Biochemical and Biophysical Research Communications 432 (2013) 116–122

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

5-Aza-dC treatment induces mesenchymal-to-epithelial transition in 1st trimester trophoblast cell line HTR8/SVneo

Ying Chen [a], Kai Wang [a,*], Richard Leach [a,b,*]

[a] Department of Obstetrics, Gynecology and Reproductive Biology, Michigan State University, Grand Rapids, MI 49503, United States
[b] Department of Obstetrics, Gynecology and Women's Health, Spectrum Health Medical Group, Grand Rapids, MI 49503, United States

ARTICLE INFO

Article history:
Received 17 January 2013
Available online 29 January 2013

Keywords:
DNA methylation
Epithelial-to-mesenchymal transition
Trophoblast invasion

ABSTRACT

Placental trophoblast invasion involves a cellular transition from epithelial to mesenchymal phenotype. Cytotrophoblasts undergo epithelial to mesenchymal transition (EMT) when differentiating into extravillous trophoblasts and gaining the capacity of invasion. In this research, we investigated the role of DNA methylation in trophoblasts during this EMT. First, using BeWo and HTR8/SVneo cell lines as models of cytotrophoblasts and extravillous trophoblasts, respectively, we analyzed the gene expression and DNA methylation status of the known epithelial marker genes, E-Cadherin and Cytokeratin7. We found that, in HTR8/SVneo cells, both genes were silenced and their promoters hypermethylated, as compared with the high-level gene expression and promoter hypomethylation observed in BeWo cells. This result suggests that dynamic DNA methylation of epithelial marker genes plays a critical role in the trophoblast EMT process. To verify these results, we treated HTR8/SVneo cells with 5-aza-dC, a known inhibitor of DNA methyltransferase, for three days. Five-Aza-dC treatment significantly increased the expression of epithelial marker genes and slightly decreased the expression of mesenchymal genes, as detected by qRT-PCR, immunocytochemistry and Western blot. Furthermore, 5-aza-dC treated HTR8/SVneo cells changed their morphology from mesenchymal into epithelial phenotype, indicating that 5-aza-dC induced mesenchymal to epithelial transition. Lastly, we examined the effect of 5-aza-dC on trophoblast migration and invasion capacity. We applied 5-aza-dC to HTR8/SVneo cells in trans-well cell migration and invasion assays and found that 5-aza-dC treatment decreased trophoblast migration and invasion capacity. In conclusion, DNA methylation of epithelial marker genes represents a molecular mechanism for the process of trophoblast EMT.

1. Introduction

Placental trophoblast invasion is associated with the transition from epithelial to mesenchymal phenotype [1,2]. In the tips of anchoring villi, cytotrophoblasts (CTs) undergo epithelial to mesenchymal transition (EMT) to differentiate into extravillous trophoblasts (EVTs), gaining the capacity to invade the decidua. Defective EMT in placental trophoblast is one of the pathologies associated with preeclampsia [2,3].

In the EMT of trophoblast invasion, silencing of epithelial genes and activation of mesenchymal genes are critical steps. E-Cadherin (gene name: CDH1) is a Ca^{2+} dependent cell–cell adhesion molecule expressed in epithelial cells. As a integral membrane glycoprotein linking to the cytoskeleton via its intracellular ligands, termed Catenins, E-Cadherin is thought to stabilize epithelial architecture. In the EMT of trophoblast invasion, down-regulation of E-Cadherin has been reported in both *in vivo* and *in vitro* trophoblast invasion models [4]. In that report, E-Cadherin is down-regulated in EVTs compared with CTs in placenta. Furthermore, it has been reported that up-regulation of E-Cadherin in EVTs is associated with the defective trophoblast EMT and shallow placentation seen in preeclamptic placenta [5]. Like E-Cadherin, Cytokeratin 7 (CK7, gene name: KRT7) is a known epithelial marker which acts as intermediate filaments within epithelia [6] and is highly expressed in placental trophoblasts.

DNA methylation is one of the major mechanisms of epigenetic gene regulation. It is well studied that DNA methylation can repress E-Cadherin expression via promotor hypermethylation, particularly in cancer cell EMT and metastasis [7,8]. DNA methylation status is maintained *in vivo* by DNA Methyltransferase (DNMTs). Treatment with 5-aza-dC, an inhibitor of DNMTs, has been shown to re-activate E-Cadherin expression in mesenchymal cancer cells and induce the transition from mesenchymal into epithelial morphology (MET) [9]. In a previous study, it has been reported that 5-aza-dC treatment induced E-Cadherin expression and decreased migration

* Corresponding authors at: Department of Obstetrics, Gynecology and Reproductive Biology, Michigan State University, Grand Rapids, MI 49503, United States.
E-mail addresses: kai.wang@hc.msu.edu (K. Wang), richard.leach@hc.msu.edu (R. Leach).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.075

Table 1
Primers for bisulfite genomic sequencing primers and luciferase reporter assays.

