TβRI/Alk5-independent TβRII signaling to ERK1/2 in human skin cells according to distinct levels of TβRII expression

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Summary

TGFβ binding to the TGFβ receptor (TβR) activates R-Smad-dependent pathways, such as Smad2/3, and R-Smad-independent pathways, such as ERK1/2. The mechanism of the TGFβ–TβRII–TβRI–Smad2/3 pathway is established; however, it is not known how TGFβ activates ERK1/2. We show here that although TGFβ equally activated Smad2/3 in all cells, it selectively activated ERK1/2 in dermal cells and inhibited ERK1/2 in epidermal cells. These opposite effects correlated with the distinct expression levels of TβRII, which are 7- to 18-fold higher in dermal cells than in epidermal cells. Reduction of TβRII expression in dermal cells abolished TGFβ-stimulated ERK1/2 activation. Upregulation of TβRII expression in epidermal cells to a similar level as that in dermal cells switched TGFβ-induced ERK1/2 inhibition to ERK1/2 activation. More intriguingly, in contrast to the equal importance of TβRII in mediating TGFβ signaling to both Smad2/3 and ERK1/2, knockdown of TβRI/Alk5 blocked activation of only Smad2/3, not ERK1/2, in dermal cells. Similarly, expression of the constitutively activated TβRI-TD kinase activated only Smad2/3 and not ERK1/2 in epidermal cells. This study provides an explanation for why TGFβ selectively activates ERK1/2 in certain cell types and direct evidence for TβRI-independent TβRII signaling to a R-Smad-independent pathway.

Key words: TGF-β, Receptors, ERK1/2, Signal transduction

Introduction

When skin is wounded and the dermal blood vessels in the wound are damaged, the resident skin cells are bathed in plasma-converted serum for the first time. A key factor in human serum, but not in plasma, is transforming growth factor-β3 (TGFβ3). TGFβ3 has a positive role in wound healing by differentially regulating the motility of epidermal and dermal cells, depending upon on their naturally occurring levels of the TGFβ receptor II (TβRII) (Bandyopadhyay et al., 2006). Therefore, we are particularly interested in the action of TGFβ3.

TGFβ signals are transmitted via a cell surface receptor complex, the TβRII and TβRI/Alk5 heterodimer. TGFβ binds to TβRII, which in turn recruits, transphosphorylates and activates TβRI, thereby achieving cross-membrane signaling to inside of the cell (Derynck and Feng, 1997; Shi and Massagué, 2003). Once the signal is inside the cell, the post-receptor signaling events are divided into R-Smad-dependent and R-Smad-independent pathways (Derynck and Zhang, 2003). The R-Smad-dependent signaling pathway mediates TGFβ signaling to transcriptional activation of target genes (Miyazono, 2000). R-Smad-independent signaling pathways, including TAK1–MEKK1, the extracellular signal-regulated kinases 1 and 2 (ERK1/2), Jun N-terminal kinase (JNK), p38-mitogen-activated kinase (p38-MAPK), phosphatidylinositol 3-kinase (PI3K) and Rho family GTPases, mediate a range of effects of TGFβ, whose outcomes are often dependent upon cell type and cellular context (Mulder, 2000; Derynck and Zhang, 2003; Moustaka and Heldin, 2005). TGFβ-induced Smad2/3 activation occurs in almost all cell types and has been thoroughly characterized. TGFβ-stimulated R-Smad-independent pathways, however, often vary in different cell types (Moustakas and Heldin, 2005). In this short report, we show that differences in the level of TβRII expression determine whether or not TGFβ activates or inhibits ERK1/2 and that TβRII alone is able to mediate TGFβ signaling to ERK1/2 without participation of TβRI/Alk5.

Results and Discussion

TGFβ3 selectively activates ERK1/2 in dermal cells, but inhibits ERK1/2 in epidermal cells, yet TGFβ3 universally activates Smad2/3

To investigate why TGFβ-stimulated activation of R-Smad-independent pathways often depends upon the cell type, we screened the three major human skin cell types that are involved in repair and regeneration: keratinocytes, dermal fibroblasts and microvascular endothelial cells, for TGFβ3-stimulated ERK1/2 activation. The three cell types were primary cells isolated from human neonatal foreskin and all express physiological levels of TβRI and TβRII. In quiescent human dermal fibroblasts (DFs) and human dermal microvascular endothelial cells (HDMECs), TGFβ3 stimulation induced a dose-dependent phosphorylation of ERK1/2 (Fig. 1Aa and c). In human keratinocytes (HKs), there was a basal level of ERK1/2 phosphorylation (Fig. 1Ae, lane 1). This was probably due to previously reported epidermal growth factor secretion and autocrine signaling in these cells (Kansra et al., 2004). However, in contrast to dermal cells, TGFβ3 stimulation

