Smooth Muscle Calponin
An Unconventional CArG-Dependent Gene That Antagonizes Neointimal Formation

Xiaochun Long, Orazio J. Slivano, Sarah L. Cowan, Mary A. Georger, Ting-Hein Lee, Joseph M. Miano

Objective—Smooth muscle calponin (CNN1) contains multiple conserved intronic CArG elements that bind serum response factor and display enhancer activity in vitro. The objectives here were to evaluate these CArG elements for activity in transgenic mice and determine the effect of human CNN1 on injury-induced vascular remodeling.

Methods and Results—Mice carrying a lacZ reporter under control of intronic CArG elements in the human CNN1 gene failed to show smooth muscle cell (SMC)-restricted activity. However, deletion of the orthologous sequences in mice abolished endogenous Cnn1 promoter activity, suggesting their necessity for in vivo Cnn1 expression. Mice carrying a 38-kb bacterial artificial chromosome (BAC) harboring the human CNN1 gene displayed SMC-restricted expression of the corresponding CNN1 protein, as measured by immunohistochemistry and Western blotting. Extensive BAC recombineering studies revealed the absolute necessity of a single intronic CArG element for correct SMC-restricted expression of human CNN1. Overexpressing human CNN1 suppressed neointimal formation following arterial injury. Mice with an identical BAC carrying mutations in CArG elements that inhibit human CNN1 expression showed outward remodeling and neointimal formation.

Conclusion—A single intronic CArG element is necessary but not sufficient for proper CNN1 expression in vivo. CNN1 overexpression antagonizes arterial injury-induced neointimal formation. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: gene expression ■ genetically altered mice ■ transgenic models ■ vascular muscle ■ serum response factor

Smooth muscle cells (SMCs) are essential constituents of the vessel wall that arise through complex cell-cell and cell-matrix signaling events at multiple sites during mouse embryogenesis. SMCs confer structural stability to endothelial cell-lining nascent blood vessels and, later in life, provide further structural support through formation of lamellar units and the elaboration of various extracellular matrix proteins. SMCs also control the caliber of the vessel wall, and thus the flow of blood, through the coordinate action of many cytoskeletal and contractile proteins. As such, SMCs in the normal adult vessel wall exhibit a quiescent, nonmotile phenotype conducive for contraction and structural support. This so-called contractile phenotype is compromised in a variety of vascular disorders, including atherosclerosis, transplant arteriopathy, hypertension, and vein graft failure following coronary artery bypass graft surgery. Such a phenotypic adaptation involves attenuated expression of numerous cytoskeletal and contractile genes, the acquisition of a growth/migratory state, or the transdifferentiation to other cell types, all of which contribute substantively to the pathogenesis of vascular disease. Hence, there has been enormous interest in elucidating the definition of vascular SMC phenotypes and, more importantly, the molecular circuitry governing these phenotypes.

One of the principal ways in which the normal adult vascular SMC phenotype is established is through the coordinate transcriptional activation of many contractile genes that are highly specific to these cells. Work over the last 20 years has uncovered a transcription factor binding code common to most SMC contractile genes. This binding code, known as a CArG box, is 10 base pairs in length and conforms to either a high affinity binding sequence of CC(A/T)GG or to any 1 of more than 1100 permutations of this consensus sequence. The aggregate collection of functional CArG boxes in the genome, known as the CAReome, binds the serum response factor (SRF) transcription factor with varying affinity. SRF is a widely expressed transcription factor that controls a variety of genes linked to the contractile apparatus and the actin cytoskeleton. There is only 1 SRF-like gene in mammalian genomes; however, myocyte enhancer factor 2 proteins share homology both in functional protein domains and DNA binding sequences, though SRF and myocyte enhancer factor-2 do not compete for one another’s binding sites. Genetic inactivation of SRF in developing...
vascular SMCs results in attenuated expression of contractile genes and a reduction in the recruitment of nascent SMCs to the dorsal aorta, both of which likely contribute to midgestation arrest of the mouse. 10 Indeed, every cell type in which SRF has been inactivated displays defective local homeostasis, with death of the animal as a frequent end point.11 Thus, CArG-SRF is viewed as a critical mediator of diverse cellular activities, including those linked to normal vascular SMC physiology.

Because SRF displays broad expression across essentially every cell type, including anucleated cells, such as platelets,12 the ability to orchestrate specific programs of gene expression hinges on its interaction with a growing number of cofactors. One such cofactor is myocardin (MYOCD), a cardiomyocyte- and SMC-restricted protein that powerfully activates a subset of SRF-dependent genes.13 Myocardin transcripts are expressed abundantly in adult vascular SMCs but invariably decrease on cell culture, where SMCs phenotypically adapt to a less contractile state.14 Forced expression of MYOCD is sufficient to activate CArG-containing SMC contractile genes14–16 and functional SMC-like contraction,17 so long as SRF is present.18 Thus, the CArG-SRF-MYOCD triad constitutes the major transcriptional switch for establishment of a functional SMC contractile phenotype. Other molecular switches exist in a supporting role, including the recently discovered microRNA143/145 gene that promotes MYOCD-dependent SMC contractile gene expression by regulating a network of transcription factors and signaling proteins.19–23

