Differential topical susceptibility to TGFβ in intact and injured regions of the epithelium: key role in myofibroblast transition

Pam Speighta, Hiroyasu Nakanob, Thomas J. Kelleyc, Boris Hinzd, and András Kapusa

aKeenan Research Centre, Li Ka Shing Knowledge Institute, St. Michael’s Hospital, and Department of Surgery, University of Toronto, Toronto, ON M5B 1W8, Canada; bDepartment of Immunology, Juntendo University School of Medicine, Tokyo 113-8421, Japan; cDivision of Pediatric Pulmonology, Case Western Reserve University, Cleveland, OH 44106; dLaboratory of Tissue Repair and Regeneration, Matrix Dynamics Group, Faculty of Dentistry, University of Toronto, Toronto, ON M5S 3E2, Canada

ABSTRACT Induction of epithelial–myofibroblast transition (EMyT), a robust fibrogenic phenotype change hallmarked by α-smooth muscle actin (SMA) expression, requires transforming growth factor-β1 (TGFβ) and the absence/uncoupling of intracellular contacts. This suggests that an “injured” epithelium may be topically susceptible to TGFβ. To explore this concept, we use an epithelial wound model in which intact and contact-deprived regions of the same monolayer can be analyzed simultaneously. We show that TGFβ elicits dramatically different responses at these two loci. SMA expression and initially enhanced nuclear Smad3 accumulation followed by Smad3 mRNA and protein down-regulation occur exclusively at the wound. Mechanistically, three transcriptional coactivators whose localization is regulated by cell contact integrity are critical for these local effects. These are myocardin-related transcription factor (MRTF), the driver of the SMA promoter; β-catenin, which counteracts the known inhibitory effect of Smad3 on MRTF and maintains MRTF protein stability and mRNA expression in the wound; and TAZ, a Hippo effector and Smad3 retention factor. Remarkably, active TAZ stimulates the SMA and suppresses the Smad3 promoter, whereas TAZ silencing prevents wound-restricted expression of SMA and loss of Smad3. Such locus-specific reprogramming might play key roles in wound healing and the susceptibility of the injured epithelium to fibrogenesis.

INTRODUCTION Epithelial–mesenchymal transition (EMT) has been implicated as a key mechanism in cell fate determination during development, regeneration, carcinogenesis, and organ fibrosis (Kalluri and Weinberg, 2009; Thiery et al., 2009; Heldin et al., 2012; Katsuno et al., 2013). The most advanced form of EMT is epithelial–myofibroblast transition (EMyT), during which epithelial cells not only lose their apico-basal polarity, polygonal shape, and strong intercellular contacts, but also acquire a myofibroblast phenotype, characterized by the expression of α-smooth muscle actin (SMA; Masszi and Kapus, 2011). The source and regulation of SMA during fibrogenesis have gained special importance for two reasons. First, the amount of SMA in fibrosing organs (e.g., lung, kidney) shows strong correlation with disease severity and progression (Jinde et al., 2001; Yang and Liu, 2001), and second, not only is SMA a marker of organ fibrosis, it is also a contributor to the process, since it enhances cell contractility (Hinz et al., 2001). Contractility is a key factor in pathological extracellular matrix remodeling and the mechanochemical activation of transforming growth factor-β1 (TGFβ), the major inducer of both EMT/EMyT and fibrosis (Wipff et al., 2007).
Although a vast literature suggests that EMT contributes to the formation of fibroblasts and myofibroblasts, the involvement and relative importance of this process in various models of fibrosis and in fibroproliferative diseases has been questioned and is a subject of intensive ongoing debate (for reviews see Zeisberg and Duffield, 2010; Quaggin and Kapus, 2011). There is no doubt that a large variety of epithelial cells possess the capacity to undergo EMT and even EMyT (Fan et al., 1999; Masszi et al., 2003, 2004; Kim et al., 2009; Humphreys et al., 2010). Thus, from a cell biological standpoint, the central challenge is to define the particular conditions or triggering factors (and the associated signaling events) that can unleash this potential, leading to full phenotypic reprogramming and ultimately the activation of a myogenic program (SMA expression) in the epithelium. In this regard, we (Masszi et al., 2004, 2010; Fan et al., 2007) and others (Kim et al., 2009; Zheng et al., 2009) have shown that TGFβ is often not sufficient to transform an intact epithelium into SMA-positive mesenchymal cells. The other key input is the disruption of intercellular contacts (Masszi et al., 2004, 2010; Fan et al., 2007; Kim et al., 2009; Zheng et al., 2009; Tamiya et al., 2010), suggesting that cell junctions are not simply passive targets, but are active regulators of EMT. Moreover, acute contact injury was mechanistically linked to SMA expression. Previous studies, including our own, indicate that contact uncoupling leads to the activation of Rho-family small GTPases, which in turn induce the nuclear translocation of myocardin-related transcription factor (MRTF; Charbonney et al., 2010). However, contact disruption itself, which was modeled by uncoupling the adherens junctions with low-calcium medium (LCM), is also insufficient to induce SMA expression, indicating that mobilization of the myogenic program requires subtle interplay between TGFβ- and contact-dependent signaling.

