A tight control of Rif1 by Oct4 and Smad3 is critical for mouse embryonic stem cell stability

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Prolonged culture of embryonic stem cells (ESCs) leads them to adopt embryonal carcinoma cell features, creating enormous dangers for their further application. The mechanism involved in ESC stability has not, however, been extensively studied. We previously reported that SMAD family member 3 (Smad3) has an important role in maintaining mouse ESC stability, as depletion of Smad3 results in cancer cell-like properties in ESCs and Smad3−/− ESCs are prone to grow large, malignant teratomas. To understand how Smad3 contributes to ESC stability, we performed microarray analysis to compare the transcriptome of wild-type and Smad3−/− ESCs. We found that Rif1 (RAP1-associated protein 1), a factor important for genomic stability, is significantly upregulated in Smad3−/− ESCs. The expression level of Rif1 needs to be tightly controlled in ESCs, as a low level of Rif1 is associated with ESC differentiation, but a high level of Rif1 is linked to ESC transformation. In ESCs, Oct4 activates Rif1, whereas Smad3 represses its expression. Oct4 recruits Smad3 to bind to Rif1 promoter, but Smad3 joining facilitates the loading of a polycomb complex that generates a repressive epigenetic modification on Rif1 promoter, and thus maintains the expression of Rif1 at a proper level in ESCs. Interestingly, Rif1 short hairpin RNA (shRNA)-transduced Smad3−/− ESCs showed less malignant properties than the control shRNA-transduced Smad3−/− ESCs, suggesting a critical role of Rif1 in maintaining the stability of ESCs during proliferation.

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Embryonic stem cells (ESCs) can serve as a rich source of differentiated cells for cell-based therapy due to their pluripotency and unlimited self-renewal capacity. However, prolonged culture of ESCs results in ESCs accumulating numerous mutations, and they gradually adopt embryonal carcinoma cell features.1–3 This prompts serious safety concerns with regard to ESC applications and also raises important questions regarding how ESCs maintain their genomic stability.

Transforming growth factor beta (TGF-β) signaling has an important role in development and homeostasis. It also functions in multiple diseases such as cancer, tissue fibrosis and diabetes.4,5 Through their respective ligand receptors, TGF-β/Activin/Nodal activates SMAD family member (Smad) 2/Smad3. The activated Smads bind to Smad4 and translocate from cytoplasm to the nucleus to regulate the downstream genes.6,7 TGF-β/Activin/Nodal signaling is crucial for maintaining self-renewal and pluripotency in human ESCs, but appears to be dispensable for the pluripotency of mouse ESCs.8,9 Instead, the activation of Activin/Nodal signaling is required for the propagation of mouse ESCs.10,11 Smad3 is a downstream factor of TGF-β/Activin/Nodal signaling. Although depletion of Smad3 leads to transient expression distortion of mesoderm markers during embryoid body (EB) formation, the final lineage formation is not affected,12 as Smad3 knockout mice are viable and fertile.13 This may be because Smad2, another downstream factor of TGF-β/Activin/Nodal signaling, has a redundant role of Smad3. It has been reported that Smad2 and Smad3 collaboratively regulate mesoderm formation during embryo development.12,14 Previously, we found that activation of Smad3 is vitally important for ESCs to maintain their genetic integrity during propagation, as depletion of Smad3 leads mouse ESCs to adopt cancer cell properties.12 To further illustrate how Smad3 contributes to ESC stability, we performed microarray assay to identify genes that show an obvious change after Smad3 depletion. Among the genes affected by Smad3 depletion, Rif1 (RAP1-associated protein 1), a factor closely associated with chromatin stability, shows the greatest upregulation.

Rif1 is first identified in budding yeast as a Rap1-interacting factor. It is recruited to the telomere by Rap1 and implicated in maintaining telomere structure and homeostasis.15,16 In mammalian cells, except the regulation

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Abbreviations: AP, alkaline phosphatase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia mutated and Rad3-related protein; BrdU, bromodeoxyuridine; Cond2, cyclin-D2; ChIP, chromatin immunoprecipitation; EB, embryoid body; ESC, embryonic stem cell; HR, homologous recombination; IgG, immunoglobulin G; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblast; NHEJ, non-homologous end joining; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; RA, retinoic acid; Rif1, RAP1-associated protein 1; RPA, replication protein A; shRNA, short hairpin RNA; Smad3, SMAD family member 3; TGF-β, transforming growth factor beta; UV, ultraviolet; WT, wild type