Formal proof of an SRF target gene’s dependence on CArG elements for normal expression requires rigorous analysis in transgenic mice. Such analyses have been done to show CArG-dependent regulation of the Tagln,24,25 Acta2,26 Myh11,27 Telokin,28 Kcnmb1,29 and Csrp130 genes. The mouse SM calponin gene (Cnn1) contains multiple intronic CArG boxes that display enhancer activity in vitro.31 These intronic CArG elements are completely conserved in sequence and space within the human CNN1 gene. We previously reported SMC-restricted expression of human CNN1 during development and in postnatal tissues using bacterial artificial chromosome (BAC) transgenic mice. The importance of intronic CArG elements, however, was not investigated.32 Here, we report that CArG-containing intron 1 sequences within the CNN1 gene are insufficient for directing proper transgene expression in SMC lineages, although orthologous sequences are necessary in the context of a Cnn1 knock out mouse. BAC transgenic mice with various CArG element mutations support the gene knockout phenotype and provide strong evidence for a critical role of a single intronic CArG element in the control of CNN1 expression in vivo. Finally, we made the unanticipated observation that overexpression of human CNN1 confers resistance to outward remodeling and neointimal formation following arterial injury.

Materials and Methods
For an expanded Materials and Methods section, please see the supplemental materials, available online at http://atvb.ahajournals.org.

Animals
Transgenic and Cnn1 knockout mice were generated through standard methods and were handled in accordance with the University of Rochester’s institutional animal care and use committee. Partial ligation injury of the carotid artery and mouse genotyping were done as described in the supplemental materials. All mice were provided water and food ad libitum.

Bioinformatics
The human and mouse CNN1 genes were subjected to comparative genomics analyses using the visualization tools for alignment (VISTA, http://genome.lbl.gov/vista/index.shtml) and the basic local alignment search tool. Sequence motifs for CArG elements were generated with a sequence logo tool.

Expression Assays
CNN1 detection was done by Western blotting and immunohistochemistry of various tissues using an antibody specific for the human antigen. Total RNA isolated from injured or noninjured carotid arteries was assessed for human and mouse CNN1 expression by quantitative reverse transcription–polymerase chain reaction.

Luciferase Assay
An upstream CArG-containing region was cloned into the pGL3 basic plasmid and transfected into cells in the presence or absence of either an SRF or myocardin expression plasmid and luciferase activity determined by luminometry.

Results

CNN1 Harbors Conserved Intronic CArG-Rich Regions
Functional transcription factor binding codes are often identical in sequence and genomic position across multiple species. We routinely use the VISTA program33 to compare orthologous gene sequences for conservation and transcription factor binding code discovery. A VISTA plot of the smooth muscle calponin locus (CNN1) shows 2 modules of high sequence identity within the first intron (Figure 1). Each module contains 2 conserved CArG elements (C2-C5, Figure 1). In a previous analysis of the mouse Cnn1 gene, we showed that 3 of the conserved intronic CArG elements (C2, C4, and C5) bind SRF and display in vitro enhancer activity to varying degrees34 based on the known sequence binding rules associated with CArG-SRF.34 C2 represents a perfect consensus CArG box and binds SRF avidly, whereas C4 and C5 deviate from the consensus CArG box by 1 bp and bind SRF weakly.34 Because our prior study was confined to in vitro analyses only,31 we set out here to evaluate these CArG elements in the context of transgenic mice.

Intronic CArG Boxes Are Necessary but Not Sufficient for Correct CNN1 Expression In Vivo
Smooth muscle calponin is transiently expressed in the heart during mouse embryogenesis but then becomes restricted to adult SMC lineages.35,36 Based on our previous in vitro analysis of 3 intronic CArG elements,31 we surmised that intron 1 of human CNN1, whose CArG elements are 100% conserved with those in mice (data not shown), would orchestrate correct spatiotemporal expression of a lacZ reporter in transgenic mouse embryos. Surprisingly, of 44 independent founder mice, 22 failed to display any detectable β-galactosidase staining, and of the remaining 22, none exhibited correct cardiovascular-restricted activity (Supplemental Figure I). We then replaced exon 1, intron 1, and exon 2 of the mouse Cnn1 gene with a lacZ reporter and removed the neomycin cassette to assess β-galactosidase staining in mice lacking all intronic CArG boxes (Figure 2A). Southern
blotting (Figure 2B), quantitative reverse transcription–polymerase chain reaction (Figure 2C), and long and accurate polymerase chain reaction (data not shown) validated correct targeting of the \( Cnn1 \) gene. No evidence of lacZ activity was observed in heterozygous embryonic (data not shown) or adult tissues (Figure 2D). Furthermore, we have been unable to generate homozygous null mice despite a previous report of viable \( Cnn1 \) knockout mice using a different targeting strategy.\(^{37}\) The basis for this result is unknown and will be pursued in future studies. Because the lacZ reporter can be silenced,\(^{38}\) we determined whether the absence of \( Cnn1 \) staining in our \( Cnn1 \) heterozygous mice resulted from methylation of lacZ sequences; however, we found no evidence of methylated lacZ sequences (data not shown). Collectively, these results suggest that intronic CArG elements within smooth muscle calponin are necessary but not sufficient for in vivo promoter/enhancer activity.

