β-Catenin and Smad3 regulate the activity and stability of myocardin-related transcription factor during epithelial–myofibroblast transition

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ABSTRACT Injury to the adherens junctions (AJs) synergizes with transforming growth factor-β1 (TGFβ1) to activate a myogenic program (α-smooth muscle actin [SMA] expression) in the epithelium during epithelial–myofibroblast transition (EMyT). Although this synergy plays a key role in organ fibrosis, the underlying mechanisms have not been fully defined. Because we recently showed that Smad3 inhibits myocardin-related transcription factor (MRTF), the driver of the SMA promoter and many other CCA(T)-rich GG element (CArG) box-dependent cytoskeletal genes, we asked whether AJ components might affect SMA expression through interfering with Smad3. We demonstrate that E-cadherin down-regulation potentiates, whereas β-catenin knockdown inhibits, SMA expression. Contact injury and TGFβ enhance the binding of β-catenin to Smad3, and this interaction facilitates MRTF signaling by two novel mechanisms. First, it inhibits the Smad3/MRTF association and thereby allows the binding of MRTF to its myogenic partner, serum response factor (SRF). Accordingly, β-catenin down-regulation disrupts the SRF/MRTF complex. Second, β-catenin maintains the stability of MRTF by suppressing the Smad3-mediated recruitment of glycogen synthase kinase-3β to MRTF, an event that otherwise leads to MRTF ubiquitination and degradation and the consequent loss of SRF/MRTF-dependent proteins. Thus β-catenin controls MRTF-dependent transcription and emerges as a critical regulator of an array of cytoskeletal genes, the “CArGome.”

INTRODUCTION

Epithelial–mesenchymal transition (EMT), a process characterized by cytoskeletal remodeling and transcriptional reprogramming, has long been known to play a key role in development and carcinogenesis (Acloque et al., 2009). In addition, EMT has emerged as a cen-
protein-1 or SMA (Wendt et al., 2009; Liu, 2010). However, TGFβ, although necessary, is usually not sufficient for EMT/EMyT. The other prerequisite, as we and others have shown, is an injury to (or absence of) the intercellular contacts (Masszi et al., 2004; Fan et al., 2007; Kim et al., 2009b; Zheng et al., 2009). Thus, whereas a fully intact epithelium is largely resistant to the EMyT-inducing effect of TGFβ, epithelial contact disruption dramatically increases susceptibility to this cytokine. On the basis of these studies, we proposed a two-hit paradigm and showed that TGFβ and contact injury synergize at the level of the SMA promoter (Masszi et al., 2004, 2010).

Although these results revealed that epithelial contacts are not simply targets but also key regulators of EMT/EMyT and the myogenic program, the molecular mechanisms remain largely undefined. Nonetheless, previous studies aimed at linking contact injury with epithelial SMA expression implicated two pathways: signaling through Rho and β-catenin.

The first mechanism involves myocardin-related transcription factor (MRTF), a recently discovered activator of serum response factor (SRF) that links cytoskeleton remodeling and the transcriptional control of cytoskeletal components (Wang et al., 2002; Olson and Nordheim, 2010). Under resting conditions, MRTF binds to monomeric (G) actin, which masks its nuclear localization signal. On F-actin polymerization (induced by various stimuli and mediated predominantly by Rho family GTPases), G-actin dissociates from MRTF, which results in nuclear translocation of MRTF (Miralles et al., 2003; Vartiainen et al., 2007). We and others showed that acute disruption of AJs stimulates Rho (Fan et al., 2007; Samarín et al., 2007) and redistributes MRTF to the nucleus (Fan et al., 2007; Busche et al., 2008). Once there, MRTF associates with SRF (Zaromytidou et al., 2006), and the complex drives gene transcription through the CCA(T/G)GG cis elements (CARG boxes) present in the promoters of a large array of muscle-type and cytoskeletal genes (the “CAR-Gome”) (Du et al., 2004; Tomasek et al., 2005; Sun et al., 2006), including SMA. Indeed, knockdown studies revealed that MRTF is indispensable for the TGFβ-induced (Morita et al., 2007; Elberg et al., 2008) and contact injury–facilitated EMyT (Fan et al., 2007; Masszi et al., 2010). However, we found that Smad3, one of the main transducers of TGFβ signaling, binds to MRTF and strongly inhibits its transcriptional activity on the SMA promoter (Masszi et al., 2010). This surprising observation implies that Smad3 is a temporary brake on EMyT, putting the process on hold. This brake is then relieved because under two-hit conditions (TGFβ plus contact disruption by low-calcium medium [LCM]) Smad3 gradually degrades, which liberates MRTF and allows for MF differentiation.

The other contact-dependent input relates to β-catenin. Given the double function of this molecule as a binding partner of E-cadherin at the AJ and as transcriptional coactivator of T cell factor/lymphoid enhancer factor (TCF/LEF) in the nucleus, β-catenin is a good candidate to link the state of AJs to transcriptional control. Indeed, β-catenin has been implicated in developmental EMT (Liebner et al., 2004), fibrogenesis (Bowley et al., 2007; Kim et al., 2009a), smooth muscle differentiation, and SMA expression (Gosens et al., 2008). However, the underlying mechanism, especially with respect to SMA expression, remains enigmatic. It is important that the SMA promoter does not contain any β-catenin-responsive (TCF/LEF) elements. On the other hand, β-catenin has been described as a binding partner of Smad3 (Tian and Phillips, 2002; Zhang et al., 2007, 2010). This fact, together with our finding that Smad3 is an inhibitor of the myogenic program, prompted us to investigate the EMyT-promoting action of β-catenin from a new angle. We asked whether β-catenin could be integrated into the recently described regulatory mechanism as a key modifier of the MRTF–Smad3 interaction. We hypothesized that β-catenin might interfere with the inhibitory action of Smad3 on MRTF.

Our results show that β-catenin is critical for the maintenance of MRTF/SRF interaction and MRTF stability. Because the MRTF/SRF complex is a master regulator of muscle and cytoskeletal genes, these results provide new insight into the mechanism by which β-catenin affects the expression of many CARG-dependent proteins, crucial for MF formation and muscle differentiation.

RESULTS
E-Cadherin down-regulation facilitates TGFβ-induced SMA expression

Our previous studies showed that disruption or absence of intercellular contacts (as induced by LCM, scratch wounding, or subconfluence) enables TGFβ to provoke SMA expression in tubular epithelial (LLC-PK1) cells (Masszi et al., 2004; Fan et al., 2007). To assess the role of AJs in this EMyT-promoting effect, we specifically targeted their chief component, E-cadherin. TGFβ failed to induce SMA expression in intact, confluent monolayers transfected with a control (nonrelated [NR]) small interfering RNA (siRNA) but provoked robust SMA expression after siRNA-mediated E-cadherin down-regulation (Figure 1, A and B). E-Cadherin silencing alone (without TGFβ stimulation) did not induce (or did so only marginally) SMA expression (Figure 1, A and B). In agreement with our previous data (Masszi et al., 2004), in subconfluent layers, TGFβ did provoke SMA expression, concomitant with ~60% decrease in E-cadherin level (Figure 1, C and D). However, when E-cadherin was fully knocked down prior to TGFβ treatment, the cytokine triggered a 3.5-fold-higher increase in SMA expression in the subconfluent cultures compared with the NR-treated controls (Figure 1, C–E). Thus the absence of E-cadherin permits TGFβ-induced SMA expression in confluent monolayers and strongly potentiates SMA expression in subconfluent cultures, implying that E-cadherin is an important regulator of the myogenic program in the epithelium.

