

The anti-motility signaling mechanism of TGF β 3 that controls cell traffic during skin wound healing

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Summary

When skin is wounded, migration of epidermal keratinocytes at the wound edge initiates within hours, whereas migration of dermal fibroblasts toward the wounded area remains undetectable until several days later. This “cell type traffic” regulation ensures proper healing of the wound, as disruptions of the regulation could either cause delay of wound healing or result in hypertrophic scars. TGF β 3 is the critical traffic controller that selectively halts migration of the dermal, but not epidermal, cells to ensure completion of wound re-epithelialization prior to wound remodeling. However, the mechanism of TGF β 3’s anti-motility signaling has never been investigated. We report here that activated T β RII transmits the anti-motility signal of TGF β 3 in full to T β RI, since expression of the constitutively activated T β RI-TD mutant was sufficient to replace TGF β 3 to block PDGF-bb-induced dermal fibroblast migration. Second, the three components of R-Smad complex are all required. Individual downregulation of Smad2, Smad3 or Smad4 prevented TGF β 3 from inhibiting dermal fibroblast migration. Third,

Protein Kinase Array allowed us to identify the protein kinase A (PKA) as a specific downstream effector of R-Smads in dermal fibroblasts. Activation of PKA alone blocked PDGF-bb-induced dermal fibroblast migration, just like TGF β 3. Downregulation of PKA’s catalytic subunit nullified the anti-motility signaling of TGF β 3. This is the first report on anti-motility signaling mechanism by TGF β family cytokines. Significance of this finding is not only limited to wound healing but also to other human disorders, such as heart attack and cancer, where the diseased cells have often managed to avoid the anti-motility effect of TGF β .

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Key words: TGF β 3, Anti-motility, PKA, Cell migration, Wound healing

Introduction

Skin wound healing is a complex process involving collaborative efforts of multiple cell types, newly synthesized extracellular matrices and growth factors. Inflammation, re-epithelialization, granulation tissue formation and remodeling are the proposed sequential events during skin wound healing under physiological conditions (Martin, 1997; Singer and Clark, 1999). Abnormalities in any of the events could result in either delayed wound healing or a wound healed with hypertrophic scars (Tredget et al., 1997). Throughout the wound healing process, cell migration is a rate-limiting event. The epidermal keratinocytes laterally migrate across the wound bed to resurface the wound, the process known as wound re-epithelialization. The dermal fibroblasts move into the wound to produce and deposit new matrix proteins. Finally, the dermal microvascular endothelial cells that have entered the wounded tissue are responsible for building new blood vessels. It has long been recognized that epidermal and dermal cells do not enter the wound bed at the same time. Following wounding, keratinocyte migration occurs within hours, whereas migration of the dermal cells remains undetectable until several days later (Singer and Clark, 1999). These observations are in accordance with the

notion that wound closure by keratinocyte migration precedes wound remodeling by the dermal cells (Clark, 1993; Falanga, 2004). However, the regulation of the sequential epidermal and dermal cell migration remained a mystery.

In intact skin, cells are bathed in interstitial fluid, largely a filtrate of plasma in blood vessels. When skin is wounded, the cells at the cut edge of the wound experience a dramatic microenvironmental transition from plasma to a new environment of serum for the first time (Henry et al., 2003). Bandyopadhyay and colleagues discovered a key factor in human serum, TGF β 3 that seems to play the “traffic control” role in migration of epidermal *versus* dermal cells. They showed that human serum selectively inhibits the migration of dermal fibroblasts and microvascular endothelial cells via TGF β 3 (not TGF β 1 or TGF β 2). Furthermore, they demonstrated that the differential responses of keratinocytes *versus* dermal fibroblasts to TGF β 3’s anti-motility signal are due to the difference in the expression levels of type II TGF β receptor (T β RII) in these cells (Bandyopadhyay et al., 2006). Therefore, this finding suggests that it is TGF β 3 in human serum that selectively halts dermal cell migration for a few days, allowing keratinocyte migration take place under the microenvironment of serum.

The three mammalian TGFβ members, TGFβ1, TGFβ2 and TGFβ3, bind to and transmit signals via the common heterodimeric complex of TβRII and TβRI serine/threonine kinases (Wrana et al., 1992; Attisano and Wrana, 1996; Piek et al., 1999; Miyazono, 2000; Derynck and Zhang, 2003; Shi and Massagué, 2003). In this classical model, TGFβ binds to TβRII, which in turn recruits, transphosphorylates and activates TβRI (Derynck and Feng, 1997; Shi and Massagué, 2003). The activated TβRII/TβRI complex then engages R-Smad-dependent and/or R-Smad-independent signaling pathways inside the cells (Miyazono, 2000; Mulder, 2000; Derynck and Zhang, 2003; Moustakas and Heldin, 2005). However, several recent studies started challenging the dogma of TβRII/TβRI signaling and suggested that TβRII and TβRI are not always required to work together to mediate TGFβ signaling. An earlier study by Zhang and colleagues provided biochemical support that TβRII under physiological expression levels are able to form homodimers in response to TGFβ stimulation (Zhang et al., 2009). Bandyopadhyay et al. showed that TβRII alone is able to mediate TGFβ signaling to ERK_{1/2}, in the absence of TβRI and TβRIII in primary human dermal fibroblasts (Bandyopadhyay et al., 2011). Moreover, Iwata et al. have recently reported that TβRI and TβRIII are able to mediate TGFβ signaling to TRAF6/TAK1/p38 pathway in TβRII^{-/-} mouse cranial neural crest cells (Iwata et al., 2012).

In the current study, we undertook a systematic approach to identify the pathway through which the anti-motility signal of TGFβ3 travels in primary human dermal fibroblasts. While our results demonstrated the necessity for the conventional TβRII/TβRI and Smad2/3/4 complexes, they also revealed a detour of the post-Smad signaling to the PKA > CREB pathway for the anti-motility signal. This is the first report on “anti-motility signaling pathway” for the TGFβ family cytokines.

Results

Profiles of TGFβ family cytokines in normal human skin

TGFβ in skin can come from two sources, filtrate of blood circulation and secretion by the skin cells. It is technically difficult to establish TGFβ profiles in unwounded intact skin, since human skin isolated by biopsy is in fact “wounded” tissue. In wounded tissues, the main source for TGFβ1 comes from degranulation of the platelets, in which TGFβ1 is the only isoform present in the α-granules (Assoian et al., 1983; Roberts and Sporn, 1996). The source for TGFβ2 and TGFβ3 remains largely unknown. There have been reports that human serum, but not plasma, contains active TGFβ3 at a concentration range of 1–2 ng/ml (Hering et al., 2001), in comparison to 10–50 ng/ml of TGFβ1 in human serum (Assoian et al., 1983; Roberts and Sporn, 1996; Grainger et al., 2000). Since the half-life of TGFβ is short (2–3 minutes), the majority of TGFβ isoforms in serum must be in complexes with carrier proteins, such as latency-associated peptide (LAP) and latent TGFβ1-binding protein (LTBP) (Roberts, 1998). These TGFβ-bound carrier proteins can be cleaved in serum and new TGFβ is made available (Roberts, 1998; Khalil, 1999; Murphy-Ullrich and Poczatek, 2000). Based on the above understanding, we examined the profiles of the three TGFβ family members using frozen section of normal adult human skin. As shown in Fig. 1, anti-TGFβ1 antibody stained the dermis of the skin significantly stronger than the epidermis (panel B *versus* panel A). As expected, anti-TGFβ2 antibody (panel D) and anti-TGFβ3 antibody (panel F) staining of the skin sections

