Pitx2 is functionally important in the early stages of vascular smooth muscle cell differentiation

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Mechanisms that control vascular smooth muscle cell (SMC) differentiation are poorly understood. We identify Pitx2 as a previously unknown homeodomain transcription factor that is rapidly induced in an in vitro model of SMC differentiation from multipotent stem cells. Pitx2 induces expression of multiple SMC differentiation marker genes by binding to a TAATC(C/T) cis-element, by interacting with serum response factor, and by increasing histone acetylation levels within the promoters of SMC differentiation marker genes. Suppression of Pitx2 reduces expression of SMC differentiation marker genes in the early stages of SMC differentiation in vitro, whereas Prx1, another homeodomain protein, regulates SMC differentiation marker genes in fully differentiated SMCs. *Pitx2*, but not *Prx1*, knockout mouse embryos exhibit impaired induction of SMC differentiation markers in the dorsal aorta and branchial arch arteries. Our results demonstrate that Pitx2 functions to regulate the early stages of SMC differentiation.

Introduction

Differentiation of smooth muscle cells (SMCs) is a critical component of formation of the cardiovascular system. During the process of arteriogenesis, endothelial cell tubes become invested with precursor cells, which subsequently differentiate into SMCs and form the medial layer of the blood vessels (for reviews see Carmeliet, 2000; Owens et al., 2004). Although the primary function of differentiated SMCs is contraction, SMCs during embryonic development also exhibit high rates of cell proliferation, migration, and production of extracellular matrix proteins that are required for vessel morphogenesis. SMC differentiation is characterized by coordinate induction of expression of a unique repertoire of SMC differentiation markers, including SM α-actin, SM22α, SM-myosin heavy chain, and h1-calponin. However, the molecular mechanisms controlling SMC differentiation during early stages of arteriogenesis are poorly understood.

Expression of SMC differentiation markers has been shown to be controlled by a combinatorial interaction of multiple transcription factors and cofactors (for review see Owens et al., 2004). The promoter-enhancer regions of many SMC differentiation marker genes contain multiple CArG [CC(AT-rich)$_6$GG] elements, and the transbinding factor serum response factor (SRF) and its coactivator myocardin play a key role in the regulation of these genes. Indeed, results of previous studies from our laboratory and others showed that *myocardin* was exclusively expressed in SMCs and cardiomyocytes and that it markedly induced the transcription of multiple CArG-containing SMC differentiation marker genes in the presence of SRF, whereas suppression of myocardin by either dominant-negative constructs or siRNA was associated with marked decreases in transcription of these genes in cultured aortic SMCs (Wang et al., 2001; Chen et al., 2002; Du et al., 2003; Yoshida et al., 2003). In addition, we showed that myocardin served as a point of convergence in mediating effects of environmental cues on expression of SMC differentiation marker genes, in that angiotensin II and platelet-derived growth factor BB regulated expression of CArG-containing SMC differentiation marker genes via changes in *myocardin* expression in cultured aortic SMCs (Yoshida et al., 2004a, 2007; Liu et al., 2005). However, myocardin does not appear to be required for the initial induction of SMC lineage because recent studies revealed that *myocardin*-null embryonic stem (ES) cells were able to differentiate into SMCs in the context of chimeric knockout mice, which were generated by injection of *myocardin*-null ES cells into the wild-type blastocysts in vivo (Pipes et al., 2005). Moreover, results of in situ hybridization assays showed that *myocardin* expression

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Abbreviations used in this paper: ACLP, aortic carboxypeptidase-like protein; ChIP, chromatin immunoprecipitation; E, embryonic day; EMSA, electrophoresis mobility shift assay; ES, embryonic stem; HDAC, histone deacetylase; RA, all transretinoic acid; SM, smooth muscle; SMC, SM cell; SRF, serum response factor; SSEA-1, stage-specific embryonic antigen–1.

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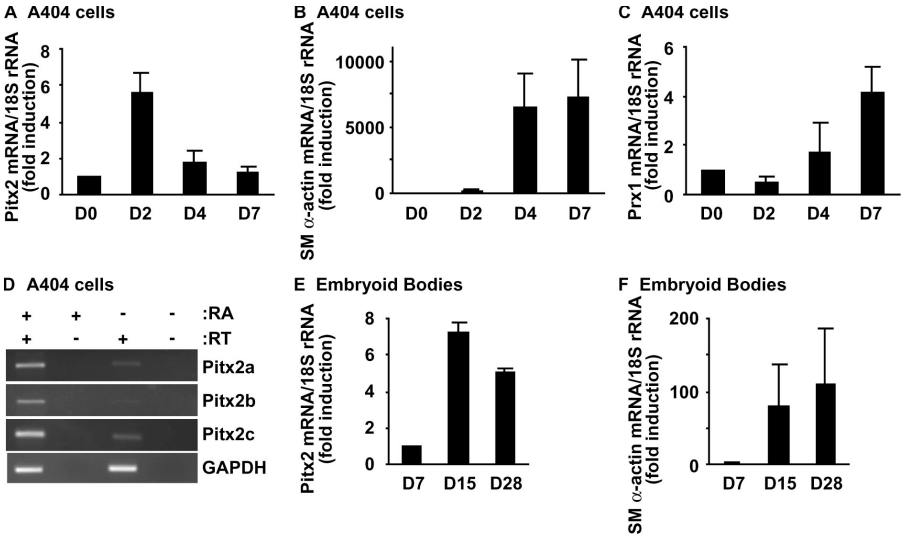

Figure 1. ***Pitx2* is induced during SMC differentiation.** (A–C) A404 cells were induced to differentiate into SMCs by treatment with 1 µg/ml RA, followed by 0.5-µg/ml puromycin treatment. Expression of *Pitx2* (A), *SM α-actin* (B), and *Prx1* (C) was determined by real-time RT-PCR from day (D) 0 to day 7. (D) Expression of *Pitx2* isoforms was determined by semiquantitative RT-PCR in undifferentiated A404 cells and RA-treated A404 cells for 48 h. PCR products are 424 bp for *Pitx2a*, 297 bp for *Pitx2b*, 234 bp for *Pitx2c*, and 230 bp for *GAPDH*. (E and F) ES cells were induced to differentiate into SMCs in an embryoid body system, and expression of *Pitx2* (E) and *SM α-actin* (F) was determined by real-time RT-PCR at days 7, 15, and 28. Values represent the mean ± SEM of three independent experiments.

was quite low or undetectable in early developmental stages of vascular SMCs (Wang et al., 2001; Du et al., 2003). As such, although the preceding results provide compelling evidence that the CArG–SRF–myocardin complex plays a key role in the control of SMC differentiation marker gene expression in differentiated SMCs, the initial induction of SMC differentiation may not be dependent on myocardin and may be regulated through alternative molecular mechanisms.

Results of our previous studies have shown that Prx1 (also called as *Prrx1* and MHox), a homeodomain protein, plays a key role in basal and angiotensin II–induced expression of SMC differentiation marker genes in adult differentiated SMCs in vitro (Hautmann et al., 1997; Yoshida et al., 2004a). Treatment with angiotensin II in fully differentiated cultured SMCs increased expression of *Prx1* as well as *SM α-actin*. In addition, siRNA-induced suppression of *Prx1* decreased basal and angiotensin II–induced expression of *SM α-actin* in differentiated SMCs. Moreover, recombinant Prx1 protein enhanced the binding of SRF to CArG elements within the *SM α-actin* promoter, although studies failed to detect the stable interaction between SRF and Prx1. These studies suggest that Prx1 is important in mediating angiotensin II–induced SMC hypertrophy in differentiated SMCs. However, there is no evidence showing that Prx1 plays a role in regulating SMC differentiation marker genes during SMC development or in SMC hypertrophy in vivo. Indeed, Bergwerff et al. [2000] found no evidence of defective SMC differentiation within developing embryos in *Prx1*, *Prx2*, or *Prx1/Prx2* knockout mice. At present, there is no direct evidence that homeodomain proteins regulate SMC differentiation in vivo.

We previously developed an in vitro inducible SMC differentiation lineage system derived from clonal lines of multipotential P19 embryonal carcinoma cells harboring an *SM α-actin* promoter-enhancer–driven puromycin-resistant gene (Manabe and Owens, 2001). One of these clonal lines, designated "A404," lacked expression of any known SMC differentiation marker gene under basal conditions but showed induction of all known SMC differentiation marker genes in response to treatment with all transretinoic acid (RA) and puromycin. It is of major significance that >80% of A404 cells showed induction of SM α-actin upon treatment with a single reagent, RA, indicating that A404 is a clonal cell line poised for induction of SMC lineage. In addition, RA-induced differentiation of A404 cells into SMCs was accompanied by increases in histone acetylation levels and SRF binding within the CArG-containing promoter regions of SMC differentiation marker genes (Manabe and Owens, 2001). Collectively, these results suggest that the A404 SMC differentiation system is a valuable model for studying early stages of induction of SMC lineage from multipotential stem cells.

In the present studies, we performed a subtractive hybridization screen between undifferentiated and differentiated A404 cells to identify factors critical for the initial induction of SMC differentiation. We discovered Pitx2 as a homeodomain transcription factor that was rapidly induced during the differentiation of SMCs, as compared with the delayed induction of Prx1. We show that Pitx2 induces expression of multiple SMC differentiation marker genes by binding to specific cis-regulatory elements, by interacting with SRF, and by increasing histone acetylation levels within the promoter regions of SMC differentiation marker genes. Moreover, we show that knockout of the *Pitx2* gene in mouse embryos virtually abolishes the induction of SMC differentiation markers in early stages of SMC differentiation, thus providing the first evidence implicating a homeodomain protein in regulation of SMC development in vivo.