β-Catenin is a crucial permissive regulator of SMA expression

E-Cadherin has been shown to mitigate Rho activation (Cho et al., 2010), which might contribute to its suppressive effect on SMA expression. In addition, E-cadherin is the major intracellular binding partner of β-catenin, and this molecule has been implicated in SMA expression (Masszi et al., 2004; Onder et al., 2008). Therefore we set out to characterize the role of β-catenin in SMA expression under myogenic (two-hit) conditions. Cells were transfected with NR or β-catenin siRNA and, after reaching confluence, exposed to LCM plus TGFβ for 48 h. β-Catenin knockdown (Figure 2A and Supplementary Figure 1A) resulted in strong suppression of SMA expression (Figure 2, A and B). Similar observations were made on another tubular cell line, NRK-52E, as well, in which the two-hit stimulation also caused marked increase in SMA expression, and β-catenin down-regulation fully blocked this response (Supplementary Figure S2, A and B). To test whether this effect manifested at the transcriptional level, we measured SMA mRNA in control and β-catenin–silenced cells after 48 h of stimulation with the combined treatment. The message for SMA was 50% less in β-catenin– siRNA-transfected versus NR-transfected cells (Figure 2C). To determine whether the presence of β-catenin affects the activity of the SMA promoter, we performed reporter assays using a 765–base pair SMA promoter coupled to firefly luciferase (pSMA-Luc; Masszi et al., 2010). Cells were first transfected with NR or β-catenin siRNA, followed 24 h later with cotransfection of pSMA-Luc and an internal control plasmid (pRL-TK). The combined treatment triggered strong
activation of the SMA promoter in control cells, whereas β-catenin silencing suppressed the (already low) basal SMA promoter activity and—of importance—prevented its stimulation-induced rise over the baseline obtained in nonstimulated control cells (Figure 2D).

Taken together, the results indicate that the absence of β-catenin dramatically reduces the activation of the SMA promoter and consequently the level of SMA mRNA and protein expression.

Having seen that β-catenin is essential for SMA expression induced by the conventional two-hit scheme, we asked whether it could be involved in the E-cadherin down-regulation–promoted SMA response as well. It is noteworthy that in our cells (similar to many other systems) E-cadherin silencing did not reduce the total β-catenin level (Figures 1C and 2E), implying that β-catenin might bind to other partners or its synthesis may keep up with its degradation. Moreover, elimination of E-cadherin resulted in a greater-than-threefold rise in nuclear β-catenin content, indicating an increase in the mobile pool of this molecule (Figure 2H). Of importance, concomitant silencing of β-catenin and E-cadherin strongly reduced the TGFβ-provoked SMA expression compared with the level observed in E-cadherin down-regulated cells (Figure 2E). This finding confirms the key role of β-catenin in the E-cadherin silencing–promoted SMA expression.

Next we sought to determine whether β-catenin plays a permissive or/and inductive role in the regulation of the SMA promoter. Overexpression of β-catenin induced a 10-fold increase in the activity of the β-catenin–responsive reporter TOP-Flash, verifying the efficacy of our expression vector (Supplementary Figure 1B). Nonetheless, β-catenin overexpression did not stimulate the SMA promoter, nor did it increase its activation provoked by TGFβ plus LCM (Figure 2I). Thus β-catenin is necessary but not sufficient for the optimal activation of the SMA promoter, in full agreement with the fact that the SMA promoter does not harbor any obvious β-catenin–responsive motifs.

Taken together these results suggest that β-catenin plays a crucial permissive role in the regulation of the SMA promoter, and the cytosolic β-catenin levels obtained after contact injury or E-cadherin down-regulation are sufficient for this permissive effect.

β-Catenin prevents the inhibitory effect of Smad3 on the MRTF-induced activation of the SMA promoter

Next we sought to gain insight into mechanism by which β-catenin facilitates the myogenic program. Because previous observations showed that β-catenin can associate with Smad3 and that Smad3 is a strong inhibitor of MRTF-dependent transcription (Masszi et al., 2010), we considered whether the Smad3/β-catenin interaction might regulate the myogenic program. We initially tested their interaction under the various conditions of the two-hit regimen after short-term stimulation, since the nuclear translocation of MRTF peaks around 30–60 min, and during this time neither β-catenin nor Smad3 levels change (Masszi et al., 2010). Coimmunoprecipitation studies revealed that in resting cells there was only a weak association between Smad3 and β-catenin, whereas stimulation with either TGFβ or LCM induced a substantial (5- to 10-fold) increase in their interaction. The combined treatment exerted an even stronger effect (Figure 3, A and B). Moreover, down-regulation of E-cadherin increased the complex formation between β-catenin and Smad3 in the absence of any stimulus, suggesting that the increased availability of β-catenin is sufficient to promote enhanced interaction between these partners (Figure 3C).

We then asked whether β-catenin could reverse the inhibitory action of Smad3 on MRTF-driven transcription. In introductory experiments we titrated the effect of Smad3 on MRTF. In agreement with our recent findings (Masszi et al., 2010), the MRTF expression–driven SMA promoter response was gradually diminished as the amount of coexpressed Smad3 was increased (Figure 4A). Under our conditions ~60% inhibition was attained with 1 μg of Smad3 DNA, and this dose was applied in the subsequent studies. As shown in Figure 4B, cotransfection with an increasing amount of β-catenin gradually restored the MRTF-induced SMA promoter activation. A detailed analysis of the β-catenin effect, using cotransfection with MRTF, Smad3, or both (Figure 4C, top) showed that overexpression of β-catenin entirely prevented the Smad3-induced
inhibition of the MRTF-triggered promoter activation, and produced only a slight (nonsignificant) decrease in the basal or MRTF-induced promoter activity. In agreement with our previous findings, overexpression of Smad3 without MRTF had no significant effect on the SMA promoter.

To test whether the β-catenin–induced protection against the inhibitory effect of Smad3 required the CArG-boxes or other cis elements in the SMA promoter, including Smad-binding element 1 (SBE1), SBE2, and the TGFβ control element (TCE), we transfected the cells with a triple mutant promoter in which each of these was inactivated (Masszi et al., 2010). Of importance, the inhibition by Smad3 and the protective effect of β-catenin remained the same as observed with the wild-type promoter (Figure 4C, middle). Similar results were obtained with a short (152 base pair) promoter.

