

Generation of Stable Myocardin Knockdown Smooth Muscle Cell Lines Using RNA Interference

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Abstract: *The transcription coactivator myocardin works with the transcription factor serum response factor (SRF) to regulate an array of smooth muscle marker genes, and is believed to be a factor necessary and sufficient for vascular smooth muscle cell (SMC) differentiation. Calcification of smooth muscle cells in artery walls is found to be one of the main hallmarks of cardiovascular disease and chronic kidney disease, and shows reduced expression of several smooth muscle marker genes [3]. In this study, we developed smooth muscle cell lines with decreased expression levels of myocardin using retroviral transduction of small double stranded RNA. These cells were characterized by analyzing the expression of myocardin, genes related to myocardin, and the genes that are downstream of myocardin and activated by the myocardin-SRF complex. These cell lines will be used in future studies to analyze the effect myocardin may have in the calcification of smooth muscle.*

1. INTRODUCTION

Cardiovascular disease (CVD) and chronic kidney disease (CKD) show ectopic calcification of tissues. Ectopic calcification, or unwanted mineralization, occurs when calcium deposits build up in soft tissues. This makes the tissue hard and severely limits tissue function. Vascular smooth muscle cells (SMCs) are the major cell type within the walls of arteries and are responsible for the maintenance and regulation of blood pressure and blood flow. Calcification of vascular SMCs has been shown to occur in CVD and CKD patients as hardened arteries which no longer regulate blood pressure adequately. When SMCs undergo calcification, many SMC marker genes are downregulated and non-SMC osteogenic genes are expressed. Mineralized SMCs show expression of Cbfa1, osteocalcin, osteopontin, and alkaline phosphatase, the proteins not present in normal SMCs [3].

Terminally differentiated SMCs, unlike most cell types, are able to switch between phenotypes such as those for proliferation and migration and contraction, allowing SMCs to respond to stress, injury, or disease [2,3]. Several SMC markers exist in differentiated SMCs, including smooth muscle (SM) α -actin, SM22 α , SM myosin heavy chain (MHC), and h₁-calponin, while none seem to be expressed exclusively in smooth muscle [2]. These SMC marker genes are controlled by the transcription factor serum response factor (SRF) that binds to a region of DNA known as the CARG box and

thus activates the transcription of SM marker genes. SRF is present in many cell types; it may control differentiation of SMCs by associating with SM specific cofactors, mainly myocardin. Both SRF and myocardin are necessary for SMC differentiation and development. However, myocardin is the one specifically involving in smooth muscle development, as illustrated by the fact that mice without a functional myocardin gene cannot survive beyond embryonic day 10.5 due to delayed development of the aorta resulting in an inadequate vascular system [1].

Although myocardin is required for SMC differentiation, it is unclear whether this protein is also involved in the alternate phenotype observed during calcification. To address this question, we developed SM cell lines with reduced expression levels of myocardin using mouse aortic SMCs and small interfering RNA (siRNA). The resulting cell lines were examined to assess the amount knockdown of myocardin and the related genes and the effect to genes downstream of myocardin. These cell lines will be used to study the effect myocardin may have in the calcification process of smooth muscle.

2. MATERIALS AND METHODS

2.1 Culture of mouse aortic SMCs

Mouse aortic SMCs were isolated from aortas of mice carrying Rosa-26 cre reporter and SM22 α -cre recombinase transgenes. The cells were cultured in Dulbecco's Modified Eagle's

Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/antimycotic. They were cultured from passage 3 to passage 6 in T-75 tissue culture flasks at 37°C with 5% CO₂ prior to retroviral transduction of myocardin siRNA.