**SMC-Restricted Expression of Human CNN1 in Mini-BAC Transgenes**

We previously demonstrated correct spatiotemporal expression of human CNN1 protein derived from a 103-kb BAC transgene.\(^ {32}\) To determine whether shorter versions of the original BAC could direct similar patterns of staining, we trimmed the 103-kb BAC to 38-kb and 19-kb lengths (Figure 3, top). Several lines of mice carrying each of these mini-BAC transgenes replicated SMC-specific CNN1 staining in such SMC-rich tissues as aorta, bladder, distal esophagus, and vessels of both cardiac and skeletal muscle (red stain in Figure 3). We also noted strong staining for human CNN1 protein in uterus, bronchiolar SMCs of the lung, and blood vessels in lung, kidney, and spleen (Supplemental Figure II). This staining was specific because nontransgenic littermates and non-SMC tissues (Figure 3 and Supplemental Figure II) showed no detectable CNN1 immunoreactivity. Substituting the CNN1 antibody with a nonimmune IgG control also revealed the absence of specific staining (Supplemental Figure III). These results demonstrate that as little as 19 kb of BAC sequence is sufficient to direct the restricted expression of CNN1 in essentially all vascular and visceral SMC lineages of adult tissues.

**A Single Intronic CArG Box Is Necessary for Human CNN1 Expression in Transgenic Mice**

We next generated a series of transgenic mouse lines with point mutated intronic CArG elements using a BAC recombineering strategy\(^ {39}\) (Figure 4A). Replacing \( \approx1 \) kb of intronic sequence comprising all 4 intronic CArG elements (C2 to C5, Figure 1) with a \( galK \) selectable marker (m1BAC38) completely abolished CNN1 protein staining in the aorta and reduced visceral SMC staining in the bladder (Figure 4B). Western blotting confirmed these expression changes (Figure 4C). On counterselection, wherein the \( galK \) cassette is replaced with wild-type human CNN1 BAC sequences containing point mutated CArG elements (m2BAC38), a similar loss in CNN1 staining was observed, indicating that attenuated SMC-specific CNN1 expression stems from loss in functional CArG elements. When only the consensus intronic CArG element (C2, Figure 1) was mutated (m3BAC38), there remained a dramatic decrease in

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**Figure 1.** Conservation of CArG sequences across the human \( CNN1 \) gene. Top, 38-kb bacterial artificial chromosome (BAC) illustrated with nucleotide coordinates relative to the transcription start site (TSS) of the \( CNN1 \) gene used throughout this study. Note that there are portions of 2 other genes (\( ECSIT \) and \( ELOF1 \)). Below this schematic is a VISTA plot showing the human \( CNN1 \) gene structure with translated and untranslated exons (E) numbered as dark blue and light blue boxes, respectively, and 5 CArG elements schematized with green vertical lines (top). The height of exonic (blue) and intronic/intergenic (pink) peaks represents the percentage of nucleotide sequence homology between the indicated species. Each of the 3 species’ plots depicts nucleotide sequence homology with human as the base sequence labeled from \( -4 \) to 16 kb at bottom (compare with 38-kb BAC at top). Bottom, Conservation of each of the 5 CArG elements is shown in sequence logos.\(^ {57}\) Note that each CArG element falls within a broader region of noncoding sequence homology (pink peaks).
CNN1 staining both in aorta and bladder (Figure 4C). These results were further supported by Western blotting studies that showed reduced human CNN1 protein in aorta, bladder, and stomach of several m3BAC38 mouse lines (Supplemental Figure IV). Finally, we examined the expression of human CNN1 in embryonic day 12.5 embryos. These results demonstrated expected human CNN1 expression in the developing heart and aortic SMCs of wild-type BAC38 mice, but loss of staining in m3BAC38 mice (Supplemental Figure V). Taken together, these results establish a necessary role for a single intronic CArG element (C2) for the expression of human CNN1 in mouse tissues. These findings are consistent with the loss in lacZ activity on deletion of endogenous mouse Cnn1 CArG elements (Figure 2D).

Identification and Functional Analysis of a Conserved CArG Box Upstream of the CNN1 Locus

Comparative sequence analysis revealed a previously undetected CArG element located \( \approx 3 \) kb upstream of the CNN1 gene (Figures 1 and 5A). This CArG element falls within a block of conserved sequence containing several putative CREB-binding sites (Figure 5A). We first determined the activity of this CArG element in cultured cells. A luciferase reporter displayed SRF- and myocardin-dependent transactivation, which was reduced on mutation of the CArG element (Figure 5B). A chromatin immunoprecipitation assay showed enriched SRF binding to the upstream CArG element in human SMCs (Figure 5B, inset). We next used BAC recombineering to replace the CArG/CREB-containing island of sequence homology with the galK cassette (m5BAC38) or counterselected and reintroduced wild-type sequences with a point mutated CArG box (m6BAC38) (Figure 5C). Each transgene was then introduced into the mouse genome to evaluate the role of this upstream, conserved CArG-containing region in human CNN1 expression. Results showed that loss of the entire conserved sequence block (m5BAC38) or mutation of just the CArG element (m6BAC38) had no effect on CNN1 expression in aortic SMCs, as well as vessels of the heart and skeletal muscle.
Moreover, there was no change in expression of CNN1 in vascular and visceral SMCs of other organs (Supplemental Figure VI). Thus, we conclude that an upstream functional CArG element (and putative CREB binding sites) is dispensable for CNN expression in vivo.

