Journal of Animal and Veterinary Advances 13 (12): 706-710, 2014
ISSN: 1680-5593

Effects of Nelin Gene Expression on Phenotypic Switch of Vascular Smooth Muscle Cells

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Abstract: To observe the effects of Nelin gene expression on phenotypic switch of Vascular Smooth Muscle Cells (VSMC). VSMC were induced from synthetic phenotype to contractile phenotype by serum starvation and Nelin mRNA expression levels of different phenotypes of VSMC were checked by using RT-PCR. siRNA was then used to silence Nelin, coomassie blue staining and Acetylcholine (Ach) stimulation were used to observe the changes of VSMC cytoskeleton and contractile functions. Nelin mRNA expression in serum stimulation and serum starvation at 24, 48 and 72 h group was 0.1940+0.0971, 0.7114+0.1265, 0.7270+0.1026 and 0.7841+0.1240. The difference was statistically significant ($p<0.05$). In serum starvation process, VSMC cytoskeleton was reconstructed into uniform, dense bundles from sparse fuzzy networks and regained contractile function. Serum starvation-induced cytoskeletal remodeling was blocked and Ach-induced cell contraction disappeared when Nelin expression was suppressed.

Key words: Nelin, vascular smooth muscle cells, phenotypic switch, cytoskeleton, acetylcholine

INTRODUCTION

Nelin gene (GenBank Accession No. AF114264) (Wang *et al*., 2005) was cloned in 1999 on the base of expressed sequence tags and heart cDNA library. Literature suggests (Zhao *et al*., 2001) Nelin is a muscle-specific gene involved in stress fibers and focal adhesion formation and plays an important role in the regulation of cell motility, migration and adhesion. According to relevant characteristics of the gene, researchers hypothesize that Nelin down regulation might be relevant to the occurrence of varicose veins.

Varicose veins are a common clinical venous disease (Kucukguven and Khalil, 2013) data shows that many factors are related to the occurrence of varicose veins such as weakness of vascular wall (Xiao *et al*., 2009) abnormal changes in the extracellular matrix (Jacob *et al*., 2001) venous defects (Karatepe *et al*., 2010), etc. But the exact role that these factors play remains unclear. Baumbach and Heistad (1989) proposed the vascular remodeling concept for the first time in 1989. Since, then other literature suggests that vascular remodeling process is extremely complex and may be related to growth factors, vasoactive substances, hemodynamic stimuli and other factors which involves integrin-mediated intracellular signal transduction. The literature also shows (Morrow *et al*., 2008) the phenotypic switch of vascular smooth muscle cells (Vascular Smooth Muscle Cell, VSMC) from the contractile to synthetic phenotype is the key of vascular remodeling. Therefore, researchers hypothesized that in the pathogenesis of varicose veins, Nelin might affect the phenotypic switch of VSMC, cytoskeletal remodeling, contraction regulation. In this study, researchers investigated the effect of Nelin expression on VSMC phenotypic switch.

MATERIALS AND METHODS

FBS and RPMI-1640 were purchase from Gibco. RNAiso-Plus was purchase from Takar. RT-PCR kit was from ShiJiKangWei (Beijing). Transfection kit sirna-mate was from Shanghai Gene Pharma Co., Acetylcholine was purchase from Sigma.

siRNA sequences and primer sequences: siRNA was synthesized by Shanghai GenePharma Co. The sequences are as follows: Nelin-siRNA; Sense: 5'-GCCCUGGUAA ACUCAAACUTT-3'; Antisense: 5'-AGUUUGAGUUUA CCAGGGCTT-3'; Negative control siRNA: Sense; 5'-UUC UCCGAACGAGUCACGUTT-3'; Antisense: 5'-ACGUGAC ACGUUCGGAGAATT-3'. Nesin primers; Forward: 5'-A GGAGTGGCTCTATTCAA-3',Reverse: 5'-GGTAAGTAA AGGCAGTAAG-3', amplicon is 611 bp. β-actin was used as internal control; Forward: 5'-TGACGTGGACATCCG CAAAG-3';Reverse: 5'-CTGGAAGGTGGACAGCGAGG-3', amplicon is 205 bp.

