Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

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

Document heading doi: 10.1016/S1995-7645(14)60093-6

Tissue factor expression and methylation regulation in differentiation of embryonic stem cells into trophoblast

Lin-Xin Liu, Hui Zeng, En-Yi Liu, Fang-Ping Chen*

Department of Hematology, Xiangya Hospital of Central South University, Changsha 410008, China

ARTICLE INFO

Article history:
Received 10 January 2014
Received in revised form 15 March 2014
Accepted 15 June 2014
Available online 20 July 2014

Keywords:
Embryonic stem cells
Trophoblast cell
Tissue factor
Methylation

ABSTRACT

Objective: To explore tissue factor (TF) expression and methylation regulation in differentiation of human embryonic stem cells (hESCs) into trophoblast. **Methods:** Differentiation of hESCs into trophoblast was induced by bone morphogenetic protein 4 (BMP4). Expression of gene, protein of TF and DNA methylation at different time points during induction process was detected by RT-PCT, Western blot, flow cytometry and MSP-PCR method. **Results:** The expression of mRNA, protein level of TF could be detected during directional differentiation of hESCs to trophoblast cells, semi methylation-semi non methylation expression appeared at TF DNA promoter region, and it showed decreased methylation level and increased non methylation level with formation of trophoblast cell and increased expression of TF. **Conclusions:** It shows that during differentiation of hESCs into trophoblast, the differential expression of TF is related with DNA methylation level, and it is changed with the methylation or non methylated degree. It provids new platform to furtherly explore the regulation mechanisms of specific expression of tissue factor in the process of the embryonic stem cell development.

1. Introduction

Balance of thrombosis and haemostasis is important in the process of development. This balance plays an important role in the protection of the normal development of embry in the womb before the formation of embryonic blood vessel and blood cell. The fertilized egg cells are differentiated into syncytiotrophoblast cells, which have high procoagulant activity so that a slight bit of bleeding in placental could be well controlled. And It is of important significance for the prevention of abortion and pregnancy massive haemorrhage[1,2]. It is also found that high procoagulant activity of syncytiotrophoblast cells are mainly due to the high expression of tissue factor (TF)[3,4].

TF is also known as coagulation factor Ⅲ, CD142, which is a membrane glycoprotein containing 263 amino acid residues. The extrinsic pathway of blood coagulation has been proved in recent ten years, and TF as the promoter of the extrinsic pathway of coagulation is considered as the most important initiation course for the physiological or pathological coagulation reaction[5,6]. TF has specific expression in perivascular vascular tissue and other important organs, such as the brain, heart and lung, while it is almost not expressed in mature blood cells, which plays an important role in the maintenance of the coagulation balance and protection of important organs[6,7]. Previous researches found that the transcription factor NF-κB, AP-1, Egr-1 were involved in the regulation of TF gene expression[8-10], but as inflammation related transcription factors, they were often not activated in normal tissues, and only mediated expression of TF in inflammatory reaction. The transcription factor Sp1 can mediate TF expression[11,12]. Sp1 is a typical housekeeping gene and is expressed widely in various tissues and cells, and it can not determine the tissue specific expression of TF. Transcription factor associated with specificity expression of TF tissue is still not founded. Therefore we assume the tissue specificity expression of TF is regulated by the regulation mode except transcription level regulation.

Epigenetics also known as post transcriptional regulation, refers to gene expression changes based on non genomic sequence. In the study of epigenetics, DNA methylation is one of the earliest discovered 13 modification pathways[13].

*Corresponding author: Fang-Ping Chen, Ph.D., Chief Physician, Department of Hematology, Xiangya Hospital of Central South University, Changsha 410008, China.

Foundation project: This research is supported by National Natural Science Fund Project (No. 81170477).

In the organism development process, as a dynamic epigenetic marks, DNA methylation not only can directing gene expression stability but also can induce methylation or demethylation according to the development and can regulate gene expression[14].

Human embryonic stem cells (hESCs) have two major characteristics: multi-directional differentiation potential and maintenance of dryness. Therefore it becomes the important experimental materials of simulation tissue and organ development. Xu *et al*[15] used inhibitors of basic fibroblast growth factor and bone morphogenetic protein (BMP) signal pathway to preserve and amplify embryonic stem cells *in vitro*, and firstly induced hESCs differentiation to the trophoblast cells by BMP4[16]. Based on the above research, this experiment induced hESCs differentiation to the trophoblast cells by BMP4 and explore the expression of TF gene and methylation during the differentiation and development.

