TGF β -stimulated Smad1/5 phosphorylation requires the ALK5 L45 loop and mediates the pro-migratory TGF β switch

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During the course of breast cancer progression, normally dormant tumour-promoting effects of transforming growth factor β (TGF β), including migration, invasion, and metastasis are unmasked. In an effort to identify mechanisms that regulate the pro-migratory TGF β 'switch' in mammary epithelial cells *in vitro*, we found that TGF β stimulates the phosphorylation of Smad1 and Smad5, which are typically associated with bone morphogenetic protein signalling. Mechanistically, this phosphorylation event requires the kinase activity and, unexpectedly, the L45 loop motif of the type I TGF β receptor, ALK5, as evidenced by studies using short hairpin RNA-resistant ALK5 mutants in ALK5-depleted cells and *in vitro* kinase assays. Functionally, Smad1/5 co-depletion studies demonstrate that this phosphorylation event is essential to the initiation and promotion of TGF β -stimulated migration. Moreover, this phosphorylation event is preferentially detected in permissive environments such as those created by tumorigenic cells or oncogene activation. Taken together, our data provide evidence that TGF β -stimulated Smad1/5 phosphorylation, which occurs through a non-canonical mechanism that challenges the notion of selective Smad phosphorylation by ALK5, mediates the pro-migratory TGF β switch in mammary epithelial cells.

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Introduction

In various breast cancer model systems, the transforming growth factor β (TGF β) signalling pathway has been shown to have disparate functions during tumorigenesis (Massague, 2008). In normal mammary epithelial cells, TGF β signalling maintains homeostasis and elicits tumour-suppressive responses such as growth inhibition and apoptosis (Siegel

and Massague, 2003). But during the course of breast cancer progression, TGF β signalling loses its tumour-suppressive function and acquires the ability to drive tumour-promoting responses such as proliferation, survival, migration, invasion, and metastasis (Jakowlew, 2006). The underlying signalling mechanisms which account for the switch in TGF β signalling responses are not well characterized and thus the subject of intense investigation.

In the canonical view of TGF β signalling, TGF β signals through a heteromeric receptor complex composed of the type II TGF β receptor kinase, T β RII, and the type I TGF β receptor kinase, ALK5 (Feng and Derynck, 2005). The presence of TGF β ligand results in receptor complex activation, thereby allowing ALK5 to phosphorylate Smad2 and Smad3, the so-called TGF β -Smads, at their C-terminal SSXS motifs. The selectivity of Smad2/3 phosphorylation by ALK5 is ensured by the kinase domain-embedded L45 loop, which is thought to serve as the docking site for Smad2/3 (Feng and Derynck, 1997; Chen *et al*, 1998; Itoh *et al*, 2003). Following the association of phosphorylated Smad2/3 with Smad4, the Smad complexes translocate to the nucleus, where they modulate transcription of TGF β -responsive genes to produce a cellular response.

Although this simplistic Smad2/3-dependent pathway accounts for many effects of TGF β signalling, it cannot adequately explain how TGF β signalling generates such a diverse and opposing set of responses. It is now recognized that the proteomic composition of a cell influences the cellular response to TGF β signalling (Massague and Chen, 2000). Thus, cellular context controls not only the output response of the Smad2/3-dependent pathway but also the activation of Smad2/3-independent pathways that can regulate additional TGF β responses (Derynck and Zhang, 2003).

This concept of context-dependent control is exemplified by studies on TGF β -stimulated migration in breast cancer model systems. For example, previous studies suggest that TGF β -stimulated migration can be Smad2/3 dependent or independent (Dumont *et al*, 2003; Tian *et al*, 2003, 2004). Moreover, the ability of TGF β to stimulate migration can be unmasked by overexpression of oncogenes such as HER2 (Seton-Rogers *et al*, 2004; Ueda *et al*, 2004). Although these studies establish that TGF β stimulates migration in breast cancer cells, few studies have investigated how cellular contexts that permit TGF β -stimulated migration affect the ability of TGF β to signal through Smad proteins.

The TGF β -related bone morphogenetic proteins (BMPs) transmit signals through Smad1, Smad5, and Smad8, the so-called BMP-Smads, by activating type I receptors containing L45 loops that specify Smad1/5/8 phosphorylation (Feng and Derynck, 2005). Interestingly, TGF β has been shown to stimulate Smad1/5 phosphorylation and regulate migration in endothelial cells (Goumans *et al*, 2002). Contrasting the canonical view of TGF β signalling, these effects have been

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attributed to lateral signalling through the Smad1/5/8-phosphorylating type I receptor, ALK1, which is thought to form a mixed ALK1–ALK5 receptor complex in the presence of TGF β (Goumans *et al*, 2003). Although these ALK1-based studies suggest that BMP-Smads have a functional role downstream of TGF β receptor complex signalling, the direct involvement of Smad1/5/8 during TGF β -stimulated migration has not been demonstrated. Moreover, apart from these endothelial cell-based studies, few studies have investigated the prevalence, regulation, and potential relevance of TGF β -stimulated Smad1/5/8 phosphorylation in other cell contexts.

Herein, we establish the mechanism and function of TGF β -stimulated Smad1/5 phosphorylation. We provide evidence of a previously unrecognized ALK5 L45 loop-dependent mechanism of TGF β -stimulated Smad1/5 phosphorylation. This phosphorylation event is essential to the initiation and promotion of potent TGF β -stimulated migration, and is particularly relevant to breast cancer progression as it occurs in permissive cellular environments such as those created by tumorigenic cells and their associated oncogenes.

