Smooth Muscle Expression of Lipoma Preferred Partner Is Mediated by an Alternative Intronic Promoter That Is Regulated by Serum Response Factor/Myocardin

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Abstract—Lipoma preferred partner (LPP) was recently recognized as a smooth muscle marker that plays a role in smooth muscle cell migration. In this report, we focus on the transcriptional regulation of the LPP gene. In particular, we investigate whether LPP is directly regulated by serum response factor (SRF). We show that the LPP gene contains 3 evolutionarily conserved CArG boxes and that 1 of these is part of an alternative promoter in intron 2. Quantitative RT-PCR shows that this alternative promoter directs transcription specifically to smooth muscle containing tissues in vivo. By using chromatin immunoprecipitation, we demonstrate that 2 of the CArG boxes, including the promoter-associated CArG box, bind to endogenous SRF in cultured aortic smooth muscle cells. Electrophoretic mobility-shift assays show that the conserved CArG boxes bind SRF in vitro. In reporter experiments, we show that the alternative promoter has transcriptional capacity that is dependent on SRF/myocardin and that the promoter associated CArG box is required for that activity. Finally, we show by quantitative RT-PCR that the alternative promoter is strongly downregulated in SRF-deficient embryonic stem cells and in smooth muscle tissues derived from conditional SRF knockout mice. Collectively, our data demonstrate that expression of LPP in smooth muscle is mediated by an alternative promoter that is regulated by SRF/myocardin. (Circ Res. 2008;102:61-69.)

Key Words: smooth muscle ■ transcriptional regulation ■ serum response factor

Migration and proliferation of smooth muscle cells (SMCs) are known to play a fundamental role in the development of atherosclerosis, which is among the leading causes of death in the Western world.1 Recently, we2 and others3 identified the LIM domain protein lipoma preferred partner (LPP) as a novel SMC marker, and in a follow-up study,4 LPP was shown to regulate SMC migration. LPP belongs to the zyxin family of LIM domain proteins that consists of 7 members: zyxin,5 TRIP6/ZRP1,6 LPP,7 ajuba,8 LIMD1,9 WTIP,10 and FBLP-1/migfilin/Cal.11,12 In cultured fibroblasts and aortic SMCs, LPP colocalizes with vinculin at focal adhesions, which are attachment sites to the extracellular matrix.3,13,14 In transverse sections of bladder smooth muscle (SM), an association of LPP with peripheral dense bodies is suggested.1 In addition to these cytoskeletal localizations, LPP also shuttles to the nucleus,13 where it acts as a coactivator for the ETS domain transcription factor PEA3.15 LPP contains multiple protein–protein interaction domains. In addition to PEA3, it interacts with α-actinin,16 vasodilator-stimulated phosphoprotein (VASP),13 Palladin,17 and Scrib.18 The proposed role of LPP in SMC migration and the coexpression of LPP and SM α-actin in the neointima after stent-induced vascular injury suggest that LPP may play an important role in for example atherosclerosis.4 The mechanisms by which LPP is regulated in physiological and pathological conditions are therefore of clinical interest. In the present study, we investigate the transcriptional regulation of the LPP gene. Many SMC marker genes are regulated by the transcription factor response factor (SRF).19 SRF belongs to the MADS box family of transcription factors20 and is involved in the regulation of disparate programs of gene expression linked to muscle differentiation and cellular growth.19 It regulates the expression of SM-specific isoforms of contractile and cytoskeletal proteins, such as SM α-actin21 and SM–myosin heavy chain (MHC),22 via cis-elements known as CArG boxes.19,23 Because SRF plays an important

Original received June 22, 2006; resubmission received April 15, 2008; revised resubmission received May 16, 2008; accepted May 16, 2008.


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DOI: 10.1161/CIRCRESAHA.108.177436
role in SMC marker gene transcription, we investigated whether the transcription of the LPP gene is directly regulated by SRF. Here, we apply mRNA expression analysis, chromatin immunoprecipitation (ChIP), electrophoretic mobility-shift assays (EMSA), reporter experiments, and mRNA expression analysis in conditional SRF knockout mice to establish that SM expression of LPP is mediated by an alternative intronic promoter that is regulated by SRF/myocardin.