Human CNN1 Antagonizes Neointimal Formation in a CArG-Dependent Manner

Most SMC differentiation proteins, including CNN1, are downregulated during atherogenesis or following various mechanical injuries to the vessel wall. We performed partial ligation injury of the carotid artery in wild-type and pan-CArG mutant BAC38 mice to ascertain whether human CNN1 would be subject to the same negative regulatory cues accompanying arterial injury as endogenous mouse SMC differentiation markers. The pan-CArG mutant BAC38 mouse was engineered to have all 5 CArG elements defined in Figure 1 mutated; we refer to this transgenic line as m8BAC38. Similar to the m3BAC38 mouse, m8BAC38 mice displayed virtually no expression of human CNN1 in adult aorta, bladder, and vascular SMCs of brain, heart, kidney, and spleen (Supplemental Figure VII). Furthermore, there was a complete absence of CNN1 immunostaining in the uninjured (data not shown) and injured (Figure 6Ad) carotid artery. In contrast, wild-type BAC38 animals exhibited strong CNN1 expression in medial SMCs of the injured carotid artery (Figure 6Ac). Results from quantitative polymerase chain reaction studies revealed similar reductions in the mRNA expression of human CNN1 and endogenous mouse Cnn1 7 days after carotid ligation injury (Supplemental Figure VIII).

Interestingly, there was a notable absence of neointimal formation in wild-type BAC38 animals compared with the m8BAC38 controls (Figure 6Ac versus 6Ad). This was also evident in an independent, human CNN1-expressing transgenic line, suggesting that the phenomenon was not simply a result of the site-of-integration or some genomic perturbation (Supplemental Figure IX). Both vessel wall area (Supplemental Figure VIIIB) and circumference (Supplemental Figure VIIIC) were significantly higher in m8BAC38 mice, suggesting that human CNN1 protein imparts resistance to both outward remodeling and neointimal formation following injury. To begin to understand the basis for this unexpected phenotype, we evaluated the growth fraction of medial SMCs 7 days after injury by Ki-67 staining; there was no difference in medial SMC growth rate (Supplemental Figure X). A summary of the 70 transgenic mice studied in this report is provided in Supplemental Table II.

Discussion

Vascular SMCs are defined by a molecular signature of gene expression that includes an array of CArG-dependent cytokerin-dependent genes governed directly by the SRF-MYOCD transcriptional switch. The importance of SRF and MYOCD in the control of SMC gene expression has been demonstrated through
gene knockout experiments. Linking specific SRF-binding CARG elements to the activity of a gene’s promoter or enhancer requires in depth analyses in transgenic mice. Such transgenic studies have demonstrated the sufficiency of 1 or more CARG elements in recapitulating correct spatial and temporal patterns of SMC-specific gene expression. However, the smooth muscle isoform of calponin (CNN1), which is expressed transiently in the developing heart before emerging as a highly restricted marker for adult SMC lineages, has been a rather unconventional SMC-specific gene. For example, the SMC calponin’s in vivo regulation necessitated an alternative approach to solve this gene’s in vivo expression control. Several years ago, we adopted a BAC transgenic strategy because BAC cloning vectors accommodate large (up to 350-kb) genomic sequences that are likely to contain most, if not all, regulatory elements controlling a gene’s expression profile. Another advantage of using BACs to elucidate the control of gene expression is the preservation of the native genomic landscape versus the out-of-genomic context that is evident when using a surrogate reporter such as the bacterial lacZ gene. We reported previously on the identification of a 103-kb BAC harboring an unadulterated (ie, no lacZ or green fluorescent protein reporter introduced) human CNN1 gene that we subsequently integrated into the mouse genome for transgenic studies. We found that human CNN1 expression reproduced the endogenous mouse gene’s pattern of expression in both embryonic and postnatal tissues. In the present study, we trimmed the original BAC down to 38 kb and demonstrated the same pattern of human CNN1 expression as documented previously. We also provide evidence for a 19-kb BAC showing the same SMC-specific staining of adult tissues, including the vasculature. These results indicate that the CNN1 gene is not under the remote control of distal regulatory elements as we initially theorized based on results of other reports and that all regulatory control elements for tissue-restricted expression of CNN1 are contained within a relatively small genomic interval.

Evidence is provided here to support a vital role for a single intronic CARG element (C2, Figure 1) in directing the complete expression profile of CNN1 in both embryonic and adult SMC lineages. This analysis, however, required that C2 be in its native genomic location within a BAC because its presence in the context of a lacZ reporter failed to direct SMC-specific lacZ activity. The results of the m3BAC38 (consensus C2 mutant) also indicate that the 3 other intronic and upstream CARG elements are insufficient for CNN1 expression within the 38-kb BAC. This result is reminiscent of single CARG element functionality within other SMC-restricted genes. For example, the Tagln1 promoter contains 2 closely spaced CARG elements originally referred to as CARG-near and CARG-far; disruption of CARG-near completely inhibited muscle-specific activity of a lacZ reporter, whereas mutating CARG-far had no effect whatsoever. The Actg2 proximal promoter has 2 similarly positioned CARG elements, but only 1 (CARG-B) directs all cell-restricted activity of a reporter in transgenic mice. Finally, the Actg2 high-level Cnn1 expression. These results suggest that intronic CARG sequences in smooth muscle calponin require strict positional interactions with other genomic modules. A less likely explanation is there are salient differences in human CNN1 intronic sequences that influence CARG element functionality in transgenic mice.

