Both TGF-β and myocardin (MYOCD) are important for smooth muscle cell (SMC) differentiation, but their precise role in regulating the initiation of SMC development is less clear. In TGF-β-induced SMC differentiation of pluripotent C3H10T1/2 progenitors, we found that TGF-β did not significantly induce Myocd mRNA expression until 18 h of stimulation. On the other hand, early SMC markers such as SM α-actin, SM22α, and SM calponin were detectable beginning 2 or 4 h after TGF-β treatment. These results suggest that Myocd expression is blocked during the initiation of TGF-β-induced SMC differentiation. Consistent with its endogenous expression, Myocd promoter activity was not elevated until 18 h following TGF-β stimulation. Surprisingly, Smad signaling was inhibitory to Myocd expression because blockade of Smad signaling enhanced Myocd promoter activity. Overexpression of Smad3, but not Smad2, inhibited Myocd promoter activity. Conversely, shRNA knockdown of Smad3 allowed TGF-β to activate the Myocd promoter in the initial phase of induction. Myocd was activated by PI3 kinase signaling and its downstream target Nkx2.5. Interestingly, Smad3 did not affect PI3 kinase activity. However, Smad3 physically interacted with Nkx2.5. This interaction blocked Nkx2.5 binding to the Myocd promoter in the early stage of TGF-β induction, leading to inhibition of Myocd mRNA expression. Moreover, Smad3 inhibited Nkx2.5-activated Myocd promoter activity in a dose-dependent manner. Taken together, our results reveal a novel mechanism for Smad3-mediated inhibition of Myocd in the initiation phase of SMC differentiation.

Vascular smooth muscle cell (SMC) differentiation is an essential component of vascular development. This complex differentiation process can be subdivided into three major regulatory stages: 1) initiation stage: selective activation of a subset of genes required for SMC functions; 2) coordinated control of SMC-selective/specific gene expression at defined times and stoichiometries; and 3) continuous regulation of gene expression through effects of local environmental cues (1, 2). Much progress has been made in recent years to identify mechanisms that control expression of the repertoire of genes specific for vascular SMC differentiation and function (2). The precise molecular mechanisms governing the early initiation of SMC differentiation, however, remain to be identified.

Myocardin (MYOCD) is a potent co-activator for serum response factor and is expressed mainly in SMCs and cardiomyocytes. Overexpression of MYOCD shows marked activation of multiple CArG-containing SMC marker genes, including smooth muscle α-actin (Acta2), myosin heavy chain, SM22α (Tagln), and SM-calponin (Cnn1) (3–6). Knock-out of Myocd in mice results in embryonic lethality at embryonic day 10.5 (E10.5) and is associated with failed SMC investment and differentiation (7). Additional studies have shown that Myocd can induce a SMC-like contractile phenotype (8), although expression of other SMC-associated genes appears to be independent of MYOCD (9, 10). Expression of early SMC marker genes such as Tagln and Acta2 emerges prior to detectable Myocd mRNA in the embryonic dorsal aorta; this may suggest that MYOCD has a minor role in the initiation of SMC differentiation in some vascular tissues (4, 11–13).

Genetic analysis in the mouse has demonstrated that TGF-β signaling is an important determinant for SMC differentiation during embryonic development. Knock-out of TGF-β receptors and downstream signaling components led to defects in embryonic angiogenesis due to loss of SMC formation (14–19). In vitro studies demonstrate that TGF-β induces SMC differentiation from several different cell types such as neural crest stem cells (20–22), mesenchymal progenitors (23), and endothelial cells (24), which parallels in vivo observations. Recent studies show that MYOCD is involved in TGF-β-induced SMC differentiation. MYOCD appears to act as a Smad3 co-activator and regulates SMC marker promoter activity via Smad-binding element in a CArG box-independent manner (25). Because MYOCD interaction with Smad3 has been observed largely by overexpression studies, the precise role of endogenous MYOCD in the initiation of SMC differentiation remains to be determined.

In this study, we found that endogenous MYOCD is not activated in a SMC progenitor cell line until 18 h after TGF-β induction. This time precedes the expression of early SMC marker genes such as Acta2 and Tagln (26), suggesting that MYOCD may not be involved in the initiation of SMC differentiation program induced by TGF-β. Additional studies show that the suppression of MYOCD is due to Smad3 sequestration of Nkx2.5, a critical transcription factor for Myocd transcription (27).
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EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—C3H10T1/2 (10T1/2) cells were cultured as described previously (22, 26). Smad3 and Smad4 expression plasmids were previously described (28, 29). Smad2 expression plasmid was a generous gift from Dr. Ying Zhang (30).