FIGURE 2: β-Catenin is a crucial permissive regulator of SMA expression. (A) Confluent LLC-PK1 cells transfected with NR or β-catenin siRNA (β-cat si, 100 nM) were exposed to combined treatment (TGFβ+ LCM) for 48 h. Cell lysates were probed by WB for the indicated proteins. (B) The effect of the β-catenin down-regulation on the two-hit stimulation–triggered SMA expression. (C) Quantification of SMA mRNA by quantitative PCR in NR or β-catenin siRNA-transfected cells. siRNAs were added 24 h prior to stimulation with the two-hit regimen for 48 h. (D) One day after transfection with NR or β-catenin siRNA (100 nM), cells were cotransfected with SMA-Luc and pRL-TK for 24 h and then exposed to LCM plus TGFβ for an additional 24 h, followed by luminometric determination of SMA promoter activity. (E) The effect of β-catenin silencing on SMA expression in E-cadherin–depleted and TGFβ-stimulated monolayers. Cells were transfected with various siRNAs alone or in combination (NR, E-cad, β-cat, or E-cad + β-cat, labeled as E/β), treated with TGFβ for 72 h, and probed by Western blotting for the indicated proteins. (F) The impact of E-cadherin down-regulation on β-catenin localization. β-Catenin was detected in nuclear extracts prepared from cells transfected with NR or E-cadherin siRNA (24 h). (G) Densitometric quantification of nuclear β-catenin normalized to total. (H) The effect of LCM plus TGFβ treatment on the cytosolic β-catenin signal. Confluent monolayers were exposed to vehicle (control) or combined stimulation for 6 h, after which cells were fixed and stained for β-catenin and DAPI (left) and the normalized cytosolic β-catenin signal was quantified (right) as described in Materials and Methods. (I) β-Catenin overexpression is not sufficient to drive the SMA promoter. SMA promoter activity was measured in cells transfected with empty vector or β-catenin (3 μg) together with the SMA-Luc/pRL-TK system (24 h) and then exposed to vehicle or the combined treatment for an additional day.
We then tested whether β-catenin down-regulation could interfere with the stimulus-induced translocation of MRTF into the nucleus. β-Catenin silencing did not prevent the fast, LCM-induced nuclear uptake of MRTF (Figure 5C). Thus β-catenin facilitates the integrity of the myogenic complex by a mechanism distal to MRTF translocation, consistent with the prevention of the inhibitory effect of Smad3.

β-Catenin and Smad3 mutually inhibit each other’s association with MRTF

We argued that if β-catenin, at least in part, acts through capturing Smad3, then overexpression of β-catenin should result in reduced Smad3–MRTF interaction. To test this, cells were transfected with HA–MRTF, Myc–Smad3, and either an empty vector or FLAG–β-catenin. Immunoprecipitation of MRTF through the HA tag (Figure 6A) brought down a substantial amount of Smad3 in empty vector-transfected cells, whereas overexpression of β-catenin strongly reduced the amount of MRTF-associated Smad3, concomitant with a dramatic increase in the SRF/MRTF interaction (Figure 6A). Intriguingly and unexpectedly, the MRTF immunoprecipitate also contained some β-catenin (see later discussion). Next we investigated the converse situation by asking whether a reduction in endogenous β-catenin could increase the MRTF–Smad3 interaction. Downregulation of β-catenin caused a substantial rise in the amount of MRTF-associated Smad3 (Figure 6B). These experiments indicate that β-catenin is a negative regulator of the Smad3/MRTF interaction.

Having observed that β-catenin itself can be in complex with MRTF, we first surmised that this interaction might be mediated through Smad3. In other words, whereas β-catenin reduces the overall Smad3 binding to MRTF, it was conceivable that the remaining Smad3–MRTF binding was responsible for the presence of β-catenin through the formation of an MRTF–Smad3–β-catenin complex. To assess this, we used an MRTF mutant, ΔB1p, that lacks a seven–amino acid sequence in the B1 region critical for Smad3 binding (Masszi et al., 2010). Indeed, coprecipitation studies revealed that ΔB1p almost entirely lost its capacity to bind Smad3 (Figure 6C). Despite this, ΔB1p retained its capacity to pull down β-catenin; in fact there was more β-catenin in complex with ΔB1p than with wild-type MRTF (Figure 6C). This implies that β-catenin does not bind to MRTF through Smad3, and that the MRTF sequence critical for Smad3 binding is not essential for the complex formation between β-catenin and MRTF.

To substantiate the reciprocal relationship between the binding of β-catenin versus Smad3 to MRTF, we down-regulated Smad3 and found an increased association between endogenous β-catenin and MRTF (Figure 6D). As expected, this was coincident with enhanced interaction between MRTF and Smad3. Taken together, the results indicate that β-catenin reduces the binding of Smad3 to MRTF, whereas Smad3 mitigates the binding of β-catenin to MRTF, and these mutually inhibitory effects do not require the same MRTF sequence. These findings imply that the Smad3–β-catenin interaction prevents these partners from accessing MRTF.

The association between β-catenin and MRTF is a novel finding that could also contribute to MRTF regulation. Although the detailed characterization of this interaction will require a separate study, as an initial step we established that this is a stimulus-regulated process, as the two-hit challenge induced a transient increase in MRTF–β-catenin association (Figure 6E).
β-Catenin maintains MRTF stability in stimulated cells by counteracting glycogen synthase kinase-3β–dependent MRTF degradation

During the course of our experiments we noticed that in β-catenin–down-regulated cells the two-hit stimulation appeared to reduce the size of the MRTF immunoreactive band. To investigate this phenomenon, we performed a detailed time course (0–120 min) in NR and β-catenin siRNA-transfected cells. Figure 7A shows that there was no obvious difference in the expression of MRTF in resting control versus β-catenin–silenced cells; in contrast, after stimulation there was a dramatic reduction in the MRTF band obtained from β-catenin–down-regulated cells. The same phenomenon was observed in NRK-52E cells as well (Supplementary Figure S2D). This decrease was apparent at times ≥60 min and became pronounced or near complete by ~120 min. It is noteworthy that in β-catenin–expressing cells stimulation induces an upward shift in the MRTF band, corresponding to the reported (multiple) phosphorylation of this protein. This often manifests as a widening of the band or the appearance of multiple bands with slightly higher molecular mass. The total density of these usually exceeds the density of the nonstimulated band, which may be due to more efficient or preferential antibody binding to the less condensed epitopes and/or the phosphorylated form(s) of MRTF. Indeed, densitometry showed an early increase in the MRTF-immunoreactive band(s) upon two-hit stimulation (Figure 7A). Because this response was readily apparent after 15 min, it likely reflects posttranslational modification (e.g., phosphorylation) and not a net rise in the MRTF protein. On stimulation, the intensity of the MRTF bands started to increase in the β-catenin–depleted cells as well, but after ~30–60 min a reversal occurred and the signal eventually dropped well below the nonstimulated level (Figure 7A). The same pattern was observed when probing with a different MRTF antibody (Figure 7A, inset). The decrease of the signal below the basal level might be due to an additional modification that masks the epitope seen by the antibody (e.g., ubiquitination) and/or the degradation of MRTF protein. To determine whether protein degradation might play a role, we followed the fate of heterologously expressed, HA-tagged MRTF using an anti-HA antibody. In NR siRNA-transfected cells the intensity of the HA signal remained constant during stimulation, indicating that the increase seen by the MRTF antibody was indeed due to some posttranslational modification (e.g., ubiquitination) and/or the degradation of MRTF protein.

**FIGURE 4:** β-Catenin counteracts the inhibitory action of Smad3 on the SMA promoter through SBE-independent, CArG-dependent mechanism. The inhibitory effect of Smad3 on the SMA promoter was titrated by cotransfecting various amounts of Smad3 expression plasmid with MRTF (0.5 μg) and the SMA-Luc/pRL-TK reporter. SMA promoter activity obtained with MRTF alone was taken as 100%. (B) Cells were cotransfected with SMA-Luc/pRL-TK, MRTF (0.5 μg), Smad3 (1 μg), and increasing amounts of β-catenin. The Smad3-induced inhibition of the SMA promoter was reversed by β-catenin in a dose-dependent manner. (C) Combinatorial expression of the indicated transcription factors along with WT and mutant SMA promoter constructs (Triple or Short mutant; see Materials and Methods). Activation of the SMA promoter by MRTF (M, 0.5 μg) was inhibited by Smad3 (S, 1 μg). This inhibitory effect was completely reversed by β-catenin (3 μg).
Glycogen synthase kinase-3β (GSK-3β) has been reported to phosphorylate and inhibit myocardin (Badoff et al., 2005) and to promote its ubiquitination (Xie et al., 2009). We therefore examined whether GSK-3β might be involved in the degradation of MRTF, an effect that might become apparent in β-catenin–depleted cells. The decrease in MRTF observed upon stimulation in β-catenin–depleted cells was efficiently mitigated by LiCl or SB-216763, two inhibitors of GSK-3β (Figure 7C). Moreover, the elimination of β-catenin strongly facilitated MRTF ubiquitination, and this phenomenon was prevented by LiCl (Figure 7D).