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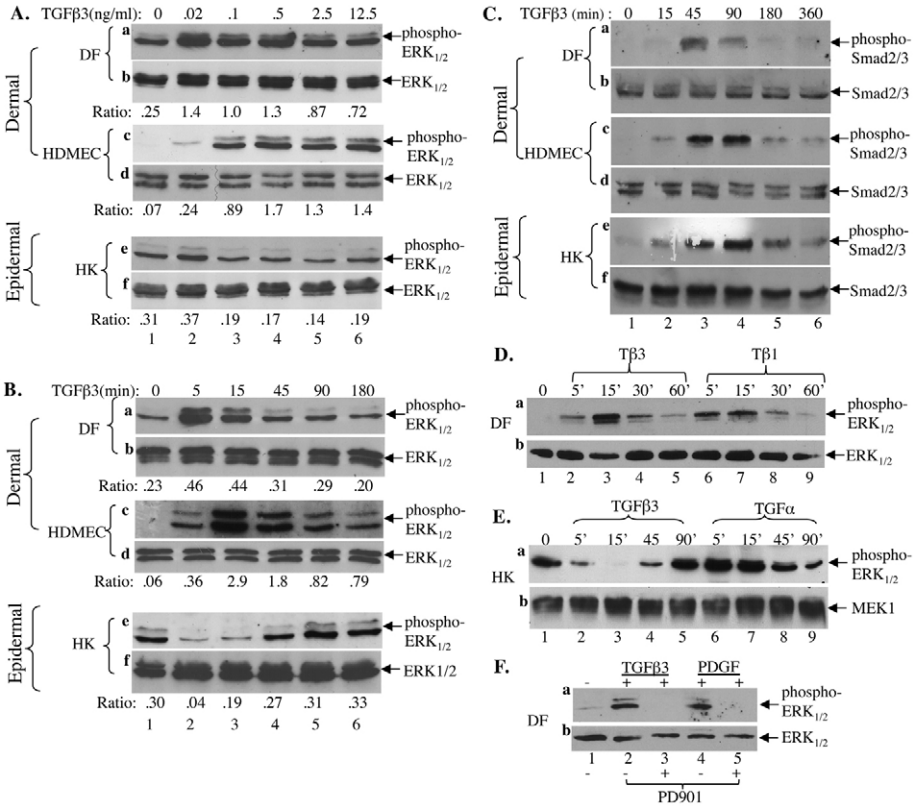

Fig. 1. Opposite effects of TGFβ3 on ERK1/2 in human dermal versus epidermal cells. HKs, DFs and HDMECs in cultures were serum-starved overnight and subjected to (**A**) dose-dependent (5 minute stimulation) or (**B**,**C**) time course (1.0 ng/ml stimulation) with recombinant human (rh) TGFβ3. Equalized cell lysates (~40 μg/lane) were subjected to western blot analysis with anti-ERK1/2-*P* and anti-ERK1/2 antibody (A,B) or anti-Smad2-*P* and anti-Smad2/3 antibodies (C). ECL results were subjected scanning densitometry to measure the ratio (fold) of ERK1/2-*P* over total ERK1/2. (**D**) Comparison of 3 ng/ml TGFβ1-stimulated and 1 ng/ml TGFβ3-stimulated kinetics of ERK1/2 phosphorylation. (**E**) Serum-starved HKs were treated without or with TGFβ3 (1.0 ng/ml) or TGFα (200 ng/ml) and subjected to western blot analysis, as indicated. (**F**) The effect of PD901 on TGFβ3- or PDGF-BB-stimulated ERK1/2 activation. DFs were pre-treated with PD901 (10 μM) for 30 minutes (and continued presence of PD901) before addition of the growth factors.

induced a transient and dose-dependent decrease in ERK1/2 phosphorylation in these cells (Fig. 1Ae, lanes 2–5). Consistently, TGFβ3 induced a time-dependent increase in ERK1/2 phosphorylation in DFs (Fig. 1Ba) and HDMECs (Fig. 1Bc), whereas it caused a transient decrease in ERK1/2 phosphorylation in HKs (Fig. 1Be).

