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Cell Division Cycle 7 Mediates Transforming Growth Factor-β-induced Smooth Muscle Maturation through Activation of Myocardin Gene Transcription*

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Background: Cell division cycle 7 (Cdc7) plays a role in cell fate determination in the early stage of smooth muscle (SM) differentiation.
Results: Cdc7 activated myocardin gene transcription by interacting with Nkx2.5, leading to the induction of a mature SM marker smooth muscle myosin heavy chain (SMMHC).
Conclusion: Cdc7 regulates SM maturation by activation of myocardin expression.
Significance: Cdc7 regulates SM differentiation and maturation through different mechanisms.

Smooth muscle (SM) development consists of several processes, including cell fate determination, differentiation, and maturation. The molecular mechanisms controlling SM early differentiation have been studied extensively. However, little is known about the mechanism underlying SM maturation. Cell division cycle 7 (Cdc7) has been shown to regulate cell fate determination in the initial phase of transforming growth factor-β (TGF-β)-induced SM differentiation. Our present study indicates that Cdc7 also regulates SM maturation. Knockdown of Cdc7 suppresses TGF-β-induced expression of SM myosin heavy chain, a late marker of SM differentiation. Cdc7 overexpression, on the other hand, enhances SM myosin heavy chain expression. Interestingly, Cdc7 activates the mRNA expression and promoter activity of myocardin (Myocd), a master regulator of SM differentiation, whose transcription is blocked in the initial phase of the differentiation because TGF-β does not induce Myocd mRNA until after the early SM markers are induced. These data suggest that Cdc7 mediates TGF-β-induced SM maturation via activation of Myocd transcription. Mechanistically, Cdc7 physically and functionally interacts with Nkx2.5 to regulate Myocd promoter activity. Cdc7 appears to enhance Nkx2.5 binding to Myocd promoter, leading to Myocd activation. Taken together, our studies demonstrate that Cdc7 regulates the initial and late phase of SM differentiation through distinct mechanisms.

Vascular smooth muscle (SM)[2] differentiation is an important process during vasculogenesis and angiogenesis, and it is recognized that alterations in SM phenotype contribute to the progression of several cardiovascular diseases, including atherosclerosis, systematic and pulmonary hypertension, and restenosis (1–7). Although the term *differentiation* generally refers to the entire process by which committed but undifferentiated SMs or SM precursors acquire their cell-specific phenotypes, this process can be divided into three phases: cell fate determination (early or initial phase), differentiation (intermediate phase), and maturation (late phase) (8). The different phases can be tracked by the expression of different contractile proteins. Smooth muscle α-actin (α-SMA) and SM22α are early markers of SM differentiation whereas calponin and smooth muscle myosin heavy chain (SMMHC) represent the intermediate and late phase markers, respectively (8–12). Although we have gained a great deal of understanding on the cell fate determination and differentiation process, molecular mechanisms governing SM maturation remain largely unknown.

Transforming growth factor-β (TGF-β) plays critical roles in the SM differentiation (13–16). TGF-β also regulates cell proliferation in different physiological contexts (8, 17–19). Our previous studies have shown that cell division cycle 7 (Cdc7), a cell cycle regulator facilitating initiation of DNA replication, regulates the initiation program of TGF-β-induced SM differentiation via interaction with Smad3 while stimulating cell proliferation (20). It is unknown, however, whether Cdc7 plays a role in SM maturation.

Myocardin (Myocd), a serum response factor (SRF) coactivator, is considered as a master regulator for SM differentiation. Overexpression of Myocd activates multiple CArG-containing SM marker genes including α-SMA, SM22α, and calponin (21–24). Myocd was found to be involved in TGF-β-induced SM differentiation (25). Paradoxically, expression of early SM markers emerges prior to the detectable level of endogenous Myocd mRNA expression in the embryonic dorsal aorta, suggesting a minor role of Myocd in the initiation of SM differentiation (22, 26–28). Moreover, expression of some SM-associated genes appears to be independent of Myocd (29) (30). However, Myocd is able to induce contractile SM phenotype (31), suggesting that Myocd plays a major role in SM maturation. The molecular mechanism underlying Myocd induction during SM maturation, however, remains largely unknown. In

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[2] The abbreviations used are: SM, smooth muscle; Cdc7, cell division cycle 7; co-IP, co-immunoprecipitation; Myocd, myocardin; NKE, Nkx2.5-binding element; qPCR, quantitative RT-PCR; α-SMA, smooth muscle α-actin; SMMHC, smooth muscle myosin heavy chain; SRF, serum response factor.

this study, we found that Myocd expression in TGF-β-induced SM maturation is regulated by Cdc7. Cdc7 appears to induce Myocd gene transcription via interaction with Nkx2.5, a critical transcription factor for Myocd gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—C3H10T1/2 (10T1/2) cells, a murine embryonic mesenchymal progenitor cell line that has been shown to differentiate into a mature SM lineage (13), were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% glutamine. For plasmid transfection, cells were cultured in 12- or 6-well plates or 6-cm dishes for 24 h with 80–90% of confluence followed by transfection using Lipofectamine LTX (Invitrogen) according to the manufacturer's instruction.

Quantitative RT-PCR (qPCR)—Total RNA from cultured cells was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using an iScript cDNA Synthesis kit (Bio-Rad). qPCR was performed in a Mx3005P qPCR machine using SYBR Green master mix (Agilent Technologies). Each sample was amplified in triplicate. Myocd primers were 5′-ACC CAT GGA CTC TGC CTA TG-3′ (forward) and 5′-AGG GGT ATT GCT CAG TGG TG-3′ (reverse). Nkx2.5 binding site and Cdc7 primers were reported previously (20).