2. Materials and methods

2.1. Main reagent and the cells

Matrixgel was purchased from Sigma Company; BMP4 was from Invitrogen Company; Rat anti TROMA-1 monoclonal antibody was from Developmental Studies Hybridoma Company; CD142-PE was purchased from BD Company; Rabbit anti TF monoclonal antibody was from Abcam Company; Mouse embryonic fibroblasts separated from ICR fetal rats with gestation 13.5 days from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. hESCs cell line H9 was gifted by professor Liang from USA National Stem Cell Bank, the State Key Medical Genetics Laboratory.

2.2. Trophoblast cells induction and differentiation

Conditioned medium was used according to the method of Xu[17-19], hESCs clones were inoculated to Matrixgel coated medium. Every other day fresh conditioned medium was replaced and added with BMP4 cytokines. Until on the 5th d, cell surface marker (immunofluorescence) was detected by reverse transcription polymerase chain reaction (RT-PCR) and methylation specific PCR (MSP-PCR).

2.3. Immunofluorescence test

Firstly cells were fixed with 4% paraformaldehyde for 30 min, added with PBS solution containing 5% FBS and 0.4% Triton for 5 min at room temperature. They were washed for 3 times with PBS-T solution and added with rat anti Troma-1 antibody diluted by protocol incubating for 1 h in 37 ℃, then the cells were washed 3 times with PBS-T. Second anti was added with the fluorescein incubation for 40 min in 37 ℃ under dark, then they were washed 3 times with PBS-T, mounted by 95% glycerol. They were observed and photographed under fluorescence microscopy (Olympus Company).

2.4. RT-PCR

RNA was obteained through splitting decomposition and differentiation the cells in different days by TRIzoI lysate (Invitrogen Company), then cDNA was synthesized by reverse transcription by ThermoScript Kit (Invitrogen Company). Concentration of cDNA was adjusted and specific primers were added in the following conditions (Table 1). The related gene expression was detected by PCR. At first they were denaturated 5 min at 95 ℃, then denaturated 30 s at 94 ℃, annealed for 30 s, at 60 ℃, extended for 30 s at 72 ℃, 32 cycle, eventually reached 10 min at 72 ℃, Samples were added to 1.5% agarose gel electrophoresis, electrophoresis with 80 V for 40 min. With GAPDH as control, molecular analyst software was used for semi quantitative analysis in the Gel Doc 1000 image analysis system (BioRad Company).

Table 1
Target gene primers of RT-PCR.

Target gene	Primer sequences
OCT4 forward	5′-TGACACTATAGAATGGGATATACAG-3′
OCT4 reverse	5′-CGACTCACTAGGGACACTCGGACCA-3′
CDX2 forward	5′-CCGAACAGGGACTTGTTTAGAG-3′
CDX2 reverse	5′-CTCTGGCTTGGATGTTACACAG-3′
TF forward	5′-ACG CTC CTG CTC GGC TGG GT-3′
TF reverse	5′-CGT CTG CTT CAC ATC CTT CA-3′
GAPDH forward	5′-AGCCACATGGCTCAGACAC-3′
GAPDH reverse	5′-GCCCAATACGACCAAATCC-3′

2.5. Counting detection with Flow cytometry

After the cells digestion, 5 mL PBS solution was added at room temperature, centrifuged for 5 min at 1 000 rpm to obtain cells resuspension. Cell concentration was adjusted into 1×10^6/mL. 300 mL cell suspension was put into isotype control antibody, 300 mL cell suspension were added with 20 μL CD142-PE streaming antibody, and were incubated for 30 min at room temperature in dark. After incubation they were added with 5 mL PBS solution at room temperature, centrifuged for 5 min at 1 000 rpm. Supernatant was discarded then 300 μL PBS solution was added for resuspension.