We were curious whether selective activation of ERK1/2 by TGFβ3 in dermal but not epidermal cells also applied to Smad2/3 phosphorylation. TGFβ3 stimulation universally induced Smad2/3 phosphorylation in DFs (Fig. 1Ca), HDMECs (Fig. 1Cc) and HKs (Fig. 1Ce), which followed a similar kinetics, with maximum Smad2/3 phosphorylation between 45 and 90 minutes. These results indicated that the cell-type-specific effects of TGFβ3 on ERK1/2 phosphorylation do not apply to TGFβ3-induced Smad2/3 phosphorylation in the same cells. Quantitatively, TGFβ1 is the most abundant TGFβ isoform in skin wounds (Bandyopadhyay et al., 2006). By comparison, we did not detect any significant differences in ERK1/2 phosphorylation in dermal cells in response to stimulation by TGFβ1 or TGFβ3, although more TGFβ1 than TGFβ3 was required (Fig. 1D). We further questioned whether the inability of TGFβ3 to activate ERK1/2 in epidermal cells was due to an intrinsic defect in the ERK1/2 pathway. We compared stimulation of ERK1/2 phosphorylation by TGFβ3 with that of TGFα (a major serum growth factor for HK growth). Although TGFβ3 induced a temporal decrease in the basal phosphorylation of ERK1/2 (Fig. 1Ea, lanes 2–4 vs lane 1), TGFα stimulation (via binding to EGFR) induced a two- to threefold increase in ERK1/2 phosphorylation over the basal level (Fig. 1Ea, lanes 6–9 vs lane 1). These results demonstrate that there is no intrinsic defect in the ERK1/2 pathway in epidermal cells. We also investigated whether TβRII directly activates ERK1/2 or acts via MEK1. We found that PD901, a specific inhibitor of MEK1, dramatically inhibited both TGFβ3- and PDGF-BB (platelet-derived growth factor-BB)-stimulated ERK1/2 phosphorylation (Fig. 1F). These results suggest that the activated TβRII also acts via the Ras–Raf–MEK1 cascade to activate ERK1/2.

TβRII expression determines how TGFβ communicates with ERK1/2

To investigate the molecular basis for differential regulation of ERK1/2 by TGFβ3 in dermal versus epidermal cells, we focused on the expression levels of TβRII and TβRI/Alk5 subunits – the first TGFβ-interacting proteins involved in cross-membrane signaling. Although variable levels of TβRI expression were found in HKs, DFs and HDMECs (Fig. 2Aa), there was no correlation between the differences in TβRI levels (Fig. 2Aa lanes 1, 3 and 4) and the selective activation of ERK1/2 in dermal, but not epidermal, cells in response to TGFβ3. Neura-crest-originated epidermal melanocytes (MCs) were also included as a control (Fig. 2Aa lane 2). By contrast, we found a strong correlation between TβRII expression levels and ERK1/2 activation. DFs and HDMECs exhibited 7- to 18-fold higher levels of TβRII expression than HKs (Fig. 2Bc, lanes 3 and 4 vs lane 1). To confirm these results, we subjected sections of normal human skin to immunostaining with three anti-TβRII antibodies against distinct epitopes and from three independent commercial sources. All three anti-TβRII antibodies showed stronger staining of TβRII in the dermis than the epidermis (Fig. 2Cb, c and d vs a). By contrast, anti-TβRI antibody showed equal staining of both dermis and epidermis, as we have previously shown (Bandyopadhyay et al., 2006). It should be noted that, unlike epidermis that is >90% composed of HKs, the sparse staining of TβRII in the dermis reflects the normal distribution of DFs in

