Arginine methylation of Smad7 by PRMT1 in TGF-β-induced epithelial-mesenchymal transition and epithelial stem cell generation

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Running Title: Smad7 methylation by PRMT1 controls TGF-b signaling and EMT

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ABSTRACT

The epithelial-to-mesenchymal transdifferentiation (EMT) is crucial for tissue differentiation in development, and drives essential steps in cancer and fibrosis. EMT is accompanied by reprogramming of gene expression, and has been associated with the epithelial stem cell state in normal and carcinoma cells. The cytokine TGF-β drives this program in cooperation with other signaling pathways and through TGF-β-activated Smad3 as major effector. TGF-β-induced Smad3 activation is inhibited by Smad7 and to a lesser extent by Smad6, and Smad6 and Smad7 both inhibit Smad1 and Smad5 activation in response to the TGF-β-related bone morphogenetic proteins (BMPs). We previously reported that, in response to BMP, the protein arginine methyltransferase PRMT1 methylates Smad6 at the BMP receptor complex, thereby promoting its dissociation from the receptors and enabling BMP-induced Smad1 and Smad5 activation. We now provide evidence that PRMT1 also facilitates TGF-β signaling by methylating Smad7, which complements Smad6 methylation. We found that PRMT1 is required for TGF-β-induced Smad3 activation, through a mechanism similar to that of BMP-induced Smad6 methylation, and thus promotes the TGF-β-induced EMT and epithelial stem cell generation. This critical mechanism positions PRMT1 as an essential mediator of TGF-β signaling that controls the EMT and epithelial cell stemness through Smad7 methylation.

Epithelial-to-mesenchymal transition (EMT) is a cellular transdifferentiation process, whereby
epithelial cells lose their apico-basal polarity, epithelial junctions and other epithelial characteristics, and acquire, to varying extents, changes in gene expression and behavior that are commonly associated with mesenchymal cells. EMT enables cells to become motile and migrate directionally, and this behavior is often accompanied by the cells’ ability to invade through extracellular matrices (1). EMT is marked by and depends on the expression and activities of one or several “master” EMT transcription factors that direct many of the changes in gene expression, yet also depends on a variety of non-transcriptional changes, including changes in cytoskeletal and membrane organization and dynamics. The EMT process results from a convergence of signaling pathways, and is often seen to be driven by, or at a minimum to depend on, TGF-β signaling (2). In this context, activation of TGF-β signaling leads to destabilization of epithelial gene expression and phenotype, and activates the expression of key EMT transcription factors such as Snail and Slug/Snail2, while also directing changes in the expression of many other genes (1,2). The EMT process plays crucial roles at many stages of tissue differentiation and development, yet also drives essential steps in pathological conditions, predominantly in cancer progression of carcinomas and in fibrosis (1).

The EMT process has also been intricately associated with the epithelial stem cell state, as it promotes or accompanies stem cell-like features in both normal epithelial and carcinoma cells (3,4). Thus, activation of genes encoding EMT transcription factors promotes the generation of cancer stem cells. For example, activation of ZEB1 is required for the tumor initiating capacity of pancreatic, colorectal and breast cancer cells (5,6) and induction of Snail expression in colorectal cancer cells increases the number of cancer stem cells (7). The Snail related transcription factor Slug and Sox9 both play central roles in the maintenance of normal breast epithelial stem cells, and perturbation of the expression of either impairs the generation of stem cells (8,9). TGF-β has been shown to promote the generation of cancer stem cells able to initiate tumor formation in breast cancer and skin squamous cell carcinomas (5,10,11).

The ability of TGF-β to activate and drive the EMT program, or any differentiation program, results primarily from the activities of TGF-β-activated Smad3 as major effector. Following ligand binding to the cell surface TGF-β receptor complex, the type I receptor C-terminally phosphorylates and thus activates Smad2 and Smad3, which then form heteromeric complexes with Smad4, translocate into the nucleus, and cooperate with DNA-binding transcription factors in the activation or repression of TGF-β/Smad target genes (12). In EMT, TGF-β-activated Smad3 activates the expression of Snail and Slug, as well as other EMT transcription factors, and then cooperates with these EMT transcription factors to induce or repress their target genes, thus initiating changes in gene expression that lead to transcriptome reprogramming and differentiation (2). The Smad-initiated gene reprogramming is complemented by non-Smad signaling pathways that are activated by TGF-β and/or other classes of ligands and receptors and contribute to the loss of epithelial phenotype and to the behavior that characterize EMT (2). In addition to the effector Smads, Smad2 and Smad3, that direct changes in expression, the cells express inhibitory Smads. These interact with the type I receptor as well as the effector Smads, thus preventing Smad activation, yet are also thought to directly repress Smad-mediated activation of target genes. Smad6 and Smad7 inhibit the activation of Smad2 and Smad3 in response to TGF-β, and of Smad1 and Smad5 in the responses to the TGF-β-related bone morphogenetic proteins (BMPs). Smad6 preferentially inhibits BMP signaling, while Smad7 inhibits TGF-β signaling more efficiently than Smad6. (13).

Protein arginine methyltransferases (PRMTs) methylate arginine residues in histones and thus control epigenetically the expression of an array of genes, yet also modify non-histone proteins, including signaling mediators, and thus control their functions. Among the PRMTs, PRMT1 is the most abundant PRMT and is responsible for 75% of all arginine methylation in cells (14). Besides the common histone 4 methylation at Arg3, PRMT1 methylates and functionally regulates an extensive variety of proteins, including components of several signaling pathways (15). Increased PRMT1 expression has been observed in a variety of carcinomas, including breast carcinomas, and has been correlated with tumor
growth and cancer progression and metastasis (16).

We reported that PRMT1 is required for BMP signaling activation. BMP induces PRMT1, in association with the type II BMP receptor BMPRII, to methylate Smad6 that is associated with the type I receptor BMPRI, leading to dissociation of methylated Smad6 from the BMP receptor complex and enabling activation of the effector Smads 1 and Smad5 (17). We now provide evidence that PRMT1 is also a critical mediator of TGF-β signaling through methylation of Smad7, which complements Smad6 methylation. PRMT1 is required for TGF-β-induced Smad3 activation, through a similar mechanism as shown for BMP-induced Smad6 methylation, and thus promotes TGF-β-induced EMT as well as epithelial stem cell generation. This study defines a novel signaling pathway, from TGF-β through PRMT1 onto Smad7, that controls EMT and epithelial stem cell maintenance through arginine methylation.

RESULTS

PRMT1 is required for TGF-β signaling

To evaluate whether PRMT1 controls TGF-β-induced Smad activation, we silenced PRMT1 expression in human skin epithelial HaCaT cells using transfected siRNAs that target the expression of all PRMT1 isoforms. Silencing PRMT1 mRNA expression with >95% efficiency dramatically decreased the TGF-β-induced activation of Smad3, detected by immunoblotting for C-terminally phosphorylated Smad3 (Fig. 1A). Furthermore, silencing PRMT1 expression repressed the TGF-β-induced mRNA expression of known TGF-β/Smad3 target genes, such as the genes encoding plasminogen activator inhibitor type 1 (PAI1) (Fig. 1B) or Smad7 (Fig. 1C). A similar inhibition of TGF-β responsiveness was apparent in human mammary epithelial HMLE cells. In these cells, silencing PRMT1 expression also impaired TGF-β-induced Smad3 activation (Fig. 1D), and repressed TGF-β-induced PAI-1 and Smad7 mRNA expression (Fig. 1E, F). These observations strongly suggest that PRMT1 is required for TGF-β-induced activation of Smad3.