Plasmid Construction—The mouse Nkx2.5 expression plasmid was generated by inserting the full-length Nkx2.5 cDNA fragment into expression vector pcDNA 3.0 at the HindIII and XbaI sites. A T7 tag was included at the 5′ end of Nkx2.5 cDNA. The cDNA and tag were verified by sequencing. A mouse Myocd promoter luciferase reporter construct (pMyoCD-luc) was generated by PCR amplification of the Myocd promoter from −1664 to +286 bp of the mapped transcription start sites (data not shown). The PCR fragment was digested with restriction enzyme HindIII and XhoI and cloned into pGL3 vector (Promega). The promoter segment was verified by sequencing. The Nkx2.5 binding site (NKE) mutation was generated by changing Nkx2.5 binding sequence TCAAGTG to TCCCTCG in pMyoCD-luc using a site-directed mutagenesis kit (Agilent) (27).

Preparation of shRNA Adenoviral Vector—Adenoviral short hairpin RNA (shRNA) target sequences were CTG TCC AAC GGG AAT for Smad3 and GCC AAG CAT TCT ACA CAT TGG TTT GTC TTG for exportin 4. Double-stranded DNAs coding Smad3 or exportin 4 shRNAs were individually cloned into pRNA-T1.1/Adeno shuttle vector (GenScript). Adenovirus was packaged in 293 cells (Agilent) and purified by CsCl2 gradient ultracentrifugation. Viral particle titer was determined by plaque assay. For adenoviral transduction, 10T1/2 cells were transduced with 100 multiplicity of infection adenoviral control or shRNA for 24–48 h.

Reverse Transcription-PCR (RT-PCR) and Quantitative PCR (qPCR)—Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad). RT-PCR was performed using a Bio-Rad C1000 thermal cycler. qPCR was performed in an MX3000P qPCR machine using SYBR Green qPCR Mastermix (Agilent). The primer sequences for exportin 4, Smad3, and Nkx2.5 were as follows: TG-3 (forward) and AGG GGT ATT GCT CAG TGG TG (reverse); Nkx2.5 (forward) and CGG AAG CAT TCT ACA CAT TGG TTT GTC TTG (reverse). The primers used for SMC markers were described previously (22, 31).

Western Blotting—Cells were lysed in radioimmune precipitation assay lysis buffer. Samples were separated on SDS-polyacrylamide gels and electrotransferred onto PVDF or nitrocellulose membranes (Bio-Rad). The membranes were incubated for 16 h at 4 °C with antibodies against ACTA2 (Millipore), TAGLN (Abcam), CNN1 (Sigma), Nkx2.5 (Santa Cruz Biotechnology), Smad3 (Santa Cruz Biotechnology), Akt (Cell Signaling), Phospho-Akt (Cell Signaling), or α-tubulin (TUBA1) (Sigma) in blocking buffer containing 5% milk followed by incubation with HRP-conjugated secondary antibody (Sigma).

Transient Transfection and Luciferase Assay—10T1/2 cells were plated at 2 × 105 cells/well in 12-well plates and incubated at 37 °C in a 5% CO2 atmosphere until 80% confluence. Cells were then transiently transfected (in triplicate) with Lipofectamine LTX according to the manufacturer’s recommendation. Luciferase assay was performed as described previously (26, 32).

Co-immunoprecipitation assay (Co-IP) and Immunoblotting Analysis—Cells were lysed with ice-cold lysis buffer with protease inhibitor mix (Sigma). The lysates were incubated with Smad3 or Nkx2.5 antibody for 1 h and then Protein-A/G agarose at 4 °C for 12 h. The immunoprecipitates were pelleted, washed, and subjected to immunoblotting using Nkx2.5 or Smad3 antibody as described previously (22, 33).

Confocal Microscopy—10T1/2 cells were treated with vehicle or TGF-β for 24 h. The cells were fixed and incubated with rabbit anti-Smad3 and mouse anti-Nkx2.5 antibodies (Zymed Laboratories Inc. and Santa Cruz Biotechnology), followed by incubation with different fluorescent dye-conjugated secondary goat anti-rabbit or anti-mouse IgG as described previously (32). The co-localization of Smad3 with Nkx2.5 was observed with confocal microscopy.