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Cell culture and grouping: VSMCs were isolated from saphenous veins abandoned from Coronary Artery Bypass Grafting (CABG) using enzymatic digestion and were cultured and passaged. VSMCs were identified by morphology observation and immunofluorescence staining. Cells at passage 4-7 were used in this study. VSMCs under serum stimulation showed synthetic phenotype and VSMCs under serum starvation showed contractile phenotype. Confluent VSMCs were serum starved and collected at 0, 24, 48, 72 h. A batch of 72 h serum-starved VSMCs were cultured in medium containing 10% serum, collected at 24, 48, 72 h and used as the model of synthetic phenotype (Ishida *et al*., 2001; Han *et al*., 2010).

RT-PCR: VSMCs were collected and total RNAs were extracted using RNAiso-Plus kit. First strand cDNA was synthesized according to the instructions of Two-step RT-PCR kit. PCR products were loaded onto a 1.5% agarose gel and electrophoresed. Bio-Best imaging system was used to take photos and Alpaview was used to analyze the absorbance. The ratio of absorbance of Nelin to absorbance of β-actin was used as the indicator of the expression level of Nelin.

Cell transfection: VSMC at logarithmic growth phase were seeded to 6 well plates 24 h before transfection. 1×10^5 to 2×10^5 cells were seeded to each well. Culture medium, siRNA and siRNA-mate transfection reagent were mixed at a certain proportion according to the manufacture's protocol and 200 μL of this transfection complex was added to 2 mL culture medium. Cells were cultured at 37°C. Cells were collect 48 h post transfection and RT-PCR was used to check the efficiency of gene silence.

Cytoskeleton observation: Slices with cells were washed with PBS, pre-fixed in 2% paraformaldehyde for 10 sec then treated in 1% TritonX-100 for 30 min after PBS washing. Fixed with 4% paraformaldehyde for 20 min, stained with 0.2% Coomassie Brilliant Blue R250 for 30 min, washed with distilled water, treated with Xylene and observed under the microscope.

Cell contractility observation: VSMCs transfected with negative control siRNA and Nelin-siRNA were grown to confluence and serum starved for 72 h then Ach was added to the medium to a final concentration of 100 nmol L^{-1}. VSMC contractile amplitude in 1 min was observed under the microscope.

Statistical analysis: Results were analyzed by SPSS 13.0, data was expressed as mean±standard deviation ($\bar{x}\pm s$). ANOVA was used for the comparison of means from multiple groups.

RESULTS AND DISCUSSION

Nelin mRNA expression in different phenotypic VSMC: RT-PCR results showed that the absorbance of serum stimulation group 24, 48 and 72 h serum starvation group and 24, 48 and 72 h post the restoration of serum stimulation group was 0.1940±0.0971, 0.7114±0.1265, 0.7270±0.1026,0.7841±0.1240,0.3208±0.1389,0.2997±0.1168 and 0.1807±0.0845, respectively. The difference between serum starvation group and serum stimulation group was statistically significant ($p<0.05$). There was no significant difference inside the serum stimulation group or serum starvation group ($p>0.05$).

Effect of Nelin expression on VSMC cytoskeleton: VSMC cytoskeleton was vague, sparse network and lightly stained before serum starvation (Fig. 1A). The cytoskeleton remodeled to a clear, dense, beam-like structure 72 h after serum starvation (Fig. 1B). siRNA was used to silence Nelin and RT-PCR showed the expression of Nelin was significantly decreased in Nelin-silenced VSMC (Fig. 2) transfection efficiency was 78.8%. When Nelin was silenced, serum starvation could not induce the

