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 (TGFβ) 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 CC(A/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."**

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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 central mechanism in the pathogenesis of organ fibrosis (Kalluri and Weinberg, 2009). During fibrogenic (type 2) EMT, epithelial cells lose their strong intercellular contacts and apicobasal polarity and acquire fibroblast-like features, such as elongated shape, increased extracellular matrix (ECM) production, and motility (Zeisberg and Neilson, 2009). Moreover, EMT can progress further toward the activation of a *myogenic program*, resulting in the transdifferentiation of epithelial cells to myofibroblasts (MFs), marked by the expression of α-smooth muscle actin (SMA) (Ng *et al.*, 1998; Masszi *et al.*, 2003). To distinguish this robust form of EMT, we introduced the term epithelial–myofibroblast transition (EMyT; Masszi *et al.*, 2010).

Transforming growth factor-β1 (TGFβ) is the chief inducer of EMT/EMyT, which contributes to all aspects of the process, including the down-regulation of epithelial cell contact molecules (e.g., the adherens junction [AJ] constituent E-cadherin), the up-regulation of ECM components (e.g., collagen, fibronectin), and the de novo induction of mesenchymal/MF proteins such as fibroblast-specific

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Abbreviations used: CArG, CC(A/T)-rich GG element; EMT, epithelial–mesenchymal transition; EMyT, epithelial–myofibroblast transition; GSK-3β, glycogen synthase kinase-3β; LCM, low-calcium medium; MF, myofibroblast; MRTF, myocardin-related transcription factor; NR, nonrelated; SBE, Smad-binding element; siRNA, small interfering RNA; SMA, α-smooth muscle actin; SRF, serum response factor.

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; Samarin *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 $CC(A/T)_6GG$ *cis* elements (CArG boxes) present in the promoters of a large array of muscle-type and cytoskeletal genes (the "CArGome") (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 cells than in the NR siRNA-transfected controls (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

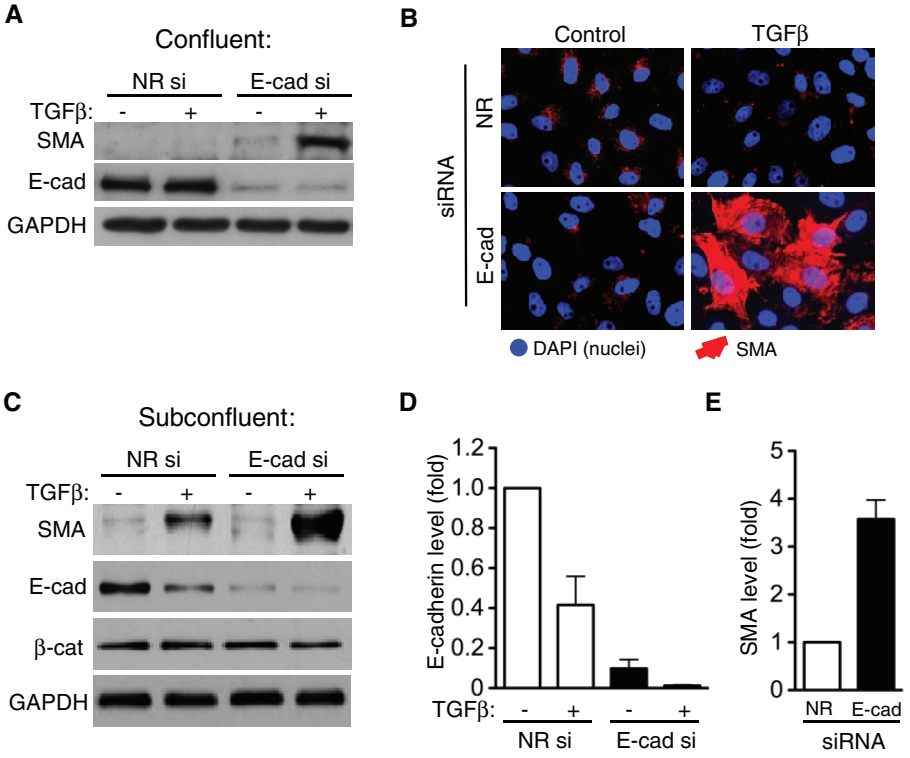

FIGURE 1: E-Cadherin down-regulation facilitates TGFβ-induced SMA expression. (A) LLC-PK1 cells were transfected with NR or E-cadherin siRNA (E-cad si, 50 nM) and 24 h later, when confluent, treated with TGFβ (10 ng/ml) for 72 h. Cell lysates were prepared and probed by Western blotting (WB) for the indicated proteins. (B) Confluent monolayers transfected with NR or E-cadherin siRNA were treated with TGFβ for 72 h, immunostained for SMA, and visualized by fluorescence microscopy. (C) After transfection with NR or E-cadherin siRNA, subconfluent monolayers (70%) were treated with TGFβ for 72 h. Cell lysates were probed for the indicated proteins. (D) Densitometric quantification of GAPDH-normalized E-cadherin levels. (E) Densitometric quantification of the effect of E-cadherin down-regulation on SMA expression in subconfluent, TGFβ-treated monolayers, as shown in C.