Results

TGF β stimulates migration and Smad1/5 phosphorylation in 4T1 cells

To explore signalling pathways that regulate TGF β -stimulated migration, we used the tumorigenic mouse mammary epithelial line, 4T1, which is known to migrate in response to TGF β (McEarchern *et al*, 2001). Although treatment of 4T1 cells with TGF β strongly promoted migration, treatment with the related ligands, activin A or BMP7, had little to no effect on migration (Figure 1A). This effect did not result from proliferative effects of TGF β as TGF β had no effect on 4T1 proliferation (data not shown). We verified that TGF β stimulated Smad3 phosphorylation, as assessed by western blot analysis using a phospho-specific antibody that detects C-terminally phosphorylated Smad3 (425 aa), Smad1 (465 aa), Smad5 (465 aa), and Smad8 (430 aa) (Figure 1B). Unexpectedly, TGF β treatment also resulted in the appearance of a robust band that was similar in size to the band corresponding to phosphorylated Smad1 and Smad5, but not phosphorylated Smad8 in lysates of BMP7-treated cells (Figure 1B). By showing that co-depletion of Smad1 and Smad5 prevented the appearance of this band, we confirmed that TGF β stimulated Smad1/5 phosphorylation (refer to Figure 3C). In contrast, activin A treatment, which stimulates Smad2 phosphorylation, resulted in only a slight increase in Smad1/5 phosphorylation (Figure 1B), suggesting that potent Smad1/5 phosphorylation by non-BMP ligands is specific to TGF β .

We next investigated the kinetics of Smad2 and Smad1/5 phosphorylation in a time course analysis. Although TGF β -stimulated Smad2 phosphorylation or BMP-stimulated Smad1/5 phosphorylation persisted for at least 195 min, TGF β -stimulated Smad1/5 phosphorylation disappeared within 195 min (Figure 1C). Although the overall duration of TGF β -stimulated Smad1/5 phosphorylation was relatively short, Smad1/5, like Smad2 and Smad3, was phosphorylated within 5 min of TGF β treatment (Supplementary Figure 1).

Figure 1 TGF β stimulates Smad1/5 phosphorylation and migration in 4T1 cells. (A) Transwell migration of cells treated for 20 h with indicated ligands. Data shown are representative of at least two independent experiments. (B) Western blot analysis of cells treated with indicated ligands for 45 min. Arrow indicates band corresponding to phosphorylated Smad8. (C) Western blot analysis of Smad phosphorylation over time in cells treated with indicated ligands.

ALK5 kinase activity is required for TGF β -stimulated migration and Smad1/5 phosphorylation in 4T1 cells

The TGF β -specific effects and rapid Smad1/5 phosphorylation described above suggested a role for the type I TGF β receptor, ALK5, during TGF β -stimulated migration and Smad1/5 phosphorylation. To test the requirement of ALK5, we generated 4T1 cells that stably express short hairpin RNA (shRNA) targeting nonspecific control sequence or ALK5 using the pSUPER-retro system. Expression of two different ALK5 shRNAs resulted in an ~65% decrease in ALK5 mRNA (Supplementary Figure 2A), potent inhibition of Smad2/3 and Smad1/5 phosphorylation following TGF β treatment (Figure 2A; Supplementary Figure 2B), and inhibition of TGF β -stimulated migration (Figure 2B; Supplementary Figure 2C). Confirming that these effects were specific to ALK5 knockdown, expression of shRNA-resistant wild-type ALK5 (ALK5 WT) in the ALK5-1 shRNA line rescued TGF β -stimulated migration and phosphorylation of Smad3 and Smad1/5 (Figure 2A and B). In contrast, expression of shRNA-resistant kinase-dead ALK5 (ALK5 KR) failed to stimulate phosphorylation of Smad3 and Smad1/5 (Figure 2A) to levels equivalent to ALK5 WT. Notably, a small increase in TGF β -stimulated Smad3 and Smad1/5 phosphorylation was

Figure 2 ALK5 kinase activity is required for TGF β -stimulated Smad1/5 phosphorylation and migration in 4T1 cells. Control or ALK5-depleted cells expressing empty vector (MSCV) or indicated shRNA-resistant HA-tagged ALK5 isoforms were subjected to (A) western blot analysis after 45 min. TGF β treatment or (B) transwell migration for 20 h in the presence or absence of TGF β . Data shown are representative of at least two independent experiments. (C) Transwell migration of cells treated for 20 h with vehicle or TGF β in the presence or absence of ALK5 kinase inhibitor, SB431542 (SB). Representative images of stained migratory cells on transwell membranes are shown. (D) Western blot analysis of cells pretreated with SB431542 for 30 min and then treated with vehicle or TGF β for 45 min.

detected in the ALK5 KR-expressing line when compared with the expression of MSCV empty vector control (Figure 2A). However, this slight increase, which is most likely attributable to recruitment, scaffolding, and activation of low-level endogenous ALK5 by ectopic ALK5, clearly failed to rescue TGF β -stimulated migration (Figure 2B). Confirming the importance of ALK5 kinase function, a small-molecule inhibitor of ALK5 kinase activity, SB431542, blocked TGF β -stimulated migration and phosphorylation of Smad2 and Smad1/5 in a dose-dependent manner (Figure 2C and D). Taken together, these data indicate that ALK5 kinase activity is required for TGF β -stimulated migration and phosphorylation of Smad2/3 and Smad1/5.

Depletion of Smad1 and Smad5 inhibits TGF β -stimulated migration in 4T1 cells

As functional inhibition of ALK5 decreased Smad1/5 phosphorylation and migration, we reasoned that Smad1/5 phosphorylation could mediate TGF β -stimulated migration. Consistent with this idea, even though BMP4 treatment alone failed to stimulate migration in MDA-MB-231 mammary epithelial cells, co-treatment of TGF β with BMP4, which produced higher levels of Smad1/5 phosphorylation than TGF β treatment alone (Supplementary Figure 3A), enhanced the ability of TGF β to stimulate migration (Supplementary Figure 3B).

To directly evaluate whether Smad1 and Smad5 functionally contribute to TGF β -stimulated migration in 4T1 cells, we

silenced the expression of Smad1 or Smad5 using the lentiviral pSicoR-puro system. Despite efficient and specific suppression by two independent Smad1-targeting shRNAs, 1A or 1B, and two independent Smad5-targeting shRNAs, 5B or 5C (Figure 3A), depletion of either Smad1 or Smad5 alone failed to block TGF β -stimulated migration (Figure 3B).

