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δEF1 Mediates TGF-β Signaling in Vascular Smooth Muscle Cell Differentiation

Go Nishimura,[1] Ichiro Manabe,[1,2,*] Kensuke Tsushima,[1] Katsuhito Fujiu,[1] Yumiko Oishi,[1] Yasushi Imai,[1] Koji Maemura,[1] Makoto Miyagishi,[3] Yujiro Higashi,[4] Hisato Kondoh,[4] and Ryozo Nagai[1]

[1] Department of Cardiovascular Medicine
[2] Nano Bioengineering Education Program
Graduate School of Medicine
[3] Department of Chemistry and Biotechnology
School of Engineering
The University of Tokyo
Bunkyo, Tokyo 113-8655
Japan
[4] Laboratory of Developmental Biology
Graduate School of Frontier Bioscience
Osaka University
Osaka 565-0871
Japan

Summary

Alteration in the differentiated state of smooth muscle cells (SMCs) is known to be integral to vascular development and the pathogenesis of vascular disease. However, it is still largely unknown how environmental cues translate into transcriptional control of SMC genes. We found that δEF1 is upregulated during SMC differentiation and selectively transactivates the promoters of SMC differentiation marker genes, *SM α-actin and SM myosin heavy chain (SM-MHC)*. δEF1 physically interacts with SRF and Smad3, resulting in a synergistic activation of *SM α-actin* promoter. Chromatin immunoprecipitation assays and knockdown experiments showed that δEF1 is involved in the control of the SMC differentiation programs induced by TGF-β signaling. Overexpression of δEF1 inhibited neointima formation and promoted SMC differentiation, whereas heterozygous *δEF1* knockout mice exhibited exaggerated neointima formation. It thus appears δEF1 mediates SMC differentiation via interaction with SRF and Smad3 during development and in vascular disease.

Introduction

Unlike striated muscle cells, smooth muscle cells (SMCs) are not terminally differentiated, even in adult blood vessels, and the plasticity they retain enables them to modify their phenotype in response to environmental cues (Owens et al., 2004). It is well documented that phenotypically modulated SMCs are centrally involved in the pathogenesis of vascular disease. Consequently, elucidation of the mechanisms that control the differentiated state of SMCs is critically important for understanding not only vascular development but also the pathology of vascular disease.

*Correspondence: manabe-tky@umin.ac.jp

A number of transcription factors have been implicated in SMC differentiation, but unlike in skeletal muscle, no single transcription factor identified so far can, by itself, induce SMC differentiation. Recent studies have clearly demonstrated that SMC gene expression is highly context dependent and is controlled not by a single SMC-specific transcription factor but by the interactions between multiple factors and cofactors, which likely form networks that selectively and coordinately control gene expression in response to environmental cues (Owens et al., 2004; Manabe and Nagai, 2003). Transcription factors and cofactors reportedly involved in such networks include SRF, GATA6, and myocardin (Miano, 2003; Owens et al., 2004); though it is still largely unknown how these factors interact within the networks to execute SMC-specific gene programs.

SMC-specific transcriptional regulatory modules very often contain the CArG element, a target element of serum response factor (SRF); indeed, in vivo expression of virtually all of the SMC-specific genes studied so far depends on CArG elements (Miano, 2003). However, SRF is ubiquitously expressed in a variety of cell-types and is necessary for expression of a large number of non-muscle genes. Thus, one critical question in the field has been: How do ubiquitously expressed factors such as SRF regulate SMC-specific transcription? One clue may come from the recent report that a transcription cofactor, myocardin, is necessary for SMC-specific SRF-dependent transcription (Wang et al., 2003). Still, it remains unclear how environmental cues translate into alterations in gene transcription that depend on SRF and myocardin.

One external factor thought to be important for control of SMC differentiation is TGF-β, which is able to induce neural crest stem cells to express SMC differentiation marker genes, such as *SM α-actin*, and to augment expression of SMC markers in cultured SMCs (Shah et al., 1996). In addition to these in vitro studies, gene knockout studies in which *TGF-β* was targeted confirmed that TGF-β signaling is essential for proper vessel formation (Dickson et al., 1995), while targeting *endoglin*, *ALK-1*, *ALK-5* (TGF-β receptors), or *Smad5* resulted in reduced SMC/pericyte recruitment and proliferation within vessels (Li et al., 1999; Oshima et al., 1996; Yang et al., 1999). TGF-β has also been shown to be crucially involved in SMC differentiation and proliferation in vascular injury models (Mallat et al., 2001). And given the importance of SRF in SMC-specific transcriptional programs, it seems likely that it, too, is involved in TGF-β-dependent transcriptional control. Indeed, one earlier report showed that two CArG boxes in the *SM α-actin* promoter were necessary for TGF-β responsiveness (Hautmann et al., 1997). The molecular basis for the effects of TGF-β on SRF-dependent SMC gene expression is not yet clear, however.

δEF1 (also called ZEB-1) is a transcription factor that contains two clusters of zinc fingers and a homeodomain and was first identified as a factor binding to an enhancer in the chicken *δ1-crystallin* gene (Funahashi et al., 1993; Postigo and Dean, 1997). In mouse embryo,

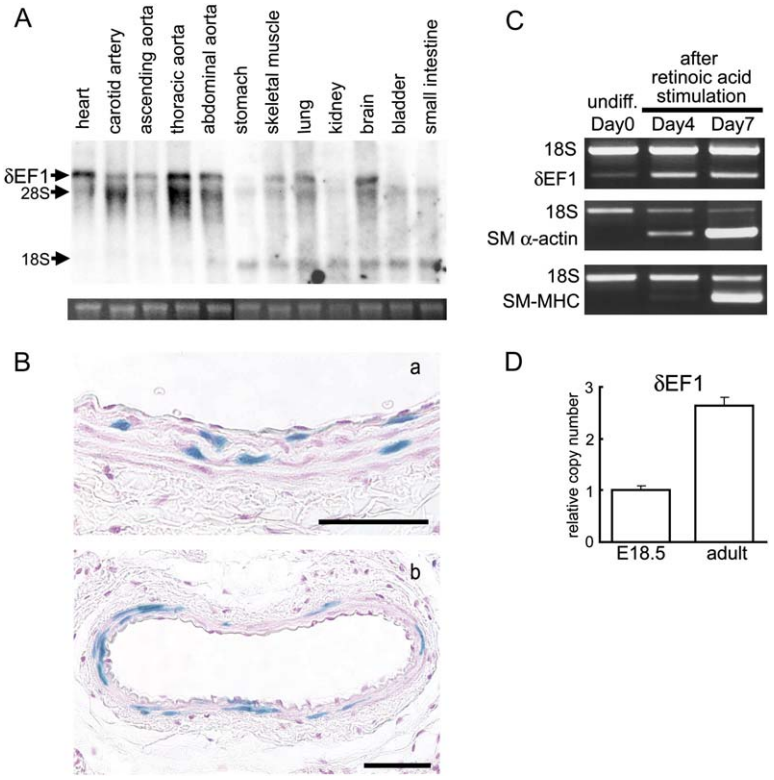

Figure 1. δEF1 Expression in Tissues and during SMC Differentiation

(A) Northern blot analysis of tissue distribution of δEF1 mRNA in adult rats.

(B) δEF1 expression in the cardiovascular system. *δEF1* knockout mice harbor a *LacZ* reporter gene controlled by the δEF1 regulatory program (Takagi et al., 1998). Tissues taken from adult $\delta EF1^{+/-}$ mice were subjected to X-gal staining and counterstained with nuclear fast red. Cross-sections of the thoracic aorta (Ba) and femoral artery (Bb) are shown. Scale bars, 50 μm.

(C) δEF1 expression during SMC differentiation of A404 cells. Expression of δEF1, SM α-actin, and SM-MHC was analyzed by semiquantitative multiplex PCR with primer sets for the gene of interest and an internal control (18S rRNA).

(D) Real-time PCR analysis of δEF1 expression in the aortas of embryos (E18.5) and adult mice. The relative numbers of δEF1 transcripts were normalized to those of 18S. Bars indicate relative copy number and SE.

