Tissue Barriers 1:1, e23699; January/February/March 2013; © 2013 Landes Bioscience

How do your contacts (or their absence) shape your fate?

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Tissue accumulation of contractile myofibroblasts is a key feature of a multitude of fibrotic diseases. Myofibroblast generation either from epithelial or mesenchymal precursors involves the activation of a myogenic program, hallmarked by the expression of α-smooth muscle actin (SMA). Recent research suggests that this robust phenotypic reprogramming requires two critical inputs: the fibrogenic cytokine transforming growth factor-β1 (TGFβ) and an injury (or absence) of intercellular junctions. This two-hit paradigm of epithelial-myofibroblast transition (EMyT) postulates that the injured (contact-deprived) epithelium is locally and selectively sensitive (topically susceptible) to the transforming effect of TGFβ, while the intact areas are quite resistant to the phenotype-changing effect of this cytokine. Searching for molecular mechanisms underlying the synergy between contact injury and TGFβ, we found that an interplay among three multifunctional transcriptional (co)activators, the junction component β-catenin, the TGFβ receptor target Smad3, and the actin cytoskeleton-regulated myocardin-related transcription factor (MRTF) controls the magnitude and timing of SMA expression.[1] Moreover, this regulation is realized not only at the transcriptional level. Notably, these factors form a pretranscriptional circuit, in which they impact each other's activity and stability. Based on this recent paper we ponder about the mechanisms of cellular plasticity in the context of EMyT. We propose that topical susceptibility to TGFβ, triggered by cell contact-modulated pretranscriptional and transcriptional control is realized through the crosstalk of a few master regulators, whose coordinated action tailors SMA expression and contributes to the major decision of whether injury leads to healing or fibrosis.

Keywords: myelofibroblast, epithelial-mesenchymal transition, β-catenin, myocardin-related transcription facr, TGFbeta signalng, Smad3, smooth muscle actin, epithelial plasticity, fibrosis

Submitted: 12/10/12

Revised: 01/18/13

Accepted: 01/21/13

Citation: Charbonney E, Speight P, Kapus A. How do your contacts (or their absence) shape your fate? Tissue Barriers 2013; 1:e23699; http://dx.doi.org/10.4161/tisb.23699

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Commentary to: Charbonney E, Speight P, Masszi A, Nakano H, Kapus A. β-catenin and Smad3 regulate the activity and stability of myocardin-related transcription factor during epithelial-myofibroblast transition. Mol Biol Cell 2011; 22:4472-85; PMID:21965288; http://dx.doi.org/10.1091/mbc.E11-04-0335.

The groundbreaking work of Elisabeth Hay called attention to the fact that the concept of "tissue" represents a continuum rather than a "quantal" endpoint and that tissue differentiation is not a one-way street but to a certain extent a reversible process.[2,3] Indeed, tissue plasticity, i.e., the phenotypic reprogramming of one tissue type into another has emerged as a central event not only in development but also in an array of physiological and pathological processes in the adult organism, including wound healing, carcinogenesis and organ fibrosis.[4,5] A prominent example of tissue plasticity is epithelial-mesenchymal transition (EMT), during which epithelial cells lose their strong intercellular junctions, acquire fibroblast-like morphology, increased motility and occasionally invasiveness and often produce excessive amounts of extracellular matrix.[6-8] The most robust form of EMT, which we termed epithelial-myofibroblast transition (EMyT)[9] is characterized by the emergence of myofibroblasts (MFs), contractile mesenchymal cells hallmarked by the expression of α-smooth muscle actin (SMA).[10] Since the accumulation of MFs and enhanced SMA expression are key features of organ fibrosis and good markers of the severity of this disease entity,[11] the origins of MFs became a central question in fibrosis research. While MFs may be formed from tissue fibroblasts and bone marrow-derived fibrocytes, the reigning paradigm in the last decade has been that the epithelium (via EMT/EMyT) represents a substantial source of