TGF-β promotes Smad7 methylation by PRMT1

We reported that PRMT1 facilitates BMP signaling through its ability to methylate Smad6, resulting in its dissociation from the type I receptor, and thus enables BMP-induced Smad1/5 activation (17). Since PRMT1 is also required for TGF-β-induced Smad3 activation (Fig. 1), and Smad7, in addition to Smad6, inhibits TGF-β signaling (18), we evaluated whether PRMT1 methylates Smad7, thus complementing the BMP-induced methylation of Smad6 (17).

For this purpose, we evaluated by in vivo 3H-methylation whether TGF-β signaling induces Smad7 methylation. This was indeed the case, as shown in transfected HaCaT epithelial cells. In the absence of TGF-β signaling, i.e. in the presence of the type 1 TGF-β receptor kinase inhibitor SB431542, Smad7 showed a low level of 3H-methylation, and TGF-β treatment enhanced the 3H-methylation of Smad7, which was blocked by the general methyltransferase inhibitor adenosine dialdehyde (Adox) (Fig. 2A). TGF-β also induced methylation of Smad6, which could be probed using specific antibodies against either of the two dimethylated Arg residues (17), with strikingly different kinetics of Arg74 and Arg81 dimethylation (Fig. 2B). The increased methylation of Smad6 and Smad7 in response to TGF-β is consistent with the notion that both Smad6 and Smad7 act as inhibitory Smads in TGF-β signaling (18). The 3H-methylation of endogenous Smad7 could not be visualized due to the low level Smad7 expression, the limited quality of available Smad7 antibodies and the long exposures required to visualize 3H-methylated Smad7. Smad7 expressed in transfected 293T cells was also 3H-methylated, but its methylation was not enhanced in response to TGF-β, which is consistent with their low levels of TGF-β receptors and poor TGF-β responsiveness of these cells to TGF-β (data not shown). Smad7 3H-methylation was decreased when the expression of PRMT1 was silenced using shRNA (Fig. 2C).
signaling, and therefore examined the interaction of PRMT1 with the TβRII and TβRI receptors. In transfected 293T cells, PRMT1 interacted with the TGF-β type II receptor, TβRII, but not with the wild-type type I receptor (Fig. 2D, lanes 1 and 2). Thr204 replacement by Asp is known to confer a partial activation of TβRI, leading to Smad2/3 activation (19), which is thought to depend on autocrine TGF-β binding to endogenous TβRII. In contrast to the lack of PRMT1 association with wild-type TβRI, PRMT1 associated with the mutant caTβRII (Fig. 2D, lane 4), which may result from its association with endogenous TβRII. Coexpression of TβRII and caTβRII, thus promoting TβRII-TβRI heteromerization, illustrated the predominant association of PRMT1 with TβRII in the receptor complexes (Fig. 2D, lane 5). This scenario is similar to the recruitment of PRMT1 to the heteromeric BMP receptor complexes, although no PRMT1 association with the activated BMPRI was seen (17). Consistent with this scenario of PRMT1 interaction with TβRII and its recruitment in the TβRII/TβRI complex, introduction of the caTβRII mutant in HaCaT cells resulted in enhanced Smad7 methylation (Fig. 2E).

**PRMT1 methylates Smad7 on Arg57 and Arg67**

To define the sites in Smad7 that are methylated by PRMT1, we first studied Smad7 methylation in vitro. Deletion analyses revealed that PRMT1 methylates Smad7 in its N-terminal region that spans amino acids 1 to 89, but not in the remaining large segment that comprises its MH2 domain and spans amino acids 90 to 427 (Fig. 3A). As control of the activity of PRMT1 in vitro, PRMT1 methylated histone 4 (Fig. 3A, lane 3).

To identify the residues of Smad7 that are methylated by PRMT1 in vivo, we carried out mutagenesis analyses, focusing on Arg residues that are adjacent to Gly and thus represent the preferred methylation sites of PRMT1 (20). Smad7 mutants, in which each of these arginines within the first 89 amino acids was individually replaced by alanine, were expressed in 293T cells, and their methylation was evaluated by in vivo ³H-methylation and immunoprecipitation (Fig. 3B). These analyses revealed that the R57A and R67A substitutions strongly decreased the methylation of Smad7 (Fig. 3B), suggesting that these two arginine residues may be methylated. Substitutions of both R57 and R67 with alanine or lysine confirmed the decreased Smad7 methylation when compared to wild-type Smad7, but did not show a cumulative decrease of ³H-methylation (Fig. 3C). Furthermore, silencing PRMT1 expression did not further decrease the ³H-methylation of the double mutant Smad7, and kept it at a level comparable to the ³H-methylation of wild-type Smad7 following PRMT1 silencing (Fig. 3D).

We also analyzed by mass spectrometry the methylation of Smad7 that was expressed in transfected 293T cells treated with TGF-β for 1h, and isolated by immunoaffinity purification. Mono- and di-methylation of R57 of Smad7 was detected in multiple independent samples (Fig. 3E, F). However, we did not detect methylation of R67 in these assays. This inability to detect R67 methylation has limited value as a negative result, but may suggest that in our methylation assays (Fig. 3B-D) R67 is required to enable R57 methylation, or that R67 methylation occurs at a later stage of TGF-β signaling, i.e. after 1 hour. Mass spectrometry also revealed lysine methylation of Smad7, which is the subject of a separate study (data not shown), and explains the persistent ³H-methylation of Smad7 after silencing PRMT1 expression (Fig. 3D).

**Arg methylation regulates Smad7 binding to TβRI**

Further drawing on the parallel with the role of Smad6 methylation in BMP signaling, we reported that BMP-induced methylation of Smad6 decreases the Smad6 association with the type I BMP receptor BMPRI, thus enabling Smad6 dissociation from the activated BMPRI (17). By analogy, we examined whether arginine methylation by PRMT1 decreases the association efficiency of Smad7 for TβRI using an in vitro binding assay (Fig. 4A). For this purpose, purified GST-Smad7, methylated in vitro by PRMT1, was compared with unmethylated GST-Smad7 for binding to immobilized, His-tagged caTβRI with its activating T204D mutation that was purified from transfected 293T cells (Fig. 4B). Unmethylated Smad7 showed efficient binding to caTβRI (Fig. 4B, right row), whereas methylated Smad7 associated with a lower efficiency,
apparent by the higher abundance of Smad7 in the unbound fraction (Fig. 4B, left row). These results provide evidence that arginine methylation decreases the binding of Smad7 to the activated TβRI, as was seen for the binding of Smad6 to BMPRI (17). They therefore suggest that Smad7 methylation by PRMT1 enables Smad7 dissociation from the activated TβRI receptor, similarly to the effect of Smad6 methylation leading to Smad6 dissociation from the BMPRI receptor. Supporting this notion, increasing PRMT1 expression decreased the interaction of Smad7 with caTβRI (Fig. 4C), again as observed for the binding of Smad6 to BMPRI (17). These results support the model that the TGF-β-induced methylation of Smad7 by TβRI-associated PRMT1 results in dissociation of Smad7 from the TβRI receptor, and thus controls the availability of TβRI receptor for subsequent Smad2 and Smad3 activation, similarly to the BMP4-induced methylation of BMPRI-associated Smad6 by BMPRII-associated PRMT1 to allow Smad1 and Smad5 activation in response to BMP (17). Protein methylation often alters protein stability, thus controlling the half-life of proteins. To determine whether Arg methylation controls the stability of Smad7, we examined the half-life of Smad7 in cycloheximide chase experiments, in the presence and absence of PRMT1 (Fig. 4D). The estimated half-life of Smad7 in control HaCaT cells was 38 min, whereas, following PRMT1 silencing, the estimated half-life was extended to 4 h, indicating that PRMT1-mediated methylation promotes degradation of Smad7. Together, these data suggest that TGF-β-induced methylation of Smad7 by TβRII-associated PRMT1 results in dissociation of Smad7 from the TβRI receptor and subsequent degradation of Smad7.