Results

Pitx2 expression was induced in multiple inducible culture models of SMC differentiation

To identify factors that play a key role in the initial induction of SMC differentiation, a subtractive hybridization screen was performed between undifferentiated A404 cells and differentiated A404 cells that were treated with RA for 48 h. 44 genes were found to be induced in differentiated A404 cells as compared with undifferentiated A404 cells (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200711145/DC1).

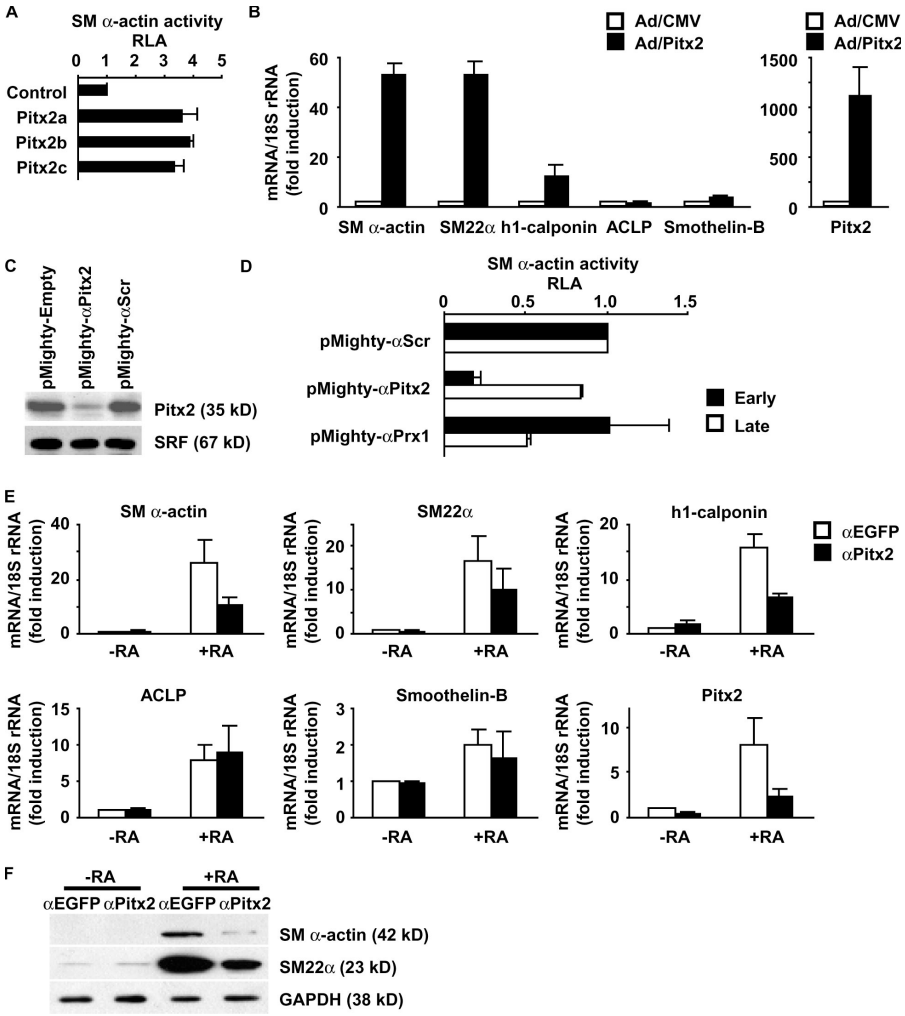

Figure 2. **Pitx2 induces expression of SMC differentiation marker genes.** (A) Luciferase assays were performed in undifferentiated A404 cells cotransfected with expression plasmids for Pitx2 isoforms and the *SM α-actin* promoter-enhancer-luciferase construct. RLA, relative luciferase activity. (B) Undifferentiated A404 cells were infected with adenovirus expressing Pitx2a or empty adenovirus, and expression of SMC differentiation marker genes was determined by real-time RT-PCR. (C) Efficiency and specificity of *Pitx2* siRNA were examined in COS cells cotransfected with Flag-Pitx2a expression plasmid and *Pitx2* siRNA expression plasmid or control plasmids. pMighty-Empty contained no target sequence and pMighty-αScr targeted scrambled sequence. (D) A404 cells were induced to differentiate into SMCs by RA treatment for 1 d (early) or by RA treatment for 3 d followed by puromycin selection for 2 d (late). Cells were transfected with the *SM α-actin* promoter-enhancer-luciferase construct and a siRNA expression plasmid for *Pitx2* (pMighty-αPitx2), *Prx1* (pMighty-αPrx1), or pMighty-αScr. Luciferase assays were performed. (E and F) A404 cells were induced to differentiate into SMCs by RA treatment and were transfected with siRNA duplexes for *Pitx2* or *EGFP*. Expression of SMC differentiation marker genes was determined by real-time RT-PCR (E) or Western blotting (F). Values represent the mean ± SEM of three independent experiments.

Among these, Pitx2 was selected for further studies, because it is expressed in vascular SMCs during embryonic development (Kitamura et al., 1999; Hjalt et al., 2000) and it is a transcription factor implicated in the development of multiple organs such as the pituitary gland, the eyes, and the heart (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999a). As an initial test of the potential importance of Pitx2 for SMC differentiation, we examined its expression in multiple inducible culture systems of SMC differentiation. Expression of *Pitx2* was rapidly induced by 5.5-fold, 48 h after RA treatment in A404 cells (Fig. 1 A). The increase in *Pitx2* expression preceded the induction of *SM α-actin* and *Prx1* (Fig. 1, B and C), and all *Pitx2* isoforms were induced (Fig. 1 D). Expression of *Pitx2* was also examined in an ES cell–derived embryoid body model of SMC differentiation, which showed induction of SMC lineage in response to treatment with RA and dibutyryl cAMP (Drab et al., 1997; Sinha et al., 2004). Importantly, ES cell–derived SMCs exhibited spontaneous contraction in the context of embryoid bodies, indicating that all regulatory pathways essential for formation of contractile SMCs are present within in this system. Expression of *Pitx2* was induced by sevenfold during the formation of embryoid bodies (Fig. 1, E and F). These results show that *Pitx2* is induced in multiple in vitro SMC differentiation models.

Pitx2 was required for the induction of SMC differentiation marker genes in multiple inducible culture models of SMC differentiation

To determine the role of Pitx2 in SMC differentiation, effects of overexpression of Pitx2 on SMC differentiation marker genes were examined. Expression plasmids for Pitx2 isoforms were cotransfected with the *SM α-actin* promoter-reporter construct into undifferentiated (non-RA treated) A404 cells. Results showed that each Pitx2 isoform increased transcriptional activity of the *SM α-actin* gene in a similar manner (Fig. 2 A). The induction of *SM α-actin* transcription by Pitx2 was also seen in 10T1/2 cells, although it was modest in NIH/3T3 cells (unpublished data). To determine if Pitx2 induces expression of endogenous SMC differentiation marker genes, an adenovirus expressing Flag-tagged Pitx2a was constructed. Adenovirus-mediated overexpression of Pitx2 dramatically induced expression of multiple SMC differentiation marker genes including *SM α-actin*, *SM22α*, and *h1-calponin* in A404 cells, although a subset of SMC differentiation marker genes, including *aortic carboxypeptidase-like protein* (*ACLP*) and *smoothelin-B*, were not induced (Fig. 2 B). Adenovirus-mediated overexpression of Pitx2 also increased cellular growth (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200711145/DC1).