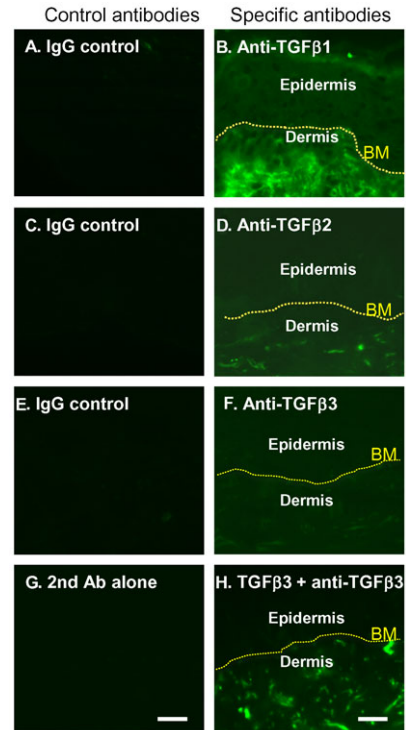


Fig. 1. TGFβ (1, 2, 3) expression profiles in normal human skin. Sections of frozen normal adult human skin from multiple independent donors were subjected to IHC staining with the antibodies against TGFβ1 (B), TGFβ2 (D) and TGFβ3 (F) or corresponding IgG controls (A,C,E,G). (H) Skin sections were incubated with TGFβ3 (5 ng/ml, 30 min, at 25°C), washed and re-blocked (with BSA) prior to anti-TGFβ3 antibody staining (Materials and Methods). The bright green color staining represents skin tissue distribution of TGFβ. Dotted yellow lines show where the basement membrane (BM) is. Scale bar, 20 μm. Similar results were obtained with skin from different donors and anti-TGFβ antibodies from independent commercial sources.

was much weaker than anti-TGFβ1 antibody staining. These data indicate that human skin contains mainly TGFβ1 and lower levels of TGFβ2 and TGFβ3, provided that the three antibodies share similar affinity toward their antigens. To test whether or not the weaker staining by anti-TGFβ3 antibody was due to its lower affinity toward TGFβ3, we incubated the skin sections with human recombinant TGFβ3 prior to anti-TGFβ3 antibody staining. We detected a strong and almost exclusively staining of the dermis (panel H). These results indicated that 1) the weaker staining of the skin sections by anti-TGFβ3 antibody (panel F) was not due to a compromise in the antibody’s affinity for TGFβ3 and 2) dermal cells are the primary targets for environmental TGFβ3, consistent with our previous finding that human dermal cells express 7–15 fold higher levels of TβRII than human keratinocytes (Bandyopadhyay et al., 2006).

Human dermal fibroblasts provide a unique cell model to define anti-motility signaling of TGFβ3

Migration of human keratinocytes (HKCs) and human dermal fibroblasts (HDFs) into the wound are two critical events during wound healing. However, a major difference between these two cell types lies in distinct responses of these cells to the anti-motility signal of TGFβ3, (Bandyopadhyay et al., 2006). As shown in Fig. 2, in the absence of TGFβ3, HDF stimulus, PDGF-bb (A), and HKC stimulus, TGFα (B), strongly stimulated

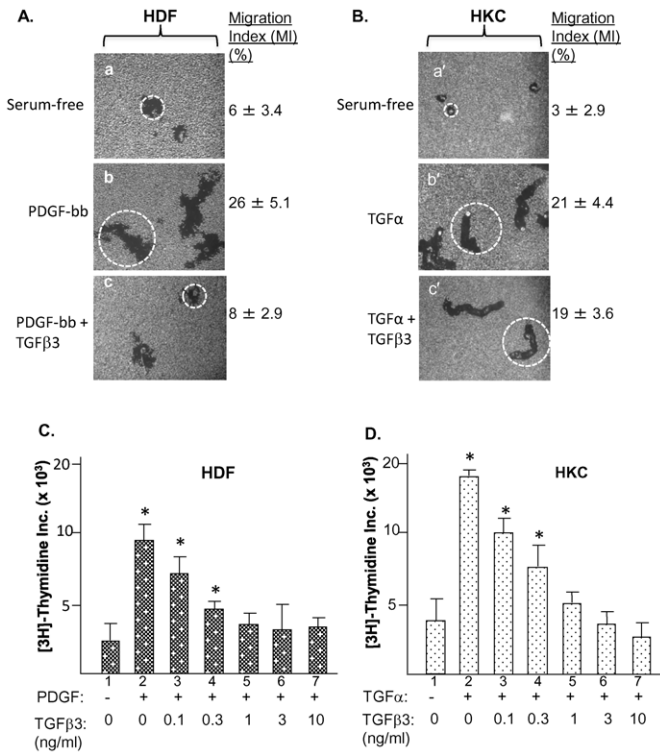


Fig. 2. Differential sensitivity of HDFs and HKCs to TGFβ3's anti-motility and anti-growth signals. HDFs and HKCs were serum starved overnight and subjected to either colloidal gold migration assay (16 hours) (A,B) or DNA synthesis assay (C,D) in response to PDGF-bb (15 ng/ml) or TGFα (20 ng/ml) in the absence or presence of indicated concentrations of TGFβ3. (A,B) Representative images of cell migration and quantitation of the migration (Migration Index, MI) are presented. Dotted circles represent an average-sized migration track under the condition. The difference in MIs in PDGF-bb-stimulated migration of HDFs with or without TGFβ3 is statistically significant ($P < 0.05$), whereas the difference in MIs in HKCs is statistically insignificant ($P < 0.01$). These experiments were repeated four to five times. (C,D) [³H]-thymidine incorporation assay was used to measure DNA synthesis under the indicated conditions. The actual cpm counts (in triplicate) from one representative experiment on HKCs and HDFs were presented here. The results of three repeated experiments were similar. *Statistically significant over the control, $P < 0.05$.

migration of HDFs (A, panel b *versus* panel a) and HKCs (B, panel b' *versus* panel a') in colloidal gold migration assay (Materials and Methods). Presence of TGFβ3, however, selectively inhibited PDGF-bb-stimulated HDF migration (A, panel c *versus* panel b), but not TGFα-stimulated HKC migration (B, panel c' *versus* panel b'). Migration Index (MI) was used to quantitate cell migration. Under similar conditions, both PDGF-bb (C) and TGFα (D) also stimulated DNA synthesis in HDFs and HKCs (bars 2 *versus* bars 1). Interestingly, in contrast to the selective inhibition of HDF migration by TGFβ3, TGFβ3 inhibited growth factor-induced DNA synthesis in both HDFs and HKCs in a dose-dependent manner (bars 3–7 *versus* bars 2). These data suggest that the TGFβ receptors in HDFs allows both anti-proliferation and anti-motility signals of TGFβ3 to enter the cells, whereas the TGFβ receptors in HKCs only transmit the anti-proliferation, but not anti-motility, signal of TGFβ3. Therefore, comparative studies between HDFs and HKCs would provide a unique model system for defining the signaling pathway by which TGFβ3 blocks growth

factor-stimulated cell migration – the anti-motility signaling mechanism by TGFβ3.

TβRII transmits the anti-motility signal of TGFβ3 in full to TβRI/AIk5

We have previously shown that it is the higher expression level of TβRII in HDFs (than in HKCs) that makes HDFs sensitive to the anti-motility signal of TGFβ3 (Bandyopadhyay et al., 2006). Therefore, we chose TβRII as the starting signaling molecule. We asked whether TβRII transmits the anti-motility signal of TGFβ3 to TβRI or independent of TβRI. We used the lentiviral shRNA-delivery system, FG12 (Qin et al., 2003), to downregulate the endogenous TβRI in HDFs. The FG12 system, as shown in Fig. 3A, offers more than 90% transduction efficiency in HDFs as indicated by a green fluorescent protein (GFP) gene expression by the vector following a single infection. Downregulation of TβRI by one of the shRNAs (shTβRI-NO. 2) is shown in Fig. 3B (panel a, lane 2 *versus* lane 1). In these cells, we observed that TGFβ3-stimulated phosphorylation of Smad3 became undetectable, while the levels of TβRII remained unaffected (Bandyopadhyay et al., 2011). When these cells were subjected to the colloidal gold migration assay, as shown in Fig. 3C, TGFβ3 was no longer able to block PDGF-bb-stimulated migration of the TβRI-downregulated cells (bar 6) in comparison to the parental HDFs (bar 3). These results indicated that the endogenous TβRI is required for transmitting the anti-motility signal of TGFβ3.