In the search for the mechanisms underlying this synergy during EMyT, our previous studies revealed that the TGFβ-regulated signal transducer Smad3 has a dual role in the process. Whereas Smad3 is indispensable for mesenchymal transition and matrix production, it is also an inhibitor of MRTF. Moreover, Smad3 expression markedly drops during EMyT, and small interfering RNA (siRNA)-mediated Smad3 silencing allows injury in the absence of TGFβ to induce SMA expression (Masszi et al., 2010). On the basis of these findings, we suggested that EMyT can be dissected into a Smad3-promoted and a Smad3-inhibited phase and that Smad3 acts as a timer/delay in the process. We also found that cell contacts regulate EMyT not only through Rho-mediated MRTF traffic, but also through the adherens junction component β-catenin (Masszi et al., 2004; Charbonney et al., 2011). Specifically, β-catenin facilitates EMyT, presumably by both preventing the direct negative effect of Smad3 on MRTF and maintaining MRTF protein expression, which in the absence of β-catenin is dramatically reduced through a Smad3-dependent process (Charbonney et al., 2011).

Taken together, these findings raise the concept of topical susceptibility to TGFβ, suggesting that the intact areas of an epithelium might be resistant, whereas the contact-deprived regions might be sensitive, to the transforming capacity of this cytokine. In this work we set out to test this idea. Our previous approach was not sufficient to prove the existence of such a phenomenon since LCM was used to disrupt the junctions, and calcium deprivation is known to alter a variety of signaling processes (e.g., integrin activation; McDowall et al., 1998). Moreover, the use of LCM did not allow us to distinguish the responsiveness of the intact and injured (contact-deprived) areas of the same epithelial layer. In addition, although our previous work indicated interplay among Smad3, β-catenin, and MRTF in SMA expression, it was unknown whether any of these key players is regulated at the transcriptional level and whether such regulation might occur in a topically differential manner.

To address these questions, we developed a cellular wound model (using normal calcium conditions), in which wound-associated and intact areas of the same epithelial layer can be analyzed simultaneously in the presence or absence of TGFβ. This enabled us to define which responses depend on the individual application of wounding, TGFβ, or combination of these stimuli. Our results show dramatic locus-specific differences in the effect of TGFβ, which manifest at the level of both gene transcription and protein stability. Furthermore, we provide evidence that the Hippo factor TAZ plays a critical role in these topical changes.

RESULTS

Differential effects of TGFβ on the expression of SMA and key regulators of the myogenic program in intact and contact-deprived regions of the epithelial monolayer

To assess the topical susceptibility of the intact and “injured” parts of the epithelium to TGFβ and study the role of cell contacts in this phenomenon, we generated a model system in which changes in gene and protein expression can be studied simultaneously at these two loci. We grew cells to confluence in a tissue culture dish, at the middle of which the formation of intercellular contacts was prevented by surgical tape (Figure 1A). This method allowed us to establish a well-defined “wound” model (or gap model), characterized by the absence of intercellular contacts without the additional effects of cellular breakage and the efflux of intracellular constituents. Once confluent, the monolayer was either left untreated or exposed to TGFβ, and at given times 2-mm-wide cellular strips were collected from regions either directly adjacent to the wound (referred to as wounded cells [W]) or from the intact area (I cells, >10 mm away from the wound). In this way, four different sample types were obtained, designated as I (intact), W (wound), I+T (intact area exposed to TGFβ), and W+T (wound exposed to TGFβ).

We then assessed the effect of wounding and TGFβ alone or in combination within the same monolayer on the transcription of selected genes, coding for markers or key regulators of the myogenic program as shown by us and others (Fan et al., 2007; Busche et al., 2008; Masszi et al., 2010). We investigated SMA, the hallmark of the myofibroblast phenotype; MRTF, a central regulator (transcriptional coactivator) of the SMA promoter; Smad3, which is critical for mesenchymal transition but delays the myogenic program by interfering with MRTF (Masszi et al., 2010); and β-catenin, which promotes both the mesenchymal and the myogenic phase of EMyT (Charbonney et al., 2011). We monitored the changes in mRNA levels both at early times (6 h) and at the peak of myofibroblast transition (72 h) after TGFβ treatment.