MFs.[12] Recently this view has been challenged, and the pericyte—a perivascular mesenchymal cell characterized by stem cell properties and strong contacts with the endothelium—has been implicated as the major fibroblast and MF precursor.[13,14] While the contribution of EMT/EMyT to the pathogenesis of fibrosis in various organs and fibroproliferatve diseases remains a subject of intensive debate,[15,16] a few important points should be emphasized. First, as verified by a plethora of papers, the epithelium undoubtedly possesses the potentiality to transform into MFs (for a review see ref. 16). The key question therefore is: Under what conditions can this potential be unleashed, i.e., what are the prerequisites to activate a transformative myogenic program in the epithelium? Second, the basic cell biology of MF generation, i.e., the set of necessary inputs and the ensuing signaling likely follow a similar overall theme, irrespective whether the original source is the epithelium or the pericyte. In fact, fibroblasts or pericytes likely have a lower threshold to undergo MF transition upon fibrogenic stimuli than the epithelium, but many of the molecular players (even some of those associated with cell contacts) appear to be common. But what are the key inputs and regulatory events that can induce EMyT?

TGFβ has long been known as the prime inducer of both tissue fibrosis and EMT/EMyT. However, previous studies by our group[9,17,18] and others[19,20] have shown that TGFβ, albeit necessary, is not sufficient to transform an intact (confluent) epithelium to MFs. The other prerequisite is the absence or disassembly of intercellular contacts, which can be achieved by subconfluence, mechanical wounding or the disruption of E-cadherin-dependent junctions by low-calcium medium (LCM).[17,18] Based on these findings we proposed a two-hit paradigm (TGFβ + contact injury) for EMyT induction.[9,18] As a corollary, we raise the concept of topical susceptibility to EMT, implying that TGFβ can elicit drastic phenotypic change only in the injured but not the intact parts of the epithelium. This view not only pictures cell contacts as active regulators (as opposed to passive targets) of EMT, but also has important pathophysiological connotations. Namely, such locally variable susceptibility might help explain the characteristic focal nature of fibrotic diseases, i.e., the "patchy" histology of fibrotic organs where myofibroblast foci are interspersed with apparently intact areas. Moreover, various forms of fibrosis-promoting tissue injury, including inflammation, have been extensively documented to induce cytoskeleton-mediated cell junction disassembly (for an excellent review see ref. 21).

To investigate the molecular mechanism underlying EMyT, we have chosen SMA expression as a key marker of MF transition. We have shown that TGFβ and contact injury synergize at the level of the SMA promoter: both inputs can drive the promoter to some extent but neither is sufficient to induce SMA expression. Their combination, however, results in multiplicative promoter activation and SMA expression.[9,17,18] Recognizing this scenario, the focus of our lab has been to understand the molecular underpinnings of this synergy, i.e., the mechanism whereby TGFβ and cell junction-originated signals collaborate to provoke EMyT.