We then tested whether TβRII transmits the anti-motility signal of TGFβ3 in full to TβRI, i.e. whether activation of TβRI alone is sufficient to replace TGFβ3. We took an advantage of the constitutively activated mutant of TβRI, the TβRI-TD mutant (Wieser et al., 1995). We reasoned that if expression of TβRI-TD alone were able to block PDGF-bb-stimulated migration even in the absence of TGFβ3, it indicates that TβRII does not need any TβRI-independent pathways for transmitting the anti-motility signal of TGFβ3. As shown in Fig. 3D, TβRI-wt (lane 2) and the TβRI-TD mutant (lane 3) genes were expressed several fold over the endogenous TβRI in HDFs by the lentiviral vector, pRRLSin (lane 1). The constitutive kinase activity of TβRI-TD was indicated by constitutive Smad3 phosphorylation even in the absence of TGFβ3 under serum-free conditions (Fig. 3E, panel c, lane 1 *versus* lanes 2, 3, 4). In TβRI-wt-expressing HDFs, however, phosphorylation of Smad3 still depended upon TGFβ3-stimulation (panel a, lanes 2, 3, 4 *versus* lane 1). Interestingly, the constitutively activated TβRI-Smad pathway was able to replace TGFβ3's anti-motility signal. As shown in Fig. 3F, TβRI-TD-expressing HDFs failed to migrate in response to PDGF-bb stimulation even in the absence of TGFβ3 (bar 5 *versus* bar 2). In contrast, the HDFs expressing TβRI-wt (inactive in the absence of TGFβ3) behaved just like the parental HDFs, with a basal migration under serum-free conditions (bars 1), enhanced migration in response to PDGF-bb (bar 2) and inhibited migration in the presence of TGFβ3 (bar 3).

Furthermore, we reasoned that if TβRI-TD was capable of carrying the full scale of the anti-motility signal of TGFβ3, expression of TβRI-TD should be able to block migration of HKCs. As shown in supplementary material Fig. S1, over-expression of TβRI-TD was sufficient to block TGFα-stimulated HKC migration even in the absence of TGFβ3 (bars 5 *versus* bar 2). In comparison, TGFα-stimulated migration of HKCs expressing the TβRI-wt remained unaffected by TGFβ3 (bar 3), just like the parental HKCs (Fig. 1). Taken together, we

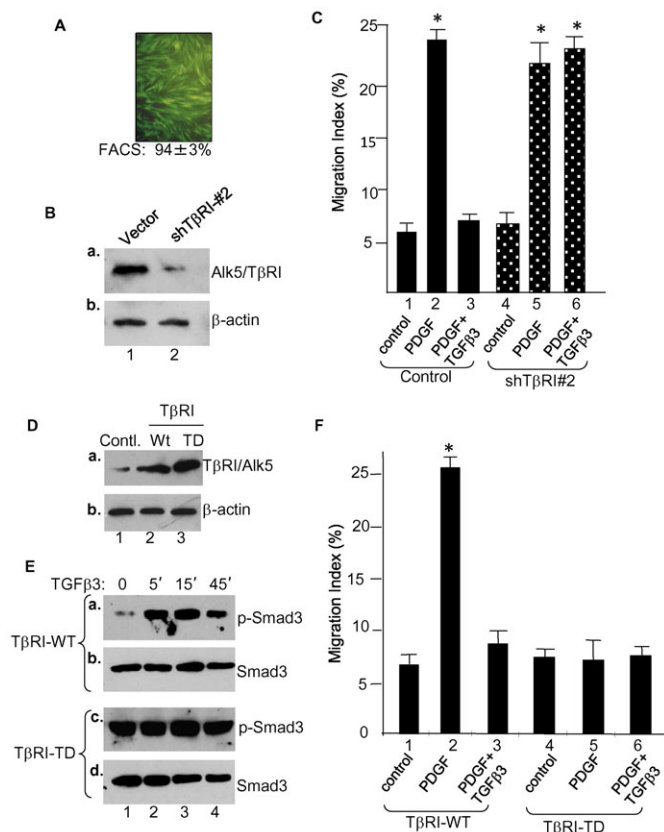


Fig. 3. TβRI mediates in full TGFβ3 anti-motility signaling in HDFs. HDFs were infected with lentiviral vector, FG-12, carrying either a GFP gene alone (under a CMV) or GFP plus an shRNA (under a U6) against the human TβRI/Alk5. (A) More than 90% gene transduction efficiency was indicated by FACS analysis of the GFP-positive cells. (B) After 48 hours, downregulation of endogenous TβRI was confirmed by anti-TβRI antibody immunoblotting (lane 2 versus lane 1). (C) The HDFs were subjected to colloidal gold migration assays in response to PDGF-bb (15 ng/ml) in the absence or presence of TGFβ3 (3 ng/ml). The migration was quantitated as Migration Index (%), $n=3$, $P\leq 0.03$. (D) In HDFs, overexpression of the WT or TD mutant of TβRI over the endogenous TβRI was shown by anti-TβRI antibody immunoblotting (lanes 2 and 3 versus lane 1). (E) Lysates of the cells untreated or treated with TGFβ3 (3 ng/ml) were immunoblotted with antibodies against the phospho-Smad3. (F) The same cells were subjected to colloidal gold migration assays in response to PDGF-bb (15 ng/ml) in the absence or presence of TGFβ3 (3 ng/ml). Migration Index (%) was shown. This experiment was repeated four times ($n=4$, $P\leq 0.05$). *Statistically significant over the control.

concluded that TβRI activation by a higher level of TβRII in HDFs is both necessary and sufficient for transmitting anti-motility signal of TGFβ3.

Smad2/3 and Smad4 are essential for mediating the anti-motility signaling of TGFβ3

The post-TβRII and TβRI signaling could use R-Smad-dependent or R-Smad-independent routes (Derynck and Zhang, 2003). We first tested the R-Smad pathway. It is known that Smad2/3 are phosphorylated by activated TβRI and in turn form a complex with Smad4. This R-Smad trimeric complex translocates to the nucleus to regulate gene expression (Shi and Massagué, 2003). To individually assess the three Smads, we designed two independent shRNAs against each Smad and introduced each of these shRNAs into HDFs by the FG-12 lentiviral shRNA system, as previously described. As shown in Fig. 4A, we

achieved nearly complete downregulation of Smad 2 (panel a, lanes 1 and 2 versus lane 3), Smad 3 (panel c, lanes 1 and 2 versus lane 3), and partial downregulation of Smad4 (panel e, lanes 1 and 2 versus lane 3). The incomplete downregulation of Smad4 may reflect the fact that a fraction of Smad4 locates in the nucleus. These cells were then subjected to the colloidal gold migration assay. As shown in Fig. 3B, TGFβ3 blocked PDGF-bb-stimulated migration of HDFs infected with vector alone, as expected (bar 3 versus bar 2). However, downregulation of Smad2 prevented TGFβ3 from inhibiting PDGF-bb-stimulated HDF migration (bar 6 versus bar 5). Similarly, downregulation of Smad3 also nullified TGFβ3's anti-motility signaling (bar 9 versus bar 8). More intriguingly, even under partial downregulation of Smad4, TGFβ3 was unable to inhibit PDGF-bb-stimulated migration of the cells (bar 12 versus bar 11). Taken together, these data indicate that Smad2/3 in complex with Smad4 are required for transmitting the anti-motility signal from TGFβ3.

