Gene 616 (2017) 22–30

Contents lists available at ScienceDirect

Gene

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

Research paper

Myocardin inhibited the gap protein connexin 43 via promoted miR-206 to regulate vascular smooth muscle cell phenotypic switch

Hui Li [a,1], Yuan Xiang [a,1], Li-Juan Fan [a], Xiao-Yu Zhang [a], Jia-Peng Li [a], Cheng-Xi Yu [a], Le-Yuan Bao [a], Dong-Sun Cao [a], Wei-Bing Xing [a], Xing-Hua Liao [a,b,*], Tong-Cun Zhang [a,b,*]

[a] Institute of Biology and Medicine, Wuhan University of Science and Technology, 430000, PR China
[b] Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education and Tianjin, College of Biotechnology, Tianjin University of Science and Technology, 300457, PR China

ARTICLE INFO

Article history:
Received 24 November 2016
Received in revised form 17 March 2017
Accepted 21 March 2017
Available online 22 March 2017

Keywords:
Myocardin
miR-206
Connexin 43
Vascular smooth muscle cell phenotypic switch

ABSTRACT

Myocardin is regarded as a key mediator for the change of smooth muscle phenotype. The gap junction protein connexin 43 (Cx43) has been shown to be involved in vascular smooth muscle cells (VSMCs) proliferation and the development of atherosclerosis. However, the role of myocardin on gap junction of cell communication and the relation between myocardin and Cx43 in VSMC phenotypic switch has not been investigated. The goal of the present study is to investigate the molecular mechanism by which myocardin affects Cx43-regulated VSMC proliferation. Data presented in this study demonstrated that inhibition of the Cx43 activation process impaired VSMC proliferation. On the other hand, overexpression miR-206 inhibited VSMC proliferation. In additon, miR-206 silences the expression of Cx43 via targeting Cx43 3′ Untranslated Regions. Importantly, myocardin can significantly promote the expression of miR-206. Cx43 regulates VSMCs' proliferation and metastasis through miR-206, which could be promoted by myocardin and used as a marker for diagnosis and a target for therapeutic intervention. Thus myocardin affected the gap junction by inhibited Cx43 and myocardin-miR-206-Cx43 formed a loop to regulate VSMC phenotypic switch.

1. Introduction

Phenotypic plasticity is one of the major cellular affairs underlying many vascular smooth muscle cell (VSMC)-related pathological situations, such as atherosclerosis, luminal stenosis, sudden cardiac death, hypertension, and tumor angiogenesis (Owens et al., 2004). Due to our failure to identify the transcriptional mechanisms of atherosclerosis, we urgent need to find therapeutic agents for handling and prevention (Prinz et al., 2011; Kinch et al., 2016; Arrowsmith, 2011; Mignani et al., 2016; Raoof and Aerssens, 2015). Unscrambling the mechanisms involved in VSMC switch between differentiated and proliferative phenotypes is a significant step toward a better understanding of the pathology of these diseases.

The myocardin/SRF complex, through its banding with the promoters containing multiple CArG boxes ($CC(A/T)_6GG$), can directly trigger a battery of contractile smooth muscle genes, including transgelin (SM22) and alpha smooth muscle actin (ACTA2) (Yoshida et al., 2003; Yoshida et al., 2004; Long et al., 2008). Our previous study has demonstrated that STAT3 regulates vascular smooth muscle cell differentiated phenotypic switch by interaction with myocardin (Liao et al., 2015), although myocardin is considered as a vital part of the contractile "switch" in VSMC, the signals that regulate myocardin remain unclear.

Connexins (Cxs), the category of proteins that form gap junction channels and hemichannels named by the size of molecular weight, are progressively recognised as modulators of leukocyte trafficking into inflamed tissues (Scheckenbach et al., 2010). Gap junctions consist of channels between neighboring cells, an intermediate to transfer of chemical molecules or electrical stimulation (Hervé and Derangeon, 2013; Alexopoulos et al., 2004). The gap junction protein connexin 43 (Cx43) has been shown to engulf VSMCs proliferation phenotypes and the development of atherosclerosis (Kwak et al., 2002). The evidences of recent studies demonstrated that intimal up-regulation of Cx43, induced VSMCs proliferation in atherosclerotic plaques through gap junction generation (Kwak et al., 2002). In addition to cardiovascular and cerebrovascular diseases, Cx43 up-regulation and coupling of smooth muscle occurred, thereby inducing change in contractile property through up-regulation of intercellular communication (Liao et al.,