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 2, F and G). Similarly, stimulation of the cells by the two-hit regimen (TGFβ plus LCM) also caused a threefold increase in cytosolic β-catenin, along with the dissipation of the peripheral β-catenin signal (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

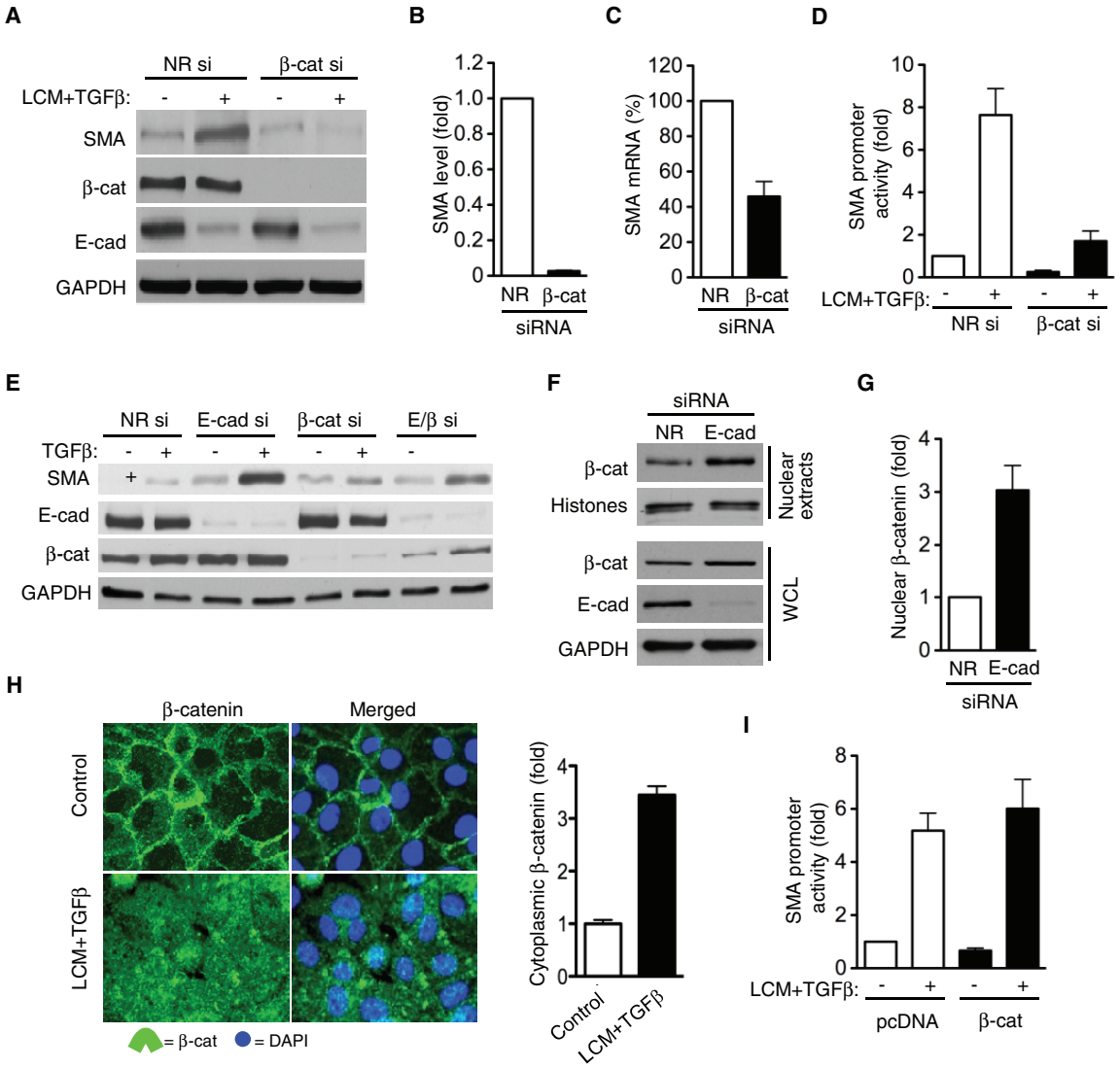

FIGURE 2: β-Catenin is a crucial permissive regulator of SMA expression. (A) Confluent LLC-PK1 cells transfected with NR or β-catenin siRNA (β-cat si, 100 nM) were exposed to combined treatment (TGFβ + LCM) for 48 h. Cell lysates were probed by WB for the indicated proteins. (B) The effect of the β-catenin 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.

