Epigenetic Regulation of Human Trophoblastic Cell Migration and Invasion

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Implantation requires a number of distinct cellular functions including attachment to the endometrial cell, spreading of the embryonic trophoblast and invasion of the trophoblastic cell into the endometrium (1, 2). Deficient trophoblastic invasion contributes to the poor success rates of in vitro fertilization (3–7) and pathologically, may result in gestational complications including miscarriage, preeclampsia, intrauterine growth restriction and preterm delivery, placental abruption, and intrauterine death (2). In contrast, excessive invasion of the trophoblastic cell into the endometrium is associated with placenta accreta and invasive carcinoma (choriocarcinoma) (8, 9). Thus, tightly regulated mechanisms govern the ability of the trophoblastic ectoderm to invade the endometrial stoma.

In this context, adherens junction (AJ) molecules [E-cadherin, α-, β-, and γ-catenin (plakoglobin)], possess a key role in trophoblast differentiation, invasion, and placental remodeling (10–14). E-cadherin is a member of a family of Ca2+-dependent cell-cell adhesion molecules. The intracellular domain of E-cadherin interacts with the actin cytoskeleton via a number of AJ proteins including β-catenin (plakoglobin) (10–14). E-cadherin is a member of a family of AJ proteins including α-, β-, and γ-catenin (plakoglobin) (10–14). Plakoglobin and β-catenin share the greatest homology and bind directly to the cytoplasmic tail of E-cadherin in a mutually exclusive manner. α-Catenin then anchors the bound E-cadherin-catenin (β-catenin or plakoglobin) to the actin cytoskeleton. This is commonly termed the AJ complex. Perturbation in the expression of the AJ complex, especially E-cadherin, has been associated with invasion and metastasis of many human cancers (15, 16). Similarly, down-regulation of E-cadherin expression has been reported in trophoblasts differentiating to an invasive phenotype (17). E-cadherin has been reported to be silenced in different cancer cells by different mechanisms, including somatic mutation, transcriptional repression, and promoter methylation (18).

The dynamic chromatin structure that influences gene expression is controlled by reversible epigenetic patterns of DNA methylation and histone modification (19). Enzymes involved in this process include DNA methyltransferases (DNMTs) (20), histone deacetylases (21), histone acetylases (22, 23), histone methyltransferases, and the methyl-binding domain protein MECP2 (24). Methylation is one of several postsynthetic modifications that normal DNA may be subject to. CpG dinucleotides are the site of DNA methylation, and the enzymes responsible for this process in mammals are DNA methyltransferases (DNMTs) (20). DNMT-1 is involved in the maintenance of specific DNA methylation patterns during DNA replication and preferentially acts on hemimethylated CpG substrates (20). In contrast, DNMT-3a and DNMT-3b exhibit de novo methyltransferase activities (25). Targeted disruption of the DNMT-1 or DNMT-3b gene produces embryonic lethality in mice (25), whereas DNMT-3a null mice die shortly after birth (26), suggestive that DNMTs are essential for normal embryonic development. We demonstrate herein that trophoblastic migration and also increased the gene promoter activity of both plakoglobin and E-cadherin. Protein levels of both plakoglobin and E-cadherin were increased by AZA, and AZA enhanced their localization to sites of intercellular contact. Forced expression of plakoglobin and E-cadherin abrogated BeWo cell migration, indicative that repression of these genes was required for BeWo cell migration. Small interfering RNA-mediated depletion of the individual DNA methyltransferase (DNMT) molecules did not affect plakoglobin and E-cadherin promoter activity or BeWo cell migration. However, increases in plakoglobin and E-cadherin promoter activity and inhibition of BeWo cell migration was achieved with small interfering RNA-mediated depletion of both DNMT-3a and DNMT-3b. Epigenetic regulation of plakoglobin and E-cadherin is therefore pivotal for appropriate trophoblastic invasion in vitro. (Endocrinology 147: 5275–5283, 2006)
invasion is an epigenetically regulated process, such that trophoblastic invasion is executed by methylation-dependent repression of both plakoglobin and E-cadherin in vitro. Repression of both gene promoters is maintained by the combined effect of both DNMT-3a and DNMT-3b. Appropriate physiological regulation of these two DNMTs may therefore be pivotal for successful placentalation and subsequent intrauterine development. Thus, based on the transformed cell line model presented, DNMT-3a and DNMT-3b may be required for appropriate epigenetic regulation of early placentalation.