After R-Smads, the anti-motility signaling of TGFβ3 detours to PKA pathway

Where next does the R-Smad complex connect the anti-motility signal to? The possibilities include i) going into the nucleus to regulate expression of migration-suppressing genes or ii) connecting with other cytosolic signaling pathway(s). We pre-set two parameters to test these possibilities. First, we focused on primary targets of R-Smads, i.e. targets that are rapidly activated by TGFβ3 in a R-Smad-dependent and protein synthesis-independent manner. Therefore, we chose 10 minutes (maximum phosphorylation of Smad2/3) as the stimulation time for TGFβ3. Second, the to-be-identified new pathway should only be activated by TGFβ3 in HDFs, but not in HKCs. Based on these parameters, we treated serum-starved HDFs and HKCs side-by-side with the same amount of TGFβ3 for 10 minutes. Cell extracts were subjected to pathway screening with the Human Phospho-Kinase Antibody Array (ARY 003, R&D), which represents 46 well-characterized protein kinase pathways (supplementary material Fig. S2A). As shown in Fig. 5A, we found that TGFβ3 induced increase in ERK1/2 phosphorylation (open dotted squares), CREB phosphorylation (a direct substrate of protein kinase A, PKA) (open dotted circles) and Src kinase phosphorylation (open dotted triangles) in HDFs (panel b versus panel a). None of these three pathways appeared to be significantly activated by TGFβ3 in HKCs (panel d versus panel c). Quantitation by phosphoimager (Materials and Methods) of these three target proteins is shown in Fig. 5B (HDFs) and Fig. 5C (HKCs). In comparisons, we detected additional three pathways, β-catenin, p53 and eNOS, which were activated in both HDFs and HKCs in response to TGFβ3 stimulation (dotted underlines, 1, 2 and 3). We decided to focus on PKA pathway for its possible role in mediating the anti-motility signaling of TGFβ3 for the following reasons. PKA has previously been shown as an antagonist of growth factor signaling (Cook and McCormick, 1993; Tokiwa et al., 1994). In contrast, both ERK1/2 and Src are known downstream pathways of the PDGF-bb signaling and required for PDGF-bb-stimulated cell proliferation and migration (Li et al., 2004a; Heldin and Westermark, 1996). It is, therefore, less likely that these two pathways are simultaneously involved in the anti-motility signaling of TGFβ3.

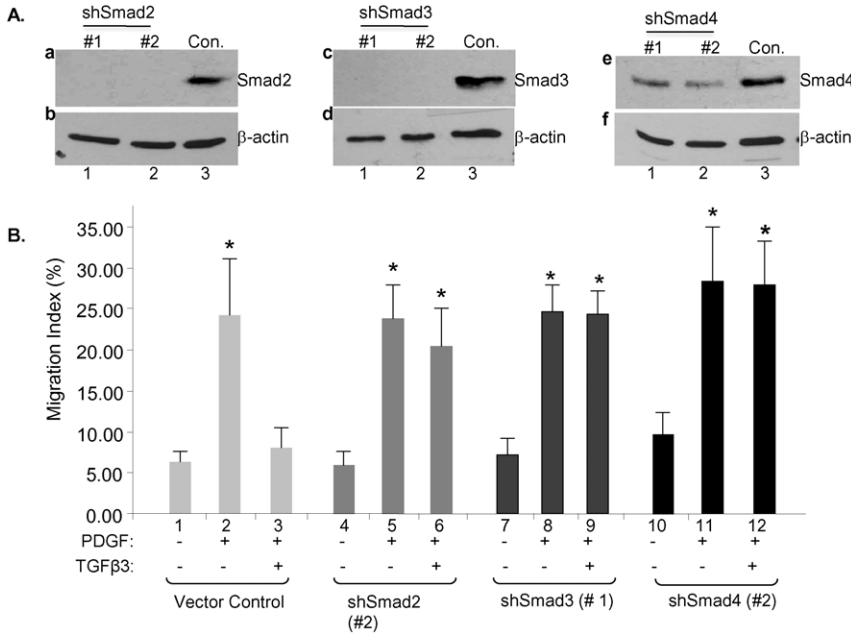


Fig. 4. Requirement for Smads 2, 3 and 4 for mediating anti-motility signaling of TGFβ3. (A) HDFs were infected with FG-12 lentivirus carrying each of two shRNAs against Smad2, Smad 3 or Smad4. After 48 hours, downregulation of the endogenous Smad2, Smad 3 or Smad4 was confirmed by immunoblotting lysates of the cells with anti-Smad2, Smad 3 or Smad4 antibodies, respectively. (B) These individual Smad-downregulated HDFs were subjected to colloidal gold migration assays in response to PDGF-bb (15 ng/ml) in the absence or presence of TGFβ3 (3 ng/ml). The migration was quantitated as Migration Index (%) ($n=3$). *Statistically significant over the control, $P < 0.05$.

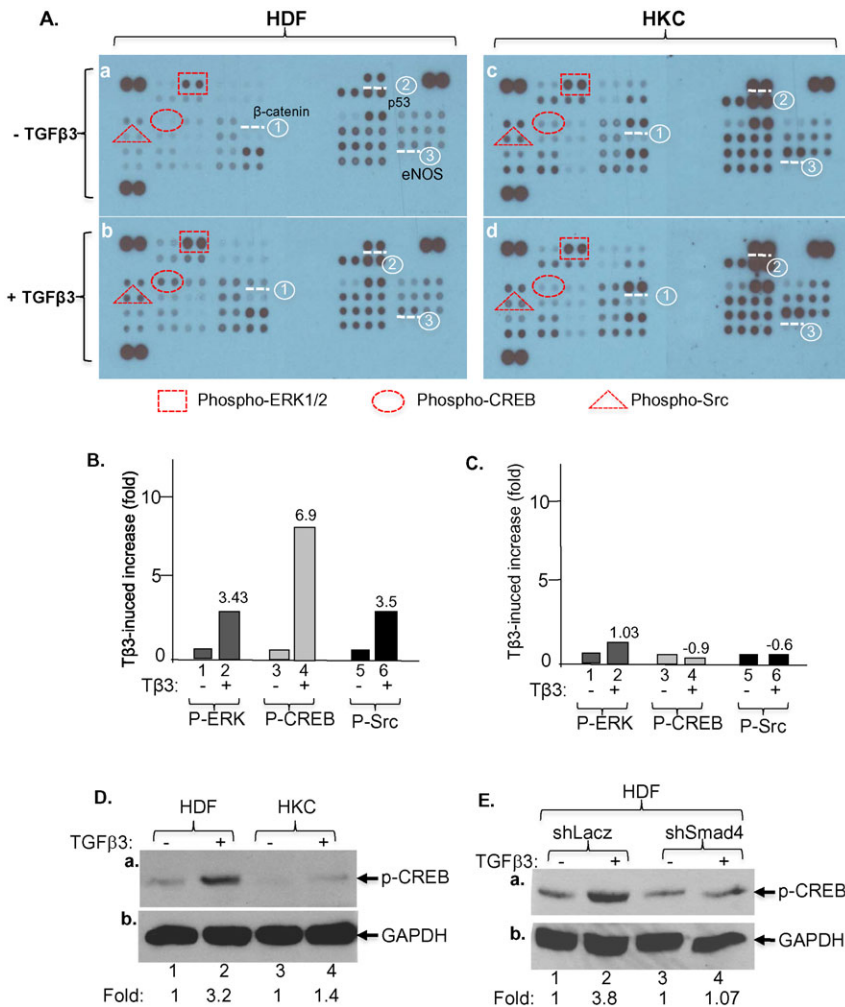


Fig. 5. TGFβ3 activates, via R-Smads, PKA pathway in HDFs. Serum-starved HDFs and HKCs in duplicate culture plates were either unstimulated or stimulated with TGFβ3 (3 ng/ml) for 10 min. The stimulation was stopped by removing the medium from the plates and rinsing of the plates three times with ice-cold PBS solution on ice. The cells were then subjected to Protein Kinase Array screening strictly following the manufacturer's procedures (Materials and Methods), including lysing cells with a special lysis buffer provided. (A) Images of ECL-developed films, where antibody identities on the spots were based on the information given by the manufacturer. (B,C) Phosphoimager quantitation of phospho-ERK, phospho-CREB and phospho-Src in HDFs and HKCs. (D) Stronger activation of PKA pathway, i.e. phosphorylation of CREB, in HDFs than in HKCs by TGFβ3 (10 min) was confirmed by Western blots (lane 2 versus lane 4). (E) Downregulation of Smad4 blocks TGFβ3-induced CREB phosphorylation (lane 4 versus lane 2). The Array experiment was repeated twice and the Western blotting experiment four times. Similar results were obtained.

