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Identification of a Klf4-dependent upstream repressor region mediating transcriptional regulation of the myocardin gene in human smooth muscle cells

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ABSTRACT

Phenotypic switching of smooth muscle cells (SMCs) plays a central role in the development of vascular diseases such as atherosclerosis and restenosis. However, the factors regulating expression of the human myocardin (Myocd) gene, the master gene regulator of SMC differentiation, have yet to be identified. In this study, we sought to identify the critical factors regulating Myocd expression in human SMCs. Using deletion/genetic reporter analyses, an upstream repressor region (URR) was localised within the Myocd promoter, herein termed PrmM. Bioinformatic analysis revealed three evolutionary conserved Klf4 sites within the URR and disruption of those elements led to substantial increases in PrmM-directed gene expression. Furthermore, ectopic expression established that Klf4 significantly decreased Myocd mRNA levels and PrmM-directed gene expression while electrophoretic mobility shift assays and chromatin immunoprecipitation (ChIP) assays confirmed specific binding of endogenous Klf4, and not Klf5 or Klf2, to the URR of PrmM. Platelet-derived growth factor BB (PDGF-BB), a potent inhibitor of SMC differentiation, reduced Myocd mRNA levels and PrmM-directed gene expression in SMCs. A PDGF-BB-responsive region (PRR) was also identified within PrmM, overlapping with the previously identified URR, where either siRNA knockdown of Klf4 or the combined disruption of the Klf4 elements completely abolished PDGF-BB-mediated repression of PrmM-directed gene expression in SMCs. Moreover, ChIP analysis established that PDGF-BB-induced repression of Myocd gene expression is most likely regulated by enhanced binding of Klf4 and Klf5 to a lesser extent, to the PRR of PrmM. Taken together, these data provide critical insights into the transcriptional regulation of the Myocd gene in vascular SMCs, including during SMC differentiation.

1. Introduction

Vascular smooth muscle cells (SMCs) are highly specialised cells that primarily function to provide support and contractility within the vascular system, regulating blood pressure, vessel diameter and blood flow distribution through maintenance of vascular tone [1,2]. Phenotypic switching, or alterations in the differentiated state of SMCs, plays a critical role in the repair of tissue damage and in the development of a variety of major human diseases including atherosclerosis, restenosis and pulmonary hypertension [3–5]. The hallmark of phenotypic modulation is the regulated and coordinate expression of smooth muscle (SM)-specific marker genes. For example, high expression of SMC-specific genes such as SM myosin heavy chain (MYH11) [6,7], calponin (CNN1) [8] and SM α-actin (ACTA2) [9] are all associated with a contractile SMC phenotype [3]. Despite the recognised importance of phenotypic plasticity, key challenges still remain in identifying the regulatory mechanisms that modulate critical transitions in SMC phenotype following vascular injury or in cardiovascular disease development.

Recent studies have made great strides towards understanding the molecular mechanisms that contribute to the regulation of SM-restricted gene expression, the most characterised being serum response factor (SRF)-dependent transcriptional regulation [10]. SRF belongs to the MADS (MCM1, Agamous, Deficiens, SRF)-box family of transcription factors that was named due to its ability to confer serum inducibility to the growth-responsive gene, *c-fos*, through binding to a DNA sequence known as a serum response element (SRE) or a CArG box [$CC(A/T)_6GG$] [11]. Mutational studies using transgenic mice identified one or more functional CArG elements within the promoter regions of most SM-specific genes. More specifically, a region of the SM22α promoter containing 445

Abbreviations: ACTA2, SM α-actin; ChIP, chromatin immunoprecipitation; CNN1, calponin; CVD, cardiovascular disease; FBS, fetal bovine serum; IgG, immunoglobulin G; Klf, Krüpple-like factor; PDGF-BB, platelet-derived growth factor-BB; pGL3E, pGL3Enhancer; pRL-TK, pRL-thymidine kinase; PRR, PDGF-BB-responsive region; RLU, relative luciferase unit; SEM, standard error of the mean; SDM, site-directed mutagenesis; SRE, serum response element; SRF, serum response factor; SMC, smooth muscle cells; SM, smooth muscle; URR, upstream repressor region