Materials and Methods

Cell culture

Choriocarcinoma cell lines BeWo, JAR, and JEG-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM/F12 (Invitrogen Life Technologies, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies), 2 mm l-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen Life Technologies) and cultured at 37 °C in a humidified 5% CO2 incubator. Cells were detached by trypsinization (1× trypsin EDTA; Invitrogen Life Technologies).

Treatment with AZA

BeWo, JAR, and JEG-3 cells were cultured in the absence or presence of 10 μm AZA (Sigma, St. Louis, MO) for 48 h. Cell morphology, motility, and invasion in presence or absence of the inhibitor was examined. Cell viability was determined by trypan blue exclusion.

Confocal laser-scanning microscopy

BeWo cells were cultured in cavity slides, fixed in 4% paraformaldehyde/PBS (pH 7.4), washed, and blocked with BBX (PBS, 0.1% Triton, 0.1% BSA, 250 mm NaCl). The cells were incubated with 5 mg/liter fluorescein isothiocyanate (FITC)-phalloidin (Sigma), at room temperature for 30 min. For localization of plakoglobin and E-cadherin, cells were fixed with methanol for 5 min at −20 °C, blocked and permeabilized as indicated above and incubated with mouse monoclonal antibody against E-cadherin (Zymed, San Francisco, CA) at 1:200 dilution, or mouse monoclonal antibody against plakoglobin (Transduction Laboratory, Lexington, KY; catalog no. 610253), at 1:500 dilution. After incubation at room temperature for 1 h, cells were again washed and blocked with BBX and incubated with secondary FITC-conjugated goat antimouse antibody (Jackson ImmunoResearch, West Grove, PA; catalog no. 18-0223) at 1:400 dilution in BBX. Cells were mounted and labeled under a Carl Zeiss (Jena, Germany) Axioplan microscope and examined under a Zeiss (Jena, Germany) Axiovert microscope equipped with epifluorescence optics microscope.

Migration and invasion assay

Assays were performed in transwell culture slides from Corning (Life Sciences, Acton, MA) according to the manufacturer’s instructions, with uncoated porous filters (12-μm pore size) for estimation of cell migration and filters precoated with Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) to examine cell invasion. BeWo, JEG-3, or JAR cell suspension was placed in the upper chamber in 0.5 ml of DMEM/F12 serum-free medium with or without 10 μm 5-Aza-2′-deoxycytidine (Sigma). DMEM/F12 was supplemented with 10% fetal bovine serum and placed in the lower chamber as a chemotactic attractant. After incubation for 48 h, cells that had migrated to the lower surface of the filters were fixed in 0.1% formaldehyde in PBS for 10 min. Cells were visualized with hematoxylin and counted. Values for the cell migration or invasion were expressed as the average number of cells per microscope field counted over four fields per filter. All experiments were performed in triplicate. For the wound migration assay, a confluent monolayer of BeWo cells was scraped with a blade, washed with PBS, and incubated in culture medium containing 10% serum with or without 5′-aza-2′-deoxycytidine (AZA) and plated at 37 °C in a humidified 5% CO2 incubator for 72 h. Three independent experiments were performed for quantitative results.

Preparation of total RNA and RT-PCR

Total RNA was isolated from BeWo cells using Trizol (Invitrogen, Auckland, New Zealand) according to the instructions of the manufacturer. RT-PCR of total RNA (1 μg) was performed using OneStep RT-PCR (QIAGEN, Valencia, CA) according to the instructions of the manufacturer. Sequences of the oligonucleotide primers used for RT-PCR were as described earlier (27) except for the plakoglobin gene (Table 1). Sequences of the oligonucleotide primers used are shown in Table 1. Amplified PCR products were visualized with ethidium bromide on a 2% agarose gel.