δEF1 expression was first detected in the headfold and the presomitic and lateral plate mesoderm on embryonic day (E) 8.5 and in the derivatives of the cranial neural crest and limb buds on E9.5 (Takagi et al., 1998). Given that vascular SMCs are derived from a variety of embryonic progenitors, including lateral mesoderm, cranial mesenchyme, and the neural crest (Majesky, 2003), the expression pattern of δEF1 is suggestive of a role in SMC biology, though the function of δEF1 in the cardiovascular system has not yet been explored. Interestingly, recent studies have shown that δEF1 interacts with Smad proteins, the basic components of intracellular TGF-β signaling pathways.

In the present study, we identified *δEF1* as a candidate gene that might control SMC differentiation, and subsequent analyses showed that δEF1 is selectively expressed in vascular SMCs and that it controls SMC-specific gene expression by mediating TGF-β signaling and SRF-dependent transcription. Overexpression of δEF1 inhibited neointima formation and promoted SMC differentiation, whereas heterozygous *δEF1* knockout mice exhibited exaggerated neointima formation. Taken together, these findings suggest that δEF1 plays an important role in the control of SMC differentiation occurring in response to environmental cues during development and in vascular diseases.

Results

δEF1 Is Preferentially Expressed in Vascular SMCs and Is Developmentally Regulated

With the aim of identifying transcription factors involved in SMC differentiation, we searched databases for transcription factors containing C_2H_2-type zinc fingers and discovered several that might be expressed in SMCs. Among these was δEF1, which in adult rats is expressed in the aorta, heart, carotid artery, brain, and skeletal muscle but not in visceral smooth muscle tissues, such as the bladder, stomach, and small intestine (Figure 1A). Because this finding suggested that δEF1 might be expressed in vascular SMCs, we next carried out a more detailed analysis of the localization of δEF1 in cardiovascular tissues. Tissues obtained from *δEF1* knockout mice harboring a *LacZ* reporter gene whose expression mimicked endogenous *δEF1* expression (Takagi et al., 1998) were stained with X-gal. As expected, positive staining was observed in SMCs located in the media of arteries (Figure 1B) as well as in cardiomyocytes (data not shown). Positively stained cells were also occasionally observed in veins (data not shown). Arterial endothelial cells were not positively stained (Figure 1B).

To analyze δEF1 expression during SMC differentiation, we utilized the A404 cell line, which is an embryonic carcinoma P19-derived in vitro SMC differentiation system (Manabe and Owens, 2001b). Undifferentiated A404 cells do not express SMC differentiation marker genes (Figure 1C), but when stimulated with retinoic acid, they rapidly acquire SMC phenotypes and express such SMC markers as SM myosin heavy chain (SM-MHC). Likewise, expression of δEF1 was upregulated during SMC differentiation in this system (Figure 1C). In the aorta, moreover, greater expression of δEF1 was seen in adult mice than in E18.5 embryos (Figure 1D). Taken together, these findings suggest that δEF1 expression is restricted to SMCs in the arterial wall, that its expression is regulated by the differentiation state of those cells, and that, perhaps, δEF1 controls the differentiation state of SMCs.

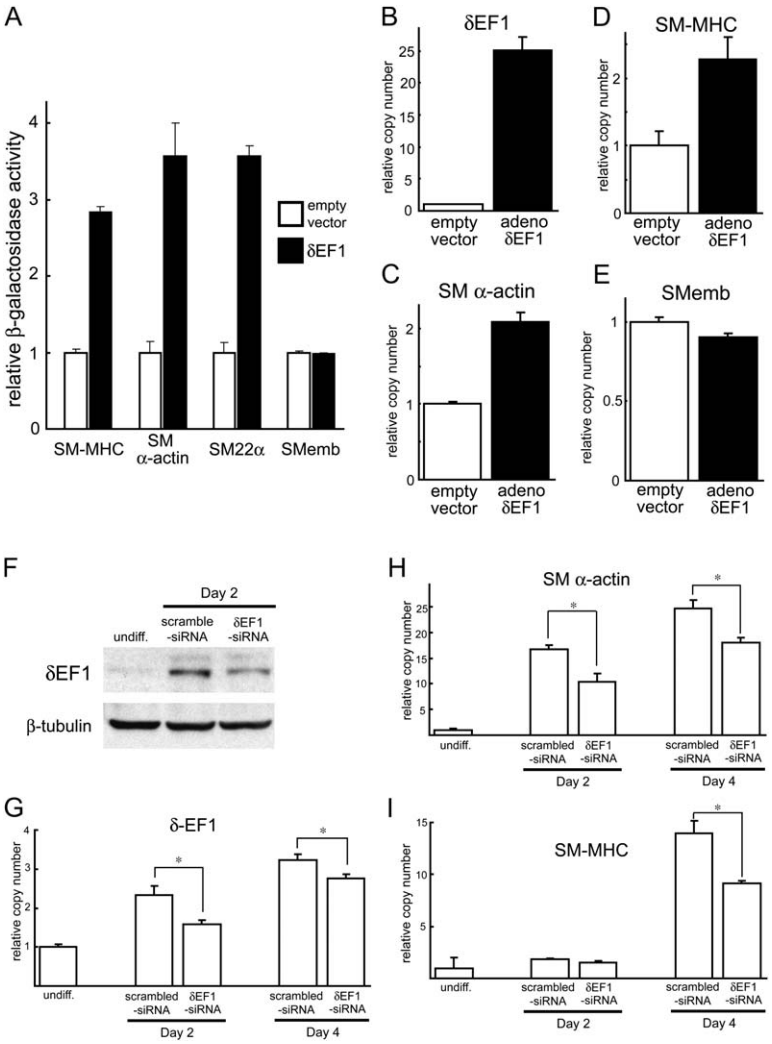

Figure 2. δEF1 Transactivated SMC Differentiation Marker Gene Promoters

(A) Effects of δEF1 on the regulatory region of SMC differentiation marker genes. Reporter genes driven by the regulatory region of indicated genes were transiently cotransfected with the δEF1 expression vector or an empty vector. The β-galactosidase activity of each reporter construct cotransfected with δEF1 expression vector was normalized to that cotransfected with the empty vector. Bars indicate relative β-galactosidase activity and SE.

(B–E) Effect of δEF1 on expression of endogenous SMC differentiation marker genes. Cultured SMCs were infected with either the δEF1 adenovirus or empty adenovirus at 20 MOI and then harvested 36 hr after infection. mRNA expression was analyzed by real-time RT-PCR. δEF1 detected in (B) included transcripts from both endogenous and exogenous genes. The copy number of each transcript was normalized to that of 18S, after which the expression was further normalized to that of cells infected with empty adenoviral vector. Bars indicate relative copy number and SE.

(F–I) Effects of δEF1 knockdown on SMC differentiation of A404 cells. A404 cells were transfected with either δEF1-siRNA or scrambled-siRNA. Twenty-four hours after transfection, the cells were treated with all-*trans* retinoic acid. Two and 4 days after the initiation of differentiation, the cells were harvested and subjected to Western analysis of δEF1 (F) and real-time PCR analysis of δEF1 (G), SM α-actin (H), and SM-MHC (I). Bars indicate relative copy number and SE.

δEF1 Controls SMC Differentiation Marker Gene Expression

To test the hypothesis that δEF1 controls the differentiated state of SMCs, we used transient transfection analysis to examine its contribution to the transcriptional regulation of SMC differentiation marker gene expression (Figure 2A). We found that δEF1 transactivated the transcriptional regulatory regions of *SM-MHC* (−4.2 to +11.6 kb) and *SM α-actin* (−2.6 to +2.7 kb), as well as the *SM22α* promoter (−441 to +41 bp), all of which have been shown to drive SMC-specific gene expression in vivo (Owens et al., 2004). By contrast, δEF1 did not activate the regulatory region (−5 to +7 kb) of *SMemb*, a marker gene expressed in embryonic and phenotypically modulated SMCs. Consistent with these effects on the promoter reporters, overexpression of δEF1 in cultured rat aortic SMCs with an adenoviral expression vector led to significant increases in the endogenous expression of *SM α-actin* (2.1-fold increase) and *SM-MHC* (2.3-fold), as compared to those seen in SMCs infected with empty adenovirus (Figures 2C and 2D). The expression level of SMemb was somewhat reduced in the δEF1-overexpressing cells (Figure 2E).

