THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 9, pp. 6860–6867, February 24, 2012

Cell Division Cycle 7 Is a Novel Regulator of Transforming Growth Factor-β-induced Smooth Muscle Cell Differentiation*[S]

Received for publication, September 21, 2011, and in revised form, January 4, 2012 Published, JBC Papers in Press, January 5, 2012, DOI 10.1074/jbc.M111.306209

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Background: Cell differentiation and proliferation are regulated simultaneously during early development, but the underlying mechanisms remain largely unknown.
Results: Cell division cycle 7 (Cdc7) regulates TGF-β-induced smooth muscle cell differentiation.
Conclusion: Cdc7 is likely a common factor regulating both cell proliferation and differentiation.
Significance: Our data provide a novel mechanism governing the synchronized regulation of cell proliferation and differentiation.

Smooth muscle cell (SMC) differentiation and proliferation occur simultaneously during embryonic development. The underlying mechanisms especially common factors regulating both processes, however, remain largely unknown. The present study has identified cell division cycle 7 (Cdc7) as one of the factors mediating both the proliferation and SMC differentiation. TGF-β induces Cdc7 expression and phosphorylation in the initial phase of SMC differentiation of pluripotent mesenchymal C3H10T1/2 cells. Cdc7 specific inhibitor or shRNA knockdown suppresses TGF-β-induced expression of SMC early markers including α-SMA, SM22α, and calponin. Cdc7 overexpression, on the other hand, enhances SMC marker expression. Cdc7 function in inducing SMC differentiation is independent of Dumbbell former 4 or Dbf4, the catalytic subunit of Cdc7 critical for cell proliferation, suggesting that Cdc7 mediates SMC differentiation through a mechanism distinct from cell proliferation. Cdc7 regulates SMC differentiation via activating SMC marker gene transcription. Knockdown of Cdc7 by shRNA inhibits SMC marker gene promoter activities. Mechanistically, Cdc7 interacts with Smad3 to induce SMC differentiation. Smad3 is required for Cdc7 function in inducing SMC promoter activities and marker gene expression. Likewise, Cdc7 enhances Smad3 binding to SMC marker promoter via supporting Smad3 nuclear retention and physically interacting with Smad3. Taken together, our studies have demonstrated a novel role of Cdc7 in SMC differentiation.

Vascular smooth muscle cells (SMCs)[2] originate from multiple sources during vascular development. The most common precursor for vascular smooth muscle is poorly defined mesenchymal cells derived from mesoderm (1). Phenotypic modulation of SMCs has been known to contribute to a number of major cardiovascular diseases including atherosclerosis, systematic and pulmonary hypertension, and restenosis (2–8). A large number of environmental cues such as growth factors/inhibitors, inflammatory mediators, cell-cell and cell-matrix interactions have been shown to regulate smooth muscle cell differentiation (2). Transforming growth factor-β (TGF-β) is among the most potent soluble growth factors that activate SMC contractile gene expression in both specified SMC and non-SMC types (9–15). It is well established that TGF-β/Smad signaling pathway plays critical roles in the SMC differentiation of pluripotent mesenchymal progenitors such as C3H10T1/2 (10T1/2) (9, 13, 16, 17).

Although cell differentiation is usually accompanied by an irreversible cell cycle exit, SMC proliferation and differentiation occur simultaneously during embryonic development (2). The molecular mechanisms governing these two somewhat uncompromised processes, however, remain largely unknown. We hypothesize that common factors regulating both the proliferation and differentiation may have coordinated these two events simultaneously. TGF-β/Smad signaling has been shown to regulate either cell growth or SMC differentiation depending on the physiological contexts (18–21). It is unknown, however, if common TGF-β downstream targets are used to regulate both SMC differentiation and proliferation concurrently.

In an Affymetrix cDNA microarray analysis of TGF-β-induced SMC differentiation of Monc-1 cells (22), we found that cell cycle regulator cell division cycle 7 (Cdc7) was up-regulated. However, it is unknown if Cdc7 is involved in SMC differentiation. Cdc7 encodes a nuclear serine/threonine protein kinase that is activated via binding to a regulatory protein called Dumbbell former 4 (Dbf4, also called ASK in mammals) (23, 24). Cdc7/Dbf4 regulates initiation of DNA replication by phosphorylating chromatin-bound mini-chromosome maintenance protein 2 (MCM2) with substantially high specific activity at the G1/S transition in eukaryotes (25–27). When the Cdc7 function is impaired in mouse embryonic stem cells (ESCs), the absence of Cdc7 kinase leads to S-phase arrest, which generates

* This work was supported, in whole or in part, by Grants HL093429 and HL107526 (to S. C.) from the National Institutes of Health.
[S] This article contains supplemental Figs. S1–S4.
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[2] The abbreviations used are: SMC, smooth muscle cell; TGF, transforming growth factor; shRNA, short hairpin RNA; qPCR, quantitative reverse transcription-PCR.

DNA structure checkpoint signals resulting in recombinational repair and eventual p53-dependent cell death (28).

Interestingly, TGF-β can stimulate SMC differentiation and proliferation of 10T1/2 cells simultaneously and up-regulates Cdc7 expression. We thus hypothesize that Cdc7 is one of the TGF-β downstream targets mediating both the differentiation and proliferation of 10T1/2 cells. Indeed, in the present study we identified Cdc7 as an important regulator in SMC differentiation in addition to its function in proliferation. Cdc7 is up-regulated and activated along with the expression of SMC markers in 10T1/2 cells. Both gain and loss of function studies showed that Cdc7 had a dramatic effect on the transcription and protein expression of early SMC markers. Importantly, Cdc7 promoted the initiation of SMC differentiation by a mechanism distinct from the proliferation. Moreover, we found that Cdc7 functionally and physically interacted with TGF-β downstream signaling molecule Smad3 to facilitate Smad3 binding to SMC marker promoter, leading to the activation of SMC marker gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—C3H10T1/2 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% glutamine. Cells were grown in serum-free medium for 16–24 h followed by incubation with TGF-β (5 ng/ml) for various times as needed. For transfections, cells were cultured in 12- or 6-well plates or 6-cm dishes, and transfected 24 h after plating with an 80 to 90% confluence using Lipofectamine LTX (Invitrogen) according to the manufacturer's instruction. In Cdc7 inhibition experiments, cells were pretreated with the 10 μM Cdc7 inhibitor PHA767491 (Tocris) for 2 h before incubation with TGF-β.