Materials and Methods

Prediction of CArG Boxes

The complete Lpp locus plus 100 kb of flanking sequences was extracted from ENSEMBL Mouse (build NCBI33). The sequence was screened for putative CArG boxes using an SRF position frequency matrix from TRANSFAC (accession no. M00186). Relative frequencies for the nucleotides in each position of the matrix were estimated by adding a pseudocount of 1 to each element. Each position in the sequence (sense and antisense) was evaluated by calculating a relative score between 0 and 1 using a uniform background model. A detection threshold (0.87) that included known CArG boxes in the Acta2 promoter was applied. Calculations were done in Matlab (MathWorks). All predicted sites were manually evaluated for conservation between species by examining the corresponding Mutlu et al. vertebrate alignment in the UCSC browser.

RT-PCR and Real-Time RT-PCR

Total RNA was isolated from tissues using the GenElute mammalian total RNA isolation kit (Sigma-Aldrich). RNA from embryonic stem (ES) cell lines E14.1, ES100, ES100-SRF-VPI6, and ES100-SRF-ΔM-VPI6 and RNA from conditional SRF knockout bladder, colon, heart, and colonic SMCs and their wild-type (WT) counterparts was isolated as described.27,28 RNA quality was confirmed using RNA6000 nanochips on a Agilent 2100 Bioanalyzer. RNA samples were quantified using a NanoDrop spectrophotometer (NanoDrop technologies) and diluted to the same concentration. Details of the real-time RT-PCR are described in the online data supplement, available at http://circres.ahajournals.org.

Chromatin Immunoprecipitation

Mouse aortic SMCs (passage 8) were fixed by adding formaldehyde to the culture medium. Cells were harvested and prepared for immunoprecipitation using the protocol of the EZ ChIP kit (Upstate) with minor modifications. To reduce nonspecific background, a sample containing 2×10⁶ cells was precleared with Salmon Sperm DNA/Protein A Agarose (Upstate), whereafter 2.5% was removed to be used as input control. One-half of the remaining sample was subsequently incubated with 5 μg of anti-SRF antibody (Santa Cruz Biotechnology), the other half was incubated in the absence of antibody overnight. Antibody–chromatin complexes were precipitated using Salmon Sperm DNA/Protein A Agarose. Precipitated complexes were washed, and histone complexes were eluted from the antibody. Histone–DNA crosslinks were reversed, and the DNA was purified using the Qiaquick PCR purification kit (Qiagen). Two microliters of each sample were subjected to PCR amplification using primer sets specific for the CArG containing regions (see online data supplement).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from cultured murine aortic SMCs (passage 7, near confluence) using the CellLytic NuCLEAR kit (Sigma-Aldrich). Double-stranded oligonucleotides corresponding to CArG boxes 8, 11, and 13 were end-labeled with [32P]γ-ATP using PNK (Roche). Four microliters of VSMC nuclear extract in binding buffer (10 mmol/L HEPES [pH 7.9], 70 mmol/L KCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 2.5 mmol/L MgCl₂, 4% Glycerol) were incubated with labeled probe for 20 minutes at room temperature. Each reaction contained 1 μg of poly(dI/dC) (Amersham Pharmacia Biotech, Little Chalfont, UK). For supershift experiments, 4 μL of anti-SRF antibody (sc-335, Santa Cruz Biotechnology) was added before incubation with labeled probe. Samples were separated on a 4% nondenaturing polyacrylamide gel in 1× Tris borate EDTA buffer. Gels were dried and exposed overnight on a BAS-MS imaging plate (FujiFilm), which was scanned using a FLA-3000 fluorescent image analyzer (FujiFilm). Oligonucleotides are listed in the online data supplement.

Cell Culture

Aortic SMCs were obtained from 12-week-old, WT, male 129SV mice. The cells were isolated from segments spanning from the aortic arch to the exit of the renal arteries.29 SMC phenotype was confirmed as outlined in Figure I in the online data supplement. The cells were cultured in DMEM (Life Technologies) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Cells were grown in a humidified incubator at 37°C and 5% CO₂.