Western blot analysis and immunoblotting

BeWo cells were lysed (50 mm Tris-HCl (pH 7.4), 1% Nonidet P-40, 150 mm NaCl, 1 mm EDTA, 1 mm Na3VO4, 1 tablet Complete protease inhibitor (Roche, Mannheim, Germany) mixture/30 ml), and total protein (40 μg) was measured using the Bradford assay (Bio-Rad, Auckland, New Zealand). Total protein extracts were diluted in 2× reducing Laemmli’s sample buffer [6.25 mm Tris (pH 6.8), 10% glycerol, 20% sodium dodecyl sulfate, 2.5% pyronin, and 100 mm dithiothreitol] and separated by 7.5% SDS-PAGE, transferred onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with PBST (3% fat-free milk in PBS containing 0.1% Tween 20), washed in PBST, and incubated for 1 h with 1:1000 dilution of mouse monoclonal antibody against human E-cadherin (Zymed), or goat polyclonal antibody against human plakoglobin (Santa Cruz Biotechnology, Santa Cruz, CA). As a secondary antibody, a conjugated horseradish peroxidase, antirabbit, or goat antigoat IgG (Sigma), was used at a 1:2000 dilution. ECL detection (Amersham) was performed according to the manufacturer’s instructions. Blots were stripped by 0.2 m NaOH for 20 min and reprobed by using anti-β-actin antibody (Santa Cruz Biotechnology).

Cell transfection and luciferase assay

Cells were transiently transfected with the expression vector containing the full-length human plakoglobin (27) and E-cadherin (gift from A. Kraemer, University of Queensland, Queensland, Australia) genes, or gene promoters for plakoglobin (gift from G. Brabant, Department of Gastroenterology and Endocrinology, Hannover, Germany) and E-cadherin (gift from E. C. Fearon, University of Michigan, Ann Arbor, MI), or small interfering RNA (siRNA) expression vectors (see footnote and E-cadherin (gift from G. Brabant, Department of Gastroenterology and Endocrinology, Hannover, Germany). Sequences of the oligonucleotide primers used for RT-PCR (QIAGEN, Valencia, CA) according to the instructions of the manufacturer. Sequences of the oligonucleotide primers used for RT-PCR were as described earlier (27) except for the plakoglobin gene (Table 1). Sequences of the oligonucleotide primers used are shown in Table 1. Amplified PCR products were visualized with ethidium bromide on a 2% agarose gel.

TABLE 1. Sequences of the oligonucleotide primers used

| Primer name      | Primer sequence                        | Size (bp) |
|------------------|----------------------------------------|-----------|
| DNMT-1 forward   | 5′-TACCTGGAGACACCTGGACCTC-3′           | 110       |
| DNMT-1 reverse   | 5′-GTTGCGCTCAACAGATGAGCA-3′            | 102       |
| DNMT-3a forward  | 5′-TATTGATGGCGCAACAAGAGACG-3′          | 110       |
| DNMT-3a reverse  | 5′-GGTGTTCCAGGTAACATTGAGG-3′           | 112       |
| DNMT-3b forward  | 5′-GGGCAAGTTTCTCCAGGTTCTTG-3′          | 112       |
| DNMT-3b reverse  | 5′-TGTTGATGGCGCAACAAGAGACG-3′          | 112       |
| Plakoglobin forward | 5′-GCGAGAGGACGTCGTCCTGAGT-3′       | 531       |
| Plakoglobin reverse | 5′-GGTGGCTGGATTATCTCAGCAT-3′         | 313       |
in the samples. At least three independent experiments were compiled for quantitative results.

Construction of siRNA expression vectors

We generated siRNA constructs in pRNAT-CMV3.1/Hygro vector from Genscript targeting at least two to three different sequences for DNMT-1, DNMT-3a, and DNMT-3b. The constructs with the following sequences, 5'-GGATATGCCATGCTTCAACAGC-3' for human DNMT-1, 5'-CTACTACATACGCAAGGCCAA-3' for human DNMT-3a, and 5'-AGATGACGGATCGCTAGGT-3' for human DNMT-3b were used in all the experiments described. Cells were transiently transfected with 2 μg of the vector containing the respective siRNA and were cultured for 24–48 h. Saint-Mix (Synvolux Therapeutics B.V.) transfection reagents were used according to the manufacturer’s instructions. A pRNA-U6.1/Hygro vector with scrambled sequences was used as control.

