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Loss of the SRF co-factor, cysteine-rich protein 1, attenuates neointima formation in the mouse

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Abstract

Objective—Cysteine-Rich Protein (CRP) 1 and 2 are cytoskeletal LIM-domain proteins thought to be critical for smooth muscle differentiation. Loss of murine CRP2 does not overtly affect smooth muscle differentiation or vascular function, but exacerbates neointima formation in response to vascular injury. Since CRP1 and CRP2 are co-expressed in the vasculature, we hypothesize that CRP1 and CRP2 act redundantly in smooth muscle differentiation.

Methods and Results—We generated *Csrp1* (gene name for CRP1)-null mice by genetic ablation of the *Csrp1* gene and found that mice lacking CRP1 are viable and fertile. Smooth muscle containing tissues from *Csrp1*-null mice are morphologically indistinguishable from wildtype and have normal contractile properties. Mice lacking both CRP1 and CRP2 are viable and fertile ruling out functional redundancy between these two highly related proteins as a cause for the lack of an overt phenotype in the *Csrp1*-null mice. *Csrp1*-null mice challenged by wire-induced arterial injury display reduced neointima formation, opposite to that seen in *Csrp2*-null mice, while *Csrp1/Csrp2* double null mice produce a wildtype response.

Conclusions—Smooth muscle CRPs are not essential for normal smooth muscle differentiation during development, but may act antagonistically to modulate the smooth muscle response to pathophysiological stress.

INTRODUCTION

During vascular development, smooth muscle cells are initially highly proliferative, then become quiescent and express proteins that allow the cells to assume a highly contractive phenotype. Unlike cardiac or skeletal muscle, smooth muscle cells are not terminally

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differentiated; instead these cells maintain plasticity where they can alternate between a contractile, “quiescent” state and a highly proliferative, “synthetic” state [1]. This phenotypic modulation is critical for smooth muscle cells to respond to pathophysiological stresses. Thus, perturbation of the complex signaling mechanisms that direct smooth muscle phenotypic modulation may contribute to the progression of vascular disease [2].

While no single transcription factor serves as a master regulator of smooth muscle differentiation, serum response factor (SRF) has a critical role in the smooth muscle transcriptional program [3]. SRF is ubiquitously expressed; therefore tissue-specific cofactors for SRF provide target specificity. Myocardin and the related proteins MRTF-A and MRTF-B are established SRF cofactors important for smooth muscle differentiation 4. The smooth muscle cysteine-rich proteins, CRP1 and CRP2, are also implicated as important SRF cofactors. Co-expression of either CRP1 or CRP2 greatly augments smooth muscle gene expression in an SRF and GATA factor dependent manner 5. Detailed characterization of CRP2 demonstrates that it can bind both SRF and GATA, and appears to bridge the two proteins, providing a potential mechanism for CRP-directed effects on transcription. A dominant negative CRP2 that cannot bind SRF blocks smooth muscle differentiation *in vitro*, strongly implicating CRP2 as a critical SRF cofactor for smooth muscle differentiation. Despite these potent effects on smooth muscle differentiation *in vitro*, analysis of mice deficient for CRP2 revealed that *Csrp2* (gene name for CRP2)-null mice are viable and fertile. However, mice lacking CRP2 display enhanced neointima formation following vascular injury, revealing a role for CRP2 in the vascular injury response [6].

Our lab originally identified CRP1 as a smooth muscle binding partner for the actin cross-linking protein α-actinin [7]. CRP1 displays two LIM domains, double zinc finger structures that serve as protein binding sites [8, 9], and is expressed prominently in both vascular and visceral smooth muscle cells 10–13. CRP2 is expressed primarily in vascular smooth muscle cells and mesenchymal derivatives, and thus displays some overlap with CRP1 11, [14]. CRP1 and CRP2 are highly related at the amino acid level (88% similar) suggesting that the two proteins carry out similar functions.

Because of the high degree of overlap in expression between CRP1 and CRP2, especially in the vasculature, the lack of a phenotype in *Csrp2*-null mice under unchallenged physiological conditions might reflect redundancy between these two family members. CRP1 is the sole family member expressed in visceral smooth muscle such as the bladder and gastrointestinal tracts. Thus, elimination of CRP1 expression would allow us to directly test whether smooth muscle differentiation can proceed in the absence of CRPs. Here we report the generation and characterization of mice in which CRP1 function has been eliminated by genetic ablation of the *Csrp1* gene. Surprisingly, mice that lack CRP1 are viable, fertile and display normal smooth muscle contractile function. In addition, $Csrp1^{-/-}$/$Csrp2^{-/-}$ double-null mice are also viable and fertile. Thus, the absence of an overt vascular phenotype in the $Csrp1^{-/-}$ or $Csrp2^{-/-}$ mice is not due to redundancy between the two genes. While CRP1 is not critical for normal development, the *Csrp1*-null mice exhibit an attenuated response to arterial injury, a response opposite to that seen in the *Csrp2*-null mice. Our data indicate a role for CRP family members in sensing or responding to pathological vascular stress, and maintaining smooth muscle homeostasis.

MATERIALS AND METHODS

Detailed methods are described in the expanded Materials and Methods in the data supplement.

Gene targeting and generation of *Csrp1*-deficient mice

We generated *Csrp1*-null mice by gene targeting. Targeted ES cells and subsequently, mice harboring a targeted allele *Csrp-1* allele were identified by standard Southern blot methods.

Northern and Western blot analysis

RNA was isolated from mouse tissues using Trizol (Invitrogen Life Technologies), following manufacturer's instructions. Northern blot procedure was carried out as described [15]. Protein extract isolation and Western blot analyses were performed as described 16.

