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Review

Epigenetic control of cell invasion – the trophoblast model

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Abstract

Trophoblast implantation and placentation allow the survival of the young embryo and its normal development inside the uterus. In order for these processes to function properly, the trophoblast has to undergo a series of characteristic changes that lead to its adhesion and invasion of the uterus. This is achieved, among other mechanisms, by inactivation of specific tumor suppressor genes, commonly by methylation of their promoters. Cell adhesion and tissue invasion are also characteristics of malignant tumors and patterns of methylation similar to that seen in trophoblast are found in various tumor types. Another important mechanism that aids trophoblast cells invasion is their transition from epithelial to mesenchymal phenotype. Such a transition is also a common characteristic of invading malignant cells. Thus, studying tissue invasion and its control mechanisms can benefit the understanding of both the trophoblast and malignant cells behavior.

Keywords: DNA methylation; epigenetic; invasiveness; placenta; trophoblast; tumor suppressor genes.

Introduction

Is there a unique epigenetic pattern specific for given cell type throughout its lifetime? If yes, and if this epigenetic pattern does not develop properly, does it indicate that the cell is potentially carcinogenic and susceptible to different factors from the environment (or from within the cell) that can disrupt its behavior and lead to autonomous proliferation?

We suggest that the investigation of trophoblast cell behavior may offer clues to halting the proliferation and invasion of tumors. Because of its ability to rapidly proliferate and invade the mother's tissue, the trophoblast is similar to malignant cancer cells; however, unlike cancer, its invasive potential is carefully regulated and finite in time and space (1). The similarities exist not only in the high proliferation rate, loss of contact inhibition, migration and invasion, but also in the activation of telomerase and tissue vascularization.

Whereas trophoblast invasion is a tightly controlled process, little is known about the mechanisms of such control in human cells. Endometrial glands secrete large number of factors, such as epidermal growth factor, vascular endothelial growth factor and various cytokines, which take part in the control of trophoblast invasion and in early stages of its differentiation (2). The trophoblast itself secretes, in autocrine and paracrine manner, factors that increase expression of protaeses, whereas decidua and extravillous trophoblast manufacture corresponding inhibitors of these proteases, thus ensuring precise regulation of uterine epithelium invasion.

Increasingly, regulatory mechanisms of invasion potential in trophoblast cells point to similarities with mechanisms of invasion in tumor cells, with epigenetics being responsible for transcriptional repression of tumor suppressor genes in a controlled (trophoblast cells) or uncontrolled (tumor cells) manner (3). Development of the embryo can be viewed as a sequence of controlled epigenetic changes in DNA methylation (4). The zygote is in a sense a 'tabula rasa' – a union of highly methylated genes of a sperm cell and (somewhat less) methylated genes of an egg cell-that proceeds through DNA demethylation steps during subsequent embryo cell divisions.

DNA demethylation of the paternal genes occurs rapidly, whereas in the maternal genes it is gradual, because of the absence of *de novo* methylation after each replication (5). Very early in the embryonic development, cells differentiate into future trophoblast (trophoectoderm) and embryoblast (inner cell mass; ICM), by means of the activation of genes characteristic for the specific type of cells (markers) (6). These markers are the homeobox protein CDX-2 (Cdx-2) in trophoblast cells and Oct4 in future embryoblast cells (7). At the beginning, Cdx-2 is present in all cells of the morula, but with the development of the blastocel it becomes concentrated in the nucleus of only the outer cells. Transcriptional factor Oct4 is also present in all cells of the morula, whereas in later development it is restricted to the ICM epiblast (8). During the first polarization in the preimplantation period of an early embryo, another transcriptional factor, Sox2, becomes active (9).