The insufficiency of 4 conserved CARG elements in the first intron of human SMC calponin to direct cardiovascular restricted activity of a lacZ reporter is unprecedented, as every other SMC-restricted gene whose regulatory sequences have been examined in vivo display at least partial duplication of the endogenous gene’s pattern of expression. This is true for Actg2, Tagln1, Acta2, Myh11, Telokin, Kcmb1, and Csrp1. As reported here, the SMC calponin’s unconventional in vivo regulation necessitated an alternative approach to solve this gene’s in vivo expression control. Several years ago, we adopted a BAC transgenic strategy because BAC cloning vectors accommodate large (up to 350-kb) genomic sequences that are likely to contain most, if not all, regulatory elements controlling a gene’s expression profile. Another advantage of using BACs to elucidate the control of gene expression is the preservation of the native genomic landscape versus the out-of-genomic context that is evident when using a surrogate reporter such as the bacterial lacZ gene. We reported previously on the identification of a 103-kb BAC harboring an unadulterated (ie, no lacZ or green fluorescent protein reporter introduced) human CNN1 gene that we subsequently integrated into the mouse genome for transgenic studies. We found that human CNN1 expression reproduced the endogenous mouse gene’s pattern of expression in both embryonic and postnatal tissues. In the present study, we trimmed the original BAC down to 38 kb and demonstrated the same pattern of human CNN1 expression as documented previously. We also provide evidence for a 19-kb BAC showing the same SMC-specific staining of adult tissues, including the vasculature. These results indicate that the CNN1 gene is not under the remote control of distal regulatory elements as we initially theorized based on results of other reports and that all regulatory control elements for tissue-restricted expression of CNN1 are contained within a relatively small genomic interval.

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proximal promoter has 4 CArG elements, but only 1 of these CArG elements is necessary for full activity in vitro.51,52 An obvious question, therefore, is what function do extra CArG elements serve in these and other multi-CArG-containing regulatory sites? One idea is that multiple SRF-binding CArG elements "bridge" myocardin homooligomers to enhance transcriptional activity.16 There is clear evidence for this model in such multi-CArG genes as Actg2, Myh11, and Tagln1.16,52 Moreover, single CArG-containing SMC genes, such as Csrp1 and Telokin, are activated by myocardin,16,53 but the level of activation may not be as strong as that of multi-CArG-containing genes. Another possible function for multiple CArG elements in a gene locus may be related to sequestration of inactive SRF that would be on "reserve" for rapid deployment when active SRF over a dominant CArG element (such as C2 in the CNN1 locus) is depleted. There may also be other functions of SRF-bound CArG elements unrelated to transcription. For example, the orthologous SRF gene in yeast (Mcm1) has been implicated to play a role in DNA replication.54 A full understanding of CArG-SRF will require identification and functional characterization of the CArGome using both informatics and a variety of wet-laboratory assays, including ChIP-Seq and RNA-Seq.

A rationale for the use of BAC clones carrying human DNA sequences is to ascertain whether human genes respond to perturbations in mouse physiology in the same manner as the orthologous mouse gene. Here, we were interested to determine whether the expression of human CNN1 would be attenuated in the neointima following arterial injury where many SMC markers, including CNN1, are downregulated. As expected, we found that human CNN1 mRNA expression was reduced to a similar extent as the endogenous Cnn1 transcript. Surprisingly, there was little to no evidence for neointimal formation in 2 independent lines of transgenic mice with wild-type BAC38-mediated human CNN1 expression. In contrast, the same BAC vector carrying mutations in C1 to C5, where no human CNN1 expression was manifest, phenocopied both the outward remodeling and neointimal tissue seen in normal FVB mice. There are reports of CNN1 displaying tumor suppressor activity in the setting of cancer,55 and at least 1 report exists demonstrating SMC growth suppression on expression of CNN1 in vitro.56 However, the medial SMC growth rate was no different between wild-type BAC38 and the m8BAC38 transgenic mice 7 days after injury. The latter findings suggest that there may be differences in the growth fraction over time following injury or some other mechanisms of action are at play, including altered SMC migration. Whatever the mechanism is, if similar protective findings are found in other species and vascular disease processes, the CNN1 gene may represent a novel target of intervention for the treatment of vascular occlusive disorders.
In summary, we have shown through BAC transgenic and gene disruption studies that a consensus intronic CArG element appears necessary for smooth muscle calponin expression in vivo, despite the presence of 4 additional CArG boxes. However, intron 1 sequences taken out of their normal genomic landscape are insufficient for driving SMC-specific expression of a reporter gene, suggesting that there may be unique structural requirements for the SRF-bound consensus CArG element to mediate transcription optimally in vivo. Future work should examine more deeply the sufficiency of the consensus intronic CArG element for CNN1 expression in the context of a BAC, as well as the remodeling phenotype seen on overexpression of human (or rodent) CNN1.

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Disclosures

None.

References


Figure 6. Overexpression of human CNN1 prevents neointimal formation and outward remodeling of the injured vessel wall. A, Representative serial cross-sections of injured carotid artery of wild-type bacterial artificial chromosome (BAC) 38 (a, c, and e) or mBAC38 (b, d, and f) mice 3 weeks after partial ligation injury were stained with Masson trichrome (a and b) or with antibodies to human CNN1 (c and d) or ACTA2 (e and f). Note the thickened neointima and attendant reduction in ACTA2 immunostaining in the mBAC38 mouse. Scale bar=30 μm for all images. Quantitative analysis showed a statistically significant increase in vessel wall area (B) and vascular circumference (C) in the mBAC38 group of mice. WT indicates wild-type.