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

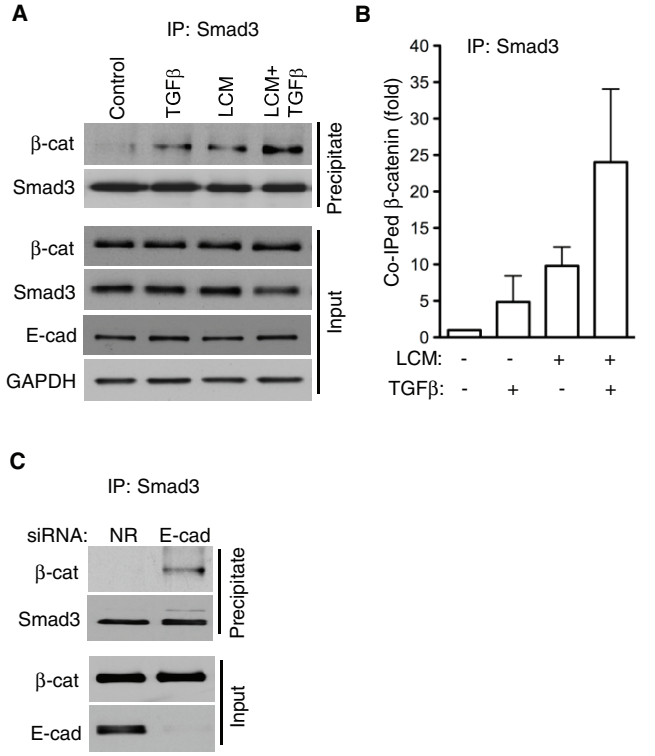

FIGURE 3: TGFβ and LCM facilitate the association of Smad3 and β-catenin. (A) Confluent cells were treated for 1 h as indicated, followed by immunoprecipitation of Smad3. The precipitates and cell lysate inputs were subjected to WB and probed for the indicated proteins. (B) Densitometric quantification of the coprecipitated β-catenin under various conditions. (C) E-cadherin silencing (50 nM siRNA for 24 h) is sufficient to facilitate the association of β-catenin with Smad3.

construct, which includes the two CArGs and the TCE but lacks both SBEs and the E-box, the target site for basic helix-loop-helix transcription factors (Figure 4C, bottom). Together these data show that β-catenin reverses the inhibitory effect of Smad3 on MRTF-driven transcription, and this effect is mediated via the CArG boxes.

β-Catenin maintains the SRF/MRTF (myogenic) complex

To investigate whether β-catenin can preserve the interaction between SRF and MRTF, cells were cotransfected with hemagglutinin (HA)-SRF and Myc-MRTF along with FLAG–β-catenin, FLAG-Smad3, or both (Figure 5A). When only MRTF and SRF were coexpressed, immunoprecipitation of MRTF (through the tag) pulled down a sizable amount of SRF (lane 4). Overexpression of Smad3 nearly abolished the association between SRF and MRTF (lane 1). Of importance, β-catenin overexpression fully restored the MRTF/SRF interaction (lanes 2 and 3).

To validate these observations with regard to the endogenous proteins, we investigated the association of SRF with MRTF in control or β-catenin–down-regulated cells with or without stimulation. Suppression of β-catenin had a dramatic effect on the myogenic complex: namely, it disrupted the association between MRTF and SRF under basal conditions and prevented any increase upon TGFβ plus LCM (30 min) stimulation (Figure 5B). Identical results were obtained using NRK-52E rat tubular cells, implying that the need for β-catenin for the SRF/MRTF association is a general phenomenon (Supplementary Figure S2C).

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 SRF. 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).