**PRMT1 controls TGF-β-induced epithelial cell de-differentiation.**

TGF-β signaling is known to repress the epithelial cell phenotype and, depending on the cell system and physiological conditions, to induce an epithelial-mesenchymal transition (EMT). In TGF-β-induced EMT, Smad3 activates the expression of EMT master transcription factors, such as Snail, and cooperates with these in the repression of epithelial genes and activation of mesenchymal genes (2). HaCaT cells are often used as model system to study the changes in gene expression during EMT. Considering the role of PRMT1 in the control of TGF-β-induced Smad3 activation, we examined its role in TGF-β-induced EMT of HaCaT cells.

TGF-β induced a loss of the cobblestone-like cell shape that characterizes the epithelial identity, and induced a more elongated EMT-like cell phenotype (Fig. 5A) with actin stress fibers rather than cortical actin organization, decreased E-cadherin immunostaining at cell junctions, decreased claudin 1 expression and increased vimentin expression (Fig. 5B, C). Silencing *PRMT1* expression using transfected siRNA inhibited this loss of epithelial phenotype (Fig. 5A, B) and largely prevented these changes (Fig. 5B, C; Suppl. Fig. 1).

A similar role for PRMT1 was observed in the differentiation of human mammary epithelial HMLE cells. Also in these cells does TGF-β induce a morphological transition from a cobblestone-like phenotype to a more elongated phenotype (Fig. 5D), although this transition in phenotype requires a longer time than in HaCaT cells. Silencing *PRMT1* expression through lentiviral expression of either of two shRNAs inhibited this morphological transition (Fig. 5D), as well as the reorganization of actin from cortical to stress fibers, and the downregulation of E-cadherin at epithelial junctions (Fig. 5E). Consistent with these findings, TGF-β induced a significant decrease in E-cadherin expression and a significant increase in the levels of the mesenchymal markers ZEB1 and ZEB2, Snail and Slug, vimentin, fibronectin, and N-cadherin, and silencing *PRMT1* expression dramatically inhibited these changes (Fig. 5F, G).

**PRMT1 controls TGF-β-induced stem cell generation.**

The acquisition of mesenchymal characteristics has been linked to the generation of epithelial and carcinoma stem cells, which, in the context of cancer progression, promotes the cancer re-seeding capacity (3,4). HMLE cells have been used as model system to study the generation of epithelial stem cells and its correlation with EMT (10). Adding to our characterization of EMT marker expression (Fig. 5E-G), we evaluated the CD44<sup>high</sup>CD24<sup>low</sup> cell population, which is the
signature for epithelial stem cells. TGF-β treatment enhanced the percentage of CD44<sup>high</sup>CD24<sup>low</sup> cells from 25.0% to 52.8%, but silencing PRMT1 expression dramatically inhibited this increase (Fig. 6A). We further evaluated the expression of CD44, KLF4, BMI1, POU5F1 and Nanog, which correlate with stemness and pluripotency in normal and malignant mammary epithelial stem cells (21-23). TGF-β induced the mRNA expression of these markers, but silencing PRMT1 expression dramatically inhibited this induction (Fig. 6B). The repression of CD44 mRNA as a result of silencing PRMT1 expression correlated, both quantitatively and over time, with a concomitant repression of induced mesenchymal marker expression, specifically of N-cadherin, fibronectin and ZEB1 mRNA (Fig. 6B and 5F, G), which are encoded by direct TGF-β/Smad3 target genes. Consistent with the repression of Smad3 activation following silencing of PRMT1 expression (Fig. 1D), silencing PRMT1 decreased the expression of PAI-1 and Smad7 mRNA, which are also transcribed from direct TGF-β/Smad responsive genes (Fig. 1E, 1F).

To examine whether PRMT1 expression controls the functional properties of these stem-like cells, we tested their efficiency of mammosphere formation. The ability to form mammospheres in serial non-adherent passages correlates with the number of stem cells that have the ability to self-renew and thus reconstruct the gland structure (24). TGF-β treatment dramatically enhanced the efficiency of mammosphere formation of HMLE cells, and silencing PRMT1 expression almost completely blocked the enhancement in primary passages (Fig. 6C, D). The requirement for PRMT1 was well maintained in secondary passages, since silencing of PRMT1 expression prevented the TGF-β-induced mammosphere formation (Fig. 6C, D). These results strongly suggest that PRMT1 controls the generation of mammary epithelial stem cells. Considering the role of PRMT1 in the dissociation of Smad7 from TβRI and, consequently, in TGF-β-induced Smad3 activation, and considering the close correlation of the inhibition of stem cell generation with the inhibition of TGF-β target gene expression and EMT, our data strongly suggest that PRMT1’s control of stem cell generation occurs through its effects on TGF-β-induced Smad activation.

**Discussion**

Smad6 and Smad7 are seen as negative feedback regulators that constrain the activities of receptor-activated Smad signaling. These inhibitory Smads were shown to bind to effector Smads and to the type I receptors, thus preventing the activation of the effector Smads through C-terminal phosphorylation by the type I receptor kinases. Smad6 is known to inhibit BMP-induced Smad activation, while Smad6 and Smad7 both target TGF-β-, activin- and BMP-induced Smad signaling (13). We previously reported that Smad6 associates with the cell surface BMP type I receptors and that PRMT1 that is associated with the BMP type II receptor methylates Smad6 on Arg74 in response to ligand, and consequently induces its dissociation. Thus, BMP-induced Smad6 methylation by PRMT1 initiates BMP-induced Smad1 and Smad5 signaling by enabling the dissociation of the inhibitory Smad6 and allowing the recruitment and activation of the effector Smads, Smad1 and Smad5 (17). Here we provide evidence that a similar and parallel mechanism controls the function of Smad7 at the TGF-β receptor, and, thus, TGF-β-induced Smad activation. Taken together, PRMT1 acts on inhibitory Smads to control both the BMP and TGF-β/activin pathways, and to enable effector Smad activation by the type I receptors. We also show that, in addition to Smad7, TGF-β induces Smad6 methylation on Arg supporting the notion that both inhibitory Smads control TGF-β-induced Smad activation.

We additionally provide evidence that PRMT1 controls the EMT program, using two epithelial cell lines. With TGF-β/Smad signaling driving the initiation and progression of this transdifferentiation program, we surmise that TGF-β signaling, through Smad7 methylation by PRMT1, is a strong determinant of EMT. Indeed, silencing PRMT1 expression, and thus inhibiting TGF-β-induced Smad3 activation, suppresses the EMT program. PRMT1 is a versatile enzyme that methylates histone and non-histone substrates, raising the possibility that it acts at multiple levels. Accordingly, PRMT1 has been shown to modulate the roles of the EMT transcription
factors Twist and ZEB1. PRMT1-mediated methylation of Twist contributes to the repression of epithelial E-cadherin expression in non-small lung cancer cells (25), while PRMT1-mediated dimethylation of histone 4 at Arg3 at the ZEB1 promoter activates ZEB1 expression in breast cancer cells, thus contributing to EMT (26). Therefore, PRMT1 may act at multiple levels in epithelial to mesenchymal transition.