Luciferase Reporter Experiments

Lpp CArG 8, 11, and 13 reporter plasmids were constructed by subcloning a PCR-amplified 0.5-kb genomic region surrounding the CArG box into the pGL3-Basic Vector (promoter reporters) or pGL3-Promoter Vector (enhancer reporters) (Promega). PCR primers are listed in the online data supplement. Identical reporters with mutated CArGs were made using the QuickChange Site-Directed Mutagenesis kit (Stratagene). All synthetic mutations, ligation sites, and PCR-amplified regions were verified by sequencing.

Reporter experiments were performed in primary aortic SMCs as described in the online data supplement. The SRF cDNA–containing vector was obtained from OriGene, and the myocardin cDNA-containing vector was a kind gift from Eric Olson (University of Texas Southwestern Medical Center, Dallas).

Results

The Lpp Gene Contains Three Evolutionarily Conserved CArG Boxes

SMC marker gene transcription is largely dependent on interactions between SRF and CArG boxes,19 which led us to investigate whether SRF is directly involved in the transcriptional regulation of LPP. As a first step, potential SRF binding sites in the mouse Lpp gene were identified with a bioinformatic approach. Lpp spans a large genomic region of nearly 600 kb (Figure 1). The complete Lpp gene and 100 kb of flanking sequence was screened for CArG boxes. In total, 35 putative CArG boxes were found scattered all over the gene. These sites were manually evaluated for species conservation, because previously identified functional CArG boxes are evolutionarily conserved.30 In this way, only 3 sites, CArG 8, CArG 11, and CArG 13, were found to be well conserved across the available species (supplemental Figure II).

CArG 8 Is Part of an Alternative Promoter Located in Intron 2

With few exceptions, known CArG elements reside within 4 kb of transcription start sites (TSSs), which probably reflects a need for SRF to be relatively close to the RNA polymerase II holoenzyme.19,30 In contrast, CArG 8 is located at 55 kb downstream of the Lpp promoter and CArG 11 and 13 reside at 85 and 100 kb downstream, respectively (Figure 2a). The distance between the promoter and CArG 8 led us to screen the Lpp gene for alternative TSSs. The DBTSS database is a collection of full-length cDNA sequences that are produced...
with the oligo-capping method.\textsuperscript{31,32} Five Lpp cDNA clones had 5’ ends that suggested a TSS located \( \sim 140 \) bp downstream of CArG 8. Moreover, the cDNA sequences included a novel first exon (hereafter denoted as exon 2b) (Figure 2a and supplemental Figure III). Analysis of the sequence region between \(-500\) and \(+200\) around this alternative TSS, using the promoter prediction program Promoter 2.0\textsuperscript{33} suggested the presence of a promoter with a TSS that precisely matches the 5’ end of the DBTSS clones. Moreover, the 250-bp region that precedes this TSS is highly conserved in 10 mammalian species, which further supports the presence of a promoter region (supplemental Figure III).

We designed RT-PCR primers to confirm the presence of the alternative transcript in SM-containing tissues. RT-PCR with a forward primer in exon 2b and a reverse primer in exon 3 produced an amplification product of the predicted size (Figure 2b). We further investigated whether exon 2b–containing transcripts are splice variants or products of an alternative promoter. RT-PCR with a forward primer in exon 2a and a reverse primer in exon 3 produced a single amplification product of the expected size, given that exon 2b was not part of the transcript (Figure 2b). In contrast, RT-PCR with a forward primer in exon 1 and a reverse primer in exon 2b did not produce an amplification product (Figure 2b). Together, these results show that the alternative transcript is not a splice variant but instead produced by an alternative promoter.

The Alternative Promoter Directs Transcription Specifically to SM-Rich Tissues

TaqMan RT-PCR assays that specifically target the exon 2b–exon 3 boundary and the exon 2a–exon 3 boundary were designed to investigate the tissue specificity of the alternative and upstream promoter, respectively. An assay that targets the exon 9–exon 10 boundary, and thereby detects transcripts from both promoters, was used for comparison. mRNA was isolated from several WT adult mouse tissues, as depicted in Figure 3.