**GSK-3β interacts with MRTF through a Smad3-dependent, β-catenin–inhibited mechanism**

We then investigated whether GSK-3β might interact with MRTF and whether this process might be modulated by β-catenin and Smad3. Of importance, a recent publication reported that Smad3 and GSK-3β can directly associate (Hua et al., 2010). This raised the intriguing possibility that Smad3 might act as an adaptor recruiting GSK-3β to MRTF and this process might be regulated by β-catenin. Probing MRTF immunoprecipitates with anti-GSK-3β revealed a weak binding of GSK-3β to MRTF that was slightly elevated by the two-hit stimulation (Figure 8A). Downregulation of β-catenin robustly potentiated the stimulus-induced association of GSK-3β with MRTF as early as 30 min after stimulation. In contrast, elimination of Smad3 prevented the basal and stimulus-induced interaction between MRTF and GSK-3β (Figure 8A). Moreover, double silencing of β-catenin and Smad3 revealed that the MRTF–GSK-3β interaction, potentiated by the absence of β-catenin, was preempted in the absence of Smad3 (Figure 8B). To substantiate the role of Smad3 as a potential recruiter of GSK-3β to MRTF, we compared the association of GSK-3β with wild-type (WT) and ∆B1p MRTF, the mutant with diminished Smad3-binding capacity. In β-catenin–depleted cells, the stimulus-induced association of ∆B1p MRTF with GSK-3β was strongly reduced compared with the WT (Figure 8C). Finally, knockdown of Smad3 significantly reduced MRTF degradation in β-catenin–depleted and stimulated cells (Supplementary Figure S3). Taken together, these results implicate β-catenin as a key factor in the maintenance of MRTF stability and suggest that the absence of β-catenin facilitates the Smad3-dependent association of GSK-3β to MRTF, which primes the latter for ubiquitination and degradation (see the scheme in Figure 9C).

**β-Catenin is a key permissive factor for the CArGome**

Having seen the importance of β-catenin in MRTF stability, we considered that β-catenin might be necessary to ensure the expression of a variety of MRTF-dependent genes in addition to SMA. We showed previously (Masszi et al., 2010) that MRTF is required for the basal and/or stimulus-induced expression of several CArG-regulated proteins, including filamin, CapZ, coflin, and SRF itself. Accordingly, we found that β-catenin depletion prevented the two-hit–induced increase in filamin, CapZ, and coflin and decreased the level of SRF both under resting conditions and after stimulation (Figure 9, A and B). These results suggest that β-catenin might be an essential regulator of the CArGome and might explain how β-catenin, independent of its transcriptional effect, can affect the expression of an array of cytoskeletal proteins.

**DISCUSSION**

Injury to the intercellular contacts has emerged as an important contributor to EMT/EMyT (Masszi et al., 2004; Fan et al., 2007; Kim et al., 2009b; Zheng et al., 2009; Tamiya et al., 2010), and the AJ component β-catenin has been shown to promote the myogenic program in the epithelium (Masszi et al., 2004) and other cell types during wound healing (Cheon et al., 2005), organ fibrosis (Surendran et al., 2002; Kim et al., 2009a), tissue specification (Liebner et al., 2004), carcinogenesis (Onder et al., 2008), and hypertrophy (Deng et al., 2008; Gosens et al., 2008). Nonetheless the underlying mechanisms remained undefined. Our studies provide evidence

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**FIGURE 5:** β-Catenin protects the SRF/MRTF myogenic complex. (A) Cells were cotransfected with vectors encoding the indicated tagged proteins for 48 h, followed by lysis and immunoprecipitation of Myc-MRTF using an anti-Myc antibody. Under basal conditions MRTF pulled down SRF (anti-HA, lane 4). Smad3 coexpression reduced the SRF/MRTF interaction, whereas β-catenin coexpression (lanes 2 and 3) restored their association. (B) Confluent monolayers transfected with NR or β-catenin siRNA (48 h) were treated for 30 min with the combined treatment (TGF-β3 + LCM), followed by the immunoprecipitation of endogenous MRTF. (C, D) To induce nuclear MRTF translocation, monolayers transfected with NR or β-catenin were treated with LCM for 30 min. Cells were then costained for MRTF and β-catenin (C) or nuclear extracts were prepared and probed for MRTF (D).
β-catenin is a strong positive regulator of MRTF, which in turn is a master regulator of cytoskeletal genes. We show that β-catenin exerts this effect via (at least) two mechanisms. First, it antagonizes the inhibitory action of Smad3 on MRTF, thereby increasing the interaction between MRTF and SRF. Second, it maintains the stability of MRTF under conditions that are able to enhance MRTF degradation. Our observations can also explain the need for the double hit for SMA expression: contact disassembly induces nuclear translocation of MRTF (Fan et al., 2007; Busche et al., 2008; Sebe et al., 2008) and elevates the level of cytosolic β-catenin (present study; Masszi et al., 2004), whereas TGFβ is required to rescue β-catenin from degradation (via multiple mechanisms) after contact injury (Masszi et al., 2004) and it is also indispensable for the efficient down-regulation of Smad3 (Masszi et al., 2010). The increasing β-catenin/Smad3 ratio then allows nuclear MRTF to exert its transcriptional effects. On the basis of these findings, we propose that β-catenin, as a key modulator of MRTF/SRF signaling, is one of the central links connecting epithelial injury to the expression of cytoskeletal and muscle-specific genes during the phenotypic reprogramming of EMyT.

Studies including our own showed that cell contact disruption induces Rho- and/or Rac-dependent nuclear translocation of MRTF (Fan et al., 2007; Busche et al., 2008; Sebe et al., 2008), but the specific contact type(s) involved were not identified. We found that down-regulation of E-cadherin rendered TGFβ able to induce SMA expression in confluent epithelia and augmented it in subconfluent layers, implicating the AJs. In line with this, recent work (Busche et al., 2010) showed that uncoupling of E-cadherin is the key trigger for MRTF translocation upon calcium switch, whereas the changes in TJ integrity do not play a role. These authors also noted that serum failed to stimulate the SRF reporter in tumor cells that had been forced to express E-cadherin, whereas the absence/dissociation of E-cadherin reestablished serum-responsiveness. Together these data suggest that E-cadherin plays two distinct roles in MRTF regulation. Acute dissociation of the AJs induces Rho/Rac activation, causing MRTF translocation. This is followed by degradation of E-cadherin (Masszi et al., 2004), which terminates this MRTF response. However, the absence of E-cadherin seems to sensitize cells for MRTF translocation or activation by other stimuli (e.g., TGFβ or serum). This could be due to the fact that E-cadherin counteracts TGFβ-induced Rho activation (Cho et al., 2010). In addition, we propose that the loss of E-cadherin contributes to MRTF activation through the liberation of β-catenin, which neutralizes Smad3, a strong inhibitor of MRTF.

