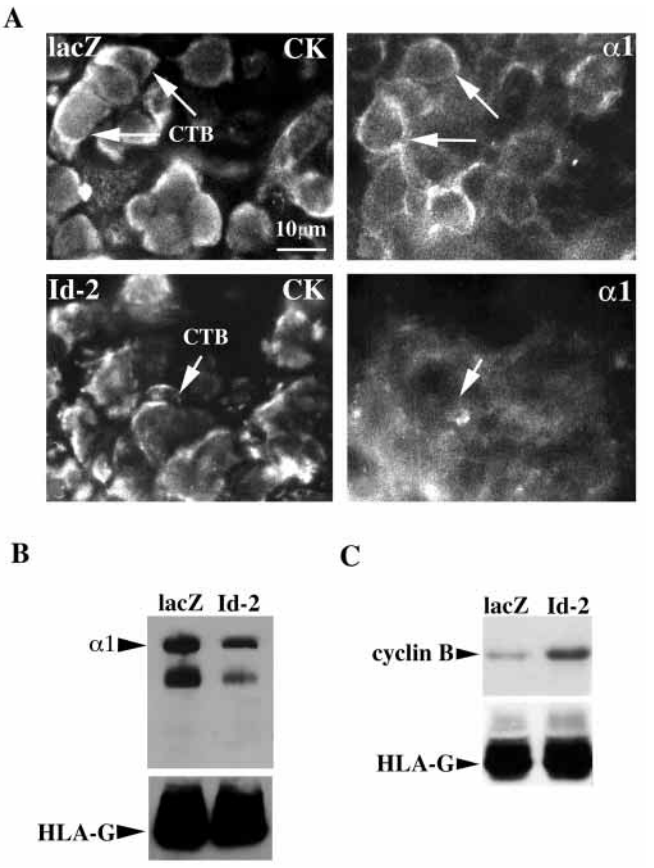


Fig. 6. Id-2 regulates cytotrophoblast integrin α1 and cyclin B expression. (A) Cytotrophoblasts were transduced with AdclacZ (top panels) or AdcId-2 (bottom panels). After 40 hours in culture, the cells were subjected to double indirect immunofluorescence with antibodies against cytokeratin (CK; left panels) and α1 integrin (α1; right panels). The plasma membranes of control AdclacZ-transduced cells stained with anti-α1 integrin (upper right panel). In contrast, cells transduced with AdcId-2 showed substantially reduced staining with the same antibody (lower right panel). (B) Levels of α1 integrin protein were assessed by immunoprecipitation. Cells forced to express Id-2 showed a 2-fold reduction in integrin α1 protein expression compared to control cells transduced with AdclacZ (top panel). 20 μl of each lysate was assayed by immunoblotting for HLA-G to confirm that the starting material contained equivalent amounts of protein (bottom panel). (C) Cytotrophoblasts were transduced with either AdclacZ or AdcId-2. After 40 hours in culture, cell lysates were prepared and analyzed by immunoblotting with an antibody that specifically recognizes cyclin B (top panel). Compared to control cells, the levels of cyclin B protein were elevated by 3-fold in cytotrophoblasts transduced with AdcId-2. The blot was stripped and re-probed with an antibody against HLA-G to confirm equal protein loading (bottom panel).

AdclacZ. In contrast, two bands, M_r 13,000 and 15,800, were detected in cells transduced with AdcId-2. The higher molecular weight band reacted with an antibody that recognizes phosphoserine (data not shown), suggesting that a fraction of the Id-2 was phosphorylated. To confirm equal loading, the blot was stripped and reprobed with an antibody to HLA-G, which identifies a broad band due to polylactosaminylation of this class Ib molecule in human cytotrophoblasts (McMaster et al., 1998).

Id-2 regulates cytotrophoblast integrin α1 protein expression

Next, we investigated the effects of transduction with AdcId-2 on cytotrophoblast expression of stage-specific antigens that mark specific transitions in the differentiation program. In normal pregnancy, the cells' onset of α1 integrin expression is strongly correlated with their invasiveness, both in vivo (Zhou et al., 1993) and in vitro (Damsky et al., 1994). To determine whether this antigen is regulated downstream of Id-2, we compared its expression, at the protein level, in cytotrophoblasts transduced with AdclacZ and AdcId-2. Cytokeratin staining showed that there were no obvious morphological differences between control and experimental cells that had been cultured on Matrigel for ~3 days (compare the upper left and lower left panels of Fig. 6A). As expected, control cells plated on Matrigel stained brightly with an antibody that specifically reacted with integrin α1 (Fig. 6A, upper right panel). The pattern of plasma membrane reactivity is compatible with the antigen's function as an ECM receptor. In contrast, cells transduced with AdcId-2 showed substantially reduced staining (Fig. 6A, lower right panel). We confirmed this result by immunoprecipitation with the same antibody. Compared to control cells transduced with AdclacZ, cells that were forced to express Id-2 showed two-fold lower integrin α1 expression (Fig. 6B). Expression of the β1 subunit, which co-immunoprecipitates, was also reduced. A portion (20 μl) of each lysate was analyzed by immunoblotting for HLA-G to confirm that the starting material contained equivalent amounts of protein.