To facilitate topological comparisons of the effects, for each investigated gene we expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—normalized mRNA levels as a fold change compared with the value obtained in the untreated, intact area (I) of the monolayer. Short-term (6 h) exposure to TGFβ in either the I or the W region did not cause any major change in the mRNA levels of these genes (Supplemental Figure S1A). However, by 72 h marked and site-specific changes developed (Figure 1B). Wounding alone did not induce SMA mRNA. TGFβ caused a large increase in SMA message, which was restricted almost exclusively to the wound (Figure 1B). Of interest, the reciprocal outcome was observed for...
Smad3 mRNA, which showed a strong (~75%) decrease only in the wound exposed to TGFβ (W+T). Wounding alone or TGFβ acting on the intact region of the monolayer had no effect. The β-catenin message was sensitive to each condition. Specifically, the wound region exhibited a significant drop in β-catenin mRNA (~50%) compared with the level detected in the intact area. TGFβ treatment induced a modest increase in the intact part of the monolayer and, of importance, it reversed the loss observed in the untreated wound region. In other words, in the presence of TGFβ the loss of cell contacts not only failed to reduce the level of β-catenin mRNA, but it also potentiated the positive, β-catenin–inducing effect of the cytokine. Because we proposed that Smad3 is a negative, whereas β-catenin is a positive, regulator of MRTF-induced SMA expression (Masszi et al., 2010; Charbonney et al., 2011), we plotted the Smad3/β-catenin mRNA ratio as observed under the various conditions against the corresponding SMA mRNA. This resulted in a near-perfect hyperbola (Figure 1C), supporting a reciprocal relationship between these parameters. We next investigated MRTF mRNA levels at the two loci in the presence or absence of TGFβ (Figure 1B). The MRTF message did not exhibit major changes compared with the level seen in the untreated, intact monolayer, suggesting that this critical regulator may not be (primarily) controlled at the transcriptional level during EMyT. It is worth noting that the investigated genes showed similar changes when contact disruption was achieved by scratch wounding (as opposed to inhibiting the formation of the contacts), although under these conditions the “wound edge” is much more difficult to define due to the unevenness of the injured area and ensuing cell migration (not shown). Taken together, these results imply that the intact and injured areas of the epithelium show dramatically different transcriptional responsiveness with respect to SMA and the most critical regulators of the myogenic program.

Topical differences in the expression of proteins associated with myofibroblast transition

Our previous results suggest that alterations in protein stability also contribute to the steady-state expression of myogenic regulators in EMyT (Charbonney et al., 2011). Therefore we sought to determine how the observed mRNA changes correlate with the expression of the corresponding proteins. To this end we performed Western blot analysis of samples obtained at both loci either from untreated layers or after short- or long-term TGFβ treatment corresponding to the early or peak phases of EMyT. No SMA protein was expressed at either location in the absence of or after 6-h TGFβ exposure, nor was any site-specific difference observed in Smad3, β-catenin, or MRTF protein levels (Supplemental Figure S1B). After 72 h (Figure 2A), we detected robust SMA expression exclusively in the TGFβ-exposed wound. Of importance, Smad3 protein expression was strongly reduced in the TGFβ-treated wound region compared with the untreated control, whereas no major change was observed in the wound without TGFβ treatment or in the intact area exposed to TGFβ. β-Catenin protein expression was markedly increased in the TGFβ-treated wound sample and appeared modestly elevated after the individual stimuli. MRTF protein levels did not seem to significantly differ under any condition.

To obtain a finer spatial resolution of the susceptible area, we determined the expression of SMA and its regulators as a function of distance from the wound edge in TGFβ-treated monolayers. All values were normalized to those detected at the wound edge in each individual experiment (Figure 2B). SMA was robustly expressed directly along the wound edge and was detectable at low levels at a distance of ~5 mm toward the intact area but not further.
Increased β-catenin expression was also restricted to the close vicinity of the wound. With this autocontrolled approach we could also detect a modest increase in MRTF protein, starting at ≤10 mm from the wound edge. Finally, as expected, Smad3 showed a robust drop at the wound compared with samples collected from areas 5 mm or more away from the edge. These sharp differences were also documented for SMA and Smad3 using immunofluorescence microscopy (Figure 2C). SMA was highly expressed and organized in thick fibers only in cells directly adjacent to the wound edge. Whereas SMA-positive patches were frequent along the wound, not the entirety of the edge showed SMA expression, indicating that even at this locus certain cells or groups of cells are more responsive than others. Analyzing these “hot spots,” we detected a sharp rise in SMA staining right at the edge encompassing a 20- to 40-μm-wide strip (one or two cell rows) and exhibiting a similarly sharp decay thereafter. Occasionally we saw areas with a slight increase in cytosolic staining without clear SMA-positive stress fibers in the subsequent row(s) as well, which likely accounts for the modest expression detected by Western blots at 5 mm from the wound. The change in Smad3 expression was more gradual, in that several rows of cells at the edge exhibited decreased Smad3 staining in the responsive areas, and the signal climbed from very low levels to a steady-state plateau in ∼120–140 μm (Figure 2C).

Taken together, these results indicate that the protein levels of SMA and key regulators of the myogenic program show strong site-specific differences, which are in good agreement with (but do not completely mirror; see Discussion) the corresponding changes observed at the transcriptional level.