The central role of β-catenin in the myogenic program is substantiated by our findings that β-catenin knockdown suppresses the SMA promoter and protein expression induced by AJ disruption or E-cadherin silencing combined with TGFβ treatment. These observations are congruent with data obtained during tumor EMT, showing that elimination of E-cadherin stabilizes free β-catenin and increases the SMA message (Onder et al., 2008). The critical question has been the mechanism by which β-catenin acts. Because extensive cross-talk exists between the β-catenin/TCF-LEF and TGFβ/Smad3 pathways (Attisano and Labbe, 2004) and β-catenin can bind to Smad3, an interaction with Smad3 was a plausible possibility. Indeed, a variety of promoters (Twist, vascular endothelial growth factor, gastrin) contain TCF sites and SBEs in close proximity, and at these loci β-catenin and Smad3 act synergistically. In addition to targeting their own sites, they form active transcriptional complexes (Lei et al., 2004; Clifford et al., 2008; Fuxe et al., 2010). Synergy can also occur when a single cis element (e.g., SBE) is occupied by the Smad3–β-catenin complex, as was reported for the SM22α promoter (Shafer and Towler, 2009). However, none of these mechanisms accounts for the stimulation of the SMA promoter in epithelial cells because 1) it does not harbor a TCF site and 2) although it contains SBEs, overexpression of Smad3, β-catenin, or both of these failed to activate the promoter.

An alternative possibility emerged from our previous studies, showing that Smad3 is a potent inhibitor of MRTF and SMA expression (Masszi et al., 2010). Initially this finding looks counterintuitive since R-Smads are considered chief mediators of fibrogenesis and EMT (Roberts et al., 2006). However, this simplified view is
β-Catenin regulates MRTF stability and ubiquitination in a GSK-3β-dependent manner. (A) Confluent monolayers transfected with NR or β-catenin siRNA (48 h) were exposed for the indicated times to the combined treatment (TGFβ + LCM) and then lysed and probed for the indicated proteins, revealing a marked reduction in the MRTF (BSAC) immunoreactive band in the β-catenin–depleted and stimulated samples. In many experiments this decrease was detectable at 60 min but became significant (and was present in all experiments) after 90 min. (B) Cells were treated as in A following transfection with HA-MRTF. Lysates were probed with an anti-HA antibody. Graphs for A and B, densitometric quantification of the MRTF signal. Inset, quantification of MRTF by another polyclonal anti–MRTF-B antibody (C-19). (C) Confluent monolayers, transfected with NR or β-catenin siRNA (48 h), were pretreated with vehicle, LiCl (15 mM), or a GSK inhibitor (30 μM, GSKi) for 30 min and then left untreated (−) or exposed to the combined treatment for 120 min. Note that inhibition of GSK-3β prevents the loss of the MRTF signal observed in β-catenin–depleted, stimulated cells. (D) Monolayers cotransfected as in B were pretreated with LiCl (15 mM, 30 min) and then exposed for 60 min to the combined treatment. Subsequently anti-HA (MRTF) immunoprecipitates were probed with a polyubiquitin antibody.

FIGURE 7: β-Catenin regulates MRTF stability and ubiquitination in a GSK-3β-dependent manner. (A) Confluent monolayers transfected with NR or β-catenin siRNA (48 h) were exposed for the indicated times to the combined treatment (TGFβ + LCM) and then lysed and probed for the indicated proteins, revealing a marked reduction in the MRTF (BSAC) immunoreactive band in the β-catenin–depleted and stimulated samples. In many experiments this decrease was detectable at 60 min but became significant (and was present in all experiments) after 90 min. (B) Cells were treated as in A following transfection with HA-MRTF. Lysates were probed with an anti-HA antibody. Graphs for A and B, densitometric quantification of the MRTF signal. Inset, quantification of MRTF by another polyclonal anti–MRTF-B antibody (C-19). (C) Confluent monolayers, transfected with NR or β-catenin siRNA (48 h), were pretreated with vehicle, LiCl (15 mM), or a GSK inhibitor (30 μM, GSKi) for 30 min and then left untreated (−) or exposed to the combined treatment for 120 min. Note that inhibition of GSK-3β prevents the loss of the MRTF signal observed in β-catenin–depleted, stimulated cells. (D) Monolayers cotransfected as in B were pretreated with LiCl (15 mM, 30 min) and then exposed for 60 min to the combined treatment. Subsequently anti-HA (MRTF) immunoprecipitates were probed with a polyubiquitin antibody.
certain cells MRTF is constitutively nuclear yet transcriptionally inactive (Elberg et al., 2008). This inactive state is likely maintained by the interaction of MRTF with its negative regulators such as intranuclear G-actin (Vartiainen et al., 2007) or Smad3 (Masszi et al., 2010). Although crucially important, only a few mechanisms have been identified that modulate these negative influences. Thus G-actin affinity of intranuclear MRTF is regulated by MAP kinase–dependent phosphorylation (Muehlich et al., 2008), whereas the present studies demonstrate that the MRTF–Smad3 interaction depends on the availability of β-catenin. Furthermore, although MRTF is indispensable for the induction of the CArGome during EMyT (Fan et al., 2007; Elberg et al., 2008), its net nuclear accumulation is transient and terminates long before SMA expression (Masszi et al., 2010). These observations suggest that contact disruption leads to an MRTF-dependent priming event, and the interactions of MRTF with its partners (e.g., Smad3) during this early, postinjury phase might be critically important for cell fate determination.

The β-catenin–Smad3 complex can exert additional effects that modify EMT and fibrogenesis. Association of β-catenin with Smad3 suppressed SBE-dependent transcription in epithelial cells (Zhang et al., 2007) and promoted TCF/LEF-dependent transcription in chondrocytes (Zhang et al., 2010). Thus the association might mitigate Smad3-dependent but enhance β-catenin–dependent gene transcription, resulting in a more proliferative and myogenic (motile, contractile) phenotype.

Perhaps our most intriguing finding is that β-catenin not only regulates the interactions of MRTF, but it also controls MRTF stability. We propose the following scenario: MRTF degradation is regulated by GSK-3β–mediated phosphorylation followed by ubiquitination. This process is very slow in the presence of normal β-catenin during this early, postinjury phase might be critically important for cell fate determination.
levels but is dramatically enhanced when β-catenin is decreased. This view is supported by our findings that 1) two-hit stimulation drastically reduces MRTF in β-catenin–down-regulated cells; 2) the same conditions provoke ubiquitination of MRTF; and 3) GSK-3β inhibitors prevent both the reduction in the MRTF band and the enhanced ubiquitination. Consistent with our proposal that GSK-3β is a key determinant of MRTF stability, deletion of GSK-3β facilitates SMA expression in fibroblasts (Kapoor et al., 2008); GSK-3β has been shown to phosphorylate myocardin, thereby reducing its transcriptional activity (Badorff et al., 2005); and the E3 ligase CHIP was reported to promote myocardin ubiquitination in a GSK-3β–dependent manner (Xie et al., 2009). Some of the implicated myocardin phosphorylation sites are conserved in MRTF, and MRTF-B dependent manner (Xie et al., 2005); and the E3 ligase CHIP was reported to promote myocardin ubiquitination in a GSK-3β–dependent manner (Xie et al., 2009), adding yet another mechanism (besides direct inhibition and enhanced degradation) by which Smad3 can antagonize myocardin signaling.