Id-2 regulates cytotrophoblast cyclin B expression

Our previous studies suggested that factors that inhibit differentiation can have opposite effects on cell cycle regulators. For example, hypoxia, which also prevents cytotrophoblast upregulation of integrin α1 expression, greatly enhances the cells' expression of cyclin B (Genbacev et al., 1997), threshold levels of which are required to advance through the G_2-M checkpoint. Therefore, we investigated the effects of AdcId-2 transduction on cytotrophoblast expression of cyclin B, using immunoblot analysis (Fig. 6C). Densitometric analysis showed that forcing cytotrophoblasts to express Id-2 resulted in a 3-fold increase in the cells' expression of cyclin B protein as compared to the control AdclacZ-transduced cells. Again, the membrane was stripped and reprobed with an antibody to HLA-G to confirm equal protein loading. In additional experiments, we found that Id-2 overexpression did not affect cyclin A levels, nor did the cells proliferate, as determined by the lack of BrdU incorporation and the failure to express mitotic markers such as phosphorylated histone H3 (data not shown). Finally, Id-2 overexpression did not induce apoptosis, as determined by Apotag staining (data not shown). These results suggest that AdcId-2 transduction increases cytotrophoblast expression of cyclin B, but that other, unrelated factors engineer completion of the cell cycle.

Id-2 overexpression inhibits cytotrophoblast invasion but promotes the cells' migration

At a functional level, one of the most important endpoints of cytotrophoblast differentiation is acquisition of tumor-like invasive properties that enable the cells to breach the uterine wall. Ultimately these fetal cells migrate to specific locations within the decidua and myometrium, where they reside for the

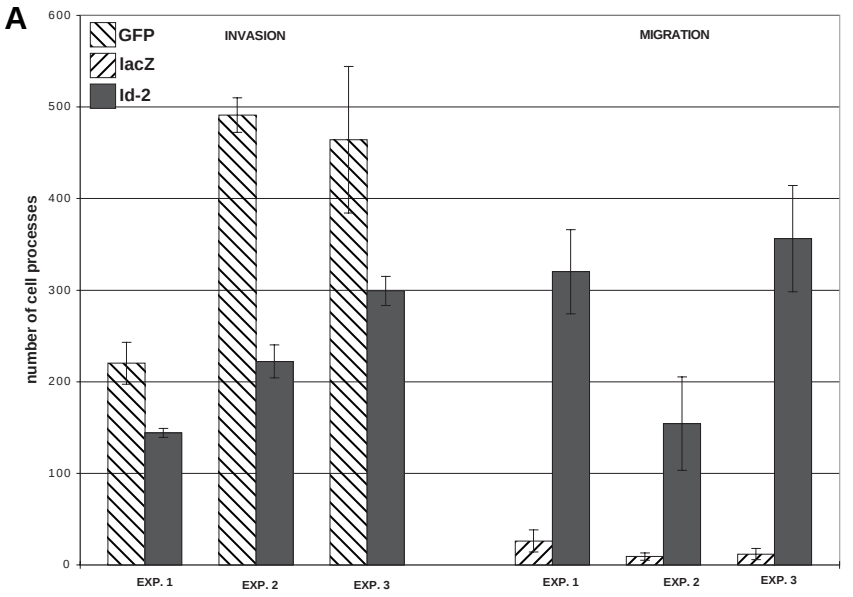
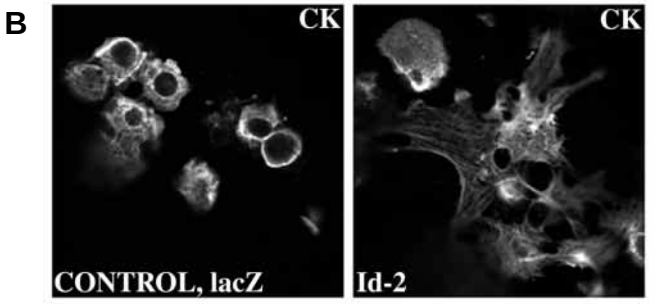