Preparation of shRNA Adenoviral Vector—Short hairpin RNA (shRNA) target sequence for Dbf4 was GCC CAC AAC CTA TTC TTC AGC TGGA. Double-stranded DNA coding Dbf4 shRNAs were cloned into pRNAT-H1.1/Adeno shuttle vector (Genscript). Adenovirus was packaged in 293 cells (Agilent) and purified by $CsCl_2$ gradient ultracentrifugation, as previously described (29). For adenoviral transduction, 10T1/2 cells were transduced with 100 multiplicity of infection of control or shRNA adenovirus for 48 to 72 h.

Immunocytofluorescent Staining—10T1/2 cells were cultured on sterile coverslips and treated with TGF-β. Cells were then fixed with 1% paraformaldehyde (PFA) for 5 min followed by methanol for 10 min at −20 °C for Smad3, phospho-Cdc7, and α-SMA staining. Anti-phospho-Cdc7 antibody was used at 1:200 in blocking buffer consisting of 3% BSA in PBS. Smad3 and α-SMA antibodies were used at 1:100. Cells were then incubated with FITC- or TRITC-conjugated secondary antibodies (1:100 and 1:50, respectively). Stained cells were imaged with a Nikon Eclipse 90i microscope and images were captured with a Nikon 12.7MP digital Sight DS-Ri1 color camera.

Cell Proliferation (MTT) Assay—5×10^3 cells/well were cultured in 96-well plates and treated as indicated. 10 μl of MTT reagent (R&D) was added to the medium and incubated in a CO_2 incubator for 3 h, and then 100 μl of detergent (R&D) was added and incubated at 37 °C for additional 2 h. Absorbance (A) was measured at 570 nm wavelength with a plate reader (Bio-Tek). The A values of the medium containing no cells were used as the blank control.

Quantitative Reverse Transcription-PCR (qPCR)—Total RNA from cultured cells was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instruction. Reverse transcription was performed using an iScript cDNA Synthesis kit (Bio-Rad). qPCR was performed in the Mx3005P qPCR machine using SYBR Green master mix (Agilent Technologies). Each sample was amplified in triplicate. Primers for the qPCR were as follows. Cdc7: 5′-TTG CAG CAG AGC TTC AGT GT-3′ (forward) and 5′-AAA TTG CTG GGC TTC ACA TC-3′ (reverse); Dbf4: 5′-CAG GAG CCT CAT GAG TGT GA-3′ (forward) and 5′-CCT CGC TTG TCT GAA AAA GG-3′ (reverse). The primers used for SMC markers were described previously (12, 22).

Western Blotting—10T1/2 cells were cultured in DMEM or treated with TGF-β or other factors as indicated. Antibodies used were: anti-Cdc7 (Santa Cruz Biotechnology), anti-phospho-Cdc7 (CycLex), anti-α-SMA (Abcam), anti-SM22α (Abcam), anti-Calponin1 (Santa Cruz Biotechnology), anti-α-tubulin (Cell Signaling), anti-Dbf4 (Santa Cruz Biotechnology). Cells were washed two times with PBS, followed by protein extraction using RIPA buffer (50 mmol/liters Tris-HCI, 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) or nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk for regular antibodies or 5% BSA for anti-phospho antibodies, and then incubated with primary antibodies in blocking buffer for 1 to 2 h followed by incubation with HRP-conjugated secondary antibody for 1 h (Sigma). Detection was performed with enhanced chemiluminescence (Millipore).

Promoter Reporter Luciferase Assay—α-SMA or SM22α promoter constructs were co-transfected into 10T1/2 cells with other plasmids as described previously (22). Cells were starved in serum-free medium for 24 h and then treated with 5 ng/ml of TGF-β for 8 h. Luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega). Experiments were repeated at least three times, and the results from representative experiments are shown with standard deviations.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assays were performed as described previously (30). Growth-arrested 10T1/2 cells were treated with TGF-β for 2 h. Chromatin complexes were immunoprecipitated with 3 μg of Smad3 antibody or IgG (negative control). Semi-quantitative PCR was performed to amplify the SM22α promoter region containing Smad binding element (SBE) using the following primer set: 5′-GGT GTT GAG CCA AGC AGA C-3′ (forward) and 5′-CGA GTT GCA TTA GCC CTG G-3′ (reverse) (31).

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

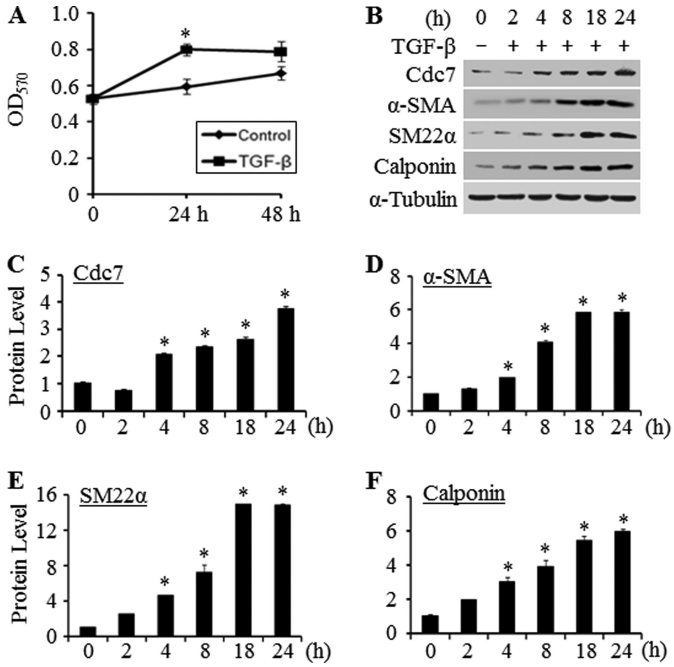

FIGURE 1. **TGF-β induced Cdc7 expression while promoting cell proliferation and SMC differentiation.** *A*, TGF-β promoted 10T1/2 cell proliferation. 10T1/2 cells were starved for 12 h followed by vehicle (Control) or TGF-β (5 ng/ml) treatment for 24 and 48 h. MTT assay was performed. *, $p < 0.05$ compared with control group ($n = 8$). *OD* refers to optical density normalized to the blank (culture medium). *B*, TGF-β induced Cdc7 expression along with SMC differentiation of 10T1/2 cells. Serum-starved 10T1/2 cells were treated with vehicle (−) or TGF-β (5 ng/ml) for the times indicated. Western blot was performed to examine the expression of Cdc7 and SMC marker proteins. *C–F*, quantitative analyses of Cdc7 and SMC marker expression. The protein expression was normalized to α-tubulin. *, $p < 0.01$ compared with vehicle-treated group (0 h) ($n = 3$).