**β-Catenin is essential for maintaining wound-specific MRTF expression**

Because MRTF is indispensable for SMA expression (Masszi et al., 2010) and our recent studies implicated β-catenin as a regulator of MRTF stability (Charbonney et al., 2011), we sought to determine whether β-catenin affects MRTF expression in our model and, if so, whether this occurs in a topically differential manner. To this end, we transfected confluent monolayers harboring a central wound region with nonrelated (NR) or β-catenin–specific siRNA for 24 h and then left them untreated or exposed to TGFβ for 72 h. The specific siRNA completely eliminated β-catenin protein expression both in the intact area and in the wound (Figure 3A). Concomitantly, it prevented TGFβ-induced SMA mRNA and protein expression and reduced MRTF protein expression in the wound (Figure 3, A and B), indicating that β-catenin is an absolute requisite for the myogenic response. To assess the kinetics of the loss of MRTF, we compared the changes in MRTF protein expression in the intact and wound areas after 24 and 72 h of TGFβ exposure (Figure 3C). In the intact region there was no substantive difference in MRTF expression after short- or long-term TGFβ treatment, irrespective of whether β-catenin was down-regulated. Whereas β-catenin silencing did not alter MRTF protein levels in the wound at 24 h, it did cause a significant reduction by 72 h, pointing to a wound-specific, gradually developing, β-catenin–counteracted loss.

Next we addressed the mechanisms by which β-catenin affects MRTF expression. We suggested in our previous work that β-catenin might stabilize MRTF by preventing Smad3 from recruiting glycogen kinase synthase-3β (GSK-3β) to MRTF. The association of GSK-3β with MRTF promotes MRTF phosphorylation, which facilitates its degradation (Charbonney et al., 2011). To test whether such a mechanism might operate specifically in the wound region, we first transfected the monolayers with nonrelated or β-catenin–specific siRNA and then exposed them to TGFβ for 24 h. This timing was chosen to ensure that the expression levels of MRTF and Smad3 were not yet affected. Subsequently we immunoprecipitated MRTF from the intact and wound areas and then probed the precipitates for cosedimenting Smad3 and GSK-3β (Figure 3D). In β-catenin–expressing (NR siRNA-transfected) cells, we observed a detectable but modest amount of both proteins in the MRTF precipitates obtained from the wound, whereas the association seen in samples collected from the intact region was missing or marginal. Remarkably, down-regulation of β-catenin caused a 6.5-fold increase in the association of GSK-3β with MRTF, indicating that even at this locus certain cells or groups of cells are more responsive than others.
with MRTF, specifically in the wound. Although these results imply that β-catenin promotes MRTF protein stability in the wound, they do not exclude the possibility that it may also affect MRTF expression. To test for such an additional mechanism, we measured MRTF mRNA as well. We noted that β-catenin depletion significantly reduced MRTF mRNA in the TGFβ-treated wound samples as compared with the corresponding control (Figure 3E). However, this cannot be the sole reason for the drop observed in MRTF protein expression, since in β-catenin-depleted and TGFβ-treated cells the reduction in mRNA was similar in the intact and wound regions (Figure 3E), yet the loss of MRTF protein was markedly more in the wounded area compared with the corresponding control (Figure 3E). However, this can be the sole reason for the drop observed in MRTF protein expression. To test for such an additional mechanism, we measured MRTF mRNA as well. We noted that β-catenin depletion significantly reduced MRTF mRNA in the TGFβ-treated wound samples as compared with the corresponding control (Figure 3E). However, this cannot be the sole reason for the drop observed in MRTF protein expression, since in β-catenin-depleted and TGFβ-treated cells the reduction in mRNA was similar in the intact and wound regions (Figure 3E), yet the loss of MRTF protein was markedly more in the latter (Figure 3C). Together these data suggest that β-catenin regulates both MRTF protein stability and transcription and these effects are critical for localized MRTF expression and TGFβ-induced myofibroblast transition at the wound.

**Differential traffic of Smad3, TAZ, and MRTF at the wound region**

Although the decrease in Smad3 expression in the TGFβ-treated wound was in good agreement with our previous studies suggesting that Smad3 is a negative regulator of MRTF, it was also somewhat surprising since low cell density or low calcium-mediated contact disruption have been reported to facilitate Smad3 signaling (Varelas et al., 2010). The proposed mechanism is that these stimuli promote the nuclear translocation of the Hippo pathway transcriptional regulators TAZ and YAP, which in turn act as nuclear retention factors for Smad2/3, thereby facilitating their accumulation and effects (Varelas et al., 2008, 2010). To test whether a similar mechanism is present in the near-wound areas and whether nuclear Smad3 levels follow a biphasic pattern, we compared TGFβ-induced Smad3 translocation at the wound edge and in the intact area after 6 h of stimulation (Figure 4A). In the absence of TGFβ, Smad3 was cytosolic in both the intact and wound regions. We often observed slight perinuclear accumulation with clear nuclear exclusion. After 6 h of TGFβ stimulation, there was a dramatic difference in the distribution of Smad3 in the wound versus the intact region. Cells along the wound showed pronounced nuclear Smad3 accumulation, whereas cells residing in the intact area exhibited only somewhat enhanced perinuclear labeling (Figure 4A). Pronounced nuclear accumulation was not restricted to a single cell row but was present in several rows behind the edge, reminiscent of the belt from which Smad3 disappeared upon long-term stimulation (Figure 2C). It is worth noting that intact epithelial monolayers are responsive to Smad3 signaling in terms of Smad3 phosphorylation, translocation, and transcriptional responses (Varelas et al., 2008, 2010; Masszi et al., 2010; Supplemental Figure S2). However, Smad3 retention is strongly potentiated by the absence of cell contacts (Varelas et al., 2008, 2010). Moreover, early, enhanced Smad3 responsiveness may underlie subsequent Smad3 down-regulation as well. Indeed, nuclear accumulation and posttranslational modification of Smad3 were recently reported to be prerequisite for Smad3 degradation (see Discussion).