Tightly connected with the Cdx2 are other potential trophoblast stem cell markers, e.g., transcriptional factors TEA domain family member (TEAD) 4 (which becomes active at an earlier stage), eomesodermin (Eomes), Elf5 and trans-acting T-cell-specific transcription factor (Gata3) (10, 11). The newly formed trophoblast has the possibility of

differentiating into several directions, determined by the type of transcriptional factors synthesized or active in the cells at that time. In this manner, in a rodent, trophoblast cells can proliferate and differentiate into spongiotrophoblasts (Tpbpα and Asc12 markers), giant trophoblast cells (prolactin family 3, subfamily d, member 1 (Prl3d)1 and Hand-1 markers) or syncytiotrophoblasts (Gcm-1) (Figure 1). It is believed that spongiotrophoblast cells are precursors of glycogen cells that are present in the second part of gestation in the rodent and are responsible for interstitional invasion (12). These cells match interstitial trophoblast cells in humans, whereas giant trophoblast cells, analogous to the endovascular trophoblast, are responsible for the endovascular invasion of spiral arteries of the mother (13, 14).

Part of the trophoblast stays undifferentiated, likely as a pool of stem cells (future trophoblast cells), that can proliferate and secure a sufficient number of differentiated cells needed for the formation of the placenta. If this hypothesis is not true, then what is the source of trophoblast stem cells? Namely, it has been shown that there are undifferentiated mononuclear precursor cytotrophoblast cells in the mature placenta from which all other trophoblast cell types can differentiate, with the number of these cells getting fewer with increasing age of gestation (15). The existence of a pool of stem cells can be explained in several ways: by extended expression of epiblast specific genes, which may retain the undifferentiated form of the stem cells for as long as possible; by cellular oncoproteins that may allow autonomous proliferation; or by cell proliferation dependent on the factors secreted by the uterus (16).

Some tumors show expression of trophoblast-specific antigens [for reviews see (17–19)], whereas normal human cytotrophoblast expresses 'functional tumor-associated genes', some of which are essential for the development of certain malignant diseases. These are mainly genes that take part in cell cycle regulation or epigenetic modification of the DNA. Changes in the expression of tumor suppressor genes were found in preeclamptic placentas, whereas aberrant methylation status is linked with gestational diseases of the trophoblast, such as mola hydatidosa and choriocarcinoma (20). Thus, in all these conditions, an important role in trophoblast invasiveness is played by DNA methylation, as the