2.2 Retroviral transduction of myocardin siRNA into mouse aortic SMCs

Four different targeting sequences from the coding sequence of mouse myocardin were used to generate myocardin siRNA retroviral constructs. The sequences used were: myosi412: GGTCCTCAACTGAATTCCATG, myosi654: GAACATTCTGCCGATGGAT, myosi1434: GGTGTCAGAGTTAAGACAA, and myosi2686: GATCATTCATGTCTTCAGA. MyosiC654 had a mismatch at nucleotide 10 of the antisense strand of myosi654. This disabled its ability to bind to myocardin mRNA and thus was used to generate oligo control cell line. All the constructs were then used to transfect the Phoenix ecotropic packaging cell line (the Nolan laboratory, Stanford University, CA) via calcium phosphate precipitation. At 48 h post transfection, virus containing culture medium was collected and used to infect SMCs described above. Infection was repeated 24 and 48 h after the initial infection. The myocardin siRNA-transduced SMCs were selected by culturing in medium containing 2.5 µg/mL puromycin for 2 days and 2.0 µg/mL puromycin for at least 2 days to achieve a pure population.

2.2 Taqman real-time reverse transcription PCR

Total RNA was extracted using the RNeasy Mini kit and genomic DNA was digested by RNase-free DNase I (Qiagen). First strand cDNA was synthesized from 1 µg total RNA using Omniscript reverse transcriptase (Qiagen). The cDNA was used to determine expression levels of myocardin and myocardin related transcription factors A and B (MRTF-A and -B) by Taqman real-time PCR using ABI Prism 7000 (Applied Biosystems, Foster City, CA). The sequences used in Taqman real-time PCR assay are: myocardin forward primer: 5'ccacccagacatcaaatcc3', myocardin reverse primer: 5'tgcatcattctgtcactttctga3', myocardin

probe: FAM-acaatccaggatctcactc-MGB; MRTF-A forward primer: 5'cagcctgaaggaggctatcatt3', MRTF-A reverse primer: 5'gaggaactgtctgtactctttgg3', MRTF-A probe: FAM-tgggccaggtaaatt-MGB; MRTF-B forward primer: 5'acctctgtgactgcaagca3', MRTF-B reverse primer: 5'gggtttcaagaattcaggaactg3', MRTF-B probe: FAM-tccagcccagtttac-MGB. All the probe sequences spanned exon-exon junctions to eliminate amplification of any residual genomic DNA. As controls of sample loading, 18S ribosomal RNA levels of samples were determined and expression of target genes was normalized to 18S ribosomal RNA.

2.3 Western blot analysis

Protein lysates were prepared from SMCs using 0.1 mol/L Tris buffer, pH 6.8, supplemented with 2% sodium dodecylsulfate (SDS), 2 µg/mL pepstatin, 1 mmol/L PMSF, 2 µg/mL leupeptin, and 2 µg/mL aprotinin. Protein content of the lysates was measured using the Micro BCA assay (Pierce Rockford, IL). Equal amounts of the protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Perkin Elmer, CA). Proteins of interest were blotted using specific antibodies followed by biotin-streptavidin amplification and Western blot chemiluminescence detection (Perkin Elmer, CA). Antibodies used are monoclonal mouse anti-human SM α-actin (1A4, Sigma; cross react with mouse SM α-actin), polyclonal goat anti-human SM22α (ab10135, Abcam, cross react with mouse SM22α), and polyclonal rabbit anti-human β-tubulin (ab6046, Abcam, cross react with mouse β-tubulin). Cellular skeletal protein β-tubulin of each sample was detected to assess loading equivalence cross the samples.

3. RESULTS

3.1 Generation of various myocardin knock-down cell lines

SMCs were seeded in six parallel plates. Five of these plates were infected with retrovirus carrying various targeting sequences of myocardin siRNA as described in "Methods." The last plate, the non-transduced control (NT-

ctrl), was not infected with retrovirus and was not selected by puromycin. The five transduced cell lines included those that were predicted to form functional siRNA constructs, myosi412, myosi654, myosi1434, and myosi2686, and one oligo control, myosiC654, as well as non-transduced SMCs, were cultured and expanded. Total RNA was extracted from each cell line and was used to detect the expression level of myocardin by Taqman real-time reverse transcription PCR. As shown in Figure 1, retroviral transduced and puromycin-selected cell lines showed increased expression of myocardin (NT ctrl vs. myosiC654), suggesting a variant of cell population may be generated in this process. However, all cell lines infected with various siRNA constructs showed reduced