To further test the involvement of δEF1 in SMC differentiation, δEF1 expression was knocked down in A404 cells with siRNA, after which the cells were induced to differentiate. Due to the low transfection efficiency of A404 cells and the long durations of culture after the siRNA transfection, the levels of knockdown of δEF1 expression were modest (Figures 2F and 2G). Nevertheless, expression of SM α-actin and SM-MHC mRNA was significantly inhibited (Figures 2H and 2I), suggesting that knockdown of δEF1 delayed differentiation of A404 cells.

δEF1 Controls the *SM α-actin* Proximal Promoter

To define how δEF1 controls SMC differentiation marker gene transcription, a set of reporter plasmids encoding various *SM α-actin* and *SM-MHC* deletion constructs were cotransfected with the δEF1 expression plasmid. The results obtained with the *SM-MHC* reporter indicated that δEF1 affects the transcriptional activity of the *SM-MHC* regulatory region (−4.2 to +11.6 kb) by acting via multiple subregions within it (data not shown). On the other hand, the initial deletion analysis of the *SM α-actin* transcriptional regulatory region (−2.6 to +2.7 kb) suggested that δEF1 mainly controlled transcription of this gene via its proximal promoter (Figure 3B). To facilitate our analysis of δEF1 activity, therefore, we focused on the *SM α-actin* proximal promoter. Deletion

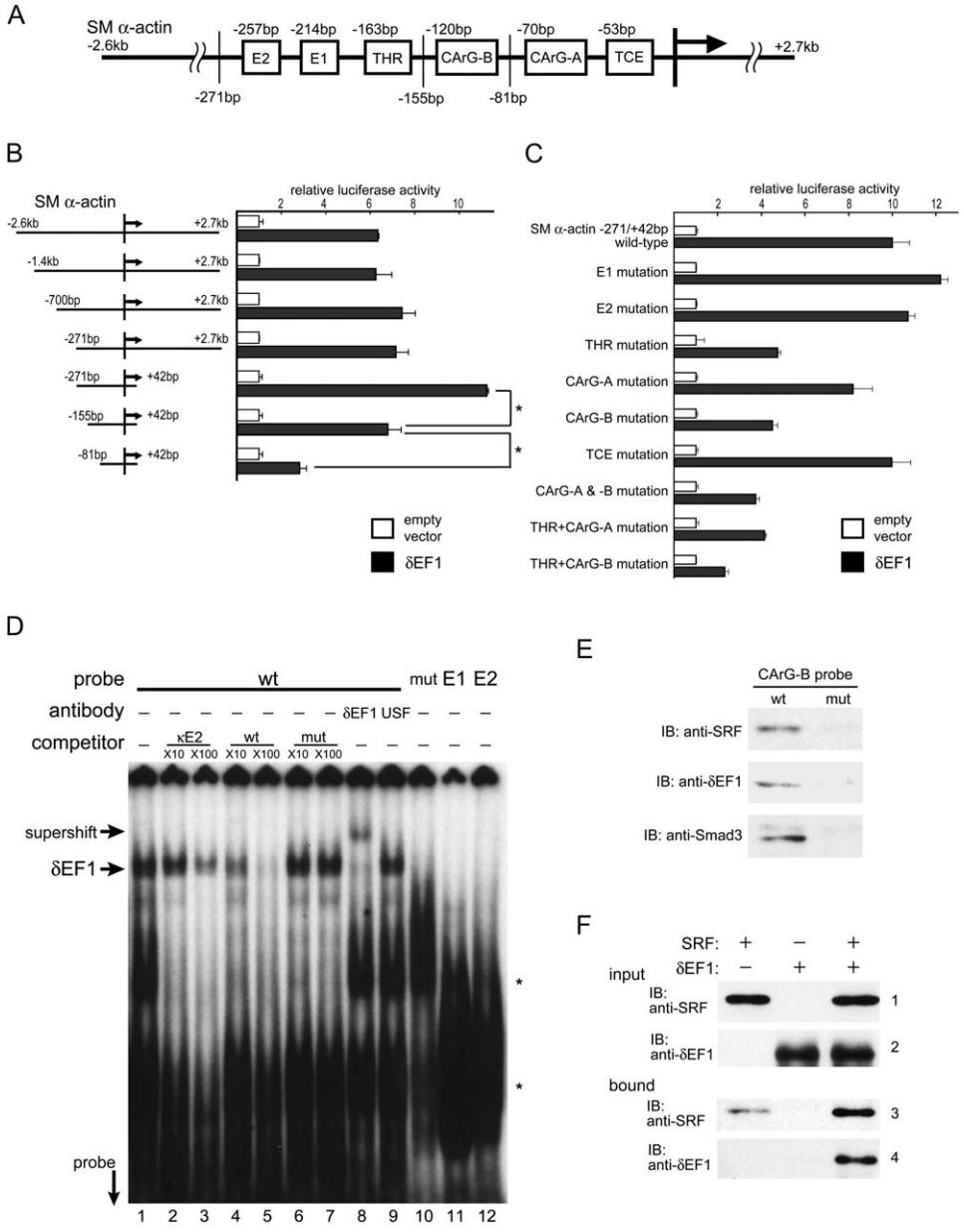

Figure 3. Analysis of δEF1-Mediated Transactivation of *SM α-actin* Promoter

(A) Schematic representation of the transcriptional regulatory regions of *SM α-actin*.

(B) Effects of δEF1 on the transcriptional activity of *SM α-actin* deletion mutants. Luciferase reporter constructs containing the indicated *SM α-actin* deletion mutants were transiently cotransfected into cultured SMCs along with either δEF1 expression vector or empty vector. Bars indicate relative luciferase activity and SE; *$p < 0.05$.

(C) Effects of mutations within the *cis*-regulatory elements on transactivation of the *SM α-actin* promoter (−271/+42) by δEF1. THR, CArGs, TCE, and E boxes were mutated within the −271/+42 bp construct. The luciferase activity of each reporter construct cotransfected with the δEF1 vector was normalized to the activity of the reporter cotransfected with empty vector. Effects of the mutations on the basal *SM α-actin* promoter activity are shown in Figure S5. Bars indicate relative luciferase activity and SE.

(D) EMSA analysis of the binding of δEF1 to the *SM α-actin* promoter. ^{32}P-labeled wild-type THR (wt), mutant THR (mut), E1, and E2 oligonucleotides were incubated with SMC nuclear extracts and subjected to EMSA. In lanes 2–7, a molar excess of the indicated cold competitor was added to the reactants; in lanes 8 and 9, anti-δEF1 or anti-USF antibody was added. Asterisks indicate nonspecific shift bands.

(E) Binding of SMC transcription factors to CArG-B. Nuclear extracts of cultured SMCs were incubated with biotinylated wild-type or mutant CArG-B probe, and the probe and bound proteins were collected with streptavidin-conjugated magnet beads. The bound proteins were subjected to SDS-PAGE and immunoblotting with anti-SRF, anti-δEF1, and anti-Smad3 antibodies.

(F) Binding of in vitro translated proteins to the CArG-B probe. SRF and/or δEF1 were incubated with the biotinylated wild-type CArG-B probe. Input proteins (1 and 2) and bound proteins (3 and 4) were immunoblotted with anti-SRF (1 and 3) or anti-δEF1 antibody (2 and 4).

of the 5′-flanking region from −2.6 kb to −271 bp had no effect on the level of transactivation by δEF1 (6.3- to 7.4-fold over the activity seen with empty vector), while deletion of the first intronic region between +43 bp and +2.7 kb augmented transactivation by δEF1 (11.4-fold with −271 to +42 bp versus 7.1-fold with −271 bp to +2.7 kb). Further deletion of the −271/−81 bp region resulted in significant reductions in δEF1-mediated reporter activity, which suggests that δEF1 affected promoter activity mainly via the proximal promoter region between −271 and +45 bp.