Analysis of smooth muscle contractility

Smooth muscle contractility analysis was performed as described [17].

Cell culture and immunofluorescence

Primary cultures of bladder and aortic smooth muscle cells were established, in parallel, from wild-type and *Csrp1*-null littermates using standard procedures.

Histological analysis of aorta and bladder

Tissue sections were obtained and stained for elastin or Masson's Trichrome (Sigma).

Femoral artery injury

Endoluminal injury to the left common femoral artery was produced by 5 passages of a 0.01″ diameter angioplasty guide wire (Guidant) essentially as described [18].

Migration assay

Boyden chamber migration assays were performed using standard methods.

Cell proliferation analysis

Cell proliferation was evaluated based on the dye incorporation/extraction assay as previously reported [19].

Apoptosis analysis

Apoptosis was evaluated based on the increase in cytoplasmic nucleosomes, using the Cell Death Detection ELISA plus kit (Roche).

Transient transfections and luciferase assays

Reporter assays were performed as described [12, 20].

Quantitative RT-PCR analysis

Specific smooth muscle gene transcript levels were analyzed by StepOne PCR system (Applied Biosystems), using total RNA.

RESULTS

Generation of *Csrp1*-deficient mice

A targeting vector was generated by replacing *Csrp1* genomic DNA that includes exons 2, 3 and 4 with a neomycin positive selection cassette (Figure 1A). A strategy that deletes exon 2 of the murine *Csrp1* gene was chosen because this exon encodes the translation initiation codon. Targeted ES cells carrying a heterozygous disruption of the *Csrp1* locus (Figure 1B) were introduced into recipient C57BL/6 blastocysts to generate chimeric mice. The resulting

chimeric animals were bred to C57BL/6 partners to generate heterozygous animals that carried one copy of the targeted *Csrp1* gene. These animals were interbred to generate homozygous mutant animals. Genotypes were confirmed by Southern analysis (Figure 1C) or by PCR (Figure 1D). Western and northern blot analysis of organ samples taken from $Csrp1^{+/+}$, $Csrp1^{+/-}$, and $Csrp1^{-/-}$ animals confirmed that no CRP1 RNA or protein is detected in the homozygous mutant mice (Figure 1E,F).

Arteries and other smooth muscle-containing organs maintain normal morphology and contractility in *Csrp1*-null mice

Genotypic analysis of the offspring derived from $Csrp1^{+/-}$heterozygous parents revealed that normal Mendelian ratios were obtained, indicating that zygotic expression of CRP1 is not required for embryonic development (Figure 2A). Interbreeding of $Csrp1^{-/-}$ animals resulted in litters of normal size. Because CRP2 is closely related to CRP1 (90% similar), and is also expressed in vascular smooth muscle [10–14], we examined whether CRP2 expression was upregulated in the $Csrp1^{-/-}$ mutant animals. By Northern analysis of RNA isolated from aorta, bladder, and lung, tissues that normally display robust CRP1 expression, we found no consistent evidence of increased CRP2 expression (Figure 1F).

We examined the vasculature in detail to determine if loss of CRP1 had any impact on organization of the arterial vessels. Labeling arterial cross sections with Verhoff's stain did not reveal any difference in the morphology or number of smooth muscle lamellar units and elastic fibers (Figure 2B,C). Trichrome staining of aortic sections also did not indicate any gross change in organization and morphology of the arterial vessels from the C$srp1^{-/-}$ mice (Figure 2D,E). Since CRP2 is also expressed in arterial smooth muscle, we also examined bladder sections, where CRP1 is the sole family member expressed. No morphological difference was detected between $Csrp1^{-/-}$ bladder and wildtype (data not shown), indicating that loss of CRP1 does not grossly impact the development of smooth muscle-containing organs.

CRP1 has been reported to bind actin and bundle actin filaments [21], suggesting that $Csrp1^{-/-}$ smooth muscle cells might be expected to exhibit an altered morphology or cytoarchitecture. We examined smooth muscle cytoskeletal organization in isolated vascular smooth muscle cells (VSMCs) from wild-type and $Csrp1^{-/-}$ animals to determine if loss of CRP1 had any impact on smooth muscle cytoarchitecture. CRP1 was associated with actin filaments and adhesion sites in wild-type cells (Figure 2F); no CRP1 protein was detected in cells derived from the mutant animals (Figure 2F′). Using smooth muscle α-actin and vinculin we observed that actin filament organization (Figure 2G,G′) and focal adhesions (Figure 2H,H′) appear morphologically indistinguishable in wild-type and *Csrp1*-null cells. Since VSMCs express both CRP1 and CRP2, we also examined primary bladder smooth muscle cells, which normally express only CRP1. Similar to that seen for vascular smooth muscle cells, we did not detect any alteration in cell cytoarchitecture in bladder smooth muscle cells derived from *Csrp1*-null mice (data not shown).

Although smooth muscle-containing organs maintain a normal morphology in the absence of CRP1, it remained possible that smooth muscle function could still be compromised in the $Csrp1^{-/-}$ mice. For example, functional analysis of muscle LIM protein (MLP), the striated muscle specific CRP isoform, revealed a critical role for MLP in cardiomyocyte contractile response [22–24]. We directly tested contractility of excised smooth muscle using standard physiology methods that delineate dose-response curves and maximal force generation. Measurements of isometric force generation of aortic smooth muscle in response to KCl or phenylephrine are summarized in Figure 3. Loss of CRP1 did not lead to any alterations in concentration-force relations with or without endothelium (repeated measures ANOVA, $p>0.5$). Maximal force generation by either KCl or phenylephrine in the endothelium-

denuded condition, which solely reflects contractility of smooth muscle layers in the aorta, did not reveal difference between wild-type and *Csrp1*$^{-/-}$ mutants (ANOVA, $p>0.5$).