The EMT program has been functionally linked to the generation of mammary epithelial stem cells and cancer stem cells. The EMT transcription factor Slug/Snail2 marks the basal mammary stem cells and cooperates with Sox9 to maintain the gland-reconstituting capacity of mammary epithelial cells (8). The expression of Snail, an EMT transcription factor related to Slug, marks the neoplastic population and is tightly associated with the tumor invasive front (9). We illustrate the role of PRMT1 in both the EMT fate transition as well as the generation of mammary epithelial stem cells.

Increased PRMT1 expression has been documented in various types of cancer including breast cancer. Its role in cancer progression has been linked to enhanced cell proliferation in breast, lung, liver, colorectal cancer, squamous cell carcinomas and leukemia (27-32). Although the underlying mechanisms remain to be further characterized, it has been proposed that PRMT1 methylates the EGFR to promote colorectal cancer growth (28). Also, PRMT1 was shown to methylate a splicing isofrom of the AML-ETO fusion protein that acts as a transcription factor, thus facilitating the expression of target genes, while also epigenetically controlling their methylation on histone 4 (31). PRMT1 also regulates cellular functions such as senescence and genomic stability (26,33).

Besides its roles in cancer, PRMT1 also controls development and tissue injury, which is consistent with its role as histone methyltransferase and its ability to methylate a variety of signaling effectors (14, 15). Consequently, many activities of PRMT1 in development may not be related to TGF-β family signaling, yet others might involve the methylation of inhibitory Smads. For example, PRMT1 inactivation in periodontal epithelium aggravates inflammatory responses and periodontal tissue injury, and these effects appear to relate to the role of PRMT1-mediated Smad6 methylation in repressing TLR-MyD88-NfkB signaling (34). Whether Smad7 plays a parallel role needs to be assessed. PRMT1 deletion in neural progenitors causes hypomyelination and defects in the central nervous system (35), which may in part relate to the roles of TGF-β family signaling in neural and neuronal cell differentiation (36). Since the TGF-β family pathways TGF-β and BMP, as well as Smad7, are also involved in craniofacial development (36,37), PRMT1-mediated Smad7 methylation may contribute to developmental defects resulting from PRMT1 inactivation (38).

Our evidence that Smad7 methylation by PRMT1 enables TGF-β signaling to regulate EMT and stemness of mammary epithelial cells now provides an additional role for PRMT1 in promoting EMT and cancer stem cell formation that may be relevant in cancer cell dissemination, and raises the expectation of normal roles in cell differentiation and development.

**Experimental procedures**

**Plasmids**

The expression plasmids pRK-5-Flag-PRMT1, pRK5-HA-PRMT1, pRK5-Flag-TβRII, pRK5-Flag-TβRIwt, pRK5-Flag-TβRlca, pRK5-His-TβRIwt, GST-PRMT1, GST-Smad7N and GST-Smad7C, pRK-5-Flag-Smad7, pRK-Myc-Smad7 have been described (17,39-41). Substitutions in the Smad7 coding sequence were introduced using the QuikChange Lightening Site-Directed Mutagenesis Kit from Agilent, thus generating the expression plasmids for Smad7 mutants, pRK5-Flag-Smad7 R57A, pRK5-Flag-Smad7 R67A, pRK5-Flag-Smad7 R57,67A, pRK5-Flag-Smad7 R57,67K, pRK5-Flag-Smad7 R15A, pRK5-Flag-Smad7 R17A, pRK5-Flag-Smad7 R38A and pRK5-Flag-Smad7 R46A.

**Reagents**

Control and PRMT1 siRNA were purchased from Qiagen. A mixture of two independent siRNAs, HRMT1L2_4 and HRMT1L2_7, was used to silence PRMT1 (17). Control shRNA and shRNAs targeting PRMT1 were purchased from Sigma and previously described (17). The 293T stable cells expressing control shRNA or shRNA targeting
PRMT1 were previously described (17). Recombinant human TGF-β1 was purchased from Humanzyme. SB431542, cycloheximide, chloroquine, and MG132 were purchased from Sigma. Core histones were purchased from Millipore.

**Cell culture**

HaCaT and 293T cells were maintained in DMEM with 10% FBS. Immortalized human mammary epithelial (HMLE) cells were maintained in MEBM (Lonza), supplemented with 10 μg/ml insulin, 10 ng/ml EGF and 0.5 μg/ml hydrocortisone.

**Antibodies, immunoprecipitation and immunoblotting**

Antibodies to PRMT1, phosphoSmad3, Smad3, claudin 1, Snail, E-cadherin and N-cadherin were purchased from Cell Signaling Technology. Antibodies to Smad7 were purchased from Abcam. Antibodies to GST, tubulin, vimentin, fibronectin, and the Flag, Myc, His and HA epitope tags were purchased from Sigma. Antibodies to GST, tubulin and GAPDH were purchased from Santa Cruz Biotechnology. Antibodies to vimentin, fibronectin, and the Flag, Myc, His and HA epitope tags were purchased from Sigma. Antibodies to GST, tubulin and GAPDH were purchased from Santa Cruz Biotechnology. Flag-conjugated M2 agarose and HA-conjugated Sepharose beads were purchased from Sigma-Aldrich. Rabbit polyclonal antibodies against asymmetric di-methyl arginine 74 of Smad6 (anti-S6R74me2) and asymmetric di-methyl arginine 81 of Smad6 (anti-S6R81me2) were previously characterized (17).

For immunoblotting, cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2mM EDTA, 0.1% NP-40, 10% glycerol, and protease inhibitor cocktail). Lysates were subjected to immunoprecipitation with anti-Myc or anti-HA antibody, and conjugation to protein G-Sepharose (GE Healthcare). Immune complexes were washed three times with IP wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2mM EDTA, 0.1% NP-40, 10% glycerol) and subjected to immunoblotting.

**Reversed-phase liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) analysis**

**In-solution digestion.** Flag-Smad7 (7-10 μg) eluted from the antibody column in 100 mM NH₄HCO₃ was concentrated by evaporation in a speed-vac system, and volume was adjusted to 25 ul. Samples were partially denatured by adding urea to a concentration of 2.7 M and reduced by incubating for 15 min at 60°C in the presence of 2.5 mM DTT. Samples were then alkylated by incubation with 3 mM iodoacetamide for 1h in the dark at room temperature. Remaining iodoacetamide was quenched by adding DTT to a final concentration of 3 mM and incubating at 37°C for 15 min. Samples were then diluted to a final concentration of urea of 2 M, and digested overnight at 37°C using 100 ng sequencing grade modified trypsin (Promega, Madison, WI). Samples were added 4% formic acid, and peptides extracted using C18 OMIX tips (Agilent Technologies) according to the manufacturer’s protocol. Eluates of the OMIX tips were vacuum-evaporated and resuspended in 20 μl 0.1% formic acid in water.