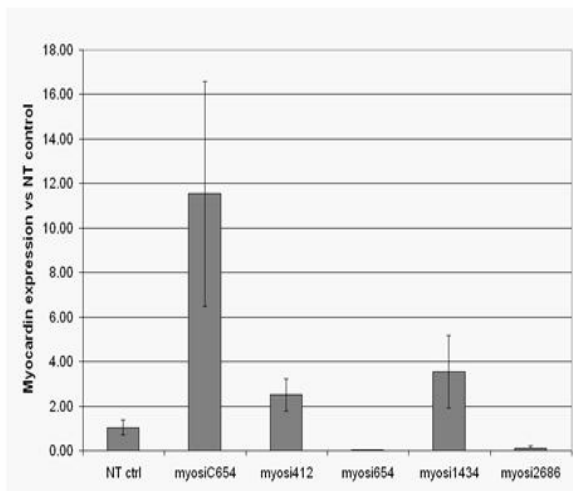


Figure 1 Myocardin expression levels in various siRNA cell lines. Total RNA was extracted from various cell lines. One μg RNA from each cell line was used to reverse transcribe to cDNA and the myocardin expression level was determined using Taqman real-time reverse transcription PCR as described in "Materials and Methods". Note that cell lines myosi654 and myosi2686 had the lowest expression levels of myocardin relative to the oligo control. NT ctrl=not infected with retrovirus and not selected with puromycin. MyosiC654=oligo control. The error bars are therefore the standard deviations calculated for the three values obtained.

expression of myocardin relative to the oligo control, myosiC654. Cell lines myosi654 and myosi2686 show complete knockdown of

myocardin expression relative to both the oligo control, myosiC654, and the non-transduction control, NT ctrl (99.7% and 99.2%, respectively). Cell line myosi412 shows 78.3% knockdown and cell line myosi1434 shows a 69.2% knockdown of myocardin expression relative to the oligo control. The cell lines myosi654 and myosi2686 will be the main focus of the following studies due to the greater success of myocardin knockdown.

3.2 Determination of other transcription factors involving in SMC differentiation

To study the specificity of the various myocardin siRNA cell lines, analysis of the mRNA transcript levels of genes related to myocardin, MRTF-A and -B, was performed (Figure 2). Compared to the oligo control, myosiC654, MRTF-A and -B were downregulated in cell line myosi654, but not in myosi2686, suggesting that the myocardin siRNA construct myosi654 is not specific to only myocardin but may have affected other related genes as well, such as MRTF-A and MRTF-B. Myosi2686 seems to be more specific to myocardin interference, as illustrated by no significant reduction of MRTF-A expression and a minor reduction of MRTF-B expression (57.1% vs. oligo control).

3.4 Functional analysis of myocardin knock-down cell lines

To assess the effect of myocardin knockdown on proteins whose expression is regulated by the SRF-myocardin transcriptional activation complex, we performed Western blot analysis to determine the expression levels of SM α -actin and SM 22 α , the two common SM marker proteins, in these cell lines. As shown in Figure 3, cell lines with disrupted expression of myocardin showed decreased levels of both SM α -actin and SM22 α . This is particularly evident in the cell lines myosi654 and myosi2686, and corresponds to the Taqman real-time RT-PCR results that indicate that these two cell lines had the highest knockdown of myocardin.