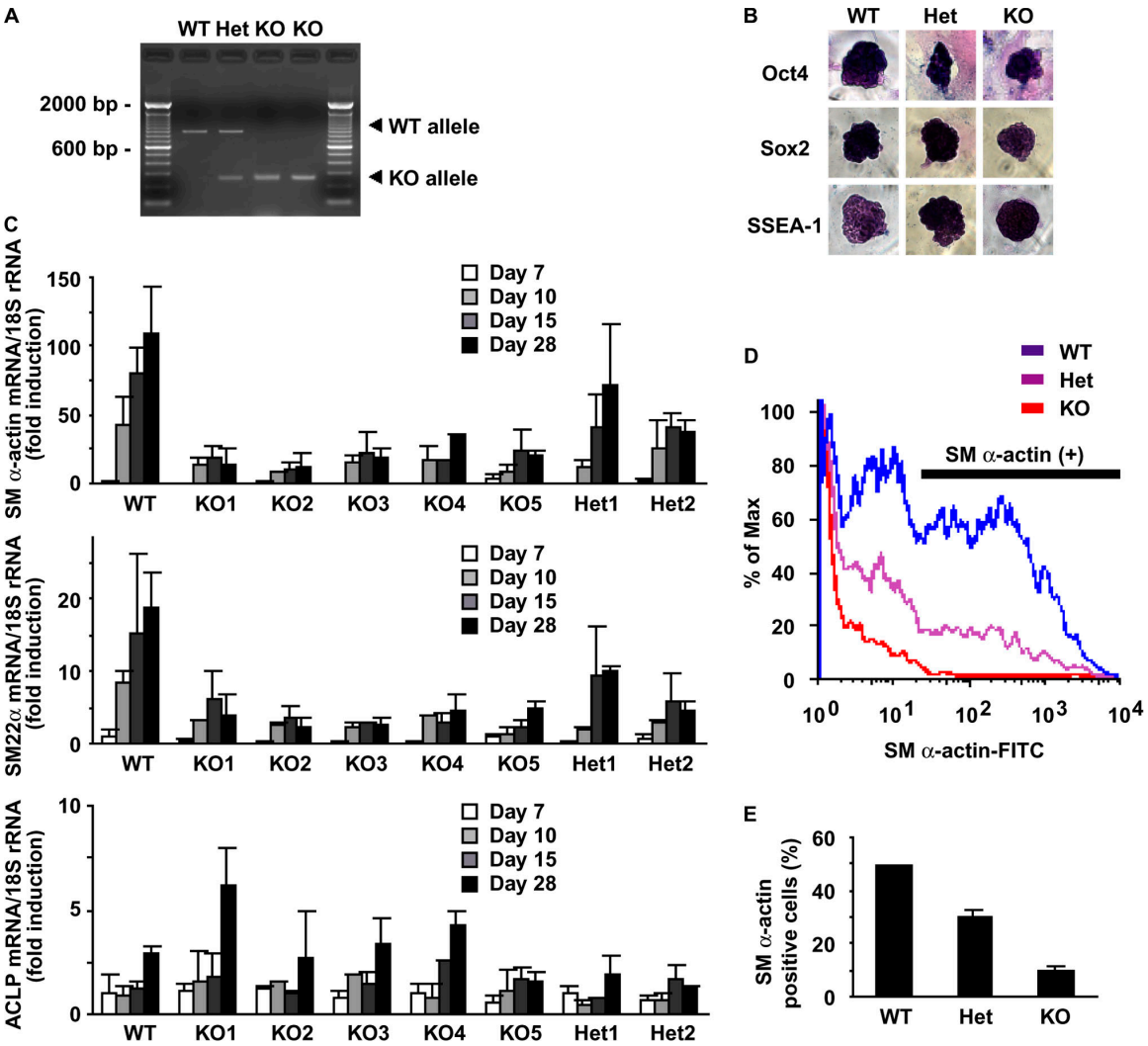

Figure 3. **Induction of SMC differentiation marker genes is attenuated in embryoid bodies derived from *Pitx2* homozygous knockout ES cells.** (A) Genotyping of *Pitx2* knockout ES cells. (B) *Pitx2* knockout ES cells were stained with Oct4, Sox2, and SSEA-1. (C–E) Five independent *Pitx2* homozygous knockout ES cells, two *Pitx2* heterozygous knockout ES cells, and wild-type ES cells were induced to differentiate into SMCs in context of embryoid bodies, and expression of *SM α-actin*, *SM22α*, and *ACLP* was determined by real-time RT-PCR (C) and flow cytometry (D and E). Representative data from flow cytometry is shown in D. WT, wild-type; Het, heterozygous; KO, knockout. Values represent the mean ± SEM from three independent experiments.

Although increased growth is usually associated with reduced expression of SMC differentiation markers in fully differentiated SMCs (for review see Owens et al., 2004), it is possible that enhanced growth and subsequent changes in cell density and cell–cell interactions may contribute to Pitx2-induced differentiation. However, the fact that Pitx2 selectively induced a subset of SMC differentiation marker genes suggests that this is not the case.

Effects of siRNA-induced knockdown of *Pitx2* on SMC differentiation marker genes were examined in differentiated A404 cells. The *Pitx2* siRNA expression plasmid, which targets a sequence that is common for all three *Pitx2* isoforms, was generated in a customized pMighty system that expresses siRNA under a mouse H1 promoter (Yoshida et al., 2003). Cotransfection of *Pitx2* siRNA expression plasmid (pMighty-αPitx2) and Pitx2a expression plasmid revealed that *Pitx2* siRNA efficiently and selectively suppressed expression of Pitx2 but not expression of a nontargeted protein, SRF (Fig. 2 C). As shown in Fig. 2 D, siRNA-induced suppression of *Pitx2* reduced the transcriptional activity of the *SM α-actin* gene by 70% in early time points of SMC differentiation in RA-treated A404 cells. In contrast, siRNA-induced suppression of *Prx1* did not decrease the *SM α-actin* activity under these conditions. However, the reduction in the transcriptional activity of the *SM α-actin* gene by the *Pitx2* siRNA was blunted at later time points of SMC differentiation in differentiated A404 cells treated with RA and puromycin, whereas *Prx1* siRNA reduced the activity more effectively at the same later time points. Transfection of *Pitx2* siRNA duplexes into A404 cells revealed that RA-mediated induction of SMC differentiation marker genes, including *SM α-actin*, *SM22α*, and *h1-calponin* but not *ACLP* and *smoothelin-B*, was attenuated by 40–60% (Fig. 2, E and F). These results suggest that Pitx2 and Prx1 play complementary roles in control of SMC differentiation marker gene expression but at different stages of SMC differentiation.

Expression levels of *myocardin* and its related factors, *MKL1* and *MKL2*, were not altered in RA-treated A404 cells in the presence or absence of *Pitx2* siRNA (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200711145/DC1). Pitx2-induced activation of the *SM α-actin* gene in A404 cells was not attenuated by siRNA-induced suppression of *myocardin*. However, it was partially reduced by siRNAs for *MKL1* or *2* alone or in combination (Fig. S2 B). These results provide evidence that Pitx2-induced activation of SMC differentiation marker genes is not dependent on myocardin but is at least partially dependent on MKL1 and 2.

Loss-of-function experiments were also performed in the ES cell–derived embryoid body SMC differentiation system. As shown in Fig. 3 A, multiple independent clones of homozygous *Pitx2* knockout ES cells were isolated from heterozygous *Pitx2* knockout ES cells using high G418 selection (Mortensen et al., 1992). Retention of pluripotency of each homozygous *Pitx2* knockout ES cell line was validated based on positive immunostaining with ES cell markers, Oct4, Sox2, and stage-specific embryonic antigen–1 (SSEA-1; Fig. 3 B). Each homozygous *Pitx2* knockout cell line was then tested for its ability to differentiate into SMCs in the context of embryoid bodies (Sinha et al., 2004). In wild-type ES cell–derived embryoid bodies, expression of SMC differentiation marker genes, including *SM α-actin* and *SM22α*, was induced by 109-fold and 18-fold, respectively (Fig. 3 C). However, induction of these SMC differentiation marker genes was dramatically attenuated in *Pitx2*-null embryoid bodies derived from all five independent lines of homozygous *Pitx2* knockout ES cells, whereas induction of *ACLP* expression was not affected. Expression of other lineage markers, including *PECAM*, *cardiac α-actin*, and *NeuroD*, was unaffected by deletion of the *Pitx2* gene (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200711145/DC1). Induction of *SM α-actin* and *SM22α* expression in heterozygous *Pitx2* knockout ES cells was also partially impaired, which is consistent with the fact that Axenfeld-Rieger syndrome, a disease caused by the mutation of the *Pitx2* gene, is a haploinsufficiency disorder (Semina et al., 1996). Furthermore, induction of SM α-actin protein expression was attenuated in *Pitx2*-null embryoid bodies as determined by flow cytometry (Fig. 3, D and E). Collectively, results from a combination of gain- and loss-of-function experiments provide compelling evidence that Pitx2 is critical for initial induction of SMC differentiation marker genes in multipotential stem cells.

Pitx2-induced expression of SMC differentiation marker genes via binding to a TAATC(C/T) element

To determine the molecular mechanisms whereby Pitx2 induces expression of SMC differentiation marker genes, various mutation constructs of the *SM α-actin* promoter-enhancer-luciferase plasmid (Fig. 4 A) were cotransfected with Pitx2a expression plasmid into A404 cells. As shown in Fig. 4 B, Pitx2 induced the transcriptional activity of the full-length −2.6/+2.8-kb *SM α-actin* promoter-enhancer-luciferase construct by 5.4-fold and the activity of the deletion construct, the −155/+21-bp *SM α-actin* promoter-luciferase, by 4.5-fold. However, Pitx2-induced activation of the −125/+23-bp *SM α-actin* promoter-luciferase construct was only 2.1-fold, suggesting that the sequence from −155 to −125 bp contains cis-regulatory elements important for Pitx2-induced activity. We previously identified an ATTA element within the *SM α-actin* promoter at −145 bp as a Prx1 binding site (Hautmann et al., 1997). We noticed that this element was highly homologous to the Pitx2 consensus DNA binding element 5′-TAATC(C/T)-3′ in the reverse orientation and therefore tested the effect of mutation of this element on the Pitx2 response. Results showed that mutation of the TAATCT element within the *SM α-actin* promoter-luciferase construct reduced the responsiveness to Pitx2 as compared with the wild-type counterpart (Fig. 4 B). A consensus TAATCC element is also located at −1071 bp within the mouse *SM22α* gene. Mutagenesis experiments revealed that this element was important for Pitx2-induced activation of the *SM22α* gene (Fig. 4 C).

To determine if Pitx2 binds to the TAATCT element within the *SM α-actin* promoter, electrophoresis mobility shift assays (EMSA) were performed using the double-stranded oligonucleotide corresponding to the −156 to −131 bp of the *SM α-actin* promoter and nuclear extracts from A404 cells. The oligonucleotide containing the TAATCT element formed a slowly migrating complex with nuclear extracts from differentiated A404 cells but not those from undifferentiated A404 cells (Fig. 4 D). This complex was eliminated by cold-competitor oligonucleotide or by addition of an anti-Pitx2 antibody, demonstrating the specific Pitx2 binding to the TAATCT element. Binding of Pitx2 to the TAATC(C/T) element-containing regions of SMC differentiation marker genes was also examined by chromatin immunoprecipitation (ChIP) assays. Results showed that RA treatment induced the association of Pitx2 with the TAATC(C/T)-containing regions within the *SM α-actin* and *SM22α* genes but not with the first intron region of the *SM22α* gene, which lacks the TAATC(C/T) element, in A404 cells (Fig. 4 E). Pitx2 binding to these regions was decreased in later stages of differentiated A404 cells when expression of SMC differentiation marker genes was not dependent on Pitx2. Collectively, results provide evidence that binding of Pitx2 to the TAATC(C/T) element within the promoter-enhancer regions of SMC differentiation marker genes is required for induction of these genes during SMC differentiation.