The alternative exon 2b containing transcript was abundantly expressed in all tissues with large proportions of SMC (Figure 3a). The distribution was overall strikingly similar to the distribution of the SMC marker SM-MHC (Myh11) (Figure 3b). In contrast, the exon 2a containing transcript was ubiquitously expressed (Figure 3c). The exon 9 containing transcripts were predominantly expressed in SMC-rich tissues (Figure 3d), which indicates that LPP transcription in SMC is primarily initiated from the alternative promoter.

We conclude that the alternative promoter directs expression specifically to SMC-rich tissues and that the alternative promoter is the primary regulator of LPP transcription under physiological conditions.

SRF Binds to Lpp CArG 8 and 13 in ChIP Assays

ChIP experiments were performed to determine, if SRF binds to the conserved endogenous CArG boxes in the LPP gene in cultured mouse aortic SMCs. Precipitation of chromatin with

![Figure 1](image-url). The LPP gene: prediction of CArG boxes. Schematic outline of the mouse Lpp genomic locus showing the positions of the 35 putative CArG boxes in relation to the 13 exons of the Lpp gene and its promoter. Asterisks indicate coding exons.

![Figure 2](image-url). An alternative promoter and first exon in intron 2. a, Schematic outline of the Lpp genomic locus showing the alternative promoter, alternative first exon 2b, and CArG 8, 11, and 13. b, RT-PCR data showing the presence of the transcript produced by the alternative promoter (exon 2b–exon 3) and the upstream promoter (exon 2a–exon 3) in RNA from aorta (A), stomach (S), and bladder (B). No transcript that includes both exon 1 and exon 2b was detected.
an anti-SRF antibody specifically enriched sequences containing Lpp CarG 8 and CarG 13 but not CarG 11 (Figure 4a). The same procedure was applied to the nonconserved CarG boxes 1 to 5 because they are located in the vicinity of the upstream promoter. No binding was recorded to any of these sites (Figure 4b). CarG 8 was used as a positive control. The results show that endogenous SRF binds to Lpp CarG 8 and CarG 13 in primary aortic SMCs.

**CArG 8, 11, and 13 Bind SRF in VSMC Nuclear Extracts**

EMSAs were used to evaluate binding of SRF to the 3 conserved CArG boxes in vitro. Nuclear extracts from cultured mouse aortic SMCs were incubated with radiolabeled double-stranded probes that contained the predicted sites. All 3 probes formed single protein-DNA complexes that were of the same size (Figure 5). The complexes were supershifted in the presence of an SRF antibody, which confirms that the CArG boxes bind SRF in vitro (Figure 5).

**CArG 8 Is an SRF/Myocardin-Responsive Element That Is Required for LPP Gene Transcription via the Alternative Promoter**

The transcriptional activity of the conserved CArG elements was tested in luciferase reporter assays. Genomic fragments containing the conserved CArG boxes were cloned into the promoter-less reporter vector pGL3basic. The CarG 8–containing construct, including the alternative transcription start, increased luciferase activity ∼40-fold relative to the empty vector in aortic SMCs (Figure 6a). Deletion of the CarG box dramatically reduced its transcriptional activation capacity (Figure 6a). In contrast, CarG 11– and CarG 13–containing genomic fragments displayed low (∼2-fold) and no promoter activity in this assay, respectively (Figure 6a). We also subcloned the 3 CarG-containing genomic fragments in the pGL3promoter vector to test their enhancer activity. CarG 8–, 11–, and 13–containing fragments induced luciferase activity by 8-, 2-, and 4-fold, respectively (Figure 6b). The activity of the CarG 8– and 11–containing fragments were not dependent on the CarG boxes, whereas deletion of CarG 13 reduced activity ∼50% (Figure 6b).

In follow-up experiments, we tested whether the CarG 8–containing fragment was responsive to SRF and myocardin. Unexpectedly, increasing amounts of the transcriptional activator SRF downregulated the transcriptional activity (Figure 6c). Deletion of the CarG 8 box completely abolished this downregulation (Figure 6c). CarG-dependent downregulation of a promoter by ectopic overexpression of SRF has previously been described for the c-fos promoter. In this case, the phenomenon was concluded to be attributable to squelching that leads to unspecific repression of transcription. We next tested the impact of myocardin. Overexpression of...
myocardin strongly induced the promoter activity, and deletion of CARG 8 completely abolished this activation (Figure 6d).