We next tested the distribution of TAZ under the same conditions (Figure 4B). TAZ was cytosolic in the intact region, with occasional peripheral accumulation, and TGFβ did not cause any significant change. In contrast, the wound region exhibited marked nuclear TAZ accumulation, which was present in several rows behind the edge, and this effect was accentuated by TGFβ. This distribution was fully consistent with enhanced nuclear Smad3 retention at early times.

Finally we examined the localization of MRTF (Figure 4C). As expected, in the intact region MRTF was cytosolic both without and with TGFβ treatment, as evidenced by the negative staining of the nuclei (nuclear exclusion). In contrast, MRTF showed a clear nuclear shift at the wound edge, which was markedly augmented by TGFβ. Strong nuclear accumulation over the cytosolic level was mostly restricted to the front row, where SMA expression was observed at

**FIGURE 3:** β-Catenin is essential for maintaining MRTF stability and mRNA expression in the wound. (A) Cells were transfected with NR or β-catenin–specific siRNA and treated with or without TGFβ for 72 h. Samples collected from the wound edge or the intact area were probed for the indicated proteins. (B) SMA mRNA analysis performed in samples prepared as in A. (C) To assess the time dependence of MRTF down-regulation in β-catenin–depleted cells, cultures were transfected as in A and treated with TGFβ for 24 or 72 h. MRTF expression, normalized to GAPDH, was quantified by densitometry. (D) After transfection with NR or β-catenin siRNA as in A, cultures were exposed to TGFβ for 24 h. Cells collected from indicated areas were subjected to immunoprecipitation using an anti-MRTF antibody. The precipitates were probed for MRTF and coprecipitating GSK-3β and Smad3. The graph shows densitometric quantification of the cosedimented GSK-3β with MRTF (mean ± SEM; n = 3). (E) MRTF mRNA analysis performed in samples prepared as in A.
alternative, we tested the effect of active and constitutively nuclear TAZ (S89A) on these promoters. For these cotransfection experiments, where the wound edge cannot be targeted specifically, we used our two-hit scheme (LCM and TGFβ; Masszi et al., 2004, 2010).

Surprisingly, expression of active TAZ induced a four- to fivefold activation of the SMA promoter and potentiated its activation to the same extent by the individual and combined effect of TGFβ and LCM, attaining an overall >40-fold stimulation under the two-hit conditions (Figure 5C). Moreover, overexpression of active TAZ inhibited the Smad3 promoter by 80% (Figure 5D).

To test whether TAZ indeed regulates SMA and Smad3 mRNA expression in the context of the wound model, we transfected the cells with NR or TAZ-specific siRNA and measured the message for these proteins at both loci with or without TGFβ treatment. As expected, the specific siRNA efficiently suppressed the message for TAZ under all conditions (Figure 6A). Remarkably, down-regulation of TAZ completely eliminated the induction of SMA mRNA (Figure 6B) and prevented the loss of Smad3 mRNA (Figure 6C) in the TGFβ-exposed wound. Thus TAZ is necessary for locus-specific SMA expression and Smad3 down-regulation.

TAZ is necessary for locus-specific SMA expression and Smad3 down-regulation

In search for the mechanism underlying the concomitant SMA mRNA expression and Smad3 mRNA suppression, first we considered that MRTF might inhibit Smad3 transcription. To assess this possibility, we overexpressed MRTF along with either an SMA or a Smad3 promoter luciferase construct. Although MRTF overexpression drove the SMA promoter 25-fold (testifying to its activity; Figure 5A), it failed to affect the Smad3 promoter (Figure 5B). As an
DISCUSSION

Our studies provide evidence that a “wound”—that is, a subcompartment of the epithelium characterized by the partial absence of intercellular contacts—is a locus distinctly susceptible to the tissue-reprogramming, myofibroblast-inducing effect of TGFβ. Compared to the intact regions of the monolayer, this site-specific sensitivity manifests in 1) spatially restricted expression of SMA; 2) initially enhanced nuclear Smad3 translocation followed by robust Smad3 down-regulation; 3) augmented and prolonged nuclear uptake of MRTF (Masszi et al., 2010); and 4) a selective need for β-catenin to maintain MRTF expression. We show that this local susceptibility is detectable at multiple levels, including transcription factor localization, pretranscriptional and transcriptional control (i.e., protein–protein interactions between transcription factors and their activity on their cis-elements), and protein stability. Because epithelial injury is a major trigger for fibrogenic changes (Chapman, 2011; Wu et al., 2013), we propose that such topical susceptibility of the contact-deprived regions of the epithelium may in part explain why fibrosis develops as a focal process in which myofibroblastic foci are interspersed with normal areas. Because many inflammatory cytokines and other injurious factors disrupt cell contacts by altering the perijunctional cytoskeleton (reviewed in Ivanov et al., 2010), they may profoundly influence the local sensitivity to fibrogenic stimuli. Of importance, the role of contacts is likely not restricted to the epithelium: our ongoing studies suggest that decreased interconnectedness and cell density promotes the transformation of pericytes as well, which have been recently proposed to be major myofibroblast precursors in certain fibrosis models (Humphreys et al., 2010; Campanholle et al., 2013). Similarly, cell density inversely correlates with SMA expression in fibroblasts (Masur et al., 1996; Michalik et al., 2011). These findings imply that the absence of contacts (even without acute contact disruption and accompanying structural injury) is sufficient to predispose cells to TGFβ-induced phenotypic transition. In addition, various cell types may exhibit a differential threshold for myofibroblast transition (lower for mesenchymal cells than the epithelium), and the contribution of the different precursors may depend on disease type and stage.