Fig. 7. Transduction with AdcId-2 inhibits cytotrophoblast invasion, but dramatically promotes the cells' migration. Forcing Id-2 expression did not change the appearance of cells plated on Matrigel (see Fig. 6A), but resulted in a 35-55% reduction in invasion (A). In contrast, striking morphological changes were observed when AdcId-2 transduced cells were plated on uncoated filters. As shown by cytokeratin staining (B), the morphology of control AdclacZ-transduced cells was essentially undistinguishable from either AdclacZ- or AdcId-2-transduced cells plated on Matrigel (see Fig. 6A). In contrast, cells that were forced to express Id-2 showed extensive spreading and highly irregular shapes. These differences were reflected by significant changes in their migratory properties. As compared with AdclacZ-transduced cells, forcing Id-2 expression produced up to a 30-fold increase in the number of cells that migrated through the filter pores (A). Exp, experiment.



remainder of pregnancy. Accordingly, we examined the effects of forcing Id-2 expression on the cells' ability to both invade and migrate. Invasiveness was assayed by plating the cells on filters coated with a ~100 μm layer of Matrigel. The results were quantified by determining the number of cell processes that reached the filter underside. In these experiments, cells transduced with control viruses (AdcGFP and AdclacZ) behaved exactly like untreated cells (data not shown). With regard to invasion through Matrigel-coated filters, forcing Id-2 expression did not change the morphology of cytotrophoblasts cultured under these conditions (Fig. 6A). Nevertheless, we found a 35-55% reduction in invasion when cells were transduced with AdcId-2 as compared to AdcGFP (Fig. 7A). This is in accord with the results of previous experiments that show blocking integrin α1 function decreases invasion by a similar amount (Damsky et al., 1994).

Migration was assayed by plating cells on filters that were coated only on the underside with fibronectin. Under these plating conditions, we noted striking morphological changes between control AdclacZ-transduced and experimental AdcId-2-transduced cells. As shown by cytokeratin staining (Fig. 7B), the morphology of control AdclacZ-transduced cells was essentially indistinguishable from that of either AdclacZ- or AdcId-2-transduced cells plated on Matrigel (Fig. 6A). In comparison, cells that were forced to express Id-2 showed extensive spreading and highly irregular shapes. These differences were reflected by significant changes in their migratory properties. As we observed previously in untransfected cells, cytotrophoblasts transduced to express AdclacZ showed very low levels of migration. In contrast, forcing Id-2 expression produced up to a 30-fold increase in the number of cells that migrated through the filter pores (Fig. 7A).

DISCUSSION

The results of these experiments offer a number of interesting insights into the role of bHLH transcription factors in human placental development. Our original hypotheses were derived from data concerning the role of HLH family members in directing the fate of murine trophoblast subpopulations. For example, Mash2, a mammalian homologue of *Drosophila* achaete/scute complex genes, is strongly expressed in diploid trophoblasts of the postimplantation embryo, but is downregulated as they differentiate into trophoblast giant cells. Mash2 is involved in the maintenance of the diploid cells' differentiation into the spongiotrophoblast layer of the ectoplacental cone (Guillemot et al., 1994; Nakayama et al., 1997; Tanaka et al., 1997). Commitment to giant cell differentiation is characterized by modulation of the expression of other HLH transcription factors. With regard to negative regulators, Id-1 and Id-2 are detectable in the extraembryonic ectoderm of the chorion, but not in the ectoplacental cone or trophoblast giant cells (Jen et al., 1997). Forced expression of Id-1 in the Rcho-1 trophoblast cell line inhibits their differentiation into giant cells (Cross et al., 1995). With regard to positive regulators, Hand1 (formerly known as Hxt, eHand and Thing1) expression is upregulated as differentiation proceeds (Cross et al., 1995). Mice carrying homozygous null mutations in Hand1 die at E7.5 with defects in trophoblast giant cells (Firulli et al., 1998; Riley et al., 1998).

Although there are many differences in the organization of the mouse and human placentas at a histological level, it is likely that these superficial characteristics hide a great many similarities at a molecular level. For example, trophoblast giant cells in mice are invasive and express many of the same adhesion molecules (e.g., integrin $\alpha_1\beta_1$; Sutherland et al., 1993)

and metalloproteinases (e.g., MMP-9; Werb et al., 1992) that are critical to human cytotrophoblast invasion (reviewed in Damsky and Fisher, 1998). We have been interested in extending these comparisons to the level of transcriptional regulators (Janatpour et al., 1999). With regard to the bHLH family, the human homologue of Mash2 is expressed in a pattern very similar to that of its murine counterpart; the highest levels are found in cytotrophoblast stem cells, which after differentiation/invasion contain nearly undetectable levels of this message. The points of divergence are equally interesting, as differentiating mouse and human trophoblasts appear to modulate expression of a different repertoire of Id family members. This observation may explain why placentation is apparently normal in *Id-2*$^{-/-}$ mice, which instead have abnormalities in peripheral lymphoid organs and greatly reduced numbers of NK cells (Yokota et al., 1999). Alternatively, placentation may be normal in these mice because the lack of Id-2 circumvents the need to turn it off appropriately. Although these studies are not yet complete, we have yet to detect Hand1 expression in human cytotrophoblasts, a result reported by other investigators (Knöfler et al., 1998). It is interesting to note that the analogies extend beyond bHLH family members, as the novel transcriptional regulator glial cells missing is highly expressed in both murine and human trophoblast populations (Basyuk et al., 1999; Janatpour et al., 1999). Finally, as in neuronal and muscle differentiation, bHLH functions, both positive and negative, are likely to intersect with complex regulatory networks that include numerous other factors (Arnold and Winter, 1998; Cepko, 1999).