Within the 271-bp 5′-flanking region, there are two E boxes, a potential *cis*-element called THR (TGF-β1 hypersensitivity region [Cogan et al., 2002]), two CArG elements, and a TCE (TGF-β control element [Hautmann et al., 1997]), which are well conserved among species (Shimizu et al., 1995). Although δEF1 reportedly binds to a subset of E box sequences (Sekido et al., 1994), the *SM α-actin* E boxes do not accommodate very well the consensus binding sequence for δEF1 (CACCTG). To investigate the involvement of the E boxes, THR, CArG, and TCE in transactivation by δEF1, they were mutated within the −271/+42 bp reporter construct. Mutation of the E boxes, CArG-A, or TCE had little effect on transactivation of the promoter by δEF1 (Figure 3C). By contrast, mutation of THR or CArG-B significantly impaired promoter activation, and double mutation of THR and CArG-B further decreased the reporter activity transactivated by δEF1. THR and CArG-B thus appear to be required for transactivation of the *SM α-actin* −271/+42 bp region by δEF1.

We next analyzed the binding of δEF1 to the THR and CArG-B in vitro. In EMSAs using nuclear extract prepared from cultured SMCs, the THR probe elicited a specific band shift (Figure 3D, lane 1). This shift was partially inhibited by a cold probe for the E box (CACCTG) within the *immunoglobulin κ* enhancer (lanes 2 and 3), but it was much more efficiently inhibited by cold probe for the THR element (lanes 4 and 5). On the other hand, cold probe in which the THR element was mutated did not inhibit the band shift (lanes 6 and 7). Moreover, the band was super shifted by the addition of anti-δEF1 antibody (lane 8), confirming that the band contained δEF1. Anti-USF1 antibody did not affect the shift band. Probes containing the *SM α-actin* E boxes did not shift bands containing δEF1 (lanes 11 and 12), indicating that δEF1 binds to the THR element but not the E boxes within the *SM α-actin* proximal promoter region. It thus appears that δEF1 transactivates the *SM α-actin* promoter at least in part via binding to the THR element.

δEF1 Interacts with SRF

The results of the reporter analyses indicate that CArG-B is also required for δEF1 action. However, the CArG-B probe did not form shift bands with in vitro translated δEF1 in EMSA (data not shown), suggesting δEF1 might be involved in the activity of CArG-B via interactions with SRF. To address this possibility, we analyzed binding proteins to CArG-B by DNA affinity binding assays (Suzuki et al., 1993). When the biotinylated CArG-B probe was incubated with SMC nuclear extracts, SRF, δEF1, and Smad3 were detected in the bound proteins (Figure 3E). To test whether δEF1 requires SRF to bind CArG-B, the biotinylated CArG-B probe was incubated with in vitro translated δEF1 protein in the presence or absence of SRF. As shown in Figure 3F, δEF1 bound the CArG-B probe only when SRF was present.

To further characterize interactions between δEF1 and SRF, we carried out coimmunoprecipitation experiments with whole-cell lysates prepared from cultured SMCs (Figures 4A and 4B). When the lysates were subjected to immunoprecipitation, we detected SRF in immunoprecipitates pulled down with anti-δEF1 antibody (Figure 4A) and detected δEF1 in those pulled down with anti-SRF antibody (Figure 4B), which indicates that endogenous δEF1 protein physically interacts with SRF protein.

We then determined which domains of δEF1 and SRF interact with one another with expression vectors encoding truncated forms of δEF1 and SRF. Coimmunoprecipitation experiments showed that the two proteins interact mainly via δEF1 regions containing two zinc-finger domains and a SRF region containing a MADS box (Figures 4C and 4D).

δEF1 Cooperates with SRF and Smad3 to Activate the *SM α-actin* Promoter

Previous studies have shown that in AKR-2B fibroblasts, the reactivity of THR can vary in in vivo footprinting evoked by TGF-β (Becker et al., 2000). Moreover, Postigo recently reported that in osteoblasts δEF1 interacts with Smads, thereby playing a key role in the regulation of transcription mediated by TGF-β signaling (Postigo, 2003). These findings suggested that δEF1 might interact with Smads and play a role in TGF-β signaling in SMCs. To test that hypothesis, we first cotransfected a luciferase reporter vector containing the *SM α-actin* regulatory region (−2.6 to +2.7 kb) with vectors encoding δEF1 and Smad3 into SMCs. Subsequent reporter analyses showed synergistic activation of *SM α-actin* reporter activity by δEF1 and Smad3 (Figure S1A). Conversely, Smad7, an inhibitory Smad, suppressed transactivation of the *SM α-actin* by δEF1, lending additional support to the idea that Smads are involved in transcriptional control by δEF1 (Figure S1B). That δEF1 physically interacts with Smads was confirmed by coimmunoprecipitation experiments with whole-cell lysates of SMCs. δEF1 was detected in immunoprecipitates pulled down with anti-Smad3 antibody (Figure 5A), and Smad3 was detected in immunoprecipitates pulled down with anti-δEF1 antibody (Figure 5B).

Our coimmunoprecipitation (Figure 4), DNA affinity binding, and reporter (Figure 3) analyses showed that δEF1 interacts with SRF in SMCs. Interestingly, SRF also interacts with Smads (Qiu et al., 2003), while CArG elements are required for TGF-β-dependent activation of *SM α-actin* (Hautmann et al., 1997), which suggests that SRF is involved in TGF-β-dependent transcriptional control. Bearing that in mind, we next tested whether δEF1, SRF, and Smad3 might all interact in the transcriptional control of *SM α-actin* expression. Our reporter analyses consistently showed that overexpressing SRF in rat aortic SMCs inhibited somewhat the activity of SMC differentiation marker gene promoters, including those of *SM α-actin* and *SM-MHC*, whereas SRF transactivated those promoters in non-SMC cells, such as NIH3T3, BALB/3T3, and COS cells. Although the exact mechanism for this SMC-selective inhibition is

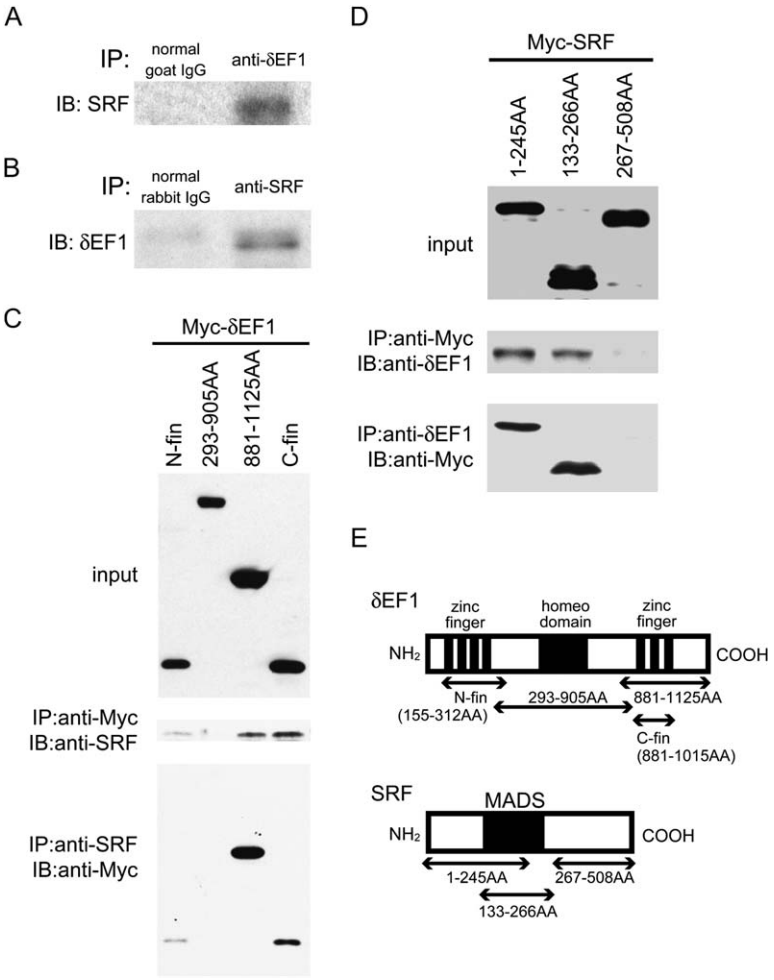

Figure 4. Interaction between δEF1 and SRF

(A and B) Physical association of δEF1 and SRF. Whole-cell lysates prepared from cultured rat aortic SMCs were incubated with anti-δEF1 antibody (A), anti-SRF antibody (B), or normal IgG (control). The reactants were immunoprecipitated with protein G (A) or anti-rabbit IgG (B) agarose-conjugated antibody, and immunoprecipitates were subjected to immunoblotting with anti-SRF antibody (A) or anti-δEF1 antibody (B).