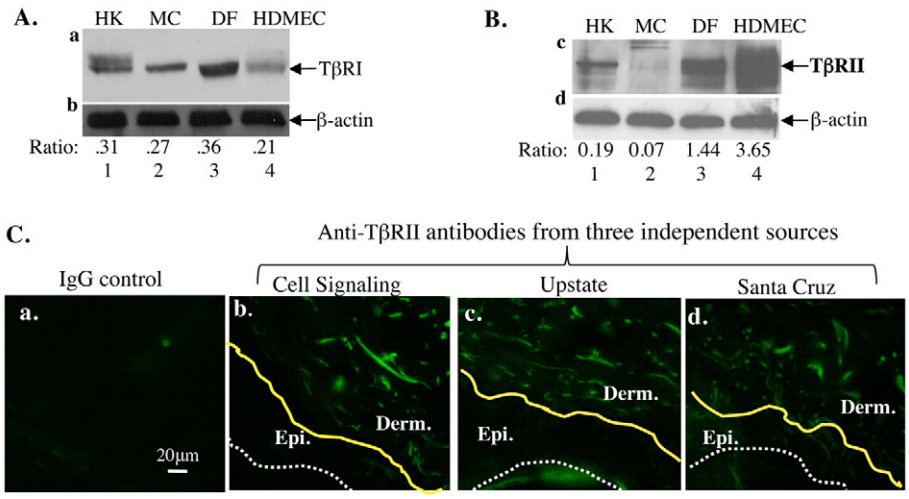

Fig. 2. In vitro and in vivo profiles of TβRII–TβRI subunit in four human skin cells. (**A**,**B**) Equalized cell lysates of HKs, DFs and HDMECs and melanocytes (MCs), were subjected to western blot analyses with antibodies against TβRI/Alk5 (A) or TβRII (B). (**C**) Indirect immunofluorescence staining of normal human skin with antibodies against TβRII from three independent sources, as indicated. Solid yellow line outlines the basement membrane. Epi, epidermis; Derm, dermis. Scale bar: 20 μm.

the dermis, where scattered DFs are embedded in large areas of connective tissue. Therefore, a given section could only reveal a few DFs.

We then studied whether the differences in TβRII levels indeed determine how TGFβ3 regulates ERK1/2 using two approaches: (1) downregulation of TβRII in DFs and (2) upregulation of TβRII in HKs. We used the lentiviral system FG-12 to deliver shRNA to knock down TβRII. FG-12 offered a >99% transduction efficiency in DFs (Fig. 3A) and the shRNA dramatically downregulated the endogenous TβRII (Fig. 3Ba, lane 3), in comparison to infections with vector alone or vector carrying a non-specific shRNA (Fig. 3Ba, lanes 1 and 2). As expected, the shRNA did not affect TβRI/Alk5 in the same cells (Fig. 3Bb). In the control cells, TGFβ3 stimulation induced a time-dependent phosphorylation of both Smad2/3 (Fig. 3Bd) and ERK1/2 (Fig. 3Bh). However, in the DFs with downregulated TβRII, TGFβ3-stimulated phosphorylation of both Smad2/3 (Fig. 3Bf) and ERK1/2 (Fig. 3Bj) was completely blocked. These results indicate that TβRII is crucial for mediation of the TGFβ3 signaling to both Smad2/3 and ERK1/2 pathways in DFs.

To further address the importance of TβRII levels in ERK1/2 activation, we carried out the reverse experiments. We reasoned that if the failure of TGFβ3 to activate ERK1/2 in HKs was due to their lower level of TβRII expression, then raising the TβRII level to that of DFs should switch the inhibition of ERK1/2 to activation of ERK1/2 in these cells. To overexpress a gene of interest, we used the lentiviral vector, pRRLsin.MCS-Deco, which also offered a >97% gene transduction efficiency in HKs (Fig. 3C). We overexpressed the wild type (WT) or a kinase-defective (DN) TβRII in HKs from the endogenous low level (Fig. 3D, lane 2) to a level that was similar to that in DFs (Fig. 3D, lanes 3 and 4 vs lane 1). We found that, in the WT TβRII-overexpressing HKs, TGFβ3 induced a rapid and time-dependent ERK1/2 activation (Fig. 3Dk), similarly to DFs. TGFβ3 still induced a decrease in ERK1/2 phosphorylation in vector control HKs (Fig. 3Di). Overexpression of the TβRII-DN mutant inhibited TGFβ3-stimulated phosphorylation of Smad2/3 (Fig. 3Dg), as expected. Furthermore, unlike WT TβRII, the TβRII-DN failed to mediate ERK1/2 activation (Fig. 3Dm). Instead, it caused a slight increase in the basal ERK1/2 phosphorylation and delayed TGFβ3-induced inhibition of ERK1/2 (Fig. 3Dm), which is the exact opposite to results when WT TβRII is overexpressed. These results indicate that the TβRII expression levels and kinase activity determine whether or not TGFβ3 activates or inhibits ERK1/2.