What are the molecular mechanisms by which the absence or disassembly of cell contacts synergizes with TGFβ-triggered myofibroblast formation? Our results suggest interplay among three contact- and cytoskeleton-dependent transcriptional coactivators and Smad3 (Figure 7). Previous studies showed that contact disruption stimulates RhoA and Rac, which in turn induce (transient) nuclear translocation of MRTF (Fan et al., 2007; Busche et al., 2008). TGFβ prolongs the nuclear accumulation of MRTF by a yet-unknown mechanism. However, the activity of MRTF on the SMA promoter is not simply regulated by Smad3, and thus additional factors are necessary to relieve this inhibition and augment SMA expression. One such factor is β-catenin, which, when released from the injured contacts (or present in the cytosol in high enough concentration), binds to Smad3 and mitigates its negative action on MRTF (Charbonney et al., 2011). In addition, β-catenin counteracts the adaptor function of Smad3, preventing it from recruiting GSK-3β, which phosphorylates and primes MRTF for degradation (Charbonney et al., 2011). Our present work shows that β-catenin, through the above-described mechanism, plays an essential permissive role in the stabilization of Smad3, preventing it from recruiting GSK-3β, which phosphorylates and primes MRTF for degradation (Charbonney et al., 2011).
The MRTF protein specifically in the wound. In addition, β-catenin is also needed for sustained MRTF mRNA expression at this locus. Such β-catenin–dependent transcriptional regulation of MRTF might explain the recent finding that Wnt2-knockout embryos show reduced MRTF expression (Goss et al., 2011). Overall β-catenin may support MRTF function via both mechanisms, explaining at least in part why β-catenin is required for SMA expression during EMyT (Charbonney et al., 2011) and fibrogenesis (Zhou et al., 2012).

Our studies also uncovered that in addition to MRTF and β-catenin, a third contact-dependent transcriptional coactivator, the Hippo effector TAZ, also plays a critical role in local EMyT. This finding is intriguing, as it can explain key events and their timing during the transition. TAZ and its counterpart YAP have been reported to translocate to the nucleus upon cell contact disassembly (Varelas et al., 2010; Halder et al., 2012). Moreover, TAZ acts as a nuclear retention factor for Smad3 (Varelas et al., 2008), a finding that underlies enhanced Smad3 translocation at the wound region and the ensuing heightened Smad3 signaling in the first phase of EMyT. Of interest, the initial nuclear accumulation of Smad3 is a main contributor to subsequent Smad3 degradation because sequential nuclear phosphorylation events prime Smad3 for NEDD4L-mediated proteasomal degradation, it does not explain our finding that the TGFβ-treated wound exhibits a dramatic drop in Smad3 mRNA. Looking for the potential mechanism, we discovered that active TAZ is a strong suppressor of the Smad3 promoter, and TAZ silencing prevents the wound-specific down-regulation of Smad3. Thus TAZ appears to play a complex role in locus (wound)-specific Smad3 signaling. Namely, it contributes to the heightened early response as well as to the subsequent down-regulation by both enhancing degradation and suppressing Smad3 transcription. The exact mechanism of the latter effect (cis-elements, potential cosuppressors) warrants further studies.

We also show that TAZ is important for site-restricted SMA expression, as the absence of TAZ prevents this response, whereas TAZ overexpression activates the SMA promoter and sensitizes it to other stimuli. Although Smad3 suppression may contribute to this effect, it is unlikely to be the sole mechanism, since TAZ also potentiates the effect of the two-hit (TGFβ + LCM) scheme, which itself suppresses Smad3 expression. There are two plausible alternatives. Our ongoing studies suggest that MRTF can associate with TAZ. This finding is consistent with a recent report showing that myocardin and YAP1 can form a complex (Xie et al., 2012). Of note, we identified a well-conserved WW domain–binding motif in members of the myocardin family, which may serve as binding sites for YAP and TAZ. It is thus conceivable that the TAZ/MRTF complex acts through the CArG box and is more potent than MRTF alone. However, YAP1 was found to inhibit the action of myocardin on the CArG box, suggesting that these factors may represent antagonistic rather than synergistic inputs through this cis-element. The other possibility regards TEAD transcription factors, the classic partners of TAZ and YAP. It is noteworthy in this regard that the SMA promoter harbors two putative TEAD-binding sequences known as MCAT elements (Yoshida, 2008). Intriguingly, MCAT elements have been proposed to be important for SMA expression in myofibroblasts but not in smooth muscle cells (Gan et al., 2007). Thus TAZ may be a myofibroblast-selective driver (and therefore target) of SMA expression. Future studies should address whether TAZ regulates the promoter through MCAT elements and whether this occurs in an MRTF-dependent or -independent manner.

