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

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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].

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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,