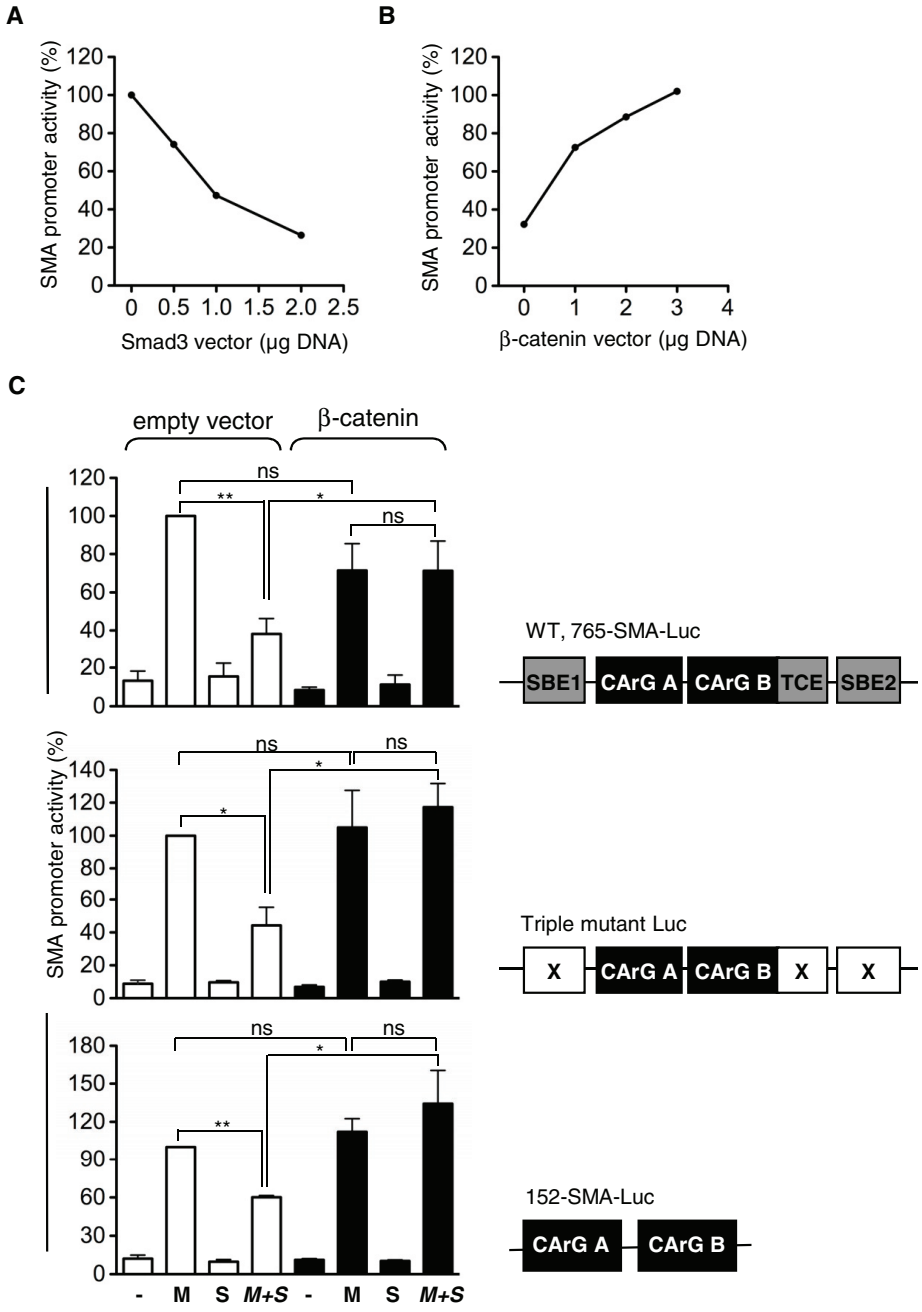

FIGURE 4: β-Catenin counteracts the inhibitory action of Smad3 on the SMA promoter through SBE-independent, CArG-dependent mechanism. The inhibitory effect of Smad3 on the SMA promoter was titrated by cotransfecting various amounts of Smad3 expression plasmid with MRTF (0.5 μg) and the SMA-Luc/pRL-TK 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).

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. In contrast, in β-catenin–silenced cells the HA signal still dropped below the level observed in the NR siRNA-transfected cells. However this decrease started later and became substantial (usually ~80%) only after ≥2 h of stimulation (Figure 7B). From these results we conclude that the early phase of the signal reduction is likely due to epitope masking by modification, whereas the later phase reflects (at least in part) reduction in MRTF protein expression.

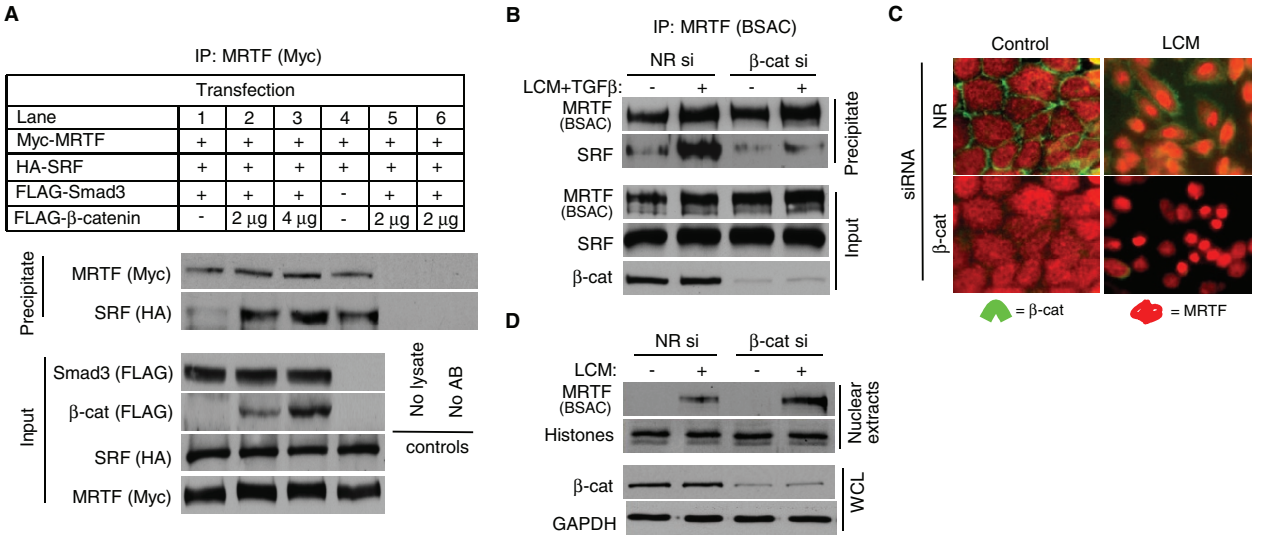

FIGURE 5: β-Catenin protects the SRF/MRTF myogenic complex. (A) Cells were cotransfected with vectors encoding the indicated tagged proteins for 48 h, followed by lysis and immunoprecipitation of Myc-MRTF using an anti-Myc antibody. Under basal conditions MRTF pulled down SRF (anti-HA, lane 4). Smad3 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 (TGFβ + LCM), followed by the immunoprecipitation of endogenous MRTF. (C, D) To induce nuclear MRTF translocation, monolayers transfected as in B 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).