2.6. Western Blot detection

After cells digestion, they were washed with PBS and cells were resuspened. They were added with 0.5 mL cell lysate, protease inhibitor respectively and mixture. They were centrifuged for 10 min at 13 000 rpm after fast shocks 5 s at 4 ℃. According to the BioRad DC Protein Assay System quantitative results, the supernatant was obtained and added with 5×denaturation loading mixture at 100 ℃. Samples were obtained by full denaturation reaction for 10 min. Prepared gel was added into electrophoresis with buffer,

the positive and negative samples was connected, then the sample was placed into the hole. Electrophoresis at 80 V was performed with the separation gel, at 120 V with stacking gel. The PVDF film was soaked in methanol for 30 s then into 1×transfer film solution, the sample gel was placed and film was transferred for 1.5 h at 260 mA. PVDF film was taken out and put into closed liquid box for 2 h at 37 ℃, washed with TBS-T. First anti was added in box shaking overnight at 4 ℃, at next day they were washed 3 times with TBS-T, then added with second anti for shaking 1.5 h at 37 ℃. The marked PVDF film was added with 2 mL ECL liquid reaction for 4 min, then developed in the darkroom. With GAPDH as control, the film was scanned and the results were analyzed by gel imaging system and image analysis software.

2.7. MSP-PCR

After cells digestion DNA was extracted by DNA Extraction Kit (TIANGEN company). DNA of the sample was modified with sodium bisulfite by EZ DNA Methylation-Gold Kit™ (Zymo Research), then the sample concentration was adjusted. Specific primers were added in the following conditions (Table 2), and the related gene expression was detected by PCR. At first, they were denaturated for 3 min at 95 ℃, then denaturated for 30 s at 95 ℃, annealed for 30 s at 57 ℃, extended for 30 s at 72 ℃, 32 cycles, extended for 5min at 72 ℃ at last. The samples were added with 2% agarose gel electrophoresis, 80V 45 min. GAPDH as the control, they were analyzed by image analysis software.

Table 2

Target gene primers of MSP-PCR.

Target gene	Primer sequences
TF-LeftM forward	5′- GATATGGAGATTTTTGTTTGGTTTC -3′
TF-RightM reverse	5′- AAACTAATACCACTCACCTAAAACG -3′
TF-LeftU forward	5′- ATATGGAGATTTTTGTTTGGTTTTG -3′
TF-RightU reverse	5′- AACTAATACCACTCACCTAAAACACC -3′
GAPDH forward	5′-AGCCACATGGCTCAGACAC-3′
GAPDH reverse	5′-GCCCAATACGACCAAATCC-3′

3. Results

hESCs was cultivated and amplified according to the methods of Xu *et al* (Figure 1), the hESCs clones were inoculated on Matrix glue trophoblast and induced to the differentiation by adding the BMP4 cell factor. Till fifth day, it showed obvious "fish eye" shaped cells, which was morphology consistent, and the size was bigger than hESCs, and the clonal boundary was clearly visible (Figure 2A). Expression of the specific marker TROMA-1 of trophoblast cell was observed by immunofluorescence staining (Figure 2B). Differentiation cells was digested respectively on 0 day and 5 day, and total RNA was extracted and detected by RT-PCR. The results showed high expression of OCT4 gene and no expression of CDX2 gene in cells on 0 day, and it remained undifferentiated characteristics of the hESCs cells. On 5 days, there was no expression of OCT4 gene and high expression of CDX2 gene in the cells, which showed cells lost undifferentiated characteristics of the stem cell induced by BMP4. Specific gene expression of CDX2 suggested that trophoblast cells were induced differentiation successfully (Figure 3).

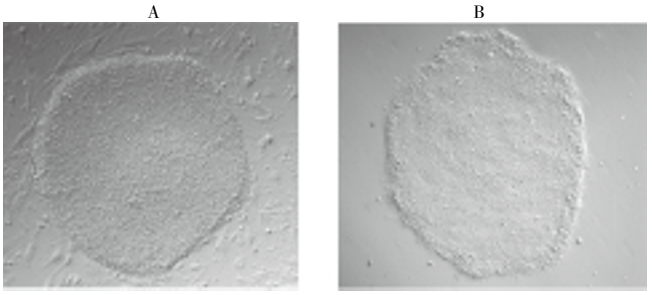

Figure 1. HESCs amplification *in vitro*.
A: The embryonic stem cell morphology of MEF trophoblast culture; B: Embryonic stem cells morphologic cultured with Matrixgel.