Western Blotting—10T1/2 cells were treated with TGF-β or other factors as indicated. Cells were washed two times with cold PBS, followed by protein extraction using radioimmunoprecipitation assay buffer (50 mmol/liter Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% w/v sodium deoxycholate, 150 mmol/liter NaCl, 1 mmol/liter EGTA, 0.1% SDS, protease inhibitors, phosphatase inhibitors). Protein concentration was measured using BCA Protein Assay reagent (Thermo Scientific). 5 or 10 μg of the lysates was resolved by SDS-PAGE and transferred to PVDF (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in TBST and then incubated with primary antibodies in blocking buffer for 1–2 h followed by incubation with HRP-conjugated secondary antibody for 1 h (Sigma). Detection was performed with enhanced chemiluminescence (Millipore). Antibodies used were anti-Cdc7 (Santa Cruz Biotechnology), anti-Nkx2.5 (Santa Cruz Biotechnology), anti-α-SMA (Abcam), anti-SM22α (Abcam), anti-SMMHC (Biomedical Technologies, Inc.), and anti-α-tubulin (Cell Signaling).

Co-immunoprecipitation (Co-IP) Assay and Immunoblotting Analysis—Cells were washed with ice-cold lysis buffer containing protease inhibitor mix (Sigma). The lysates were incubated with Cdc7 or Nkx2.5 antibody for 1 h and then with protein A/G-agarose beads at 4 °C for 12 h. The immunoprecipitates were pelleted, washed, and subjected to immunoblotting using Nkx2.5 or Cdc7 antibody as described previously (32, 33).

Promoter Reporter Luciferase Assay—Myocd promoter constructs were co-transfected into 10T1/2 cells with other plasmids as described previously (34). Cells were starved in serum-free medium for 24 h and then treated with 5 ng/ml TGF-β for 24 h. Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega). Experiments were repeated at least three times, and the results from representative experiments are shown ± S.D.

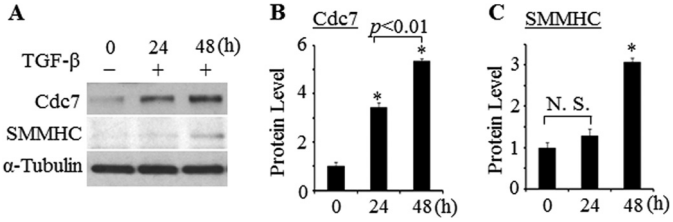

FIGURE 1. **TGF-β induced Cdc7 expression while promoting SM maturation in 10T1/2 cells.** *A*, TGF-β induced Cdc7 expression along with maturation of 10T1/2 cells. Serum-starved 10T1/2 cells were treated with vehicle (−) or TGF-β (+, 5 ng/ml) for the times indicated. Western blotting was performed to examine the expression of Cdc7 and SM maturation marker SMMHC. *B* and *C*, quantitative analyses of Cdc7 and SMMHC expression. The protein expression was normalized to α-tubulin. *, $p < 0.01$ compared with vehicle-treated group (0 h) ($n = 3$). *N.S.*, not significant.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assays were performed as described previously (35). Growth-arrested 10T1/2 cells were treated with TGF-β for 24 h. Chromatin complexes were immunoprecipitated with 1 μg of Nkx2.5 antibody or IgG (negative control). Semiquantitative PCR and qPCR were performed to amplify the Myocd promoter region containing Nkx2.5-binding element (NKE) using the following primer set: 5′-GTT CAG CAC TGC TTG TGG AA-3′ (forward) and 5′-TTT TCC CAT TCT CTG GGT TG-3′ (reverse).

Statistical Analysis—All values are expressed as mean ± S.E. Data were evalutated using analysis of variance with pairwise comparisons between groups. A p value <0.05 was considered statistically significant.

RESULTS

Cdc7 Expression Is Enhanced during TGF-β-induced SM Maturation—Our previous study has shown that Cdc7 plays a role in initiating TGF-β-induced SM differentiation (20). To determine whether Cdc7 is important for SM maturation, we examined the expression of Cdc7 along with the mature SM marker SMMHC in cells treated with TGF-β for up to 48 h, a time past the initial stage of SM differentiation. As shown in Fig. 1, a very low level of SMMHC was expressed at 24 h after TGF-β treatment, but a significantly higher level of SMMHC expression was evident 48 h after TGF-β induction, indicating the formation of a mature SM phenotype. Cdc7 expression was significantly induced 24 h after TGF-β treatment (Fig. 1, *A* and *B*), consistent with its role in the initial phase of SM differentiation. Interestingly, Cdc7 expression was further increased by TGF-β during the SM maturation process (Fig. 1), suggesting that in addition to the initiation of SM differentiation, Cdc7 may also be involved in SM maturation.

Cdc7 Is Essential for TGF-β-induced SM Maturation—To determine whether Cdc7 plays a role in SM maturation, we tested whether Cdc7 is required for the induction of SMMHC. As shown in Fig. 2, *A* and *B*, when Cdc7 expression was blocked by its shRNA, TGF-β-induced SMMHC expression was significantly attenuated, indicating that Cdc7 is essential for TGF-β-induced SM maturation. To further determine the role of Cdc7 in mature SM marker induction, we overexpressed Cdc7 in serum-starved cells without TGF-β addition and found that forced expression of Cdc7 in 10T1/2 cells induced a 2.5-fold increase in SMMHC expression (Fig. 2, *C–E*), indicating that Cdc7 alone is sufficient for promoting SM maturation.