Chromatin Immunoprecipitation Assay (ChiP)—ChiP assays were performed as described previously (34). 10T1/2 cells were treated with TGF-β for 0, 2, and 18 h. Chromatin complexes were immunoprecipitated with 3 μg of Nkx2.5 antibody or IgG (negative control). Semi-quantitative PCR and qPCR were performed to amplify the Myocd promoter region containing NKE using the following primer set: 5′-GTT CAG CAC TGC TTG TGG AA-3′ (forward) and 5′-TTT CAC TCT TCT CGT TG-3′ (reverse).

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

RESULTS

TGF-β Induces Myocd Expression following SMC Early Marker Activation—To determine whether MYOCD plays a role in initiating SMC differentiation, we first examined MYOCD activation in TGF-β-induced SMC differentiation from pluripotent mesenchymal 10T1/2 cells. TGF-β is known to be an important determinant for SMC lineage. Weak Myocd mRNA expression was observed at 8 h after TGF-β induction, but the activation was not significant until 18 h (Fig. 1, A and B). Interestingly, Myocd expression is undetectable at 48 h of TGF-β induction (data not shown), consistent with a previous finding (6). We have shown that the Tagln promoter was activated at 4 h and reached the highest level at 8 h of TGF-β induction in 10T1/2 cells (26). To confirm the early marker activation, we examined TGF-β-induced endogenous activation of Acta2, Tagln, and Cnn1. As shown in Fig. 1, A and B, mRNA expression of Acta2, Tagln, and Cnn1 was activated at 2 or 4 h after TGF-β treatment. The expression reached the highest level at 8 h after induction, which is consistent with the promoter analysis (26). SMC early marker protein expression was also detected as early as 2 (CNN1), 4 (TAGLN), or 8 h (ACTA2) following TGF-β stimulation (Fig. 1C), times that precede those seen when Myocd activation is observed.

Because a reliable antibody detecting endogenous levels of MYOCD is not readily available (35), Myocd promoter-driven reporter luciferase gene expression (pMyoCD-luc) was used to
reflect Myocd expression indirectly. As shown in Fig. 1D, the Myocd promoter was not activated in 10T1/2 cells until 18 h of TGF-β stimulation, consistent with endogenous MYOCDD activation. These data suggest that Myocd expression was blocked during the early activation of SMC marker genes.

**Smad Signaling Inhibits TGF-β-induced MYOCDD Activation**—TGF-β signaling is mediated predominantly by Smad proteins. Smad signaling is activated immediately after TGF-β stimulation. Activated Smads require binding to a common Smad called Smad4 to translocate into the nucleus where they regulate gene transcription. TGF-β function is mediated mainly by Smad2 and Smad3. Smad2 and Smad3 activation is negatively regulated by the inhibitory Smad7. To determine the role of Smad signaling in MYOCDD activation, we used dominant negative Smad4 (∆Smad4, with deletion of Smad4 activation domain) to block Smad2 and Smad3 nuclear translocation (36), or Smad7 to block Smad2 and Smad3 activation. To our surprise, both ∆Smad4 and Smad7 significantly increased TGF-β-induced Myocd promoter activity (Fig. 2A), suggesting that Smad signaling inhibits the Myocd promoter. To determine whether Smad signaling inhibits the endogenous Myocd mRNA expression induced by TGF-β, we overexpressed ∆Smad4 in 10T1/2 cells to block Smad2 and Smad3 activity and then treated the cells with TGF-β. We found that ∆Smad4 increased the endogenous Myocd mRNA level by 1.8-fold (Fig. 2B), confirming that Smads inhibit Myocd expression. Because both Smad2 and Smad3 can mediate TGF-β function, we sought to determine which Smad is responsible for the inhibition of MYOCDD activation. Thus, we overexpressed Smad2 or Smad3 in 10T1/2 cells and then treated the cells with vehicle or TGF-β.