(C and D) Analysis of the interaction between domains within δEF1 and SRF proteins. Myc-tagged δEF1 or SRF deletion constructs were transfected into cultured SMCs. Lysates of the cells were incubated with the indicated antibodies (IP), and immunoprecipitates were subjected to immunoblot with the indicated antibodies (IB).

(E) The domains are schematically illustrated.

unknown, competition for coactivators has been proposed as a possible mechanism (Prywes and Zhu, 1992). Because of this inhibitory effect of SRF, we used NIH3T3 cell in the following experiments. In those cells, SRF transactivated the full-length (−2.6 to +2.7 kb) *SM α-actin* reporter, eliciting a 13.8-fold increase in activity (Figure 5C). By themselves, Smad3 or δEF1 each only slightly increased the luciferase activity of the full-length (−2.6 to +2.7 kb) *SM α-actin* reporter (3.2-fold and 2.2-fold, respectively) (Figure 5C). Interestingly, introduction of a constitutively active form of the TGF-β type 1 receptor, caALK5, increased basal reporter activity (8.2-fold increase versus control without caALK5) (Figure 5C versus Figure 5D), and under those conditions, Smad3 and δEF1 clearly augmented transactivation of the reporter (6.4- and 5.6-fold increase over the control with caALK5), suggesting that the activities of both the *SM α-actin* promoter and δEF1 are dependent upon Smad signaling. Cotransfection of SRF and either Smad3 or δEF1 resulted in synergistic activation of the reporter activity, irrespective of the inclusion of caALK5 (Figures 5C and 5D). Moreover, cotransfection of constructs for SRF, Smad3, and δEF1 resulted in further activation of the reporter. We found similar patterns of activation of the −271/+42 bp construct (Figure S2), although overexpression of SRF increased the reporter activity to a much greater extent. Thus, SRF, δEF1, and Smad all appear to be important for *SM α-actin* transcription, and the synergistic activation observed with combinations of these transcription factors suggests that they interact to control promoter activity.

δEF1 Is Involved in TGF-β-Dependent Transcriptional Control of *SM α-actin*

The involvement of δEF1 in Smad3-dependent transcriptional control in SMCs suggests that δEF1 may be required for TGF-β-induced *SM α-actin* transcription. To test this idea, we first examined TGF-β-evoked δEF1 expression. Levels of δEF1 protein were increased by TGF-β stimulation, peaking within 12 hr and then returning to baseline within 24 hr (Figure 5E). Levels of δEF1 mRNA were also increased by TGF-β (Figure 5G).

We then analyzed the effect of δEF1 knockdown with siRNA (Figures 5F and 5G). δEF1-siRNA or a control-scrambled siRNA was transfected into cultured SMCs, after which the cells were stimulated with TGF-β. δEF1-siRNA successfully knocked down expression of δEF1 at both the mRNA (Figure 5F) and protein (Figure 5G) levels. In cells transfected with control siRNA, TGF-β induced δEF1 expression that peaked within 4 hr after stimulation (Figure 5G). In those cells, SM α-actin expression was clearly increased within 4 hr and peaked within 8 hr after TGF-β stimulation. In cells transfected with δEF1-siRNA, by contrast, the TGF-β-induced

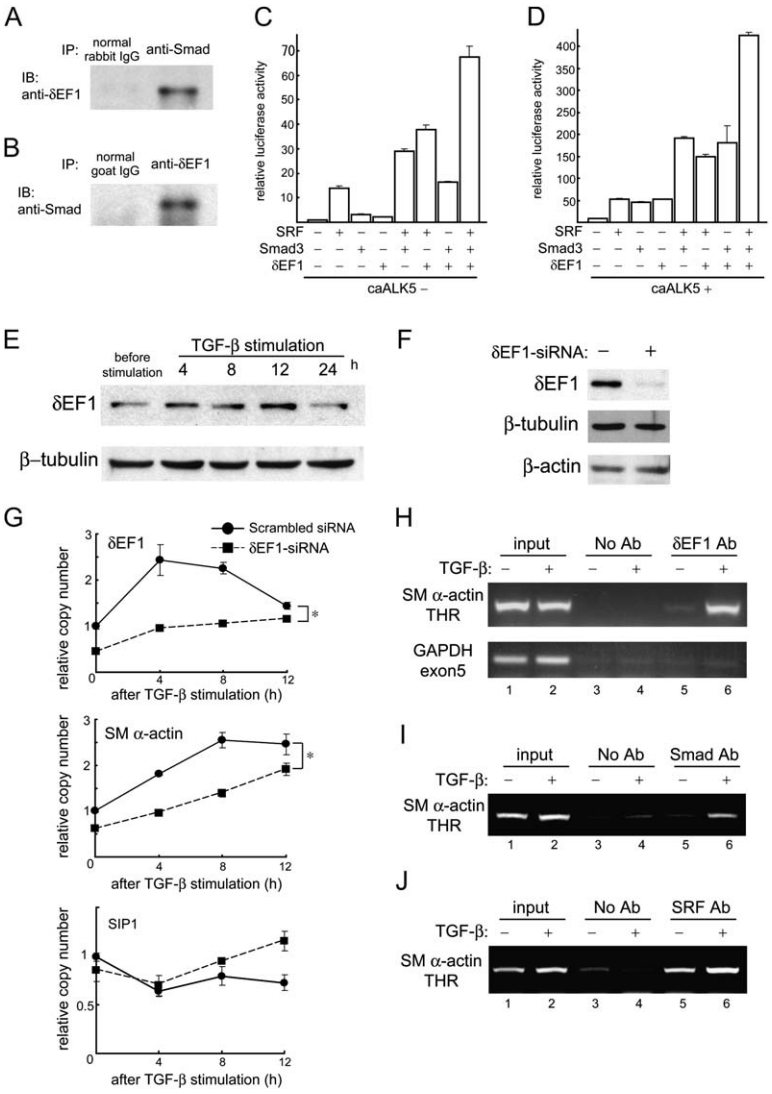

Figure 5. δEF1 in TGF-β Signaling

(A and B) Physical association between Smad and δEF1. SMC lysates were incubated with the indicated antibodies (IP) and immunoprecipitated. δEF1 (A) or Smad3 (B) in the immunoprecipitate was detected with the corresponding antibodies.

(C and D) Effects of SRF, Smad3, and δEF1 on the *SM α-actin* promoter. NIH3T3 cells were cotransfected with full-length *SM α-actin* luciferase plasmid and expression vectors encoding SRF, Smad3, and/or δEF1, with or without constitutively active ALK5 (caALK5), as indicated. The luciferase activity was normalized to that of the *SM α-actin* reporter cotransfected with empty plasmid. Bars indicate relative luciferase activity and SE.

(E) Induction of δEF1 protein by TGF-β stimulation. Rat aortic SMCs were cultured in serum-free defined medium for 4 days, after which TGF-β (2.5 ng/ml) was added to the medium. Whole-cell lysates were subjected to Western analyses of δEF1 and β-tubulin.

(F) Cells transfected with either δEF1-siRNA or control siRNA and incubated in serum-free medium for 24 hr were harvested, after which their whole-cell lysates were subjected to Western analyses.

(G) Effects of δEF1 knockdown on *SM α-actin* expression. Either δEF1-specific siRNA or control scrambled siRNA was transfected into SMCs. Twenty-four hours later, the medium was changed to serum-free defined medium for an additional 24 hr, after which TGF-β (2.5 ng/ml) was added for the indicated times. mRNA expression was analyzed by real-time PCR. Solid lines represent gene expression in cells transfected with the scrambled siRNA; dotted lines, expression in cells transfected with δEF1-siRNA. Error bars represent SE from triplicate culture wells; *$p < 0.05$ (two-way ANOVA).

