The role of DNA methyltransferases in smooth muscle cell phenotypic switch

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Abstract: Phenotypic switching of vascular smooth muscle cells (VSMCs) is a common pathological process of multiple cardiovascular diseases. DNA methylation plays a key role in atherosclerosis, but its mechanism is not clear. DNA methyltransferases (DNMT3a and DNMT1) could be involved in the development of phenotypic switching process of VSMCs. Therefore, the purpose of this research is to probe the action of DNA methyltransferases on phenotypic switching process of VSMCs. First, Treatment with platelet-derived growth factor-BB (PDGF-BB) induced human aorta VSMC (HA-VSMC) morphological changes from contractile to synthetic phenotype. EdU staining confirmed that PDGF-BB could induce HA-VSMC proliferation. PDGF-BB remarkably augmented the mRNA and protein level of proliferation marker gene Cyclin D1 and osteopontin (OPN) in HA-VSMCs. The mRNA and protein level of differentiation marker SM22 and SM-α-actin were obviously upregulated in HA-VSMCs after PDGF-BB treatment. Next, the level of DNMT1 and DNMT3a were examined in PDGF-BB-treated HA-VSMCs by RT-PCR and western blotting. The high level of DNMT1 and DNMT3a was observed in PDGF-BB-induced proliferation model. Therefore, DNMT3a and DNMT1 could be involved in phenotypic switch of vascular smooth muscle cells.

1. Introduction
Phenotypic switch of vascular smooth muscle cells (VSMCs) plays an important role in the initial stage of vascular proliferative disease. In adult tissue, VSMCs presents a physiological contractile phenotype, which is characterized by high expression of contractile markers and decreased proliferation and migration of cells[1]. After the injury to the vessel, VSMCs undergo phenotypic modulation from a contractile phenotype back to a proliferative phenotype, which is related to the significant decreased selective marker gene expression and the promoted proliferation and migration.

Platelet-derived growth factor-BB (PDGF-BB) is considered to be the key regulator of VSMC phenotypic switching, which stimulates phenotype regulation from differentiated phenotype to proliferative phenotype[2]. PDGF-BB is one of the most effective mitogens and chemokines in VSMCs. PDGF-BB has also been showed to activate a variety of signal transduction pathways, including extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT and p38 mitogen-activated protein kinase (MAPK) pathways by binding with PDGF receptor [3].

Epigenetic modification, such as DNA methylation, plays an essential role in the regulation of gene expression. De novo methyltransferases DNMT3A and DNMT3B mainly catalyze the methylation of unmodified DNA, and DNMT1 is essential to maintain CpG DNA methylation [4]. Hypomethylation of CpG islands in the promoter region leads to gene expression, while hypermethylation results in gene suppression.
silence. In this study, we investigated the role of DNA methyltransferase in phenotypic transformation of VSMCs\cite{5}.

2. Materials and methods

2.1. Cell culture

Human aortic VSMCs (HA-VSMCs) were preserved by our laboratory. HA-VSMCs were incubated in dulbecco's minimum essential medium-high glucose (DMEM-HG) containing 10% FBS and 1% penicillin-streptomycin, in humified air with 5% CO₂.

2.2. EDU assay

To explore the pro-proliferation role of PDGF-BB on HA-VSMCs, $1 \times 10^5$ cells were cultured with 40 ng/mL PDGF-BB for 24h, and then EdU (5-ethynyl-2'-deoxyuridine) incorporation test was carried out with Cell-Light EdU imaging detection kit. Mainly, the cells were incubated with EdU, followed by fixation and permeabilization. EdU was detected by click reaction using Alexa Fluor 488 azide. The nuclear DNA was visualized by DAPI. In the four random high power fields each well, the incorporation rate of EdU is expressed as the percentage of EdU positive nucleus in the total nucleus.

2.3. Real-time RT-PCR

Total RNA was extracted from VMSCs using Trizol reagent (Solarbio) according to the manufacturer’s instructions. RNA was reverse-transcribed into cDNA with random primers using M-MLV reverse transcriptase. Thermal cycling conditions were as follows: 1 cycle at 95°C for 2 min, 40 cycles at 95°C for 10 s, 60°C for 34s, and 95°C for 1min. The PCR primers used are as follows: CyclinD1: F-5’GCTGTGCATCTACACCGACAACTC’, R-5’TTGCGGATGATCTGTTTTGTCTCCT’; OPN: F-5’AGTACCCTGATGCTACAGACGG3’, R-5’CGTTCATAACTGTCCCTTCCCCAC3’, DNMT1: F-5’TATCCGAGGAGGGCTACCTGGGC3’, R-5’TGGGCTAGGTGAAGGTTCAGGC3’; DNMT3a: F-5’TATTGATGACGCAAGAGAGAC3’, R-5’GGGTGTTCCAGGTAAACATTGAG3’; GAPDH: F-5’ATGGAAATCCCATCACCATCTT-3’, R-5’CGCCCACTTGTATTGCG3’.

2.4. Western blotting

The total protein was extracted from cells using extraction buffer (0.5% Triton X-100, EDTA, phenylmethyl sulfonylfluoride, and complete protease inhibitors). Cell extracts were separated by SDS-PAGE and then proteins were transferred onto PVDF membranes. The membranes were blocked with 5% skim milk powder, probed with anti-β-actin (1:200 dilution, Santa Cruz), anti-Cyclin-D1 (1:600 dilution, Santa Cruz), anti-OPN (1:600 dilution, Santa Cruz), anti-DNMT1 (1:500 dilution, Santa Cruz) and anti-DNMT3a (1:500 dilution, Santa Cruz) antibodies overnight at 4°C, followed by incubation with IRDyeTM-800 conjugated secondary antibodies for 30 min at RT. After washing, protein bands were visualized using an Odyssey infrared imaging system (Gene Company, Lincoln, NE, USA).