Pitx2 and SRF synergistically enhanced the transcriptional activity of SMC differentiation marker genes

Results thus far have shown that Pitx2 induces a subset of SMC differentiation marker genes that contain CArG elements within their promoter-enhancers. Thus, we tested the hypothesis that Pitx2 regulates SMC differentiation marker genes by interacting with the CArG–SRF complex. First, cotransfection experiments were performed using expression plasmids for Pitx2a and SRF as well as the *SM α-actin* promoter-luciferase construct. Results showed that Pitx2 and SRF synergistically induced the *SM α-actin* transcriptional activity in A404 cells (Fig. 5 A). Second, effects of mutation of the CArG elements on Pitx2-induced activation of the *SM α-actin* gene were tested in A404 cells. Mutation of CArG B, CArG A, or double mutation of

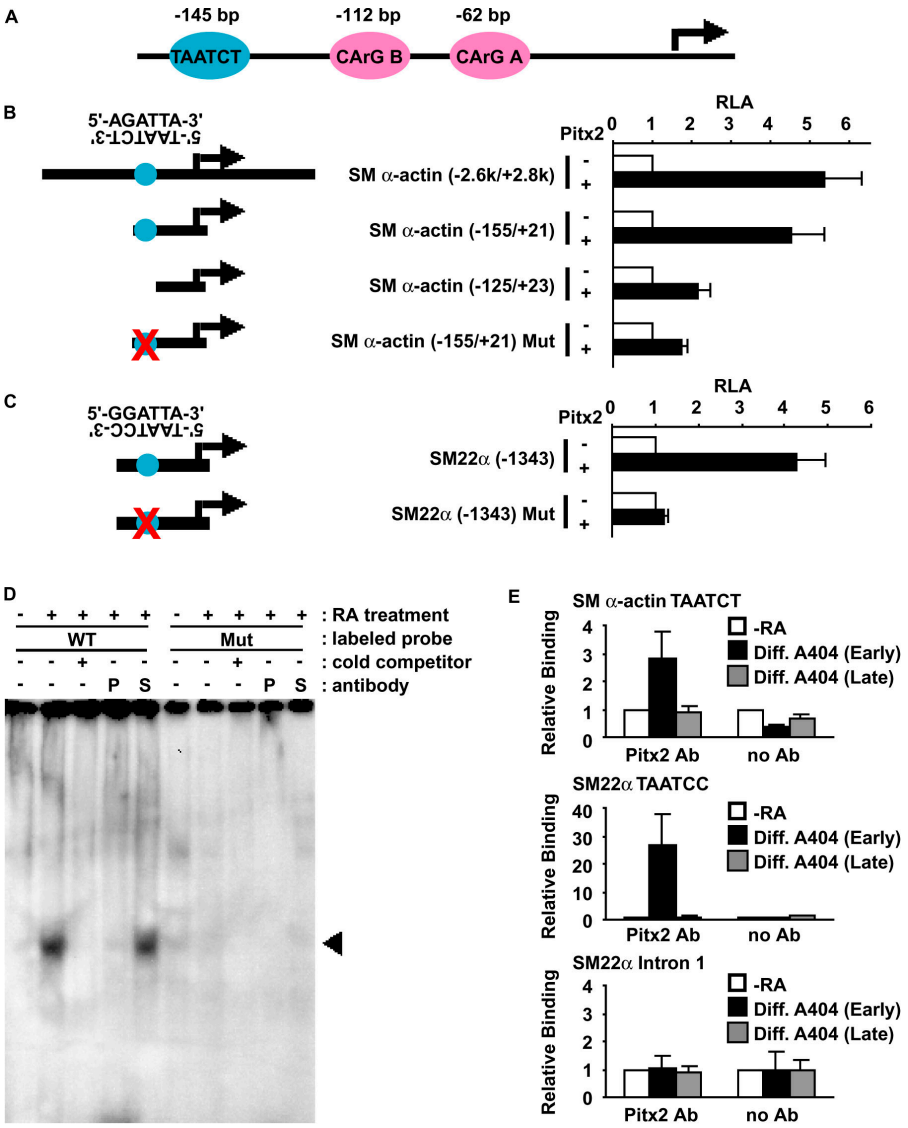

Figure 4. **Pitx2 induces SMC differentiation marker genes by binding to the TAATC(C/T) element.** (A) Schematic structure of the *SM α-actin* promoter. CArG B, CArG A, and a TAATCC element are shown. (B and C) Luciferase assays were performed in A404 cells cotransfected with Pitx2a expression plasmid and the promoter-enhancer-luciferase construct of the *SM α-actin* (B) and *SM22α* (C) genes. Schematic representation of mutation constructs is shown on the left. Values represent the mean ± SEM from three independent experiments. (D) EMSA was performed using the ^{32}P-labeled double-stranded oligonucleotide containing the TAATCT element of the *SM α-actin* gene and nuclear extracts from undifferentiated and differentiated A404 cells. Antibodies for Pitx2 (P) or SRF (S) were used to test interference. Arrowhead indicates a labeled oligonucleotide–Pitx2 protein complex. (E) Association of Pitx2 with the TAATC(C/T) region of the *SM α-actin* and *SM22α* genes or the first intron region of the *SM22α* gene was determined by ChIP assays in undifferentiated A404 cells, differentiated A404 cells treated with RA for 2 d (early), and differentiated A404 cells treated with RA for 3 d followed by puromycin selection for 2 d (late). Values represent the mean ± SEM from three independent experiments.

CArGs B and A each decreased the response to Pitx2 (Fig. 5 B). These results suggest that Pitx2-induced *SM α-actin* transcription is mediated in part through the CArG–SRF complex. However, the TAATCT element was not required for SRF-induction of the *SM α-actin* gene (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200711145/DC1).

To determine if the synergistic effect of Pitx2 and SRF was caused by the direct interaction of these two proteins, coimmunoprecipitation assays were performed using expression plasmids for Flag-tagged Pitx2a and SRF. Interaction was readily detectable between Pitx2a and SRF (Fig. 5 C). The domain of Pitx2a required for SRF interaction was then determined by using Pitx2a deletion mutants. Although deletions from aa 101 to the carboxy terminus (Pitx2a [aa 1–208], Pitx2a [aa 1–189], and Pitx2a [aa 1–101]) did not affect SRF interaction, Pitx2a (aa 1–49), which lacked the homeodomain and the carboxy-terminal domain, failed to interact with SRF. In addition, although a deletion of the amino terminus to aa 38 (Pitx2a [aa 39–271]) did not affect SRF interaction, Pitx2a (aa 98–271), which lacked the homeodomain and the amino-terminal domain, did not exhibit SRF interaction. Moreover, a deletion mutant, Pitx2a (aa 39–100), which only contained the homeodomain, interacted with SRF, indicating that the homeodomain of Pitx2, which is common for all three Pitx2 isoforms, is sufficient for the physical interaction with SRF.

The domain of SRF required for Pitx2 interaction was determined by GST pulldown assays and mammalian two-hybrid assays. Although the full-length SRF bound to Pitx2, neither SRF (aa 1–222) nor SRF (aa 1–132) truncation mutants bound to Pitx2 as determined by GST pulldown assays (Fig. 5 D). In support of this, results of mammalian two-hybrid assays showed that the domain of SRF from aa 266 to 414 was required for Pitx2 interaction (Fig. 5 E). Higher basal activities observed in GAL4-SRF (aa 168–508), GAL4-SRF (aa 266–508), and GAL4-SRF (aa 414–508) constructs may be caused by the lack of amino-terminal domain, which inhibits the activation domain of SRF (Johansen and Prywes, 1993). Collectively, these results suggest that the homeodomain of Pitx2 (aa 39–100) directly binds to the carboxy-terminal domain (aa 266–414) of SRF.