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of telomere homeostasis.\textsuperscript{17,18} Rif1 mediates the ATM (ataxia telangiectasia mutated)/53BP1 (tumor suppressor p53-binding protein 1) signaling after DNA damage to repress break resection and promote the non-homologous end joining (NHEJ) mechanism in G1 phase.\textsuperscript{19–23} In addition, Rif1 globally regulates the replication-timing program in both yeast fission and mammalian cells.\textsuperscript{24–27} Rif1 localizes to the stalled replication forks in response to ATR activation and serves as a component of the DNA replication checkpoint.\textsuperscript{28–31} Rif1 is also highly expressed in the pluripotent stem cells.\textsuperscript{32–34} Knockdown of Rif1 by RNA interference in mouse ESCs leads to ESC differentiation.\textsuperscript{35}

In this study, we determine that Rif1 is an important contributor to ESC stability during its propagation. Rif1 expression level is tightly controlled by Smad3 and Oct4. Reduction of Rif1 by RNA interference leads Smad3\textsuperscript{−/−}−ESC to show less malignant properties than control shRNA knockdown Smad3\textsuperscript{−/−}−ESCs, suggesting that upregulation of Rif1 is a key factor in the transformation of Smad3\textsuperscript{−/−}−ESC.

Results

Rif1 is a direct downstream target of Smad3. Previous, we reported that depletion of Smad3 in mouse ESCs produced cancer cell-like features.\textsuperscript{12} To understand the underlying mechanism, cDNA microarray analysis was performed to compare the transcriptome between wild-type (WT) and Smad3\textsuperscript{−/−}−ESCs. Genes with more than a 1.5-fold difference between WT and Smad3\textsuperscript{−/−}−ESCs were selected by Partek software to generate a heat map. On the basis of the microarray data, the expression of Smad3 and Lefty1 was markedly reduced in Smad3\textsuperscript{−/−}−ESCs. Besides, validation of eight randomly picked genes by real-time PCR further suggests that the microarray result was reliable. Among the genes that show different expression after Smad3 depletion, Rif1 ranked as the highest upregulated gene in Smad3\textsuperscript{−/−}−ESCs (Figure 1a and Supplementary Figure S1A). Real-time PCR and western blot analysis confirmed the upregulation of Rif1 at both mRNA and protein level in Smad3\textsuperscript{−/−}−ESCs (Figures 1b and c). Furthermore, overexpression of Smad3 in Smad3\textsuperscript{−/−}−ESCs could significantly downregulate Rif1 expression, but upregulate Lefty1 expression (Figure 1d). As Smad3 is a downstream factor of the Activin pathway in mouse ESCs,\textsuperscript{10} we treated ESCs with Activin A (25 ng/ml) and Activin A inhibitor SB431542 (10 μM), respectively, to examine the expression of Rif1. As expected, the expression of Rif1 was decreased by Activin A treatment, but increased by SB431542 treatment. The expression of Lefty1 and Lefty2 was regulated conversely, confirming that Lefty1 and Lefty2 are positively regulated by Activin/Smad3 pathway, whereas Rif1 is negatively regulated by this pathway (Figures 1e and f). On the basis of Mullen’s chromatin immunoprecipitation (ChiP)-seq data, there are two Smad3-binding sites (SBS1 and SBS2) at the promoter region of Rif1.\textsuperscript{4} Therefore, we designed primers to quantitate Rif1-1 and Rif1-2 regions that cover SBS1 and SBS2, respectively. Examining ChiP-enriched DNA by real-time PCR, we found that Smad3 specifically bound to the Rif1-1 and Rif1-2 regions (Figure 1g). To further examine whether Rif1 promoter activity was affected by Smad3 depletion, a luciferase assay was performed with the Rif1 promoter containing the Smad3-binding sites. The result showed that Rif1 promoter activity was enhanced in Smad3\textsuperscript{−/−}−ESCs compared with WT ESCs (Figure 1h). Taken together, all these data demonstrated that Rif1 is a target of the Activin/Smad3 pathway, and that Smad3 represses Rif1 expression in mouse ESCs.