As Smad1 and Smad5, which are 90% homologous at the amino-acid level, are functionally redundant in some contexts (Arnold *et al*, 2006), we reasoned that functional compensation could be occurring. Consistent with this notion, we detected comparable levels of phosphorylated Smad1/5 in TGF β -treated control, Smad1, and Smad5 shRNA-expressing cells (Figure 3A).

Accordingly, we examined the effect of Smad1/5 co-depletion on TGF β -stimulated migration. 5B + 1B shRNA expression resulted in complete and specific reduction of Smad1/5 expression and TGF β -stimulated Smad1/5 phosphorylation (Figure 3C). This reduction in Smad1/5 expression resulted in an ~85% decrease in BMP4-induced transcriptional activity from the Smad1/5/8-responsive BRE-luciferase reporter (Supplementary Figure 4), confirming potent functional inhibition of Smad1/5 activity. Importantly, 5B + 1B shRNA expression resulted in an ~65% decrease in TGF β -stimulated migration when compared with control shRNA expression (Figure 3D), suggesting a partial requirement for Smad1/5 activity during TGF β -stimulated migration. In 1A + 5C or 1B + 5C shRNA cells, we observed complete inhibition of Smad1 expression, but incomplete inhibition of Smad5

Figure 3 Depletion of Smad1/5 but not Smad2/3 inhibits TGF β -stimulated migration in 4T1 cells. Puromycin-selected cells expressing empty vector (pSicoR-puro), Smad1 targeting shRNA (1A or 1B), or Smad5 targeting shRNA (5B or 5C) were subjected to (A) western blot analysis after 45 min. TGF β treatment or (B) transwell migration for 20 h in the presence or absence of TGF β . Asterisk indicates detection of Smad1 by Smad5 antibody. Puromycin-selected, GFP $^{+}$ cells expressing empty vector (pSicoR-puro + GFP), 1A + 5C shRNA, 5B + 1B shRNA, and 1B + 5C shRNA were subjected to (C) western blot analysis after 45 min. TGF β treatment or (D) transwell migration for 20 h in the presence or absence of TGF β . Two-tailed Student's *t*-test was performed (***P* = 0.05, ****P* = 0.01, *****P* = 0.11). Cells expressing control or S3 + S2 shRNA were subjected to (E) western blot analysis after 45 min. TGF β treatment or (F) transwell migration for 20 h in the presence or absence of TGF β . Migration assays represent the average of at least three independent experiments.

expression and Smad5 phosphorylation (Figure 3C). This partial Smad1/5 co-depletion corresponded to a moderate decrease (~25–40%) in TGF β -stimulated migration when compared with control cells (Figure 3D). Taken together, these data indicate that maximal TGF β -stimulated migration in 4T1 cells is dependent on the total level of Smad1/5 expression and phosphorylation.

As Smad2 and Smad3 may also contribute to TGF β -stimulated migration, we tested the requirement of Smad2 or Smad3 using the lentiviral FG12 and pLKO.1 shRNA systems. Smad2 or Smad3 depletion alone failed to block TGF β -stimulated migration (Supplementary Figure 5A and B). Likewise, efficient Smad2/3 co-depletion, as assessed by western blot analysis and Smad2-responsive ARE-luciferase or Smad3-responsive SBE-luciferase activity (Figure 3E; Supplementary Figure 5C and D), did not inhibit TGF β -stimulated migration (Figure 3F), suggesting that TGF β -stimulated Smad1/5 phosphorylation and migration are independent of Smad2/3 activity. Consistent with this view, expression of 5B + 1B shRNA did not significantly alter TGF β -stimulated translocation of Smad3 to the nucleus or TGF β -induced Smad3 transcriptional activity (Supplementary Figure 5E and F). Taken together, these data indicate that Smad1/5 phosphorylation mediates TGF β -stimulated migration in a Smad2/3-independent manner.

Selective Smad1/5 phosphorylation by ALK5 partially restores TGF β -stimulated migration in ALK5 shRNA-expressing 4T1 cells

To confirm the involvement of Smad1/5 phosphorylation during TGF β -stimulated migration, we tested whether selec-

tive Smad1/5 phosphorylation could rescue TGF β -stimulated migration in the ALK5 shRNA background. To do this, we expressed a previously characterized 'swap' mutant of ALK5 (ALK5 3/6), in which the L45 loop of ALK5 was replaced by the corresponding L45 loop of ALK3 or ALK6, thereby permitting Smad1/5, but not Smad2/3 phosphorylation by ALK5 (Figure 4A). Consistent with published 'swap' mutant data (Feng and Derynck, 1997; Chen *et al*, 1998), expression of ALK5 3/6, but not ALK5 WT permitted TGF β -induced BRE-luciferase activity (Figure 4B), which correlated with the ability of ALK5 3/6 to prolong TGF β -stimulated Smad1/5 phosphorylation when compared with ALK5 WT (Supplementary Figure 6). Importantly, ALK5 3/6 expression preferentially increased TGF β -stimulated Smad1/5 phosphorylation when compared with ALK5 KR, and partially restored TGF β -stimulated migration when compared with ALK5 WT (Figure 4C and D). These data reinforce the notion that Smad1/5 phosphorylation promotes TGF β -stimulated migration in 4T1 cells. Moreover, when taken together with the partial inhibition of TGF β -stimulated migration in Smad1/5 co-depleted cells, these data indicate that Smad1/5 phosphorylation cooperates with other ALK5 L45 loop-derived signaling events to promote maximal TGF β -ALK5-stimulated migration.