**Reverse-phase LC-MS/MS analysis.** The digests were separated by nano-flow liquid chromatography using a 75-μm x 150-mm reverse phase 1.7 μm BEH 130 C18 column (Waters) at a flow rate of 600 nL/min in a NanoAcquity™ Ultra performance UPLC system (Waters). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Following equilibration of the column in 5% solvent B, an aliquot of each digest (5 μL) was injected, then the organic content of the mobile phase was increased linearly to 40% over 60 min, and then to 50% in 1 min. The liquid
chromatography eluate was coupled to a hybrid linear ion trap-Orbitrap mass spectrometer (either a LTQ-Orbitrap XL or a LTQ Orbitrap Velos, Thermo Scientific, San Jose, CA) equipped with a nanoelectrospray ion source. Spraying was from an uncoated 15-µm-inner diameter spraying needle (New Objective, Woburn, MA). Peptides were analyzed in positive ion mode and in information-dependent acquisition mode to automatically switch between MS and MS/MS acquisition. MS spectra were acquired in profile mode using the Orbitrap analyzer in the m/z range between 300 and 1800. For each MS spectrum, the 6 most intense multiple charged ions over a threshold of 1000 counts were selected to perform CID experiments. Product ions were analyzed on the linear ion trap in centroid mode. The CID collision energy was automatically set to 30%. A dynamic exclusion window of 0.5 Da was applied that prevented the same m/z from being selected for 60 s after its acquisition. Peak lists were generated using PAVA in-house software (42). The peak lists were searched against the murine and human subset of the SwissProt database as of July 6, 2011 using in-house ProteinProspector version 5.8.0 (a public version is available online).

Peptide tolerance in searches was 20 ppm for precursor and 0.8 Da for product ions, respectively. Peptides containing two mis-cleavages were allowed. Carbamidomethylation of cysteine was allowed as constant modification; acetylation of the N terminus of the protein, pyroglutamate formation from N terminal glutamine and oxidation of methionine, were allowed as variable modifications in initial searches. Some searches allowed for mono and dimethylation of arginine and acetylation, mono, di and trimethylation of lysine as variable modifications. In these cases systematic error of the peptides masses observed on the initial searches was used to correct the search parameters. The number of modifications was limited to two per peptide. Protein hits were considered significant when two or more peptide sequences matched a protein entry and the Prospector score was above the significance level.

A minimal ProteinProspector protein score of 20, a peptide score of 15, a maximum expectation value of 0.05 and a minimal discriminant score threshold of 0.0 were used for initial identification criteria. For identifications of posttranslational modification sites, the MS/MS spectrum was reinterpreted manually by matching all the observed fragment ions to a theoretical fragmentation obtained using MS Product (Protein Prospector) (43).

**In vitro GST binding assays**

Recombinant GST-tagged Smad7 that is not methylated was generated in *E. coli*, which is deficient in Arg methylation. Immunopurified GST-tagged Smad7 was methylated *in vitro* using purified recombinant PRMT1 in the presence or absence of methylation cofactor S-adenosylmethionine (SAM). His-tagged TßRIca was purified from transfected 293T cells and conjugated to anti-His antibody-conjugated Sepharose beads. Then, methylated or non-methylated Smad7 was incubated with His-tagged TßRIca for 4 hours with agitation. After incubation, the beads-bound and flow-through fractions were separated by centrifugation, and subjected to SDS-PAGE and immunoblotting.

**In vitro methylation assay**

GST-tagged Smad7, Smad7 mutants, and Smad7 N-terminal and C-terminal segments were generated in *E. coli* transformed with pGEX expression vectors and purified using glutathione Sepharose 4B (GE Healthcare). Flag-tagged PRMT1 was expressed in 293T cells, immunopurified and incubated with GST-Smad7 or core histone (Millipore), in reaction buffer (50 mM Tris-HCl pH 8, 20 mM KCl, 5 mM MgCl2, 1 mM DTT, 1 mM PMSF and 10% glycerol), in the presence of 2 µCi ³H-labeled S-adenosylmethionine (SAM) (Perkin-Elmer) at 30°C for 90 min. The reaction mixture was quenched with 5x SDS buffer and separated by SDS-PAGE. The gel was stained with GelCode Blue (Pierce), scanned (Canoscan 9000F), fluorographed, dried and exposed to Kodak film at -80°C.

**In vivo methylation assay**

Transfected 293T cells were pretreated with the proteasomal degradation inhibitor MG132 (20-50 µM) and the lysosomal degradation inhibitor chloroquine (100 µM) for 2-6 h. The medium was replaced with DMEM without methionine, containing the same inhibitors and supplemented...
with the protein synthesis inhibitor cycloheximide (100 µg/ml). After 30 min, 10 µCi/ml of L-[methyl-3H]methionine (Perkin-Elmer) was added and incubated with the cells for an additional 1-6 h. The cells were lysed in lysis buffer. The Smad7 complexes were immunopurified with anti-Flag M2 (Sigma-Aldrich) and separated by SDS-PAGE. The protein gel was then stained with GelCode Blue, scanned, fluorographed, dried, and exposed to Kodak film at -80°C.

**Immunocytochemistry**

Cells plated on chamber slides were fixed with ice-cold methanol for 20 min, permeabilized in PBS containing 0.3% Triton X-100 (PBT) for 15 min, and incubated in PBT and 5% serum blocking solution for 1 h. The slides were incubated with anti-E-cadherin antibody at 4 °C overnight and then stained for 2 h with Alexa Fluor secondary antibodies (1:500; Invitrogen), or incubated with Alexa Fluor 488 or 568 phalloidin for 1-3 h. The slides were then mounted using Prolong Gold anti-fade reagent and stained with DAPI to visualize nuclei (Invitrogen). The cells were viewed with an inverted light microscope (DMI5000, Leica Microsystems) or a laser scanning confocal microscope (SP5, Leica Microsystems).

**qRT-PCR analysis**

To quantify mRNA expression, cells were treated as indicated, and RNA was isolated with Trizol (Invitrogen) and used as a template for reverse transcriptase. The mRNAs of interest were quantified by real-time PCR with IQ Sybr Green Supermix (Bio-Rad), and normalized against RPL19 mRNA. The primer sequences are listed in Table 1.

**Flow Cytometry**

Antibodies for the human CD24 (Clone ML5) and human CD44 (Clone G44-26) were purchased from BD Biosciences. Cells were dissociated into single cells and stained with antibodies for CD24 and CD44 in PBS. Flow cytometry analysis was done using a Gallios Flow Cytometer (Beckman Coulter).

**Mammosphere assay**

Mammosphere formation assays were performed as described (10). Cells were seeded in ultra-low attachment plates at a density of 5000 cells/well in MEGM medium (Lonza) supplemented with B27, 10 ng/ml bFGF, 20 ng/ml EGF and 1% methyl cellulose. After incubating the cells for 6 days, the mammospheres were observed by phase-contrast microscopy and quantified. For secondary sphere formation, primary spheres were collected by centrifugation, dissociated into single cells by trypsinization, and replated in ultra-low attachment plates at 2000 cells/well.
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Conflict of interests
The authors declare that they have no conflicts of interests.