Aortic smooth muscle cells express both CRP1 and CRP2, so we repeated the contractility analysis on bladder tissue derived from wildtype and *Csrp1*-null mice, since bladder expresses only CRP1. Force generation, elicited by either KCl (Figure 3D) or carbachol (data not shown), was similar for both wildtype and *Csrp1*-null bladder samples. We conclude from these analyses that CRP1 is not critical for normal smooth muscle contraction.

Smooth muscle cell differentiation appears normal in *Csrp1*-null mice

CRPs have been shown to synergize with SRF and GATA factors and potently upregulate smooth muscle gene expression [5]. To directly address whether the loss of CRP1 has an impact on the steady-state expression of smooth muscle-specific proteins, we compared the expression of smooth muscle markers in tissues from wildtype and *Csrp1*-null mice. By immunoblot analysis, we did not detect any alteration in the levels of smooth muscle α-actin or smooth muscle myosin heavy chain in aortic lysates from *Csrp1*-null or *Csrp2*-null mice (Figure 4A). Vinculin, a prominent dense plaque component critical for smooth muscle cell adhesion, was also present at wildtype levels. Likewise, smooth muscle marker expression was not altered in mesenteric artery samples (Figure 4A), indicating that different vessel types (*e.g.*, elastic vs. muscular) did not show an altered response to loss of *Csrp1*. We also examined smooth muscle marker expression in bladder and lung, two tissues in which CRP1 is the sole CRP family member expressed; no difference in steady-state expression of representative smooth muscle-specific proteins was observed (data not shown). Thus, despite the potent capacity of CRP1 to stimulate smooth-muscle specific gene expression *in vitro*, smooth muscle gene expression persists in mice lacking *Csrp1*.

To further assess the impact of loss of CRP1 on smooth muscle transcription, we determined the capacity of isolated VSMCs to activate an SRF-dependent reporter when stimulated with TGF-beta. The smooth muscle α-actin (SMA) promoter-luciferase construct is a commonly used reporter, which has been shown to be TGF-beta responsive and CArG-dependent [25]. Both *Csrp1*$^{-/-}$ and wildtype cells displayed a similar response and upregulated the SMA-luciferase reporter approximately 2-fold after TGF-beta stimulation (Figure 4B). Interestingly, *Csrp2*$^{-/-}$ cells appear to be refractory to TGF-beta stimulation. The ability of the wildtype and *Csrp1*$^{-/-}$ cells to activate the SMA-reporter was SRF-dependent, as a SMA-reporter with a mutated CArG was not induced in either cell type by TGF-beta. Consistent with our studies with the SMA reporter, a luciferase reporter containing 3 copies of the intronic CArG element from the *Csrp1* gene [12, 26] was also upregulated by TGF-beta in the WT and *Csrp1*$^{-/-}$ cells, while the *Csrp2*$^{-/-}$ cells were unresponsive (supplementary data, Figure S1, panel A).

To determine if the lack of activity observed in *Csrp2*$^{-/-}$ cells was due to an inability to respond to TGF-beta, we examined induction of smooth muscle gene expression by measuring endogenous RNA. Both *Csrp1*$^{-/-}$ and *Csrp2*$^{-/-}$ cells exhibited a similar induction of *smooth muscle α-actin, SM-22α,* and *calponin* in response to TGF-beta (Figure 4C and supplementary data, Figure S1, panels B–C). These data indicate that *Csrp2*-null cells are not defective in TGF-beta signaling *per se*, but have compromised SRF-dependent transcriptional activity, consistent with the normal expression of SMA and other smooth muscle proteins in the isolated tissues (Figure 4A).

Mice lacking both CRP1 and CRP2 are viable and fertile

Our observation that loss of CRP1 did not overtly affect smooth muscle development or contractility, even in tissues in which it is the sole CRP expressed, strongly suggests that CRPs are not essential for smooth muscle development. To provide further support for this conclusion, we crossed the *Csrp1*$^{-/-}$ and *Csrp2*$^{-/-}$ mice to create animals that were null for both *Csrp1* and *Csrp2*. Like the *Csrp1*$^{-/-}$ or *Csrp2*$^{-/-}$ null animals, mice lacking both CRP1 and CRP2 were viable (Figure 5A). Moreover, interbreeding of double-null *Csrp1*$^{-/-}$ *Csrp2*$^{-/-}$ animals resulted in normal-sized litters (~8 pups/litter). Together, these results indicate that the smooth muscle CRPs, CRP1 and CRP2, are not essential for smooth muscle development.

Mice lacking CRP1 show attenuated response to guide wire-induced arterial injury

While the principal function of unchallenged smooth muscle is contraction, smooth muscles also play a role in maintaining and repairing injured tissue. For example, in response to arterial injury, smooth muscle cells quickly switch from a quiescent phenotype to a proliferative and migratory phenotype, leading to neointima formation [2, 27]. The involvement of CRP2 in neointima formation prompted us to determine if the *Csrp1*$^{-/-}$ mice display an altered response to vascular injury. Since CRP1 and CRP2 are highly related at the amino acid level, we anticipated a similar response to the wire-induced arterial injury (*e.g.* increased neointima formation).