Inverse expression profiles between Smad3 and Rif1. To further examine the correlation between Smad3 and Rif1, we examined the expression profiles of these two genes in mouse ESCs, mouse embryonic fibroblasts (MEFs), teratoma cells and mouse ESC-differentiated cells. Smad3 showed higher expression in MEFs and teratoma cells than in ESCs. Conversely, Rif1 was expressed at a lower level in MEFs and teratoma cells than in ESCs (Supplementary Figures S1B, S1C and S2A). In addition, we also traced the expression changes of Smad3 and Rif1 during ESC differentiation using monolayer culture and EB formation (Supplementary Figures S1D and S2B). ESC differentiation was accompanied by gradual downregulation of pluripotent markers and expression of lineage markers (Supplementary Figures S1E and S2C). During ESC differentiation, the expression of Smad3 mRNA was gradually increased, whereas the expression of Rif1 mRNA was decreased (Supplementary Figures S1F and S2D). Interestingly, although the expression of Rif1 was decreased in Smad3\textsuperscript{−/−}−ESC-formed EBs, the Rif1 level was always
higher in Smad3−/− ESC-formed EBs than in WT ESC-formed EBs at the same stage, suggesting that Smad3 is one of the key components regulating Rif1 expression during ESC differentiation (Supplementary Figure S2E). These expression profiles also confirm previous reports that Rif1 is a factor associated with pluripotency.32,35
Oct4 is required for Smad3 to bind to Rif1 promoter. Multiple Oct4-bound genes are found to be co-occupied by Smad3 and respond to TGF-β signaling. To find out whether Rif1 is among these genes, we first knocked down the expression of Pou5f1 by RNA interference. The expression level of Rif1 was significantly decreased after Pou5f1 knockdown (Figure 2a). This is consistent with the luciferase activity of Rif1 promoter being reduced to 20% after Pou5f1 knockdown, suggesting that Rif1 is regulated by Oct4 (Figure 2b). Furthermore, overexpression of Pou5f1 in mouse ESCs can enhance the expression of Rif1 at both mRNA and protein level (Figures 2c and d). To determine whether Oct4 co-binds with Smad3 on Rif1 promoter region, we performed ChIP assay with Oct4 antibody. We found that Oct4 was highly enriched on the promoter regions of Rif1 where Smad3 binds (Figure 2e). These data support previous reports that Oct4 positively regulates Rif1 expression in mouse ESCs.

Through co-immunoprecipitation and sequential ChIP experiments, Mullen et al. discovered that Oct4 can form a complex with Smad3 and recruit Smad3 to a Lefty1 enhancer to regulate Lefty1 expression in mouse ESCs. Prompted by the opposed regulatory roles of Smad3 and Oct4 on Rif1, we first examined whether Smad3 is required for Oct4 to bind to Rif1 promoter. As the Oct4 protein level is similar in WT and Smad3−/− ESCs, we directly performed a ChIP assay with Oct4 antibody. We found that Oct4 enrichment on Lefty1, Lefty2 and Rif1 in WT and Smad3−/− ESCs was not obviously affected by the depletion of Smad3 (Figure 2f). We then investigated whether Oct4 is required for recruiting Smad3 to bind to Rif1. We knocked down Pou5f1 by shRNA. After 1-day selection with puromycin, the mRNA level of Pou5f1 was significantly reduced, but the expression of Smad3, pluripotent marker Nanog, lineage markers Cdx2, Cxcr4 and T were not significantly changed and ESCs still maintained the colony morphology (Supplementary Figure S3A). Meanwhile, the Oct4 protein level was obviously decreased, whereas the Smad3 was not affected (Supplementary Figure S3B). We performed ChIP assay with Smad3 antibody using cells at this stage and found that the binding efficiency of Smad3 on Lefty1, Lefty2 and Rif1 was significantly reduced after Pou5f1 knockdown (Figure 2g). To further confirm that Oct4 and Smad3 co-bind to Rif1 promoter, we performed re-ChIP assay with a Smad3 antibody after Oct4 ChIP and discovered that Smad3 and Oct4 do bind to Rif1 promoter simultaneously (Figure 2h). These data suggested that Oct4 is required for Smad3 to bind to Rif1 promoter, but Smad3 is not required for Oct4 binding.

Rif1 promoter shows Smad3-dependent H3K27 methylation. Mullen et al. reported that Activin could induce both upregulation and downregulation of the expression of Oct4 and Smad3 co-occupied genes, indicating that Oct4 and Smad3 regulate their targets by sophisticated regulatory mechanisms. To uncover how Oct4 and Smad3 regulate Rif1, we performed the ChIP assay with histone modification markers, H3K9me2, H3K9me3, H3K4Me3 and H3K27Me3. H3K9me2 and H3K9me3 label the heterochromatin and H3K4Me3 and H3K27me3 are the bivalent markers that label genes related to pluripotency and differentiation. Rif1 promoter was enriched by H3K4me3 and H3K27me3, but not by H3K9me2 and H3K9me3 (Figures 3a and d). Depletion of Smad3 did not affect H3K4me3 enrichment (Figure 3c), but seriously affected H3K27me3 enrichment. However, H3K27me3 level at Lefty1 and Lefty2 was not obviously affected after Smad3 depletion (Figure 3d). This result implies that Rif1 expression is controlled by a specific epigenetic modification involving Smad3. As Suz12 is the key component of the polycomb repressive complex (PRC2) and contributes to H3K27me3, we performed a ChIP assay with a Suz12 antibody. We found that Suz12 enrichment on Rif1 is significantly reduced after Smad3 depletion, but its enrichment on Lefty1 and Lefty2 was stable (Figure 3e). Taken together, these data suggest that, although Oct4 and Smad3 co-bind to Lefty1, Lefty2 and Rif1, the mechanism used to regulate Rif1 is different to that used to regulate Lefty1 and Lefty2. In the Rif1 regulatory complex, Smad3 has a critical role in loading PRC2 to regulate the expression of Rif1 through H3K27 methylation.