We directly confirmed that TGFβ3 activates PKA pathway in HDFs, but not HKCs. Lysates of TGFβ3-treated HDFs and HKCs were subjected to Western blot analysis with anti-phospho-CREB antibodies. As shown in Fig. 5D, TGFβ3 stimulation clearly caused an increased CREB phosphorylation in HDFs (panel a, lane 2 *versus* lane 1), and this induction occurred in a time dependent manner (supplementary material Fig. S2B). In contrast, a much weaker, if any, TGFβ3-stimulated CREB phosphorylation was detected in HKCs (lanes 3 and 4). This weaker induction was reproducible in time-course experiments (supplementary material Fig. S2B). More importantly, we found that the TGFβ3-stimulated CREB phosphorylation in HDFs depended upon the Smad pathway, since Smad4 downregulation prevented the TGFβ3-stimulated CREB phosphorylation (Fig. 5E, panel a, lane 4 *versus* lane 2). Phosphoimager scanning showed TGFβ3-induced fold increases in CREB phosphorylation in HDFs *versus* HKCs. Therefore, PKA appeared to be a specific downstream effector of the Smads in HDFs in response to TGFβ3.

PKA mediates anti-motility signaling of TGFβ3

We undertook two complementary approaches to examine whether PKA acts downstream R-Smads to mediate TGFβ3's anti-motility signal. First, we tested the effect of a chemical inhibitor, H-89, and a chemical activator, forskolin, of PKA pathway. As shown in Fig. 6A, PDGF-bb greatly enhanced HDF migration under serum-free conditions in the absence of TGFβ3 (panel b *versus* panel a). The presence of TGFβ3 completely nullified the stimulatory effect of PDGF-bb (panel c). However, in the presence of H89, TGFβ3 was no longer able to block PDGF-bb-stimulated HDF migration (panel d *versus* panel c). Consistently, H89 inhibited TGFβ3-induced phosphorylation of CREB (Fig. 6B, panel a, lane 3 *versus* lane 2). In comparison, H89 did not affect TGFβ3-stimulated phosphorylation of Smad3 in the same cells (panel b, lane 3 *versus* lane 2), consistent with the notion that R-Smads are upstream of PKA in response to TGFβ3. Similarly, the PKA activator, forskolin, recapitulated the effect of TGFβ3. As shown in Fig. 6C, the presence of forskolin

caused increased phosphorylation of CREB under serum-free and TGFβ3-free conditions (insert) and treatment with forskolin alone was able to block PDGF-bb-stimulated HDF migration in a dose-dependent manner (bars 3 to 7 *versus* bar 2).

Second, since few chemical inhibitors/activators could claim absolute specificity, we next directly targeted the catalytic subunit of human PKA, the PKA-Cα, with shRNA technology. We designed two shRNAs against the human PKA-Cα and individually delivered them by the FG-12 lentiviral system into HDFs. It is shown in Fig. 7A that PKA-Cα was clearly detected in FG-12 vector-infected HDFs (panel a, lane 1). However, PKA-Cα expression was dramatically downregulated in cells infected with shRNA-NO. 1 or shRNA-NO. 2 against PKA-Cα (panel a, lanes 2 and 3). The effect of PKA-Cα downregulation was also confirmed by blockade of forskolin-stimulated CREB phosphorylation in these cells (Fig. 7B, panel a, lane 4 *versus* lane 2). In contrast, in the same cells, TGFβ3 was still able to induce Smad2/3 phosphorylation (Fig. 7C). We then tested whether TGFβ3 was still able to block PDGF-bb-stimulated migration of the cells. As shown in Fig. 7D, in control HDFs, PDGF-bb stimulation caused dramatically increased migration (panel b *versus* panel a), but failed to do so in the presence of TGFβ3 (panel c). However, downregulation of the PKA-Cα by either shRNA-NO. 1 or shRNA-NO. 2 completely blocked anti-motility signaling of TGFβ3 (panels f and i *versus* panels e and h, respectively). Quantitation of the migration, Migration Index, was shown in Fig. 7E. Taken together, results of this study demonstrated that the "TβII > TβI > R-Smads > PKA" pathway mediates TGFβ3's anti-motility signaling in HDFs, which represents a key cells traffic regulator during wound healing.

Discussion

The tumor suppressing effect of the TGFβ family cytokines includes anti-proliferation and anti-motility. These two functions of TGFβ also take an important part in the process of wound healing. A cell could only choose migration or proliferation, but not both, at a time. Immediately following the injury, the

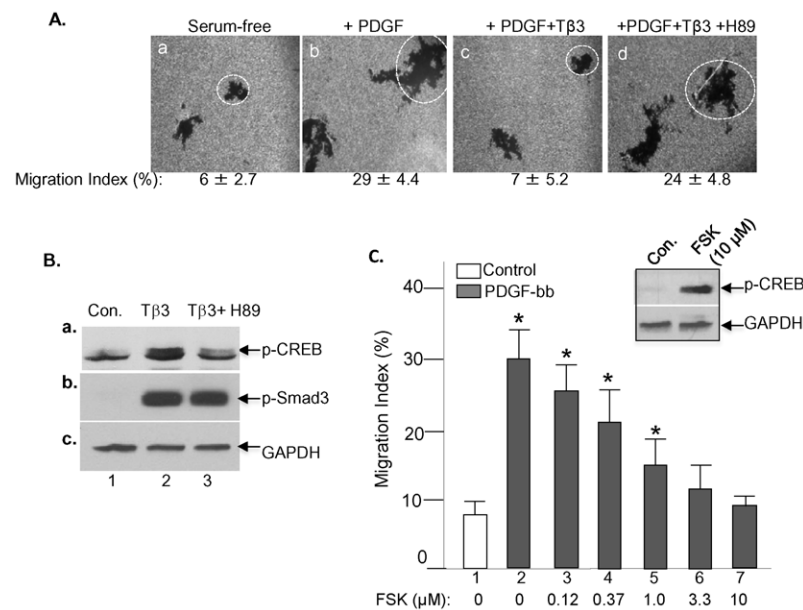


Fig. 6. Activation of PKA alone replaces the anti-motility signal of TGFβ3 in HDFs.

(A) HDFs were serum starved overnight and subjected to colloidal gold migration assays in the absence or presence of PDGF-bb (15 ng/ml), TGFβ3 (3 ng/ml) and PKA inhibitor, H89 (1.0 μM). Representative images of migrated cells under the indicated conditions are shown (a to d). Quantitative analyses of the migration tracks are shown underneath as Migration index (MI) (%). Average size migration tracks are marked with dotted circles. This experiment was repeated multiple times and similar results were obtained. (B) Serum-starved HDFs were treated without or with TGFβ3 or TGFβ3 plus H89. Total cell lysates were subjected to immunoblotting analyses with indicated antibodies. (C) Serum-starved HDFs were subjected to colloidal gold migration assay, as previously described, in the absence or presence of PDGF-bb (bar 1 and 2) or PDGF-bb plus increasing concentrations of forskolin, an activator of PKA (bars 3 to 7). The effectiveness of forskolin (10.0 μM) on HDFs is indicated by causing an increased phosphorylation of CREB (insert image) in the cells. These experiments were repeated three times. *Statistically significant over the control, $P < 0.05$.