Surprisingly, neointima formation in *Csrp1*$^{-/-}$ mice was significantly decreased as compared to wild-type mice (Figure 5B,C). This result was in striking contrast to the enhanced neointima formation in *Csrp2*$^{-/-}$ mice (Figure 5D; 6). Attenuated neointima formation in *Csrp1*$^{-/-}$ mice after the arterial injury led us to ask if the *Csrp1*$^{-/-}$ *Csrp2*$^{-/-}$ double null mice would produce a “balanced” response, and thus show less neointima formation than *Csrp2*$^{-/-}$ mice. Indeed, double null mice showed statistically significant decrease in the thickness of neointima compared to what we observed for *Csrp2*$^{-/-}$ mice (Figure 5E,F, $p<0.05$ by ANOVA and Bonferroni test).

The increased neointima formation seen in the *Csrp2*$^{-/-}$ mice is thought to be due, at least in part, to increased vascular smooth muscle migration during neointima formation [6]. We assessed the migration of *Csrp1*$^{-/-}$ and wildtype VSMCs towards the chemoattractant PDGF-BB, and found that both cell types exhibited a similar migratory capacity (Figure 6A). Smooth muscle proliferation also contributes to neointima formation, so we measured proliferation rates of isolated *Csrp1*$^{-/-}$ and wildtype VSMCs over a 6-day period. Both cell types displayed similar proliferation rates over the entire time course (Figure 6B). Studies by Latonen *et al.* indicate that CRP1 may have anti-apoptotic properties [28]; if loss of CRP1 resulted in increased cell death, this could account for the attenuated neointima formation seen in the *Csrp1*-null mice. To test this hypothesis, we determined whether the *Csrp1*$^{-/-}$ cells undergo increased apoptosis under subconfluent and confluent growth conditions. Wildtype, *Csrp1*$^{-/-}$ and *Csrp2*$^{-/-}$ cells all exhibit an equivalent, low level of apoptosis, as detected by accumulation of cytoplasmic nucleosomes (Figure 6C). Together these results indicate that loss of CRP1 does not impact vascular smooth muscle proliferation, migration or apoptosis, at least under these assay conditions.

Another aspect of phenotypic modulation is the redifferentiation of smooth muscle cells after neointima formation. We examined the redifferentiation capacity of isolated VSMCs by allowing cultures to become confluent, and assaying expression of smooth muscle myosin heavy chain (SM-MHC). When first isolated, cultured VSMCs lose expression of SM-MHC and other smooth muscle markers, but regain expression when cultures become confluent [29]. In newly confluent cultures, we still detect abundant non-muscle myosin (data

not shown) and also begin to see expression of SM-MHC (Figure 6D). By two-days post-confluency, wildtype cells have continued to upregulate SM-MHC, but the *Csrp1*$^{-/-}$ cells do not display the same degree of SM-MHC upregulation (Figure 6D). Freshly isolated aortas from both wildtype and *Csrp1*$^{-/-}$ mice express similar levels of SM-MHC (Figure 4A), suggesting that the rate of re-expression of SM-MHC or maximal smooth muscle redifferentiation is compromised in the *Csrp1*$^{-/-}$ cells.

DISCUSSION

The cysteine-rich proteins are muscle-enriched, actin-associated proteins strongly implicated in muscle gene regulation and differentiation [5, 7, 13, 22, 30, 31]. Tissue culture based experiments have shown that all three CRPs can greatly augment muscle gene transcription, and disruption of CRP function *in vitro* can block muscle differentiation. Therefore it was unexpected that loss of the smooth muscle CRPs or striated muscle CRP (CRP3/MLP) has no overt affect on muscle development *in vivo*. However, these studies of CRP-deficient mice have revealed important roles for CRPs in maintaining normal muscle function. Loss of either CRP1 (this study) or CRP2 [6] alters neointima formation in response to arterial injury, while MLP/*Csrp3*-deficient mice develop dilated cardiomyopathy post-natally [22]. Collectively these data demonstrate that CRPs are not essential for muscle development, and instead have important functions in responding to pathophysiological stress and maintaining muscle homeostasis.

CRPs have the capacity to drastically potentiate smooth muscle gene expression, leading to the hypothesis that smooth muscle gene expression would be compromised in the absence of CRPs. We tested this hypothesis directly by generating single and double *Csrp1* and *Csrp2* null-mice. The resulting mice are viable and fertile with apparently normal smooth muscle function and gene expression. However, a more detailed analysis revealed that isolated *Csrp2*$^{-/-}$ cells do not upregulate an SRF-dependent reporter in response to TGF-beta (Figure 4B), indicating that loss of CRP2 can influence transcription. In addition, expression of smooth muscle myosin heavy chain was attenuated in post-confluent *Csrp1*$^{-/-}$ cells (Figure 6D). Our results suggest that although CRPs are not absolutely essential *in vivo* for smooth muscle gene expression to proceed, their loss can impact smooth muscle gene expression under specific physiological or pathological conditions. Studies of smooth muscle gene regulation specifically during neointima formation in *Csrp1*$^{-/-}$ mice should help elucidate the contribution of CRP1 to smooth muscle gene expression *in vivo*.

The cytoskeletal role of CRPs has potential to influence both cell morphology and gene expression. CRP1 and CRP2 have been shown to bind and bundle actin filaments [21, 32], and thus may participate in actin remodeling that occurs during smooth muscle phenotypic modulation. Indeed, over-expression of CRP1 increases actin stress fiber thickness, and CRP1 translocates with actin to membrane ruffles in PDGF-treated fibroblasts [21]. This observation is especially intriguing given that PDGF is a potent humoral trigger of neointima formation *in vivo* [33]. CRP1-mediated actin-bundling could influence the G-actin:F-actin ratio, a critical regulator of SRF-dependent smooth muscle gene expression and differentiation [34–36]. Identification of the mechanism by which CRPs affect actin stress fiber dynamics should help elucidate its potential contribution to smooth muscle cell behavior during vascular injury.