Finally we consider the pathophysiological implications of these findings. β-Catenin levels show characteristic changes in fibroblasts during wound healing or fibrosis (Cheon et al., 2002, 2005; Surendran et al., 2005), with a postinjury rise followed by a gradual decrease if tissue restoration occurs. These changes might be accentuated in the epithelium, where AJ injury can also increase free β-catenin. The fate of free β-catenin depends on its stability, which is promoted by fibrogenic and myogenic stimuli, such as Wnt proteins and TGFβ. We propose that an initial rise in β-catenin keeps fibrogenic Smad3 signaling in check and maintains MRTF activity. This could contribute to normal healing by facilitating wound closure (contractility; Tomasek et al., 2006) and reepithelialization. Later the decrease in β-catenin allows the termination of MRTF signaling. However, during dysregulated healing, β-catenin–stabilizing inputs become persistent, sustaining MRTF. Indeed, we found that the expression of several CArGome proteins depends on β-catenin. Because β-catenin– and MRTF-dependent promoters drive many genes involved in matrix production and MF differentiation, the final outcome (healing vs. fibrosis) might depend on the delicate interplay among β-catenin, Smad3, and MRTF/SRF signaling.

In summary, we have defined novel mechanisms by which the integrity of intercellular contacts, through a network of β-catenin–controlled interactions, regulates MRTF-dependent transcription and thus the expression of a multitude of key cytoskeletal proteins. These mechanisms likely play key roles in normal healing, EMT/EMyT, and tissue fibrosis.

**MATERIALS AND METHODS

**Reagents**

The GSK-3β inhibitor SB-216763 was purchased from Sigma-Aldrich (St. Louis, MO) and TGFβ from R&D Systems (Minneapolis, MN). Commercially available antibodies were obtained from various sources as follows: MRTF-B (C-19), SRF (G-20), HA (Y-11), c-Myc (9E10), β-catenin (C-18), GSK-3α/β (0011-A), and GAPDH (0411), Santa Cruz Biotechnology (Santa Cruz, CA); FLAG (M2) and SMA (1A4), Sigma-Aldrich; E-cadherin, CapZ α, and filament A (clone 5/ A5B-280), BD Transduction Laboratories (Lexington, KY); SMAD3, Abcam (Cambridge, MA); HA.11 clone 16B12, Covance (Berkeley, CA); histones (clone P152.C25.WJ1) and ubiquitin (P4D1), Millipore...
Cell culture and treatment

LLC-PK1 (Cl 4) cells, a porcine proximal tubular epithelial cell line (a kind gift from R. C. Harris, Vanderbilt University School of Medicine, Nashville, TN) were cultured in low-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin solution (Invitrogen) as in our previous studies (Masszi et al., 2004, 2010). Rat proximal tubular cells (NRK-52E) were purchased from American Type Culture Collection (Manassas, VA) and cultured in high-glucose DMEM containing the same supplements as mentioned. Cells were incubated under serum-free conditions for at least 3 h before various treatments. To induce cell contact disassembly, cells were thoroughly washed with phosphate-buffered saline (PBS; Invitrogen) and cultured in nominally calcium chloride-free DMEM (LCM; Invitrogen). Where indicated, cells were treated with TGFβ (10 ng/ml for luciferase reporter assays and 4–10 ng/ml for other experiments).

Plasmids and transfection

The p765-SMA-Luc reporter construct containing the proximal 765–base pair portion of the rat SMA promoter in a pGL3-basic vector (WT), the constructs harboring an inactivating mutation at the SBE1 or SBE2 sites (SBE1mut and SBE2mut) (Hu et al., 2003), and the p152-SMA-Luc reporter (provided by S. H. Phan, University of Michigan, Ann Arbor, MI) were described in our previous studies (Masszi et al., 2010). Using SBE1mut as a template, we performed PCR-based mutagenesis to create subsequent mutations in SBE2 and the TCE resulting in the triple-mutant Luc construct, as described (Masszi et al., 2010). Briefly, the mutations (in parentheses) and the corresponding primer pairs were as follows:

- SBE2 (C\(^{+15}/T\), A\(^{+16}/G\), and G\(^{+17}/C\)), 5'–CCACCCACCTGCAGTG-GAGAACCCACGC–3' and 5'–CTGGGCTTCTCCACTGGCAGGT-GGGTGTG–3'
- TCE (T\(^{−53}/C\), G\(^{−52}/T\), and G\(^{−50}/C\)), 5'-TGAGACGAGGGGACGCAGGGATCACAGCA–3' and 5'-TGGTCTGATCCCCTGCAGCTCG-GTTCACC–3'

The thymidine kinase minimal promoter-driven Renilla luciferase internal control plasmid, pRL-TK, was purchased from Promega (Madison, WI). The LEF/TCF reporter plasmid, TOPFlash, was obtained from Upstate (Millipore). The N-terminally Myc- or FLAG-tagged Smad3 expression constructs (in pcMV5B) were a kind gift from L. Attisano (University of Toronto). The FLAG-tagged β-catenin was provided by E. R. Fearon (University of Michigan, Ann Arbor, MI) (Kolligs et al., 1999). The FLAG-tagged MRTF-B plasmid was provided by E. N. Olson (University of Texas Southwestern Medical Center, Dallas, TX). The coding region of MRTF-B was amplified by PCR and cloned into pcDNA3.1/Myc–His A to obtain the myc-MRTF-B expression vector. pcDNA3.1/HA-MRTF-B was generated by engineering a 2xHA tag at the N-terminus of MRTF-B using standard PCR methodology. The B1 region–deletion mutant of HA-MRTF-B (ΔB1) was then constructed using primer pairs complementary to regions upstream and downstream of the specific deletion, as described previously (Masszi et al., 2010). The final construct contained a seven–amino acid (S279–P285 inclusive) deletion. PCR reactions were performed using PfuTurbo (Agilent Technologies, Santa Clara, CA). All constructs were verified by sequencing. The pCGN-SRF plasmid encoding HA-tagged human SRF generated by the Prywes lab (Johansen and Prywes, 1993) was obtained through Addgene (Cambridge, MA). Depending on the experiment, cells were transfected using FuGENE 6 (Roche Applied Science, Indianapolis, IN), Lipofectamine 2000 (Invitrogen), or jetPRIME (Polyplus-transfection SA, Illkirch, France) reagents as in our previous studies (Masszi et al., 2004, 2010; Fan et al., 2007) and (for jetPRIME) according to the manufacturer’s recommendation.

Luciferase reporter assays

Luciferase reporter assays were performed as described in our previous studies (Masszi et al., 2003, 2004) using 0.5–μg/well luciferase construct, 0.05–μg/well pRL-TK, and varying amounts of empty carrier or expression vector. Sixteen hours later, cells were serum starved for 3 h, treated for 24 h (if not indicated otherwise), and lysed and the luciferase activity was determined using the Dual Luciferase Reporter Assay System Kit (Promega). For each condition, treatments were performed in duplicate, and experiments were repeated at least three times. From each sample, the firefly luciferase activity corresponding to a specific promoter construct was normalized to the Renilla luciferase activity of the same sample. Results are expressed as fold changes compared with the mean firefly/Renilla ratio of the untreated controls taken as a unit.

RNA interference

Optimal target sequences were determined using the siRNA Target Finder program (Applied Biosystems, Foster City, CA). The siRNA sequences used in the present experiments were as follows: pig β-catenin siRNA, 5’-AAGUAACAUCCACCUACUCG-3’; pig SMAD3 siRNA, 5’-AAGATTCACTGCCACATTCT-3’; and pig E-cadherin siRNA, 5’-CTCCTGCTGTTGTGATTAT-3’. The validated siRNA against rat β-catenin was obtained from Dharmacon (Lafayette, CO). Alternative siRNAs were also designed and used for the down-regulation of each of the aforementioned proteins. The siRNAs directed against different sequences of the corresponding mRNA gave identical experimental results. Silencer Negative Control #2 siRNA was purchased from Applied Biosystems. LLC-PK1 cells were cultured in antibiotic-free growth medium and transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen). DNA/siRNA cotransfections were performed using jetPRIME.