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Supplement Material

Materials and Methods

Comparative Genomics

We used the VISTA algorithm \(^1\) to analyze and plot orthologous 20-kb blocks of sequence from human, Rhesus monkey (\(Mmul\)), dog (\(Cfa\)), and mouse (\(Mmu\)) genomes in order to define the positions and sequences of CArG elements as well as overall sequence homology. A detailed protocol for performing VISTA plots is available upon request.

Transgenic Mouse Studies

All mouse studies were approved by the local Institutional Animal Care and Use Committee. Transgenic mice (FVB/N) were generated carrying human \(CNN1\) intron 1 controlling a hsp68-lacz reporter and embryos were stained for beta galactosidase and cleared with a 2:1 (v/v) solution of benzyl benzoate and benzyl alcohol as described \(^2\). A previously defined 103-kilobase (kb) BAC containing human \(CNN1\) \(^3\) was trimmed to 38-kb and 19-kb (Figure 3) and linearized DNA was injected into fertilized eggs of strain FVB/N mice. Transgenic mice were identified through PCR genotyping (primers in Supplemental Table I). Founder mice were bred to the F\(_1\) generation for developmental and postnatal tissue analysis of CNN1 protein expression. Southern blotting was done with specific probes to ascertain the integrity and copy number of each BAC transgene.

\(CNN1\) Knockout Mouse

We designed a targeting vector to replace the protein coding first exon, the entire first intron with all 4 CArG elements, plus the second exon and a portion of the second intron with a lacZ reporter gene (Figure 2A). To obtain this targeting vector, we PCR cloned two homology arms from genomic liver DNA of SV129EV mice using a high fidelity polymerase (primers in Supplemental Table I). The left homology arm, encompassing the entire 5’ untranslated sequence
and ~2-kb promoter sequence, was cloned into the BamHI and XhoI sites of the PKI-lacZ vector. In a similar manner, the right homology arm, encompassing most of intron 2 and exon 3 was cloned into SalI and NotI of the same vector. Sequence analysis confirmed high fidelity amplification of both homology arms. The resultant plasmid DNA was linearized with NotI and submitted to the University of Rochester’s Transgenic Core facility for electroporation into embryonic stem cells. Southern blot analysis of more than 100 independent embryonic stem cell clones revealed two potentially targeted cell lines. One of these clones was amplified and cells were injected into the blastocyst of several C57BL/B6 females to generate chimera. We obtained a total of 8 chimeras, two of which were bred to the germline. We then bred heterozygous Cnn1 null mice to a CMV-Cre mouse to excise the pgk-Neo cassette (Figure 2A). PCR and sequence analysis of genomic DNA confirmed that the Neo cassette was removed. We verified correct targeting of Cnn1 by two means. First, Southern blotting of XhoI or NheI digested DNA using a 5’ probe (outside the left homology arm) revealed correct wildtype and gene targeted bands (Figure 2B). Second, long and accurate PCR with a Neo cassette primer and a primer outside the right homology arm followed by sequence analysis revealed that the right homology arm was correctly targeted (primers in Supplemental Table I). N6 heterozygous mice (back-crossed to C57BL/B6) were then interbred and genotyped (during development and in adulthood) for wildtype, heterozygous, and homozygous null mice.

**BAC Recombineering**

Point mutations were introduced so as to abolish SRF binding to the CArG element as defined previously. DNA replacement or point mutations in each of the intronic or upstream CArG elements were generated within the 38-kb BAC containing human CNN1. The procedure involves two sequential steps as schematized in Figure 4A (protocol available upon request). Briefly, wildtype BAC DNA was electroporated into electro-competent SW102 bacterial cells
carrying the lambda prophage recombineering system (induced by heat shock at 42°C for 15 min) and a deleted galK gene and streaked on LB plates containing 12.5 μg/ml chloramphenicol. In the first step, 50 bp oligonucleotides corresponding to BAC sequences flanking each CArG element followed by galK sequences (Supplemental Table I) were used to amplify the galK cassette so as to create a PCR product with long (50 base pair) homology arms to the desired BAC sequence to be modified. The resultant PCR product was treated with DpnI (to remove methylated parental galK vector), gel purified by overnight low-voltage agarose gel electrophoresis and then electroporated in 0.1 cm cuvettes containing SW102 cells (plus wildtype BAC DNA) using a BioRad machine set with the following settings: 25 μF, 1.75 kV, and 200 ohms. Following 1 hr recovery in M9 salts and growth for 3 days at 32°C on M63 minimal media plates containing galactose, leucine, biotin, and chloramphenicol, bacterial colonies were streaked on MacConkey plates containing galactose and 12.5 μg/ml chloramphenicol. Resultant bright red colonies (representing probable clones that underwent successful recombination with the galK PCR product) were amplified and the BAC DNA purified for validation studies. To validate galK recombination into the BAC, we did exhaustive analyses to demonstrate (a) correct targeting of the BAC sequence and (b) fidelity of the remaining BAC sequences. Our analyses included, restriction digestion of wildtype versus mutant BAC DNA with Mmel, BamHI, and SacI followed by gel electrophoreses; Southern blotting using the galK sequence as a probe to demonstrate single integration events at correct loci; and PCR and sequence analysis across the recombined segment. In the second (counter-selection) step, the galK BAC was electroporated with a PCR product containing the original BAC sequence (replaced with galK) only the intervening CArG(s) carried point mutations shown previously to abolish SRF binding (See Figure 4A). The same exhaustive validation assays described above were done following the counter-selection step, including a final sequence analysis to confirm correct mutation
of CArG elements. Final mutant BAC DNA was linearized with EcoRI and injected in fertilized eggs for generating transgenic mice. Note that sequence analysis of the galK cassette failed to reveal any CArG-like elements (data not shown). See Supplemental Table II in conjunction with Figures 1, 4, and 5 for the BAC mutants studied in this report.