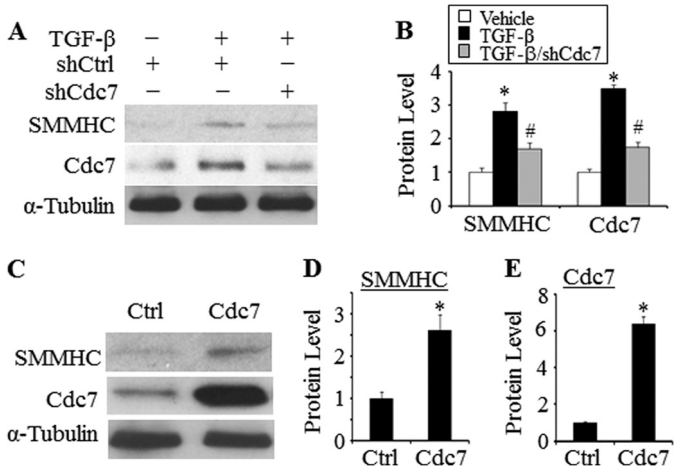

FIGURE 2. **Cdc7 is required for TGF-β-induced SM maturation of 10T1/2 cells.** *A*, Cdc7 knockdown suppression of TGF-β-induced SM maturation. 10T1/2 cells were transfected with control (shCtrl) or Cdc7 shRNA (shCdc7) plasmid followed by vehicle (−) or TGF-β (+, 5 ng/ml) treatment for 48 h. Western blotting was performed to detect SMMHC and Cdc7 expression. *B*, quantitative analyses of the protein expression in *A*. The protein expression was normalized to α-tubulin. *, $p < 0.01$ compared with shCtrl-transfected group with vehicle treatment. #, $p < 0.01$ compared with shCtrl-transfected group with TGF-β treatment ($n = 3$). *C*, Cdc7 sufficiency for SM maturation. 10T1/2 cells were transfected with control or Cdc7 plasmid followed by serum starvation for 24 h. Cell lysates were collected for Western blot analysis of the proteins indicated. *D* and *E*, quantitative analyses of the protein expression in *C*. *, $p < 0.01$ compared with the control plasmid-transfected group ($n = 3$). *Error bars*, S.E.

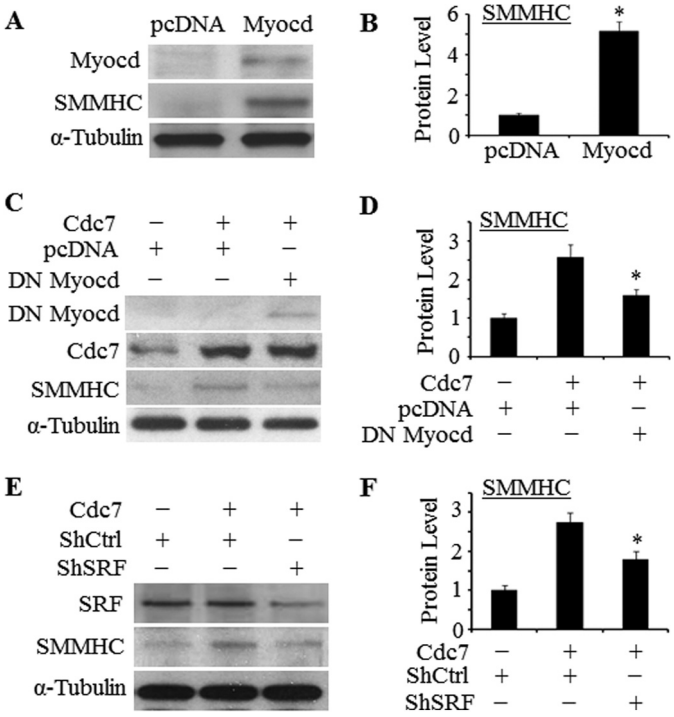

FIGURE 3. **TGF-β induced SM maturation in a Myocd/SRF-dependent manner.** *A*, Myocd promoted SM maturation. 10T1/2 cells were transfected with pcDNA (control) or Flag-Myocd plasmid followed by serum starvation for 24 h. Cell lysates were collected for Western blot analysis of Flag and SMMHC. *B*, quantitative analyses of the protein expression in *A*. *, $p < 0.01$ compared with the control group. *C*, blockade of Myocd function inhibited SM maturation. Cells were transfected with Cdc7, pcDNA, or dominant negative (*DN*) Myocd mutant plasmid followed by serum starvation for 24 h. Cell lysates were collected for Western blot analysis. *D*, quantitative analyses of the protein expression in *C*. *, $p < 0.01$ compared with Cdc7-transfected group without dominant negative Myocd ($n = 3$). *E*, suppression of SM maturation by blockade of SRF function. Cells were transfected with Cdc7, shCtrl, or shSRF followed by serum starvation for 24 h. Western blotting was performed to detect the expression of proteins indicated. *F*, quantitative analyses of the protein expression in *E*. *, $p < 0.01$ compared with Cdc7-transfected group without shSRF ($n = 3$). *Error bars*, S.E.

Cdc7 Induces Mature SM Marker through Myocd/SRF—Myocd is a master regulator for SM differentiation and is expressed mainly in SMs and cardiomyocytes. Myocd can induce a contractile SM phenotype (31). Indeed, Myocd alone can induce the mature SM marker SMMHC expression (Fig. 3, *A* and *B*), consistent with previous reports (24, 36). Because Cdc7 played a very important role in regulating mature SM marker gene expression, and Cdc7 was induced much earlier than Myocd by TGF-β (20), we sought to determine whether Cdc7 induces mature SM marker expression through Myocd. As shown in Fig. 3, *C* and *D*, Cdc7 induced SMMHC expression; however, dominant negative Myocd significantly inhibited Cdc7-induced SMMHC expression, indicating that Myocd is a downstream effector of Cdc7 during SM maturation. Because Myocd is a coactivator of SRF, we tested whether SRF is also important for Cdc7 function in SM maturation. As shown in Fig. 3, *E* and *F*, knockdown of SRF by shRNA blocked Cdc7-induced SMMHC expression. These data demonstrate that Cdc7 induces SM maturation in a Myocd/SRF-dependent manner.

Cdc7 Mediates TGF-β-induced Myocd Expression—Our previous studies have shown that TGF-β did not induce Myocd expression until 18 h of TGF-β treatment, preceding SM maturation (37), supporting the role of Myocd in regulating SM maturation. Interestingly, Cdc7 appeared to be essential for TGF-β-induced Myocd expression because knockdown of Cdc7 by shRNA blocked Myocd expression induced by TGF-β (Fig. 4, *A* and *B*). Conversely, overexpression of Cdc7 stimulated Myocd expression (Fig. 4, *C* and *D*). Moreover, knockdown of Cdc7 by shRNA significantly blocked TGF-β-induced Myocd promoter activity (Fig. 4*E*), suggesting that Cdc7 plays a role in Myocd gene transcription. Indeed, Cdc7 can activate Myocd promoter in a dose-dependent manner (Fig. 4*F*).