Results

Inhibition of methylation alters the migratory phenotype of trophoblast-derived cells and inhibits trophoblast-derived cell migration and invasion

The migratory cell phenotype is associated with coordinated changes in cell morphology. To examine the potential epigenetic regulation of trophoblastic cell function, we therefore first determined whether pharmacological inhibition of methylation would alter the morphology of trophoblastic derived choriocarcinoma cells. The trophoblast-derived choriocarcinoma BeWo cell line was cultured on plastic slides in the absence or presence of 10 μM of the DNA methyltransferase inhibitor AZA for 48 h and filamentous actin visualized with FITC-phalloidin. Vehicle-treated BeWo cells were arranged in islands with multiple interconnecting cellular bridges (Fig. 1, A and B). Cells located at the periphery of the clumps were flat and elongated and possessed large cellular protrusions indicative of actively motile cells. The absence of a prominent localization of filamentous actin to cortical regions of the cell and stress fibers traversing the cell also indicated a motile phenotype (Fig. 1C). In contrast, AZA-treated BeWo cells were rounded, exhibited smaller colony sizes due to decreased motility, formed tight clumps with little extension, and individual cells at the periphery of clumps exhibited few cellular protrusions (Fig. 1, D and E). In addition, AZA treatment of BeWo cells resulted in prominent localization of filamentous actin to cortical regions of the cell and the generation of prominent stress fibers traversing the cell (Fig. 1F). Thus, treatment of BeWo cells with AZA resulted in a conversion of BeWo cell morphology to a nonmotile phenotype.

We next examined the ability of AZA to abrogate the migratory and invasive properties of BeWo cells using wound-healing (Fig. 2A), and transwell migration assays (Fig. 2B), respectively. In the wound-healing assay, cells were cultured to confluence and a section of the cell layer removed with a sharp razor. The marking of the razor on the plastic served as the migratory start line. The cleared space was subsequently inspected microscopically over 48 h for the ability of cells to migrate across and fill the wounded area. Vehicle-treated BeWo cells rapidly migrated across the demarcation line and largely filled the wounded area after 48 h. In contrast, treatment of BeWo cells with AZA largely abrogated the ability of BeWo cells to migrate across the demarcation line. Concordantly, reduced migration and invasion of BeWo cells treated with AZA (Fig. 2C), in comparison with nontreated cells, was observed in the transwell assay. BeWo cells exhibiting a nonmigratory phenotype after AZA treatment were unable to pass through matrigel as do vehicle-treated control cells (Fig. 2C).

To generalize our observations on BeWo cells, we also examined the effect of AZA on the ability of two other choriocarcinoma cell lines, JEG-3 and JAR (28) to migrate in the transwell chamber assay. Migration of both JEG-3 and JAR cells was inhibited by 63% ± 4.3% and respective 29.3 ± 1.75% after treatment with AZA (data not shown), indicative that cellular methylation is required for trophoblastic cell migration. It has previously been reported that AZA affects cell viability (29) and reduced cellular viability could potentially contribute to the decrease in migration observed with AZA. To obviate this possibility, we examined the motility of single identified BeWo cells over a period of time in the presence of AZA. AZA dramatically altered the morphology and inhibited the motility of individual BeWo cells compared with single identified vehicle-treated cells (data not shown). Thus, events dependent on cellular methylation are required for trophoblastic migration and invasion.

FIG. 1. Inhibition of methylation produces a less invasive phenotype in BeWo cells. BeWo cell morphology was examined by phase-contrast microscopy in cells treated in the absence (vehicle) or presence of 10 μM AZA (A). FITC-phalloidin was used to visualize the organization of actin filaments in response to AZA under ×10 (B and E) and ×40 magnification (C and F).
Inhibition of methylation increases plakoglobin and E-cadherin expression in BeWo cells

A migratory phenotype is associated with disassembly of the AJ complex (27). We therefore examined the mRNA expression of components of the AJ complex after AZA treatment in BeWo cells. Treatment of BeWo cells with AZA did not affect the mRNA levels of β- and α-catenin. However, the mRNA levels of plakoglobin and E-cadherin were up-regulated by 51.8% and 41.7% respectively in BeWo cells after AZA treatment (Fig. 3A).

AZA treatment of BeWo cells concordantly increased the protein levels of both plakoglobin by 50% and E-cadherin by 44.6% ± 4.5%, as observed by Western blot analysis (Fig. 4A). Again, β-actin was used as expression and loading control and was not altered between the experimental conditions. Appropriate localization of both plakoglobin and E-cadherin to sites of intercellular contact is required for proper function of the AJ (30). We therefore used confocal laser scanning microscopy to examine the effect of inhibition of DNA methyltransferases with AZA on the localization of both plakoglobin and E-cadherin in BeWo cells. We observed that treatment of BeWo cells with AZA enhanced the localization of both plakoglobin and E-cadherin to sites of intercellular contact (Fig. 4B).