Glycogen synthase kinase-3β (GSK-3β) has been reported to phosphorylate and inhibit myocardin (Badorff *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 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 ΔB1p MRTF, the mutant with diminished Smad3-binding capacity. In β-catenin–depleted cells, the stimulus-induced association of ΔB1p 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, cofilin, and SRF itself. Accordingly, we found that β-catenin depletion prevented the two-hit–induced increase in filamin, CapZ, and cofilin 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

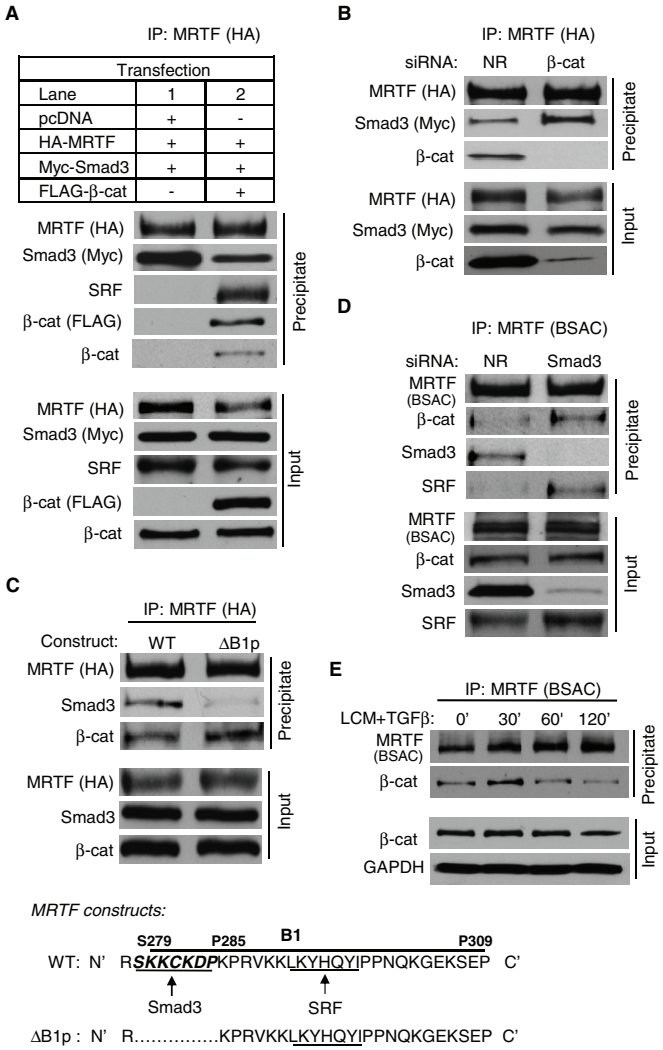

FIGURE 6: β-Catenin inhibits the association of Smad3 with MRTF. (A) Cells were cotransfected with the indicated vectors for 24 h, followed by immunoprecipitation of HA-MRTF. The precipitates and the input were probed for the indicated proteins. (B) Cells were transfected with NR or β-catenin siRNA for 24 h and then cotransfected with the indicated vectors for another 24 h. Similar coprecipitation experiments were performed as in A. (C) WT or ΔB1p HA-MRTF (which has impaired Smad3-binding capacity) were expressed and immunoprecipitated by an anti-HA antibody. Coprecipitating proteins were detected by WB. (D) Endogenous MRTF was precipitated with the BSAC antibody from cells that had been pretreated with NR or Smad3 siRNA (50 nM) for 48 h. Coprecipitating proteins were detected by WB. (E) Confluent cells were exposed to TGFβ plus LCM for the indicated times, followed by MRTF immunoprecipitation. The precipitates were probed for β-catenin.