Abbreviations: VSMC, vascular smooth muscle cell; Cx43, connexin 43; CNN1, calponin; SM22, transgelin; ACTA2, alpha smooth muscle actin; MYH11, smooth muscle myosin heavy chain; STAT3, signal transducer and activator of transcription 3; Cxs, connexins; SRF, serum response factor; siCx43, siRNA-Cx43; EdU, 5-ethynyl-2′-deoxyuridine; N.C, negative control; 3′ UTR, 3′ untranslated region; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; PCNA, proliferating cell nuclear antigen; Ki67, Antigen identified by monoclonal antibody.

* Corresponding authors at: Institute of Biology and Medicine, Wuhan University of Science and Technology, Hubei 430081, PR China.
E-mail addresses: xinghualiao@hotmail.com (X.-H. Liao), zhangtongcun@wust.edu.cn (T.-C. Zhang).

[1] These authors contributed equally to the project.

http://dx.doi.org/10.1016/j.gene.2017.03.029
0378-1119/

2007; Rocha et al., 2008; Imamura et al., 2009). These findings collectively suggest that alterations in the regulation of Cx43 expression could underlie VSMC dysfunction.

The SRF-Myocardin transcriptional signaling has been shown to modulate several microRNAs, including the miR143/145 bicistronic gene (Cordes et al., 2009). MiRNAs have been crucial for development or implicated in diverse pathological condition, thereby the role in regulating cell proliferation, angiogenesis, invasion, and metastasis of breast cancer via targeting genes (Baranwal and Alahari, 2010). In myocardial cells of a coronary atherosclerosis mouse model, miR-1 affected Cx43 expression (Yang et al., 2007; Yang et al., 2011). Some other miRNAs such as miR-206, miR-133, miR-130, miR-143 and miR-145 were reported to be pivotal in cardiac development (Martignani et al., 2011). According to the results of TargetScan and PicTar, Cx43 has two possible binding sites for miR-206. Cx43 may be one of the target genes of miR-206 (Yang et al., 2007; Bagga et al., 2005). These lines of evidence have suggested that miR-206 may regulate Cx43 protein synthesis. Of specific interest in this study is the molecular machinery of myocardin-miR-206-Cx43 axis in VSMCs proliferation.

The refreshing finding here is a new role for myocardin as a repressor of Cx43 in smooth muscle cell. Myocardin has been known as a key trimmer for VSMC and cardiac contractile genes but its regulation of non-contractile genes remain uncertain. Interestingly one recent study demonstrated that myocardin induced the expression of a VSMC-restricted ion channel gene with a coincident increase in electrical current in vitro (Long et al., 2009). Our data in this study demonstrate that myocardin indirectly down-regulates Cx43 through its repressive action on miR-206.

2. Materials and methods

2.1. Cell lines

T/G HA-VSMC (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

2.2. Plasmids

The vector pCDNA3.1 (Promega) alone was used as a negative control. The pcDNA3.1-myocardin expression plasmid encoding mouse myocardin has been described previously (Liao et al., 2011). The promoter regions of Cx43 (−643 to +103) and miR-206 (−1158 to +103) were amplified by PCR followed by cloning into pGL3 luciferase reporter vector, respectively. The primers used to create Cx43-luc and miR-206-luc was as follows: Cx43: F-5′ CGGGGTACCTGAAAGATGGATTGGGTAT 3′ and R-5′ TGTGCTAGCGAACTCCTTGGAGGATGA 3′; hsa-miR-206: F-5′ GCCGGTACCCTAAGCCACCTCCATCAA 3′ and R-5′ GGGGCTAGCTCGGG AAGCAGTGTCATC 3′, the vector pGL-3 (Promega) was used as a control.