Several factors influence neointima formation, including smooth muscle cell apoptosis, proliferation and migration [37]. Our *in vitro* studies of primary VSMCs from the *Csrp1*-null mice did not indicate a defect in these aspects of smooth muscle cell behavior, at least under our experimental conditions. Instead, we show that cultured *Csrp1*$^{-/-}$ vascular smooth muscle cells express lower levels of smooth muscle myosin heavy chain (SM-MHC) under

post-confluent conditions. Under these conditions, wildtype cells re-express late differentiation markers, such as SM-MHC, and become quiescent, essentially entering a redifferentiation program. During neointima formation, smooth muscle cells initially dedifferentiate, but as the neointima becomes established, the smooth muscle cells that invaded the intimal region redifferentiate and express smooth muscle markers [2]. If smooth muscle redifferentiation is compromised, the neointima may be unstable, resulting in loss or regression of the vascular occlusion.

Alternatively, the attenuated SM-MHC expression in *Csrp1*$^{-/-}$ cells may indicate a defect in rates of redifferentiation and dedifferentiation. Recent work by Sayers *et al.* showed that loss of the FAK inhibitor FRNK, lead to attenuated smooth muscle marker expression during postnatal growth and after vascular injury, with no overt effect on vascular function under normal physiological conditions [38]. After vessel maturation, aortas from FRNK−/− mice expressed smooth muscle markers at normal levels, indicating that the rate of smooth muscle differentiation is compromised by loss of FRNK. These observations suggest that cellular factors can regulate the rate of smooth muscle phenotypic modulation, which may be critical for timing cellular events leading to neointima formation.

Vascular phenotypes revealed by this study for CRP1 and the study by Wei *et al* for CRP2 [6] highlight intriguing features of CRP family members expressed in smooth muscle. *Csrp1*$^{-/-}$ mice show attenuated neointima formation in response to the arterial injury, while *Csrp2*$^{-/-}$ mice show an excessive response. These results were surprising, given that CRP1 and CRP2 are highly similar (88%). However, CRP1 and CRP2 also possess regions of dissimilarity that are evolutionarily conserved, suggesting the possibility of some uniqueness in the binding partner repertoire for CRP1 and CRP2. Thus, the smooth muscle CRPs may have common properties (*e.g.* actin binding) as well as unique protein partners in smooth muscle that allow them to perform specific functions. For example, our studies indicate that CRP1 and CRP2 have distinct effects on SRF-dependent smooth muscle gene regulation. We demonstrate that *Csrp2*$^{-/-}$ VSMCs do not activate a CArG-luciferase or SMA-luciferase reporter in response to TGF-beta stimulation, while the *Csrp1*$^{-/-}$ cells retain this capacity (Figure 4B, and Supplemental Figure 1).

The observation that animals lacking both CRP1 and CRP2 have a more “balanced” response to arterial injury strongly suggests that the two proteins somehow antagonize each other, or are involved in different cellular responses activated by arterial injury. Thus, co-expression of CRP1 and CRP2 may act to fine-tune the response of the vascular smooth muscle cell to pathological stresses, such as arterial injury. The molecular programs initiated during vascular injury are still poorly understood. Our findings highlight the importance of further characterizing the molecular functions of CRPs in normal and pathologic smooth muscle, and will hopefully contribute to our understanding of the pathophysiology of vascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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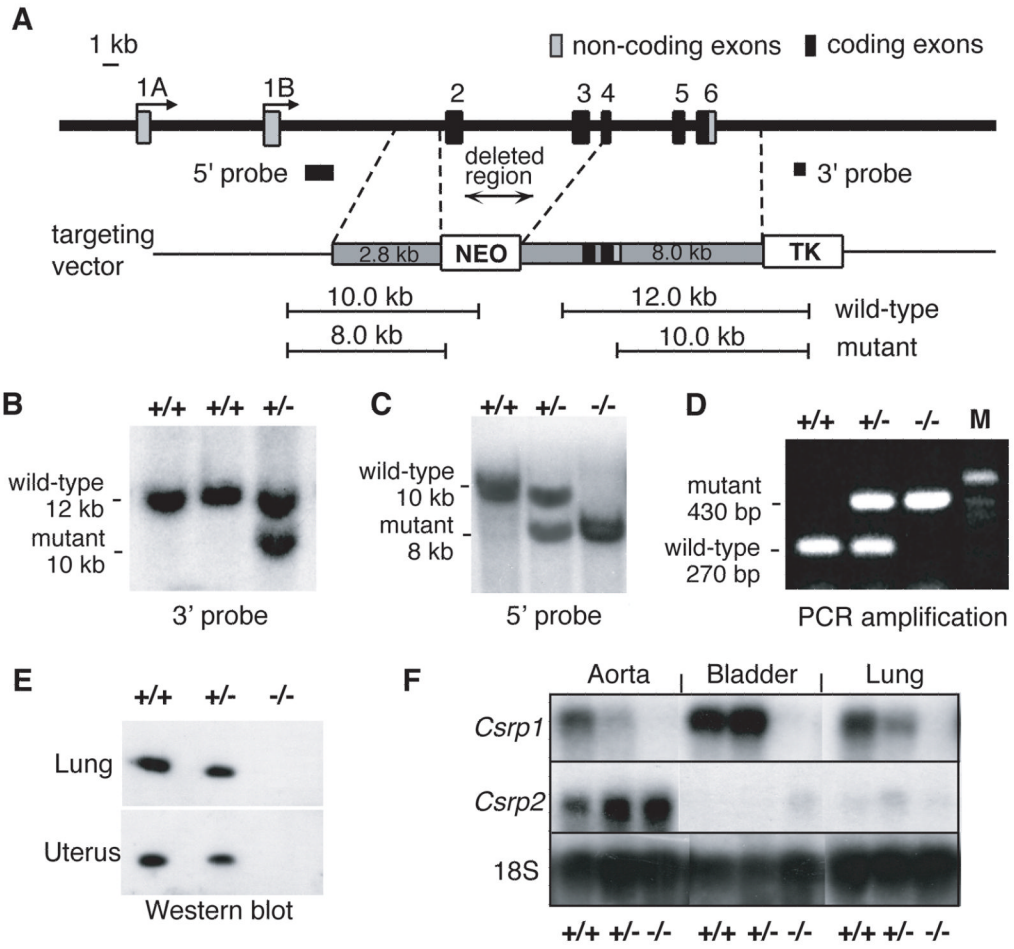