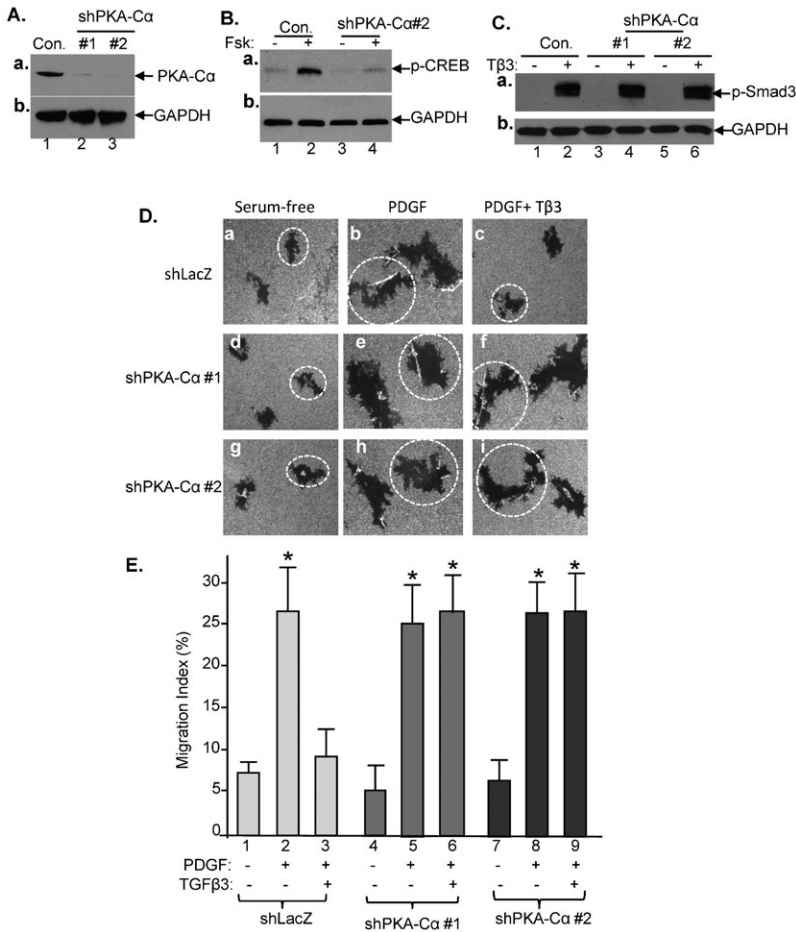


Fig. 7. PKA acts downstream R-Smads to mediate anti-motility signaling of TGFβ3. (A) HDFs were infected with FG-12 lentivirus carrying either a GFP control gene or a shRNAs against PKA-Cα. After 48 hours, downregulation of the endogenous PKA-Cα was confirmed by immunoblotting the lysates of the cells with an anti-PKA-Cα antibody. (B) Downregulation of PKA-Cα blocks forskolin-stimulated CREB phosphorylation (lane 4 versus lane 2). (C) Downregulation of PKA-Cα showed little effect on TGFβ3-stimulated phosphorylation of Smad3 (lanes 4 and 6 versus lane 2). (D) Two sets of PKA-Cα-downregulated HDFs were subjected to colloidal gold migration assay in response to PDGF-bb (15 ng/ml) in the absence or presence of TGFβ3 (3 ng/ml). Representative images of the migrated cells under the indicated conditions are shown (a to i). (E) The computer-assisted quantitative analyses of the migration tracks are shown as Migration index (MI) (%). The experiment was repeated four times ($n=3$, $P \leq 0.05$). *Statistically significant over the control.

anti-proliferation effect of TGFβ3 from serum halts the proliferation mode of both epidermal and dermal cells and migration mode of only dermal cells at the wound edge, in order for epidermal keratinocytes to migrate toward the wound first (Sarret et al., 1992; Bandyopadhyay et al., 2006). Following keratinocyte migration to re-epithelialize the wound, the dermal fibroblasts and dermal microvascular endothelial cells start to migrate into the wound bed. These findings provide a molecular mechanism for how keratinocyte migration occurs within hours, whereas the dermal cell migration is undetectable until 3–4 days later following the injury (reviewed by Singer and Clark, 1999). While the anti-proliferation effect of TGFβ has been studied in various cell culture models, the anti-motility signaling of TGFβ has largely been overlooked over the years. In the current study, we took an advantage of the differential responses of epidermal keratinocytes and dermal fibroblasts to the anti-motility signal of TGFβ3 to identify the pathway that mediates the anti-motility signal of TGFβ3. As schematically shown in Fig. 8, both anti-proliferation and anti-motility signals of TGFβ3 enter HDFs via the higher expression level of TβRII, whereas only the anti-proliferation, but not anti-motility, signal of TGFβ3 was able to enter keratinocytes due to a lower expression of TβRII. In HDFs, TGFβ3 uses the “TβRII > TβRI > R-Smads > PKA” pathway to execute its anti-motility signal.

Our finding of the R-Smads > PKA pathway to execute anti-motility signal of TGFβ3 is in line with several previous reports

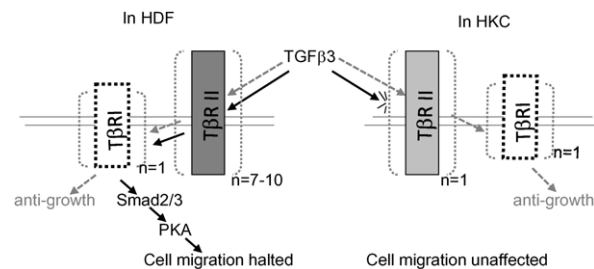


Fig. 8. A schematic representation of anti-motility versus anti-growth signaling by TGFβ3 in two human skin cell types critical for wound healing. While TGFβ3 blocks proliferation of both HDFs and HKCs, it selectively inhibits migration of HDFs, but not HKCs, due to 7 to 10 fold higher expression of TβRII (Bandyopadhyay et al., 2006). TGFβ3 uses TβRII/TβRI/Smad2/3/Smad4/PKA pathway to transmit its anti-motility signal. The anti-proliferation signaling by TGFβ is beyond the scope of this study. The numbers in “n =” are arbitrary to reflect the differences in TβR expression in fold between HDFs and HKCs.

that the canonical R-Smad pathway can cross talk with the PKA pathway in cells. Zhang et al. showed that TGFβ activates PKA via interaction between Smad2/3 and the regulatory subunit (R) of PKA, resulting in release of the catalytic subunit from the PKA holoenzyme in Mv1Lu cells (Zhang et al., 2004). Chowdhury and colleagues reported that TGFβ/PKA pathway is part of TGFβ’s

tumor suppressing function that decreases the survival and inhibits metastasis of colon cancer cells (Chowdhury et al., 2011). Moreover, consistent with our current finding, Giannouli and Kletsas reported that TGFβ activates PKA in adult dermal fibroblasts (Giannouli and Kletsas, 2006). Two groups also reported that PKA and CREB mediate TGFβ-stimulated gene expression in the human keratinocyte cell line, HaCat, and other cell lines of epithelial origin (Schiller et al., 2003; Hayashida et al., 2006). However, we detected weak CREB phosphorylation in response to TGFβ3 stimulation and TGFβ3 did not block migration of HKCs under these conditions. The discrepancy may be due to differences between the immortalized cell lines used by others and primary HKCs used in our study. It should be pointed out that, while the migration of HKCs is unaffected by TGFβ3, HKCs, like HDFs, are equally sensitive to TGFβ3's anti-proliferation signal (Fig. 1B). Therefore, the anti-motility signaling and anti-proliferation signaling of TGFβ3 must take distinct signaling routes in the same cells. Our observation that the constitutively active TβRI-TD mutant was sufficient to convert HKCs to HDF-like cells toward the anti-motility signal of TGFβ3 suggests that the "degrees" of TβRI activation (by the upstream TβRII) are different between HKCs and HDFs, which would determine transmission of either anti-proliferation signal alone or both anti-proliferation or anti-motility signals of TGFβ3 into the cells.