Overall, these results show that the predicted alternative promoter has strong transcriptional activation capacity in aortic SMC. They also show that the alternative promoter is SRF/myocardin-responsive and that the CARG 8 box is critical for this activity.

The Alternative Promoter Is Robustly Downregulated in SRF-Deficient Embryonic Stem Cells and SM Tissues

To confirm the physiological relevance of our observations, we investigated whether the exon 2b transcript levels were affected by the absence of endogenous full-length SRF and/or the overexpression of modified SRF molecules.

We first looked into an SRF-deficient ES cell system (ES100). This system allows strong induction of SRF target genes, independent of signal transduction, on overexpression of the constitutively active SRF fusion protein SRF-VP16. SRF-ΔM-VP16, lacking the MADS box and, therefore, defective in DNA binding, was used as a negative control. Exon 2b transcript levels were downregulated 70% in SRF-ΔM ES cells as compared with WT ES cells (Figure 7a), and SRF-VP16 strongly induced expression 10-fold relative to SRF-ΔM-VP16 (Figure 7a). In contrast, exon 2a transcript levels were similar in WT ES cells and SRF-ΔM ES cells (Figure 7a), and SRF-VP16 only induced expression 1.7-fold relative to SRF-ΔM-VP16 (Figure 7a). These results indicate that the exon 2b transcript is the primary target of changes in SRF activity in this system.

We next investigated transcript levels in SM-specific SRF knockout mice. In these mice, a floxed Srf allele was conditionally deleted in adult mice by the CreERT2 recombinase expressed via a SM-specific SM22α promoter. Cre recombinase activity is subsequently induced by treatment of animals with tamoxifen, which activates the CreERT2 protein. SRF was robustly downregulated in colon, bladder,
colonic SMCs (CSMCs) from the conditional SRF knockout mice, whereas SRF levels in heart were similar to controls as expected (Figure 7b). Exon 2b transcript levels were downregulated in all SRF knockout SM tissues and cells: a 90% reduction in colon, 85% in bladder, and 75% in CSMCs (Figure 7b). Interestingly, exon 2a transcript levels were also downregulated but to a lesser extent: 60% reduction in colon and bladder and 55% in CSMCs (Figure 7b).

Overall, these results show that the alternative promoter, producing the exon 2b–containing transcript, is the primary target of SRF regulation in SMC under physiological conditions.

### Discussion

Here, we present the first experimental investigation of the transcriptional regulation of the LPP gene. We show that the LPP gene contains 3 evolutionarily conserved CArG boxes and that 1 of these is part of an alternative promoter located in intron 2. This promoter directs expression specifically to SM-containing tissues. We further demonstrate that SRF occupies 2 of the 3 CArG boxes in primary aortic SMC, including the promoter-specific CArG box. The alternative promoter has strong transcriptional activation capacity that requires an intact CArG box in reporter experiments, and this activity is critically regulated by SRF/myocardin. We finally show that the alternative promoter is strongly downregulated in SRF knockout ES cells and in SM tissues from conditional

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<th>Figure 6. Reporter gene analysis of promoter activity in CArG 8, 11, and 13 regions in Lpp. The promoter (a) and enhancer (b) activity of genomic regions surrounding CArG 8, 11, and 13 was evaluated in reporter assays by transient transfection of luciferase reporter plasmids in primary aortic SMC. The influence of SRF and myocardin on the activity of the CArG 8–containing alternative promoter was tested by cotransfection of 10 or 100 ng of SRF cDNA–containing vector (c) and 100 or 500 ng of myocardin cDNA-containing vector (d). The impact of the CArG boxes was evaluated by deleting it (del). The luciferase activity is presented as relative fold change compared with empty vector. Error bars show SEM (n=3).</th>
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show that myocardin also regulates transcription of the alternative LPP transcript. This is in agreement with an earlier report that showed that LPP transcription in the SMC-like cell line A404 is activated by myocardin. Many SMC-specific genes contain 2 or 3 CArG boxes, and cooperation between multiple CArG elements, the 2-CArG model, has been proposed to contribute to SMC-specific transcription by myocardin. However, the alternative promoter in LPP contains a single CArG box, and the other conserved CArG elements, CArG 11 and 13, are located 30 and 45 kb downstream of CArG 8. The interactions that are referred to in the “2-CArG model” occur over short distances, and interactions between the conserved LPP CArG boxes therefore seem unlikely. Interestingly, the 2 most closely related LPP family members, zyxin and TRIP6, are also likely to be regulated by SRF: Schratt et al reported that zyxin is