**Immunohistochemistry**

Sections (5 μm) from adult transgenic and wildtype littermates were obtained from most organs, including aorta and carotid artery as well as embryos for immunodetection of the human calponin protein (CNN1) using an antibody raised against the human protein. Adult mice were perfusion fixed with 10% neutral buffered formalin and tissues dissected for immersion fixation in the same fix for at least 24 hr. Tissues were processed and embedded in paraffin wax for micrometry. Sections were deparaffinized in two changes of xylene, rehydrated through a graded series of ethanol washes, and then treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. CNN1 protein (from human BAC) was detected with an antibody raised to human CNN1 (DAKO, M3556) at a dilution of 1:500 incubated overnight at 4°C followed by a 30 min room temperature (RT) incubation with a 1:2000 dilution of biotinylated horse anti-mouse secondary antibody and vector red alkaline phosphatase substrate kit (Vector Labs, BA-2000, Burlingame, CA). Staining with this antibody appears red throughout the figures. Smooth muscle alpha actin was detected by a 1 hr RT incubation using a monoclonal mouse anti-human antibody (DAKO, M0851) at a dilution of 1:1000, followed by a 30 min RT incubation with biotinylated horse anti-mouse secondary antibody (1:400) and 3,3’-diaminobenzidine substrate (brown color). In some cases, adjacent sections were stained with hematoxylin and eosin or Masson Trichrome stain to reveal the elastic lamellae of the vessel wall.
Western Blotting

Tissue homogenates were prepared from BAC transgenic mice to further validate expression of SMC markers and human CNN1 using antibodies we have used in a previous publication².

Luciferase Assay

A 3,204 nucleotide mouse Cnn1 promoter construct encompassing CArG1 was prepared by ligating a 1.2-kb Smal/EcoRV genomic fragment of the mouse Cnn1 upstream region to an MluI/EcoRV digest of a ~2-kb Cnn1 promoter construct in the pGL3 basic vector. CArG1 was mutagenized (Stratgene) with oligonucleotides (Supplemental Table I) and sequence verified. The rat PAC1 SMC line was co-transfected with SRFVP16⁵ or Myocd_v5⁷ and either the wildtype or mutant Cnn1 promoter plasmids. Quadruplicate samples were processed for luciferase activity as described previously²;⁵.

Chromatin Immunoprecipitation Assay

ChIP assays were carried out with EZ-ChIP kit (Millipore, 17-371) as described previously² using growing BC₃H₁ cells stably transfected with the wildtype human BAC38-kb transgene. Briefly, cells were cross-linked with 1% formaldehyde and protein-chromatin complexes were incubated overnight with 1 μg of SRF antibody (Santa Cruz, G-20) or rabbit IgG control. DNA-protein complexes were reverse cross-linked with 5 M NaCl at 65°C for 5 hr and DNA was then column purified. Equal amounts of input, IgG, or anti-SRF samples were PCR amplified with primers flanking CArG1 located upstream of the human CNN1 gene.

Partial Carotid Artery Ligation

Transgenic mice with either wildtype BAC38 or m8BAC38 (carrying mutations in CArG1-CArG5) transgenes underwent partial ligation of the left carotid artery essentially as described⁸ and
animals were perfusion fixed and carotid arteries harvested either 1 or 3 weeks following injury. Vessels at 1 week were immunostained with an antibody to Ki-67 and the growth fraction of medial SMC was calculated. In a separate experiment, 1 week injured vessels (or contra-lateral control vessels) were combined (n=2 in two studies) for total RNA isolation to assess endogenous mouse *Cnn1* and human *CNN1* mRNA by qPCR analysis. Fixed carotid arteries isolated 3 weeks following injury underwent special staining (H&E or Masson Trichrome), immunostaining for CNN1 and ACTA2, or determination of vessel wall area and circumference using Image Pro (version 6.2). Data were imported into GraphPad Prism (4.0) and statistically analyzed with an unpaired t-test (p<0.05 was considered significant).

References


Supplemental Figure Legends

Figure I. Lack of cardiovascular-restricted lacZ activity with human CNN1 intronic CArG elements. (A) Schematic of transgenic construct containing intron 1 of human CNN1. The 4 CArG elements in intron 1 are depicted as green vertical lines. (B) A sample of 6 independent founder embryos carrying the construct in panel A. Note the variable beta galactosidase staining that was also seen in the remaining 16 positive embryos. A total of 22 embryos showed no staining whatsoever.

Figure II. Human CNN1 protein staining in BAC38 transgenic mouse tissues. Human CNN1 immunodetection (red stain) in aorta (A), uterine SMC (B), esophagus (C), lung (D), kidney (E), and spleen (F). Staining of CNN1 is present in SMC of the lamina propria and surrounding SMC bundles of the distal esophagus near entrance to stomach (C). The inset in panel C shows absence of CNN1 staining in skeletal muscle of proximal esophagus. Note frequent CNN1 positivity in blood vessels around aorta (A) and within kidney (E) and spleen (F) parenchyma (arrows). Scale bar is 100 μm for panels A and B and 200 μm for panels C-F.