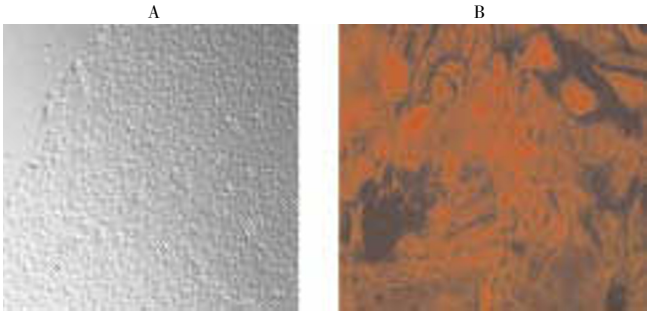

Figure 2. hESCs of BMP4 treated after 5 days.
A: Cell morphology of BMP4 treated after 5 days (40×); B: TROMA-1 expression of BMP4 treatmed after 5 days (red fluorescence, 200×) .

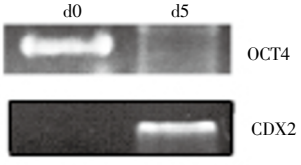

Figure 3. Embryonic stem cell pluripotency markers(OCT-4) and trophoblast cell specific markers(CDX2) in process of HESCs differentiation to the trophoblast cells (RT-PCR).

Differentiation cells were collected, TF gene and protein expression level were detected by RT-PCR, Western blot and flow cytometry. At the same time, methylation degree and its change of TF gene promoter DNA regional were measured by specific MSP-PCR. The results showed no expression of TF in trophoblast cells at 0 day and TF high expression when they were differentiated and induced on 5 day (Figure 4). But on 0 day half and non-half methylation expression of DNA promoter of TF gene was observed, non methylated degree was increased with decreasing methylation level in the

formation of trophoblast cells (Figure 5).

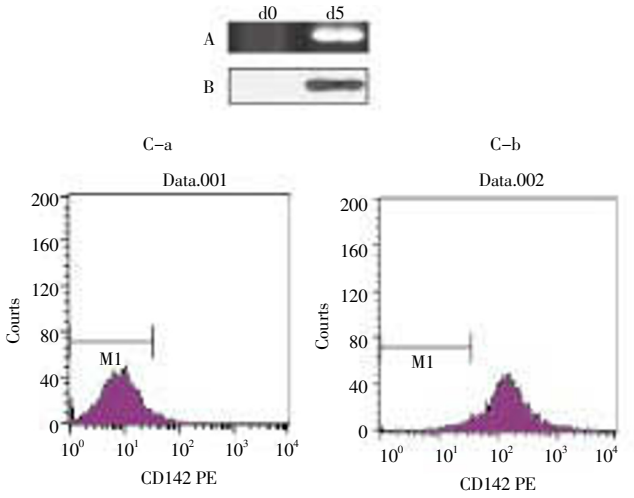

Figure 4. Expression change of TF in process of hESCs was induced to the trophoblast cells differentiation.
A. Expression change of TF gene; B. The expression change of TF protein level; C. The expression change of TF (CD142); C-a: CD142 expression of BMP4 untreated; C-b: CD142 expression of BMP4 treated after 5 days.

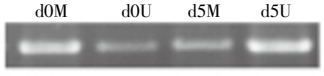

Figure 5. Methylation level of TF promoter region in process of HESCs differentiation to the trophoblast cells.
U represents as the non methylation level, M represents as the methylation level.

4. Discussion

Epigenetics includes DNA methylation, histone acetylation, genomic imprinting and X-chromosome inactivation, *etc*[13], which plays a key role in the development and differentiation of various cells and organisms. The apparent genetic status would affect the environment changes and the organism aging, which induces different biological characteristics[20,21].

During organism development, DNA methylation not only leads to changes of chromatin structure, but also causes the changes of DNA conformation, DNA stability and DNA protein interaction[14]. DNA methylation is important to regulate genome function, it is also the base for the establishing and maintaining other epigenetic phenomena[22]. In October in 2000, Human Epigenome Consortium launched the human epigenome project to study DNA methylation patterns of human tissues[23,24]. DNA methyltransferase is associated with DNA methylation[25]. The America research team found that DNA methyltransferase 3a (Dnmt3a) gene can induce methylation of some genes to maintain the stem cell characteristics. It can cause corresponding protein expression and the transcription of RNA blocked. By this way, gene inactivation and differentiation, Dnmt3a gene loss or mutation could lead to hematopoietic blood stem cells differentiation barriers[26]. It is also reported that discovery of DNA transferase 1 on hematopoietic stem cell and its premise cells also plays an important regulating role[27].