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**Figure 7.** The alternative promoter is regulated by SRF in vivo. a, TaqMan RT-PCR was applied to mRNA from WT E14.1 ES cells and SRF−/− ES100 ES cells (left) and from ES100 ES cells overexpressing SRF-VP16 or SRF-VP16 with a deleted MADS box (ΔM) (right). b, TaqMan RT-PCR was applied to mRNA from colon, bladder, heart, and CSMCs derived from conditionally WT and SRF KO mice. Primers amplify mRNA products that are specific for the alternative Lpp promoter (Lpp 2b), the upstream Lpp promoter (Lpp 2a), the total Lpp transcripts (Lpp 9 to 10), and SRF. The diagrams display relative expression levels for each transcript. Error bars show SEM (n=4 in a, n=3 in b).
downregulated in SRF−/− ES cells, and Sun et al. reported that the TRIP6 gene has a CArg box that competes with SRF binding to a radiolabeled CArg element in EMSA experiments. This suggests that not only LPP but also its most closely related family members are regulated by SRF.

The function of the evolutionarily conserved CArg 11 and 13 boxes remains elusive. One possibility would be that CArG 11 and 13 are regulatory elements of yet another alternative promoter in the LPP gene. There is, however, no evidence for additional transcriptional start sites in the vicinity of these elements and neither CArg 11 nor CArg 13 displayed promoter activity in luciferase experiments. CArg 13 displayed some enhancer activity, but the physiological relevance of this is not clear. In genes containing several functional CArG boxes, individual CArG boxes may be required or dispensable depending on the tissue under investigation. It is possible that CArg 11 and 13 would display functionality if experimental conditions were adapted or if binding were tested in another cell type. Another intriguing possibility refers to similar sequences in yeast that bind the SRF ortholog Mcm1. These sequences mediate gene transcription as well as DNA replication. Collectively, the SRF binding experiments, the reporter experiments, and the expression analyses of LPP transcripts in SRF-modulated cells and mice show that the alternative promoter is regulated directly by SRF in SMCs. Interestingly, the upstream transcript also showed consistent, although weaker, regulation by overexpression or absence of SRF. This may indicate that SRF interacts with the upstream promoter or with enhancer elements in the vicinity of the promoter. We did, however, not find any conserved CArG elements in this region of the gene and failed to show binding of SRF to any of the nonconserved CArG elements in ChiP experiments. Another possibility refers to the global effects of SRF overexpression or depletion. More than 160 genes are known to be regulated by SRF, and modulation of its activity may have profound effects on the cell phenotype that indirectly effects LPP transcription. Further studies will be necessary to discriminate among these possibilities.

Taken together, our data establish that LPP transcription is initiated by multiple promoters and that the exon 2b promoter directs transcription specifically to SMC-rich tissues. Moreover, we show that the exon 2b promoter is regulated by SRF/myocardin and that a conserved CArg box is required for this regulation.

Acknowledgments

We thank the Göteborg Genomics Resource Center at Swegene for supplying equipment and support.

Sources of Funding

This work was supported by the Swedish Cancer Foundation and the Swedish Research Council. E.L. was supported by Lymphangio-genomics, an Integrated Project funded by the European Commission within its FP6 Programme, under the thematic area “Life sciences, genomics and biotechnology for health” (contract no. LSHG-CT-2004-503573). M.M.R.P. was a postdoctoral fellow of the Fund for Scientific Research (Flanders, Belgium) (F.W.O.-Vlaanderen) and received a 1-year Mobility grant from the F.W.O.-Vlaanderen to perform this study. At present, M.M.R.P. is supported by GOA/2008/16. A.N. was supported by the Deutsche Forschungsgemeinschaft through grant SFB446.

Disclosures

None.