TβRI is not required for TGFβ signaling to ERK1/2

The widely accepted dogma for TGFβ signaling is the sequential events of TGFβ–TβRII–TβRI–intracellular signaling pathways, as previously mentioned. However, we found little correlation between TβRI expression levels and the differential effects of TGFβ3 on ERK1/2. We questioned, therefore whether TβRI had any role in mediating TGFβ3 signaling to ERK1/2. First, to answer this question in DFs, we used two independent approaches: (1) downregulation of endogenous TβRI to an undetectable level, which became technically feasible with the lentiviral RNAi system, FG-12 (Qin et al., 2003), and (2) inhibition of TβRI kinase. Two independent shRNAs (RNAi1 and RNAi2) either dramatically or completely downregulated the endogenous TβRI/Alk5 (Fig. 4Ab′, lanes 1 and 3 vs lane 2), but did not affect TβRII in the same cells (Fig. 4Aa′, lanes 1 and 3 vs lane 2). We chose to use the RNAi2-infected DFs, where the TβRI was reduced to an undetectable level (Fig. 4Ab′, lanes 1). As expected, TGFβ3 was no longer able to induce any detectable Smad2/3 phosphorylation (Fig. 4Af′) in these cells, in comparison with the control cells (Fig. 4Ad′). However, the TGFβ3-induced ERK1/2 phosphorylation remained unchanged in these cells (Fig. 4Aj′), in comparison with the control cells (Fig. 4Ah′). To confirm this surprising finding, we used a TβRI kinase-specific inhibitor, SB431542 (inhibiting Alk4, Alk5 and Alk7) (DaCosta Byfield et al., 2004). Treatment of DFs with increasing concentrations of SB431542 led to a complete blockade of TGFβ3-stimulated Smad2/3 phosphorylation (Fig. 4Ba′, lanes 3–5 vs lane 2). However, SB431542 showed little inhibition of TGFβ3-stimulated ERK1/2 activation at any of the concentrations used in the same cells (Fig. 4Bb′, lanes 3–5 vs lane 2).

Second, to answer the same question in HKs, we took an advantage of the constitutively activated TβRI mutant, TβRI-TD, which would initiate TGFβ signaling in the absence of upstream activators. The idea was that, if TGFβ3 signaling to ERK1/2 goes through TβRI, similarly to Smad2/3, the TβRI-TD mutant should induce a constitutive activation of both Smad2/3 and ERK1/2 even in the absence of TGFβ. There was a four- to sixfold increase in TβRI-WT and TβRI-TD mutant expression over the endogenous TβRI (Fig. 4Ca′, lanes 2 and 3 vs lane 1). As expected, in TβRI-TD-overexpressing HKs, Smad2/3 phosphorylation became