Smad3−/− ESCs show higher cell proliferation and DNA repair capacities than WT ESCs after ultraviolet irradiation. Our previous studies showed that Smad3 depletion enhanced the anti-apoptosis capacity of ESCs. To further substantiate this observation, we set out to examine the response of WT and Smad3−/− ESCs to DNA damage in detail. Before ultraviolet (UV) irradiation, WT and Smad3−/− ESCs showed no obvious difference after propidium iodide (PI) staining. Further, labeling the cells with bromodeoxyuridine (BrdU) revealed that more Smad3−/− ESCs showed active DNA replication than WT ESCs. WT and Smad3−/− ESCs were then exposed to UV irradiation (40 mJ/cm²) to induce DNA damage. Five hours later, the cells were pulse labeled with BrdU and cultured for another 30 min. Subsequently, the cells were collected and stained with PI and FITC-conjugated BrdU antibody for flow cytometry analysis. About 7% Smad3−/− ESCs showed active DNA replication compared with only about 3% for the WT cells (Figures 4a and b). This result demonstrated that Smad3−/− ESCs have a higher cell proliferation capacity than WT ESCs after DNA damage.

In responding to the DNA damage after UV irradiation, ATR-Chk1 and ATM-Chk2 are activated to modulate checkpoint, DNA repair, apoptosis and cell senescence. Replication protein A (RPA) is an ssDNA-binding protein in eukaryotes and prevents ssDNA from forming hairpin structures or re-annealing when the DNA is under repair, replication or recombination. RPA is upregulated after DNA damage and it is essential for the ATR-mediated DNA damage checkpoint. Smad3−/− ESCs expressed a significantly higher level of RPA than WT ESCs at 1 h and 3 h after UV irradiation (Figure 4c). Consistent with this, phosphorylated Chk1 was significantly higher in Smad3−/− ESCS than WT ESCS, whereas phosphorylated Chk2 was only slightly increased in Smad3−/− ESCs (Figure 4d). These data imply an elevated DNA damage response in Smad3−/− ESCs.

H2AX, a histone H2A variant, is phosphorylated by ATM after DNA damage. It binds to the damaged DNA and attracts more proteins to join the DNA repair, thus it is a good indicator of DNA damage. Significant amounts of H2AX were detected in both WT and Smad3−/− ESCs 1 h after UV irradiation. However, H2AX level in Smad3−/− ESCs was not obviously increased at 3 h after UV irradiation (Figure 4e). This result indicated that Smad3−/− ESCs have a lower DNA damage response than WT ESCs.
Figure 2  Oct4 positively regulates Rif1 and is indispensable for Smad3 to bind to Rif1 promoter regions. (a) Quantitative real-time PCR to examine the mRNA levels of Pou5f1 and Rif1 after transfection with pSuper control and pSuper-Pou5f1-shRNA plasmids. Actin was analyzed as a control. The data are shown as the mean ± S.D. (n = 3). (b) Luciferase assay to examine Rif1 promoter activity in mouse ESCs transfected with pSuper control and pSuper-Pou5f1-shRNA plasmids. Renilla was analyzed as an internal control. The data are shown as the mean ± S.D. (n = 3). (c) Quantitative real-time PCR to examine the mRNA levels of Pou5f1, Lefty1 and Rif1 after transfection with pCAG-GFP and pCAG-Pou5f1 plasmids. Actin was analyzed as a control. The data are shown as the mean ± S.D. (n = 3). (d) Western blot analysis of the protein levels of Oct4 and Rif1 in mouse ESCs transfected with pCAG-GFP and pCAG-Pou5f1 plasmids. Gapdh was analyzed as a control. Arrow indicates the overexpression band of Flag-Oct4. (e) ChIP-qPCR to examine the DNA enrichment of Oct4 and control IgG at the Smad3-binding sites on the promoter of Rif1 in mouse ESCs. Enrichment of studied proteins on Actin was analyzed as a control. The data are shown as the mean ± S.D. (n = 3). (f) ChIP-qPCR to examine the Oct4 enrichment at the Lefty1, Lefty2 and Rif1 in WT and Smad3−/− ESCs. Actin was analyzed as a control. The data are shown as the mean ± S.D. (n = 3). (g) ChIP-qPCR to examine Smad3 enrichment at the Lefty1, Lefty2 and Rif1 at 1-day puromycin selection after mouse ESCs were transfected with pSuper control and pSuper-Pou5f1-shRNA plasmids. Smad3 enrichment at Actin was analyzed as a control. The data are shown as the mean ± S.D. (n = 3). (h) Sequential ChIP assay was performed to examine Smad3 and IgG enrichment on Oct4-enriched DNAs. The quantity of enriched Lefty1, Lefty2 and Rif1 (Rif1-1 and Rif1-2) fragments was checked by real-time PCR. The data are shown as the mean ± S.D. (n = 3). Statistically significant differences, calculated through student's t-tests, are indicated (*P < 0.05; **P < 0.01; ***P < 0.001).
irradiation, suggesting substantial DNA damage in these cells (Supplementary Figure S5A). However, 6 h after UV irradiation, phosphorylated H2AX was significantly less in Smad3−/− ESCs than WT ESCs (Figure 4e), indicating that, at this point in time, less-damaged DNA remained in the Smad3−/− ESCs. All these observations confirm that Smad3−/− ESCs have an enhanced DNA repair ability after UV irradiation. Rif1 has been demonstrated to be highly associated with UV-induced DNA damage response and checkpoint activation. We analyzed the phosphorylation level of Chk1 in Smad3−/− ESCs after being transfected with Rif1 shRNA and treated with UV irradiation. Phosphorylated Chk1 was significantly reduced in the Rif1 knockdown Smad3−/− ESCs compared with the control knockdown Smad3−/− ESCs (Supplementary Figure S5B), suggesting that upregulation of Rif1 in Smad3−/− ESCs mediates the enhanced DNA damage response after UV irradiation.