References


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*Circ Res.* 2008;103:61-69; originally published online May 29, 2008; doi: 10.1161/CIRCRESAHA.108.177436

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Expanded Materials and methods

RT-PCR and real-time RT-PCR
Reverse transcription was done on 200 ng of total RNA using the ABI High Capacity cDNA archive kit with random primers. The following primers were used for RT-PCR: LPP exon 2b-3, 5’-AGTGAAGGCCAGCTCTCTGT-3’ and 5’-GGGGGAACGGTGAAAAAGTA-3’; LPP exon 2a-3, 5’-AGAGCTGCTTGGGACTCCTC-3’ and 5’-GGAACAATGCTTCCCTCCA-3’; LPP exon 1-2b, 5’-CCCAGGATAAGTAGCGTTAG-3’ and 5’-CTGACTCCCTCTCACAAGC-3’. For real-time quantitative PCR, the following TaqMan probes were used in combination with the above described primers: LPP exon 2b-3, 6FAM-ACCTCCTCAGTGGAACACAGGCTC-MGB; LPP exon 2a-3, 6FAM-CTCCCAAGGAGACTGGGGAAC-MGB. Predesigned TaqMan assays for LPP exon 9–10 (Mm00724478_m1) and SM-MHC (Mm00443013_m1) were ordered from Applied Biosystems. Real-time PCR was carried out on an Applied Biosystems 7900 Sequence Detector under standard cycling conditions.

Chromatin immunoprecipitation
Primer sets specific for the region containing CArG 1 (5’-AAGCCAGACAATGAATCAGC-3’ and 5’-GCCACTAAGACTTGTACGCACC-3’), CArG 2 (5’-CAGCCACTCTGTGTTTCCCTTCATGGAAC-3’), CArG 3-4 (5’-ATGCCAGGCTTGGGAAGTA-3’ and 5’-AGCAGCTCTTTGTCTGCCTC-3’), CArG 5 (5’-GACAACCCTTGTGCAAGCACC-3’ and 5’-AATACCTGAAAGTGGGAGCTG-3’), CArG 8 (5’-AAGGAGGTCTGCCTGTGGAC-3’ and 5’-GAGTCACCCGCTCCCTTCCCTTCC-3’), CArG 11 (5’-TGAGAGCGAACAAGCAAA-3’ and 5’-TGACCGAGAGATGCAACAG-3’), and CArG 13 (5’-CTCCTCTCTTTCTGCTCCTCCTTCT-3’).

Electrophoretic mobility shift assays (EMSA)
The following oligonucleotides were used: LPP CArG 8, 5’-CCTCTCCATATAAGGAAAATGT-3’ and 5’-ACATTTCTTATATGGGAGAAGG-3’; LPP CArG 11, 5’-TTGAGGTCCATATAAGGGCACGCT-3’ and 5’-
AGCTGCCCTTATATGGACACAA; LPP CArG 13, 5’-TTATTACCATATTAGGCACTGA-3’ and 5’-TCAGTGCCTAATATGGTAATAA-3’.

Construction of reporter plasmids
The following primers were used with appropriate flanking restriction sites added: CArG 8, 5’-ATTGGGAGGCTGTTTCTGG-3’ and CTGACTCCCTCTCACCAAGC; CArG 11, 5’-TGCAGCAAGCTATACCAGTGA-3’ and 5’-TCTGACACACAGGCACAACA-3’; CArG 13, 5’-TCCTCACACACACGCTTCCATCCA-3’ and 5’-TCCTTAGTCCCATCCCATGC-3’.

Luciferase reporter experiments
Twenty-four hours after seeding, semiconfluent VSMC cells in 24-well plates were transiently cotransfected using Fugene HD (Roche) with 1 µg of luciferase reporter construct, and 5 ng of pRL-SV40 (Promega). 10 or 100 ng of human SRF cDNA-containing vector (Origene) and 100 or 500 ng of mouse Myocardin cDNA-containing vector (kind gift from Dr Eric N. Olson) were used for co-transfection experiments. The total mount of DNA in each transfection was kept constant by adding an empty pcDNA3.1-vector (Invitrogen). Cell lysates were prepared 48 h after transfection and assayed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega). For each experiment, luciferase activity was determined in duplicate wells, and each experiment was repeated at least three times.
Online Figure I. Two batches of primary SMCs were generated. The first batch was used for EMSA and ChIP experiments and the second batch for reporter experiments. 