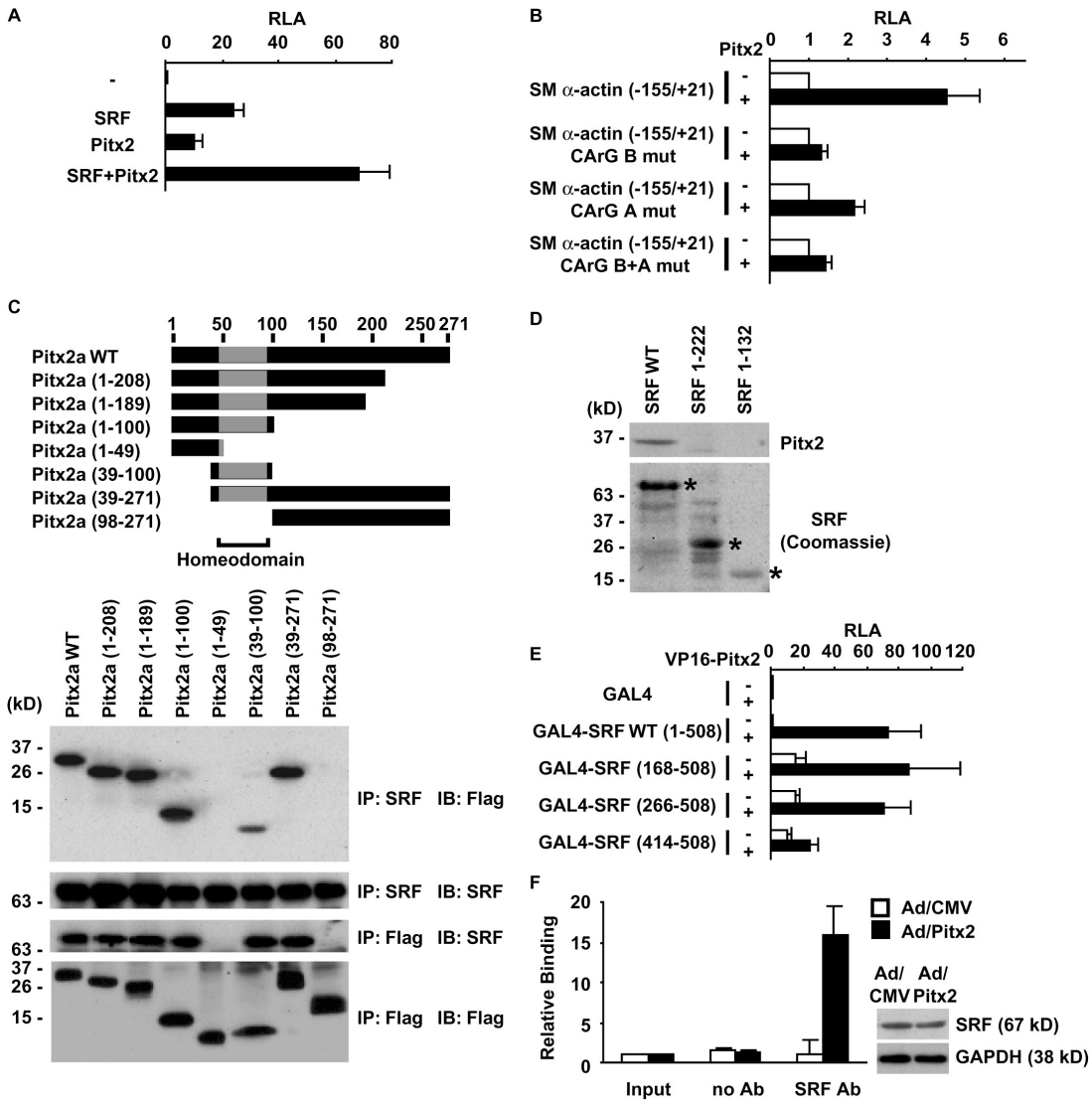

Figure 5. **Pitx2 and SRF synergistically induce SMC differentiation marker genes.** (A) Luciferase assays were performed in A404 cells cotransfected with expression plasmids for Pitx2a and SRF and the *SM α-actin* promoter-enhancer-luciferase construct. (B) Luciferase assays were performed in A404 cells cotransfected with Pitx2a expression plasmid and mutation constructs of the *SM α-actin* gene. (C) Coimmunoprecipitation assays were performed in COS cells cotransfected with SRF expression plasmid and wild-type Pitx2a expression plasmid or deletion mutants. Schematic representation of Pitx2a deletion constructs is shown (top). Numbers indicate the position of amino acids. (D) GST pulldown assays were performed using Myc-tagged Pitx2a protein and GST-SRF fusion protein or its deletion mutants. Asterisks indicate GST-SRF protein and its deletion mutants. (E) Mammalian two-hybrid assays were performed in Balb/c 3T3 cells. (F) A404 cells were infected with adenovirus expressing Pitx2a or empty adenovirus, and the association of SRF with the CArG-containing region of the *SM α-actin* gene was determined by ChIP assays. Values represent the mean ± SEM of three independent experiments. Expression of SRF and GAPDH protein in A404 cells infected with adenovirus expressing Pitx2a or empty adenovirus are also shown.

Because results of our previous studies showed that SRF was associated with CArG elements of SMC differentiation marker genes in differentiated, but not in undifferentiated A404 cells (Manabe and Owens, 2001), we tested if Pitx2 alters the binding affinity of SRF with CArG elements of SMC differentiation marker genes. Adenovirus-mediated overexpression of Pitx2 did not change SRF expression levels, but it greatly enhanced the association of SRF with the CArG-containing region of the *SM α-actin* promoter in A404 cells, as determined by ChIP assays (Fig. 5 F). Results suggest that Pitx2 induces expression of SMC differentiation marker genes, in part by interacting with SRF and increasing the association of SRF with the CArG elements within the promoter regions of SMC differentiation marker genes.

Pitx2 induced acetylation of histone H4 at the promoter regions of SMC differentiation marker genes through the exchange of histone deacetylase (HDAC) 2 and HDAC5 with p300

Previous studies from our laboratory showed that RA-induced SMC differentiation in A404 cells was accompanied by the increase in the acetylation levels of histone H4 at the promoter regions of SMC differentiation marker genes (Manabe and Owens, 2001). The acetylation status of histones at the promoter regions of SMC differentiation marker genes has been shown to be regulated by the histone acetyltransferase p300 and multiple HDACs (Qiu and Li, 2002; Yoshida et al., 2007). We thus tested

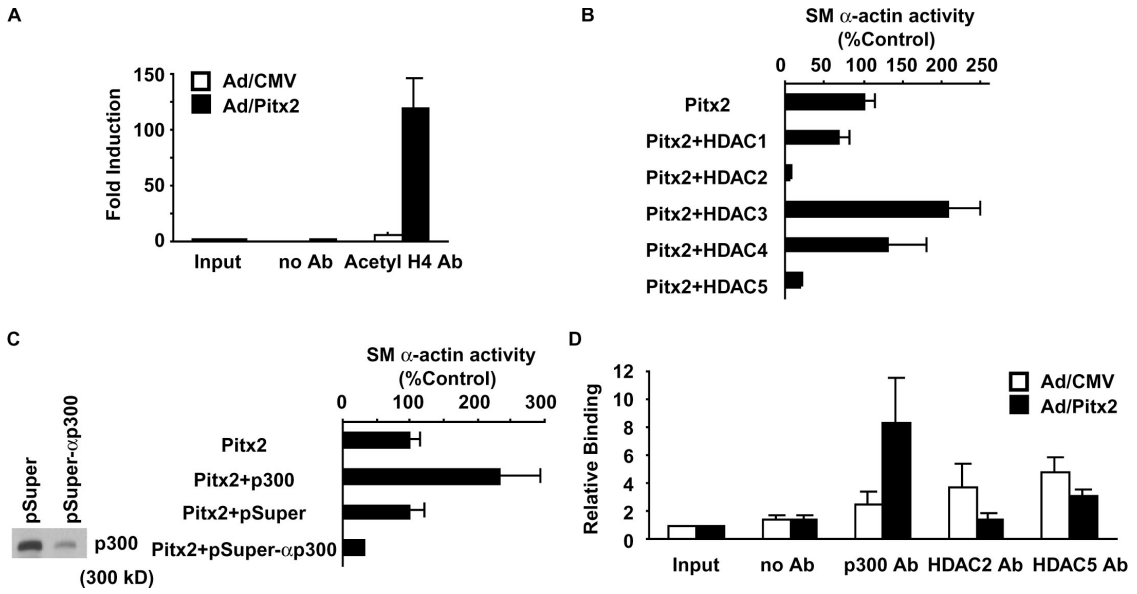

Figure 6. **Pitx2 induces acetylation of histone H4 at the promoter regions of SMC differentiation marker genes.** (A) A404 cells were infected with adenovirus expressing Pitx2a or empty adenovirus, and acetylation levels of histone H4 at the promoter region of the *SM α-actin* gene were determined by ChIP assays. (B) Luciferase assays were performed in A404 cells cotransfected with HDAC expression plasmids, Pitx2a expression plasmid, and the *SM α-actin* promoter-enhancer-luciferase construct. (C, left) Efficiency of *p300* siRNA was tested by Western blotting. (C, right) Luciferase assays were performed in A404 cells cotransfected with Pitx2a expression plasmid, expression plasmid for p300 or *p300* siRNA, and the *SM α-actin* promoter-enhancer-luciferase construct. (D) A404 cells were infected with adenovirus expressing Pitx2a or empty adenovirus, and the association of p300, HDAC2, and HDAC5 with the CArG-containing region of the *SM α-actin* gene was determined by ChIP assays. Values represent the mean ± SEM of three independent experiments.

the hypothesis that changes in the histone acetylation status, through control of the balance between p300 and HDACs at the promoter regions of SMC differentiation marker genes, contribute to Pitx2-induced activation of SMC differentiation marker genes in A404 cells. This hypothesis was supported by the following experiments. First, results of ChIP assays showed that adenovirus-mediated overexpression of Pitx2 increased the acetylation levels of histone H4 within the promoter region of the *SM α-actin* gene in A404 cells (Fig. 6 A). Second, cotransfection experiments revealed that p300 enhanced Pitx2-induced activation of the *SM α-actin* gene, whereas HDAC2 and 5 markedly repressed it in A404 cells (Fig. 6, B and C). Third, siRNA experiments showed that knockdown of *p300* decreased Pitx2-induced activation of the *SM α-actin* gene (Fig. 6 C). Finally, results of ChIP assays showed that adenovirus-mediated overexpression of Pitx2 increased the association of p300 with the promoter region of the *SM α-actin* gene, whereas it decreased the association of HDAC2 and 5 in A404 cells (Fig. 6 D). Collectively, these results suggest that Pitx2-induced activation of SMC differentiation marker genes is mediated, at least in part, through the exchange of HDAC2 and 5 with p300 at the promoter regions of SMC differentiation marker genes, which in turn results in increased acetylation levels of histone H4 and enhanced transcription of SMC differentiation marker genes.