We found that Smad2 did not affect Myocd promoter activity. However, Smad3 significantly inhibited Myocd promoter activity (Fig. 2C). Smad3 appeared to inhibit both the basal and TGF-β-induced Myocd promoter activity (Fig. 2C). TGF-β induced Smad3 activation in 10T1/2 cells within 15 min of stimulation (26), but did not significantly activate Myocd mRNA until 18 h after induction (Fig. 1, A, B, and D), suggesting that the inactivation of Myocd at the early stage of SMC differentiation may be due to Smad3 activation. To test this, we used shRNA expressed by adenovirus to block Smad3 expression (Fig. 2D). Smad3 knockdown efficiently blocked Smad3-dependent 3TP-Lux promoter activity (Fig. 2E) (37) and resulted in a significant increase of Myocd promoter activity and endogenous mRNA.
level at the early stage (4 and 8 h) of TGF-β treatment (Fig. 2, F and G). These data demonstrate that the Myocd transcriptional machinery is present but is blocked by Smad3 during the initiation stage of SMC differentiation. Interestingly, Smad3 knockdown also significantly increased Myocd promoter activity in cells treated with vehicle or TGF-β for 18 h (Fig. 2F), suggesting that Smad3 inhibits both basal and early MYOCD activation. Smad3 has been shown to shuttle consistently in and out of the nucleus through the nuclear pore of the cells (38, 39). The small amount of nuclear Smad3 may be sufficient to block Myocd promoter activity in the basal state. In the early TGF-β induction stage, the increased amount of nuclear Smad3 may have prevented the full activation of Myocd expression.

Blocking Nuclear Exportation of Smad3 Inhibits Myocd Promoter Activity at the Late Stage of TGF-β Induction—To confirm whether nuclear Smad3 caused the inhibition of Myocd expression, we used shRNA to block exportin 4, a protein responsible for Smad3 nucleocytoplasmic shuttling (38). Knockdown of exportin 4 expression caused retention of the majority of Smad3 in the nuclei of 10T1/2 cells (Fig. 3, A and B), which led to the inhibition of Myocd promoter activity and thus the mRNA level at the late phase (18 h) of TGF-β induction (Fig. 3C and 3D). These data demonstrate that the inhibition of Myocd gene activation in the early stage of TGF-β induction is due to Smad3 nuclear translocation.

PI3K Kinase (PI3K) Signaling Pathway Mediates TGF-β-induced Myocd Activation—In addition to Smad signaling, several other signaling pathways have been shown to mediate TGF-β function as well, including p38 MAPK, p42/44 MAPK, PI3K, and RhoA (40). Because Smad signaling is not responsible for TGF-β-induced Myocd activation, we sought to determine whether the other pathways are important. We used specific inhibitors to block these pathways in 10T1/2 cells and then examined Myocd promoter activity. As shown in Fig. 4A, p38 MAPK (SB203580), ERK (U0126), and Rho kinase (Y27632) inhibitors did not affect the TGF-β-induced Myocd promoter expression. The PI3K inhibitor (LY294002), however, significantly inhibited the promoter activity, suggesting that the PI3K signaling pathway is responsible for TGF-β induction of the Myocd promoter.

To confirm whether the PI3K pathway is important for endogenous Myocd activation, we treated 10T1/2 cells with the pathway inhibitors prior to TGF-β stimulation and then examined Myocd mRNA expression. As shown in Fig. 4, B and C, TGF-β-induced endogenous Myocd expression was not affected by p38, ERK, or Rho kinase inhibitors. However, the PI3K inhibitor blocked the Myocd mRNA expression. These data further demonstrate that Myocd activation is mediated by PI3K pathway. Activation of PI3K downstream target Akt suggests that TGF-β may induce Myocd through PI3K downstream target genes (Fig. 4D). Because Smad3 suppressed and PI3K activated Myocd expression (Figs. 2, C and F, and 4, A–C), we sought to determine whether Smad3 suppresses PI3K activity in the early phase of TGF-β induction. As shown in Fig. 4E, shRNA knockdown of Smad3 did not affect TGF-β-induced Akt phosphorylation, suggesting that Smad3 does not alter PI3K pathway activation.
Previous studies have shown that the cardiac transcription factor Nkx2.5 transactivates the Myocd promoter in cardiac myocytes (27). In addition to cardiac myocytes, Nkx2.5 is also expressed in some cultured SMC types (41). Nkx2.5 is a downstream target of PI3K (42). Because Myocd activation in 10T1/2 cells was mediated by PI3K, we sought to determine whether Nkx2.5 is responsible for TGF-\(\beta\)-induced Myocd promoter activity. We first determined whether TGF-\(\beta\) induces Nkx2.5 expression in 10T1/2 cells. As shown in Fig. 5, A–C, both Nkx2.5 mRNA and protein expression were activated as early as 2 h after TGF-\(\beta\) induction. To determine whether Nkx2.5 activates the Myocd promoter, we co-transfected Nkx2.5 and the Myocd promoter into 10T1/2 cells and found that Nkx2.5 significantly activated the Myocd promoter (Fig. 5D). To confirm the importance of Nkx2.5, we mutated the NKE in the Myocd promoter (27) and found that the NKE mutation diminished TGF-\(\beta\)-induced Myocd promoter activity (Fig. 5E), suggesting that Nkx2.5 is required for TGF-\(\beta\) function in inducing the Myocd promoter. Although Nkx2.5 was able to activate the Myocd promoter containing intact NKE, it failed to activate the Myocd promoter with the NKE mutation (Fig. 5F), demonstrating that Nkx2.5 binding to NKE is essential for Myocd promoter activation.