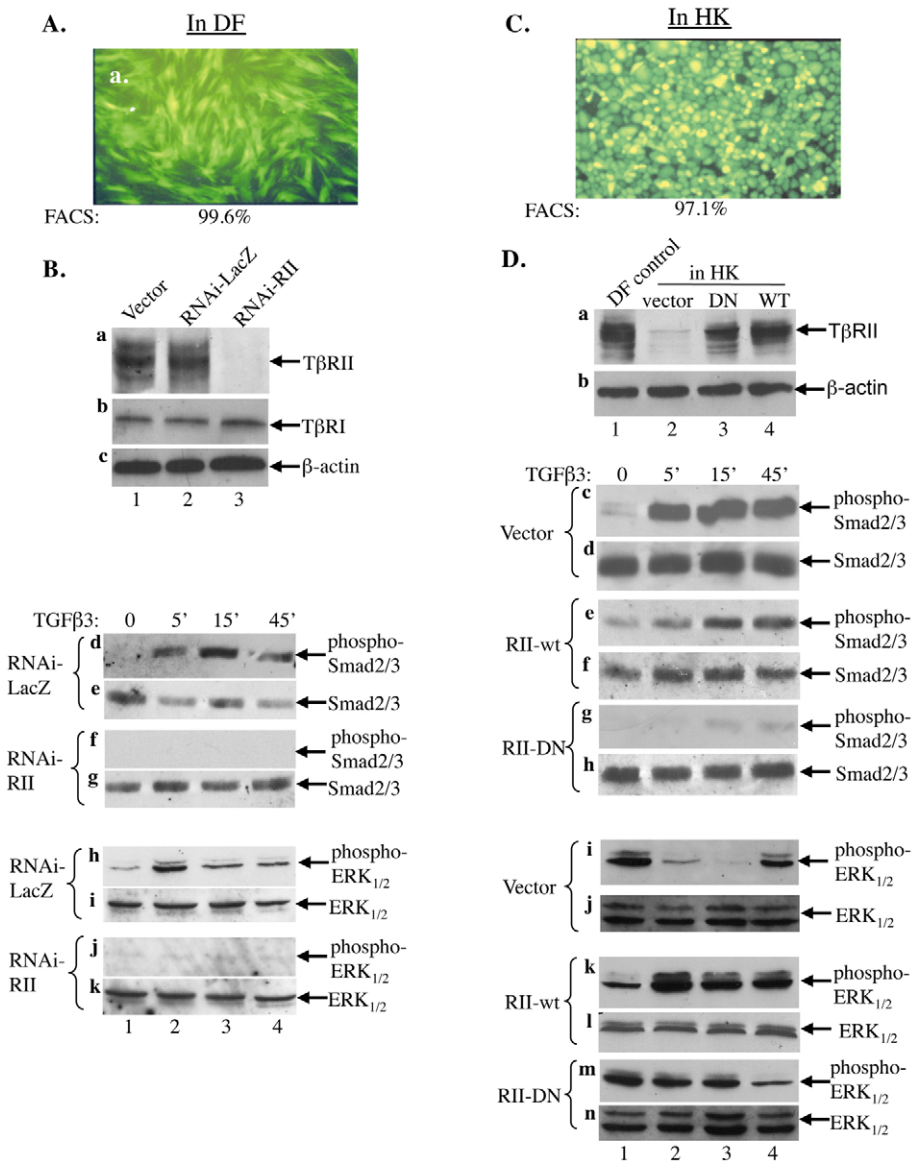

Fig. 3. TβRII expression levels determine how TGFβ regulates ERK1/2. (**A**) Lentiviral gene transduction efficiency, indicated by GFP expression, in DFs. (**B**) Downregulation of endogenous TβRII (a, lane 3 vs lanes 1 and 2) but not TβRI (b, lane 3). Equalized lysates of TGFβ3 stimulated DFs were subjected to anti-Smad2-*P* (d and f), anti-Smad2/3 protein (e and g) antibodies, anti-ERK1/2-*P* (phospho-ERK1/2) (h and j) or anti-ERK protein (ERK1/2) (i and k) antibody immunoblotting analyses. (**C**) Lentiviral gene transduction efficiency in HKs. (**D**) Overexpression of the wt or DN mutant of TβRII in HKs (a, lanes 3 and 4 vs lane 2), with DF lysate as a comparison (lane 1). Western blot analyses with anti-Smad2-*P* (c,e,g), anti-Smad2/3 protein (d,f,h), anti-ERK1/2-*P* (i,k,m) or anti-ERK1/2 protein (j,l,n) antibodies.

constitutive even in the absence of TGFβ3 stimulation (Fig. 4Cg′, lanes 2–4 vs lane 1). Therefore, the TβRI-TD mutant worked as expected. However, in the same cells, TβRI-TD was unable to either cause ERK1/2 activation over its basal level or rescue the TGFβ3-induced inhibition of ERK1/2 (Fig. 4Cm′ vs i′ and k′), in sharp contrast to the effect of overexpressed WT TβRII (see Fig. 3C). In addition, the negative results with the constitutively activated TβRI-TD kinase on ERK1/2 also ruled out any possible involvement of other Alk isoform kinase(s) that might be present in HKs. Taken together, the above findings provide direct evidence for TβRI-independent signaling by TβRII to a R-Smad-independent pathway, as schematically represented in Fig. 4D.