Acute junction disruption activates the small GTPases Rho and Rac,[18,22,23] a finding that suggested a potential link to the SMA promoter. Namely, Rho/Rac activation results in F-actin polymerization, which in turn regulates myocardin-related transcription factor (MRTF), a transcriptional co-activator of serum response factor (SRF).[24] The latter is a direct driver of the SMA promoter, which acts through the $CC(A/T)_6GG$ cis-element, the CArG box.[25] Under resting condition MRTF binds G-actin, which upon actin polymerization dissociates from MRTF. This unmasks MRTF's nuclear localization sequence thereby promoting its nuclear entry[26] and binding to SRF. Indeed, MRTF was shown to be necessary for the induction of SMA and an array of other cytoskeleton components during EMyT,[9] and it also plays a key role in fibrogenesis in vivo.[27-29] Nonetheless, MRTF is not enough for EMyT. Considering the other arm, i.e., TGFβ signaling, the obvious candidate mediator was the transcription factor Smad3, which is a direct target of the TGFβ receptor kinase and a key inducer of a variety of fibrogenic genes.[30] In addition Smad3 was shown to bind to MRTF, and the complex induces (through modified Smad-binding elements) the expression of Snail2, which in turn suppresses E-cadherin.[31] This means that MRTF and Smad3 collaborate in the suppression of epithelial features, and it appeared plausible that they do so in promoting MF characteristics as well. However, life is rarely that simple. We have shown that Smad3 is actually an inhibitor of MRTF or at least the action of MRTF on the CArG box.[9] Moreover, in our two-hit model Smad3 eventually degrades, which augments the effect of MRTF.[9] Interestingly (and in agreement with our counterintuitive finding), reduction in Smad3 levels has been observed in (the late phases of) experimental fibrosis[32,33] (and reviewed in ref. 34). We also found a drastic drop in Smad3 expression in the fibrosing lungs of rats infected with TGFβ-encoding adenovirus (Gauldie J and Kapus A, unpublished observation).

At this point, however, two important considerations must be made. First, Smad3 cannot be depicted just as an overall negative regulator of SMA expression. Rather, it is a timer or fine-tuner of EMyT and has a critically important role in the regulation of the temporal pattern (sequence of events) during the process. Accordingly we could dissect EMyT into a mesenchymal phase (promoted by Smad3) and a myogenic phase (delayed by Smad3). Further, while Smad3 counteracts the effect of MRTF on the SMA promoter, it may have (indirect) positive effects on SMA expression as well. Elegant studies from the laboratory of Joe Miano[35] and Akiko Hata[36] revealed that in smooth muscle cells both MRTF and Smad3 stimulate the transcription of micoRNA 143/145 via their corresponding cis-elements present in the promoter of this miRNA. Intriguingly, miRNA 143/145 downregulates Krüppel-like factor-4, which is a suppressor of the SMA promoter and a strong inhibitor of smooth muscle differentiation.[36] These complexities clearly imply that Smad3 and MRTF can collaborate with or antagonize each other in various EMyT events, which allows a delicate, temporal regulation of the phenotypic reprogramming.

Second, it is becoming clear that such fine-tuning is often realized through interactions of transcription factors, prior to

and independent of their transcriptional effects. This type of regulation, which we term as "pretranscriptional control" should be distinguished from the action of transcription factor complexes exerted on cis-elements (specific for one or the other factor or their complex) in a promoter. Pretranscriptional interactions may modify the access of transcription factors to their target sequence but may also impact the long-term fate (e.g., stability) of the partners.

Our recent study[1] describes such a pretranscriptional circuit in EMyT, which can provide an additional link between the state of cell contacts and MF generation (**Fig. 1**). One of the enigmas of EMyT has been the mechanism of action of the AJ component β-catenin. The dual nature of β-catenin (as a contact element and transcriptional co-activator) renders this protein another good candidate to connect contact state with gene expression. Indeed, we and others have shown that β-catenin regulates SMA expression.[1,17,37-39] Both the two hit condition-induced and the E-cadherin downregulation-promoted SMA expression are strongly mitigated by β-catenin-silencing.[1] However, the SMA promoter does not harbor a β-catenin responsive element, and β-catenin overexpression itself could not drive the promoter. These findings suggested an important permissive role through an indirect mode of action. Our observation that Smad3 is an inhibitor of MRTF allowed us to propose a pretranscriptional mechanism of action and integrate β-catenin into the regulation of SMA. Since Smad3 and β-catenin are well-known interactors,[40] we hypothesized that β-catenin might abolish the negative effect of Smad3 on MRTF. In agreement with this assumption we found that EMyT-induction is accompanied by enhanced β-catenin/Smad3 association. Moreover, overexpression of β-catenin eliminates the inhibitory effect of Smad3 on the MRTF-induced activation of the SMA promoter. Intriguingly, the presence of β-catenin is necessary for the formation of the SRF/MRTF myogenic complex. Smad3 (by binding to MRTF) displaces MRTF from SRF; β-catenin (by binding to Smad3) displaces Smad3 from MRTF and allows it to complex with SRF. In addition, β-catenin can also bind to MRTF (independent of Smad3 binding), an observation whose structural basis and functional significance warrants further studies. During the characterization of the above-described "displacement-type" mechanism, we made an unexpected observation, which suggested that β-catenin might play a different and even more important role in MRTF regulation. We found that upon β-catenin silencing MRTF is rapidly degraded in cells in which EMyT was induced. In other words, β-catenin is necessary for the maintenance of the myogenic program. Notably, not only SMA but a whole array of MRTF-dependent "CArGome" proteins is lost or will not get upregulated if the cell does not contain sufficient amount of β-catenin. Looking for the underlying mechanism we found that Smad3 can recruit glycogen synthase kinase-3 to MRTF, which in turn likely phosphorylates MRTF. This then leads to the ubiquitnation and subsequent proteasomal degradation of MRTF. Indeed, previously both ubiquitination and sumoylation have been shown to alter the stability and/or activity of members of the