that 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

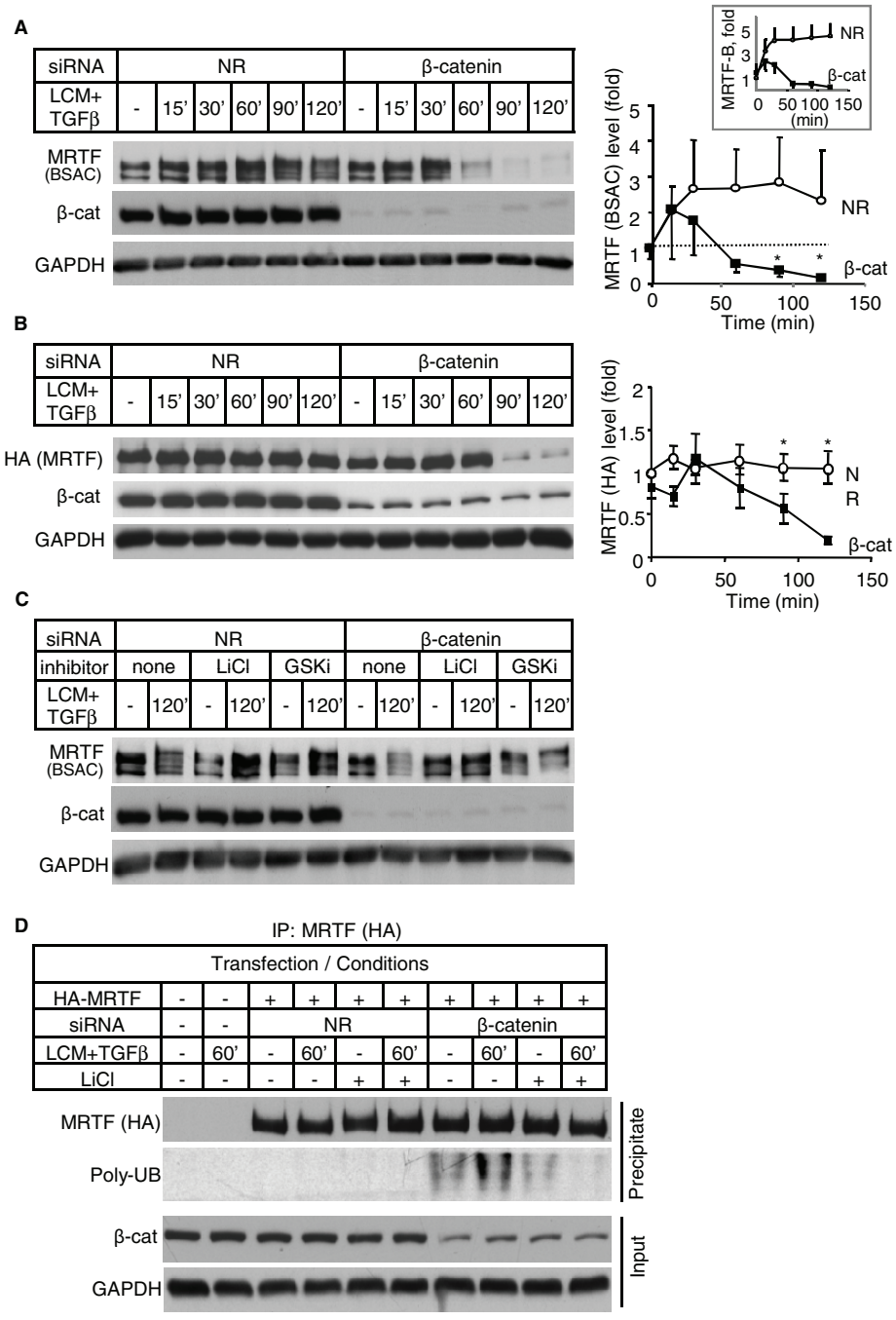

FIGURE 7: β-Catenin regulates MRTF stability and ubiquitination in a GSK-3β–dependent manner. (A) Confluent monolayers transfected with NR or β-catenin siRNA (48 h) were exposed for the indicated times to the combined treatment (TGFβ + LCM) and then lysed and probed for the indicated proteins, revealing 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.

controversial since the progression of EMT, fibrosis, and MF accumulation is often accompanied by a *reduction* in Smad3 (or Smad2) levels (Zhao and Geverd, 2002; Poncelet *et al.*, 2007; Meng *et al.*, 2010), and suppression of Smad3 can actually facilitate SMA expression (Masszi *et al.*, 2010); for a review see Masszi and Kapus (2011). These facts and the demonstration that Smad3 can bind to (Morita *et al.*, 2007) and inhibit (Masszi *et al.*, 2010) MRTF led to the new view that Smad3 is an inducer of the fibrogenic phase but an inhibitor/delayer of the myogenic phase of EMyT. Accordingly, our present work shows that β-catenin acts by preventing the Smad3-mediated inhibition of MRTF. β-Catenin and Smad3 bind together and mutually inhibit each other's interactions with MRTF. Conversely, the absence of β-catenin enhances the MRTF–Smad3 binding and destroys the myogenic complex (SRF–MRTF). It was recently reported (Zhang *et al.*, 2010) that the N-terminal and armadillo domain of β-catenin associates with the C-terminal region of Smad3. It is intriguing that the same C-terminal region is critical for MRTF binding (Morita *et al.*, 2007).

Although both TGFβ and LCM enhanced β-catenin–Smad3 association, the underlying mechanisms remain to be elucidated. Beside the possibility of increased β-catenin availability, C-terminal or linker-region phosphorylation of Smad3 (Kamaraju and Roberts, 2005) and/or serine dephosphorylation or tyrosine phosphorylation of β-catenin (Doble and Woodgett, 2003) might also contribute. Because total E-cadherin levels do not change within the first hour after the combined treatment, at this early time post-translational modification of β-catenin and/or Smad3 may be the predominant signal. Later the absence of E-cadherin likely contributes to the increase in the available β-catenin pool. Of interest, tyrosine phosphorylated β-catenin has been shown to associate with phospho-Smad2 during contact injury and integrin-dependent EMT, and these proteins colocalized in epithelium-derived MFs in the lungs of patients with pulmonary fibrosis (Kim *et al.*, 2009a, 2009b). However, the mechanism by which this complex would promote MF generation was not defined. Our findings raise the possibility that under the same conditions Smad3 or the Smad3/Smad2 complex can also associate with β-catenin, which leads to the disinhibition of MRTF.

Consistent with these views, MRTF translocation is necessary but not sufficient for the induction of MRTF/SRF target genes, as in