Cdc7 Regulates Myocd Transcription via Interaction with Nkx2.5—Nkx2.5 is a transcription factor regulating Myocd expression (38). It is also involved in TGF-β-induced Myocd promoter activity in 10T1/2 cells (37, 38). Because both Cdc7 and Nkx2.5 are essential for Myocd promoter activity, we sought to determine whether there is a functional interaction between Cdc7 and Nkx2.5 in Myocd gene transcription. As shown in Fig. 5*A*, although Cdc7 or Nkx2.5 alone significantly up-regulated Myocd promoter activity, the combination of Cdc7 and Nkx2.5 dramatically and synergistically increased the promoter activity by 3.7- and 10.2-fold compared with Cdc7 and Nkx2.5 alone group, respectively. To determine whether Cdc7 is required for Nkx2.5 activity, we knocked down Cdc7 by shRNA and found that blockade of Cdc7 expression suppressed Nkx2.5-induced Myocd activation in both vehicle- and TGF-β-treated cells (Fig. 5*B*). TGF-β did not induce a dramatic increase of Myocd promoter activity in Nkx2.5-overexpressed cells probably because TGF-β induced Myocd expression through the induction of Nkx2.5. Cdc7 did not affect Nkx2.5 expression because neither overexpression nor knockdown of

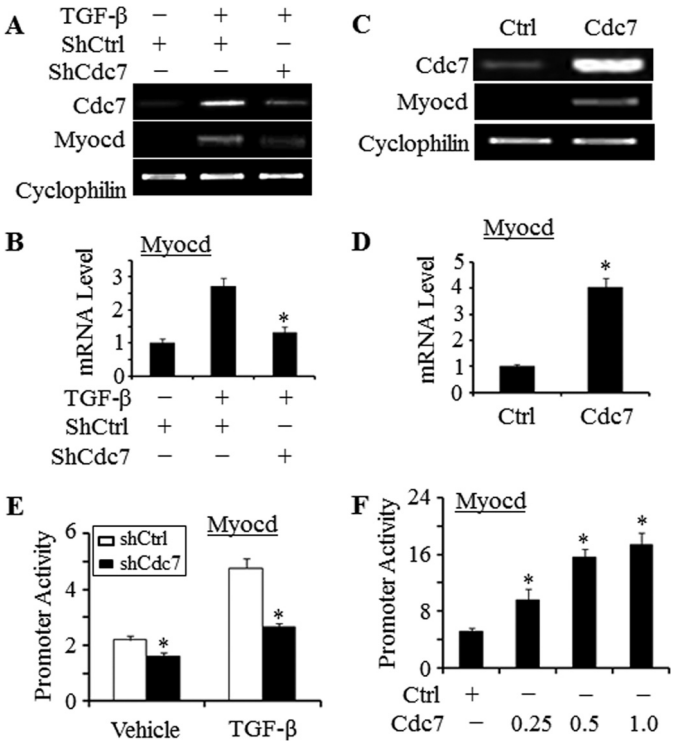

FIGURE 4. **Cdc7 is required for Myocd transcription and activation.** *A* and *B*, Cdc7 is essential for TGF-β-induced Myocd expression. 10T1/2 cells were transfected with shCtrl or shCdc7 followed by vehicle (−) or TGF-β (+, 5 ng/ml) treatment for 24 h. Myocd mRNA expression was examined by semiquantitative RT-PCR (*A*) and qPCR (*B*). Cyclophilin is an internal control. *, $p < 0.01$ compared with shCtrl-transfected group with TGF-β treatment ($n = 3$). *C* and *D*, Cdc7 overexpression induced Myocd mRNA expression. 10T1/2 cells were transfected with control or Cdc7 plasmid followed by serum starvation for 24 h. Myocd mRNA expression was examined by semiquantitative RT-PCR (*C*) and qPCR (*D*). *, $p < 0.01$ compared with the control group ($n = 3$). *E*, Cdc7 knockdown reduced TGF-β-induced Myocd promoter activity. 10T1/2 cells were co-transfected with Myocd promoter construct and shCtrl or shCdc7 followed by vehicle or TGF-β treatment for 24 h. Luciferase assays were performed. *, $p < 0.01$ compared with corresponding shCtrl-transfected group ($n = 3$). *F*, Cdc7 enhanced Myocd promoter activity. 10T1/2 cells were transfected with control (1 μg) or the indicated amount of Cdc7 plasmid in 12-well plates followed by serum starvation for 24 h. Luciferase assays were performed. *, $p < 0.01$ compared with control group ($n = 3$). *Error bars*, S.E.