References
1. Lamouille, S., Xu, J., and Derynck, R. (2014) Molecular mechanisms of epithelial-mesenchymal transition. Nat. Rev. Mol. Cell Biol. 15, 178-196
2. Xu, J., Lamouille, S., and Derynck, R. (2009) TGF-β-induced epithelial to mesenchymal transition. Cell Res. 19, 156-172
3. Polyak, K., and Weinberg, R. A. (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat. Rev. Cancer 9, 265-273
4. Shibue, T., and Weinberg, R. A. (2017) EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nature reviews. Clinical oncology 14, 611-629
5. Chaffer, C. L., Marjanovic, N. D., Lee, T., Bell, G., Kleer, C. G., Reinhardt, F., D’Alessio, A. C., Young, R. A., and Weinberg, R. A. (2013) Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. Cell 154, 61-74
6. Wellner, U., Schubert, J., Burk, U. C., Schmalhofer, O., Zhu, F., Sonntag, A., Waldvogel, B., Vannier, C., Darling, D., zur Hausen, A., Brunton, V. G., Morton, J., Sansom, O., Schuler, J., Stemmner, M. P., Herzberger, C., Hopt, U., Keck, T., Brabletz, S., and Brabletz, T. (2009) The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat. Cell Biol. 11, 1487-1495
7. Hwang, W. L., Jiang, J. K., Yang, S. H., Huang, T. S., Lan, H. Y., Teng, H. W., Yang, C. Y., Tsai, Y. P., Lin, C. H., Wang, H. W., and Yang, M. H. (2014) MicroRNA-146a directs the symmetric division of Snail-dominant colorectal cancer stem cells. Nat. Cell Biol. 16, 268-280
8. Guo, W., Keckesova, Z., Donaher, J. L., Shibue, T., Tischler, V., Reinhardt, F., Itzkovitz, S., Noske, A., Zurrer-Hardi, U., Bell, G., Tam, W. L., Mani, S. A., van Oudenaarden, A., and Weinberg, R. A. (2012) Slug and Sox9 cooperatively determine the mammary stem cell state. Cell 148, 1015-1028
9. Ye, X., Tam, W. L., Shibue, T., Kaygusuz, Y., Reinhardt, F., Ng Eaton, E., and Weinberg, R. A. (2015) Distinct EMT programs control normal mammary stem cells and tumour-initiating cells. Nature 525, 256-260
10. Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Brisken, C., Yang, J., and Weinberg, R. A. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133, 704-715
11. Oshimori, N., Oristian, D., and Fuchs, E. (2015) TGF-β promotes heterogeneity and drug resistance in squamous cell carcinoma. Cell 160, 963-976
12. Xu, P., Lin, X., and Feng, X. H. (2016) Posttranslational Regulation of Smads. Cold Spring Harb. Perspect.Biol. 8
13. Miyazawa, K., and Miyazono, K. (2017) Regulation of TGF-β Family Signaling by Inhibitory Smads. Cold Spring Harb. Perspect Biol. 9
14. Bedford, M. T., and Clarke, S. G. (2009) Protein arginine methylation in mammals: who, what, and why. Mol. Cell 33, 1-13
15. Blanc, R. S., and Richard, S. (2017) Arginine Methylation: The Coming of Age. Mol. Cell 65, 8-24
16. Yang, Y., and Bedford, M. T. (2013) Protein arginine methyltransferases and cancer. Nat. Rev. Cancer 13, 37-50
17. Xu, J., Wang, A. H., Oses-Prieto, J., Makhijani, K., Katsuno, Y., Pei, M., Yan, L., Zheng, Y. G., Burlingame, A., Bruckner, K., and Derynck, R. (2013) Arginine Methylation Initiates BMP-Induced Smad Signaling. Mol. Cell 51, 5-19
18. Feng, X. H., and Derynck, R. (2005) Specificity and versatility in TGF-β signaling through Smads. Ann. Rev. Cell Dev. Biol. 21, 659-693
19. Wieser, R., Wrana, J. L., and Massague, J. (1995) GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-β receptor complex. EMBO J. 14, 2199-2208
20. Thandapani, P., O’Connor, T. R., Bailey, T. L., and Richard, S. (2013) Defining the RGG/RG motif. Mol. Cell 50, 613-623
21. Visvader, J. E., and Stingl, J. (2014) Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev. 28, 1143-1158
22. Yu, F., Li, J., Chen, H., Fu, J., Ray, S., Huang, S., Zheng, H., and Ai, W. (2011) Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. Oncogene 30, 2161-2172
23. Itoh, F., Watabe, T., and Miyazono, K. (2014) Roles of TGF-β family signals in the fate determination of pluripotent stem cells. Sem. Cell Dev. Biol. 32, 98-106
24. Dontu, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J., and Wicha, M. S. (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev. 17, 1253-1270
25. Avasarala, S., Van Scoyk, M., Karuppusamy Rathinam, M. K., Zerayesus, S., Zhao, X., Zhang, W., Pericande, M. R., Borgia, J. A., De Gregori, J., Port, J. D., Winn, R. A., and Bikkavilli, R. K. (2015) PRMT1 is a Novel Regulator of Epithelial-Mesenchymal-Transition in Non-small Cell Lung Cancer. J. Biol. Chem. 290, 13479-13489
26. Gao, Y., Zhao, Y., Zhang, J., Lu, Y., Liu, X., Geng, P., Huang, B., Zhang, Y., and Lu, J. (2016) The dual function of PRMT1 in modulating epithelial-mesenchymal transition and cellular senescence in breast cancer cells through regulation of ZEB1. Sci. Rep. 6, 19874
27. Chuang, C. Y., Chang, C. P., Lee, Y. J., Lin, W. L., Chang, W. W., Wu, J. S., Cheng, Y. W., Lee, H., and Li, C. (2017) PRMT1 expression is elevated in head and neck cancer and inhibition of protein arginine methylation by adenosine dialdehyde or PRMT1 knockdown downregulates proliferation and migration of oral cancer cells. Oncol. Rep. 38, 1115-1123
28. Nakai, K., Xia, W., Liao, H. W., Saito, M., Hung, M. C., and Yamaguchi, H. (2017) The role of PRMT1 in EGFR methylation and signaling in MDA-MB-468 triple-negative breast cancer cells. Breast cancer (Tokyo, Japan)
29. Zhou, W., Yue, H., Li, C., Chen, H., and Yuan, Y. (2016) Protein arginine methyltransferase 1 promoted the growth and migration of cancer cells in esophageal squamous cell carcinoma. Tum. Biol. 37, 2613-2619
30. Liao, H. W., Hsu, J. M., Xia, W., Wang, H. L., Wang, Y. N., Chang, W. C., Arolid, S. T., Chou, C. K., Tsou, P. H., Yamaguchi, H., Fang, Y. F., Lee, H. J., Lee, H. H., Tai, S. K., Yang, M. H., Morelli, M. P., Sen, M., Ladbury, J. E., Chen, C. H., Grandis, J. R., Kopetz, S., and Hung, M. C. (2015) PRMT1-mediated methylation of the EGF receptor regulates signaling and cetuximab response. J. Clin. Invest. 125, 4529-4543
31. Shia, W. J., Okumura, A. J., Yan, M., Sarkeshik, A., Lo, M. C., Matsuura, S., Komeno, Y., Zhao, X., Nimer, S. D., Yates, J. R., 3rd, and Zhang, D. E. (2012) PRMT1 interacts with AML1-ETO to promote its transcriptional activation and progenitor cell proliferative potential. *Blood* 119, 4953-4962

32. Zheng, S., Moehlenbrink, J., Lu, Y. C., Zalmas, L. P., Sagum, C. A., Carr, S., McGouran, J. F., Alexander, L., Fedorov, O., Munro, S., Kessler, B., Bedford, M. T., Yu, Q., and La Thangue, N. B. (2013) Arginine methylation-dependent reader-writer interplay governs growth control by E2F-1. *Mol. Cell* 52, 37-51