Finally, how TGFβ3's anti-motility signal overrides PDGF-bb's pro-motility signal remains to be studied. For instance, where exactly the two opposite signals meet with each other, where they "fight" and TGFβ3's anti-motility effect wins over PDGF-bb's pro-motility effect, is the next important question for investigation. Results of our recent experiments pointed to the important focal adhesion protein, paxillin, as a center of this battlefield. It is known that extracellular signals regulate paxillin function by two ways: its phosphorylation status and its levels of expression. Changes in either of the two parameters could greatly affect cell morphology and motility (Turner, 2000). While we observed that neither PDGF-bb nor TGFβ3 significantly affect the protein levels of paxillin in HDFs, we found that PDGF-bb and TGFβ3 regulate the serine and tyrosine phosphorylation of paxillin in opposite ways (A.H. and W.L., unpublished data). To functionally define the effects of the distinct phosphorylation levels and sites awaits further and technically challenging studies. In these studies, one would need to first deplete the endogenous wild type paxillin (permanently) and then to introduce various paxillin mutants on phosphorylation sites into these cells. We are currently testing if these opposite effects in paxillin phosphorylation are the reason by which TGFβ3 executes its anti-motility effect over PDGF-bb's stimulatory effect.

Materials and Methods

Primary human neonatal HKCs and HDFs were purchased from Clonetics (San Diego, CA). HKCs were cultured in EpiLife medium with added human keratinocyte growth supplements (HKGS). HDFs were cultured in DMEM supplemented with 10% fetal bovine serum. The third or fourth passages of cells were used for experiments. rhPDGF-bb, rhTGFα, rhTGFβ3, anti-phospho-CREB (Ser-133) antibody and anti-Smad4 antibody were purchased from R&D System (Minneapolis, MN). The sources of human TβR (I and II) and other Smad reagents, including TβRI-TD, TβRII-KD cDNAs, antibodies against TβRI/Alk5 and TβRII, anti-phospho-Smad2 (ser465/467) antibody and anti-Smad2/3 antibody were as previously described (Bandyopadhyay et al., 2006; Bandyopadhyay et al., 2011). Anti-phospho-Smad2/3 or anti-phospho-Smad3 antibodies were from Abcam (Cambridge, MA). PD0325901 (PD901) was from Calbiochem (San Diego, CA). Human Phospho-Kinase Antibody Array (ARY 003) was purchased from R&D System. Anti-PKA Cα antibody, H-89 (dihydrochloride) and forskolin

were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Rat type I collagen was purchased from BD Biosciences (San Jose, CA). Anti-β actin antibody and anti-GAPDH antibody were from Cell Signaling Technology, Inc. (Beverly, Massachusetts). XL-10 Gold Ultra competent cells (XL-10 Gold) (for lentiviral vectors) were from Stratagene (La Jolla, CA).

Immunostaining human skin with anti-TGFβ antibodies

For immunostaining with anti-TGFβ antibodies, we used a diamond cutter to make a highly visible circle around the tissue on the back of the slides with tissue specimen, washed and air-dried the slides. The tissue specimen on slides was fixed with 25 μl acetone for 5 min, washed with PBS, and blocked in 25 μl of the blocking reagent (1:10 dilution of normal goat serum, 0.05% Tween 20, 0.05 Triton X-100, and 1% BSA in PBS) for 60 min in a H₂O humidified chamber. The slides were washed with PBS and incubated with a primary antibody for two hours. The slides were washed three times (7 min each) in PBS, incubated with FITC-conjugated secondary antibody (added directly onto the tissue area) and covered to prevent exposing to light (for the rest of the procedures). Slides were subjected to final wash twice in PBS, air-dried and mounted. The results were analyzed under a microscope (100× at -20°C; TE-2000U Eclipse; Nikon) with application of green (FITC) fluorochrome in the absence of any imaging medium. The images were recorded in JPEG by an attached camera (TE-2000U; Nikon) using MetaMorph software (version-6.2rb; Universal Imaging). The detailed procedures are as previously described (Woodley et al., 2007). This experiment was repeated with tissue specimen from multiple (>6) independent donors.

Cell migration assay and DNA synthesis assay

The colloidal gold migration assay was reported previously (Albrecht-Buehler, 1977) and modified and described in details by us (Li et al., 2004b). Data from multiple independent experiments on HKCs or HDFs were calculated as Migration Index (MI), i.e. the percentage (%) of cell-migrated area over total areas under microscope, in response to corresponding stimulus/inhibitor over the baseline control (mean ± s.d.), $P < 0.01-0.05$. The [³H]-thymidine incorporation assay to measure *de novo* DNA synthesis followed our previously published protocol (Bandyopadhyay et al., 2006).

Lentiviral FG-12 and pRRLsinh-CMV systems

The lentivirus-derived FG-12 system for shRNA delivery, shRNA cloning and viral infection were carried out as previously described (Qin et al., 2003) and then modified (Guan et al., 2007). The sense oligonucleotide shRNA sequence for TβRI, Smad2, Smad3, Smad4, and the PKA catalytic subunit (Cα) are as follows: Smad2, 5'-GGACTGAGTACACCAAATA-3' and 5'-GCAGAAGTATCTCCTACTA-3', Smad3, 5'-GCCTGGTCAAGAACTCAA-3' and 5'-GGTGCTCATCTCCTACTA-3', Smad4, 5'-GGTGGAGAGAGTGAACAT-3' and 5'-GAATCCATATCACTACGAA-3'. PKA-Cα, 5'-GGGTCTCCATCAATGAGA-A-3' and 5'-CCATCCAGATCTATGAGAA-3'. The oligonucleotide shRNA sequence (5'-GACCACAGACAAAGTTATA-3') for TβRI was previously reported (Bandyopadhyay et al., 2011). The usage of the lentivirus-derived vector, pRRLsinh-CMV, for overexpressing genes of interest was as described in details (Li et al., 2004a). Down- or up- regulation of genes was verified by Western blotting with corresponding antibodies (Bandyopadhyay et al., 2006).

Human Phospho-Kinase Array assay

Passage 3 to 4 subconfluently-grown primary HDFs and HKCs in 15 cm tissue culture plates were serum-starved (with 0.2% FBS) overnight. The cells were treated with TGFβ3 for 10 min under serum-free conditions. The cells were lysed (in commercially provided lysis buffer) and subjected to the Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN) strictly according to the manufacturer's instructions, including preparation of cell lysates (750 μl lysis buffer, scraped and centrifuged in a 1.5 ml Eppendorf tube at 13,000 g for 3 min at 4°C), time and temperature for incubation with array membrane, washing, secondary antibodies and ECL. Results were verified by Western blotting experiments. Data were quantitated using an imager, ChemiDoc™ MP with Imagemag Version 4.0.1 software. For the phosphoarray's phosphoimager data, fold = +TGFβ/-TGFβ. For the Western blot data, fold = -TGFβ/its own GAPDH ÷ +TGFβ/its own GAPDH.

Statistics

Data are presented as mean ± standard deviation (s.d.). Statistical significance for comparisons was determined by the Student's two-tailed t-test. A *p* value equal or less than 0.05 was considered statistically significant (Woodley et al., 2007).

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Competing Interests

The authors have no competing interests to declare.