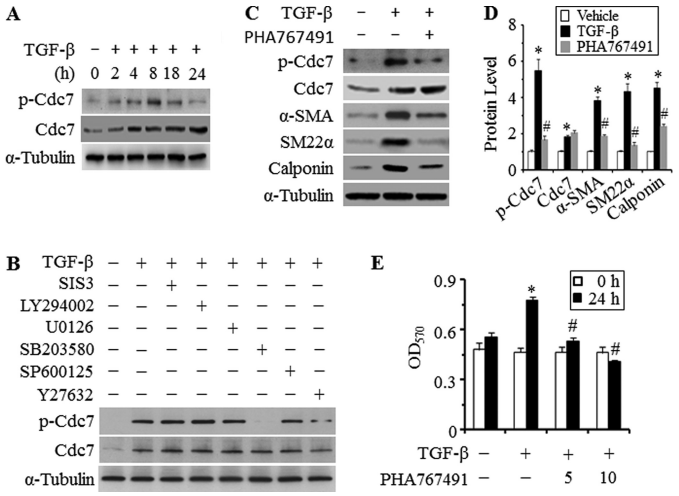

FIGURE 2. **Blockade of Cdc7 activation (phosphorylation) suppressed TGF-β-induced SMC differentiation and proliferation of 10T1/2 cells.** *A*, Cdc7 phosphorylation was induced in the early phase of TGF-β (5 ng/ml) treatment. The phosphorylation reached the peak at 8 h after TGF-β induction. *B*, TGF-β induced Cdc7 phosphorylation through p38 MAPK and RhoA pathways. Serum-starved 10T1/2 cells were treated with Smad3 inhibitor (SIS3, 10 μM), PI3 kinase inhibitor (LY294002, 10 μM), MEK1/2 inhibitor (U0126, 10 μM), p38 MAPK inhibitor (SB203580, 10 μM), JNK inhibitor (SP600125, 10 μM), or Rho kinase inhibitor (Y27632, 10 μM) for 2 h prior to vehicle or TGF-β (5 ng/ml) treatment for 8 h. Western blot was performed to detect phospho- and total Cdc7. *C*, Cdc7-specific inhibitor PHA767491 (10 μM) blocked TGF-β-induced Cdc7 phosphorylation and the expression of SMC early markers. 10T1/2 cells were treated with vehicle (−) or TGF-β (5 ng/ml) for 8 h with (+) or without (−) the addition of PHA767491. *D*, quantitative analysis of the protein expression shown in *panel C* ($n = 3$). *, $p < 0.01$ compared with the vehicle-treated groups, #, $p < 0.01$ compared with the TGF-β-treated group without PHA767491. *E*, PHA767491 blocked TGF-β-induced growth of 10T1/2 cells. 10T1/2 cells were starved for 4 h followed by vehicle (−) or TGF-β (5 ng/ml) induction with (5 μM or 10 μM) or without (−) PHA767491 addition for 24 h. MTT assay was performed ($n = 8$). *, $p < 0.01$ compared with the vehicle-treated group. #, $p < 0.01$ compared with TGF-β-treated group (24 h) without PHA767491.

RESULTS

Cdc7 Expression Is Increased in TGF-β-induced Proliferation and SMC Differentiation—TGF-β is known to be an important determinant for SMC lineage. Whether or not TGF-β coordinates both the proliferation and SMC differentiation, a unique phenomenon observed during embryonic development, however, remains to be determined. We found that TGF-β stimulated both the proliferation (Fig. 1*A*) and SMC differentiation of the embryonic mesenchymal 10T1/2 cells (Fig. 1, *B–F*) in the early phase of the induction (24 h), indicating that the initiation of SMC differentiation and proliferation occur simultaneously, which mimics the process found in the early embryonic development. Interestingly, cell cycle regulator Cdc7 expression was significantly increased along with the activation of SMC early markers (Fig. 1*B*). TGF-β induced a 4-fold increase of Cdc7 expression after 24 h of treatment (Fig. 1*C*). These data suggest that Cdc7 may be involved in both the proliferation and TGF-β-induced early SMC differentiation.

Blockade of Cdc7 Activation Suppresses both SMC Marker Expression and Cell Proliferation—As a kinase, Cdc7 is activated via phosphorylation at residue Thr-376 (26). To determine if TGF-β activates Cdc7, we examined the time-dependent phosphorylation of Cdc7. As shown in Fig. 2*A*, Cdc7 was activated as early as 2 h after TGF-β treatment, which was prior to the activation of SMC early marker α-SMA, SM22α or calponin (Fig. 1, *B–F*). Cdc7 phosphorylation reached the highest level at 8 h after TGF-β induction (Fig. 2*A*). It appeared that TGF-β induced Cdc7 phosphorylation through p38 mitogen-activated protein kinase (MAPK) and RhoA signaling pathways because both p38 and Rho kinase specific inhibitors blocked TGF-β-induced Cdc7 activation (Fig. 2*B*). To test if Cdc7 activation is required for TGF-β-induced SMC differentiation, we treated the serum-starved 10T1/2 cells with Cdc7-specific inhibitor PHA767491 in the presence of TGF-β. PHA767491 effectively blocked TGF-β-induced Cdc7 phosphorylation. Importantly, PHA767491 also blocked TGF-β-induced α-SMA, SM22α, and calponin expression by 51%, 69%, and 46%, respectively (Fig. 2, *C* and *D*), suggesting that Cdc7 plays an important role in TGF-β-induced SMC differentiation. Immunostaining of TGF-β-treated 10T1/2 cells confirmed that Cdc7 is required for TGF-β-induced SMC marker expression (supplemental Fig. S1). Cdc7 activity appeared to be essential for the α-actin filament reorganization and SMC morphology because PHA767491 inhibited α-SMA expression, reduced actin filament-formed stress fibers, and altered the morphology of TGF-β-induced cells (supplemental Fig. S1). In addition to its role in SMC marker expression, Cdc7 activity was also indispensible for TGF-β-induced cell proliferation. PHA767491 inhibited 10T1/2 cell growth in a dose-dependent manner (Fig. 2*E*). 5 or 10 μM of PHA767491 completely blocked the cell growth (Fig. 2*E*), consistent with its function in the initiation of