(A) The first batch was characterized with real-time RT-PCR for expression of SMC markers. The levels were compared to undifferentiated mouse embryonic stem cells. All markers were expressed at markedly higher levels in primary SMC compared to ES cells. The presence of serum in the culture medium is known to suppress the expression of SMC marker genes. We therefore tested the primary SMCs for induction of SMC marker expression in response to serum starvation and TGF-beta1 stimulation. All markers were prominently induced in response to both stimuli. SM-MHC was for example up-regulated 22 folds in response to TGF-beta1. We conclude that the cells express SMC markers and that the expression is markedly induced in response to serum starvation and TGF-beta1 stimulation. 

(B) The second batch was characterized for mRNA expression of SMC markers and SRF. The levels were compared to a macrophage cell lineage. All SMC markers were expressed at high levels in the primary SMC compared to the macrophage cell lineage.

![Graph A](image1.png)

![Graph B](image2.png)
Online Figure II. Sequence alignments for CArG box 8, 11, and 13 across the available species.

CArG 8:
Mouse: AGCCGCTTTCTCATATAGGAAATGTTTACCC
Human: AGCCTGCTTTCTCATATGGAAATGTTTACCC
Rat: AGCCGCTTTCTCATATGGAAATGTTTACCC
Rabbit: AGCCGCTTTCTCATATGGAAATGTTTACCC
Chimp: AGCCGCTTTCTCATATGGAAATGTTTACCC
Macaque: AGCCGCTTTCTCATATGGAAATGTTTACCC
Cow: AGCCGCTTTCTCATATGGAAATGTTTACCC
Dog: AGCCGCTTTCTCATATGGAAATGTTTACCC
Elephant: AGCTGGTATCTCCATATGGAAATGTTTACCC
Tenrec: AGCTGGTATCTCCATATGGAAATGTTTACCC
Chicken: AGCTGGTATCTCCATATGGAAATGTTTACCC

CArG 11:
Mouse: AGCAAAGCTGCCCTTATATGGACACAAAGC
Human: GATGAAGCTGTCCTTATATGGGCAGGAAAAACT
Rat: AGTGGAGCTGCCCTTATATGGAGGCGAAAGC
Rabbit: GAGGCAGCTGCCCTTATATGGGCGGCAAGAAGC
Chimp: GATGAAGCTGCCCTTATATGGGCGGCAAGAAGC
Macaque: GATGAAGCTGCCCTTATATGGGCGGCAAGAAGC
Cow: GATGAAGCTGCCCTTATATGGGCGGCAAGAAGC
Dog: GATGAAGCTGCCCTTATATGGGCGGCAAGAAGC

CArG 13:
Mouse: GGAACTCAGTGCCCTTTATATGGGAAATGGGA
Human: AGAGCTCAGTGCCCTTTATATGGGAAATGGGA
Rat: GGAACTCAGTGCCCTTTATATGGGAAATGGGA
Rabbit: GGAACTCAGTGCCCTTTATATGGGAAATGGGA
Chimp: AGAGCTCAGTGCCCTTTATATGGGAAATGGGA
Macaque: AGAGCTCAGTGCCCTTTATATGGGAAATGGGA
Cow: AGAGCTCAGTGCCCTTTATATGGGAAATGGGA
Dog: AGAGCTCAGTGCCCTTTATATGGGAAATGGGA
Elephant: TGAGCTCAGTGCCCTTTATATGGGAAATGGGA
Tenrec: TGAGCTCAGTGCCCTTTATATGGGAAATGGGA
Online Figure III. Predicted alternative promoter region in intron 2. The black graph shows the phastCons score\(^1\), a measure of the evolutionarily conservation across 17 vertebrates. This score was obtained using the UCSC Table Browser tool.\(^2\) The transcription start site, as indicated by several 5'-oligo capped cDNA clones in the DTBSS database, is indicated with an arrow. Exon sequence is shown in bold. Putative cis-regulatory elements, identified using TRANSFAC are shaded.
References