(H–J) ChIP analysis of δEF1, SRF, and Smad3 binding to the endogenous *SM α-actin* promoter region containing the THR under TGF-β1 stimulation. SMCs were cultured in defined serum-free medium for 4 days and then treated with TGF-β1 (2.5 ng/ml) for 12 hr. Chromatin samples prepared from these cells were subjected to ChIP analysis. PCR was carried out to detect the *SM α-actin* promoter. Lanes 1 and 2 show amplification of total input DNA. Lanes 3 and 4 show PCR amplification of control samples precipitated with no antibody. Lanes 5 and 6 show amplification of target sequences within the immunoprecipitates. The sequence of GAPDH, which does not contain a δEF1 binding sequence, was also amplified to confirm the specificity of the assay (H).

upregulation of δEF1 was clearly suppressed (Figure 5G), as were the level of SM α-actin expression at 0 hr and its subsequent upregulation. Thus, knocking down δEF1 in SMCs attenuated the upregulation of SM α-actin otherwise seen following TGF-β stimulation, indicating δEF1 plays an important role in the TGF-β-dependent transcriptional control of *SM α-actin* expression. In addition, we also found that knocking down Smad3 resulted in inhibition of *SM α-actin* expression (Figure S3).

To confirm that endogenous δEF1, SRF, and Smad bind to the endogenous *SM α-actin* promoter in intact chromatin, cross-linked chromatin samples prepared from SMCs were subjected to chromatin immunoprecipitation (ChIP) assays. The *SM α-actin* promoter region was pulled down with antibodies against δEF1 or Smad3 in SMCs treated with TGF-β1 (Figures 5H and 5I) but was almost undetectable in the precipitants of untreated cells. The binding of SRF to the promoter was also augmented by TGF-β stimulation (Figure 5J). Clearly, TGF-β stimulation induces binding of δEF1, Smad3, and SRF to the endogenous *SM α-actin* promoter.

δEF1 Plays a Role in SMC Differentiation In Vivo

We then examined the in vivo expression of SMC differentiation marker genes in the aortas of *δEF1* knockout mice (Figure 6). We observed no apparent gross abnormalities in the cardiovascular systems of either $\delta EF1^{-/-}$ or $\delta EF1^{+/-}$ mice. Given the lethality of the $\delta EF1^{-/-}$ genotype, we decided to examine expression of SMC differentiation marker genes in aortas collected from embryos on E18.5. Using real-time PCR, we found that the levels of expression of SM α-actin, SM22α, and SM-MHC were lower in both $\delta EF1^{-/-}$ and $\delta EF1^{+/-}$ embryos than in wild-type embryos (Figures 6C–6F), while expression of

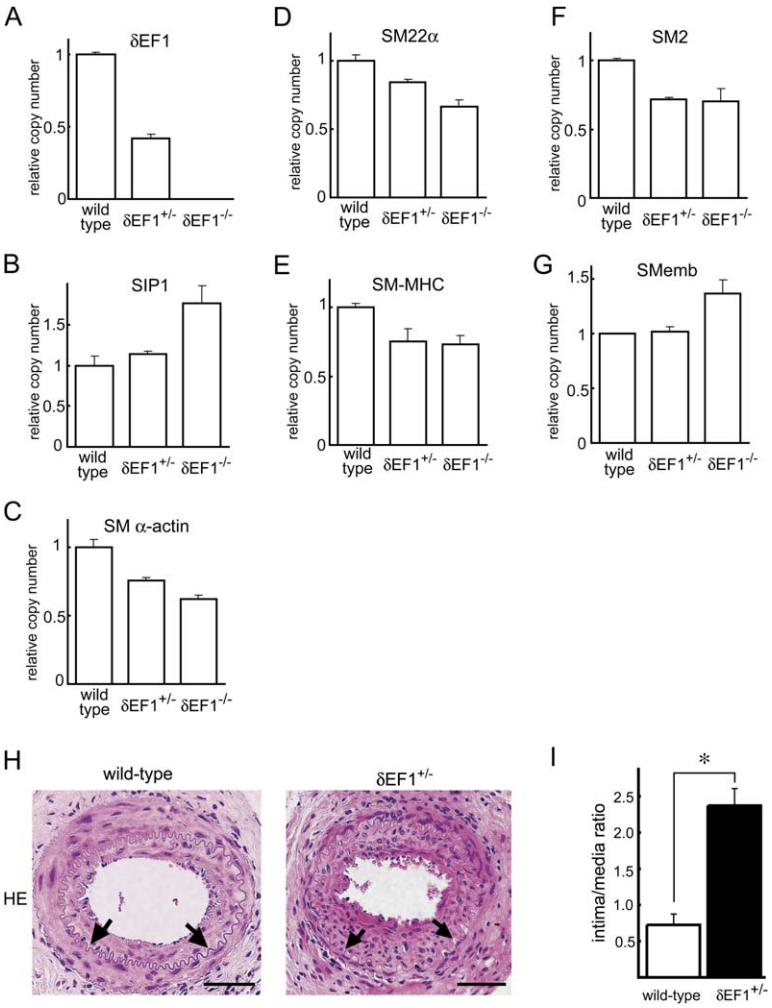

Figure 6. Effects of Haploinsufficiency of *δEF1* on Vascular Lesion Formation

(A–G) Real-time PCR analysis of the expression of δEF1, SIP1, and SMC markers in the aortas of wild-type, $\delta EF1^{+/-}$, and $\delta EF1^{-/-}$ embryos. Aortas were collected from embryos of each genotype (n = 3, 4, and 3, respectively), after which total RNA was isolated from the collected samples, and real-time PCR was carried out. The relative numbers of transcripts were normalized to those of 18S, after which expression of the transcript for each genotype was further normalized to that of the wild-type embryo. Error bars represent SE in real-time PCR data.

(H) Representative photomicrographs of transverse sections of femoral arteries harvested from wild-type and $\delta EF1^{+/-}$ mice 4 weeks after injury with a catheter guide wire. The sections were stained with hematoxylin-eosin.

(I) Neointimal/medial wall area ratios; bars are means ± SE; n = 4 for each genotype.

SMemb was higher in $\delta EF1^{-/-}$ embryos than in wild-type or $\delta EF1^{+/-}$ embryos (Figure 6G).

It was interesting to us that although aortic SMCs from $\delta EF1^{-/-}$ embryos exhibited retarded differentiation on E18.5, SMC differentiation marker genes were still expressed in $\delta EF1^{-/-}$ embryos, albeit at a lower level. In that regard, SIP1/ZEB-2, a transcription factor closely related to δEF1, was expressed in the aortas of E18.5 embryos and adult mice, and its expression was higher in $\delta EF1^{-/-}$ than in wild-type embryos (Figure 6B), suggesting that SIP1 might have acted to compensate for the lack of δEF1 function in the knockout mice (see Discussion).

$\delta EF1^{+/-}$ mice do not exhibit a clearly abnormal arterial phenotype, but we hypothesized that injury might elicit abnormal responses, as has been seen when other transcription factor genes were targeted (Manabe and Nagai, 2003). Moreover, previous studies have demonstrated that alteration of the differentiated state of SMCs plays an important role in vascular lesion formation and that changes in the course of phenotypic modulation and redifferentiation of SMCs during lesion formation may affect the outcome after vascular injury (Owens et al., 2004; Wiegman et al., 2000). With that in mind, we carried out vascular injury experiments in $\delta EF1^{+/-}$ knockout mice in which the femoral arteries were injured by using catheter guide wires. In $\delta EF1^{+/-}$ mice, neointimal lesions observed 4 weeks after the injury were much more prominent than in wild-type mice (Figure 6H), leading to significantly ($p < 0.001$) higher intimal/medial area ratios in $\delta EF1^{+/-}$ mice (Figure 6I). Moreover, as expected, we found much reduced expression of SM α-actin and SM-MHC proteins in the lesions of $\delta EF1^{+/-}$ mice (Figure S4), indicating redifferentiation of SMCs was disrupted in those animals. By contrast, we did not find clear differences in the numbers of $CD45^+$ leukocytes within the lesions.