References

- Albrecht-Buehler, G. (1977). The phagokinetic tracks of 3T3 cells. *Cell* **11**, 395-404.
- Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M. and Sporn, M. B. (1983). Transforming growth factor-β in human platelets. Identification of a major storage site, purification, and characterization. *J. Biol. Chem.* **258**, 7155-7160.
- Attisano, L. and Wrana, J. L. (1996). Signal transduction by members of the transforming growth factor-β superfamily. *Cytokine Growth Factor Rev.* **7**, 327-339.
- Bandyopadhyay, B., Fan, J., Guan, S., Li, Y., Chen, M., Woodley, D. T. and Li, W. (2006). A "traffic control" role for TGFβ3: orchestrating dermal and epidermal cell motility during wound healing. *J. Cell Biol.* **172**, 1093-1105.
- Bandyopadhyay, B., Han, A., Dai, J., Fan, J., Li, Y., Chen, M., Woodley, D. T. and Li, W. (2011). TβRI/Alk5-independent TβRII signaling to ERK1/2 in human skin cells according to distinct levels of TβRII expression. *J. Cell Sci.* **124**, 19-24.
- Chowdhury, S., Howell, G. M., Rajput, A., Teggart, C. A., Brattain, L. E., Weber, H. R., Chowdhury, A. and Brattain, M. G. (2011). Identification of a novel TGFβ/PKA signaling transduceome in mediating control of cell survival and metastasis in colon cancer. *PLoS ONE* **6**, e19335.
- Clark, R. A. (1993). Regulation of fibroplasia in cutaneous wound repair. *Am. J. Med. Sci.* **306**, 42-48.
- Cook, S. J. and McCormick, F. (1993). Inhibition by cAMP of Ras-dependent activation of Raf. *Science* **262**, 1069-1072.
- Derynck, R. and Feng, X.-H. (1997). TGF-β receptor signaling. *Biochim. Biophys. Acta* **1333**, F105-F150.
- Derynck, R. and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature* **425**, 577-584.
- Falanga, V. (2004). The chronic wound: impaired healing and solutions in the context of wound bed preparation. *Blood Cells Mol. Dis.* **32**, 88-94.
- Giannouli, C. C. and Kletsas, D. (2006). TGF-β regulates differentially the proliferation of fetal and adult human skin fibroblasts via the activation of PKA and the autocrine action of FGF-2. *Cell. Signal.* **18**, 1417-1429.
- Grainger, D. J., Mosedale, D. E. and Metcalfe, J. C. (2000). TGF-β in blood: a complex problem. *Cytokine Growth Factor Rev.* **11**, 133-145.
- Guan, S., Chen, M., Woodley, D. and Li, W. (2007). Nckβ adapter controls neuriteogenesis by maintaining the cellular paxillin level. *Mol. Cell. Biol.* **27**, 6001-6011.
- Hayashida, K., Johnston, D. R., Goldberger, O. and Park, P. W. (2006). Syndecan-1 expression in epithelial cells is induced by transforming growth factor β through a PKA-dependent pathway. *J. Biol. Chem.* **281**, 24365-24374.
- Heldin, C.-H. and Westermark, B. (1996). Role of platelet-derived growth factor *in vivo*. In *The Molecular And Cellular Biology Of Wound Repair*, 2nd edition (ed. R. A. F. Clark), pp. 249-274. New York, NY: Plenum Press.
- Henry, G., Li, W., Garner, W. and Woodley, D. T. (2003). Migration of human keratinocytes in plasma and serum and wound re-epithelialisation. *Lancet* **361**, 574-576.
- Hering, S., Isken, F., Janott, J., Jost, C., Pommer, A., Muhr, G., Schatz, H. and Pfeiffer, A. F. (2001). Analysis of TGFβ3 gene expression and protein levels in human bone and serum. *Exp. Clin. Endocrinol. Diabetes* **109**, 107-115.
- Iwata, J., Hacia, J. G., Suzuki, A., Sanchez-Lara, P. A., Urata, M. and Chai, Y. (2012). Modulation of noncanonical TGF-β signaling prevents cleft palate in *Tgfb2* mutant mice. *J. Clin. Invest.* **122**, 873-885.
- Khalil, N. (1999). TGF-β: from latent to active. *Microbes Infect.* **1**, 1255-1263.
- Li, W., Fan, J., Chen, M., Guan, S., Sawcer, D., Bokoch, G. M. and Woodley, D. T. (2004a). Mechanism of human dermal fibroblast migration driven by type I collagen and platelet-derived growth factor-BB. *Mol. Biol. Cell* **15**, 294-309.
- Li, W., Henry, G., Fan, J., Bandyopadhyay, B., Pang, K., Garner, W., Chen, M. and Woodley, D. T. (2004b). Signals that initiate, augment, and provide directionality for human keratinocyte motility. *J. Invest. Dermatol.* **123**, 622-633.
- Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. *Science* **276**, 75-81.
- Miyazono, K. (2000). Positive and negative regulation of TGF-β signaling. *J. Cell Sci.* **113**, 1101-1109.
- Moustakas, A. and Heldin, C.-H. (2005). Non-Smad TGF-β signals. *J. Cell Sci.* **118**, 3573-3584.
- Mulder, K. M. (2000). Role of Ras and Mapks in TGFβ signaling. *Cytokine Growth Factor Rev.* **11**, 23-35.
- Murphy-Ullrich, J. E. and Poczatek, M. (2000). Activation of latent TGF-β by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev.* **11**, 59-69.
- Piek, E., Heldin, C. H. and Ten Dijke, P. (1999). Specificity, diversity, and regulation in TGF-β superfamily signaling. *FASEB J.* **13**, 2105-2124.
- Qin, X. F., An, D. S., Chen, I. S. and Baltimore, D. (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc. Natl. Acad. Sci. USA* **100**, 183-188.
- Roberts, A. B. (1998). Molecular and cell biology of TGF-β. *Miner. Electrolyte Metab.* **24**, 111-119.
- Roberts, A. B. and Sporn, M. B. (1996). Transforming growth factor-β. In *The Molecular And Cellular Biology Of Wound Repair*, 2nd edition (ed. R. A. F. Clark), pp. 275-308. New York, NY: Plenum Press.
- Sarret, Y., Woodley, D. T., Grigsby, K., Wynn, K. and O'Keefe, E. J. (1992). Human keratinocyte locomotion: the effect of selected cytokines. *J. Invest. Dermatol.* **98**, 12-16.
- Schiller, M., Verrecchia, F. and Mauviel, A. (2003). Cyclic adenosine 3', 5'-monophosphate-elevating agents inhibit transforming growth factor-β-induced SMAD3/4-dependent transcription via a protein kinase A-dependent mechanism. *Oncogene* **22**, 8881-8890.
- Shi, Y. and Massagué, J. (2003). Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.
- Singer, A. J. and Clark, R. A. F. (1999). Cutaneous wound healing. *N. Engl. J. Med.* **341**, 738-746.
- Tokiwa, G., Tyers, M., Volpe, T. and Futcher, B. (1994). Inhibition of G1 cyclin activity by the Ras/cAMP pathway in yeast. *Nature* **371**, 342-345.
- Tredget, E. E., Nedelec, B., Scott, P. G. and Ghahary, A. (1997). Hypertrophic scars, keloids, and contractures. The cellular and molecular basis for therapy. *Surg. Clin. North Am.* **77**, 701-730.
- Turner, C. E. (2000). Paxillin and focal adhesion signalling. *Nat. Cell Biol.* **2**, E231-E236.
- Wieser, R., Wrana, J. L. and Massagué, J. (1995). GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J.* **14**, 2199-2208.
- Woodley, D. T., Remington, J., Huang, Y., Hou, Y., Li, W., Keene, D. R. and Chen, M. (2007). Intravenously injected human fibroblasts home to skin wounds, deliver type VII collagen, and promote wound healing. *Mol. Ther.* **15**, 628-635.
- Wrana, J. L., Attisano, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F. and Massagué, J. (1992). TGFβ signals through a heteromeric protein kinase receptor complex. *Cell* **71**, 1003-1014.
- Zhang, L., Duan, C. J., Binkley, C., Li, G., Uhler, M. D., Logsdon, C. D. and Simeone, D. M. (2004). A transforming growth factor β-induced Smad3/Smad4 complex directly activates protein kinase A. *Mol. Cell. Biol.* **24**, 2169-2180.
- Zhang, W., Jiang, Y., Wang, Q., Ma, X., Xiao, Z., Zuo, W., Fang, X. and Chen, Y. G. (2009). Single-molecule imaging reveals transforming growth factor-β-induced type II receptor dimerization. *Proc. Natl. Acad. Sci. USA* **106**, 15679-15683.