Reduced cell proliferation by knockdown of Rif1 in Smad3−/− ESCs. To check whether the upregulation of Rif1 is a major factor for Smad3−/− ESC-enhanced proliferation, two shRNAs targeting to two different regions of Rif1 gene were constructed. Both of them could efficiently reduce Rif1 at both mRNA and protein level. Knockdown of Rif1 caused ESC differentiation with downregulation of pluripotent markers alkaline phosphatase (AP) and Oct4 and upregulation of differentiation markers Cdx2, Gata6, T and Fgf5 (Supplementary Figures S4A–S4D). Next we transfected Rif1 shRNA into the Smad3−/− ESCs. We found that the expression level of Rif1 in Smad3−/− ESCs could be reduced to about the Rif1 level in WT ESCs by Rif1 shRNA after selection with puromycin for 3 days (Figures 5a and b). To constantly reduce Rif1 expression in Smad3−/− ESCs, we transduced Smad3−/− ESCs with lentivirus to stably express Rif1 shRNA. By picking a number of single-cell colonies, we were able to select Smad3−/− ESCs with stably reduced Rif1. Rif1 shRNA-transduced Smad3−/− ESCs expressed significantly lower Rif1 than Smad3−/− ESCs and control shRNA-transduced Smad3−/− ESCs, but a similar level of Rif1 to WT ESCs (Figure 5c). These Rif1 shRNA-transduced Smad3−/− ESCs could still proliferate. The expression of Ccnd2 (cyclin-D2), which is increased in Smad3−/− ESCs, was significantly decreased by Rif1 reduction (Figure 5d). Besides, BrdU integration assay revealed that proliferating cell number in Rif1 knockdown Smad3−/− ESCs was significantly reduced to about the WT ESC level (Figures 5e and f), suggesting that upregulation of Rif1 promoter shows Smad3-dependent H3K27 methylation ChIP-qPCR to examine (a) H3K9me2, (b) H3K9me3, (c) H3K4Me3, (d) H3K27Me3 and (e) Suz12 enrichment at Lefty1 and Lefty2 enhancer and Rif1 promoter in WT and Smad3−/− ESCs. Enriched Actin was analyzed as a negative control. The data are shown as the mean±S.D. (n=3). Statistically significant differences, calculated through student’s t-tests, are indicated (*P<0.05; **P<0.01; ***P<0.001)
Figure 4  Smad3−/− ESCs show enhanced cell proliferation and DNA repair capacity after UV irradiation. (a) Representative flow cytometry dot plot of BrdU-integrated WT (WT1 and WT2) and Smad3−/− (Smad3−/−1 and Smad3−/−2) ESCs at 0 and 6 h after UV irradiation. x axis represents DNA content through PI stain and y axis represents BrdU-FITC-labeled cells. (b) Statistical analysis of the BrdU-positive cell percentage of a from two independent experiments. The data are shown as the mean ± S.D. (n = 2). (c) Western blot and densitometric analyses of the expression of RPA2 in WT (WT1 and WT2) and Smad3−/− (Smad3−/−1 and Smad3−/−2) ESCs before, and at 1 and 3 h after, UV (40 mJ/cm²) irradiation. Gapdh expression level was used as an internal control. The densitometric data are shown as the mean ± S.D. (n = 2). (d) Western blot and densitometric analyses of the expression of pChk1 (Ser345) and pChk2 (Thr68) in WT (WT1 and WT2) and Smad3−/− (Smad3−/−1 and Smad3−/−2) ESCs before, and at 1 and 3 h after, UV (40 mJ/cm²) irradiation. Gapdh expression level was used as an internal control. The densitometric data are shown as the mean ± S.D. (n = 2). (e) Western blot and densitometric analyses of the expression of pH2AX (Ser139) in WT (WT1 and WT2) and Smad3−/− (Smad3−/−1 and Smad3−/−2) ESCs before, and at 6 h after, UV (40 mJ/cm²) irradiation. Gapdh expression level was used as an internal control. The data are shown as the mean ± S.D. (n = 2). Statistically significant differences, calculated through student’s t-tests, are indicated (*P<0.05; **P<0.01; ***P<0.001)
Knockdown of Rif1 in Smad3−/− ESCs can attenuate cell proliferation. (a) Morphological appearances of WT ESCs, Smad3−/− ESCs and control shRNA- and Rif1 shRNA-transduced Smad3−/− ESCs. Scale bar = 200 μm. (b) Quantitative real-time PCR to examine the mRNA level of Rif1 in WT ESCs and Smad3−/− ESCs after being transfected with control shRNA and Rif1 shRNA plasmids. Actin was analyzed as an internal control. The data are shown as the mean ± S.D. (n = 3). (c) Western blot and densitometric analyses of the expression of Rif1 in WT, Smad3−/− ESCs and control shRNA- and Rif1 shRNA-transduced Smad3−/− ESCs. Gapdh expression level was used as an internal control. The data are shown as the mean ± S.D. (n = 2). (d) Quantitative real-time PCR to examine the mRNA level of Ccnd2 in WT ESCs and control shRNA- and Rif1 shRNA-transduced Smad3−/− ESCs. Actin was analyzed as an internal control. The data are shown as the mean ± S.D. (n = 3). Statistically significant differences, calculated through student’s t-tests, are indicated (*P < 0.05; **P < 0.01; ***P < 0.001). (e) Immunofluorescence staining with BrdU antibody to examine BrdU integration in WT ESCs and control shRNA- and Rif1 shRNA-transduced Smad3−/− ESCs. Cells were pulse labeled with BrdU for 30 min, and then fixed for BrdU staining. The nuclei were stained with DAPI. Scale bar = 200 μm. (f) Flow cytometry analysis of BrdU integration percentage (pulse labeled with 30 min) in WT, control shRNA- and Rif1 shRNA-transduced Smad3−/− ESCs. x axis represents BrdU-FITC cell percentage and y axis represents cell counts.
Rif1 in Smad3−/− ESCs is one of the main factors enhancing ESC proliferation.