Thus, inhibition of methylation in BeWo cells resulted in increased expression of both plakoglobin and E-cadherin by transcriptional activation of their respective promoters.

Concomitant expression of plakoglobin and E-cadherin is necessary for inhibition of BeWo cell motility

Expression of both plakoglobin and E-cadherin are negatively associated with cellular migration (31, 32) and forced expression of either protein has been demonstrated to revert cells to an epithelial phenotype and prevent migration (27, 33). The increased expression of plakoglobin and E-cadherin observed upon AZA treatment of BeWo cells may therefore be responsible for the observed inhibition of BeWo cell migration by AZA. We therefore examined the effect of forced expression of plakoglobin and E-cadherin on BeWo cell motility. BeWo cells were transiently transfected with expression vectors encoding human plakoglobin and E-cadherin, and assayed for their ability to migrate in a transwell chamber. Forced expression of plakoglobin or E-cadherin resulted in a modest but significant inhibition of cell motility, whereas
concomitant forced expression of both plakoglobin and E-cadherin significantly inhibited the ability of BeWo cells to migrate in the transwell chamber (Fig. 5A). The forced expression of both plakoglobin and E-cadherin resulted in an increase in protein expression by 49.8% and 2.3% respectively, as determined by Western blot analysis (Fig. 5B). Thus, simple increased expression of both plakoglobin and E-cadherin is sufficient to inhibit trophoblastic cell migration.

Plakoglobin and E-cadherin promoter activity is regulated by the combined actions of DNMT-3a and DNMT-3b

DNA methylation within the cell is mediated by specific enzymes termed DNA methyltransferases of which there are three functional members. We therefore constructed siRNA vectors to specifically deplete each of the three functional DNMTs in BeWo cells. Transient transfection of siRNA to each of the individual DNMTs, (DNMT-1, DNMT-3a and DNMT-3b) resulted in the specific depletion of DNMT-1 mRNA by 48.4% ± 3.3%, of DNMT-3a mRNA by 51.9 ± 4.2% and of DNMT-3b mRNA by 47.5% ± 1.1% compared with the vector with scrambled sequence control (Fig. 6A). siRNA-mediated individual depletion of DNMT-1, DNMT-3a, or DNMT-3b in BeWo cells had no significant effect on the promoter activity of plakoglobin (Fig. 6B), nor E-cadherin (Fig. 6C). However, concomitant depletion of both DNMT-3a and DNMT-3b resulted in a dramatic increase in the promoter activity of both plakoglobin and E-cadherin (Fig. 6, B and C). Concomitant depletion of DNMT-1, DNMT-3a, and DNMT-3b did not result in a further increase in the promoter activity of the PLAKOGLOBIN and E-CADHERIN genes. E-CADHERIN and PLAKOGLOBIN gene promoter activity are therefore regulated specifically by DNMT-3a and DNMT-3b. To demonstrate that the increases observed in the activity of the PLAKOGLOBIN and E-CADHERIN promoters upon concomitant siRNA-mediated depletion of DNMT-3a and DNMT-3b (see Fig. 6, B and C) correlated with increased expression of the endogenous protein we performed Western blot analysis for plakoglobin and E-cadherin protein expression. We observed increased expression of endogenous plakoglobin and E-cadherin after concomitant depletion of DNMT-3a and DNMT-3b compared with the scrambled sequence control (Fig. 6D).

Depletion of DNMT-3a and DNMT-3b inhibits BeWo cell migration

To determine whether DNMT-3a and DNMT-3b were consequently required for BeWo cell migration, we next examined the effect of siRNA-mediated depletion of the individual DNMT molecules on the ability of BeWo cells to migrate in a transwell chamber. Concordant with the effect of DNMT depletion on E-CADHERIN and PLAKOGLOBIN gene promoter activities we observed that depletion of the individual DNMT molecules did not alter the ability of BeWo cells to migrate. We did, however, observe inhibition of BeWo cell migration upon the concomitant depletion of both DNMT-3a and DNMT-3b. Depletion of all three DNMT molecules did not result in further inhibition of cell migration in addition to that observed with concomitant depletion of DNMT-3a and DNMT-3b (Fig. 7). Human trophoblastic cell migration is therefore dependent on DNMT-3a and DNMT-3b.