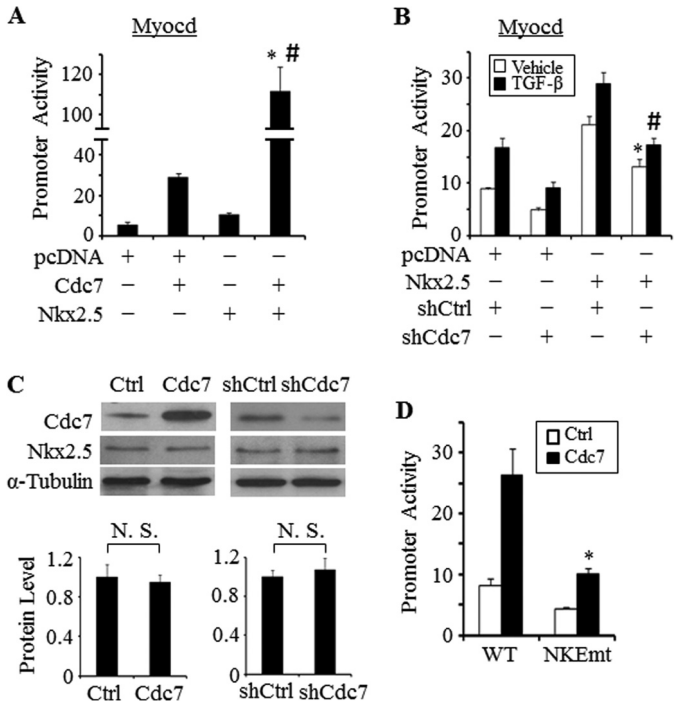

FIGURE 5. **Cdc7 functionally interacted with Nkx2. 5 to regulate Myocd transcription.** *A*, Cdc7 overexpression dramatically promoted Nkx2.5-induced Myocd promoter activity. 10T1/2 cells were co-transfected with Myocd promoter construct and the plasmids indicated followed by serum starvation for 24 h. Luciferase assays were performed. *, $p < 0.01$ compared with Cdc7 alone-transfected group; #, $p < 0.01$ compared with Nkx2.5 alone-transfected group ($n = 3$). *B*, Cdc7 knockdown diminished Nkx2.5-induced Myocd promoter activity. 10T1/2 cells were co-transfected with Myocd promoter construct and the plasmids indicated followed by vehicle (−) or TGF-β treatment for 24 h. Luciferase assays were performed. *, $p < 0.01$ compared with Nkx2.5-transfected group without shCdc7 and TGF-β; #, $p < 0.01$ compared with Nkx2.5-transfected group without shCdc7 but treated with TGF-β ($n = 3$). *C*, Cdc7 had no effect on Nkx2.5 expression. 10T1/2 cells were transfected with the plasmid indicated followed by serum starvation for 24 h. Western blotting was performed. *D*, Cdc7 failed to activate NKE-mutated Myocd promoter. 10T1/2 cells were co-transfected with wild type (*WT*) or NKE-mutated (*NKEmt*) Myocd promoter and Ctrl or Cdc7 expression plasmid. The cells were then starved for 24 h followed by luciferase assay. *, $p < 0.01$ compared with Cdc7-transfected WT group ($n = 3$). *Error bars*, S.E.

Cdc7 had any effect on the Nkx2.5 protein level (Fig. 5*C*). These results led us to hypothesize that Cdc7 regulates Nkx2.5 function by modulating Nkx2.5 interaction with Myocd promoter. To test this hypothesis, we detected whether NKE mutation (38), which blocks Nkx2.5 binding to Myocd promoter, affects Cdc7 activity in activating the Myocd promoter. As shown in Fig. 5*D*, the NKE mutation markedly diminished Cdc7-induced Myocd activation by 62%, demonstrating that Cdc7-induced Myocd transcription depends on the interaction of Nkx2.5 with NKE in the Myocd promoter.

Cdc7 Physically Interacts with Nkx2.5—Because Cdc7 functionally interacted with Nkx2.5, and Cdc7 function was dependent on Nkx2.5 binding to Myocd promoter, we sought to examine whether Cdc7 physically interacts with Nkx2.5. A co-IP assay using endogenous proteins extracted from 10T1/2 cells treated with or without TGF-β showed that Nkx2.5 was co-immunoprecipitated with Cdc7 (Fig. 6*A*), and Cdc7 was co-immunoprecipitated with Nkx2.5 (Fig. 6*B*), indicating that Cdc7 physically interacted with Nkx2.5 in 10T1/2 cells. TGF-β appeared to enhance their interaction (Fig. 6, *A* and *B*).

Our previous studies have shown that the interaction of Nkx2.5 with Smad3 blocks Nkx2.5 binding to NKE in Myocd promoter in the early phase of SM differentiation (37). To determine whether the interaction of Nkx2.5 with Smad3 prevents Nkx2.5 from interacting with Cdc7, we performed co-IPs of Nkx2.5 with Smad3 and Cdc7 at early (4 h) and later (24 h) times following TGF-β treatment. As shown in Fig. 6, *C* and 6*D*, in the initial phase of TGF-β induction, 79% of Nkx2.5 interacted with Smad3. However, at the later phase, 77% of Nkx2.5 interacted with Cdc7. These data suggest that the interaction of Nkx2.5 with Smad3 and that with Cdc7 are mutually exclusive.

Cdc7 Is Essential for Nkx2.5 Binding to Myocd Promoter—Because Cdc7 functionally and physically interacted with Nkx2.5, we further explored the mechanism underlying Cdc7 function in SM maturation by testing whether Cdc7 modulates Nkx2.5 interaction with Myocd promoter. ChIP analysis showed that Nkx2.5 bound weakly to the NKE in Myocd promoter in the basal state (Fig. 7). TGF-β markedly increased the binding of Nkx2.5 to the NKE (Fig. 7). Blockade of Cdc7 expression by shRNA, however, significantly attenuated the TGF-β-en-

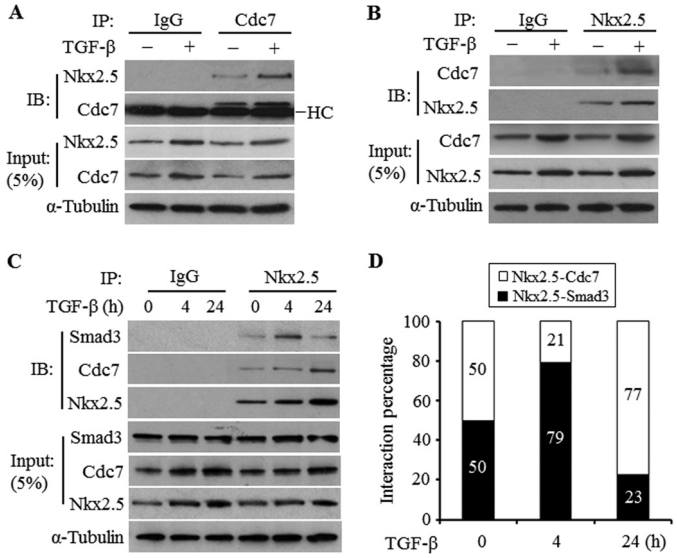

FIGURE 6. **Cdc7 physically interacted with Nkx2.** 5. *A* and *B*, endogenous co-IP indicated that Cdc7 physically interacted with Nkx2.5. Serum-starved 10T1/2 cells were treated with vehicle (−) or TGF-β (+) for 24 h. Cell lysates were immunoprecipitated with normal IgG, Cdc7 (*A*), or Nkx2.5 (*B*) antibody. The immunoprecipitates were blotted (*IB*) with Nkx2.5 (*A*) or Cdc7 (*B*) antibody. The interaction between Cdc7 and Nkx2.5 was enhanced by TGF-β induction. *HC* indicates the IgG heavy chain. *C*, interactions of Nkx2.5 with Smad3 and that with Cdc7 were mutually exclusive. 10T1/2 cells were treated with vehicle (0 h) or TGF-β for 4 or 24 h. Cell lysates were immunoprecipitated with normal IgG or Nkx2.5 antibody. The immunoprecipitates were blotted with Smad3 or Cdc7 antibody. *D*, the percentages of Nkx2.5 interacted with Smad3 or Cdc7 at different time points were calculated based on three independent experiments.