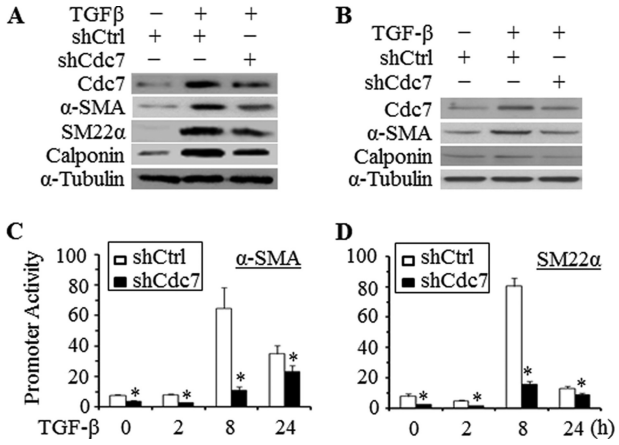

FIGURE 3. **Cdc7 is essential for TGF-β-induced SMC differentiation.** *A* and *B*, Cdc7 knockdown diminished TGF-β-induced expression of SMC early markers. 10T1/2 (A) and Monc-1 cells (*B*) were transfected with control (*shCtrl*) or Cdc7 shRNA (shCdc7) followed by vehicle (−) or TGF-β (5 ng/ml) treatment for 18 h. Western blot was performed to detect Cdc7 and SMC marker protein expression. *C* and *D*, Cdc7 knockdown blocked promoter activity of SMC marker genes. 10T1/2 cells were cotransfected with α-SMA (*C*) or SM22α (*D*) promoter construct and shCtrl or shCdc7 followed by TGF-β treatment for the times indicated. Luciferase assays were performed. *, $p < 0.01$ compared with shCtrl group in the same time points ($n = 3$).

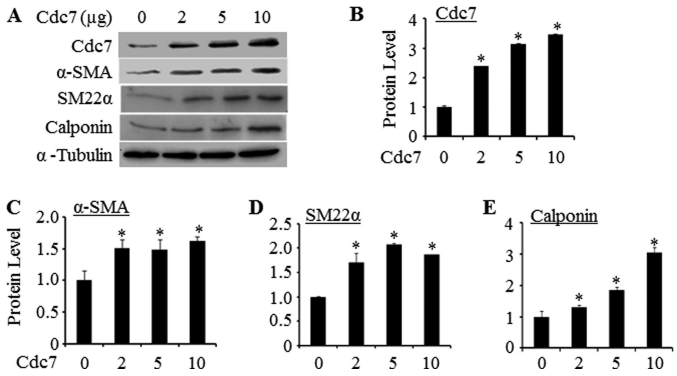

FIGURE 4. **Cdc7 overexpression induced SMC differentiation of 10T1/2 cells.** *A*, Cdc7 enhanced SMC early marker expression. 10T1/2 cells were transfected with control (10 μg) or the indicated amount of Cdc7 expression plasmid in 60 mm cell culture dishes followed by serum-starvation for 24 h. Cell lysates were then collected for Western blot analysis of the proteins indicated. *B–E*, quantitative analysis of the protein expression in *panel A*. Cdc7 and SMC marker protein levels were normalized to α-tubulin. *, $p < 0.05$ compared with the control plasmid-transfected group ($n = 3$).

DNA replication (25–27, 32). These data suggest that Cdc7 mediates both the cell proliferation and SMC differentiation induced by TGF-β.

Specific Knockdown of Cdc7 Blocks TGF-β-induced SMC Differentiation—Although PHA767491 blocks Cdc7 activation, it also has inhibitory activity against cyclin-dependent kinase 9 (CDK9) with a higher IC_{50} (33). To prove that the inhibition of SMC differentiation by PHA767491 was due to the blockade of Cdc7 activity, we used Cdc7-specific shRNA to knockdown Cdc7 expression. As shown in Fig. 3, *A* and *B* and supplemental Figs. S2 and S3, knockdown of Cdc7 diminished TGF-β-induced protein expression of α-SMA, SM22α, and calponin in both 10T1/2 (Fig. 3*A*) and Monc-1 neural crest cells (Fig. 3*B*), demonstrating the essential role of Cdc7 in SMC differentiation. Cdc7 shRNA specifically targeted Cdc7 because it did not affect the expression of other key Cdc family members such as Cdc6 and Cdk9 (supplemental Fig. S2). Cdc6 also participates in the DNA replication (34).

SMC differentiation is initiated by the activation of SMC marker gene transcription. To determine if Cdc7 is involved in the initiation process of SMC differentiation, we examined if Cdc7 regulates SMC marker gene promoter. We found that knockdown of Cdc7 markedly reduced TGF-β-induced α-SMA and SM22α promoter activity at various times of TGF-β treatment (Fig. 3, *C* and *D*). Cdc7 shRNA blocked α-SMA and SM22α promoter activity by 83 and 80%, respectively, at 8 h of TGF-β induction as compared with the control shRNA (Fig. 3, *C* and *D*). These results demonstrate that Cdc7 plays a critical role in the transcriptional activation of SMC marker genes during the initial phase of SMC differentiation.