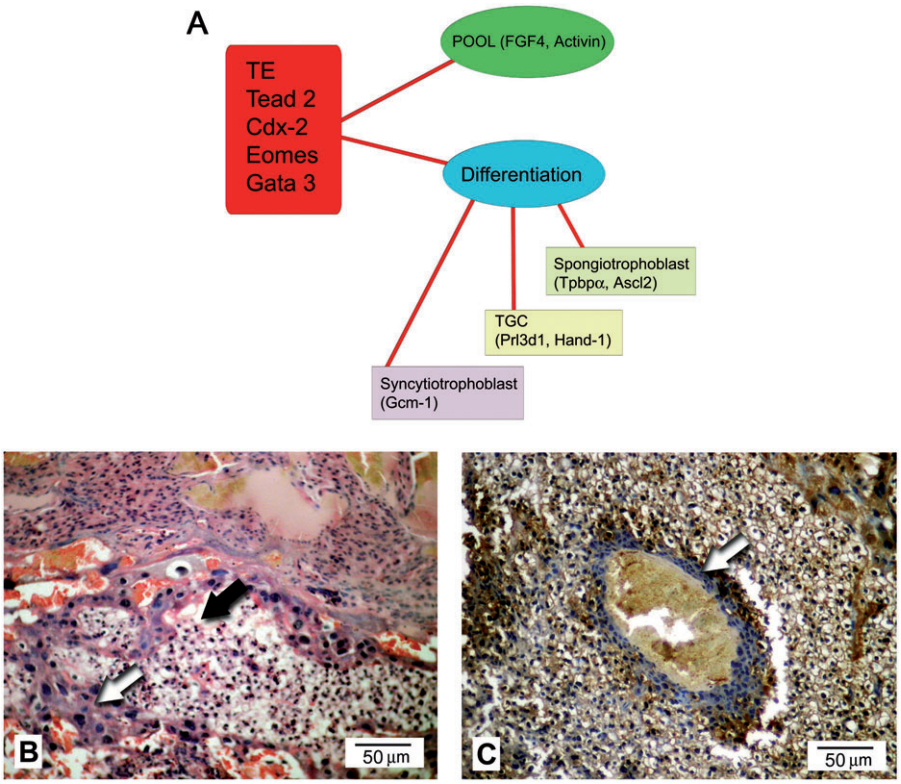

Figure 1 Trophoblast cell differentiation in rodents.
(A) Trophoblast cell differentiation in rodents. Trophoblast cells can proliferate and differentiate into spongiotrophoblasts (Tpbpα and Ascl2 markers), giant trophoblast cells (TGC; Prl3d1 and Hand-1 markers) or syncytiotrophoblasts (Gcm-1). A pool of trophoblast stem cells is retained, controlled by the action of specific growth factors. (B) Spongiotrophoblasts of the rat placenta, with glycogen containing cells (black arrow) and trophoblast giant cells (TGC) (white arrow). (C) Endovascular invasion of the mother's blood vessel by trophoblast cells (white arrow) (Fisher rat). Tpbpα, trophoblast specific protein alpha; Ascl2, Achaete-scute complex like 2; Prl3d, prolactin family 3, subfamily d, member 1; Hand1, heart and neural crest derivatives – expressed protein 1; Gcm1, glial cells missing homolog 1; TE, trophoectoderm; TEAD, TEA domain family member; Cdx-2, homeobox protein CDX-2; Eomes, eomesodermin; GATA3 – trans-acting T-cell-specific transcription factor.

inhibition of DNA methylation by demethylation agents such as 5-azacytidine (AZA), disrupts invasion and trophoblast migration potential (21). In hydatidiform moles, expression of four microRNAs (miRNA) (miR-517a, miR-517b, miR-518b, miR-519a) was found to be lower than in healthy placentas, pointing to their potential role in development of this disease (22). Namely, besides DNA and histone methylation, epigenetic regulation of gene expression can be accomplished with miRNAs. Primary transcipts of a miRNA gene are cleaved in the nucleus yielding pre-miRNA, which is exported in cytoplasm and further cleaved by Dicer to produce a double stranded RNA duplex (containing mature miRNA and its antisense strand) (23). Recent findings suggest that hypoxia, which is necessary for trophoblast differentiation, does not influence the expression of proteins involved in miRNA production. It is believed, however, that hypoxia influences the expression of individual miRNA genes directly, without affecting the protein machinery of miRNA synthesis (24). An example of miRNA-dependent regulation is the trophoblast-specific C19MC (chromosome 19 cluster) miRNA, which may play an important role in placento-maternal communication, thus ensuring adaptation of the mother to pregnancy (25). In addition, trophoblast-derived exosomes containing C19MC miRNA are hypothesized to be involved in immune response suppression during pregnancy (26).

Whereas the inactivation of tumor suppressor genes and epithelial to mesenchymal cell transition as mechanisms of trophoblast invasion discussed here are subject of intense study, some of the other key regulatory factors committing invasive trophoblast are still largely unknown. For example, immune regulation of trophoblast invasion and the action of various immunomodulatory factors have only recently started to gain attention. The definitive picture is far from clear but it points to a complicated network of cytokines, chemokines and growth factors that are strong inducers of trophoblast migration within the deciduas (27). Immune reactivity is modified during trophoblast invasion. For example, in order to attenuate immune response of the mother's T cells, placenta modifies the immune reactivity by producing trophoblast specific HLA molecules (28).

Expression of tumor suppressor genes in the trophoblast

Epigenetic mechanisms of cell invasion and proliferation control are utilized by both trophoblast and tumor tissues. Efficient trophoblast invasion into changing decidual tissue of the uterus is dependent on the changes in cell adhesion, specifically on the function and expression of adhesion junction molecules such as E-cadherin (29). Lowered E-cadherin expression has been documented in a number of human tumor metastases, as well as in trophoblast cells during invasion. Thus, epigenetic repression of tumor suppressor genes transcription could be a vital mechanism for controlling the invasion potential of both trophoblast tissue and malignant tumors. The reduction of functional E-cadherin could potentially be useful as an epigenetic biomarker in early identification of pathological pregnancies and cancers, and could also lead to new therapeutic strategies for those aiming to correct aberrant epigenetic states in various tissues (30).