δEF1 Is Involved in Controlling the Differentiated State of SMCs during Neointima Formation In Vivo

We further analyzed the role played by δEF1 in vascular pathology with a rat vascular injury model that enabled analysis of gene expression and SMC phenotypes. The left common carotid arteries of rats were subjected to balloon injury, after which an adenoviral vector encoding δEF1 (Ad-δEF1) or empty adenovirus was injected into the lumens of the injured arteries to infect mural cells. As evident from arteries exposed to empty adenovirus, expression of endogenous δEF1 protein was barely detectable in neointimal cells except those located adjacent to the tunica media (Figures 7Ac). The neointimal cells in the arteries exposed to empty adenovirus also

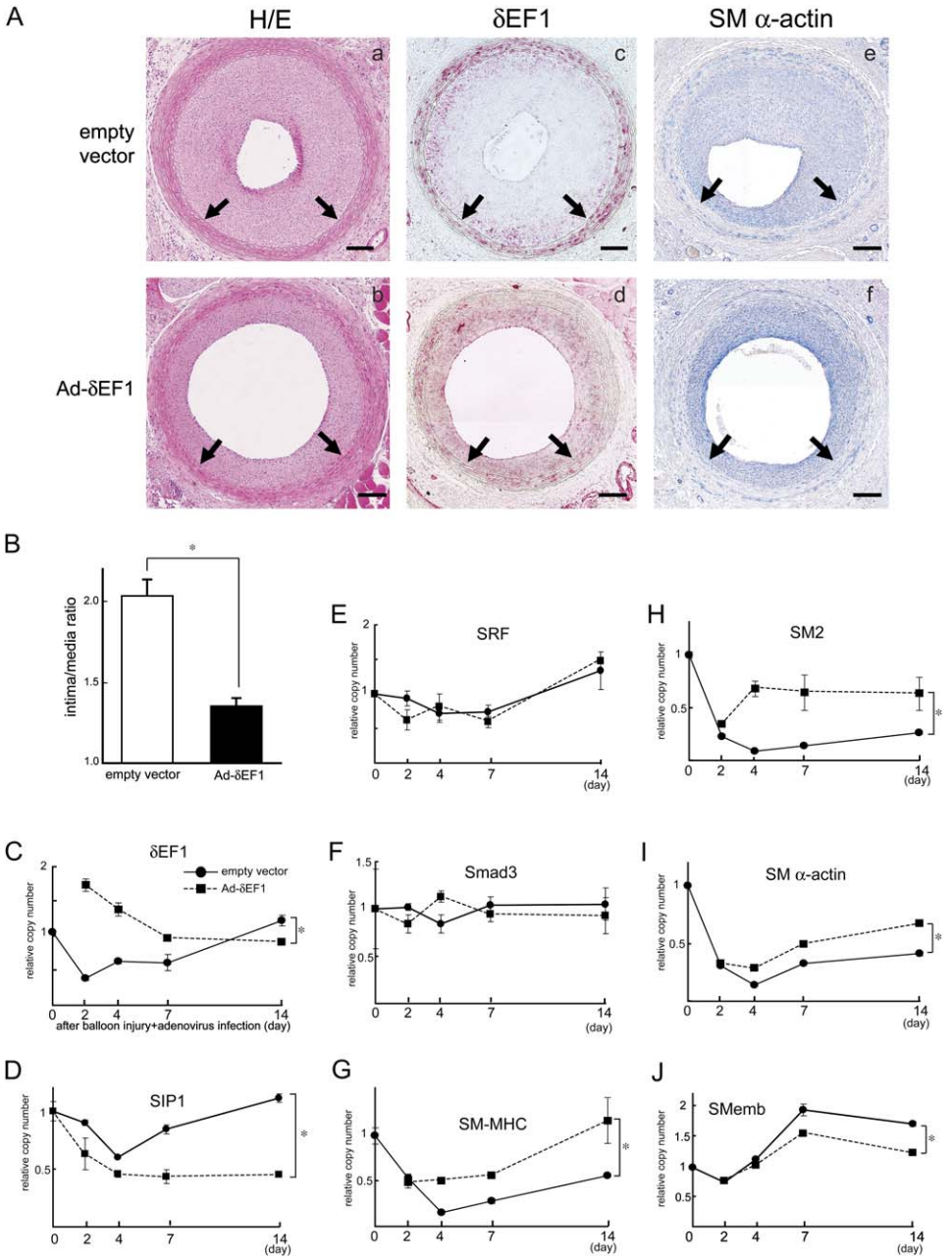

Figure 7. Effects of Adenoviral Overexpression of δEF1 on Vascular Injury Responses

(A) Representative photomicrographs of transverse sections of rat common carotid arteries harvested 2 weeks after balloon injury and injection of empty adenoviral vector (Aa, Ac, and Ae) or Ad-δEF1 (Ab, Ad, and Af). (Aa and Ab) The sections were stained with hematoxylin-eosin. (Ac and Ad) Immunohistochemical staining of δEF1 protein (red). (Ae and Af) Immunohistochemical staining of SM α-actin protein (blue). Nuclei were counterstained with nuclear fast red. Arrows indicate the internal elastic laminae. Scale bars represent 100 μm.

(B) Neointimal/medial wall area ratios; bars are means ± SE; n = 8 for each group.

(C–J) Real-time PCR analysis of mRNA expression. Solid lines represent mRNA expression in arteries infected with empty adenovirus; dotted lines, expression in arteries infected with Ad-δEF1; n = 3 in each group. Copy number of each transcript was normalized to that of 18S, after which the expression was further normalized to that in arteries without balloon injury and adenovirus injection. Graphs indicate relative copy number and SE; *p < 0.05 (two-way ANOVA).

exhibited weaker staining for SM α-actin than was seen in medial SMCs (Figure 7Ae), indicating that the neointimal cells were phenotypically modulated. Downregulation of δEF1 was also seen at the mRNA level (Figure 7C).

Ad-δEF1 infection markedly attenuated neointima formation (Figure 7Aa versus 7Ab), so that neointimal/medial wall area ratios were significantly ($p < 0.001$) lower in arteries infected with Ad-δEF1 than in those infected with empty adenovirus (Figure 7B). In the Ad-δEF1-infected arteries, δEF1 was detected in both the media and the neointima, and similar levels of SM α-actin staining were detected in medial and neointimal cells (Figure 7A). This suggests that at this point in time, neointimal cells in the Ad-δEF1-treated arteries exhibit a more

differentiated phenotype than those in arteries treated with empty adenovirus.

We then analyzed mRNA expression in the injured carotid arteries (Figures 7C–7J) and found that in arteries infected with empty adenovirus, levels of δEF1 transcript were clearly diminished by day 2 after injury and gradually returning to baseline by day 14 (Figure 7C). This reduction in δEF1 expression was accompanied by reductions in SM-MHC and SM α-actin expression (Figures 7G–7I). In the Ad-δEF1-infected arteries, by contrast, overexpression of δEF1 was observed until day 7, and the level of suppression of SM-MHC and SM α-actin was much attenuated (Figures 7G–7I). Expression of SMemb, which was induced by injury, was moderately, but significantly, decreased in the arteries infected with Ad-δEF1 (Figure 7J). Thus, judging from the observed expression of SMC phenotype marker genes, SMCs in Ad-δEF1-infected arteries are in a more differentiated state than those in control arteries. We also analyzed expression of other transcription factors. Interestingly, overexpression of δEF1 resulted in a reduction in SIP1 expression (Figure 7D), whereas expression levels of the δEF1 partners, SRF and Smad3, were not significantly affected by Ad-δEF1 infection (Figures 7E and 7F).