**Smad3 Physically Interacts with Nkx2.5 during TGF-\(\beta\)-induced Smad3 Activation**—Because Smad3 inhibited, but Nkx2.5 activated Myocd expression, we sought to determine whether Smad3 interacts with Nkx2.5. Immunostaining showed TGF-\(\beta\)-induced Smad3 nuclear translocation at 2, 4, and 8 h (Fig. 6A). At 18 h of TGF-\(\beta\) stimulation, most Smad3 shuttled back to the cytoplasm (Fig. 6A). TGF-\(\beta\)-induced Smad3 phosphorylation, and Nkx2.5 can enter the nuclei in the early and late time of TGF-\(\beta\) induction (Fig. 6A). Smad3 physically interacted with Nkx2.5 during TGF-\(\beta\) induction (Fig. 6B). To confirm this interaction, we performed co-immunoprecipitation experiments. As shown in Fig. 6C, Smad3 co-immunoprecipitated with Nkx2.5 in 10T1/2 cells at 2, 4, and 8 h after TGF-\(\beta\) treatment (Fig. 6A). At these times, Myocd expression is inactive (Fig. 1). At 18 h after the TGF-\(\beta\) induction, Smad3 was translocated to the cytoplasm; no nuclear co-localization between Smad3 and Nkx2.5 was observed (Fig. 6A). At this time, Myocd expression was significantly activated (Fig. 1). These data suggest that Smad3-Nkx2.5 interaction may lead to the activity of Myocd in the initiation phase of TGF-\(\beta\)-induced SMC differentiation. To test whether Smad3 indeed
interacts with Nkx2.5 physically, co-IPs using endogenous proteins were performed. As shown in Fig. 6B, Smad3 co-immunoprecipitated with Nkx2.5. Nkx2.5 also co-immunoprecipitated with Smad3 (Fig. 6C). These data demonstrate a physical interaction between Smad3 and Nkx2.5. To determine whether the Smad3-Nnkx2.5 interaction depends on Smad3 activation, cells were treated with or without TGF-β for 8 h, and co-IPs were performed. As shown in Fig. 6D, the interaction between Smad3 and Nkx2.5 was undetectable in untreated cells probably due to the low basal level of Smad3 and Nkx2.5 in the nuclei. TGF-β induction, however, resulted in detectable interaction (Fig. 6D). The intensity of the Smad3-Nnkx2.5 interaction was significantly reduced by the ALK5 inhibitor, demonstrating that only activated (nuclear) Smad3 interacted with Nkx2.5, consistent with their co-localization in nuclei (Fig. 6A).

Smad3 Blocks Nnkx2.5 Binding to the Myocd Promoter and Inhibits Nnkx2.5-mediated Myocd Promoter Activation in a Dose-dependent Manner—Because Nkx2.5 activated Myocd through binding to its promoter, we sought to determine whether Smad3 inhibited Myocd activation by blocking Nkx2.5 interaction with the Myocd promoter. ChIP analysis showed that TGF-β increased binding of Nkx2.5 to the Myocd promoter in the late (18 h) but not in the early phase (2 h) of induction (Fig. 7, A and B, left panels), consistent with the induction of Myocd promoter activity (Fig. 1D). However, shRNA knockdown of Smad3 enriched Nkx2.5 binding to the Myocd promoter in the early phase of TGF-β stimulation (Fig. 7, A and B, right panels), indicating that Smad3 blocked the interaction of Nkx2.5 with the Myocd promoter during the early stage of smooth muscle differentiation. To determine functionally whether the Smad3-Nnkx2.5 interaction is important for the silencing of the Myocd promoter, we tested whether Smad3 inhibits Nnkx2.5 activation of the Myocd promoter directly. The Myocd promoter was co-transfected with Nkx2.5 with or without different amounts of Smad3 cDNA into 10T1/2 cells. Cells were then treated with TGF-β for 4 h to allow Smad3 activation. As shown in Fig. 7C, low levels of Smad3 did not prevent Myocd promoter activation induced by Nkx2.5. However, higher doses of Smad3 significantly blocked Nnkx2.5 function (Fig. 7C). These data further demonstrate that Myocd blockade in the initiation stage was due to nuclear Smad3 sequestration of Nkx2.5, which prevents Nnkx2.5-mediated Myocd activation (Fig. 8).