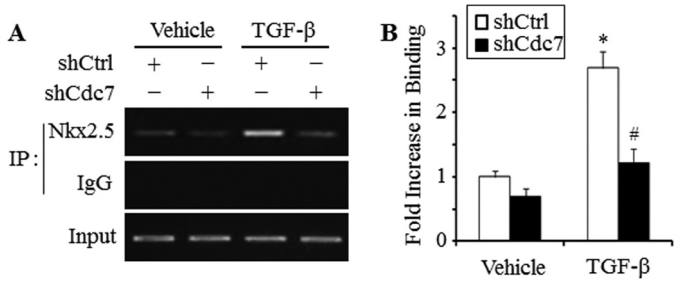

FIGURE 7. **Cdc7 enhanced Nkx2. 5 activity by promoting Nkx2.5 binding to Myocd promoter in a chromatin setting.** ChIP assay showed that the binding of Nkx2.5 to NKE in Myocd promoter was markedly enhanced by stimulation of TGF-β (24 h). However, TGF-β-induced binding was diminished by shCdc7. Representative semiquantitative PCR (*A*) and qPCR (*B*) were performed.*, $p < 0.01$ compared with vehicle-treated group. #, $p < 0.01$ compared with shCtrl-transfected group treated with TGF-β ($n = 3$).

hanced Nkx2.5 binding to the promoter (Fig. 7), indicating that Cdc7 is essential for Nkx2.5 interaction with Myocd promoter. These data demonstrate that Cdc7 regulates SM maturation through enhancing Nkx2.5 binding to Myocd promoter, leading to increased expression of Myocd, which in turn activates the expression of mature SM marker SMMHC.

DISCUSSION

SM maturation is a process by which multipotential cells in the developing organism acquire contractile SM characteristics that distinguish them from other cell types (4, 8). At present, the two marker proteins that provide the best definition of a mature contractile SM phenotype are SMMHC and smoothelin. SMMHC expression has never been detected in non-SMs *in vivo* and is the only marker protein that is SM-specific during embryogenesis (39). Our results show that TGF-β induces SMMHC expression in 10T1/2 cells after 48 h of stimulation while enhancing Cdc7 expression (Fig. 1). Because TGF-β downstream intermediate signaling molecules Smad2/3 are translocated to cytoplasm 18 h after TGF-β stimulation when they have completed their role in inducing early stage of SM differentiation (32, 37), molecular mechanisms other than Smad2/3 signaling pathway are required for mediating TGF-β-induced SM maturation. Our data demonstrate that Cdc7/Nkx2.5-mediated Myocd expression is one of these mechanisms. First, Cdc7 is essential for TGF-β-induced SMMHC expression (Fig. 2). Second, Cdc7 activates Myocd expression (Fig. 4). Finally, Myocd/SRF mediates Cdc7 function in inducing SMMHC expression because dominant negative Myocd or SRF shRNA blocks Cdc7-induced SMMHC expression (Fig. 3). These data establish a novel mechanism by which Cdc7 induces SM maturation via the induction of Myocd.

Cdc7 is not a transcription factor. Therefore, Cdc7 may serve as a coactivator in regulating Myocd expression. Nkx2.5 is a downstream transcription factor of PI3K signaling and is essential for Myocd expression in cardiac myocytes (38). Cdc7 appears to be an Nkx2.5 coactivator because Cdc7 and Nkx2.5 synergistically up-regulate Myocd transcription (Fig. 5). Moreover, Cdc7 physically interacts with Nxk2.5 (Fig. 6). Their interaction is enhanced by TGF-β probably because the enhanced expression of both Cdc7 and Nkx2.5 is induced by TGF-β. Most importantly, Cdc7 is required for TGF-β-enriched Nkx2.5 binding to Myocd promoter because knockdown of Cdc7 blocks the enhanced Nkx2.5 interaction with Myocd promoter (Fig. 7). Cdc7 may recruit more Nkx2.5 for binding to the NKE in the Myocd promoter in TGF-β-treated cells or enhance the binding affinity of Nkx2.5.

The present study together with our recently published results (20, 37) provide a model for Cdc7 function in the initial and late phase of TGF-β-induced SM differentiation, *i.e.* upon TGF-β stimulation, Smad3 is activated and translocated to nucleus where it binds to Cdc7 and Nkx2.5. Smad3 interaction with Cdc7 promotes transcription of the early SM markers such as α-SMA and SM22α (within 18 h of TGF-β stimulation). Smad3 interaction with Nkx2.5 blocks Nkx2.5 binding to NKE in Myocd promoter. After 18 h of TGF-β stimulation, Smad3 dissociates with Nkx2.5 and shuttles back to the plasma possibly due to the dephosphorylation of Smad3 by protein phosphatase 1A (40), which makes Nkx2.5 available for Cdc7. Cdc7 then interacts with Nkx2.5 to enhance Nkx2.5 binding to NKE in Myocd promoter, leading to the activation of Myocd transcription, which in turn promotes SMMHC expression (Fig. 8).