A large number of studies showed that epigenetics plays an important role in maintaining the function of the trophoblast and placenta[28-30]. Dnmt3L knockout female mice (Dnmt3Lmal-/-) after pregnancy, fetal rat died before the mid of pregnant, which showed that DNA methyltransferase Dnmt3L plays an important role in the maternal establishment of methylation imprint[31,32], while the defects of Dnmt3Lm-/- placenta is mainly induced by imprinted methylation abnormally regulated by DNMT3L[33]. Studies had found that human growth hormone gene activation plays an important role in the formation of human trophoblast cells into the cells of syncytiotrophoblast, and histone methylation and acetylation induced activation play the key role in the process of activation induced programmed[34,35]. In addition, it is reported that imprinted genes of placenta plays an important role in trophoblast cells proliferation, differentiation, angiogenesis and placental nutrient transport function[36]. For example, IGF2 imprinted genes is very important for the nutrient transport of placenta and nutritional needs of the fetal[37]. Epigenetic regulation disorders could also lead to pregnancy related diseases[38,39], such as intrauterine growth delay[40], preeclampsia[41], and etc.

DNA methylation and other epigenetic factors have influence on the regulation of gene expression[42], but it has not been reported if TF is regulated by epigenetic regulation during differentiation of hESC into trophoblast cells. Study on methylation specific PCR on differentiation of TF gene proved that TF expression is related with DNA promoter region methylation level in differentiation of hESC into into trophoblast cells, and it is changed with the methylation or non methylated degree. But MSP is only a rough study if the expression from the DNA promoter region methylation exists. Accurate detection of the methylation level, and regulation of the expression of TF and other epigenetic factors still need further study.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Lanir N, Aharon A, Brenner B. Procoagulant and anticoagulant mechanisms in human placenta. *Semin Thromb Hemost* 2003; **29**(2):175-184.
[2] Aharon A, Brenner B, Katz T, Miyagi Y, Lanir N. Tissue factor and tissue factor pathway inhibitor levels in trophoblast cells: implications for placental hemostasis. *Thromb Haemost* 2004; **92**(4):776-786.
[3] Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of bolld coagulation in hemostasis and thrombosis. *Arterioscler Thromb Vasc Biol* 2007; 27(8):1687-1693.