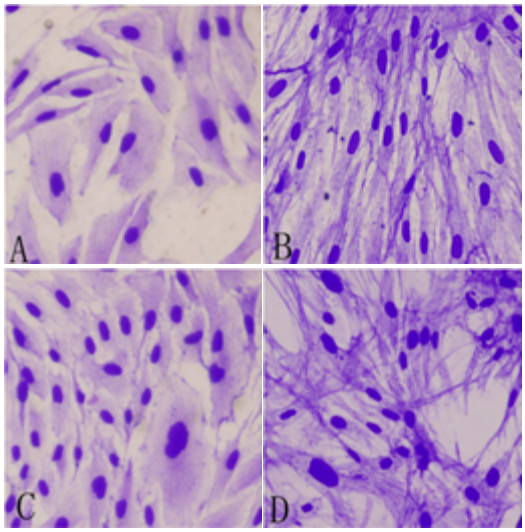

Fig. 1: Observation of VSMC cytoskeleton stained with Coomassie blue (200x). A) 10% serum stimulation; B) serum starvation; C) 72 h serum starvation after transfection of Nelin-siRNA; D) 72 h serum starvation after transfection of negative control-siRNA

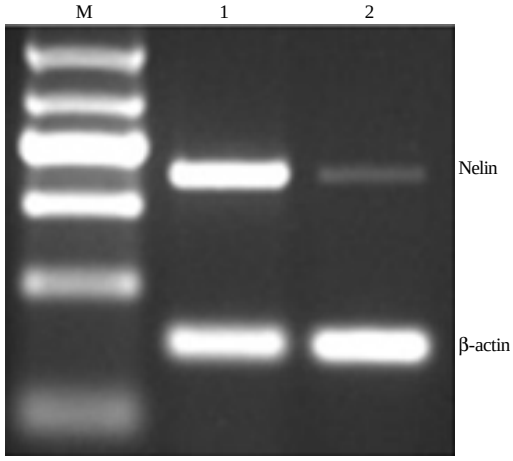

Fig. 2: Transfection efficiency of Nelin-siRNA. M: Maker; 1: Transfection of negative control-siRNA; 2: Transfection of Nelin-siRNA

change of cytoskeleton from sparse network to beam-like structure (Fig. 1C). In contrast, siRNA negative control group did not stop the change of cytoskeleton (Fig. 1D).

Relationship between Nelin expression and VSMC contraction: Contractile VSMC can contract in response to stimulants. The results showed that VSMC transfected with negative control siRNA contracted upon Ach stimulation after serum starvation. Cell length was shortened and the cell gap was widened (Fig. 3A and B). In contrast, VSMC transfected with Nelin-siRNA did not contract upon Ach stimulation after serum starvation (Fig. 3C and D).

VSMC phenotypic switch and the consequent proliferation and migration are the common pathophysiological processes of varicose veins and other vascular remodeling diseases (Majesky, 2007; Zhao *et al*., 2011). VSMC has two phenotypes, contractile type and synthetic type. Under normal conditions, VSMCs are contractile and there are abundant muscle bundles and fewer synthetic organelles in cytoplasm to maintain the vessel wall tension and contract blood vessels. Synthetic VSMCs have very few muscle bundles and abundant synthetic organelles which have significant proliferative properties (Nagel *et al*., 2006). The process that contractile VSMCs change to synthetic phenotype is called phenotypic switch which can be affected by growth factor stimulation, mechanical force, neurological factors and cell-cell interactions (Hirano, 2007). When contractile VSMCs change to synthetic VSMCs, metabolic disorder occurred, matrix protein increased, thereby caused fibrosis, shrink and stretch units broken therefore led to vascular remodeling.