The limitation of this study is that it is unclear why Cdc7 does not bind to Nkx2.5 in the early phase of SM differentiation. The possible explanation is that Smad3 has a stronger binding affinity to Nkx2.5. In addition, Smad3 was translocated into nuclei within 10 min after TGF-β induction. Therefore, Smad3 has the advantage to preoccupy any available Nkx2.5 in the nuclei. Cdc7 is not induced until 2 h after TGF-β induction (20) and requires a high level of expression in inducing SMMHC expression, suggesting that Cdc7 may be less competitive in binding

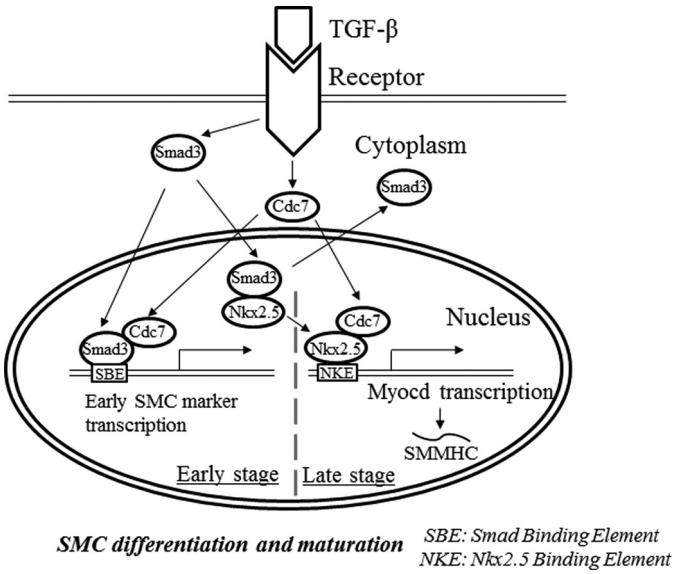

FIGURE 8. **Schematic mechanism by which Cdc7 regulates the early and late SM differentiation.** Upon TGF-β stimulation, Smad3 enters the nucleus and binds to SBE. Smad3 also binds to Nkx2.5 to prevent its interaction with Myocd promoter. Cdc7 binds to Smad3 complex to enhance the early SM marker gene transcription. 18 h after TGF-β stimulation, Smad3 is translocated out of the nucleus, and thus Nkx2.5 is released. Cdc7 then interacts with Nkx2.5 and promotes its binding to NKE in Myocd promoter, which activates Myocd transcription, leading to the increased SMMHC expression and SM maturation.

with Nkx2.5 compared with Smad3, which can be further studied in the future.

REFERENCES

1. Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362,** 801–809
2. Kocher, O., and Gabbiani, G. (1986) Cytoskeletal features of normal and atheromatous human arterial smooth muscle cells. *Hum. Pathol.* **17,** 875–880
3. Kocher, O., Gabbiani, F., Gabbiani, G., Reidy, M. A., Cokay, M. S., Peters, H., and Hüttner, I. (1991) Phenotypic features of smooth muscle cells during the evolution of experimental carotid artery intimal thickening: biochemical and morphologic studies. *Lab. Invest.* **65,** 459–470
4. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.* **84,** 767–801
5. Aikawa, M., Sivam, P. N., Kuro-o, M., Kimura, K., Nakahara, K., Takewaki, S., Ueda, M., Yamaguchi, H., Yazaki, Y., and Periasamy, M. (1993) Human smooth muscle myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis. *Circ. Res.* **73,** 1000–1012
6. Glukhova, M. A., Kabakov, A. E., Frid, M. G., Ornatsky, O. I., Belkin, A. M., Mukhin, D. N., Orekhov, A. N., Koteliansky, V. E., and Smirnov, V. N. (1988) Modulation of human aorta smooth muscle cell phenotype: a study of muscle-specific variants of vinculin, caldesmon, and actin expression. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 9542–9546
7. Libby, P. (2001) Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation* **104,** 365–372
8. Owens, G. K. (1995) Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75,** 487–517
9. Guo, X., and Chen, S. Y. (2012) Transforming growth factor-β and smooth muscle differentiation. *World J. Biol. Chem.* **3,** 41–52
10. McKee, J. A., Banik, S. S., Boyer, M. J., Hamad, N. M., Lawson, J. H., Niklason, L. E., and Counter, C. M. (2003) Human arteries engineered *in vitro*. *EMBO Rep.* **4,** 633–638
11. Yao, Y., Zebboudj, A. F., Torres, A., Shao, E., and Boström, K. (2007) Activin-like kinase receptor 1 (ALK1) in atherosclerotic lesions and vascular mesenchymal cells. *Cardiovasc. Res.* **74,** 279–289
12. Ko, Y. S., Yeh, H. I., Haw, M., Dupont, E., Kaba, R., Plenz, G., Robenek, H., and Severs, N. J. (1999) Differential expression of connexin43 and desmin defines two subpopulations of medial smooth muscle cells in the human internal mammary artery. *Arterioscler. Thromb. Vasc. Biol.* **19,** 1669–1680
13. Hirschi, K. K., Rohovsky, S. A., and D'Amore, P. A. (1998) PDGF, TGF-β, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J. Cell Biol.* **141,** 805–814
14. Sinha, S., Hoofnagle, M. H., Kingston, P. A., McCanna, M. E., and Owens, G. K. (2004) Transforming growth factor-β1 signaling contributes to development of smooth muscle cells from embryonic stem cells. *Am. J. Physiol. Cell Physiol.* **287,** C1560–1568
15. Chen, S., Kulik, M., and Lechleider, R. J. (2003) Smad proteins regulate transcriptional induction of the SM22α gene by TGF-β. *Nucleic Acids Res.* **31,** 1302–1310
16. Kennard, S., Liu, H., and Lilly, B. (2008) Transforming growth factor-β (TGF-β1) down-regulates Notch3 in fibroblasts to promote smooth muscle gene expression. *J. Biol. Chem.* **283,** 1324–1333
17. Stouffer, G. A., and Owens, G. K. (1994) TGF-β promotes proliferation of cultured SMC via both PDGF-AA-dependent and PDGF-AA-independent mechanisms. *J. Clin. Invest.* **93,** 2048–2055
18. Edlin, R. S., Tsai, S., Yamanouchi, D., Wang, C., Liu, B., and Kent, K. C. (2009) Characterization of primary and restenotic atherosclerotic plaque from the superficial femoral artery: potential role of Smad3 in regulation of SMC proliferation. *J. Vasc. Surg.* **49,** 1289–1295
19. Lien, S. C., Usami, S., Chien, S., and Chiu, J. J. (2006) Phosphatidylinositol 3-kinase/Akt pathway is involved in transforming growth factor-β1-induced phenotypic modulation of 10T1/2 cells to smooth muscle cells. *Cell. Signal.* **18,** 1270–1278
20. Shi, N., Xie, W. B., and Chen, S. Y. (2012) Cell division cycle 7 is a novel regulator of transforming growth factor-β-induced smooth muscle cell differentiation. *J. Biol. Chem.* **287,** 6860–6867
21. Chen, J., Kitchen, C. M., Streb, J. W., and Miano, J. M. (2002) Myocardin: a component of a molecular switch for smooth muscle differentiation. *J. Mol. Cell. Cardiol.* **34,** 1345–1356
22. Du, K. L., Ip, H. S., Li, J., Chen, M., Dandre, F., Yu, W., Lu, M. M., Owens, G. K., and Parmacek, M. S. (2003) Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. *Mol. Cell. Biol.* **23,** 2425–2437
23. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001) Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell* **105,** 851–862
24. Yoshida, T., Sinha, S., Dandré, F., Wamhoff, B. R., Hoofnagle, M. H., Kremer, B. E., Wang, D. Z., Olson, E. N., and Owens, G. K. (2003) Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. *Circ. Res.* **92,** 856–864
25. Qiu, P., Ritchie, R. P., Fu, Z., Cao, D., Cumming, J., Miano, J. M., Wang, D. Z., Li, H. J., and Li, L. (2005) Myocardin enhances Smad3-mediated transforming growth factor-β1 signaling in a CArG box-independent manner: Smad-binding element is an important cis element for SM22α transcription *in vivo*. *Circ. Res.* **97,** 983–991
26. Li, L., Miano, J. M., Cserjesi, P., and Olson, E. N. (1996) SM22α, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. *Circ. Res.* **78,** 188–195
27. Zhang, J. C., Kim, S., Helmke, B. P., Yu, W. W., Du, K. L., Lu, M. M., Strobeck, M., Yu, Q., and Parmacek, M. S. (2001) Analysis of SM22α-deficient mice reveals unanticipated insights into smooth muscle cell differentiation and function. *Mol. Cell. Biol.* **21,** 1336–1344
28. Hoofnagle, M. H., Neppl, R. L., Berzin, E. L., Teg Pipes, G. C., Olson, E. N., Wamhoff, B. W., Somlyo, A. V., and Owens, G. K. (2011) Myocardin is differentially required for the development of smooth muscle cells and cardiomyocytes. *Am. J. Physiol. Heart Circ. Physiol.* **300,** H1707–1721
29. Yoshida, T., Kawai-Kowase, K., and Owens, G. K. (2004) Forced expression of myocardin is not sufficient for induction of smooth muscle differentiation in multipotential embryonic cells. *Arterioscler. Thromb. Vasc. Biol.* **24,** 1596–1601