The cell migration capacity of Smad3−/− ESC-differentiated cells is reduced by knockdown of Rif1. Smad3−/− ESC-differentiated cells show enhanced cell migration. To examine whether upregulation of Rif1 contributes to cell migration (Supplementary Figure S6A), we performed wound-healing and transwell assays. After induction of ESC differentiation by withdrawing 2i and leukemia inhibitory factor (LIF), a wound scratch was generated. Three hours after the scratch, there was no obvious difference in the wound gap between control and Rif1 shRNA-transduced Smad3−/− ESC-differentiated cells. But at 12 h after the scratch, the wound gap of the control sample was narrower than Rif1 knockdown sample, suggesting a reduced cell migration capacity caused by Rif1 knockdown. This difference was more obvious at 24 h (Figure 6a). In addition, a transwell assay also revealed that Rif1 knockdown Smad3−/− ESC-differentiated cells had lower migration capacity than the control (Supplementary Figure S6B). This is consistent with the cell migration markers Mmp2 and Mmp9 being significantly downregulated in Rif1 knockdown Smad3−/− ESC-differentiated cells compared with the control knockdown samples (Supplementary Figure S6C).

Rif1 shRNA-transduced Smad3−/− ESCs form less-malignant teratomas. To investigate whether upregulation of Rif1 is responsible for Smad3−/− ESCs forming malignant teratoma, we subcutaneously injected WT, Smad3−/− ESCs and Rif1 shRNA-transduced Smad3−/− ESCs into SCID mice. Four weeks later, all the injected ESCs had formed tumors. Smad3−/− ESCs formed the largest tumors and Rif1 shRNA-transduced Smad3−/− ESCs formed the smallest tumors (Figure 6b). The same result was obtained by a repeat injection assay. Hematoxylin and eosin staining revealed that all these tumors were teratomas with tissues of three germ layers (Supplementary Figure S6D). Genotyping of tumor samples confirmed the integration of Rif1 shRNA in the teratoma cells produced by Rif1 shRNA-transduced Smad3−/− ESCs (Figure 6c). Furthermore, the cell proliferation gene Ccnd2 and cell migration genes Mmp2 and Mmp9 were significantly reduced in the teratomas formed by Rif1 shRNA-transduced Smad3−/− ESCs compared with the teratoma formed by control Smad3−/− ESCs (Figure 6d). Staining the teratomas with proliferating cell nuclear antigen (PCNA) antibody showed that Smad3−/− ESC-formed teratomas contained more PCNA-positive cells than WT ESC and Rif1 shRNA-transduced Smad3−/− ESC-formed teratomas (Figures 6e and f). Collectively, these results demonstrated that upregulation of Rif1 is one of the main factors in Smad3−/− ESC transformation.