Since the initial reports that TGFβ activates ERK1/2 in epithelial cells and breast cancer cells (Hartsough and Mulder, 1995), ERK1/2 activation has been linked to a number of TGFβ-regulated cellular events, including CKIs $p21^{Cip1}$ and $p27^{Kip1}$ gene expression and growth arrest (Hartsough et al., 1996; Frey and Mulder, 1997), the epithelial-mesenchymal transition (EMT) (Zavadil et al., 2001) and breast cancer cell motility (Dumont et al., 2003). An important question was how the TβRI–TβRII complex activates ERK1/2. Lee and colleagues reported that, following TβRII activation, TβRI in the TβRI–TβRII complex recruits and phosphorylates ShcA, an SH2 adapter protein. Tyrosine-phosphorylated ShcA in turn recruits the Grb2/Sos complex that activates the Ras–Raf–Mek1–ERK1/2 cascade (Lee et al., 2007). By contrast, Imamichi and co-workers showed that the TβRI inhibitor SB431542 could not fully block TGFβ-induced ERK1/2 activation, suggesting that TβRI is not involved in ERK1/2 activation (Imamichi et al., 2005). However, none of these studies specifically addressed the roles of TβRII and TβRI in TGFβ signaling to ERK1/2. Using FG12-mediated complete knockdown of the endogenous TβRs and pRRLsin-mediated overexpression of the constitutively activated TβRI-TD mutant, the current study provides direct evidence that (1) the differences in TβRII expression levels determine ERK1/2 activation or inhibition and (2) TβRII is crucial and TβRI/Alk5 is dispensable for TGFβ signaling to ERK1/2.

This finding appeared to be at odds with a previous notion that TβRII is constitutively activated even in the absence of TGFβ. If