Discussion

In the present study, we have demonstrated that the key proteins involved in trophoblastic (choriocarcinoma) cell invasion are epigenetically regulated by components of the de novo DNA methylation pathway with DNMT-3a and DNMT-3b in BeWo cells had no significant effect on the promoter activity of plakoglobin (Fig. 6B), nor E-cadherin (Fig. 6C). However, concomitant depletion of both DNMT-3a and DNMT-3b resulted in a dramatic increase in the promoter activity of both plakoglobin and E-cadherin (Fig. 6, B and C). Concomitant depletion of DNMT-1, DNMT-3a, and DNMT-3b did not result in a further increase in the promoter activity of the PLAKOGLOBIN and E-CADHERIN genes. E-CADHERIN and PLAKOGLOBIN gene promoter activity are therefore regulated specifically by DNMT-3a and DNMT-3b. To demonstrate that the increases observed in the activity of the PLAKOGLOBIN and E-CADHERIN promoters upon concomitant siRNA-mediated depletion of DNMT-3a and DNMT-3b (see Fig. 6, B and C) correlated with increased expression of the endogenous protein we performed Western blot analysis for plakoglobin and E-cadherin protein expression. We observed increased expression of endogenous plakoglobin and E-cadherin after concomitant depletion of DNMT-3a and DNMT-3b compared with the scrambled sequence control (Fig. 6D).
DNMT-3b possessing a pivotal role in this process. The trophoblastic ectoderm of the developing embryo is required for the initial attachment to, and later invasion into, the endometrial layer of the uterus; and the extent of the primary trophoblastic invasion determines later placental efficiency, fetal viability and performance (34). Significantly, only 22.8% of conceptive matings appear to result in a live birth and a large percentage of those matings not progressing to live birth is due to failure of embryonic implantation (35). Thus, delineation of the molecular mechanisms regulating trophoblastic cell migration is crucial to understand the appropriate regulation of placentation.

In epithelial cells, AJs play a significant role in the maintenance of cell-cell adhesion, and loss of AJ function results in cell-cell dissociation (30) and is strongly implicated in tumor progression and metastasis (32, 36). In neoplastic tissues, repression of plakoglobin and E-cadherin gene expression is an important step in enabling tumor cells to migrate and invade the surrounding tissues (27, 33, 37–39). In this context, the hypermethylation of E-cadherin and recently plakoglobin promoters is well documented in many cancer models including human lung cancer (40), ovarian cancer (41), prostate cancer (42) and renal cell carcinoma (43), resulting in decreased expression of PLAKOGLOBIN and E-CADHERIN genes (39, 42, 44).

Trophoblastic invasion at the human implantation site resembles, in many aspects, the invasion of malignant tumors (45). Concordantly, down-regulation of plakoglobin and E-cadherin expression is commonly associated with the morphological alterations that occur during the differentiation of intermediate trophoblasts (46, 47). Similar to that observed in invasive cancer, we have observed methylation-
dependent regulation of plakoglobin and E-cadherin in trophoblast cells. We therefore observed that AZA, widely used as a DNA methyltransferase inhibitor (48), up-regulates the expression of plakoglobin and E-cadherin in trophoblastic cells. This was accompanied by elevated localization of plakoglobin and E-cadherin to sites of intercellular contact, which consequently abrogated the motile and invasive behavior of choriocarcinoma cells. Thus, repression of PLAKOGLOBIN and E-CADHERIN gene expression may represent one mechanism that trophoblastic cells use to invade the maternal uterine wall. Given that placentation could be described as a recapitulation of the invasive mechanisms in cancer models, we postulate that DNA methylation may be a fundamental mechanism used to govern the invasive characteristics observed in placentation. Indeed, our results are concordant with Karmakar and Das (49), who report that IL-1α decreases in E-cadherin expression is associated with enhanced invasiveness of human choriocarcinoma cell line JEG-3, whereas TGF-β1-mediated up-regulation of E-cadherin expression is associated with reduced invasiveness, along with an altered cellular morphology.