DISCUSSION

MYOCD is considered to be a master regulator for SMC differentiation and is expressed in both SMC and cardiomyocytes during embryonic and postnatal development. Interestingly, although MYOCD is a very potent activator, some progenitor cells such as A404 expressing a low level of MYOCD are not converted to SMC phenotype without retinoic acid induction (6). Conversely, other SMC progenitors such as 10T1/2 cells that do not express MYOCD can be converted to SMC phenotype by overexpression of MYOCD (6), suggesting that a threshold level of MYOCD is required for SMC differentiation. Because MYOCD function was usually examined at a late stage of SMC differentiation or in mature SMCs, its role in the initiation of SMC differentiation remains to be clearly defined.

The present studies have revealed that Myocd mRNA expression is blocked by Smad3 at the initiation stage of SMC differentiation. Smads have been shown to activate SMC differentiation from several different progenitors (22, 26, 43, 44) although Smad3 was recently reported to inhibit MRTF-induced ACTA2 expression in epithelial-myofibroblast transition (45). It appears that only Smad3, but not Smad2, mediates the suppression of Myocd expression. Smad3 blockade of Myocd in the initial phase of TGF-β induction is due to Smad3 nuclear location. Retention of Smad3 in the nuclei at the late phase (18 h) leads to a prolonged inhibition of the promoter. This silencing of Myocd is likely an important mechanism for the initiation of SMC development because the initial conversion of SMC phenotype needs to be regulated precisely. MYOCD is a very strong activator for SMC genes, and its expression normally causes dramatic changes, which may prevent the progenitor cells from a fine-tuned and accurate initiation of SMC differentiation. Instead, Smad proteins, e.g., Smad3 in 10T1/2 cells, are mild regulators and thus can activate SMC genes in a gentle but precise way. Once the cells pass the initi-
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Collectively, our data provide a model for Myocd activation during TGF-β-induced initiation of SMC differentiation from mesenchymal progenitor 10T1/2 cells. Upon TGF-β stimulation, Smad3 is activated and translocated to nucleus where it binds to Nkx2.5, which blocks Nkx2.5 binding to the Myocd promoter and thus inhibits the mRNA expression of Myocd. After the initiation stage, Smad3 is shuttled back to the cytoplasm, which releases Nkx2.5 and makes it available to the Myocd promoter. Nkx2.5 and other transcription factors work together to activate MYOCD expression to the threshold level, which accelerates the SMC differentiation process (Fig. 8). This model provides novel insights for the initiation of SMC differentiation from at least some of the progenitors including 10T1/2 cells and neural crest cells because Myocd is also not activated in Monc-1 cells until a late stage (2 days after TGF-β induction; data not shown). However, for other progenitor cells such as A404, Myocd is present before retinoic acid treatment. Retinoic acid RA stimulation significantly enhances Myocd mRNA expression (6). Therefore, the initiation of retinoic acid-mediated SMC differentiation from A404 must be regulated by a different mechanism. The existence of distinct mechanisms for different progenitors is consistent with the distinct Myocd expression pattern in subsets of vascular SMCs in vivo during embryonic development (4).

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FIGURE 8. Mechanism by which TGF-β controls Myocd transcription during the initiation stage of SMC differentiation. During the initiation stage of SMC differentiation, Smad3 is activated by TGF-β and translocated to nucleus where it binds to Nkx2.5. This interaction blocks Nkx2.5 binding to the Myocd promoter and thus inhibits the expression of Myocd. After the initiation stage, Smad3 is shuttled back to the cytoplasm, which releases Nkx2.5, resulting in Myocd activation. Because MYOCD is an extremely strong activator for SMC marker genes, inhibition of its expression in the initial phase of TGF-β induction allows a temperate and precise regulation of the initiation of the SMC differentiation program. Smad3 serves as a mild activator for SMC genes. Once the platform is established, MYOCD activation accelerates the differentiation process.

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