Figure 1. Targeted disruption of the *Csrp1* gene

(A) Schematic representation of the *Csrp1* gene and targeting strategy. Exons 2 through 4 were deleted and replaced with the neomycin resistance gene (NEO). The thymidine kinase gene (TK) was inserted downstream of the 3 ′ region of homology (3 ′ arm). Wild-type and mutant (targeted) alleles were detected by Southern blot hybridization using *Csrp1* genomic sequences residing outside the targeted region. The 5 ′ probe detects a 10-kb wild-type allele and 8-kb mutant allele. The 3 ′ probe detects a 12-kb wild-type allele and 10-kb mutant allele. (B) Southern blot of DNA isolated from embryonic stem (ES) cells shows a targeted clone. (C) Southern blot analysis of tail DNA from mice generated from targeted ES cells. (D) Genotyping by PCR amplification of tail DNA detects both wild-type and *Csrp1* mutant alleles. (E) Western blot to detect CRP1 protein in tissue extracts isolated from wild-type, heterozygous, and null mice. An equivalent amount of each tissue extract was added per lane. (F) Northern blot to evaluate *Csrp1* and *Csrp2* expression in wild-type, heterozygous and null mice. Total RNA from designated tissues was probed for the presence of *Csrp1* and *Csrp2* transcripts using radio-labeled cDNA probes. Hybridization and detection of 18S ribosomal RNA was used as a control to determine relative amount of RNA in each lane. The original autorad was scanned, and two other tissue sets were cropped out of the final image for brevity. The *Csrp2* band intensity for each sample was normalized to the 18S signal and quantified to demonstrate equivalent expression between genotypes (data not shown).

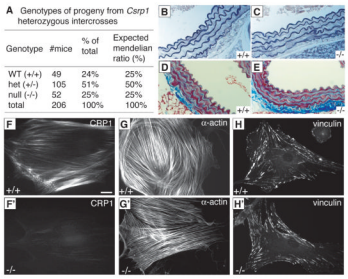

Figure 2. *Csrp1*-null mice are viable and have a grossly-normal vasculature

(A) Analysis of genotypes from *Csrp1* heterozygous intercross. Expected mendelian ratios were seen for the three genotypes. (B–E) Histological analysis of aortic sections from wildtype (+/+) and *Csrp1*-null (−/−) mice. Sections in panels B and C are stained with Verhoff's stain to delineate the elastin fibers between the smooth muscle in the medial region of the vessel. Sections in D and E are stained with Trichrome; the smooth muscle layers label red, alternating with the blue labeling of the elastin fibrils. (F–H) Wild-type (+/+) and (F′–H′) *Csrp1*-null (−/−) aortic smooth muscle cells labeled with antibodies indicated. Antibodies used for immunodetection: anti-CRP1 (F,F′); anti-smooth muscle α-actin (G,G′), and anti-vinculin (H,H′). Note lack of labeling with anti-CRP1 serum in cells derived from *Csrp1*-null mice. (scale bar in F=100 μm.)

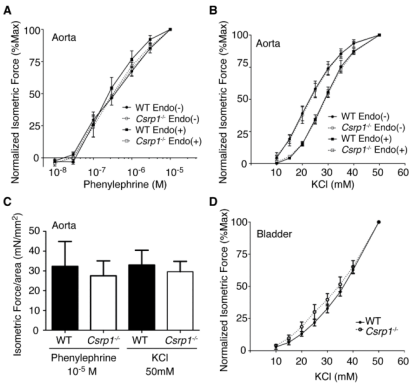

Figure 3. *Csrp1*-null smooth muscle displays a normal contractile response to phenylephrine or KCl

(A) Dose response curve of the isometric contractile force in response to phenylephrine, a α 1 adrenergic agonist. Values were normalized based on the maximal force generation at the highest concentration. WT: wild type; Endo: presence (+) or absence (−) of endothelium. Note that no differences were detected between wild type and *Csrp1*$^{-/-}$ genotypes regardless of the presence of endothelium (repeated measures ANOVA, $p>0.5$). (B) Responses to KCl, which induce smooth muscle contraction by membrane depolarization, were also unaltered by the loss of CRP1. (C) Maximal isometric force development was compared in the absolute, and no statistical differences were detected (ANOVA, $p>0.5$). (D) Dose response curve of the isometric contractile force in response to KCl, assessed for bladder tissue. No difference was detected between the two genotypes (repeated measures ANOVA, $p>0.5$).