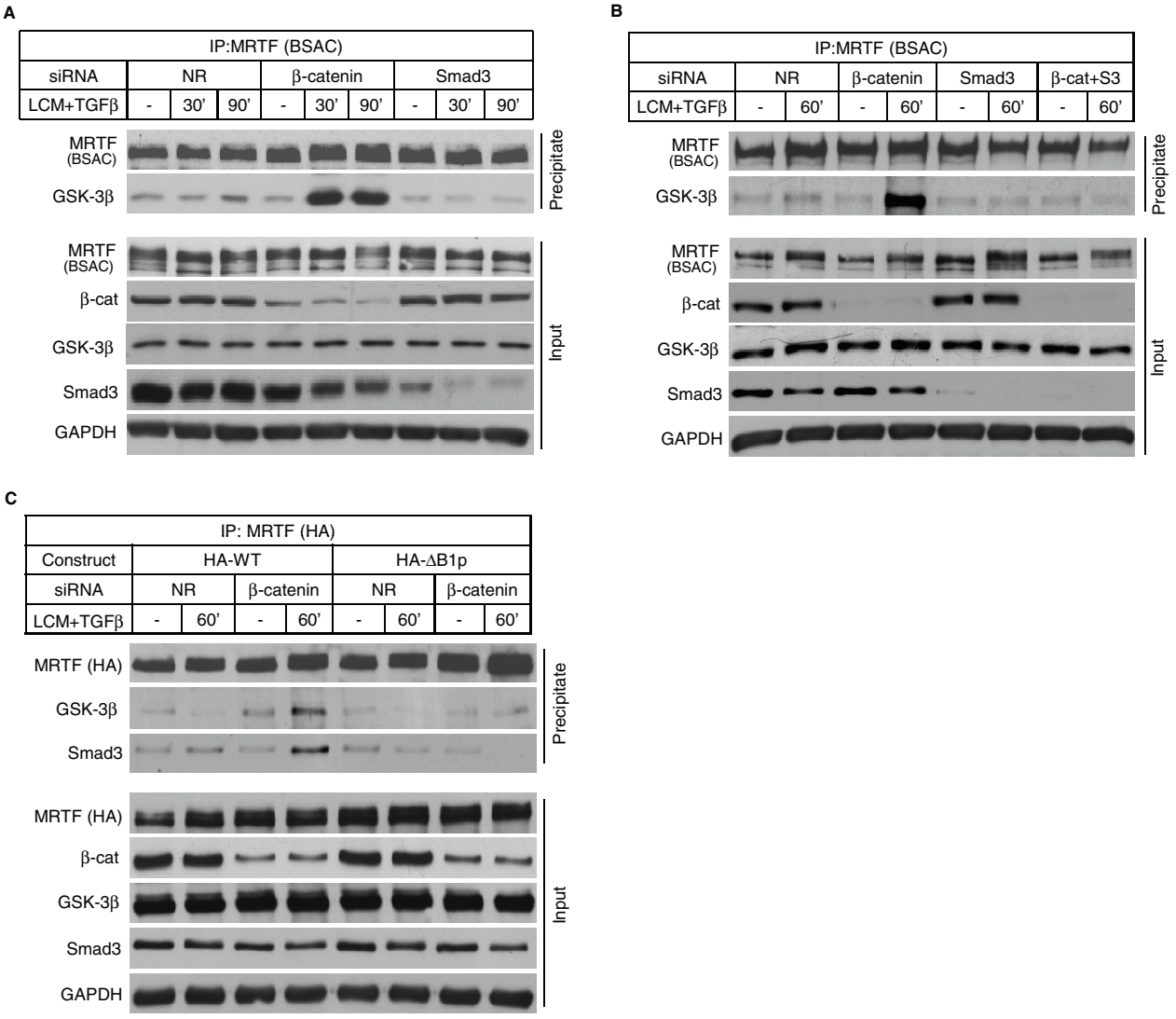

FIGURE 8: Smad3 recruits GSK-3β to MRTF and is necessary for MRTF degradation. (A) Monolayers transfected with NR, β-catenin, or Smad3 siRNA (48 h) were exposed for various times to the combined treatment. Cells were then lysed, MRTF was immunoprecipitated, and the precipitates and inputs were probed for GSK-3β and other indicated proteins. (B) Endogenous MRTF was immunoprecipitated from cells that had been transfected with various siRNAs (NR, β-catenin, Smad3, or β-catenin + Smad3) and then treated with vehicle (–) or TGFβ plus LCM for 60 min. Note that the MRTF/GSK-3β association, seen in β-catenin–depleted and stimulated cells, is abolished by the concomitant down-regulation of Smad3. (C) Smad3-binding capacity of MRTF is critical for the recruitment of GSK-3β to MRTF. Cells transfected with NR or β-catenin siRNA and WT or ΔB1p (Smad3-binding–deficient mutant) HA-MRTF were exposed for 60 min to the combined treatment. Anti-HA immunoprecipitates and inputs were probed for the indicated proteins.