Knockout of the *Pitx2* gene in mice abolished expression of SMC differentiation markers in vessels during the early embryogenesis

Knockout of the *Pitx2* gene in mice has been shown to result in embryonic lethality by embryonic day (E) 14.5 because of multiple abnormalities, including the failure of ventral body wall closure, right pulmonary isomerism, altered cardiac positioning with valvular and atrial septation defects, and abnormal morphogenesis of the eyes, teeth, and pituitary gland (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999a). To extend our preceding results from cultured cells to the in vivo circumstances, the role of Pitx2 in SMC differentiation was examined in *Pitx2* knockout mouse embryos. Heterozygous *Pitx2* mice were intercrossed, and expression of SMC differentiation markers, including SM α-actin and SM22α, was examined in *Pitx2* knockout mouse embryos by immunohistochemistry (Figs. 7 and 8). Expression of both SM α-actin (Fig. 7 C and Fig. 8 C) and SM22α (Fig. 7 F) was virtually abolished in both the left and right sides of the third, fourth, and sixth branchial arch arteries and the dorsal aorta of homozygous *Pitx2* knockout embryos at E11.5, as well as at E10.5 and 12.5 (not depicted). Expression of these SMC differentiation markers was also decreased in vessels of heterozygous *Pitx2* knockout mouse embryos (Fig. 7, B and E; and Fig. 8 B), as compared with the wild-type embryos (Fig. 7, A and D; and Fig. 8 A). In contrast, expression of ACLP was not decreased in *Pitx2* knockout mouse embryos (Fig. 7, G, I, and K; and Fig. 8, D–F). Pitx2 expression was seen in SMCs of wild-type mouse embryos, whereas it was abolished in *Pitx2* knockout mouse embryos (Fig. 7, M–O; and Fig. 8, J–L). Expression of PECAM, an endothelial marker, was not altered in *Pitx2* knockout embryos (Fig. 7, H, J, and L; and Fig. 8, G–I), suggesting that the formation of the endothelial tube is not impaired and that deletion of the *Pitx2* gene exhibits a selective effect on SMC differentiation rather than the general effects on vessel formation. At E13.5, expression of SMC differentiation markers, including

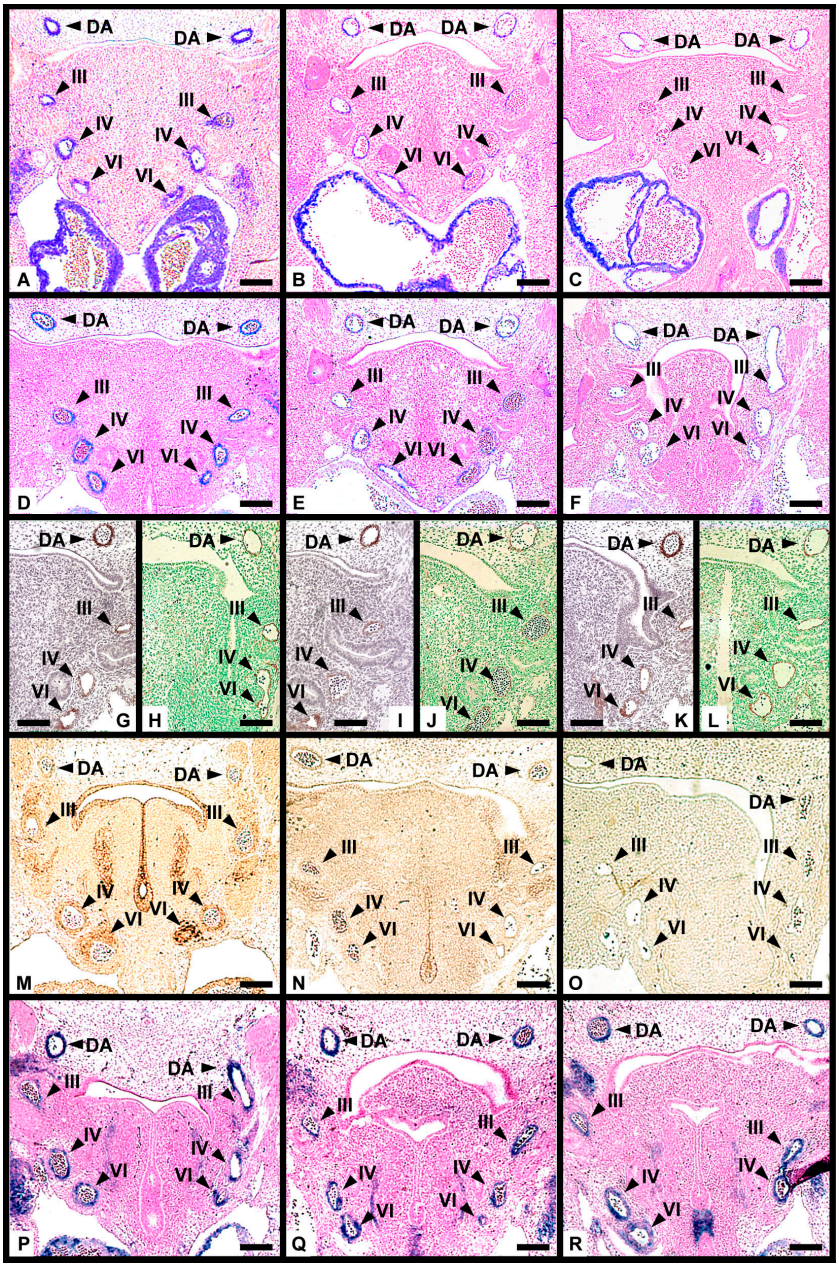

Figure 7. **Expression of SMC differentiation markers is nearly abolished in vessels of *Pitx2* knockout mouse embryos.** (A–O) Expression of SM α-actin (A–C), SM22α (D–F), ACLP (G, I, and K), PECAM (H, J, and L), and Pitx2 (M–O) was examined by immunohistochemistry in wild-type (A, D, G, H, and M), heterozygous *Pitx2* knockout (B, E, I, J, and N), and homozygous *Pitx2* knockout (C, F, K, L, and O) mouse embryos at E11.5. (P–R) Expression of SM α-actin was examined by immunohistochemistry in wild-type (P), heterozygous *Prx1* knockout (Q), and homozygous *Prx1* knockout (R) mouse embryos at E11.5. III, third branchial arch artery; IV, fourth branchial arch artery; VI, sixth branchial arch artery; DA, dorsal aorta. Original magnification, 40×. Bars, 100 μm.

SM α-actin and SM22α, was not decreased in any vessels in *Pitx2* knockout mouse embryos (unpublished data). Moreover, analysis of *Prx1* knockout mouse embryos revealed that SM α-actin expression was not decreased in any vessels at E11.5 (Fig. 7, P–R). These results suggest that Pitx2, but not Prx1, is required for the initial induction of SMC differentiation during early embryonic development but can be compensated by other factors at later developmental time points.

Discussion

Pitx2 is a *bicoid*-related homeodomain transcription factor that plays a key role in the development of multiple organs, including the heart, lung, eyes, teeth, and pituitary gland (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999a). In addition, the Pitx2c isoform has been shown to be a critical factor for directing the left-right asymmetry (Liu et al., 2001). Indeed, mutation of the *Pitx2* gene in human causes Axenfeld-Rieger syndrome, an autosomal-dominant disorder characterized by ocular anterior chamber anomalies causing glaucoma, dental hypoplasia, craniofacial dysmorphism, umbilical stump abnormalities, and abnormal development of the heart and pituitary gland (Semina et al., 1996). As such, although it is clear that Pitx2 is required for proper embryonic development, the role and mechanisms by which Pitx2 influences differentiation of the vascular system were unclear. In the present studies, we provide several lines of evidence indicating that Pitx2 plays a key role in the initial induction of SMC differentiation. First, overexpression of Pitx2 induced expression of multiple SMC differentiation marker genes, including *SM α-actin*, *SM22α*, and *h1-calponin*, whereas knockout of the *Pitx2* gene attenuated the induction of these SMC differentiation marker genes in