33. Yu, Z., Chen, T., Hebert, J., Li, E., and Richard, S. (2009) A mouse PRMT1 null allele defines an essential role for arginine methylation in genome maintenance and cell proliferation. *Mol. Cell Biol.* 29, 2982-2996

34. Zhang, T., Wu, J., Ungvijanpunya, N., Jackson-Weaver, O., Gou, Y., Feng, J., Ho, T. V., Shen, Y., Liu, J., Richard, S., Jin, J., Hajishengallis, G., Chai, Y., and Xu, J. (2018) Smad6 Methylation Represses NFkappaB Activation and Periodontal Inflammation. *J Dent Res* 22034518755688

35. Hashimoto, M., Murata, K., Ishida, J., Kanou, A., Kasuya, Y., and Fukamizu, A. (2016) Severe Hypomyelination and Developmental Defects Are Caused in Mice Lacking Protein Arginine Methyltransferase 1 (PRMT1) in the Central Nervous System. *J. Biol. Chem.* 91, 2237-2245

36. Krampert, M., Chirasani, S. R., Wachs, F. P., Aigner, R., Bogdahn, U., Yingling, J. M., Heldin, C. H., Aigner, L., and Heuchel, R. (2010) Smad7 regulates the adult neural stem/progenitor cell pool in a transforming growth factor beta- and bone morphogenetic protein-independent manner. *Mol. Cell Biol.* 30, 3685-3694

37. Tang, S., Snider, P., Firulli, A. B., and Conway, S. J. (2010) Trigenic neural crest-restricted Smad7 over-expression results in congenital craniofacial and cardiovascular defects. *Dev. Biol.* 344, 233-247

38. Gou, Y., Li, J., Wu, J., Gupta, R., Cho, I., Ho, T. V., Chai, Y., Merrill, A., Wang, J., and Xu, J. (2018) Prmt1 regulates craniofacial bone formation upstream of Msx1. *Mech Dev*

39. Choy, L., Skillington, J., and Derynck, R. (2000) Roles of autocrine TGF-β receptor and Smad signaling in adipocyte differentiation. *J. Cell Biol.* 149, 667-682

40. Muthusamy, B. P., Budi, E. H., Katsumo, Y., Lee, M. K., Smith, S. M., Mirza, A. M., Akhurst, R. J., and Derynck, R. (2015) SheA Protects against Epithelial-Mesenchymal Transition through Compartmentalized Inhibition of TGF-β-Induced Smad Activation. *PLoS Biol.* 13, e1002325

41. Xu, P., Bailey-Bucktrout, S., Xi, Y., Xu, D., Du, D., Zhang, Q., Xiang, W., Liu, J., Melton, A., Sheppard, D., Chapman, H. A., Bluestone, J. A., and Derynck, R. (2014) Innate antiviral host defense attenuates TGF-β function through IRF3-mediated suppression of Smad signaling. *Mol. Cell* 56, 723-737

42. Guan, S., Price, J. C., Prusiner, S. B., Ghaemmaghami, S., and Burlingame, A. L. (2011) A data processing pipeline for mammalian proteome dynamics studies using stable isotope metabolic labeling. *Mol. Cell Proteomics.* 10, M111 010728

43. Clauser, K. R., Baker, P., and Burlingame, A. L. (1999) Role of accurate mass measurement (+/-10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal. Chem.* 71, 2871-2882
Figure legends

Figure 1. PRMT1 is required for TGF-β-induced Smad3 activation. (A) Downregulating PRMT1 expression in HaCaT cells using transfected siRNA attenuates TGF-β-induced Smad3 activation, assessed by immunoblotting (IB) for C-terminally phosphorylated Smad3. HaCaT cells were transfected with PRMT1 siRNA or control siRNA, and treated with the TGF-β signaling inhibitor SB431542 or with TGF-β1 for the indicated times, or were left untreated. (B, C) Downregulating PRMT1 expression using siRNA reduced TGF-β-induced PAI1 (B) and SMAD7 (C) mRNA expression, assessed by qRT-PCR. HaCaT cells were transfected with siRNA and treated with TGF-β for the indicated times. * p<0.01 vs. control siRNA at the same time point. (D) Downregulating PRMT1 expression in HMLE cells using lentiviral shRNA attenuates TGF-β-induced Smad3 activation, assessed by immunoblotting (IB) for C-terminally phosphorylated Smad3. HMLE cells were infected with lentiviral constructs expressing one of two different PRMT1 shRNAs #1 or #3, or a control shRNA, and were treated with the TGF-β signaling inhibitor SB431542 or TGF-β1 for the indicated times, or were left untreated. (E, F) Downregulating PRMT1 expression using lentiviral shRNA reduced TGF-β-induced PAI1 (E) and SMAD7 (F) mRNA expression, assessed by qRT-PCR. HMLE cells were infected with shRNAs and treated with TGF-β for 6 d. # p<0.01 vs. untreated. * p<0.01 vs. control shRNA.

Figure 2. Role of Smad7 in the control of TGF-β-induced Smad activation by PRMT1. (A) TGF-β treatment enhanced Smad7 methylation in HaCaT cells, determined by in vivo labeling using 3H-methionine and fluorography, and this methylation was blocked by Adox. (B) TGF-β treatment enhanced Smad6 methylation in HaCaT cells, determined by antibodies specific for Smad6 dimethylation at R74 and R58. (C) Smad7 methylation is decreased following repression of PRMT1 expression. (D) PRMT1 associates with TbRII, but not with wild-type (wt) TbRI; however, PRMT1 associates with an activated form of TbRI (TbRIca). HA-tagged PRMT1 was co-expressed with wild-type (wt) or activated (ca) Flag-tagged TbRI, or TbRII or both, in transfected 293T cells. PRMT1 was immunoprecipitated, and the associated TbRII or TbRI were visualized by immunoblotting. (E) Expression of a constitutively activated TbRI (TbRIca) enhances Smad7 methylation in HaCaT cells expressing Flag-Smad7, as determined by in vivo labeling using 3H-methionine and fluorography. Flag-Smad7 was immunoprecipitated, and subjected to SDS-PAGE, staining and autoradiography to visualize 3H incorporation.