Intriguingly, the nuclear uptake of TAZ and MRTF is regulated by strikingly similar inputs, including contact disassembly, increased actin polymerization, cellular tension, and myosin-based contractility (Olson and Nordheim, 2010; Halder et al., 2012). Although both proteins are mechanosensitive and cytoskeleton regulated (Gomez et al., 2010; Dupont et al., 2011), the mechanism responsible for the actin sensitivity of TAZ/YAP transport is unknown. Binding between MRTF and TAZ might confer actin sensitivity to TAZ through a putative “piggyback” mechanism. Future studies should discern whether such coupling exists or the two factors are regulated independently and similar stimuli elicit partially overlapping but distinct transcriptional programs. In any case, our results support the notion that TAZ plays an important role in EMT (Zhang et al., 2009) and allow new insight into the underlying mechanisms. Further, TAZ may participate in the wound healing process as well, since loss of Smad3 abolishes the antiproliferative effect of TGFβ (Nicolas et al., 2003) and may facilitate reepithelialization (Ashcroft et al., 1999), while TAZ may directly stimulate proliferation. In this regard, slightly elevated MRTF activity might support wound contraction by increasing myosin expression (Masszi et al., 2010), whereas long-lasting or overwhelming MRTF activity together with upregulated β-catenin signaling might lead to EMyT and fibrogenesis. A subtle balance between these critical factors might determine whether the outcome is healing or fibrosis.
Finally, it is worth mentioning that although Smad3 is an inhibitor of the direct effect of MRTF on the SMA promoter, it cannot be regarded as an overall negative regulator of the myogenic program. Rather, it is a timer and fine tuner of EMT. Enhanced early Smad3 signaling not only induces the mesenchymal phase of EMT, but it also prepares for the myogenic one, and MRTF and Smad3 can collaborate in these processes. For example, the Smad3-MRTF complex may be shown to induce Snail2, an important suppressor of epithelial markers (Morita et al., 2007). Further, recent elegant studies suggest that MRTF and Smad3 induce miRNA 143/145 through their respective cis-elements in its promoter. miRNA 143/145 in turn suppresses Krüppel factor-4, a strong inhibitor of MRTF and serum response factor (Davis-Dusenberg et al., 2011; Long and Miano, 2011). Thus Smad3 helps eliminate one inhibitor of MRTF, and then its level decreases, which facilitates MRTF signaling. Moreover, our ongoing studies point out some differences between the signaling mechanisms of EMT when induced by LCM plus TGFβ or wound-inducing TGFβ. Specifically, the first model is predominantly MRTF dependent with much less of a role for TAZ, whereas the second (pathophysiologically more relevant) model is both MRTF and TAZ dependent. Accordingly, the role of Smad3 also differs somewhat. In the first model, Smad3 down-regulation augments and accelerates SMA expression, which is driven mostly by MRTF. In the wound model, complete elimination of Smad3 before TGFβ addition also speeds up the expression of SMA (possibly due to the increased MRTF effect), but this response is transient, and at later times SMA expression ceases. The potential explanation is that some Smad3 expression is required for the effect of TAZ. Indeed, we found that transfection with a low amount of Smad3 facilitates the stimulatory effect of TAZ on the SMA promoter. The intriguing possibility that myofibroblast generation involves a gradual switch from an MRTF- to a TAZ-driven SMA expression remains to be tested.

In summary, we described the phenomenon of topical susceptibility of the injured epithelium to TGFβ and showed that the underlying mechanisms involve the interplay of three cell contact–dependent cotranscriptional activators. This circuitry may help explain the focal nature of fibrotic diseases.

MATERIALS AND METHODS

Reagents and antibodies
TGFβ was purchased from R&D Systems (Minneapolis, MN). Commer- commercially available antibodies were from the following sources: β-catenin, GSK-3β, and GAPDH, Santa Cruz Biotechnology (Santa Cruz, CA); SMA, Sigma-Aldrich (St. Louis, MO); TAZ, BD Biosciences (Franklin Lakes, NJ); and Smad3, Cell Signaling Technology (Beverly, MA). The MRTF (BSAC) antibody was used as previously described (Masszi et al., 2010). All horseradish peroxidase–labeled and fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI) was from Lonza (Basel, Switzerland).

Cell culture and treatment
Experiments were conducted using a kidney proximal tubule cell line (LLC-PK1) as described in previous studies (Fan et al., 2007; Masszi et al., 2010). Cultures were maintained in low-glucose DMEM supplemented with 10% fetal bovine serum. Cells were incubated under serum-free conditions for at least 3 h before treatments. TGFβ was used at 5–10 ng/ml, and where indicated cell–cell contact disassembly was induced by thoroughly washing cells with phosphate-buffered saline (PBS) and then culturing in modified DMEM, which contained only nominal calcium (designated as LCM).