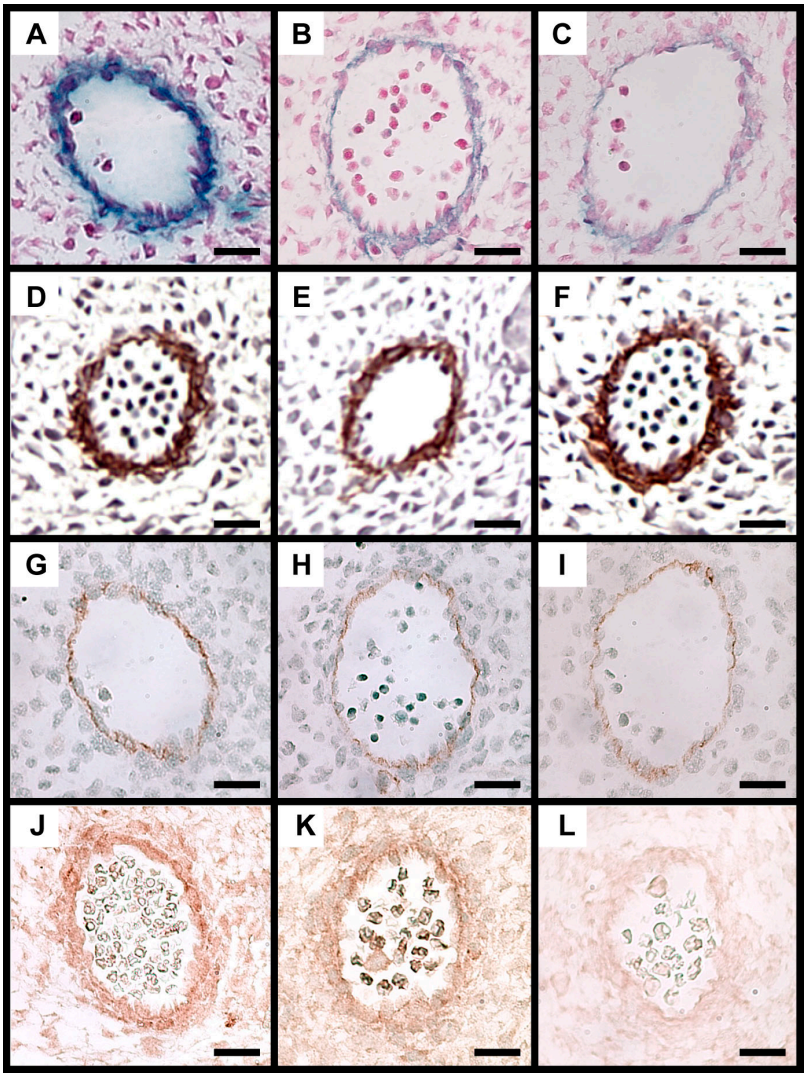

Figure 8. **Expression of SMC differentiation markers is nearly abolished in vessels of *Pitx2* knockout mouse embryos.** Expression of SM α-actin (A–C), ACLP (D–F), PECAM (G–I), and Pitx2 (J–L) was examined in the dorsal aorta of wild-type (A, D, G, and J), heterozygous *Pitx2* knockout (B, E, H, and K), and homozygous *Pitx2* knockout (C, F, I, and L) mouse embryos at E11.5. Original magnification, 400×. Bars, 20 μm.

multiple in vitro SMC differentiation model systems. Second, Pitx2-induced activation of SMC differentiation marker genes was mediated by a combination of mechanisms, including the following: the binding of Pitx2 to TAATC(C/T) cis-elements within the promoter regions of SMC differentiation marker genes; the interaction between Pitx2 and SRF; and Pitx2-mediated exchange of HDACs with p300 to increase the histone H4 acetylation levels at the promoter regions of SMC differentiation marker genes. It is of note that the TAATC(C/T) cis-elements within the *SM α-actin* and the *SM22α* promoters were shown to be functional, and a similar TAATCA sequence was found at +336 bp within the first intron of the mouse *h1-calponin* gene. Third, knockout of the *Pitx2* gene in mice virtually abolished the initial induction of SMC differentiation markers in branchial arch arteries and the dorsal aorta during embryogenesis. Indeed, our demonstration of impaired induction of SMC differentiation markers in *Pitx2* knockout mouse embryos provides the first evidence that a homeodomain protein regulates SMC differentiation in vivo. Although we previously showed that Prx1 plays a key role in angiotensin II–induced hypertrophy in cultured SMCs (Hautmann et al., 1997; Yoshida et al., 2004a), *Prx1* knockout had no effect on expression of SMC differentiation markers at the same time point during embryogenesis. Moreover, no evidence of defective SMC differentiation was found in *Prx1*, *Prx2*, or *Prx1/Prx2* knockout mice (Bergwerff et al., 2000). Collectively, our results provide evidence that Pitx2 and Prx1 may play complementary roles in modulating SMC differentiation marker gene expression but operate at different developmental stages.

Results of the present studies showed that knockout of the *Pitx2* gene was associated with marked decreases in expression of SMC differentiation marker genes that contain multiple CArG elements in their promoter-enhancers, including *SM α-actin* and *SM22α*. Although several previous studies, including our own, have reported that the CArG–SRF–myocardin complex plays an important role in the regulation of CArG-containing SMC differentiation marker genes in differentiated SMCs (Chen et al., 2002; Du et al., 2003; Yoshida et al., 2003), our present results suggest a novel mechanism wherein the initial induction of CArG-containing SMC differentiation marker genes is dependent on a combination of Pitx2 and SRF rather than a combination of myocardin and SRF, based on the following observations. First, we showed that the induction of SMC differentiation marker genes was dramatically attenuated

during SMC differentiation in the context of embryoid bodies derived from *Pitx2* knockout ES cells, whereas our previous studies showed that homozygous knockout of the *myocardin* gene exhibited little effect on SMC differentiation in the same embryoid body system (Pipes et al., 2005). Second, although recent studies showed the transcriptional activity of the *myocardin* gene during early embryonic development (Long et al., 2007), *myocardin* mRNA was not readily detectable in vessels, including the descending aorta, until E12.5, as determined by in situ hybridization assays (Wang et al., 2001; Du et al., 2003). Third, results of recent studies (Pipes et al., 2005) showed that *myocardin*-null ES cells were able to differentiate into SMCs in the context of chimeric knockout mice that were generated by injection of *myocardin*-null ES cells into the wild-type blastocysts. As such, these findings support the hypothesis that the initial induction of SMC differentiation is not dependent on myocardin but is regulated through alternative molecular mechanisms including Pitx2 and SRF. It is of interest that results of our recent studies showed that *SM α-actin* expression during early stages of embryonic development is dependent on two MCAT elements located at −184 and −320 bp within rat *SM α-actin* promoter (Gan et al., 2007). Indeed, mutation of MCAT elements in transgenic mice harboring an *SM α-actin* promoter-enhancer-LacZ construct revealed that MCAT elements are required for *SM α-actin* expression at E10.5 and 12.5 but dispensable after E13.5 through adulthood. It is thus interesting to postulate that MCAT elements and their binding factor RTEF-1 may cooperatively regulate *SM α-actin* expression with Pitx2. However, Pitx2 effects on the transcriptional activity of the *SM α-actin* gene were not affected by mutation of MCAT elements (unpublished data). Moreover, MCAT elements are not present in the promoter regions of other SMC differentiation marker genes (Yoshida, 2008). Collectively, our results suggest that Pitx2 and RTEF-1/MCAT elements may regulate early induction of SMC differentiation markers through independent mechanisms.

Results of recent studies have provided evidence for diversity in transcriptional control of SMC differentiation among cells derived from different embryological origins. For example, knockout of the *MKL2* (also called *MRTF-B*) gene in mice resulted in defective formation of neural crest–derived branchial arch arteries and severe reduction in SM α-actin expression selectively in these vessels (Li et al., 2005; Oh et al., 2005). These defects were rescued by neural crest–specific restoration of MKL2/MRTF-B expression, suggesting that MKL2/MRTF-B plays a critical role in regulating differentiation of neural crest cells into SMCs. In addition, results of studies in chick embryos also showed the diversity in differentiation of SMCs among distinct embryological origins in that they showed differences in morphology, growth, and responsiveness to transforming growth factor β between local mesoderm-derived SMCs and neural crest–derived SMCs (Topouzis and Majesky, 1996). However, our results suggest that Pitx2 is required for SMC differentiation in at least two distinct embryological sources of SMCs. Indeed, expression of SMC differentiation markers in *Pitx2* knockout mouse embryos was abolished in neural crest–derived SMCs located at the third, fourth, and sixth branchial arch arteries, as well as in the mesoderm-derived SMCs present in the dorsal aorta. These findings are consistent with results of previous studies showing that Pitx2 functioned in tissues from multiple embryological origins (Evans and Gage, 2005; Ai et al., 2006). For example, studies using the cardiac second lineage–specific *Pitx2* knockout mice showed that deletion of the *Pitx2* gene in the cardiac second lineage, which was derived from mesoderm, caused severe defects in alignment of the cardiac outflow tract (Ai et al., 2006). In contrast, studies using neural crest–specific *Pitx2* knockout mice revealed that Pitx2 expression in neural crest was required for optic stalk and ocular anterior segment development (Evans and Gage, 2005). As such, the function of Pitx2 is unlikely to be restricted to the single embryological origin. Further analyses of expression of SMC differentiation marker genes in conditional *Pitx2* knockout mice will be required to better clarify the cell type–specific requirement of Pitx2 for SMC differentiation from different embryological origins.

Our results provide evidence that Pitx2-induced activation of SMC differentiation marker genes is mediated, at least in part, by promoting exchange of HDAC2 and 5 with p300 within the promoter regions of SMC differentiation marker genes, thereby increasing acetylation levels of histone H4 and chromatin relaxation. Consistent with this model, we previously showed that platelet-derived growth factor BB–induced suppression of SMC differentiation marker genes was caused, in part, by HDAC-mediated hypoacetylation of histone H4 within these promoters in cultured SMCs (Yoshida et al., 2007). Pitx2-induced hyperacetylation of histones has also been reported previously (Kioussi et al., 2002). The studies showed that Pitx2 activated transcription of the *cyclin D2* gene by increasing the acetylation levels of histone H4 at the gene promoter. They presented evidence that these changes were dependent on exchange of HDAC1 with p300, which was mediated through association of Pitx2 and Wnt-activated β-catenin within the *cyclin D2* promoter. In addition, the interaction between Pitx2 and p300 has been shown by coimmunoprecipitation assays (Kioussi et al., 2002), although it is possible that other factors are required for this interaction. During the embryonic development, SMCs have been shown to exhibit a high rate of proliferation while at the same time activating expression of SMC differentiation marker genes. It is interesting to speculate that Pitx2 may play a key role in regulating both of these processes by cooperating with distinct partner proteins such as β-catenin and SRF, respectively.

In summary, results of the present studies provide novel evidence that Pitx2 plays a critical role in the induction of SMC differentiation during the early embryogenesis. Further studies are needed to determine whether Pitx2 contributes to the pathophysiology of vascular diseases such as atherosclerosis, hypertension, and restenosis, where SMC phenotypic switching plays a key role.

Materials and methods

Plasmid constructs and siRNA duplexes

Rat −2.6/+2.8-kb *SM α-actin* promoter-enhancer-luciferase, −155/+21-bp *SM α-actin* promoter-luciferase, and −125/+23-bp *SM α-actin* promoter-luciferase constructs were described previously (Yoshida et al., 2003, 2004a).