Figure III. Lack of human CNN1 staining in BAC transgenic mice using IgG control antibody. Sections of indicated tissues incubated with either non-immune IgG (top panels) or an equal concentration of the antibody against human CNN1 (bottom panels). Note positive blood vessels in brain (lower). The scale bar is 100 μm.

Figure IV. Attenuated expression of human CNN1 in SMC-containing tissues of m3BAC38 transgenic mice. Shown are a series of Western blots from a wildtype BAC38 mouse or each of 3
non-transgenic or m3BAC38 (consensus intronic CArG box, C2, mutated) mice probed for human CNN1 protein. Note uniform reduction of CNN1 staining in each independent m3BAC38 transgenic line. The apparent CNN1 signal seen in bladders from non-Tg-1 and m3BAC38-2 is due to bleed-over from adjacent lanes. Parallel blots with the same amount of protein loaded were probed for alpha tubulin to demonstrate equal input of protein extract.

Figure V. Human CNN1 staining in mouse embryos. Sagittal sections of day 12.5 embryos derived from non-transgenic FVB (panels A, D), wildtype BAC38 (panels B, E), and m3BAC38 (panels C, F) mice and stained for human CNN1 (brown). Note abundant expression of CNN1 only in the WTBAC38 embryonic heart (B) and vascular SMC of dorsal aorta (arrows in E). Little or no CNN1 staining is seen in SMC of the other aortic samples (arrows). The scale bar in panel A is 100 μm for all three hearts and the scale bar in panel D is 50 μm for the dorsal aorta images.

Figure VI. Expression of CNN1 in the absence of a functional upstream CArG box. Sections of brain (Br), stomach (St), and proximal esophagus (Es) from indicated transgenic line stained for human CNN1 (red). Note CNN1 staining of SMC in vessels of brain (arrows), visceral SMC bundles in stomach, and lamina propria of esophagus. Neither mutant altered expression of human CNN1. Scale bar is 50 μm (m5BAC38) or 100 μm (m6BAC38).

Figure VII. Loss of human CNN1 expression in pan-CArG mutant (m8BAC38) transgenic mice. There is little to know CNN1 staining in the indicated tissues derived from m8BAC38 mice. Arrows point to vessels in each tissue. The dotted line in bladder (Bl) coincides with a bundle of SMC. A coronary arteriole from a wildtype (no CArG mutations) BAC38 mouse heart (He) is
clearly stained positive. Scale bar at lower right of spleen (Sp) panel is 30 μm for all m8BAC38 tissue samples.

**Figure VIII. Expression of SM calponin mRNA following arterial injury.** WTBAC38 mice underwent partial ligation injury and 7 days later injured and contralateral (uninjured control) carotid arteries were processed for total RNA isolation and qPCR. Shown are two independent experiments (n= 2 animals each) revealing decreases in both human CNN1 and endogenous mouse Cnn1 mRNA following arterial injury.

**Figure IX. Lack of outward remodeling and neointimal formation in a second CNN1 expressing transgenic mouse.** Hematoxylin and eosin stained sections of carotid artery obtained 3 weeks following partial ligation injury in non-transgenic FVB, m8BAC38, and m6BAC38 (expressing human CNN1, Figure 5D) mice. Each of these sections were derived from different mice than those shown in Figure 6A. The scale bar is 50 μm. Note the similarity between the FVB mouse and the m8BAC38 mouse that does not express human CNN1.

**Figure X. SMC growth rate following arterial injury.** Groups of WTBAC38 (panels A-C) and m8BAC38 (panels D-F) mice underwent partial ligation injury and 7 days later processed for Ki-67 staining (panels A, D), CNN1 staining (panels B, E), or IgG control staining (panels C, F). The insets in panels B and E represent the corresponding contralateral vessel stained for CNN1. Scale bar at bottom of panel F measures 100 μm for all panels. Quantitative measures of the medial SMC growth rate were determined by counting the number of nuclei staining positive for Ki-67 and expressing that number as a percent of the total number of nuclear profiles (G, n=3 mice per group).
Long et al, Supplemental Fig. III

Aorta  Brain  Kidney  Spleen
Long et al, Supplemental Fig. IX

FVB | m8 | WT
Long et al, Supplemental Fig. X

G

Medial SMC Growth Rate

WT BAC38  m8BAC38

p = 0.752
### Key PCR Primers Used

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Supplemental Table II
Summary of Transgenic Mouse Studies

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<tr>
<th>Transgenic Line</th>
<th>Ratio of Expressers</th>
<th>Notes</th>
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<tr>
<td>CNN1-Int1-hsp68-lacZ</td>
<td>22/44</td>
<td>None recapitulated endogenous expression in e12.5 embryos</td>
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<tr>
<td>WT BAC38</td>
<td>5/5</td>
<td>All 5 showed correct expression of human CNN1</td>
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<tr>
<td>WT BAC19</td>
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<td>Both lines showed same pattern of expression as WT BAC38</td>
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<td>m1BAC38 (galK replaced intron 1)</td>
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<td>All lines exhibited no CNN1 expression in aorta; weak in bladder</td>
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<td>All lines showed virtually undetectable expression of CNN1</td>
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<td>Pattern of expression was identical to WT BAC38 and BAC19</td>
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<td>m8BAC38 (mutation of C1-C5)</td>
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<td>No CNN1 expression despite higher than average copy number</td>
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Please consult Figure 1 for the position of each CArG element (labeled C1-C5 in Figure 1).