RASSF1

In addition to E-cadherin, there are a number of other tumor suppressor genes that show similar methylation patterns and are expressed in cancer and trophoblast cells (Table 1). One of these is RASSF1 (31), a tumor suppressor gene whose promoter is hypermethylated in a large number of tumors, as well as in a placenta. The hypermethylated RASSF1 can be detected in a cell free fetal DNA (cffDNA) isolated from the mother's plasma using one of the recently developed prenatal non-invasive diagnostic tests (32). Thus, hipermethylation of this gene could serve as a universal marker of fetal DNA, independent of gender and genetic variation, and also potentially be valuable in early diagnosis of preeclampsia development (33).

APC

The APC gene, mutations of which are linked with familiar adenomatous polyposis, is another tumor suppressor that is hypermethylated in placental villi (34). The loss of function of APC is directly related to the invasiveness of colorectal neoplasms, making yet another potential link between regulation of invasiveness in human trophoblast and tumor tissues (35).

Maspin

Maspin (serpin B5) is a tumor suppressor gene that is differentially expressed in the human placenta, i.e., its expression rate grows in the early second trimester and stays high for the entire third trimester. Although its true role in a placenta is unknown, *in vitro* data indicate that it could be a regulator of trophoblast invasion (36). Hypermethylation of the CpG island maspin promoter is linked with the inhibition of its transcription in breast cancer (37) and ovarian cancer cell lines (38). Addition of AZA (DNA demethylation agent) into placental explants does not change its expression, whereas addition of trichostatin A (TSA) increases its expression up to 20 times, thus indicating that Maspin expression is regulated by histone modification rather than by DNA methylation (39).

KiSS-1

Metastasis suppressor gene KiSS-1 encodes the kisspeptin peptide and was initially discovered as a metastasis suppressor in human melanoma and breast cancer (its original name was metastin), followed by the discovery of the similar function in other tumors (40). It is hypothesized that KiSS-1 gene also has a role in the repression of trophoblast invasion, following binding with its receptor (Kiss-1R). In the human placenta, the expression of KiSS-1 and KiSS-1R is highest in the first trimester of gestation (12.5 day in the rat), a period

Table 1 Genes expressed in trophoblast and cancer cells.

Gene	Function/characteristics	References
Cdx2	Trophoblast stem cell marker	(7)
Oct4	Embryoblast cell marker	(8)
TEAD4, *Eomes*, *Elf5* and *GATA3*	Potential trophoblast stem cell markers, transcriptional factors	(10, 11)
Tpbpα, *Asc12*	Spongiotrophoblast cell markers	(12)
Prl3d1, *Hand-1*	Trophoblast giant cell markers	(12)
Gcm-1	Syncytiotrophoblast cell marker	(12)
RASSF1	Tumor suppressor gene whose promoter is hypermethylated in a placenta	(31)
APC	Tumor suppressor gene, hypermethylated in placental villi, linked with familiar adenomatous polyposis	(34)
Maspin (Serpin B5)	Tumor suppressor gene (its true role in a placenta is unknown, *in vitro* data indicate that it could be a regulator of trophoblast invasion)	(36)
KiSS-1	Metastasis suppressor gene, KiSS1 and its receptor are present in invasive trophoblast giant cells	(40)
NECC1 gene (also known as HOP-homeodomain-only protein)	Suppressor of choriocarcinoma	(42)
Wnt3A	Stimulates invasion and migration of trophoblast	(45)
TCF/LEF	β-catenin dependent transcription factors activated in invasive trophoblast	(49)
cycline D1, *c-myc*, *MMP-7* and *MT1-MMP*	Genes responsible for cell growth and invasion	(49)
SFRP	Gene familiy of Wnt signaling pathway antagonist, responsible for reducing activity of metalloproteinases and activation of β-catenin	(52, 53)
WIF1	Tumor suppressor gene (in the placenta, it is imprinted on maternal chromosome)	(34)
DKK	Wnt signaling pathway antagonist, causes reduction of cytotrophoblast proliferation	(50)
DLX4 homeotic gene	Preeclampsia susceptibility gene	(55)
STOX1	Preeclampsia susceptibility gene (paternally imprinted)	(56, 57)