The ALK5 L45 loop is required for TGF β -stimulated Smad1/5 phosphorylation and migration

We next investigated potential mechanisms of TGF β -stimulated Smad1/5 phosphorylation. On the basis of the requirement of ALK5 activity, we hypothesized that TGF β -stimulated Smad1/5 phosphorylation arises from indiscriminate ALK5

Figure 4 Expression of various ALK5 isoforms in ALK5-1 shRNA cells differentially affects TGFβ-stimulated Smad1/5 phosphorylation and migration. (A) Summary of wild-type and mutant ALK5 amino-acid sequences and previously reported Smad phosphorylation activities. Underlined residues indicate substitutions. ALK5-depleted 4T1 cells expressing the indicated shRNA-resistant HA-tagged ALK5 isoforms were subjected to (B) luciferase assay as described in Supplementary data, (C) western blot analysis after 45 min. TGFβ treatment or (D) transwell migration for 20 h in the presence or absence of TGFβ. Data shown are representative of at least two independent experiments. (E, F) *In vitro* kinase assay measuring GST-Smad phosphorylation by anti-HA immunoprecipitates derived from 293T cells co-expressing the indicated TGFβ receptors. The western blot analysis of assay samples using the indicated antibodies is shown. Coomassie-stained GST-Smad serves as a GST-Smad loading control.

L45 loop activity. To investigate this possibility, we expressed in the ALK5 shRNA background two different shRNA-resistant ALK5 mutants (ALK5 D266A and ALK5 3A). These previously characterized mutants possess alanine substitutions within the L45 loop, thereby rendering ALK5 defective in Smad2/3 recruitment and phosphorylation (Figure 4A; Itoh *et al*, 2003). As with the ALK5 KR mutant, expression of the D266A and 3A mutants did not restore TGFβ-stimulated Smad3 phosphorylation to levels equivalent to the expression of ALK5 WT (Figure 4C).

Notably, expression of the D266A and 3A mutants of ALK5 failed to restore TGFβ-stimulated Smad1/5 phosphorylation (Figure 4C). These mutants also failed to rescue TGFβ-stimulated migration (Figure 4D), demonstrating that TGFβ-stimulated Smad1/5 phosphorylation and migration require the ALK5 L45 loop. When considered alongside our Smad protein depletion data, these data suggest that ALK5 L45 loop-dependent migration does not result from L45 loop-mediated Smad3 activation; rather, it stems from L45 loop-mediated Smad1/5 phosphorylation.

As ALK5 L45 loop-mediated Smad1/5 phosphorylation contrasts the widely accepted notion of selective Smad2/3 phosphorylation mediated by the ALK5 L45 loop, we sought to extend this observation to additional cell systems. As shown in Supplementary Figure 7, TGFβ-stimulated Smad1/5 phosphorylation was also dependent on the ALK5 L45 loop in Mv1Lu-derived R1B mink lung epithelial cells and PY-4-1 endothelial cells.

The requirement for the ALK5 L45 loop during TGFβ-stimulated Smad1/5 phosphorylation suggested that ALK5 could directly phosphorylate Smad1 and Smad5. To address this possibility, we performed *in vitro* kinase assays using TGFβ receptor complexes immunoprecipitated from 293T cells co-expressing HA-tagged TβRII and HA-tagged ALK5. As shown in Figure 4E, GST-Smad3, GST-Smad1, and GST-Smad5 were C-terminally phosphorylated by anti-HA immunoprecipitates containing ALK5 WT, but not ALK5 KR, suggesting that ALK5 directly phosphorylates Smad3, Smad1, and Smad5 in a kinase activity-dependent manner. Importantly, anti-HA immunoprecipitates containing ALK5

D266A (Figure 4F) could not phosphorylate GST-Smad1, indicating that an intact wild-type ALK5 L45 loop is required for direct phosphorylation of Smad1/5 by ALK5. That GST-Smad1 phosphorylation occurred even in the absence of TGF β treatment most likely reflects the ability of 293T cells to support high levels of basal Smad1/5 phosphorylation, which is due in part to ALK5 kinase activity (Supplementary Figure 8A; Daly *et al*, 2008), and the ability of co-expressed T β RII and ALK5 to signal in a ligand-independent manner (Feng and Derynck, 1996).

TGF β -stimulated Smad1/5 phosphorylation and migration in 4T1 cells is independent of BMP signalling and lateral type I receptor signalling

Although our data demonstrate the direct involvement of ALK5 during TGF β -stimulated Smad1/5 phosphorylation, additional mechanisms may be involved. Therefore, we asked whether TGF β -stimulated Smad1/5 phosphorylation and migration are secondary to activation of an autocrine BMP signalling loop. To test this possibility, we treated 4T1 cells with TGF β in the presence or absence of soluble chimaeric antibody antagonists that neutralize the function of BMPs (BMPRIa-Fc) or TGF β (T β RII-Fc). Although BMPRIa-Fc and T β RII-Fc effectively blocked BMP-stimulated Smad1/5 phosphorylation and TGF β -stimulated Smad3 phosphorylation, respectively (Figure 5A), TGF β -stimulated migration and phosphorylation of Smad3 and Smad1/5 were blocked by T β RII-Fc, but not by BMPRIa-Fc (Figure 5A and B). These data indicate that TGF β -stimulated Smad1/5 phosphorylation and migration are independent of BMP-derived signals. Additionally, the translation inhibitor, cycloheximide, did not block TGF β -stimulated Smad1/5 phosphorylation (Supplementary Figure 9), suggesting that *de novo* synthesis of secreted Smad1/5/8-activating ligands is not required for this effect.

We next asked whether lateral signalling through Smad1/5/8-phosphorylating type I receptors was involved in TGF β -stimulated Smad1/5 phosphorylation and migration. ALK1, ALK2, and ALK3, but not ALK6 mRNA expression was detected in 4T1 cells (Supplementary Figure 10). However, unlike endothelial cells, which are known to express high levels of ALK1 mRNA, 4T1 cells expressed low levels of ALK1 mRNA (Figure 5C). Indeed, we determined that 4T1 cells express three orders of magnitude less ALK1 mRNA than PY-4-1 endothelial cells, but relatively similar levels of ALK5 mRNA (Figure 5C). As such low ALK1 levels were beyond the limit of precise quantitation by real-time RT-PCR (data not shown), thereby precluding our ability to assess and confirm ALK1 suppression using shRNA, we determined that ALK1 was unlikely to have a significant function.