Discussion

In this study, we found that Rif1, a factor involved in genomic stability, is tightly regulated by Oct4 and Smad3 in mouse ESCs. Oct4 recruits Smad3 to the Rif1 promoter and facilitates the loading of the PRC2. To maintain Rif1 expression at a proper level in ESCs, Oct4 activates Rif1 expression, but Smad3 is involved in repressing it (Figure 7). Mullen et al. reported that Nanog, Oct4 and Sox2 form a complex and tend to co-bind with Smad3 on many genes. From the ChIP-seq data of their report, we found that Nanog, Oct4 and Sox2 all bind to the SBE of Rif1. It is therefore likely that Rif1 is synergistically regulated by these core transcription factors in ESCs. From past reports and our own studies, it seems very important to keep Rif1 expressed at a suitable level to sustain ESC pluripotency and stability. Low level of Rif1 leads to mESC differentiation. However, high level of Rif1 is also deleterious to ESCs by driving malignant transformation of Smad3−/− ESCs. Comparison of the expression of Smad3 and Rif1 between teratoma cells and teratocarcinoma cell lines F9 and P19 revealed that Smad3 was significantly lower in teratocarcinoma cells than teratoma cells, whereas Rif1 is significantly higher in teratocarcinoma cells than teratoma cells (Supplementary Figures S7A and S7B). These data support our findings and indicate that a disturbance in the expression of Smad3 and Rif1 may be one of the underlying mechanisms for teratocarcinoma formation.

It is reported that Rif1 colocalizes with DNA double-strand breaks (DSBs) and is involved in DNA repair. Recent studies revealed that Rif1 contributes to the inhibition of 5′ end resection of DSBs, the first step of homologous recombination (HR). As a result, NHEJ, an error-prone DNA repair is promoted with Rif1 presence. In the absence of Rif1, the level of misjoined chromosomes is significantly reduced, which is deleterious to ESCs by driving malignant transformation of Smad3−/− ESCs. Comparison of the expression of Smad3 and Rif1 between teratoma cells and teratocarcinoma cell lines F9 and P19 revealed that Smad3 was significantly lower in teratocarcinoma cells than teratoma cells, whereas Rif1 is significantly higher in teratocarcinoma cells than teratoma cells (Supplementary Figures S7A and S7B). These data support our findings and indicate that a disturbance in the expression of Smad3 and Rif1 may be one of the underlying mechanisms for teratocarcinoma formation.

The role of Rif1 in mouse ES cell stability

P Li et al

Cell Death and Disease
Materials and methods

Cell culture and differentiation. Derivation of WT and Smad3−/− mouse ESCs was described in previous reports.12,54 The ESCs were maintained on feeders under the normal ESC medium, which is composed of DMEM with high glucose (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 15% ES culture grade FBS (Gibco), 0.1 mM non-essential amino acids (NEAA; Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and streptomycin (Gibco) and 1000 U/ml LIF (Millipore, Billerica, MA, USA). To obtain feeder-free ESC lines, ESCs were passaged 2–3 times to completely get rid of the feeder cells and grown on a 0.2% gelatin (Sigma, St. Louis, MO, USA)-coated dish in ES medium containing LIF and 2i (1 μM PD325901 and 3 μM CHIR99021).

For ESC differentiation assay, 2i and LIF were removed and 1 μM retinoic acid (RA) was added in the culture medium to induce ESC differentiation. For EB formation assay, monolayer undifferentiated WT ESCs and Smad3−/− ESCs were