Cdc7 Is Sufficient for Early SMC Differentiation—To further determine Cdc7 function in SMC differentiation, we tested if Cdc7 overexpression induces SMC marker gene expression. As shown in Fig. 4, *A–E*, forced expression of Cdc7 in 10T1/2 cells promoted α-SMA, SM22α, and calponin protein expression. These results indicate that Cdc7 alone is able to activate the SMC early markers.

Cdc7 Induces SMC Differentiation Independent of Dbf4—Dbf4 is the regulatory subunit of Cdc7. Cdc7 kinase activity is activated by Dbf4, which stimulates DNA replication and cell proliferation (34). To test if Dbf4 is involved in Cdc7-induced SMC differentiation, we constructed adenoviral vector expressing Dbf4 shRNA. Dbf4 shRNA effectively blocked Dbf4 expression in 10T1/2 cells as assessed by both mRNA and protein expression (Fig. 5*A*). Knockdown of Dbf4 significantly diminished the growth of 10T1/2 cells (Fig. 5*B*), consistent with the biological activity of Dbf4 in cell cycle. Blockade of Dbf4, however, had no effect on Cdc7-induced expression of α-SMA, SM22α, or calponin (Fig. 5*C*). Dbf4 shRNA also did not interfere with Cdc7 expression (Fig. 5*D*). These data demonstrate that Cdc7-induced SMC differentiation is independent of Dbf4.

To determine whether or not Cdc7-induced SMC marker gene expression was an indirect effect due to the altered cell cycle or cell density, we blocked TGF-β-induced cell proliferation using RO-3306, a specific inhibitor of another cell cycle regulator Cdk1. As shown in Fig. 5*E*, blockade of Cdk1 activation by RO-3306 effectively blocked TGF-β-induced cell growth, but did not alter TGF-β-induced SMC marker gene expression (Fig. 5*F*). These data further demonstrate that Cdc7-induced SMC differentiation is a process independent of cell cycle.

Cdc7-induced SMC Differentiation Is Smad3-dependent—Previous studies have established that Smad3 plays a major role in TGF-β-induced SMC differentiation of 10T1/2 cells (16, 35). Although Smad2 also mediates TGF-β function, it is not involved in the differentiation of 10T1/2 cells (16, 31, 36). Since knockdown of Cdc7 inhibited TGF-β-induced SMC differentiation (Fig. 3), we sought to determine how Cdc7 activity is linked to Smad3 function in TGF-β-mediated differentiation. To determine whether or not Cdc7-induced differentiation is related to Smad3 activity, we used both Smad3 shRNA (Fig. 6) and Smad3-selective inhibitor SIS3 (supplemental Fig. S4) to treat 10T1/2 cells with overexpression of Cdc7. Smad3 shRNA effectively knocked down Smad3 expression (Fig. 6*A*). Cdc7

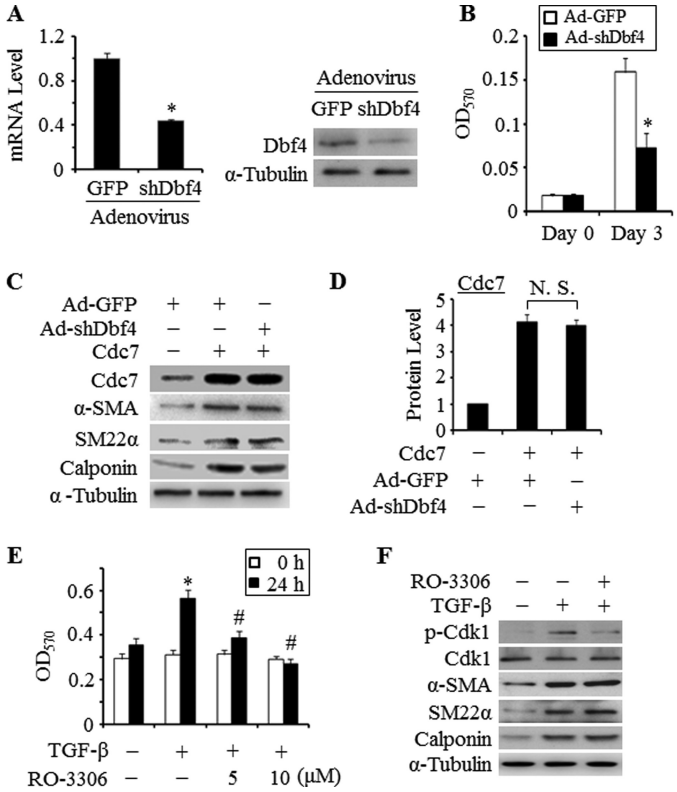

FIGURE 5. **Cdc7 induced SMC differentiation independent of Dbf4.** *A*, Dbf4 expression was effectively knocked down by Dbf4 shRNA as shown by both mRNA and protein expression. *, $p < 0.01$ compared with GFP group ($n = 3$). *B*, knockdown of Dbf4 markedly inhibited 10T1/2 cell proliferation as measured by MTT assay. *, $p < 0.01$ compared with Ad-GFP group ($n = 8$). *C*, knockdown of Dbf4 had no effect on Cdc7-induced expression of SMC early markers. Ad-GFP or Ad-shDbf4-transduced cells were transfected with 5 μg of control (−) or Cdc7 plasmid in 60-mm cell culture dishes. Cells were then serum-starved for 24 h. Western blots were performed to examine Cdc7 and SMC marker expression. *D*, quantitative analysis of Cdc7 expression shown in *panel C* by normalized to α-tubulin level. No significant difference (*N.S.*) was detected in Cdc7 expression between Ad-GFP and Ad-shDbf4-treated groups. *E*, Cdk1 inhibitor (RO-3306, 5 or 10 μM) suppressed TGF-β-induced 10T1/2 cell proliferation as measured by MTT assay ($n = 8$). *, $p < 0.01$ compared with the vehicle-treated group. #, $p < 0.01$ compared with TGF-β-treated group (24 h) without RO-3306. *F*, Cdk1 inhibitor had no effect on the expression of SMC markers. Serum-starved 10T1/2 cells were treated with vehicle (−) or TGF-β (5 ng/ml) for 24 h with (+) or without (−) the addition of RO-3306 (10 μM). Western blot was performed to examine the expression of phospho-Cdk1 (Thr-161), total Cdk1, and SMC markers as indicated.