Western blotting and coimmunoprecipitation

Following treatments, cells were lysed with Triton x-100 buffer (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 100 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 20 mM NaF, and 1% Triton X-100) supplemented with 1 mM Na\(_2\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, and Complete Mini Protease Inhibitor Cocktail (Roche). SDS–PAGE and Western blotting were performed on equivalent protein loads, as determined using BCA Protein Assay Reagents (Thermo Scientific, Waltham, MA). Prior to coimmunoprecipitation studies, cell lysates were spun at 12,000 rpm for 5 min to remove cell debris. Precleared supernatants were incubated with appropriate antibodies, and immunocomplexes were captured on protein G–agarose beads (Thermo Scientific). Bound proteins were eluted from the washed beads and analyzed by Western blotting. Antibody-free and lysate-free controls were routinely included to confirm specificity of the immunoprecipitated proteins. Aliquots of each input were run in parallel to monitor expression levels. Densitometry was performed with a GS800 densitometer using Quantity One software (Bio-Rad Laboratories, Hercules, CA).
Nuclear extraction

Nuclear extracts were prepared from confluent layers of LLC-PK1 cells grown on 6-cm dishes using the NE-PER Nuclear Extraction Kit (Thermo Scientific). Equivalent amounts of protein from each extract were analyzed by Western blotting. Equal loading of nuclear protein was monitored by probing with an anti-histone antibody.

Immunofluorescence microscopy

Cells plated on glass coverslips were fixed with 4% paraformaldehyde (Canemco & Marivac, Gere, Canada) for 30 min, washed with PBS, and quenched with 100 mM glycine/PBS for 10 min. Cells were permeabilized for 20 min in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA), blocked in 3% BSA for 1 h, and incubated with primary antibody for an additional 1 h. Washed coverslips were incubated with the corresponding fluorescently labeled secondary antibody, which included the addition of 4',6-diamidino-2-phenylindole (Lonza, Basel, Switzerland) for nuclear labeling. When staining for E-cadherin, cells were fixed with cooled methanol for 5 min, washed with PBS, and blocked with 3% BSA prior to immunostaining. Coverslips were mounted on slides using fluorescent mounting medium (Dako, Grostrup, Denmark). Samples were analyzed using a microscope (IX81; Olympus, Center Valley, PA) with a UPlan S-Apo 60x 1.42 numerical aperture oil objective (Olympus) coupled to a camera (Evolution QX1 Monochrome; Media Cybernetics, Bethesda, MD) controlled by imaging software (QED In Vivo; Media Cybernetics). Images were processed using ImagePro Plus software (3DS 5.1; Media Cybernetics). Modifications were restricted exclusively to minor adjustments of brightness/contrast. Cytoplasmic beta-catenin staining was quantified using the MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA). Briefly, the mean fluorescence intensity was determined in circular regions (6.5 μm diameter) randomly placed in the cytoplasmic (extranuclear) area in 50 cells. Data were normalized to the background fluorescence determined in a cell-free area on the same coverslip. The average ratio of all measurements in untreated controls was taken as 1 and compared with the ratio obtained in cells exposed to the combined treatment (LCM plus TGFβ) for 6 h.

mRNA analysis

LLC-PK1 cells were transfected with pig specific beta-catenin or NR siRNA using Lipofectamine RNAiMAX. After 48 h, cells were serum deprived for 3 h and treated with TGFβ and LCM for an additional 48 h. RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA), and cDNA was synthesized from 1 μg of total RNA using iScript reverse transcriptase (Bio-Rad Laboratories). SYBR green–based real-time PCR was used to evaluate gene expression of SMA, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference standard. Primer pairs designed against known pig sequences were as follows:

- SMA, 5’-TGTGACAAATGGTTGGCTGTTGT-3’ and 5’-CTTGGT-CACCCACGTAGCTGTTTT-3’
- GAPDH, 5’-GCCAAGTTGACATTTGTCGCCCATCA-3’ and 5’-AG-CTTCCATTCTCAGCCTGACT-3’

Statistical analysis

Data are presented as representative blots or images from at least three similar experiments or as the means ± SEM for the number of experiments indicated. Statistical significance was determined by one-way analysis of variance (Tukey or Dunn post hoc testing for parametric and nonparametric analysis of variance, as appropriate), using Prism and the InStat software GraphPad, La Jolla, CA. p < 0.05 was accepted as significant; *p < 0.05 and **p < 0.01.

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REFERENCES

Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA (2009). Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. J Clin Invest 119, 1438–1449.
Artisano L, Labbe E (2004). TGFbeta and Wnt pathway cross-talk. Cancer Metastasis Rev 23, 53–61.
Badorff C, Seeger FH, Zeiher AM, Dimmeler S (2005). Glycogen synthase kinase 3beta inhibits myocardin-dependent transcription and hypertrophy induction through site-specific phosphorylation. Circ Res 97, 645–654.
Bowley E, O’Gorman DB, Gan BS (2007). Beta-catenin signaling in fibroproliferative disease. J Surg Res 138, 141–150.
Busche S, Desco A, Julien S, Genth H, Poser G (2008). Epithelial cell-cell contacts regulate SRF-mediated transcription via Rac-actin-MAL signaling. J Cell Sci 121, 1025–1035.
Busche S, Kremmer E, Poser G (2010). E-cadherin regulates MAL-SRF-mediated transcription in epithelial cells. J Cell Sci 123, 2803–2809.
Cheon S, Poon R, Yu C, Khoury M, Shenker R, Fish J, Alman BA (2005). Prolonged beta-catenin stabilization and tcf-dependent transcriptional activation in hyperplastic cutaneous wounds. Lab Invest 85, 416–425.
Cheon SS, Chew AH, Turley S, Nadesan P, Poon R, Clevens H, Alman BA (2002). beta-Catenin stabilization dysregulates mesenchymal cell proliferation, motility, and invasiveness and causes aggressive fibromas and hyperplastic cutaneous wounds. Proc Natl Acad Sci USA 99, 6973–6978.
Cho U, Kim YW, Han CY, Kim EH, Anderson RA, Lee YS, Lee CH, Hwang SJ, Kim SG (2010). E-Cadherin antagonizes transforming growth factor beta1 gene induction in hepatic stellate cells by inhibiting RhoA-dependent Smad3 phosphorylation. Hepatology 52, 2053–2064.
Clifford RL, Deacon K, Knox AJ (2008). Novel regulation of vascular endothelial growth factor-A (VEGF-A) by transforming growth factor beta; requirement for Smads, beta-CATENIN, and GSK3beta. J Biol Chem 283, 35337–35353.
Deng H, Dokshin GA, Lei J, Goldsmith AM, Bitar KN, Fingar DC, Hershenson MB, Bentley JK (2008). Inhibition of glycogen synthase kinase 3beta is sufficient for airway smooth muscle hypertrophy. J Biol Chem 283, 10198–10207.
Doble BW, Woodgett JR (2003). GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci 116, 1175–1186.
Du KL, Chen M, Li J, Lepore JJ, Mericok P, Parmacek MS (2004). Megakaryoblastic leukemia factor-1 transduces cytoskeletal signals and induces smooth muscle cell differentiation from undifferentiated embryonic stem cells. J Biol Chem 279, 17578–17586.
Elberg G, Chen L, Elberg D, Chan MD, Logan CJ, Turman MA (2008). MKL1 mediates TGF-beta-1-induced alpha-smooth muscle actin expression in human renal epithelial cells. Am J Physiol Renal Physiol 294, F1116–F1128.
Fan L et al. (2007). Cell contact-dependent regulation of epithelial-myofibroblast transition via the rho–rho kinase–phospho-myosin pathway. Mol Biol Cell 18, 1083–1097.
Fuxe J, Vincent T, de Herreros AG (2010). Transcriptional crosstalk between TGFbeta and stem cell pathways in tumor cell invasion: role of EMT promoting Smad complexes. Cell Cycle 9.
Gosens R, Meurs H, Schmidt M (2008). The GSK-3/beta-catenin-signalling pathway promotes Smad complexes. Cell Cycle 9.
Hua F, Zhou J, Liu J, Zhu C, Bai B, Lin H, Liu Y, Jin W, Yang H, Hu Z (2010). Prolonged beta-catenin stabilization and tcf-dependent transcriptional activation in hyperplastic cutaneous wounds. Lab Invest 85, 416–425.
Johansen FE, Prywes R (1993). Identification of transcriptional activation and inhibitory domains in serum response factor (SRF) by using GAL4-SRF constructs. Mol Cell Biol 13, 4640–4647.
Kalluri R, Weinberg RA (2009). The basics of epithelial-mesenchymal transition. J Clin Invest 119, 1420–1428.