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

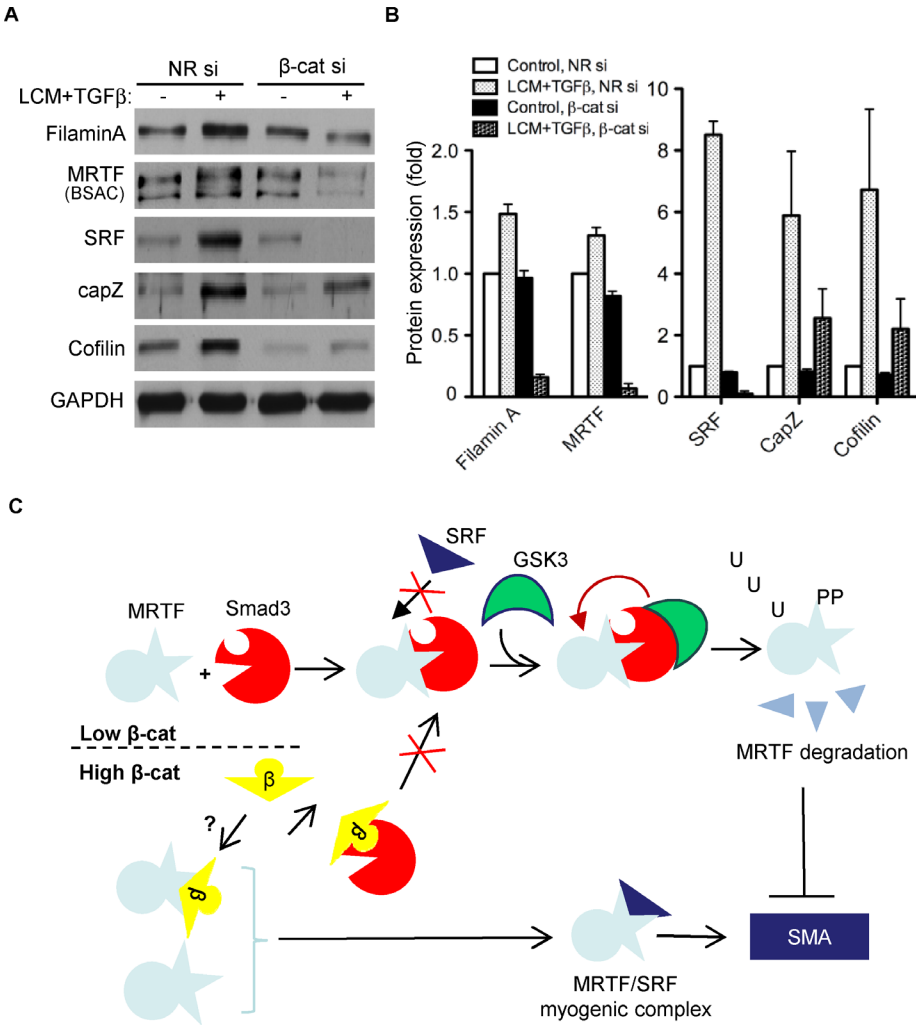

FIGURE 9: β-Catenin maintains expression of CArG-dependent proteins. Cells transfected with NR or β-catenin siRNA (100 nM, 24 h) were left untreated or exposed for 48 h to TGFβ plus LCM. Cell lysates were prepared and probed for a panel of CArG-dependent proteins. (B) Densitometric quantification of the implicated CArG proteins. (C) Proposed model of the β-catenin– and Smad3-dependent regulation of MRTF. At high β-catenin levels, Smad3 associates with β-catenin, which prevents the Smad3-induced disruption of the myogenic complex (MRTF/SRF) and maintains MRTF activity. At lower β-catenin levels, Smad3 associates with MRTF and inhibits the formation of the myogenic complex. Moreover, Smad3 recruits GSK-3β, which in turn promotes MRTF degradation.

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 contains other potential GSK-3β consensus motives as well. Verification of these and the relevant E3 ligase(s) warrants further studies.

How does a reduction in β-catenin promote MRTF degradation? We propose that Smad3 recruits GSK-3β to MRTF, and this process is counteracted by β-catenin. Indeed, Smad3 has been reported to bind to GSK-3β through its MH2 domain (Hua *et al.*, 2010), and we found that the absence of Smad3 suppresses GSK-3β/MRTF association and subsequent MRTF degradation in β-catenin–depleted cells. Recently Smad3 was shown to suppress the myocardin gene (Xie *et al.*, 2011), 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 filamin A (clone 5/ABP-280), BD Transduction Laboratories (Lexington, KY); SMAD3, Abcam (Cambridge, MA); HA.11 clone 16B12, Covance (Berkeley, CA); histones (clone F152.C25.WJJ) and ubiquitin (P4D1), Millipore

(Billerica, MA); and cofilin, Cell Signaling Technology (Beverley, MA). Rabbit polyclonal anti-MRTF (BSAC) was described previously (Sasazuki *et al.*, 2002). All secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

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 Medical School, 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′-CCACCCACCTGCAGTGGAGAAGCCCAGC-3′ and 5′- CTGGGCTTCTCCACTGCAGGTGGGTGGT-3′

TCE (T^{-53}/C, G^{-52}/T, and G^{-50}/C), 5′-TGGGAAGCGAGCTGCAGGGGATCAGACCA-3′ and 5′-TGGTCTGATCCCCTGCAGCTCGCTTCCCA-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-tagged 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′-AAGUACAUACACCAUACUACG-3′; pig SMAD3 siRNA, 5′-AAGAGTTCACTCCACATTCTC-3′; and pig E-cadherin siRNA, 5′-CTCTGCTGGTGTTTGATTATT-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 lysis 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_3VO_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, Gore, 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, Glostrup, Denmark). Samples were analyzed using a microscope (IX81; Olympus, Center Valley, PA) with a UPlan S-Apo 60× 1.42 numerical aperture oil objective (Olympus) coupled to a camera (Evolution QEi Monochrome; Media Cybernetics, Bethesda, MD) controlled by imaging software (QED InVivo; 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 β-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 cytosplasmic (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′-TGTGACAATGGTTCTGGGCTCTGT-3′ and 5′-TTCGTCACCCACGTAGCTGTCTTT-3′
GAPDH, 5′-GCAAAGTGGACATGGTCGCCATCA-3′ and 5′-AGCTTCCCATTCTCAGCCTTGACT-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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