DNMT-1 has a central role in copying the pattern of DNA methylation after replication, which is one manifestation of epigenetic inheritance (50). DNMT-3a and -3b are actively expressed in male and female germ lines (51, 52). DNMT-3a appears to be the primary de novo methyltransferase involved in germ-line establishment of genomic imprinting, whereas DNMT-3b is likely to have a role in methylation of centromeric satellite repeats (25). Overexpression of DNMT-3b has been suggested to play a significant role in the development of endometrial cancer development, with increased DNMT-3b promoter gene activity in poorly differentiated endometrial cancer cell lines (53). The principal effector mechanisms of AZA is believed to be the covalent and irreversible binding of DNA-DNMT adducts rather than secondary DNA demethylation effects due to enzyme depletion (54, 55). Recently, it has been demonstrated that DNMT-3a and DNMT-3b are required concomitantly to mediate the effect of AZA (56). This report is consistent with what we have observed in that concomitant depletion of DNMT-3a and DNMT-3b mimics the effect of AZA in releasing the repression of plakoglobin and E-cadherin expression and subsequent facilitation of migration in BeWo cells. However, the molecular mechanisms mediating DNMT-3a and DNMT-3b repression of plakoglobin and E-cadherin remain to be elucidated. Several recent reports have emphasized the role of transcription factors in mediating the action of DNMTs (57, 58). For example, the Ets transcription factor PU.1, normally essential for the development of myeloid and B-cell lineages, has been reported to interact with DNMT-3a and DNMT-3b in repression of p16 (INK4A) gene expression (57). Expression of small heterodimer protein (SHP)-1 phosphatase, a key negative regulator of cell signaling, is lost in T-cell lymphomas and other malignancies due to DNA methylation of the SHP-1 promoter. It has also been reported recently, that STAT3, a cell signal transducer and activator of transcription, may transform cells by inducing epigenetic silencing of SHP-1 in cooperation with DNMT1 and histone deacetylase 1 (58). The transcription factors Snail and Slug are important in cell migration during development and also during tumor metastasis and are implicated in repression of E-cadherin and β-catenin gene expression (59). In this regard it would be interesting to examine whether Snail or Slug or other transcription factors associate with DNMT-3a and DNMT-3b in repression of plakoglobin and E-cadherin expression in BeWo cells. Alterations in DNMT expression may therefore directly alter methylation of the plakoglobin/E-cadherin promoters or alter their activity indirectly by affecting expression of transcription factors required for regulation of these promoters. The relevant mechanism(s) remain to be elucidated.

Imprinted genes are methylated according to whether they are inherited from the mother or the father. A substantial proportion of imprinted genes are involved in the control of fetal growth, and in general paternally expressed imprinted genes enhance fetal growth, whereas maternally expressed ones suppress it (60, 61). Of the approximately 60 imprinted genes identified so far in the mouse genome, around half have been examined for placental expression and all have been found to be expressed. Effects of knockouts of imprinted genes in the placenta have shown that these genes may play a major role in the development and function of the placenta. Imprinted genes could affect the function of the placenta by regulating nutrient supply in two principal ways: 1) by affecting overall growth of the placenta or of particular structures (such as the labyrinthine trophoblast); or 2) by affecting specific transport systems (62). Thus, epigenetic defects may account for a significant proportion of abortions that are not associated with chromosomal aberrations of the placenta and the embryo. Epigenetic changes in uteri may therefore predispose to sub optimal health outcomes and disease later in life. Numerous studies suggest that delivery of smaller babies is associated with increased risks to that infant of diabetes and other pathologies in adult life (63–65). There may be several mechanisms that operate at different levels that may account for this association. As postulated by Gluckman and Hanson (66), epigenetic changes may alter long-term gene expression programs. Even tissue differentiation may be altered by allocation of blastocyst stem cells to the inner cell mass or trophoectoderm lineage that influences the relative growth of placenta and fetus. Thus, delineation of the epigenetic mechanisms that alter long-term gene expression programs in trophoblastic cells may assist in the prediction of later health outcomes.

Delineation of epigenetic mechanisms involved in placenta-tion will allow for a better understanding of the complex molecular events associated with human implantation. This study has demonstrated epigenetic regulation of key proteins essential for trophoblastic migration and invasion. However, the physiological mechanisms involved in the regulation of de novo methylation during early human placental development remains to be elucidated, and may ultimately provide clues into the biology of diseases associated with abnormal placentation. An improved understanding of epigenetic regulation of early stages of gestation will open new avenues to improve or abrogate this key reproductive event leading to healthy fetal outcome.
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