We next investigated the potential involvement of ALK2 or ALK3. First, we verified significant ALK2 mRNA suppression by shRNA (Supplementary Figure 11A). In ALK2-4 shRNA cells, we observed a significant reduction of Smad1/5 phosphorylation in response to BMP7, a known ALK2 ligand, but not to TGF β (Figure 5D), thereby confirming specific suppression of ALK2. However, ALK2-4 shRNA did not block TGF β -stimulated migration (Figure 5E). In ALK3-23 shRNA cells, we verified ALK3-specific mRNA suppression (Supplementary Figure 11B), and observed a significant reduction of BRE-luciferase activity in response to BMP4, a known ALK3 ligand (Figure 5F). However, a decrease in

TGF β -stimulated Smad1/5 phosphorylation or migration was not detected (Figure 5G and H). Taken together, these data suggest that lateral signalling through ALK1, ALK2, or ALK3 is not required for TGF β -stimulated Smad1/5 phosphorylation and migration in 4T1 cells. Consistent with this notion, we could not detect ALK3 in anti-HA immunoprecipitates used to phosphorylate GST-Smad1/5 *in vitro* (Supplementary Figure 8B).

Tumorigenic mammary epithelial cells provide a permissive context for TGF β -stimulated Smad1/5 phosphorylation

TGF β treatment has been shown to activate overexpressed Smad1 in Hs578T mammary epithelial cells (Liu *et al*, 1998). However, the prevalence of TGF β -stimulated Smad1/5 phosphorylation in mammary epithelial cells, particularly in the endogenous context, is unknown. Thus, we tested whether this phosphorylation event occurs in a small panel of mammary epithelial cell lines, including hTERT-immortalized DU9910 cells, spontaneously immortalized MCF10A cells, and tumorigenic MDA-MB-231, 67NR, and MIV cells. Interestingly, we detected TGF β -stimulated Smad1/5 phosphorylation in tumorigenic, but not immortalized cells (Figure 6), suggesting that potent TGF β -stimulated Smad1/5 phosphorylation is dependent on mammary epithelial cell context.

TGF β -stimulated Smad1/5 phosphorylation is essential for establishing sensitivity to TGF β -stimulated migration

On the basis of preferential TGF β -stimulated Smad1/5 phosphorylation in tumorigenic mammary epithelial cells, we reasoned that breast cancer cells could provide a permissive environment for TGF β -stimulated Smad1/5 phosphorylation through amplification and activation of breast cancer-associated oncogenes such as HER2. To address this possibility, we evaluated TGF β -stimulated Smad1/5 phosphorylation in control MCF10A cells or MCF10AN cells, which stably express a chimaeric HA-tagged HER2 receptor, the dimerization and activation of which can be induced by the synthetic molecule, AP1510 (Muthuswamy *et al*, 2001). In control MCF10A cells, we observed low levels of TGF β -stimulated Smad1/5 phosphorylation in the presence or absence of AP1510 (Figure 7A). Although we detected slightly elevated levels of TGF β -stimulated Smad1/5 phosphorylation in untreated MCF10AN cells, which is most likely due to leaky chimaeric HER2 dimerization as evidenced by low-level Erk phosphorylation (Figure 7A), we noticed potent TGF β -stimulated Smad1/5 phosphorylation in AP1510-treated MCF10AN cells (Figure 7A). We also observed HER2-enabled TGF β -stimulated Smad1/5 phosphorylation in independently generated MCF10A cells that overexpress full-length human HER2 (Figure 7B), suggesting that this effect did not result from artefactual chimaeric HER2 activity. Taken together, these data demonstrate that HER2 overexpression or dimerization facilitates potent TGF β -stimulated Smad1/5 phosphorylation.

Previous work has shown that HER2 overexpression or dimerization provides a permissive cellular context for TGF β -stimulated migration (Seton-Rogers *et al*, 2004; Ueda *et al*, 2004). Confirming this result, treatment of control MCF10A cells with TGF β in the presence or absence of AP1510 did not

Figure 5 TGF β -stimulated Smad1/5 phosphorylation and migration are independent of BMP signalling and lateral type I receptor signalling in 4T1 cells. (A) Western blot analysis of cells co-treated for 45 min with indicated ligands and soluble antagonists. (B) Transwell migration of cells after 20-h co-treatment with indicated ligands and soluble antagonists. Asterisks denote untested conditions. (C, left) Thirty-five-cycle qualitative RT-PCR analysis with or without reverse transcriptase (RT) or (C, right) quantitative real-time RT-PCR analysis of relative mRNA levels in PY-4-1 (PY) and 4T1 cells. Control or ALK2-depleted cells were subjected to (D) western blot analysis after 45-min treatment with indicated ligands or (E) transwell migration for 20 h in the presence of vehicle or TGF β . Arrow indicates band corresponding to phosphorylated Smad8. Control or ALK3-23 shRNA-expressing cells were subjected to (F) BRE-luciferase assay in the presence or absence of BMP4 as described in Supplementary data, (G) western blot analysis after 45-min TGF β treatment, or (H) transwell migration for 20 h in the presence of vehicle or TGF β .

Figure 6 Tumorigenic cells provide a permissive context for TGF β -stimulated Smad1/5 phosphorylation. Western blot analysis of cells after 45-min TGF β treatment.

result in a significant change in migration, whereas treatment of MCF10AN cells with TGF β in the presence, but not the absence of AP1510 led to a significant increase in migration (Figure 7C). Interestingly, the magnitude of TGF β -stimulated migration was closely correlated with the potency of TGF β -stimulated Smad1/5 phosphorylation (compare Figure 7A with C), suggesting that Smad1/5 phosphorylation mediates the ability of HER2 to unmask an otherwise latent pro-migratory function of TGF β .