Discussion

One of the major findings of the present study is that δEF1 promotes the expression of SMC differentiation marker genes. δEF1 expression was upregulated during SMC differentiation of A404 cells (Figure 1C) and in mouse aorta (Figure 1D). δEF1 transactivated promoters of the SMC differentiation marker genes (Figure 2A) and upregulated expression of endogenous *SM-MHC* and *SM α-actin* in cultured SMCs (Figures 2B–2E). Moreover, knockdown of δEF1 resulted in inhibition of SM α-actin and SM-MHC induction during SMC differentiation of A404 cells (Figures 2H and 2I). Taken together, these findings demonstrate that δEF1 expression is regulated during vascular development and that, once expressed, δEF1 controls expression of various SMC differentiation marker genes and is thus actively involved in SMC differentiation. The role of δEF1 in the control of SMC differentiation was indicated further by the observations that differentiation of aortic SMCs was delayed in δEF1 knockout mice (Figure 6 and Figure S4) and that differentiation is promoted by δEF1 overexpression in cultured SMCs (Figure 2) and in injured arteries (Figure 7).

Another major finding of the present study is the involvement of δEF1 in TGF-β-dependent SMC transcriptional programs. TGF-β is thought to be one of the most important environmental cues controlling SMC differentiation (Owens et al., 2004), but little was known about how TGF-β affects the molecular machinery of SMC transcriptional programs. We were able to show in the present study (1) that δEF1 expression is regulated by TGF-β (Figure 5E); (2) that δEF1 physically interacts with Smad3 to synergistically enhance *SM α-actin* reporter activity (Figures 5A–5D); (3) that δEF1 also physically interacts with SRF (Figure 4); (4) that TGF-β induces binding of δEF1, SRF, and Smad3 to the endogenous *SM α-actin* promoter region (Figures 5H–5J); and (5) that knocking down δEF1 reduces TGF-β-induced upregulation of endogenous *SM α-actin* expression in SMCs (Figure 5G). Collectively, then, our findings suggest that δEF1 mediates TGF-β signaling by binding to both SRF and Smad3, which is consistent with earlier observations showing that CArG elements are indispensable for TGF-β-dependent transcriptional activation. Although further studies are needed to confirm whether δEF1 forms multiprotein complexes (enhanceosomes) by simultaneously binding SRF, Smad3, and other factors on the *SM α-actin* promoter, the results of the present study provide compelling evidence for the involvement of δEF1 in TGF-β-dependent SMC-specific transcriptional regulation mediated by SRF and Smad.

The finding of an interaction between δEF1 and SRF provides insight into SMC transcriptional regulatory programs that need to respond to diverse environmental cues (Owens et al., 2004). Identification of a number of the factors that interact with SRF has led to the hypothesis that protein-DNA complexes comprised of multiple factors control SMC-specific gene regulation (Miano, 2003; Owens et al., 2004). Recently, a coactivator called myocardin was reported to be specifically expressed in SMCs and cardiomyocytes and to dramatically activate SRF-dependent SMC promoters, suggesting it may be myocardin that determines the SMC-specificity of the SRF-dependent transcriptional programs. On the other hand, myocardin, alone, does not appear to be sufficient to control SMC transcriptional programs in response to diverse environmental cues (Parmacek, 2004; Pipes et al., 2005). δEF1 is interesting in this regard, as it is selectively expressed in vascular SMCs, is regulated by TGF-β, and interacts with both SRF and Smad. δEF1 thus possesses several characteristics necessary to mediate context-dependent combinatorial regulation of SMC genes. Future studies will be needed to address the role of myocardin in δEF1-mediated transcriptional control.

The attenuated expression of SMC differentiation marker genes in the aorta of *δEF1*$^{-/-}$ mice is indicative of delayed SMC differentiation there. Still, although the level was reduced, some expression of SMC differentiation marker genes did occur in *δEF1*$^{-/-}$ mice, and despite the lethality of the genotype, there were no clear morphological abnormalities within their cardiovascular systems. One possible reason for the mildness of the phenotype is that the loss of δEF1 is compensated for by other factors. Indeed, SIP1/ZEB-2, a closely related transcription factor, is also expressed in the aorta, and the level of its expression was higher in *δEF1*$^{-/-}$ mice than in wild-type mice (Figure 6B). This is consistent with earlier studies showing that *δEF1*$^{-/-}$ mice exhibit no clear phenotype in the central nervous system, skeletal muscle, or heart, where both δEF1 and SIP1 are expressed (Postigo and Dean, 2000; Takagi et al., 1998). δEF1 and SIP1 bind to similar sequences, and we found that SIP1 is also capable of transactivating SMC differentiation marker gene promoters (G.N. and I.M., unpublished data). That said, it was recently shown that δEF1 and SIP1 have distinctly different functions under certain conditions (Postigo et al., 2003), so further studies will be required to clarify the functional relationship between δEF1 and SIP1.

Finally, we found that expression of δEF1 is downregulated in neointimal SMCs (Figure 7A) and in rat carotid

arteries during the first week after balloon injury (Figure 7C) and that expression of SM α-actin, SM1, and SM2 is also downregulated during the same period (Figures 7G–7I). In addition, overexpression of δEF1 in injured arteries significantly reduced neointima formation (Figure 7B), whereas neointimal areas were significantly larger in the injured arteries of *δEF1*$^{+/-}$ mice than in those of wild-type animals (Figure 6I). The precise mechanism by which δEF1 expression affects neointima formation is not yet clear, but there are several possibilities. First, activation of differentiation programs by δEF1 may affect SMC proliferation, as differentiation and proliferation are inversely correlated in many cell types. Indeed, proliferating SMCs in vascular diseases are known to exhibit modulated phenotypes characterized by downregulation of the SMC differentiation marker genes and upregulation of *SMemb* (Manabe and Nagai, 2003). Gene expression patterns of δEF1-treated arteries and *δEF1*$^{+/-}$ animals suggest that δEF1 inhibits phenotypic modulation and/or promotes differentiation of SMCs (Figure 7 and Figure S4). It has also been shown that transcription factors that control differentiation programs directly affect cell-cycle proteins and vice versa. In that regard, TGF-β is known to control SMC proliferation in a context-dependent manner, and Postigo recently showed that δEF1 acts synergistically with TGF-β to arrest proliferation of Mv1Lu epithelial cells (Postigo, 2003). Second, in addition to contractile protein genes, δEF1 may regulate genes involved in other functions of SMCs. Neointima formation is a complex process, in which SMCs migrate, proliferate, and produce extracellular matrices and paracrine factors, thereby mediating tissue remodeling. The clear suppression of neointima formation by adenovirus-mediated δEF1 expression suggests that δEF1 may modulate various functions of SMCs in that process. In that regard, it was recently reported that δEF1 controls *collagen type 1* expression in the A7r5 cultured SMC line (Ponticos et al., 2004), which is noteworthy because collagen fibers and other extracellular matrix proteins and matrix proteases are known to affect SMC function and the pathology of blood vessels. However, we did not find that Ad-δEF1 infection significantly affected collagen deposition in the injured carotid arteries (G.N. and I.M., unpublished data). It would be important to identify additional δEF1 target genes to further clarify its role in neointima formation.

δEF1 is among only a few transcription factors that have been shown to affect neointima formation after vascular injury (Perlman et al., 1998). This suggests that modulation of δEF1 function may represent a strategy for treating vascular conditions such as the restenosis that can occur after percutaneous coronary intervention, as well as unstable plaque. Moreover, definition of the transcription factor networks of which δEF1 is one component could reveal additional potential targets for therapeutic intervention in the treatment of vascular disease.

Experimental Procedures

Plasmids

ZEB-1 constructs were kindly provided by Dr. D.C. Dean, Smad3 and ALK5 constructs were by Dr. K. Miyazono, and *SM-MHC* and *SM α-actin* reporter constructs were by Dr. G.K. Owens.

Animal Models

All animal procedures strictly adhered to the guidelines for animal experiments of the University of Tokyo.

Chromatin Immunoprecipitation

Confluent SMCs were maintained in serum-free medium for 4 days, after which TGF-β1 (2.5 ng/ml) was added to the medium. After incubating for an additional 12 hr in the presence of TGF-β1, the cells were fixed and harvested, and the samples were subjected to chromatin immunoprecipitation (ChIP) assays (Manabe and Owens, 2001a). An enhanced Supplemental Experimental Procedures section is available online.

Supplemental Data

Supplemental data include an enhanced Experimental Procedures section and supplemental figures and are available at http://www.developmentalcell.com/cgi/content/full/11/1/93/DC1/.

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