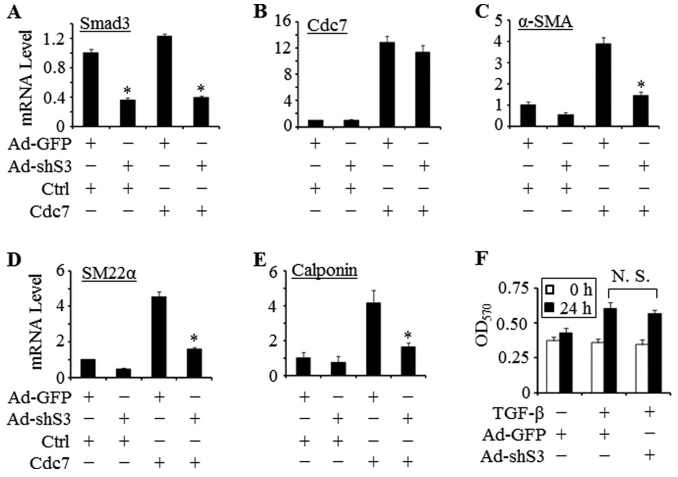

FIGURE 6. **Cdc7 induced SMC differentiation via Smad3 signaling pathway.** *A*, 10T1/2 cells were transduced with Ad-GFP or Ad-Sh Smad3 (Ad-ShS3) for 24 h, and followed by transfection with control or Cdc7 plasmid for additional 36 h. qPCR was performed to detect Smad3 mRNA expression. *, $p < 0.01$ compared with corresponding Ad-GFP group ($n = 3$). *B*, knockdown of Smad3 had no effect on Cdc7 expression. *C–E*, knockdown of Smad3 significantly inhibited Cdc7-induced mRNA expression of SMC marker genes as indicated. *, $p < 0.01$ compared with Cdc7-transfected and Ad-GFP-transduced group ($n = 3$). *F*, MTT assay showed that knockdown of Smad3 did not alter TGF-β-stimulated cell proliferation ($n = 8$).

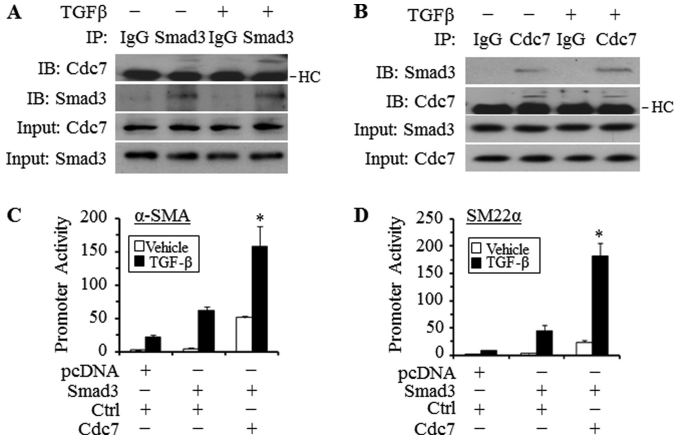

FIGURE 7. **Cdc7 physically and functionally interacted with Smad3.** *A* and *B*, endogenous Co-IP indicated that Cdc7 physically interacted with Smad3. Serum-starved 10T1/2 cells were treated with vehicle (−) or TGF-β for 2 h. Cell lysates were immunoprecipitated with normal IgG, Smad3 (*A*), or Cdc7 (*B*) antibody. The immunoprecipitates were blotted with Cdc7 (*A*) or Smad3 (*B*) antibody. The interaction between Cdc7 and Smad3 was enhanced by TGF-β induction. *HC* indicates the IgG heavy chain. *C* and *D*, Cdc7 markedly enhanced Smad3-mediated SMC marker gene promoter activity. 10T1/2 cells were cotransfected with α-SMA (*C*) or SM22α (*D*) promoter construct and control (Ctrl), Smad3, or Cdc7 expression plasmid as indicated followed by vehicle or TGF-β treatment for 16 h. luciferase assay was performed. *, $p < 0.01$ compared with Smad3-transfected group without Cdc7 but treated with TGF-β ($n = 3$).

significantly activated α-SMA, SM22α, and calponin mRNA expression (Fig. 6, *B–E*). Knockdown of Smad3 or SIS3, however, blocked the Cdc7-induced effects (Fig. 6, *C–E* and supplemental Fig. S4). Smad3 shRNA did not alter TGF-β-induced proliferation of 10T1/2 cells (Fig. 6*F*). These results indicate that Smad3 activity is essential for Cdc7 induction of SMC differentiation.

Cdc7 Physically and Functionally Interacts with Smad3—Because Cdc7 function in SMC differentiation relies on Smad3 activity, we sought to determine if Cdc7 interacts with Smad3. Co-IP assay with endogenous proteins extracted from 10T1/2 cells treated with or without TGF-β showed that Cdc7 was coimmunoprecicipated with Smad3 (Fig. 7*A*), and Smad3 was coimmunoprecipitated with Cdc7 (Fig. 7*B*), suggesting that Cdc7 physically interacts with Smad3 in 10T1/2 cells. TGF-β induction appeared to enhance their interaction (Fig. 7, *A* and *B*). To test whether or not the physical interaction between Cdc7 and Smad3 has functional significance, we co-transfected Smad3 and Cdc7 expression plasmids individually or in combination with α-SMA or SM22α promoter construct into 10T1/2 cells. Promoter analyses showed that Cdc7 significantly increased Smad3-mediated promoter activity even in the basal states (Fig. 7, *C* and *D*). TGF-β induction markedly enhanced Smad3 activity, which was dramatically augmented by Cdc7 (Fig. 7, *C* and *D*). These results demonstrate that Cdc7 interacts with Smad3 both physically and functionally to induce SMC marker promoter activity.