Kamaraju AK, Roberts AB (2005). Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-beta-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo. J Biol Chem 280, 1024–1036.

Kapoor M, Liu S, Shi-wen X, Huh K, McCann M, Denton CP, Woodgett JR, Abraham DJ, Leask A (2008). GSK-3 beta in mouse fibroblasts controls wound healing and fibrosis through an endothelin-1-dependent mechanism. J Clin Invest 118, 3279–3290.

Kim KK et al. (2009a). Epithelial cell alpha3beta1 integrin links beta-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. J Clin Invest 119, 213–224.

Kim Y, Kugler MC, Wei Y, Kim KK, Li X, Brumwell AN, Chapman HA (2009b). Integrin alpha3beta1-dependent beta-catenin phosphorylation links epithelial Smad signaling to cell contacts. J Cell Biol 184, 309–322.

Kolligs FT, Hu G, Dang CV, Fearon ER (1999). Neoplastic transformation of RK3E by mutant beta-catenin requires deregulation of Tcf/LeF transcription but not activation of c-myc expression. Mol Cell Biol 19, 5696–5706.

Lei S, Dubeykovskiy A, Chakladar A, Wojtukiewicz L, Wang TC (2004). The murine gastrin promoter is synergistically activated by transforming growth factor-beta/Smad and Wnt signaling pathways. J Biol Chem 279, 42492–42502.

Liebner S, Cattelino A, Gallini R, Rudini N, Iurlaro M, Piccolo S, Dejana E (2004). Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. J Cell Biol 166, 359–367.

Liu Y (2010). New insights into epithelial-mesenchymal transition in kidney fibrosis. J Am Soc Nephrol 21, 212–222.

Masszi A, Di Ciano S, Siromgany M, Arthur WT, Rotstein OD, Wang J, McIlloch CA, Rossilv L, Mucsi I, Kapus A (2003). Central role for Rho in TGF-beta1-induced alpha-smooth muscle actin expression during epithelial-mesenchymal transition. Am J Physiol Renal Physiol 284, P119–P122.

Masszi A, Fan L, Rossilv L, McIlloch CA, Rotstein OD, Mucsi I, Kapus A (2004). Integrity of cell-cell contacts is a critical regulator of TGF-beta 1-induced epithelial-to-myofibroblast transition: role for beta-catenin. Am J Pathol 165, 1955–1967.

Meng XM, Huang XR, Chung AC, Qin W, Shao X, Igarashi P, Ju W, Bottinger EP, Lan HY (2010). Smad2 protects against TGF-beta/Smad3-mediated renal fibrosis. J Am Soc Nephrol 21, 1477–1484.

Miralles F, Posem G, Zaronytidou AI, Treisman R (2003). Actin dynamics control SRF activity by regulation of its coactivator MAL. Cell 113, 329–342.

Morita T, Mayanagi T, Sobue K (2007). Dual roles of myocardin-related transcription factors in epithelial-mesenchymal transition and proliferation of retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 51, 2755–2763.

Tian YC, Phillips AO (2002). Interaction between the transforming growth factor-beta type II receptor/Smad pathway and beta-catenin during transforming growth factor-beta1-mediated adhered junction disassembly. Am J Pathol 160, 1619–1628.

Tomasek JJ, McRae J, Owens GK, Haakmsa CJ (2005). Regulation of alpha-smooth muscle actin expression in granulation tissue myofibroblasts is dependent on the intrinsic CARG element and the transforming growth factor-beta1 control element. Am J Pathol 166, 1343–1351.

Tomasek JJ, Vaughan MB, Kropp BP, Gabbiani G, Martin MD, Haakmsa CJ, Hinz B (2004). Contribution of myofibroblasts in granulation tissue is dependent on Rho/Rho kinase/myosin light chain phosphatase activity. Wound Repair Regen 12, 313–320.

Vartainen MK, Guettler S, Larjani B, Treisman R (2007). Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL. Science 316, 1749–1752.

Wang DZ, Li S, Hockemeyer D, Sutherland L, Wang Z, Schratt G, Richardson JA, Nordheim A, Olson EN (2002). Potentiation of serum response factor activity by a family of myocardin-related transcription factors. Proc Natl Acad Sci USA 99, 14855–14860.

Wendt MK, Allington TM, Schiemann WP (2009). Mechanisms of the epithelial-mesenchymal transition by TGF-beta. Future Oncol 5, 1145–1168.

Xie R, Fan Y, Zhang H, Zhang Y, She M, Gu D, Patterson C, Li H (2009). CHIP represses myocardin-induced smooth muscle cell differentiation via ubiquitin-mediated proteasomal degradation. Mol Cell Biol 29, 2398–2408.

Xie WB, Li Z, Miano JM, Long X, Chen SY (2011). Smad3-mediated myocardin silencing, a novel mechanism governing the initiation of smooth muscle differentiation. J Biol Chem 286, 15050–15057.

Zaronytidou AI, Miralles F, Treisman R (2008). MAL and ternary complex factor use different mechanisms to contact a common surface on the serum response factor DNA-binding domain. Mol Cell Biol 28, 6302–6313.

Zhang M, Wang M, Tan X, Li TF, Zhang Y, Chen D (2010). Smad3 prevents beta-catenin degradation and facilitates beta-catenin nuclear translocation in chondrocytes. J Biol Chem 285, 8703–8710.

Zhao Y, Geer DA (2002). Regulation of Smad3 expression in bleomycin-induced pulmonary fibrosis: a negative feedback loop of TGF-beta signaling. Biochem Biophys Res Commun 294, 319–326.

Zhu X et al. (2003). Linking actin dynamics and gene transcription to drive cellular motile functions. Nat Rev Mol Cell Biol 4, 317–330.

Samasaj T et al. (2002). Identification of a novel transcriptional activator, BSAC, by a functional cloning to inhibit tumor necrosis factor-induced cell death. J Biol Chem 277, 28853–28860.