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base pairs including CArG elements was found to be sufficient to direct the specific expression of a lacZ transgene in mouse embryos in the vascular smooth, cardiac, and skeletal muscle lineages in a similar pattern to that of the endogenous SM22α gene [12]. Furthermore, deletion of this same region (containing the two CArG elements) resulted in a six-fold reduction in SM22α promoter activity in SMCs *in vitro* [12,13]. Mack and Owens (1999) also demonstrated that while a specific region of the SM α-actin promoter (−2560 to +2784) could recapitulate expression patterns of endogenous SM α-actin expression *in vivo*, simple mutation of any of the three highly conserved CArG elements completely abolished this effect [14].

Myocardin is a transcriptional co-factor of SRF and is a crucial component of the SM differentiation programme [15,16]. Myocardin belongs to the SAP (SAF-A/B, Acinun, PIAS) family of transcription factors and mediates its activity through a direct association with SRF, which in turn binds to CArG elements found within the regulatory regions of SMC gene regulatory regions. Ectopic expression of Myocd in non-SMCs is sufficient to promote a SMC-like phenotype, activating several endogenous SMC-restricted genes including SM myosin heavy chain, calponin, SM α-actin and SM22α, all of which are CArG-dependent [15,17,18]. While alterations in Myocd mRNA levels have been shown to disturb homeostasis of the SMC contractile gene programme *in vitro* and *in vivo* [19], genetic ablation of Myocd impairs SMC differentiation and normal function [20]. Thus, Myocd has been termed the master gene transcriptional regulator governing the SMC differentiation programme.

Despite these studies and a wealth of knowledge regarding mechanisms regulating the expression of SMC-specific markers and mediating phenotypic switching of SMCs in response to vascular injury, surprisingly, to date the human myocardin gene remains largely uncharacterized and the factors regulating its expression have yet to be identified. To address this knowledge deficit, the central aim of this study was to characterise the human myocardin gene, seeking to identify the *cis*-acting element(s) and *trans*-acting factor(s) determining its expression within SMCs.

Platelet-derived growth factor (PDGF-BB) is a potent inhibitor of SMC differentiation and its levels have been shown to dramatically increase in response to vascular injury *in vivo*, identifying it as a key mediator of SMC phenotypic switching [3,6,21]. Hence, given the critical role of myocardin expression during phenotypic modulation, this current study also sought to investigate the influence of PDGF-BB treatment on myocardin gene expression within human SMCs. Herein, we identify a critical role for three evolutionarily conserved Krüppel-like factor (Klf) 4 *cis*-acting elements within an overlapping upstream repressor region (URR) and PDGF-BB-responsive region (PRR), localised within the myocardin promoter, in mediating the transcriptional repression of myocardin gene expression in SMCs. This study represents the first detailed characterisation of the human myocardin promoter and provides a strong molecular and genetic basis for understanding the modes of regulation of myocardin gene expression in response to vascular injury and in the development of vasoproliferative diseases.

2. Materials and methods

2.1. Materials

Dual Luciferase® Reporter Assay System, pGL3Enhancer (pGL3E), pRL-Thymidine Kinase (pRL-TK) and FuGENE® HD transfection reagent were from Promega. Lipofectamine 2000 and SuperScript® III reverse transcriptase were from Invitrogen. Antibodies *Anti*-GKLF (H-180)X (Klf4; sc-20691 X), *anti*-BTEB2 (H-300)X (Klf5; sc-22797 X), *anti*-LKLF (H-60)X (Klf2; sc-28675 X), normal rabbit IgG (sc-2027), goat *anti*-rabbit horseradish peroxidase (HRP; sc-2357) and goat *anti*-mouse HRP (sc-2005) were from Santa Cruz. Antibody *anti*-Myocd (MAB4028) was from R&D systems. M199 media and *Anti*-β-actin antibody were from Sigma. pCMV-SPORT6 vectors encoding Klf4 (IRATp970H0848D), Klf2 (IRAKp961B0998Q) and Elk-1 (IRATp970A0181D) were from GenomeCube (SourceBiosciences). Fetal bovine serum (FBS) was from Gibco. Platelet-derived growth factor BB (PDGF-BB) was from Peprotech.