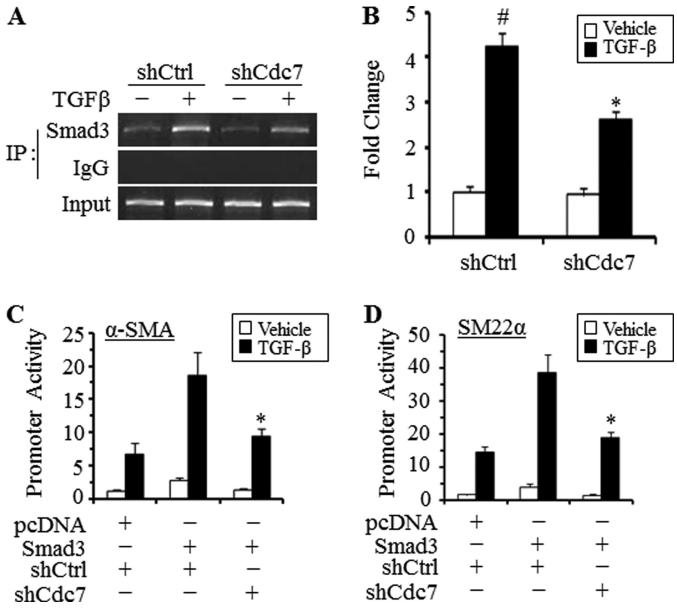

FIGURE 8. **Cdc7 enhanced Smad3 activity by promoting Smad3 binding to SMC marker gene promoter in a chromatin setting.** *A*, ChIP assay showed that the binding of Smad3 to SBE in SM22α promoter was markedly enhanced by stimulation of TGF-β. However, the TGF-β-induced binding was diminished by Cdc7 shRNA (shCdc7). *B*, quantitative analysis of Smad3 binding to SM22α promoter shown in *panel A* ($n = 3$). #, $p < 0.01$ compared with vehicle-treated group; *, $p < 0.01$ compared with control shRNA (shCtrl)-transfected group treated with TGF-β. *C* and *D*, Cdc7 knockdown significantly reduced Smad3-stimulated SMC marker promoter activity. 10T1/2 cells were cotransfected with Smad3 cDNA, shCtrl, shCdc7, α-SMA (*C*), and/or SM22α (*D*) promoter construct as indicated followed by vehicle or TGF-β treatment for 16 h. Luciferase assays were performed. *, $p < 0.01$ compared with TGF-β-treated group with Smad3 and shCtrl transfection ($n = 3$).

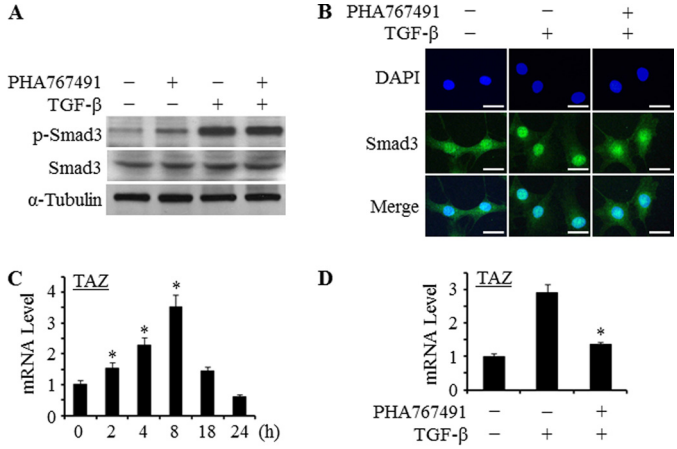

FIGURE 9. **Cdc7 enhanced Smad3 binding to SMC marker gene promoter by regulating the expression or stability of Smad nuclear retention factor TAZ.** *A*, Cdc7 inhibitor had no effect on Smad3 phosphorylation. Serum-starved 10T1/2 cells were pretreated without (−) or with (+) Cdc7 inhibitor (PHA767491, 10 μM) followed by vehicle (−) or TGF-β (+) induction for 1 h. Western blot was performed to examine phospho-Smad3 and total Smad3 expression. *B*, Cdc7 inhibitor diminished Smad3 nuclear translocation. 10T1/2 cells were treated with (+) or without (−) TGF-β (5 ng/ml) or PHA767491 (10 μM) as described in *panel A*. Immunostaining was performed to detect Smad3 nuclear location. DAPI stains nuclei. Scale bar, 50 μm. *C*, TGF-β induced a time-dependent expression of TAZ. *, $p < 0.01$ compared with vehicle-treated group (0 h) ($n = 3$). *D*, Cdc7 inhibitor blocked TGF-β-induced TAZ expression. 10T1/2 cells were pretreated without (−) or with (+) PHA767491 (10 μM) followed by vehicle (−) or TGF-β (+) incubation as indicated for 8 h. qPCR was performed to examine TAZ expression. *, $p < 0.01$ compared with TGF-β-treated group without PHA767491 ($n = 3$).

Cdc7 Is Essential for Smad3 Binding to SMC Marker Promoter—Smad3 regulates SMC differentiation by binding to SBE in SMC marker gene promoter (31). To further explore the mechanism underlying Cdc7 function in SMC differentiation, we tested if Cdc7 plays a role in Smad3 interaction with SMC marker gene promoter. Previous studies have shown that Smad3 binding to a SBE that is functionally important to SM22α promoter activity both *in vitro* and *in vivo* (16, 31, 37). To determine if Cdc7 affects Smad3 binding to this SBE, ChIP assay was performed. We found that Smad3 weakly bound to the SBE in the basal state (Fig. 8*A*); TGF-β increased the binding of Smad3 to SM22α promoter (Fig. 8, *A* and *B*). shRNA knockdown of Cdc7, however, significantly attenuated the TGF-β-enhanced Smad3 binding to the promoter (Fig. 8, *A* and *B*), indicating that Cdc7 is essential for Smad3 binding to SMC marker gene promoter in TGF-β-induced SMC differentiation. Functionally, knockdown of Cdc7 inhibited TGF-β/Smad3-mediated α-SMA and SM22α promoter activity by 49 and 51%, respectively, (Fig. 8, *C* and *D*), suggesting that Cdc7-mediated Smad3 binding is critical for the transcriptional activation of SMC markers.