To test this idea, we suppressed Smad1 and Smad5 expression in MCF10AN cells by transiently transfecting control or Smad1/5 duplex siRNA. To assess the requirement of Smad3 in this cellular context, we also transfected Smad3 siRNA. These siRNAs specifically and efficiently suppressed target Smad protein expression (Figure 7D). Confirming the functional effect of Smad3 siRNA and the known requirement for Smad3 in the TGF β cytosolic response (Siegel and Massague, 2003), Smad3-depleted cells were defective in TGF β -stimulated growth inhibition (Figure 7E). Conversely, Smad1/5 depletion had no effect on TGF β -stimulated growth inhibition (Figure 7E). We then tested the effect of Smad depletion on migration of AP1510-treated MCF10AN cells. Although TGF β treatment led to a significant increase in migration in control siRNA-transfected cells, TGF β treatment had little to no effect in Smad3 or Smad1/5 siRNA-transfected cells (Figure 7F), indicating that Smad3 and Smad1/5 are required for TGF β -stimulated migration in MCF10AN cells. Taken together, these data demonstrate that HER2 establishes sensitivity to TGF β -stimulated migration by facilitating potent TGF β -stimulated Smad1/5 phosphorylation.

Figure 7 TGF β -stimulated Smad1/5 phosphorylation is essential for the initiation of sensitivity to TGF β -stimulated migration. (A) Western blot analysis of cells co-treated as indicated for 45 min. (B) Western blot analysis of cells cultured in growth media and treated with TGF β for 45 min. (C) Transwell migration of cells and co-treated as indicated for 20 h. (D) Western blot analysis of cells transfected with control, Smad3 (S3), or Smad1/5 (S1 + S5) targeting duplex siRNA co-treated as indicated for 45 min. (E) TGF β -stimulated growth inhibition in siRNA-transfected MCF10AN cells as described in Materials and methods. (F) Transwell migration of siRNA-transfected, AP1510-treated MCF10AN cells following 20h vehicle or TGF β treatment. TGF β -stimulated migration is shown. Data from (E, F) represent average of at least three independent experiments.

Discussion

In this report, we provide evidence for the existence, regulation, and pro-migratory function of TGF β -stimulated Smad1/5 phosphorylation in breast cancer model systems. We present two main conclusions. First, TGF β -stimulated Smad1/5 phosphorylation occurs through an ALK5 L45 loop-dependent mechanism. Second, Smad1/5 phosphorylation is critical to the initiation and promotion of sensitivity to TGF β -stimulated migration in mammary epithelial cells.

TGF β -stimulated Smad1/5 phosphorylation occurs through an ALK5 L45 loop-dependent mechanism

We and others have clearly demonstrated in different cell types that ALK5 is required for TGF β -stimulated Smad1/5 phosphorylation (Goumans *et al*, 2003; Daly *et al*, 2008).

However, the precise involvement of ALK5 remains controversial. TGF β has been proposed to signal laterally through a mixed ALK1/2/3-ALK5 complex (Goumans *et al*, 2003; Daly *et al*, 2008). However, this proposed mechanism is under increased scrutiny due to recent evidence indicating that direct formation of mixed ALK5 receptor complexes is unlikely due to differences in ALK receptor-binding modes (Groppe *et al*, 2008). Indeed, our data indicate that lateral type I receptor signalling is unlikely to be involved during TGF β -stimulated Smad1/5 phosphorylation, as evidenced by low-level expression of ALK1 in 4T1 and MCF10A-derived cells (data not shown) and by the inability of ALK2 or ALK3 depletion to block TGF β -stimulated Smad1/5 phosphorylation. Notably, pretreatment of 4T1 cells with dorsomorphin, a small-molecule inhibitor of BMP signalling that blocks Smad1/5/8 phosphorylation by ALK2/3/6 (Yu *et al*, 2008),

partially blocked TGF β -stimulated Smad1/5 phosphorylation (Supplementary Figure 11C). Although this result appears to suggest a requirement for ALK2/3/6, our data from ALK5 3/6 mutant studies suggest that the effect of dorsomorphin may not be attributable to the inhibition of ALK2/3/6 kinase activity, but rather to the prevention of Smad1/5 recruitment to the L45 loop of ALK receptors (Supplementary Figure 11D).

We provide evidence of a direct role for ALK5 in TGF β -stimulated Smad1/5 phosphorylation. The ALK5 L45 loop is required for TGF β -stimulated Smad1/5 phosphorylation in mammary epithelial, lung epithelial, and endothelial cells, suggesting that ALK5 L45 loop dependence is a conserved feature of TGF β -stimulated Smad1/5 phosphorylation in other cell types. Consistent with this idea, a TGF β receptor complex composed of wild-type T β RII and ALK5 is sufficient for Smad1/5 phosphorylation *in vitro*. Moreover, an intact ALK5 L45 loop is required for Smad1 phosphorylation *in vitro*. Although our results depart from the long-standing view of selective Smad2/3 phosphorylation mediated by the ALK5 L45 loop, it corroborates the central role of the L45 loop during Smad recruitment and phosphorylation. Therefore, we conclude that ALK5 has a more direct function than previously recognized during TGF β -stimulated Smad1/5 phosphorylation.

The factors that regulate ALK5 L45 loop-mediated Smad1/5 phosphorylation are unknown, but Smad2 and Smad3 are candidates based on their known L45 loop-dependent recruitment to ALK5. However, Smad3 depletion in MCF10AN cells or Smad2/3 co-depletion in 4T1 cells did not affect TGF β -stimulated Smad1/5 phosphorylation. Moreover, TGF β -stimulated Smad1/5 phosphorylation is detected in MCF10A-HER2 cells despite significantly downregulated levels of Smad3 expression relative to control MCF10A-vector cells (see Figure 7B). Thus, it is unlikely that Smad2/3 regulates TGF β -stimulated Smad1/5 phosphorylation.