30. Yoshida, T., and Owens, G. K. (2005) Molecular determinants of vascular smooth muscle cell diversity. *Circ. Res.* **96,** 280–291
31. Long, X., Bell, R. D., Gerthoffer, W. T., Zlokovic, B. V., and Miano, J. M. (2008) Myocardin is sufficient for a smooth muscle-like contractile phenotype. *Arterioscler. Thromb. Vasc. Biol.* **28,** 1505–1510
32. Chen, S., and Lechleider, R. J. (2004) Transforming growth factor-β-induced differentiation of smooth muscle from a neural crest stem cell line. *Circ. Res.* **94,** 1195–1202
33. Briones, V. R., Chen, S., Riegel, A. T., and Lechleider, R. J. (2006) Mechanism of fibroblast growth factor-binding protein 1 repression by TGF-β. *Biochem. Biophys. Res. Commun.* **345,** 595–601
34. Li, F., Luo, Z., Huang, W., Lu, Q., Wilcox, C. S., Jose, P. A., and Chen, S. (2007) Response gene to complement 32, a novel regulator for transforming growth factor-β-induced smooth muscle differentiation of neural crest cells. *J. Biol. Chem.* **282,** 10133–10137
35. Kawai-Kowase, K., Kumar, M. S., Hoofnagle, M. H., Yoshida, T., and Owens, G. K. (2005) PIAS1 activates the expression of smooth muscle cell differentiation marker genes by interacting with serum response factor and class I basic helix-loop-helix proteins. *Mol. Cell. Biol.* **25,** 8009–8023
36. Raphel, L., Talasila, A., Cheung, C., and Sinha, S. (2012) Myocardin overexpression is sufficient for promoting the development of a mature smooth muscle cell-like phenotype from human embryonic stem cells. *PLoS One* **7,** e44052
37. Xie, W. B., Li, Z., Miano, J. M., Long, X., and Chen, S. Y. (2011) Smad3-mediated myocardin silencing: a novel mechanism governing the initiation of smooth muscle differentiation. *J. Biol. Chem.* **286,** 15050–15057
38. Ueyama, T., Kasahara, H., Ishiwata, T., Nie, Q., and Izumo, S. (2003) Myocardin expression is regulated by Nkx2.5, and its function is required for cardiomyogenesis. *Mol. Cell. Biol.* **23,** 9222–9232
39. Miano, J. M., Cserjesi, P., Ligon, K. L., Periasamy, M., and Olson, E. N. (1994) Smooth muscle myosin heavy chain exclusively marks the smooth muscle lineage during mouse embryogenesis. *Circ. Res.* **75,** 803–812
40. Lin, X., Duan, X., Liang, Y. Y., Su, Y., Wrighton, K. H., Long, J., Hu, M., Davis, C. M., Wang, J., Brunicardi, F. C., Shi, Y., Chen, Y. G., Meng, A., and Feng, X. H. (2006) PPM1A functions as a Smad phosphatase to terminate TGFβ signaling. *Cell* **125,** 915–928