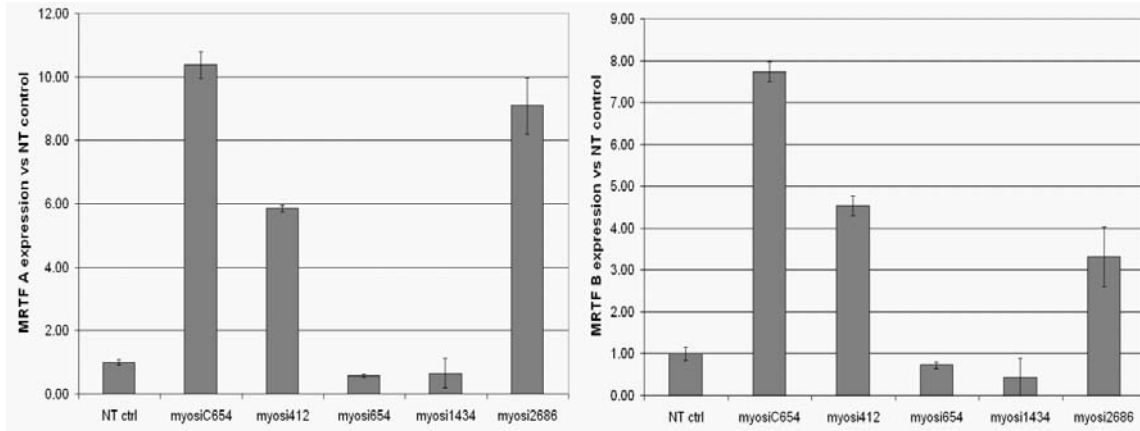


Figure 2 Expression levels of myocardin-related transcription factors A and B (MRTF-A and -B) in myocardin siRNA cell lines. Complementary DNA was generated as described in legend to Figure 1. MRTF A and B expression were determined by Taqman real-time reverse transcription PCR. Myosi654 shows reduced expression of both MRTF-A and -B relative to the oligo control, while cell line myosi2686 shows no significant reduction of MRTF-A expression and 57.1% reduction of MRTF-B expression. NT ctrl=not infected with retrovirus and not selected with puromycin. MyosiC654=oligo control. The error bars are therefore the standard deviations calculated for the three values obtained.

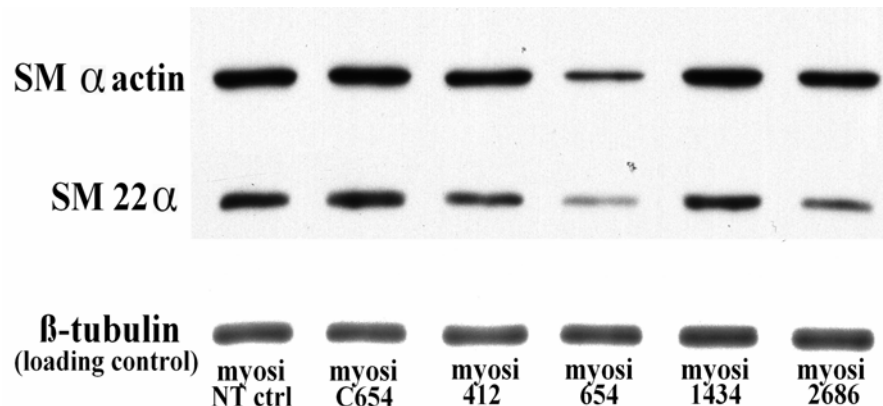


Figure 3 Western blot analysis of proteins whose expression is controlled by the myocardin-SRF complex. Five μ g cell lysate was used to detect smooth muscle marker genes SM22 α and SM α -actin by Western blot analysis. β -tubulin was used as sample loading controls of the analysis.

4. DISCUSSION

We have generated several myocardin knockdown smooth muscle cell lines via retroviral transduction of myocardin-specific siRNA constructs in the current study. The generated cell lines, myosi654 and myosi2686, showed over 99% reduction of myocardin expression in an mRNA level. In addition, knockdown of the myocardin expression in the

SMCs leads to significant inhibition of downstream SM marker genes, SM α -actin and SM22 α , as determined by Western blot analysis. Finally, myosi2686 cell line showed higher specificity than myosi654 cell line according to Taqman real-time RT-PCR analysis of other smooth muscle transcription factors, MRTF-A and MRTF-B. Thus, cell line myosi2686 had levels comparable to the oligo control cell line,

and cell line myosi654 had a marked decrease in the expression levels of MRTF-A and MRTF-B in addition to myocardin. These cell lines will be useful tools to determine the role of transcription coactivator myocardin and its related transcription factors in the calcification of smooth muscle in future studies.

Our data also showed that expression levels of SM marker genes correlated with the expression level of myocardin, but not necessarily correlating to the expression levels of MRTF-A and -B. This indicates that myocardin is the main coactivator responsible for the transcription of SM marker genes. Further studies will need to verify these findings.

ACKNOWLEDGMENTS

Many thanks to the Giachelli lab and the REU UWEB program at the University of Washington. This research was funded by NSF Grant #EEC 9529161 and NIH Grant 1 R01 HL081785-01 to Dr. Giachelli.

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