[4] Tilley R, Mackman N. Tissue factor in haemostasis and thrombosis. *Semin Thromb Hemost* 2006; **32**(1): 5–10.
[5] Davidson EH, Rast JP, Oliveri P, Ransick A, Calestani C, Yuh CH, et al. A genomic regulatory network for development. *Science* 2002; **295**(5560): 1669–1678.
[6] Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* 2007; **447**(7143): 425–432.
[7] Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001; **293**(5532): 1089–1093.
[8] Mackman N. Regulation of the tissue factor gene. *Thromb Haemost* 1997; **78**(1): 747–754.
[9] Bea F, Puolakkainen MH, McMillen T, Hudson FN, Mackman N, Kuo CC, et al. Chlamydia pneumonia induces tissue factor expression in mouse macrophages via actibation of Egr-1 and the MEK-ERK1/2 pathway. *Cir Res* 2003; **92**: 394.
[10]Mackman N. Role of tissue factor in hemostasis and thrombosis. *Blood Cells Mol Dis* 2006; **36**(2): 104–107.
[11]Li L, He S, Sun JM, Davie JR. Gene regulation by Sp1 and Sp3. *Biochem Cell Biol* 2004; **82**(4): 460–471.
[12]Wierstra I. Sp1: emerging roles-beyond constitutive activation of TATA-less housekeeping genes. *Biochem Biophys Res Commun* 2008; **372**(1): 1–13.
[13]Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001; **293**(5532): 1089–1093.
[14]Ann MB, Lena V, Shabnam K, Huska MR, Mancini E, Scheller M, et al. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. *Nat Genetics* 2009; **41**(1): 1207–1215.
[15]Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods* 2005; **2**(3): 185–190.
[16]Levenstein ME, Ludwig TE, Xu RH, Llanas RA, VanDenHeuvel-Kramer K, Manning D, et al. Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells* 2006; **24**(3): 568–574.
[17]Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, et al. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 2002; **20**(12): 1261–1264.
[18]Xu RH. *In vitro* induction of trophoblast from human embryonic stem cells. *Methods Mol Med* 2006; **121**(1): 189–202.
[19]Lin G, Martins-Taylor K, Xu RH. Human embryonic stem cell derivation, maintenance, and differentiation to trophoblast. *Methods Mol Biol* 2010; **636**(1): 1–24.
[20]Beerman I, Bock C, Garrison BS, Smith ZD, Gu H, Meissner A, et al. Proliferation-dependent alterations of the DNA methylation landscape underlie hematopoietic stem cell aging. *Cell Stem Cell* 2013; **12**(4): 413–425.
[21]Herzog E, Galvez J, Roks A, Stolk L, Verbiest M, Eilers P, et al. Tissue-specific DNA methylation profiles in Newborns. *Clin Epigenetics* 2013; **5**(1): 8.
[22]Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 2003; **33**(1): 245–254.
[23]Bradbury J. Human epigenome project-up and running. *PLoS Biol* 2003; **1**(3): E82.
[24]Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, et al. DNA methylation profiling of human chromosames 6, 20 and 22. *Nat Genet* 2006; **38**(12): 1378–1385.
[25]Haggarty P, Hoad G, Horgan GW, Campbell DM. DNA methyltransferase candidate polymorphisms, imprinting methylation, and birth outcome. *PLos One* 2013; **8**(7). DOI: 10.1371/journal.pone.0068896
[26]Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genetics* 2011; **44**(1): 23–31.
[27]Jennifer JT, Jonathan WS, Jonghwan K. DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells. *Cell Stem Cell* 2009; **5**(4234): 442–449.
[28]Novakovic B, Gordon L, Wong NC, Moffett A, Manuelpillai U, Craig JM, et al. Wide-ranging DNA methylation differences of primary trophoblast cell populations and derived cell lines: implications and opportunities for understanding trophoblast function. *Mol Hum Reprod* 2011; **17**(6): 344–353.
[29]Hogg K, Price EM, Robinson WP. Improved reporting of DNA methylation data derived from studies of the human placenta. *Epigenetics* 2014; **6**(3): 333–337.
[30]Schroeder DI, Blair JD, Lott P, Yu HO, Hong D, Crary F, et al. The human placenta methylome. *Proc Natl Acad Sci USA* 2013; **110**(5): 6037–6042.
[31]Rugg-Gunn PJ. Epigenetic features of the mouse trophoblast. *Reprod Biomed Online* 2012; **25**(1): 21–30.
[32]Hata K, Okano M, Lei H, Li E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development* 2002; **129**(8): 1983–1993.
[33]Arima T, Hata K, Tanaka S, Kusumi M, Li E, Kato K, et al. Loss of the matemal imprint in Dnmt3Lmat-/- mice leads to a differentiation defect in the extraembryonic tissue. *Dev Biol* 2006; **297**(2): 361–373.
[34]Kimura AP, Sizova D, Handwerger S, Cooke NE, Liebhaber SA. Epigenetic activation of the human growth hormone gene cluster during placental cytotrophoblast differentiation. *Mol Cell Biol* 2007; **27**(18): 6555–6568.
[35]Nelissen EC, van Montfoort AP, Dumoulin JC, Daunay A, Evers JL, Tost J. Epigenetics and the placenta. *Hum Reprod Update* 2011; **17**(3): 397–147.
[36]Lambertini L. Genomic imprinting: sensing the environment and driving the fetal growth. *Curr Opin Pediatr* 2014; **26**(2): 237–242.
[37]St-Pierre J, Hivert MF, Perron P, Poirier P, Guay SP, Brisson D, et al. IGF2 DNA methylation is a modulator of newborn′s fetal growth and development. *Epigeneticss* 2012; **7**(10): 1125–1132.
[38]Koukoura O, Sifakis S, Spandidos DA. DNA methylation in the human placenta and fetal growth. *Mol Med Rep* 2012; **5**(4): 883–889.
[39]Tomizawa S, Sasaki H. Genomic imprinting and its relevance to congenital disease, infertility, molar pregnancy and induced pluripotent stem cell. *J Hum Genet* 2012; **57**(2): 84–91.
[40]Himes KP, Koppes E, Chaillet JR. Generalized disruption of inherited genomic imprints leads to wide-ranging placental defects and dysregulated fetal growth. *Dev Biol* 2013; **373**(1): 72–82.
[41]Anderson CM, Ralph JL, Wright ML, Linggi B, Ohm JE. DNA methylation as a biomarker for preeclampsia. *Biol Res Nurs* 2013; **61**(4): 235–241.
[42]Schroeder DI, LaSalle JM. How has the study of the human placenta aided our understanding of partially methylated genes? *Epigenomics* 2013; **5**(6): 645–654.