Mutations (TAATCT element at −145 bp, AGATTA to AGCAGT; CArG B at −112 bp, CCCTATATGG to AACTATATAA; and CArG A at −62 bp, CCTTGTTTGG to AATTGTTTAA) were introduced into −155/+21-bp *SM α-actin* promoter-luciferase construct as described previously (Yoshida et al., 2004a). Mouse 1343-bp *SM22α* promoter-luciferase construct was provided by L. Li (Wayne State University, Detroit, MI) and mutation (GGATTA to GGCCCA) was introduced at −1071 bp by site-directed mutagenesis (Li et al., 1996). An expression plasmid for Flag-tagged Pitx2a was constructed by inserting *Pitx2a* cDNA into pCMV7.1 3×FLAG vector (Sigma-Aldrich). Deletion constructs of Flag-tagged Pitx2a were made by PCR. Expression plasmids for Myc-tagged Pitx2a, Pitx2b, and Pitx2c isoforms and SRF were described previously (Ganga et al., 2003; Liu et al., 2005). A siRNA expression plasmid targeting all *Pitx2* isoforms was constructed by inserting specific oligonucleotide (AGAAACCGCTACCCAGACA) into pMighty-Empty (Yoshida et al., 2003). siRNA expression plasmids for *Prx1*, *myocardin*, *MKL1*, and *MKL2* were described previously (Yoshida et al., 2003, 2004a, 2007). siRNA duplexes targeting *Pitx2* and *EGFP* were purchased from MWG Biotech. Expression plasmids for HDAC1–5 were provided by S.L. Schreiber (Harvard University, Cambridge, MA) and E. Seto (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL). An expression plasmid for p300 was purchased from Millipore. A siRNA expression construct for *p300*, pSUPER-αp300, was provided by D.K. Granner (Vanderbilt University, Nashville, TN). GST-tagged SRF expression plasmid and its deletion mutants were provided by B.P. Herring (Indiana University, Indianapolis, IN). Expression plasmids for GAL4-SRF and truncated mutants were provided by R. Prywes (Columbia University, New York, NY). Expression plasmid for VP16-Pitx2a fusion protein was constructed as previously described (Yoshida et al., 2004a).

Cell culture, transient transfection, and adenovirus infection

A404 cells were cultured and induced to differentiate into SMCs as described previously (Manabe and Owens, 2001). 10T1/2 cells and NIH/3T3 cells were cultured as described previously (Yoshida et al., 2003). Transfection of DNA plasmids was performed by using FuGene6 (Roche). Replication-deficient adenovirus expressing Flag-tagged Pitx2a was generated and purified by ViraQuest Inc. Infection of adenovirus expressing Flag-Pitx2a or empty adenovirus was performed at 50 MOI as previously described (Yoshida et al., 2003).

Generation of *Pitx2* homozygous knockout ES cells

To obtain *Pitx2* homozygous knockout ES cells, *Pitx2* heterozygous knockout ES cells (Lu et al., 1999a) were cultured with 2 mg/ml G418 for 21 d (Mortensen et al., 1992). After 21 d, 192 independent clones were picked up, and five clones were found to be *Pitx2* homozygous knockout ES cells by PCR genotyping. Two *Pitx2* heterozygous knockout ES cell clones that survived the selection protocol were also used for control. Pluripotency of these ES cells was confirmed by immunostaining of ES cell markers including Oct4, Sox2, and SSEA-1 (Millipore). Procedures for differentiation of ES cells into SMCs in the context of embryoid bodies were described previously (Sinha et al., 2004).

Subtraction hybridization screen

Subtraction hybridization screen was performed by using the PCR-select cDNA subtraction kit (BD Biosciences) according to the manufacturer's instruction.

RT-PCR and luciferase assays

Total RNA prepared from cultured cells was used for semiquantitative or real-time RT-PCR analyses. Primer and probe sequences for *SM α-actin*, *SM22α*, *h1-calponin*, *ACLP*, *smoothelin-B*, *myocardin*, *MKL1*, *MKL2*, *Prx1*, *PECAM*, *cardiac α-actin*, *NeuroD*, *GAPDH*, and *18S ribosomal RNA* were described previously (Sinha et al., 2004, 2006; Yoshida et al., 2004a,b, 2007). Primer and probe sequences for *Pitx2* were as follows: Pitx2 sense, 5′-GCCAGCCTGAGACTGAAAGC-3′; Pitx2 antisense, 5′-CTGCATACTGGCAAGCACTCA-3′; and Pitx2 probe, 5′-GATTTCTTCGCGAGTGGAC-3′. Sequences for Pitx2 isoform-specific primers were described previously (Ganga et al., 2003). Luciferase assays were performed as previously described (Yoshida et al., 2003).

Western blotting and coimmunoprecipitation assays

Western blotting and coimmunoprecipitation assays were performed as previously described (Yoshida et al., 2007). Antibodies used were as follows: SM α-actin (1A4; Sigma-Aldrich), SM22α (Abcam), GAPDH (Millipore), Flag (M2; Sigma-Aldrich), SRF (Santa Cruz Biotechnology, Inc.), and Pitx2 (provided by T. Hjalt, Lund University, Lund, Sweden).

EMSA and ChIP assays

EMSA was performed as described previously (Yoshida et al., 2003) using a double-stranded oligonucleotide (5′-CACCCAGATTAGAGAGTTTTGTG-3′) or a mutated oligonucleotide (5′-CACCCAGCAGTGAGAGTTTGTG-3′). ChIP assays were performed as previously described (Yoshida et al., 2007). Antibodies used were the following: SRF, Pitx2, acetyl histone H4 (Millipore), p300 (Santa Cruz Biotechnology, Inc.), HDAC2 (Invitrogen), and HDAC5 (Cell Signaling Technology). Real-time PCR was performed to amplify the promoter region of the *SM α-actin* gene (Manabe and Owens, 2001), the TAATCC-containing region of the *SM22α* gene (5′-TGGGATTAAAGGCGTGTGCCCA-3′ and 5′-ATCCACTACAGCCAGCCTGGCT-3′), or the first intron region of the *SM22α* gene (5′-AAGAAGGTGAGGGGCTAAGTTC-3′ and 5′-TATCCCAGGCTAGTCTGAAGGA-3′).

GST pulldown assays and mammalian two-hybrid assays

Myc-tagged Pix2a protein was translated in TnT Quick coupled transcription/translation system (Promega). GST-tagged SRF and its truncated mutants were induced in *Escherichia coli* by the addition of 0.5 mmol/L isopropyl-1-thio-β-D-galactopyranoside for 1 h at 37°C. Lysates were prepared by sonicating bacterial pellets in PBS containing 10% glycerin. Cleared lysates were incubated with Glutathione Sepharose 4B beads (GE Healthcare) for 1 h at 4°C and washed three times with cold PBS containing 10% glycerin. Beads–protein complex was incubated with Myc-tagged Pitx2a protein for 2 h at 4°C and washed three times. Beads-bound proteins were eluted by boiling and analyzed by Western blotting with anti-Myc antibody (Santa Cruz Biotechnology, Inc.). Mammalian two-hybrid assays were performed as previously described (Yoshida et al., 2004a).

Flow cytometry

Embryoid bodies were washed with PBS and incubated with 1 mg/ml collagenase type IV and 0.125% trypsin for 20 min at 37°C. Cells were fixed in 2% PFA, permeabilized by ice-cold methanol, and incubated with FITC-conjugated SM α-actin antibody (Sigma-Aldrich) or FITC-conjugated IgG as a negative control. Cells were analyzed using a FACSCalibur dual-laser benchtop cytometer (BD Biosciences).

Immunohistochemistry in mouse embryos

Heterozygous *Pitx2*$^{+/-}$ mice (Lu et al., 1999a) were purchased from the Mutant Mouse Regional Resource Center. *Prx1* knockout mice were described previously (Lu et al., 1999b). Heterozygous knockout mice were intercrossed to generate homozygous knockout mouse embryos. Embryos were harvested, fixed in 10% neutral buffered formalin, and embedded into paraffin. Yolk sacs were used for genotyping. The 5-μm sections were deparaffinized, rehydrated, and stained with antibodies for SM α-actin, SM22α, Pitx2, ACLP (Yoshida et al., 2004b), and PECAM (BD Biosciences). Staining was visualized by DAB or the Vector Blue Alkaline Phosphatase Substrate kit (Vector Laboratories) and counterstained by nuclear FastRed, hematoxylin or methylgreen. Images were obtained using a microscope (Axioskop 2; Carl Zeiss, Inc.) and camera (SPOT; Diagnostic Instruments, Inc.). Animal protocols were approved by the University of Virginia Animal Care and Use Committee. E0.5 was defined as noon of the day a vaginal plug was detected after overnight mating.

Online supplemental material

Fig. S1 shows growth rates of A404 cells infected with adenovirus expressing Pitx2 or control adenovirus. Fig. S2 shows involvement of MKL1, MKL2, and myocardin in Pitx2-induced activation of SMC differentiation marker genes in A404 cells. Fig. S3 shows expression of *PECAM*, *cardiac α-actin*, and *NeuroD* in embryoid bodies derived from *Pitx2* homozygous knockout ES cells, *Pitx2* heterozygous ES cells, and wild-type ES cells. Fig. S4 shows mutational effects of a TAATCT element on SRF-induced activation of the *SM α-actin* gene. Table S1 shows a list of genes preferentially expressed in RA-treated A404 cells as compared with undifferentiated A404 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200711145/DC1.

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