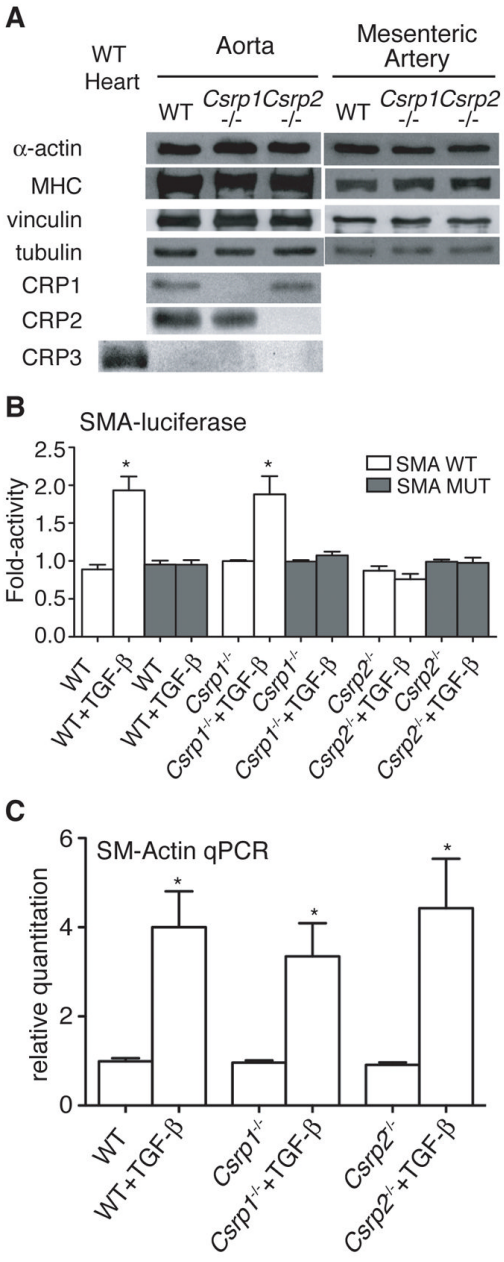

Figure 4. Smooth muscle lacking CRP1 expresses markers indicative of normal smooth muscle differentiation

(A) Western blot to detect protein expression in smooth muscle tissues of wild-type, *Csrp1*-null and *Csrp2*-null mice. An equivalent amount of each tissue extract was added per lane. Protein levels were examined by immunoblotting with antibodies against smooth muscle α-actin (α-actin), smooth muscle myosin heavy chain (MHC), vinculin, and tubulin (as a loading control). Blots were also probed with antibodies specific for CRP1, CRP2, and CRP3 to confirm genotypes and demonstrate lack of upregulation other family members in the null mice. (B) *Csrp2*$^{-/-}$ VSMCs do not upregulate a SMA-luciferase reporter in response to TGF-beta. Values are mean ± SEM of 3 experiments. *p<0.05, compared to no TGF-beta control. (C) Quantitative RT-PCR analysis of SM α-actin in WT, *Csrp1*$^{-/-}$ or

Csrp2$^{-/-}$ VSMCs in response to TFG-beta stimulation. *p<0.05, compared to no TGF-beta control.

A Progeny of *Csrp1*$^{+/-}$*Csrp2*$^{-/-}$ intercrosses

Genotype	#mice	% of total	Expected mendelian ratio (%)
Csrp1$^{-/-}$ *Csrp2*$^{-/-}$	7	21%	25%
Csrp1$^{+/-}$ *Csrp2*$^{-/-}$	21	64%	50%
Csrp1$^{+/+}$ *Csrp2*$^{-/-}$	5	15%	25%
total progeny	33	100%	100%

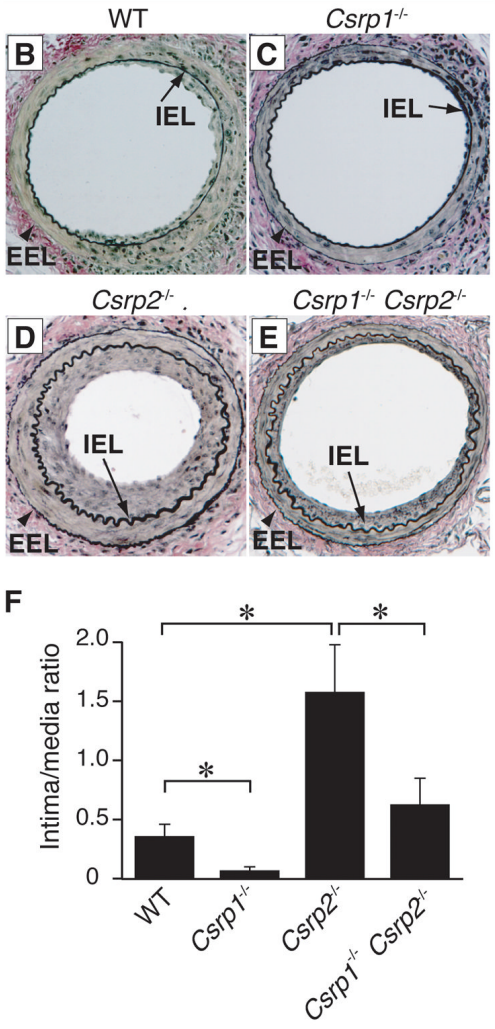

Figure 5. Loss of CRP1 attenuates neointima formation in response to guide-wire induced arterial injury