Ascl2, Achaete-scute complex like 2; APC, adenomatous polyposis coli; DKK, Dickkopf; DLX4, distal-less homeobox 4; Eomes, eomesodermin; GATA3 – trans-acting T-cell-specific transcription factor; Gcm-1, glial cells missing homolog 1; Hand-1, heart and neural crest derivatives-expressed protein 1; NECC1, not expressed in choriocarcinoma clone; 1Prl3d, prolactin family 3, subfamily d, member 1; SFRP, secreted Frizzled-related protein; TEAD, TEA domain family member; Tpbpα, trophoblast specific protein alpha; WIF1, Wnt inhibitory factor 1.

when placental invasion is at its most intense. It is thought that KiSS1 is made by the syncytiotrophoblast cells and that its receptor is expressed on the extravillious trophoblast, indicating that the syncytiotrophoblast regulates invasion through paracrine signaling. In the rat, expression of KiSS1 and its receptor are present in invasive trophoblast giant cells (TGC) (41).

NECC1

The villi of a healthy placenta express the NECC1 gene (not expressed in choriocarcinoma clone 1; also known as HOP-homeodomain-only protein), which is a suppressor of choriocarcinoma, and therefore is not expressed in the choriocarcinoma cell lines (42). Its product is an ubiquitine protein expressed in the placenta and many other organs, such as lungs, smooth muscle cells, uterus, urinary bladder and kidneys (43). NECC1 expression is regulated by epigenetic mechanisms, because the treatment of cells with 5-azadeoxycytidine demethylation agent and trichostatin A histone acetylase inhibitor reactivates its expression in esophageal squamous cell carcinoma (44).

Epithelial-mesenchymal transition of trophoblast cells

Invasive trophoblast differentiation displays certain common characteristics with epithelial-mesenchymal transition (EMT), a process involved in early-stage malignant cell transformation (45). During EMT, epithelial cells undergo a series of biochemical changes that enable them to acquire the characteristics of mesenchymal cell phenotype, such as enhanced migratory capacity, invasiveness, decreased sensitivity to apoptosis, highly increased extracellular matrix (ECM) production (46) and decreased expression of cell-cell adhesion molecules (e.g., E-cadherin) (47).

Similar to EMT, invasive trophoblast shows a transient decrease in E-cadherin, as well as membrane bound β-catenin expression in proximal invasive anchoring villi. Areas of cell membranes that lack E-cadherin point to partial EMT in invasive trophoblast, a process that seems to be mediated by Wnt ligands and β-catenin/TCF signaling pathway activation (45). Wnt3A stimulates the invasion and migration of trophoblast (through matrigel), during which trophoblast cells lose the expression of epithelial polarizing markers (e.g., integrin α6β5), transiently lower E-cadherin expression, and increase the expression of matrix metalloproteinases and receptors

for fibronectin and collagens (such as integrins α5β1 and α1β1).

Wnt signaling pathway

The Wnt signaling pathway is important in a variety of biological processes during embryonal development, whereas its aberrant signaling contributes to cancer growth. It is a conserved pathway that is involved in the maintenance of cell polarirty and in cell proliferation control (48). The Wnt pathway molecules bind to heterodimer receptor composed of transmembrane proteins Frizzled membrane receptor and lipoprotein receptor-related protein 5/6 (Fzd/LRP) (49). Binding of Wnt ligand onto Fzd/LRP receptor causes the translocation of β-catenin into the nucleus and activation of β-catenin dependent transcription factors TCF/LEF. These, in turn, activate a number of genes responsible for cell growth and invasion, such as cycline D1, c-myc, MMP-7 and MT1-MMP. Accumulation of nuclear β-catenin is frequently seen in human epithelial carcinomas, as well as in invasive trophoblasts of the complete hydatidiform moles (45).