Figure 3. PRMT1 methylates Smad7. (A) PRMT1 methylates Smad7 in vitro in the N-terminal 89 amino acid sequence (Smad7N), but not in the remaining segment spanning amino acids 90 to 427 (Smad7C). GST-Smad7N and GST-Smad7C, or core histones were incubated with PRMT1 in the presence of 3H-S-adenosyl-methionine, separated by SDS-PAGE, and visualized by Gelcode blue staining and 3H-radiography. (B) Replacement of Arg57 (R57A) or Arg67 (R67A), but not other arginines, with alanine decreased Smad7 methylation in vivo. 293T cells expressing wild-type or mutant Flag-tagged Smad7 were labeled in the presence of 3H-methionine, immunoprecipitated with anti-Flag antibody, and visualized by SDS-PAGE, gel staining and 3H-autoradiography. (C) Replacement of Arg57 and/or Arg67 with alanine or lysine decreased Smad7 methylation in vivo, as determined in (B) using 3H-methionine labeling and autoradiography. (D) Downregulating PRMT1 expression using transfected siRNA decreased the methylation of Flag-tagged wild-type Smad7, but not the double mutant Smad7. Wild-type or mutant Smad7 were transfected in 293T cells with PRMT1 siRNA or control siRNA, and labeled using 3H-methionine, immunoprecipitated with anti-Flag antibody, and visualized by SDS-PAGE, gel staining and 3H-autoradiography. (E, F) CID tandem mass spectrometry identified Arg57 monomethylation (E) and dimethylation (F) in 293T cells expressing Flag-Smad7. CID tandem mass spectra obtained from precursor ions with m/z=827.3800+2 (Panel E) or 556.5971+3 (Panel F), corresponding to the mono- and dimethylated Arg57 forms of the peptide spanning residues Ala-47 to Lys-64 of Smad7. Theoretical masses and measured mass errors: 827.3828+2, 3.4 ppm (E); and 556.5962+3, 1.6 ppm (F). The observed sequence ions are labeled in the figure and over the sequence. C*, carboxamidomethyl cysteine residues. IP, immunoprecipitation; IB, immunoblotting.
Figure 4. Smad7 methylation decreases the association of with the TβRI receptor. (A) Flow chart of the experiments aimed at evaluating the effect of PRMT1-mediated methylation of Smad7 on the association of Smad7 with activated (ca) TβRI. Bacterially expressed GST-fused Smad7 was purified using glutathione-Sepharose, and incubated or not with purified PRMT1 in the presence or absence of 3H-S-adenosyl-methionine, thus generating Arg-methylated (Met) or unmethylated (unMet) GST-Smad7. In parallel, His-tagged caTβRI, expressed in 293T cells was immunoprecipitated and coupled to Ni-Sepharose. The methylated and unmethylated GST-Smad7 were then incubated with the Sepharose-bound caTβRI. Both the bound GST-Smad7 and non-bound Smad7 were analyzed by immunoblotting. (B) Results of an experiment shown in (A). Methylation by PRMT1 decreases the binding efficiency of GST-Smad7 for caTβRI. With equal amounts of GST-Smad7 used, methylation by PRMT1 decreased the amount of Smad7 bound to caTβRI, when compared to unmethylated Smad7, and increased the fraction of non-bound Smad7 (flow through). The lower panels show methylation of Smad7, assessed by 3H-labeling. (C) Increased PRMT1 expression decreased the association of Smad7 with caTβRI in 293T cells transfected to express Flag-tagged caTβRI and Myc-tagged Smad7. caTβRI association was revealed by immunoblotting. (D) Downregulation of PRMT1 expression prolonged the half-life of Smad7. HaCaT cells, transfected with control or PRMT1 siRNA for 48 h, were treated with 50 μg/ml of cycloheximide (CHX) for the indicated times. Smad7 was detected by immunoblotting, and its relative expression level was quantified by densitometry, and plotted against time to determine the half-life of Smad7. IB, immunoblotting; IP, immunoprecipitation.

Figure 5. PRMT1 regulates TGF-β-induced EMT. (A) Repression of PRMT1 expression using transfected siRNA attenuated the TGF-β-induced EMT morphology of HaCaT cells. Cells transfected with PRMT1 siRNA or control siRNA were treated with TGF-β for 48 or 72 h to induce EMT, or were left untreated. (B, C) Depletion of PRMT1 expression using transfected siRNA prevented the TGF-β-induced change in actin organization and downregulation of E-cadherin expression, shown by immunofluorescence (B), as well as the EMT-associated decrease in claudin 1 expression and increase in vimentin expression, shown by immunoblotting (C). HaCaT cells were transfected with PRMT1 siRNA or control siRNA, and treated with TGF-b as in (A). (D) Downregulating PRMT1 expression using lentiviral shRNA reduced the TGF-β-induced EMT morphology in HMLE cells. HMLE cells were infected with lentiviral vectors expressing one of two different PRMT1 shRNAs, #1 and #3, or a control shRNA, and were treated with TGF-β for 6 d to induce EMT. (E, F) Downregulating PRMT1 expression using lentiviral shRNA reduced TGF-β-induced changes in EMT marker expression. The HMLE cells expressing PRMT1 shRNA or control shRNA were treated with TGF-β as in (D). Downregulation of PRMT1 expression reduced the expression of epithelial E-cadherin, shown by immunofluorescence (IF) (E), and attenuated the decreased expression of epithelial E-cadherin and the increased expression of mesenchymal N-cadherin, fibronectin, vimentin and ZEB1, as shown by immuno blotting (F). (G) Downregulating PRMT1 expression using lentiviral shRNA reduced the TGF-β-induced expression of EMT markers, assessed by qRT-PCR analyses of mRNA. The HMLE cells expressing PRMT1 shRNA or control shRNA were treated with TGF-β as in (D). Downregulation of PRMT1 reduced the suppression of epithelial marker gene E-cadherin mRNA, and induction of mRNAs encoding mesenchymal markers, N-cadherin, fibronectin, vimentin, ZEB1, ZEB2, Snail and Slug. # p<0.01 vs. no treatment. * p<0.01 vs. control shRNA.

Figure 6. PRMT1 controls stem cell generation. (A) FACS analysis for the expression of the cell surface markers CD44 and CD24 in HMLE cells infected with lentiviral vectors expressing PRMT1 shRNA #1 or #3, or control shRNA. HMLE cells were infected with lentiviral shRNA vectors, and treated with TGF-β for 6 d. Downregulating PRMT1 expression decreased the TGF-β-induced enhancement of the CD44<sup>high</sup>/CD24<sup>low</sup> population. The fractions of the CD44<sup>high</sup>/CD24<sup>low</sup> cell populations against the total viable cell population are shown in the panels and the graph below the panels. # p<0.01 vs. untreated. *
p<0.01 vs. control shRNA. (B) Downregulating PRMT1 expression using lentiviral shRNA reduced the TGF-β-induced expression of stemness markers, assessed by qRT-PCR analyses of mRNA. HMLE cells, infected with lentiviral constructs as shown, were treated with TGF-β as in (A). Downregulation of PRMT1 reduced the induction of stem cell marker gene CD44 and the pluripotency genes POU5F1, Nanog, KLF4, Sox2 and BMI1. # p<0.01 vs. no treatment. ## p<0.05 vs. no treatment. * p<0.01 vs. control shRNA. (C) Downregulating PRMT1 expression using lentiviral shRNA reduced the mammosphere formation. HMLE cells were infected with lentiviral shRNA constructs and treated with TGF-β as in (A). Downregulation of PRMT1 reduced the mammosphere formation in primary and secondary passages. # p<0.01 vs. untreated. * p<0.01 vs. control shRNA.
Figure 1. Katsuno et al.
Figure 2. Katsuno et al.
Figure 4. Katsuno et al.
Figure 5. Katsuno et al.
Figure 6. Katsuno et al.

**A**

- Untreated vs. TGF-β treated conditions.
  - CD44 and CD24 expression levels.
  - PRMT1 shRNA treatment effects.

**B**

- mRNA expression levels for CD44, KLF4, BMI1, and POU5F1.
  - Relative expression in control shRNA, PRMT1 shRNA #1, and PRMT1 shRNA #3 conditions.

**C**

- Mammosphere formation in passage 1 and passage 2.
  - Comparison between untreated and TGF-β treated conditions.

**D**

- Quantitative analysis of mammospheres formed in passage 1 and passage 2.
  - Statistical significance indicated by symbols.*
Arginine methylation of Smad7 by PRMT1 in TGF-β-induced epithelial-mesenchymal transition and epithelial stem cell generation

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