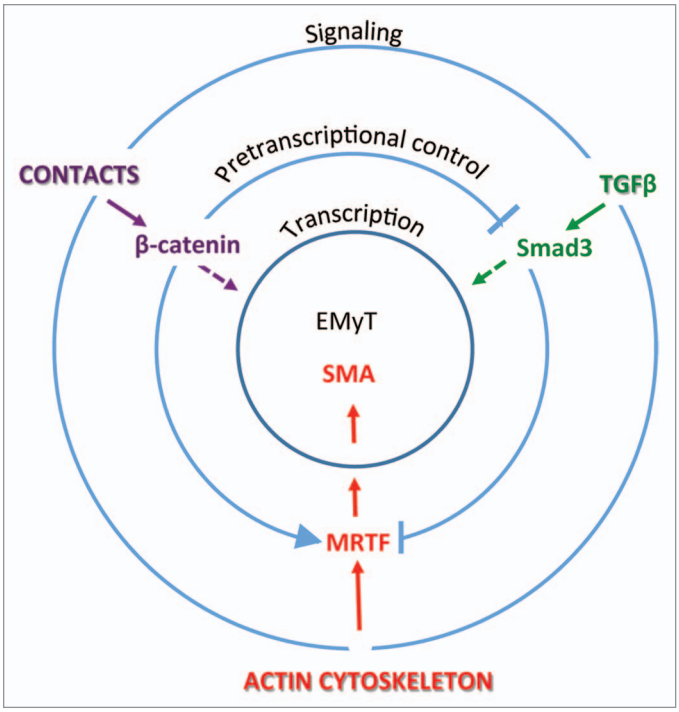

Figure 1. Pretranscriptional and transcriptional control during epithelial-myofibroblast transition (EMyT). The induction and temporal coordination of SMA expression is realized by key inputs emanating from the cells contacts, the TGFβ receptor and the cytoskeleton. Signals initiated from each of these sensory systems impact on the other systems. For example contact disruption triggers Rac and Rho signaling that modifies the cytoskeleton; TGFβ signaling can also modulate small GTPase activation. Conversely, the cytoskeleton, which also receives integrin-mediated and mechanical signals, modifies cell contacts. Such interactions are represented by the outermost "Signaling" circle. In addition, each input directly stimulates a corresponding transcription factor/coactivator (β-catenin for the cell contacts, Smad3 for TGFβ and MRTF for the actin skeleton. These transcription factors exert "pretranscriptional control" on each other (middle circle). As detailed in the text, Smad3 inhibits the action of MRTF on the α-smooth muscle actin (SMA) promoter, whereas β-catenin binds to Smad3 and counteracts its MRTF-inhibitory action. By capturing Smad3, β-catenin not only prevents the direct inhibitory effect of Smad3 on MRTF but also rescues MRTF from degradation. This occurs because Smad3 also works as an adaptor protein, which recruits Glycogen synthase kinase-3 to MRTF, resulting in MRTF's ubiquitination and proteasomal degradation. β-catenin can bind to MRTF as well, but the significance of this phenomenon remains to be elucidated. Finally each transcription factor (alone or in complex) can bind to its cognate cis-elements in various promoters (innermost circle labeled "transcription") facilitating different events in EMyT. With regard to the SMA promoter, MRTF appears to be the prime integrator of the various inputs whereas the direct effects of Smad3, β-catenin or the Smad3/β-catenin complex in this promoter are much weaker or absent.