The Dickkopf (DKK) family of proteins, together with secreted Frizzled-related protein (SFRP) and Wnt inhibitory factor 1 (WIF1), function as Wnt signaling pathway antagonists. The addition of Dickkopf-1 results in the reduction of basal migration, invasion and cytotrophoblast proliferation (50). Whereas Dickkopf-1 reduces invasive growth of cultured human cell lines, it has an opposite effect when secreted by decidual cell in the mouse, where it enhances trophoblast invasion during placentation (51).

The Wnt pathway antogonists DKK proteins and sFRP show a reduced expression in several types of carcinomas, which is frequently associated with an unfavorable clinical outcome. They are thought to be epigenetically regulated through DNA methylation, as melanoma cells treated with 5′-aza-deoxycitidine show reduced methylation in the first exon of the *SFRP* gene and, consequently, increased production of SFRP3 proteins (48). SFRP3 reduces the activity of metalloproteinases and activation of β-catenin and thus inhibits EMT seen in several cancer types (52). In the endometrium, increased levels of SFRP1 mRNA are seen in endometriosis, where SFRP1 seems to be responsible for induction of angiogenesis (53). All SFRP proteins contain highly conserved, cysteine rich, domain (CDR) with which they bind Wnt signaling molecules, resulting in the inhibition of Wnt signaling pathway (54). SFRP can also block Wnt pathway by binding to the Fzd receptor directly and forming inhibitory complex.

The EMT inhibition is seen also with WIF1, another secreted Wnt signaling pathway antagonist. Expression of WIF1 is reduced in most prostate cancer cell lines, due to the hypermethylation of its promoter. Its ectopic expression in prostate cancer bone metastases results in increased expression of epithelial cell markers (E-cadherin, keratins 8 and 18) and decreased expression of mesenchymal cell markers (N-cadherin, fibronectin, vimentin), suggesting the reversal of EMT. In addition, WIF1 expression results in reduced motility and invasiveness in cultured cells and xenograft tumor growth (50). WIF1 is a tumor suppressor gene which, in the placenta, is imprinted on the maternal chromosome, so that only the paternal allele is active, and its promoter remains hypermethyated throughout gestation, from 7 weeks until birth (34).

EMT in preeclampsia and IUGR

Incomplete EMT has been linked with two important gestational diseases – preeclampsia and intrauterine growth restriction (IUGR). Trophoblast growth, migration and invasion into endometrium are essential for successful placentation and they all appear to be defective in both disorders.

One of the genes that significantly contributes to EMT in trophoblast tissue is the DLX4 (distal-less homeobox 4) homeotic gene. Its decreased expression results in reduced motility and invasiveness of choriocarcinoma cells (JEG-3 cell line) and in lower expression of E-cadherin. Therefore, low levels of DLX4 in preeclamptic placentas has been associated with the pathogenesis of preeclampsia due to its inhibition of EMT in trophoblast cells (55).

STOX1 is another preeclampsia susceptibility gene, identified in a population of Dutch families. Its allelic form Y153H has been associated with negative regulation of trophoblast invasion and with increased expression of adhesion protein α-T-catenin (56). It is paternally imprinted and maternally expressed and the expression is dependent on DNA methylation, which has been shown to take place in columns of extravillous trophoblast cells developing in the area of the anchoring villus (57).

Conclusion

The controlled invasive behavior of trophoblast cells allow us to broaden the understanding of cell invasion in normal and pathologic conditions, such as during embryonic development and tumor spread, respectively. Activation of oncogenes, inactivation of tumor suppressor genes and constellation of molecular and morphological changes that characterize epithelial mesenchymal transition in trophoblast cells are key to understanding the mechanisms of their invasion, and likely that of cancerous cells as well. Additionally, mechanisms that underlie uncontrolled trophoblast invasion, with resulting pathological placental development, add valuable insights into the mechanisms responsible for the transition of normal cells into malignant and metastatic ones.

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