Although we detected TGF β -stimulated Smad1/5 phosphorylation, we did not observe TGF β -induced transcriptional activity from the Smad1/5/8-responsive BRE-luciferase reporter (Supplementary Figure 4). The relatively short duration of TGF β -stimulated Smad1/5 phosphorylation most likely accounts for the lack of BRE-luciferase activity. Supporting this view, optimal activation of a Smad-responsive reporter requires at least 3–4 h of continuous ALK5 activity or Smad phosphorylation (Inman *et al*, 2002). Additionally, the ALK5 3/6 ‘swap’ mutant, which prolongs TGF β -stimulated Smad1/5 phosphorylation when compared with ALK5 WT, enables TGF β -induced BRE-luciferase activity. Importantly, the lack of TGF β -induced BRE-luciferase activity suggests that Smad1/5-dependent TGF β -stimulated migration does not require some classical Smad1/5-dependent BMP target genes. Indeed, BMP4, but not TGF β upregulates Id1 and Id2 mRNA expression in 4T1 cells (data not shown). We are currently conducting microarray analysis to identify potential Smad1/5-dependent TGF β target genes as part of a larger effort to explore mechanisms of Smad1/5-mediated migration.

Smad1/5 phosphorylation is critical to the initiation and promotion of sensitivity to TGF β -stimulated migration in mammary epithelial cells

Previous work has shown that activation of oncogenes, such as Ras and HER2, can unmask the pro-metastatic, pro-inva-

sive, and pro-migratory functions of TGF β signalling (Oft *et al*, 1998; Seton-Rogers and Brugge, 2004), but few mechanisms regulating this TGF β switch have been identified. One potential mechanism involves prolonged signalling through receptor-tyrosine kinase effectors, such as Rac1 and ERK (Seton-Rogers *et al*, 2004; Wang *et al*, 2006). Another mechanism involves the subversion of Smad3 tumour-suppressive function to pro-invasive function (Sekimoto *et al*, 2007). We demonstrate the importance of a third mechanism involving TGF β -stimulated Smad1/5 phosphorylation.

One advantage of the TGF β -stimulated Smad1/5 phosphorylation response for pre-malignant mammary epithelial cells is that it helps initiate the pro-migratory response to TGF β . Indeed, in MCF10AN cells, the ability of HER2 to elevate TGF β -stimulated Smad1/5 phosphorylation to functionally relevant levels underlies the known ability of HER2 to unmask TGF β -stimulated migration. Notably, HER2 is but one factor that promotes TGF β -stimulated Smad1/5 phosphorylation as functional inhibition of HER2 in 4T1 cells using shRNA-mediated mRNA depletion or small molecule ErbB kinase inhibitors fails to block TGF β -stimulated Smad1/5 phosphorylation (data not shown).

A second advantage of the TGF β -stimulated Smad1/5 phosphorylation response is that it provides a Smad2/3-independent pathway, which is capable of stimulating migration or invasion (Supplementary Figure 12). This may be of particular relevance to breast cancer progression as at least a subset of breast cancer cells can acquire resistance to the tumour-suppressive or pro-migratory functions of Smad3 (Chen *et al*, 2001; Dumont *et al*, 2003; Gomis *et al*, 2006). This notion is supported by our observation of Smad3-dependent TGF β -stimulated migration and growth inhibition in MCF10AN cells, which are thought to model early-stage cancer cells (Muthuswamy *et al*, 2001), and Smad2/3-independent TGF β -stimulated migration and resistance to the TGF β cytostatic response in tumorigenic 4T1 cells.

As a possible response to increasing resistance to Smad3-dependent functions, some cancer cells may promote sensitivity to TGF β -stimulated migration through non-canonical pathways (Bakin *et al*, 2000, 2002; Dumont *et al*, 2003; Northey *et al*, 2008). Our data from 4T1 cells indicate that the ALK5 L45 loop is required for two such pathways. One is Smad1/5-dependent; the other is Smad1/5-independent. The basis for Smad1/5-independent pathways is not known but could involve XIAP and Dab2, which associate with ALK5 in an L45 loop-dependent manner (Itoh *et al*, 2003). Thus, in the tumorigenic 4T1 cell context, Smad1/5 phosphorylation cooperates with additional ALK5 L45 loop-derived TGF β signals to promote maximal sensitivity to TGF β -stimulated migration.

The detection of high levels of TGF β -stimulated Smad1/5 phosphorylation in tumorigenic, but not immortalized, mammary epithelial cells suggests that elevated levels of Smad1/5 phosphorylation are functionally relevant during breast cancer progression. Supporting this notion, a recent study has reported intense phospho-Smad1/5 staining in primary and metastatic tumour tissues derived from breast cancer patients with bone metastasis, yet little to no phospho-Smad1/5 staining in non-cancerous mammary epithelial tissue from the same patients (Katsuno *et al*, 2008). Although such immunohistochemical analysis cannot resolve whether phospho-Smad1/5 staining results from TGF β , BMPs, or other

ligands, that intense phospho-Smad2 staining is detected in the same primary and metastatic tumour tissues, but not in the non-cancerous mammary epithelial tissue, strongly suggests that Smad1/5 phosphorylation is due, at least in part, to active TGF β signalling in malignant mammary epithelia. Thus, this study supports the possibility that Smad1/5-dependent TGF β -stimulated migration and invasion occur *in vivo* during breast cancer progression.

In summary, we propose that in permissive environments created by cancer cells or oncogene activation, TGF β -stimulated Smad1/5 phosphorylation requires the ALK5 L45 loop, promotes maximal TGF β -stimulated migration, and mediates the pro-migratory TGF β switch. We are particularly interested in confirming the role of the TGF β –ALK5–Smad1/5 axis in physiologically and pathologically relevant model systems in the light of our observation that the pro-migratory and growth inhibitory functions of TGF β are separable by Smad1/5 (Figure 7). This interesting observation raises the possibility that TGF β –ALK5–Smad1/5 axis-targeting inhibitors can selectively block the pro-tumorigenic functions of TGF β .