Gene symbol	NCBI GI/ID		Primer sequence (5′–3′)	Position to TSS
E-Cadherin	6615	OutF	GTTGATTGGTTGTGGTC(T)GGTAGGTG	−98
		InF	GAATTGTAAAGTATTTGTGAGTTTG	+11
		InR	AATACCTACAACAACAACAACAAC	+117
		OutR	CAAATCAAAAAATCCG(A)AAATACC	+194
CK7	6591	OutF	TTTTGTTTGGATTGAAAGTTTGG	−100
		InF	TTTTAGGAGGGGTTTGGTAGTAGAGAA	−40
		InR	TAACTAAACC(G/A)AAATAAACCTAAC	+100
		OutR	AAAACTAAAATAAATAAACATAATAACTA	+150
CK7 Promoter		F	CCAGGGCCTCAAGGCAACAG	−2000
		R	GGTGGCTGGGCCGGGATGGAC	+127

in the trophoblast cell line BeWo (CT-like) [10]. However, to date, no studies have been published examining the molecular mechanism of the 5-aza-dC treatment regulating EMT related genes in trophoblasts. Therefore, in this research, we investigated the DNA methylation status of the epithelial genes (E-Cadherin and CK7) in trophoblast EMT using the human cell lines, HTR8/SVneo (EVT-like) and BeWo (CT-like), as a trophoblast EMT model. This study shows that 5-aza-dC treatment in HTR8/SVneo cells induces a mesenchymal to epithelial transition (MET) change and suggests that DNA methylation plays a critical role in EMT.

2. Materials and methods

2.1. Cell culture and 5-aza-dC treatment

Two trophoblast cell lines, BeWo (ATCC) and HTR8/SVneo (gift from Dr. C. Graham), were cultured in DMEM/F12 supplemented with 10% FBS, 2 mmol/L L-glutamine and 1% Pen/Strep. For 5-aza-dC treatment, 5×10^3 BeWo and HTR8/SVneo cells were plated in wells of 24-well dishes before treatment. Doses ranging from 0.1 to 5 μM of 5-aza-dC (Sigma) were added to the culture media for 3 days. Medium was refreshed every other day for 3 days prior to harvesting cells for RNA, DNA and protein analysis.

2.2. Sodium bisulfite genomic sequencing

Genomic DNA from cultured cells was extracted as previously described [11]. In brief, the DNA methylation-Gold kit (Zymo Research) was used according to manufacturer's instructions to bisulfite treat genomic DNA, followed by amplification via nested PCR (2 rounds of 30 cycles) using primers listed in Table 1. PCR products were then cloned into pTopo TA vector (Invitrogen), and ten to twelve clones from each sample were selected for sequencing.

2.3. Combined bisulfite restriction analysis (COBRA) of E-Cadherin and CK7 proximal promoter regions

For E-Cadherin and CK7 proximal region, 10 μl of PCR products was added to a 10 μl of mixture containing 1 μL of HinfI or *BsuUI* (5 units) (NEB) and 2 μL of restriction buffer (10×). After gentle mixing, the mixtures were incubated for overnight at 37 °C (for *HinfI*) and 65 °C (for *BstUI*) respectively. After running these digested PCR products on a 2% agarose gel, the images were analyzed using Quantity One software (Bio-Rad).

2.4. Luciferase reporter assay

Luciferase reporter assay was performed according to published procedure [12]. Briefly, CK7 gene promoter regions, −2000 to +127 relative to its transcription start site (TSS), was cloned into pGL3-Basic vector (Promega), upstream of firefly luciferase coding sequence via PCR and subsequent ligation. Primers are listed in Table 1. *In vitro* methylation of pGL3-CK7 plasmids was performed by incubation with *Sss*I methylase (New England Labs) for 3 h. The resistance of methylated plasmids to *BstUI* digestion was used to verify the success of *in vitro* methylation. HTR8/SVneo cells plated at 5×10^4 cells per well in a 96-well plate were transiently transfected with 0.1 μg of methylated and non-methylated plasmids, along with 0.005 μg of the internal control plasmid (pRL-TK vector, expressing *Renilla* luciferase using the Lipofectimine 2000 transfection reagent (Invitrogen, CA). Twenty four hour post-transfection, the activities of both luciferases were determined using the Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. Assays were performed 3 times each in duplicate and promoter activity determined by the ratio of the two luciferase activities.

2.5. Invasion and migration assay

2×10^4 HTR8/SVneo cells were seeded in the upper side of transwell chambers with or without Matrigel-coating (BD Biosciences) for invasion and migration assay respectively. The medium used in upper well was: DMEM/F12 with 0.1% BSA with or without 2.5 μM 5-aza-dC whereas in bottom well: DMEM/F12 with 20% FBS. 22 h later, cells were stained with 0.09% Crystal violet solution. Non-invading/migrating cells on the upper side of the

Table 2
Primers for quantitative RT-PCR.

Gene symbol	NCBI GI/ID		Primer sequence (5′–3′)	Position
E-Cadherin	GI:169790842	F	TGACTACTTGAACGAATGGG	2701
		R	GGAAGGGAGCTGAAAAACCAC	2885
Beta-Catenin	GI:148233337	F	GTTCGCCTTCACTATGGACT	1721
		R	AGGTGCATGATTTGCGGGAC	1846
KRT7	GI:67782364	F	GCCAGTCGCAGGAGTGCCCG	1511
		R	TGGGACCAGGCATGGGAGGC	1620
VIM	GI:240849334	F	GACGGTTGAAACTAGAGATGGAC	1748
		R	CTTGCGCTCCTGAAAAACTGC	1925
DSC3	GI:148539845	F	GGAAGAGGATCTCCAGCTGG	2721
		R	ATTGTAGCACTGTGACATTATCTC	2863
JGD4	GI:145699104	F	AGGAAGTCTGAGTGGGTGTG	1251
		R	TCAAACGATCCTCCTGCCTG	1353
SPTAN1	GI:306966130	F	CTCTCACTTTCCACTGTAACCT	7649
		R	CCCCACAGTGACATGATGATTG	7762
Actin	GI:168480144	F	CAGCAGATGTGGATCAGCAAG	1141
		R	TTGTCAAGAAAGGGTGTAACGC	1252
Cadherin18	NM_001167667	F	GAGAGATGGGCGTGTGCGGA	2201
		R	TTGCCAGGAGAATGAGAACAC	2310