To determine how Cdc7 enhances Smad3 binding to SBE in SMC marker gene promoter, we first detected if Cdc7 affects Smad3 phosphorylation. As shown in Fig. 9*A*, Cdc7 inhibitor PHA767491 did not alter Smad3 phosphorylation. However, PHA767491 inhibited TGF-β-induced Smad3 nuclear translocation (Fig. 9*B*), suggesting that Cdc7 may regulate Smad3 nuclear location independent of Smad3 phosphorylation. Previous studies demonstrate that Smad3 nuclear location is controlled by Smad nuclear retention factor tafazzin (TAZ) (38). We found that TGF-β induced TAZ mRNA expression in 10T1/2 cells in a time-dependent manner (Fig. 9*C*). Importantly, PHA767491 blocked TGF-β-induced TAZ mRNA expression (Fig. 9*D*), suggesting that Cdc7 may enhance Smad3 binding to SBE via regulating TAZ expression level, by either the enhanced transcription or mRNA stability.

DISCUSSION

Exit from cell cycle into G1/G0 is considered as a necessary, or at least frequent, prelude to differentiation of different cell types (39–41). Several other studies, on the other hand, have found that differentiation and cell proliferation can be regulated simultaneously (40, 42, 43). During embryonic development, SMC differentiation and proliferation also occur concurrently. The molecular mechanisms, especially the genes or protein factors that regulate both proliferation and differentiation, however, remain largely unknown. Our present studies have shown that during the initial phase of SMC differentiation of 10T1/2 cells (within 24 h of TGF-β treatment), TGF-β induces both the proliferation and differentiation of the progenitor cells simultaneously. Thus, TGF-β stimulation of 10T1/2 cells is an ideal model to study the molecular mechanisms underlying the synchronization of these two different cellular processes.

Cell cycle regulators have the potential to regulate both the proliferation and differentiation. Our studies have revealed that cell cycle regulator Cdc7 is able to regulate both proliferation and differentiation of SMC progenitor 10T1/2 cells. Cdc7 is induced and activated by TGF-β along with the proliferation

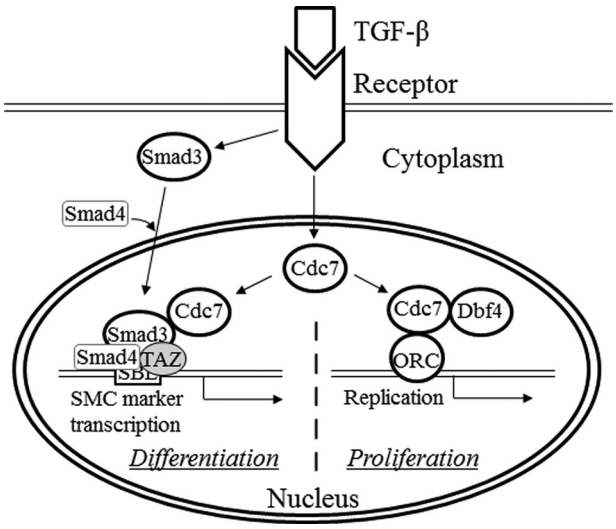

FIGURE 10. **A schematic mechanism by which Cdc7 regulates SMC differentiation and proliferation.** Upon TGF-β stimulation, both Cdc7 and Smad3 are translocated to the nucleus where Cdc7 stimulates Smad3 binding to SBE in SMC marker gene promoter by interacting with Smad3 and regulating TAZ expression level thus enhancing Smad3 nuclear retention, leading to the activation of SMC marker transcription and SMC differentiation. If Cdc7 binds to Dbf4 and origin recognition complex (*ORC*), Cdc7 will stimulate DNA replication, resulting in cell proliferation.

and differentiation of 10T1/2 cells. Blockade of Cdc7 activity through inhibition of its phosphorylation or knockdown of Cdc7 expression attenuates both the proliferation and SMC differentiation. Cdc7 itself appears to be able to induce SMC marker gene expression to a much lesser extent as compared with TGF-β induction (Fig. 4 *versus* Fig. 1), indicating that other factors such as Smad3 are required for TGF-β-induced SMC differentiation.

Cdc7 appears to regulate SMC differentiation by modulating SMC marker gene transcription. Knockdown of Cdc7 dramatically blocks the promoter activities of SMC early marker genes α-SMA and SM22α. Cdc7 function in the transcription requires intact Smad3 activity because knockdown of Smad3 or blockade of Smad3 activation by Smad3-selective inhibitor markedly inhibits Cdc7-induced mRNA expression of SMC markers. Mechanistically, Cdc7 physically interacts with Smad3 to promote Smad3 binding to SBE in the promoter of SMC marker genes, leading to activation of marker gene transcription. Knockdown of Cdc7 impairs Smad3 binding to SBE, resulting in the attenuation of SMC marker promoter activity and transcription. Interestingly, Cdc7 does not affect Smad3 phosphorylation but regulates Smad3 nuclear location, which is due to Cdc7 function in regulating the expression or stability of Smad nuclear retention factor TAZ.

Cdc7 regulates cell proliferation and SMC differentiation through distinct mechanisms. Dbf4, the catalytic subunit of Cdc7 and key regulator of cell cycle, is not important for SMC differentiation because shRNA knockdown of Dbf4 has no effect on SMC marker gene expression. However, Dbf4 is critical for proliferation of 10T1/2 cells. Based on our results in 10T1/2 cells, Cdc7 appears to coordinate the proliferation and SMC differentiation simultaneously via the following two distinct mechanisms: Upon TGF-β stimulation, both Cdc7 and Smad3 are induced and/or activated, and translocated into nuclei where Cdc7 regulates Smad3 binding to SBE on SMC marker gene promoter via physically interacting with Smad3 and enhancing TAZ expression level, leading to the activation of SMC marker transcription and SMC differentiation. When Cdc7 binds to Dbf4 and origin recognition complex (ORC), DNA replication will be activated, leading to cell proliferation and growth (Fig 10).

In summary, we have identified a novel role of Cdc7 in regulating SMC differentiation in addition to its role in cell proliferation. Cdc7 is likely to be one of many factors that simultaneously control cell proliferation and SMC differentiation. Whether Cdc7 facilitates these two processes during embryonic development will be an important and interesting area for future study.

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