Wound model
To create a “wound” in the cell monolayer, we placed strips of surgical tape (3M Transpare; 3M, St. Paul, MN) in the appropriate culture dishes to generate identical areas in which the cells were unable to adhere when plated. Once confluent, the tape was gently removed, leaving well-defined and undamaged rows of cells at each edge. After the specified treatments, cells were collected from very narrow (~2 mm) regions along the wound edge and from one (or several) areas at measured distances from this edge in the adjacent intact monolayer. These areas were previously marked on the underside of each dish to enable standardization of the sample collection between large numbers of dishes and experiments. In some cases, samples collected from multiple experiments were pooled in order to generate enough material for analysis.

Plasmid transfection and luciferase reporter assays
Wild-type and mutant (S89A) TAZ constructs were gifts from Kunliang Guan (plasmids 32839 and 32840; Addgene, Cambridge, MA). The full-length (~1892 to +13) human SMAD3 promoter construct (SMAD3p-Luc) was described previously (Lee et al., 2004). The 765–base pair rat SMA–luciferase construct (pSMA-Luc) was described in our previous studies (Masszi et al., 2004, 2010). Cells were transfected using X-tremeGENE 9 (Roche Applied Science, Indianapolis, IN), with a mixture of the promoter construct of interest along with either an empty plasmid control or the specific expression vector to be tested. At 16 h later, cells were serum starved for 3 h and treated as indicated for 24 h. Luciferase activity in cell lysates was determined with the Dual Luciferase Reporter Assay System Kit (Promega, Madison, WI) measured in a Berthold Lumat 9507 luminometer (Berthold Technologies, Oak Ridge, TN). Each sample was normalized to the thymidine kinase Renilla luciferase activity of cotransfected pRL-TK plasmid. Treatments were performed in dupli- cate, and experiments were repeated at least three times.

siRNA-induced gene silencing
Cells were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) with 20–50 nM porcine-specific siRNA as listed in Supplemental Table S1. Control cells were transfected under the same conditions using NR siRNA (Silencer Negative Control #2) pur- chased from Applied Biosystems (Foster City, CA). Any subsequent treatments were performed 48 h after siRNA transfection.

Western blot analysis and coimmunoprecipitation
Cells collected from specific regions 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 Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and Complete Mini protease inhibitor cocktail (Roche). Protein concentrations were determined using BCA Protein Assay Reagents (Thermo Scientific, Waltham, MA), and equal amounts of protein per lane were subjected to SDS gel electrophoresis and subsequent Western blot analysis. For coimmunoco- precipitation studies, cells were harvested after the indicated treatments, and lysates were spun at 12 000 rpm for 5 min to remove cell debris. Precleared supernatants were incubated with the precipitating antibody, and immunocomplexes were captured on protein G–agarose beads (Thermo Scientific). Bound proteins were eluted from the washed beads and analyzed by Western blotting. Anti- body-free and lysate-free controls were used to confirm specificity of the (co)immunoprecipitation procedure. Aliquots of each input were run in parallel to monitor expression levels. Immuno detection was performed using either ECL or ECL Plus reagents (GE Healthcare
Life Sciences, Piscataway, NJ), and densitometric analysis was performed using a GS800 densitometer and Quantity One software (Bio-Rad, Hercules, CA).

RNA extraction and quantitative real-time PCR
Total RNA from collected cells was extracted using the RNeasy Kit (Qiagen, Valencia, CA), and cDNA was synthesized using iScript reverse transcriptase (Bio-Rad). SYBR green–based real-time PCR was used to evaluate gene expression of SMA, Smad3, β-catenin, MRTF, and TAZ using GAPDH as the reference standard. Primer pairs were designed against known porcine sequences and are listed in Supplemental Table S1. Each experimental condition was analyzed in triplicate. Results were calculated using the relative standard curve method, and experiments were performed a minimum of three times.

Microscopy
Cells were grown on glass coverslips, and surgical tape was adhered across the center of the coverslip in those experiments in which a wound was to be generated. After treatment as indicated, cells 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 also containing DAPI to counterstain cell nuclei. Coverslips were mounted on slides using fluorescent mounting medium (Dako, Carpinteria, CA). Images were captured using an Olympus IX81 microscope (Olympus, Tokyo, Japan) coupled to an Evolution QEF monochrome camera (Media Cybernetics, Silver Spring, MD) and MetaMorph Premiere software (Molecular Devices, Sunnyvale, CA). Tools within the program were used to analyze intensity differences between the wound edge and the intact monolayer on the same coverslip. Line scans were generated to graph the intensity values along a selected line placed perpendicular to the wound edge.

Statistical analysis
Data are presented as representative blots or images from at least three similar experiments or as means ± SEM for the number of experiments indicated. Statistical significance was determined by Student’s t test or one-way analysis of variance (Tukey post hoc testing) as appropriate using Prism 6 software (GraphPad, La Jolla, CA). *p < 0.05; **p < 0.01, ***p < 0.001.

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