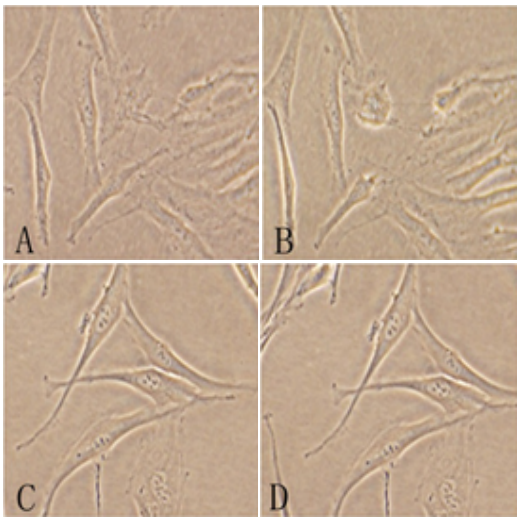

Fig. 3: VSMC contraction induced by Ach. A, B) transfection of negative control-siRNA; C, D) transfection of Nelin-siRNA; A, C) before Ach stimulation; B, D) after Ach stimulation

Nelin gene was a new gene cloned from human cDNA library, located on chromosome 1p31~1p32. The analysis of the structure of Nelin proteins found three functional domains: 36-75 amino acid residues formed a coiled-coil like domain; 76-245 amino acid residues can interact with F-actin protein binding domain; 354-440 amino acid residues formed an immunoglobulin-like domain which has interaction with F-actin and can interact with the cytoskeletal protein Filamin (Wang *et al*., 2004). It is speculated that Nelin is related to VSMC cytoskeleton and it may directly or indirectly involve in cell phenotype, morphology, contraction function and regulations of other biological behavior.

In order to study the effect of Nelin expression on VSMC phenotypic switch, researchers studied the dynamic change of Nelin expression in this process using serum starvation-induced synthetic VSMC formation from contractile phenotype. The results showed that the expression of Nelin in serum stimulated synthetic VSMCs. The expression of Nelin mRNA was increased rapidly by serum starvation and maintained at a high level and decreased rapidly when serum was provided (Fig. 4). At the same time, VSMC regained contractile function with the emergence of VSMC cytoskeleton remodeling. Therefore, Nelin expression was higher in the contractile phenotypic VSMC than that of the synthetic type, suggesting Nelin involved in the VSMC phenotypic switch.

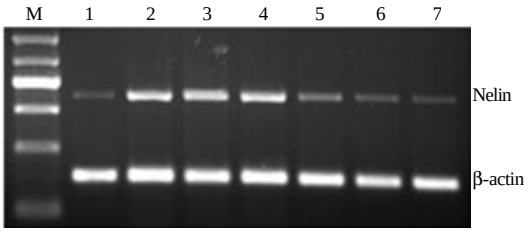

Fig. 4: Nelin mRNA expression under serum stimulation or serum starvation. M: Marker; 1: 10% serum stimulation; 2: Serum starvation; 3: Serum starvation for 48 h; 4: Serum starvation for 72 h; 5: 10% serum stimulation for 24 h after serum starvation; 6: 10% serum stimulation for 48 h after serum starvation; 7: 10% serum stimulation for 72 h after serum starvation

To further explore the effect of Nelin expression on VSMC phenotypic switch, researchers silenced Nelin first then researchers serum starved VSMC to study the change of cytoskeleton and contractility. It was found that skeletal reconstruction was inhibited and Ach could not lead to cell contraction when Nelin expression is suppressed indicating that there is a direct causal relationship between Nelin expression and VSMC skeleton remodeling and contractile function. The down-regulation of Nelin inhibited the conversion to contractile VSMC. Therefore, Nelin is considered an important regulatory gene involved in VSMC phenotypic switch and plays an important role in maintenance its phenotype. The differentiation of VSMC is reversible. And the VSMC phenotypic switch is the key process of vascular remodeling. The study showed that Nelin plays a very important role in this process. Nelin down regulation-induced defects of cytoskeleton remodeling and contractibility could affect cell function and lead to vascular remodeling. This could shed light on finding of new targets to intervene VSMC phenotypic switch which has great impact on reversing vascular remodeling, prevention of varicose veins, atherosclerosis and other vascular diseases.

CONCLUSION

Nelin involves in VSMC skeleton remodeling and adjusts its contractile function. Nelin plays an important role in the regulation of VSMC phenotypic switch and the maintenance of contractile phenotype.

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