From the beginning, we expected the consequences of maintaining Id-2 expression in differentiating cytotrophoblasts to mimic the known actions of HLH proteins in trophoblasts, in particular, and other cells in general. Hallmarks of their actions include regulating competence and/or other aspects of cell fate determination (Cepko, 1999). Thus, many of their downstream targets include molecules that play pivotal roles in balancing the mitotic capacity of progenitor stem cell populations with the differentiative capacity of their progeny. With regard to mitotic capacity, recent work has shown that bHLH proteins interact with components of cell cycle machinery (Molkentin and Olson, 1996). For example, Id-1 overexpression in NIH 3T3 cells inhibits p21 expression (Prabhu et al., 1997). In human osteosarcoma cells, Id-2 binds retinoblastoma protein family members and promotes cell proliferation (Iavarone et al., 1994). Therefore, we considered the possibility that Id-2 might play a role in regulating the cytotrophoblast cell cycle. Forcing Id-2 expression increased cyclin B levels, without other discernable effects. These results suggest that in cytotrophoblasts, other transcription factors operate in concert with Id family members to maintain the mitotic potential of the stem cell population.

With regard to differentiative capacity, we found that Id-2 levels were highest in stem cells. Furthermore, inhibition of cytotrophoblast differentiation, e.g. as a consequence of hypoxia in vitro and preeclampsia in situ, was also associated with relatively high Id-2 levels. These results suggested that maintaining Id-2 expression could alter the ability of the cells to invade. In these experiments, we focused on the stage-specific antigen integrin α1. We chose this marker because of the tight positive correlation between its expression and the differentiative state of the cells, as well as its central role in mediating invasion. Specifically, cytotrophoblasts upregulate integrin α1 expression as they invade both in utero (Damsky et al., 1992) and in vitro (Damsky et al., 1994). Furthermore, antibody perturbation of the molecule's function in vitro inhibits invasion (Damsky et al., 1994), an observation that is in accord with significantly reduced α1 staining associated with invasive cytotrophoblasts in preeclampsia (Zhou et al., 1993). We found that maintaining Id-2 levels in cytotrophoblasts as they differentiated in vitro significantly downregulated their integrin α1 expression. Functional data also supported this theory: maintaining Id-2 expression inhibited the cells' ability to invade a laminin-rich Matrigel substrate. These data are consistent with the hypothesis that this negative regulator controls important aspects of the differentiation pathway that leads to cytotrophoblast invasion.

In contrast to the results discussed thus far, we were very surprised by the striking effects that maintaining Id2 expression had on cytotrophoblast migration in the absence of an exogenous substrate. Under these conditions the cells interact with their own ECM. Production of this matrix, which consists of ligands for many of the cells' ECM receptors, is upregulated in concert with adhesion molecule switching during invasion in situ and in vitro (Damsky et al., 1992, 1994). This situation models the cell-ECM interactions of cytotrophoblasts in cell columns, where only homotypic adhesive interactions occur (see Fig. 2). These results suggest that Id-2 expression, which is highest in regions of column initiation, could facilitate the cells' departure from the anchoring villus, analogous to the role of the bHLH protein MesP1 in directing mesodermal cell migration from the primitive streak (Saga et al., 1996). These results also suggest that downstream effector pathways regulated by Id-2 include molecules that are critical to migration.

We found that Id-2 expression in situ was dramatically modulated once the cells entered the uterine wall. Two distinct zones were observed. Cells at the uterine surface no longer expressed Id-2. These results are in accord with our observation that Id-2 expression is normally downregulated during invasion in vitro; however, expression reappeared in cytotrophoblasts that occupied the deeper portions of the uterus, where invasion stops. We speculate that forcing Id-2 expression in the context of a Matrigel substrate may be analogous to the latter situation. In this regard it is interesting to note that the net effect was to inhibit invasion.

In summary, we have demonstrated that downregulation of Id-2 expression is an important component of the transcriptional regulatory pathway that governs cytotrophoblast differentiation along the pathway that leads to uterine invasion. Our results also imply the potential importance of tissue-specific basic HLH transcription factors in this differentiation process. Future experiments will be directed toward identifying these factors, as well as other transcriptional networks with which they interact.

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