2.5 Statistical analysis

Data were expressed as the means ± standard deviation, and subsequently analyzed by t-test. The difference was statistically significant (P < 0.05).
3. Results

3.1. PDGF-BB promoted the proliferation of HA-VSMCs confirmed by EdU staining
To explore the pro-proliferative role of PDGF-BB in HA-VSMCs, 40 ng/mL PDGF-BB was used to treat HA-VSMCs for 24 hours, and EdU staining was carried out. As shown in Figure 1, PDGF-BB-treated group had more EdU positive cells compared to control group, suggesting that smooth muscle cells stimulated with PDGF-BB are in a proliferative state. The results showed that PDGF-BB could induce the proliferation of HA-VSMCs.

![Figure 1. PDGF-BB promoted the proliferation of HA-VSMCs confirmed by EdU staining](image)

3.2. PDGF-BB induced the upregulation of proliferative markers CyclinD1 and OPN in HA-VSMCs
In the early stages of atheromatosis, vascular smooth muscle cells undergo the loss of contract markers and gain of uncontrolled proliferative activity. Cyclin D1 is a cell cycle regulator necessary for G1 phase progression and is also a marker of SMC proliferation\(^6\). OPN is a pleiotropic cytokine involved in SMC proliferation, which is upregulated in phenotypic switch of SMCs from a contract phenotype to a pathological synthetic phenotype\(^7\). To study the effect of PDGF-BB on the cyclin D1 and OPN, HA-VSMCs were incubated with 40ng/mL PDGF-BB for 48h, and then the RNA and protein levels were tested by RT-PCR and western blotting. The increased mRNA and protein level of cyclin D1 and OPN were observed in PDGF-BB-stimulated HA-VSMCs (Figure. 2). These data further illustrated that PDGF-BB could stimulate the proliferation of SMCs.

![Figure 2. PDGF-BB could induce the upregulation of proliferative markers CyclinD1 and OPN in HA-VSMCs.](image)

3.3. PDGF-BB inhibited the expression of differentiated markers SM-α-actin and SM22 in HA-VSMCs
To investigate the effect of PDGF-BB on the expression of differentiated markers SM22 and SM-α-actin, HA-VSMCs were incubation with 40 ng/mL PDGF-BB for 48 h, and then the mRNA and protein level of SM22 and SM-α-actin were detected by RT-PCR and western blotting. As shown in
Figure 3, the decreased level of SM22 and SM-α-actin were observed in HA-VSMCs stimulated by PDGF-BB, suggesting the loss of differentiation phenotype.

![Graph showing the decreased level of SM22 and SM-α-actin](image)

Figure 3. PDGF-BB inhibited the expression of differentiated markers SM22 and SM-α-actin in HA-VSMCs. (a) The mRNA level of SM22 and SM-α-actin were tested by RT-PCR. (b) The protein level of SM22 and SM-α-actin were detected by western blotting.

3.4. The expression of DNMT3a and DNMT1 were upregulated in PDGF-BB-processed HA-VSMCs

To investigate the effect of PDGF-BB on the expression of DNMT1 and DNMT3a, the mRNA level of DNMT1 and DNMT3a were tested in PDGF-BB-treated HA-VSMCs by RT-PCR and the protein expression were detected by western blotting. The data were illustrated in Figure 4. Treatment with PDGF-BB could lead to the added expression of DNMT3a and DNMT1 in HA-VSMCs. These result illustrated that DNMT1 and DNMT3a could play important roles in phenotypic switch of smooth muscle cells.

![Graph showing the level of DNMT3a and DNMT1](image)

Figure 4. The level of DNMT3a and DNMT1 were upregulated in PDGF-BB-treated HA-VSMCs. (a) The mRNA level of DNMT3a and DNMT1 were tested by RT-PCR. (b) The protein level of DNMT3a and DNMT1 were detected by western blotting.

4. Conclusion

PDGF-BB could promote the proliferation of SMCs and inhibit its differentiation, confirmed by markedly upregulating the expression of SMC proliferative markers, such as cyclin D1, OPN and PCNA and downregulating the expression of SMC contract markers, including calponin, αSMA and SM22 in HA-VSMCs [8]. Similar to other studies, we found PDGF-BB could induce the proliferation of SMCs. DNA methylation happens on the fifth carbon of cytosine through DNA methyltransferase enzymes (DNMT1, several DNMT3B isoforms, and two isoforms of DNMT3A) [9]. CpG methylation of key regions can regulate gene expression. Our results showed that DNMT1 and DNMT3a could play essential roles in phenotypic switch of smooth muscle cells. The increased expression of DNMT1 and DNMT3a in PDGF-BB-treated HA-VSMCs could be associated with the decrease of differentiation marker express.

5. Discussion

The abnormal proliferation and migration of VSMCs is a common pathological feature of the occurrence and development of various cardiovascular diseases, such as pulmonary hypertension, hypertension, atherosclerosis, et al, and VSMCs phenotype transformation plays a key role in the process of VSMCs proliferation and migration. Therefore, understanding the mechanism of VSMC
phenotype transformation is of great significance for the prevention and treatment of cardiovascular diseases. The influencing factors of phenotype transformation of VSMCs include the surrounding environment, including mechanical force, extracellular matrix, neuroregulation, cytokines and their inhibitors. Cytokines can also activate different signaling pathways to induce the phenotype transformation of VSMCs, but the specific mechanism needs further study.

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