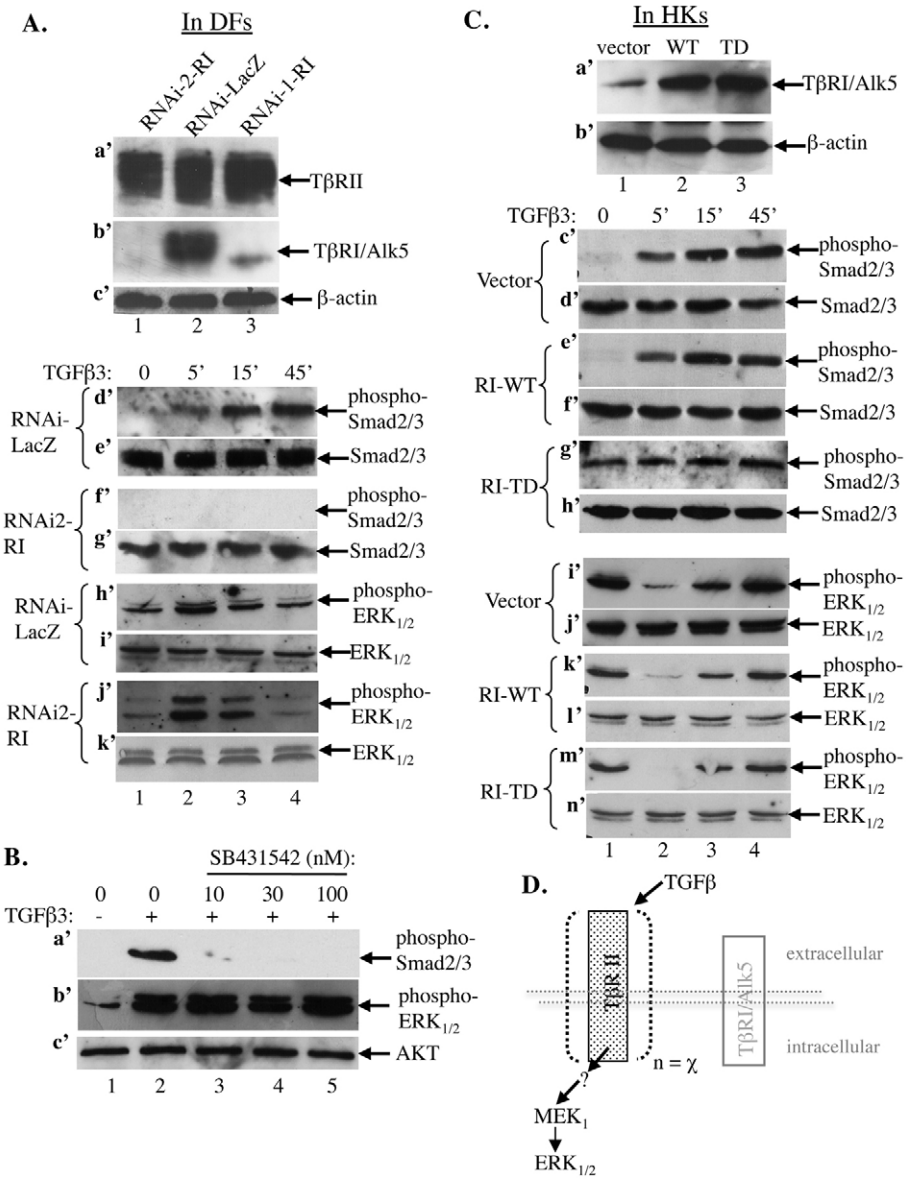

Fig. 4. TβRI is dispensable for TGFβ signal to ERK1/2. (**A**) Downregulation of the endogenous TβRI (b′, lanes 1 and 3 vs lane 2), but not TβRII (a′), by two shRNAs to knock down TβRI. TGFβ3-stimulated Smad2-*P* (d′ and f′), Smad2 protein (e′ and g′), ERK1/2-*P* (h′ and j′) and ERK protein (i′ and k′) were analyzed. (**B**) In DFs, rhTGFβ3 (1.0 ng/ml, 45 minutes) stimulated, in the absence or presence of various concentrations of SB431542, Smad2-*P* (a′) or ERK1/2-*P* (b′). To use the same membrane for all antibodies, anti-AKT blot was used. (**C**) In HKs, overexpression of the WT or TD mutant of TβRI over the endogenous TβRI (a′, lanes 2 and 3 vs lane 1). Lysates of TGFβ3-stimulated cells were subjected to Smad2-*P* (c′,e′,g′) or ERK1/2-*P* blots (i′,k′,m′), as previously described. (**D**) A schematic representation of the main findings in this study. The question mark indicates an unknown linker. The '*n*' in *x*=*n* was not defined by a specific number, but low in HKs and higher in DFs and HDMECs.

this is true, how can ERK1/2 activation still remain sensitive to TGFβ stimulation? It should be pointed out that previous studies were based on exogenously overexpressed TβRs and/or in vitro biochemical kinase assays. Under similar conditions, even tyrosine kinase receptors such as HER2 in breast cancer cells and EGFR in the carcinoma cell line A431 become constitutively active. Recently, Chen's group has shown that under physiological expression levels TβRII forms dimers in response to TGFβ stimulation (Zhang et al., 2009). Thus, the first likely explanation is that the 'excess' of TβRII (which do not have TβRI molecules to form heterodimers at the cell surface) in DFs and HDMECs form homodimers among themselves. This homo-dimerization leads to activation of TβRII but not TβRI, and activation of ERK1/2. Second, the excess TβII receptors might form a complex with a new gene product. For instance, Qiu and colleagues have recently reported that TβRII can form a complex and directly phosphorylate the parathyroid hormone type I receptor (PTH1R), which is important for bone production and absorption (Qiu et al., 2010).

Materials and Methods

Cells and antibodies

Primary human neonatal human keratinocytes (HKs), melanocytes (MCs), dermal fibroblasts (DFs) and human dermal vascular endothelial cells (HDMECs) were purchased from Clonetics (San Diego, CA) and cultured as previously described (Bandyopadhyay et al., 2006). Human recombinant TGFβ1, TGFβ2 and TGFβ3 were purchased from the R&D Systems. Anti-ERK1/2 antibody (03-6600) was from Zymed Laboratories (South San Francisco, CA). Anti-ERK1/2-*P* antibody (V803A) was from Promega (Madison, WI). The sources of human TβR (I and II), including TβRI-TD, TβRII-KD cDNAs, antibodies against TβRI/Alk5 and TβRII (Santa Cruz, SC-400, Upstate, 06-277 and Cell Signaling, #3713), anti-Smad2-*P* (Ser465/467) antibody, anti-Smad2/3 antibody, anti-Akt antibody, anti-MEK1 antibody and anti-β actin antibody were used as previously described (Bandyopadhyay et al., 2006). Anti-Smad3-*P* antibody (#52903) was from Abcam (Cambridge, MA). PD0325901 (PD901) was from Calbiochem (San Diego, CA).

Lentiviral gene-overexpressing and gene-downregulating systems

Lentivirus-derived vectors, FG-12 and pRRLsinh-CMV were used as described elsewhere (Qin et al., 2003; Bandyopadhyay et al., 2006).

Measurement of gene expression and activation levels

Protein expression of infected genes was detected and quantified as described (Li et al., 2004a; Li et al., 2004b).

Staining human skin tissue or cells with anti-TβR antibodies

The protocols for staining frozen human skin sections with the indicated antibodies were described previously (Bandyopadhyay et al., 2006).

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