(A) Analysis of genotypes from *Csrp1*$^{+/-}$ *Csrp2*$^{-/-}$ intercrosses demonstrates that *Csrp1*$^{-/-}$ *Csrp2*$^{-/-}$ mice are viable. (B–E) Sections of femoral arteries from mice subjected to guide-wire induced arterial injury, sacrificed 2 weeks after injury. As compared to wild type (B), *Csrp1*-null mice (C) showed significantly reduced neointima formation. Increased neointima formation in *Csrp2*-null mice is also shown as reference (D; 6). Note that neointima formation in *Csrp1*$^{-/-}$ *Csrp2*$^{-/-}$ double null was decreased (E), compared to *Csrp2*-null. (F) Quantification of the intima/media ratio demonstrates a statistically significant difference in the response of *Csrp1*-null (n=16) and *Csrp2*-null (n=11) verses wildtype (n=12), and *Csrp2*-null verses *Csrp1*$^{-/-}$ *Csrp2*$^{-/-}$ double-null (n=10). *p<0.05 by ANOVA.

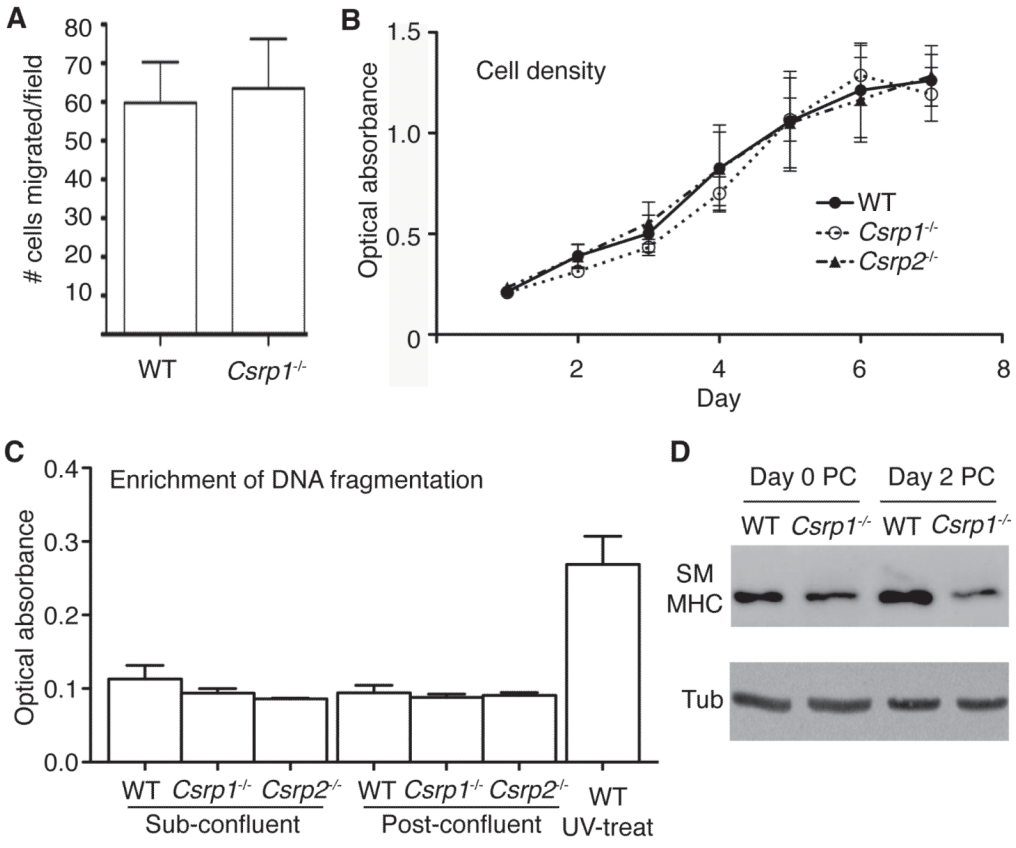

Figure 6. Isolated *Csrp1*$^{-/-}$ vascular smooth muscle cells show reduced smooth muscle myosin heavy chain upon redifferentiation

(A) Boyden chamber migration assay with 10 ng/ml PDGF-BB reveals no difference in migratory capacity of isolated VSMCs from *Csrp1*$^{-/-}$ and wildtype mice. Graph shows average number of migrated cells in a defined field. Values are mean ± SEM of 3 experiments, counting 3 fields per filter and averaging. (B) Wildtype, *Csrp1*$^{-/-}$ and *Csrp2*$^{-/-}$ VSMCs display similar proliferative capacities. Equivalent numbers of cells were plated in 6 60 mm dishes, and one dish harvested each day. Cell quantification performed by violet dye incubation, which was measured by absorbance. Data presented as mean ± SEM of 3 experiments. (C) Wildtype, *Csrp1*$^{-/-}$ and *Csrp2*$^{-/-}$ VSMCs display similar levels of apoptosis. Apoptosis was assayed by presence of cytoplasmic nucleosomes, quantified using an ELISA-based detection method for histones. Data presented as mean ± SEM of 3 experiments. (D) *Csrp1*$^{-/-}$ VSMCs have attenuated smooth muscle myosin (SM-MHC) upregulation upon redifferentiation. Western blot analysis of SM-MHC protein from postconfluent VSMC cultures. Day 0 is first day confluent and Day 2 is two days after. Experiment was performed for three independent cultures, and a representative blot is shown. Tubulin (Tub) is shown as a loading control.