2.2. Cell culture and PDGF-BB treatment

Human aortic smooth muscle cells (SMC), obtained from Health Protection Agency Culture Collections, were cultured in M199, 5% FBS. Cells were grown at 37 °C in a humid environment with 5% CO_2 and were confirmed to be free of mycoplasma contamination. Routinely cultured SMCs were serum-starved overnight (16 h) prior to assays or being stimulated with PDGF-BB (0–40 ng/ml, as indicated) or vehicle (10 mM acetic acid and 2 μg/ml BSA) for 24 h prior to analysis. In the context of basal Klf4 expression levels, and consistent with our findings (Supp. Fig. 2B), serum-stimulation resulted in the destabilisation of Klf4. A process thought to be mediated, at least in part, through an ubiquitin-proteasome pathway [22].

2.3. Construction of luciferase-based genetic reporter plasmids

The myocardin promoter (PrmM; nucleotides −4016 to −105, relative to the translational start codon at +1) luciferase reporter construct was generated by PCR amplification of DNA fragments from human genomic DNAs incorporating *Bam*HI restriction endonuclease sites using the following primer set: forward 5′-AAAGACCTGAATT CTATCAATAACC-3′; and reverse 5′-ACAACCCAGGATCCATCCAACTCT CCGTGA-3′. The 3.9 kb PCR amplified fragment was cloned in linearized pGL3Enhancer plasmid (pGL3E; Promega) *Bgl*II to *Bgl*II (*Bam*HI cohesive termini compatible) to generate pGL3E:PrmM. Truncated PrmM variants PrmM1 (−2226 to −105), PrmM2 (−1377 to −105), PrmM3 (−807 to −105) and PrmM4 (−135 to −105) were excised from pGL3E:PrmM and re-cloned into pGL3E (*Bgl*II, *Hind*III, *Sma*1, *Kpn*I to *Bgl*II, respectively) to generate pGL3E:PrmM1, pGL3E:PrmM2, pGL3E: PrmM3 and pGL3E:PrmM4. All constructs were verified by sequencing.

2.4. Site-directed mutagenesis

Bioinformatic analysis was initially used to identify putative binding sites and was performed using Genomatrix MatInspector software [23]. Site-directed mutagenesis (SDM) was carried out using the Quik-Change™ method (Agilent). The identities of the PrmM elements subjected to SDM, with their starting positions in brackets, the nucleotides that were changed in underlined bold, templates used and names of the corresponding plasmids generated, as well as the sequences of the specific primers used are listed below.

1. Klf4 #1 (−3788), from agCAAAGga to agCAA**CT**ga using template pGL3B:PrmM to generate pGL3B:PrmM$^{Klf4\#1*}$. Primers: forward 5′-CAGGTAACTAACAGCAACTGAGAGAAAGCCAAAA-3′ and reverse 5′-TTTTGGCTTTCTCTCAGTTGCTGTTAGTTACCTG-3′.
2. Klf4 #2 (−3765), from aaGGGGaa to aa**GTA**Gaa using template pGL3B:PrmM to generate pGL3B:PrmM$^{Klf4\#2*}$ and using pGL3B: PrmM$^{Klf4\#1*}$ to generate pGL3B:PrmM$^{Klf4\#1\&2*}$. Primers: forward 5′-GAAAGCCAAAATGAAGTAGAAAGGGACAGAATTGC-3′ and reverse 5′-GCAATTCTGTCCCTTTCTACTTCATTTTGGCTTTC-3′.
3. Klf4 #3 (−3693), from gaAGGGtc to gaA**TA**Gtc using template pGL3B:PrmM to generate pGL3B:PrmM$^{Klf4\#3*}$ and using pGL3B: PrmM$^{Klf4\#1\&2*}$ to generate pGL3B:PrmM$^{Klf4\#1,2\&3*}$. Primers: forward 5′-CAGGGGAAGTAAAAAGAATAGTCCAAAACACACCA-3′ and reverse 5′-TGGTGTGTTTTGGACTATTCTTTTTACTTCCCCTG-3′.