2.3. VSMC proliferation assays

Cell viability and proliferation were tested by 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay. Five hundred microliters of 0.5 mg/mL MTT was added to the 24-well plate, and 500 μL DMSO was added after incubating for 4 h at 37 °C. The mixture was then agitated for 10 min, and transferred into a 96-well plate. The absorbance was recorded at 492 nm. Experiments were conducted in triplicate. Results were expressed as an invasive inhibition rate. Invasive inhibition rate (%) = (blank control group OD-transfection group OD) / blank control group OD.

2.4. 5-Ethynyl-2′-deoxyuridine (EdU)-labeling in cultured cells

The EdU-labeling procedure was previously reported (Chehrehasa et al., 2009; Limsirichaikul et al., 2009). EdU (5-Ethynyl-2′-deoxyuridine), a nucleoside analog of thymidine, can be incorporated into the newly synthesized DNA and subsequently detected by Apollo® Dye via "click" reaction between EdU alkyne and fluorescent azide. Based on EdU-fluorescent labeling, cell proliferation activities could be accurately detected. EdU incorporation Cells on a 96-well plate were treated 2 h as follows: siRNA-Cx43 (siCx43), miR-206-mimic and its paired negative control (N.C.) by 24 h pretreatment. DNA synthesis was determined using a Cell-Light™ EdU In Vitro Cell Proliferation Imaging Kit (RIBOBIO, China), measuring incorporation of the nucleotide 5-ethynyl-2′-deoxyuridine (EdU). The sections and cultured cells were counterstained with DAPI and mounted with Vectashield. Statistical analyses were performed using Microsoft Excel software.

2.5. siRNA gene silencing

For functional studies of Cx43, a siRNA-based silencing approach was used. Customized siCx43 (sense: CAGUCUGCCUUUCGUUGUA dTdT, antisense: UACAACGAAAGGCAGACUG dTdT) were purchased from RIBOBIO. HA-VSMCs were transfected using Lipofectamine® 2000 Reagen (Life Technologies) according to the manufacturer's instructions. Transfected cells were pre-incubated for 48 h before further use. The efficiency of the siRNA-mediated Cx43 knockdown was verified by western blot.

2.6. Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR)

RT-PCR and qRT-PCR analysis were carried out as described previously (Liao et al., 2014a). Briefly, total cellular RNA was extracted from cultured cells with Trizol reagent (Invitrogen) and 2 μg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. The thermal cycle profile was as follows: denaturation for 30 s at 95 °C, annealing for 45 s at 52–58 °C depending on the primers used, and extension for 45 s at 72 °C. Each PCR reaction was carried out for 28–32 cycles. PCR products were visualized on 2% agarose gels stained with ethidium bromide (EB) under UV trans-illumination. qRT-PCR was performed in Applied Biosystems StepOne™ Real-Time PCR System. Fast SYBR®Green Master Mix was obtained from Applied Biosystems. Expression levels of proliferating cell nuclear antigen (PCNA) and Antigen identified by monoclonal antibody Ki67, and Cx43 were examined with conventional PCR or qPCR. Data were presented as relative level normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The PCR primer sequences are as follows,
GAPDH: F-5′ ATTCAACGGCACAGTCAAGG 3′,
R-5′ GCAGAAGGGGCGGA GATGA 3′;
PCNA: F-5′ ATGAAATGAAGTTGATGGAT 3′,
R-5′ TTGAAGAGAGTGGAGTGGCT 3′;
Ki67: F-5′ CAACTATCCTCGTCTGTCC 3′,
R-5′ GGTCCCTAAAGATGTGCT 3′;
Cx43: F-5′ ATCATGTTTGAGACCTTCAACA 3′,
R-5′ CATCTCTTGCTCGAAGTCCA 3′.

2.7. Antibodies, western blotting

Western blotting was performed as described previously (Liao et al., 2011; Liao et al., 2014b). Cells were lysed homogeneously using RIPA Lysis buffer (Beyotime) with freshly added protease inhibitor cocktail. After 30 min on ice and then centrifugation at 13,000 × *g* for 30 min, the resulting suspension was mixed with 2 × SDS sample buffer and boiled for 5 min. Samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (PALL Life Science, USA). The membranes were blocked using 5% non-fat milk for 1 h, then incubated for 1 h at ambient temperature with purified primary antibody in 5% non-fat milk. After three washes with Tris-buffered saline containing 0.05% Tween-20 (TBST), the membranes were incubated for 1 h at ambient