Materials and methods

Cell culture, antibodies, plasmids, and reagents

A detailed description of these materials can be found in Supplementary data.

Generation of shRNA and/or ALK5-expressing stable cell lines

Detailed information regarding retroviral and lentiviral expression vectors or shRNA vectors and sequences are available in Supplementary data. Two rounds of 24-h viral transductions were performed using 0.45 μ m-filtered, 8 μ g/ml polybrene-spiked supernatants acquired 24 and 48 h after transfection of 293T cells with shRNA and helper constructs using Eugene6 (Roche). After 24 h recovery in appropriate culture media, infected cells were selected by treatment with 2 μ g/ml puromycin or by FACS for GFP⁺ populations. GFP sorting typically resulted in 90–95% GFP⁺ cell populations.

siRNA reagents and MCF10AN transfection

Duplex siRNA including two non-silencing controls, NSC-1 and NSC-2, Smad1–7, Smad5–8, and Smad3 smartpool were purchased from Dharmacon, and diluted to 20 nM stocks. MCF10AN cells (3×10^5) were plated overnight in six wells in growth media, and starved in 1.5 ml assay media (–p/s) immediately before two rounds of 5-h Lipofectamine 2000 (L2000)-based transfections with 500 μ l of an siRNA:L2000:optiMEM mix. The siRNA:L2000 ratios were as follows: control knockdown (3 μ l NSC-1:3 μ l NSC-2:6 μ l L2000), Smad1/5 knockdown (3 μ l Smad1–7:3 μ l Smad1–8:6 μ l L2000), and Smad3 knockdown (6 μ l Smad3:6 μ l L2000). Cells were replenished with growth media, and used in subsequent assays 24 h later. ~95% transfection efficiency was achieved under these conditions.

Western blot analysis

Here, $2\text{--}3 \times 10^5$ cells were seeded in six-well format, and treated with vehicle (4 mM HCl+1 μ g/ μ l BSA in water), 100 pM TGF β , 20 ng/ml activin A, 33 ng/ml BMP2 or BMP4, and 100 ng/ml BMP7 in the presence or absence of 200 ng/ml T β RII-Fc or 1 μ g/ml BMPRIa-Fc. In MCF10A-based studies, cells were starved for 24 h in assay media before treatment with vehicle or 200 pM TGF β unless noted otherwise. Lysates were harvested in ULB pH 7.5 (50 mM Tris, 150 mM NaCl, 50 mM NaF, 0.5% NP-40 supplemented with 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM DTT, and protease cocktail inhibitor), subjected to SDS-PAGE and western blot using the appropriate primary and secondary antibodies. In some exposures, vertical hash marks were added to indicate position of cropped, irrelevant bands.

Transwell migration assay

4T1 cells were trypsinized, resuspended in culture media, and loaded into the upper chamber at $2\text{--}4 \times 10^4$ cells per 24-well transwell insert (8 μ m pore size; Corning). Transwell inserts were incubated at 37°C for 20 h in lower chamber culture media containing vehicle or 100 pM TGF β . For MCF10A-based studies, cells were starved for 24 h in assay media before loading into transwells. Lower chamber was filled with assay media containing vehicle or 200 pM TGF β with or without 500 nM AP1510. After removing non-migrating cells using cotton swabs, inserts were washed in PBS, fixed in 4% PFA, and stained with 0.5% toluidine blue in 4% PFA. For each condition, quantitation of cell migration was determined by averaging the total cell count of five random fields at $\times 100$ magnification or by densitometry from triplicate membranes. Error bars represent standard error of the mean.

In vitro kinase assay

Assays were performed as described earlier (Macias-Silva *et al*, 1996). Briefly, TGF β receptor complexes were immunoprecipitated from 293T cells transiently transfected with T β RII-HA and ALK5-HA WT, ALK5-HA KR, or ALK5-HA D266A using anti-HA antibody and protein-G sepharose beads, washed three times with lysis buffer, pH 7.5 (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 supplemented with 1 mM PMSF, 1 mM sodium orthovanadate, 2 mM sodium fluoride, 2 mM β -glycerophosphate, and protease cocktail inhibitor), then washed two times with kinase buffer, pH 7.4 (5 mM Tris, 1 mM magnesium chloride, and 0.1 mM calcium chloride). Receptor complexes were then incubated for 30 min at room temperature in kinase buffer containing 50 μ M ATP and bacterially expressed GST-Smad. Samples were mixed with 4 $\times$ loading buffer, boiled, and then subjected to SDS-PAGE and western blot analysis using the appropriate primary and secondary antibodies.

Real-time RT-PCR

Total RNA (1 μ g), extracted from cells using Trizol (Invitrogen) or RNeasy Plus (Qiagen) kit, was used to generate cDNA using the Superscript III cDNA synthesis kit (Invitrogen). Real-time RT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad).

Growth inhibition assay

siRNA-transfected MCF10AN cells were starved in assay media for 24 h. Then, 4×10^4 cells were plated into 12 wells in assay media containing 500 nM AP1510. After 24 h, cells were treated with vehicle or 200 pM TGF β , incubated at 37°C for 24 h, and pulsed with 2 μ Ci tritiated ³H-thymidine for the final 4 h. After washing cells with PBS and fixing with 10% TCA, incorporated ³H-thymidine was extracted with 0.2 N NaOH, added to liquid scintillation cocktail, and quantitated in a scintillation counter. For each condition, thymidine incorporation was quantitated by averaging the counts per minute (c.p.m.) of triplicate wells. Normalized TGF β -stimulated growth inhibition was determined by first calculating the percentage difference in c.p.m. between TGF β -treated and vehicle-treated cells, then normalizing percentage differences to that of control cells. Error bars represent standard error of the mean.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

None declared.

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