myocardin family.[41-44] Importantly, this "destructive" role of Smad3 is antagonized by β-catenin. While many details (e.g., the exact site of phosphorylation, the identity of the ubiquitin ligase) await clarification, this mechanism clearly implicates Smad3 as an adaptor protein for MRTF degradation and β-catenin as an inhibitor of this adaptor function. These are key roles for interacting transcription factors, completely independent of transcription. This mechanism does not exclude "conventional" modes of action as was suggested for the β-catenin/Smad3 complex, which was proposed to work through Smad-binding elements.[39] Since we did not observe similar direct promoter effects, this may be a cell type-specific phenomenon.

Returning to our starting point, how then do TGFβ and injury synergize to provoke EMyT? TGFβ is necessary for the initial Smad3 response (mesenchymal phase) as well as for the subsequent Smad3 degradation, which potentiates the effect of MRTF. It is also needed—possibly by activating Akt—to preserve or augment β-catenin levels.[17] Upon contact injury (or wounding), in the absence of TGFβ, β-catenin is lost by proteolysis.[17] TGFβ rescues β-catenin, which in turn is required for the ensuing MF transformation because it keeps MRTF alive. Contact injury, on the other hand, triggers the nuclear translocation of MRTF. In addition, it also reprograms TGFβ signaling, which signifies an exciting new aspect of the problem. Specifically, Smad3 translocation was shown to be enhanced by contact injury, possibly because it triggers the nuclear accumulation of TAZ, a hippo pathway-regulated transcription factor.[45] Remarkably, TAZ acts as Smad3 nuclear retention factor.[46] Our ongoing studies (manuscript in preparation) implicate TAZ both in the early enhanced Smad3 signaling (mesenchymal phase) and in the subsequent elimination of Smad3 (myogenic phase) and the ensuing SMA expression.

Taken together, we propose that a wound, i.e., a locus with missing or injured intracellular contacts shows remarkable topical susceptibility to the fibrogenic and MF-generating effects of TGFβ. This state is brought about by injury- and TGFβ-activated transcription factors, which in addition to their transcriptional effects exert pretranscriptional control on each other, thereby fine-tuning the kinetics of the reprogramming. Undoubtedly, a multitude of mechanisms affect MF generation, including the emerging role of microRNAs and epigenetic control.[47] Nonetheless the recent identification of the four Yamanaka factors as the critical set for pluripotency (an exceedingly complex feature) holds promise that the chief factors governing MF transition might also be graspable. We propose that the interplay among MRTF, Smad3, β-catenin and possibly TAZ has a decisive role in tissue restoration after injury. These factors, integrating a plentitude of inputs, may determine whether seamless healing or fibrosis will ensue. Evaluation and validation of these cellular mechanisms in animal models of fibrosis or MF accumulation seems equally challenging and rewarding.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Original research described by the authors was supported by grants from the Canadian Institute of Health Research (CIHR) (MOP-86535 and MOP-106625) and the Kidney Foundation of Canada to A.K. E.C. was a recipient of a stipend from the Cell Signals Training Program of CIHR (TGF-53877, 2010-12). We thank Dr K. Szászi for critical reading of the manuscript.

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