Figure 6  Knockdown of Rif1 in Smad3−/− ESCs can attenuate cell migration. (a) Image (upper) and histogram (lower) of scratch wound healing of WT ESCs and control shRNA- and Rif1 shRNA-transduced Smad3−/− ESC-differentiated cells at 3, 12 and 24 h after scratch. The data are shown as the mean ± S.D. (n = 2). (b) Picture of SCID mice with tumors at 4 weeks after WT ESCs, Smad3−/− ESCs and Rif1 shRNA-transduced Smad3−/− ESCs were subcutaneously injected into SCID mice. (c) Genotyping of ES cell formed tumors to confirm Rif1 shRNA integration in Rif1 shRNA transduced Smad3−/− ESCs. (d) Quantitative real-time PCR to examine the mRNA levels of Smad3, Rif1, Ccn2, Mmp2 and Mmp9 in tumors grown from WT ESCs, Smad3−/− ESCs and Rif1 shRNA-transduced Smad3−/− ESCs. Actin was analyzed as an internal control. The data are shown as the mean ± S.D. (n = 3). (e) Immunofluorescence staining with anti-PCNA antibody to examine PCNA expression in WT ESC, control Smad3−/− ESC and Rif1 shRNA-transduced Smad3−/− ESC-formed teratomas. The nuclei were stained with DAPI. Scale bar = 200 μm. (f) Quantification of the PCNA-positive cell percentage compared with DAPI in WT ESC, control Smad3−/− ESC and Rif1 shRNA transduced Smad3−/− ESC-formed teratomas. Statistically significant differences, calculated through student’s t-tests, are indicated (*p < 0.05; **p < 0.01; ***p < 0.001)
instructions. The cycle (SYBR Premix Ex Taq (TaKaRa, Shiga, Japan) according to the manufacturer's Real-time PCR assay.

*Plasmid constructs.* Two shRNA constructs targeting Rif1 were generated according to previous reports with pSuper.puro vector (Addgene, Cambridge, MA, USA). To generate lentiviral vector for express shRNA by lentivirus, Rif1 shRNA sequences together with the H1 promoter were cut from pSuper plasmid with EcoRI and Clai and sub-cloned to pLVTH plasmid. To construct the Rif1 promoter reporter plasmid, a 2000 bp fragment, encompassing the Smad3-binding sites, was amplified by primers from the genomic DNA of mouse ESCs and cloned into a pGL3 vector (Promega, Madison, WI, USA) at the MluI and Xhol sites. To construct the pCAG-GFP, pCAG-Smad3 and pCAG-Pou5f1 plasmids, the ORF sequences of these genes were amplified from the cDNA of mouse ESCs, digested and inserted into pCAG-Flag vector (Addgene) at BglII-XhoI (GFP and Smad3) and MluI-Xhol (Pou5f1) sites. All the amplification primers have been added to Table 1 and these recombinant vectors have been sequenced.

**Real-time PCR assay.** Real-time PCR analysis was conducted using the ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA) analysis machine with SYBR Premix Ex Taq (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. The cycle (Ct) values of target genes were first normalized against the Ct value of an internal control (Actin gene) and then normalized against the Ct value of corresponding transcripts of the control sample. The DNA primer sequences used for the real-time PCR assay are listed in Table 1. For each pair of the primer, only one correct size band and one peak were detected. All the real-time PCR assays comprised triplicate data with samples from three independent experiments.

**Western blot.** WT and Smad3−/− ESCs were collected and subjected to RIP A protein lysis buffer, containing 0.2 M NaCl, 1% SDS, 1 mM PMSF inhibitor and 0.1 M DTT. Then, the SDS-PAGE was used to separate the proteins. After separation, the proteins were transferred to PVDF membranes (Pall Corporation, Port Washington, NY, USA). Subsequently, the PVDF membranes were blocked in 5% nonfat milk (BD Biosciences, Franklin Lakes, NJ, USA) at 37 °C for 45 min to remove DNA contamination, and then the proteins were transferred for reverse transcription, labeled and hybridized to an Affymetrix mouse exon 1.0 STArray. The antibody-enriched DNAs were decrosslinked and purified using DNAse I (New England Biolabs, Beverly, MA, USA). The antibody-enriched DNAs were then blotted with the corresponding antibodies. The antibody-enriched DNAs were then blotted with the corresponding antibodies. The antibody-enriched DNAs were decrosslinked and purified with phenol–chloroform (Ambion, Applied Biosystems). The precipitated DNA was dissolved in TE buffer and analyzed by agarose gel electrophoresis. The microarray data is GSE57985.
Table 1 Primers used in the study

| Primers used in the study | Real-time PCR primers |
|--------------------------|-----------------------|
| Actin F                  | 5′-ACCACTCGAGGACATAGTGGA-3′ |
| Actin R                  | 3′-CTGCGCTACTCTTGAAGATG-5′ |
| Smad3 F                  | 5′-CATCCTGGTGAGAGGACCTG-3′ |
| Smad3 R                  | 3′-AAAGCTTGCAAGACGGAACAA-5′ |
| Oct4 F                   | 5′-ATCCCGCGTTATGCTGCTCT-3′ |
| Oct4 R                   | 3′-GACACTCCACTTCAGCATATA-5′ |
| Nanog F                  | 5′-GGGTATCGTGGGAGGACGGAAG-3′ |
| Nanog R                  | 3′-AACTGAAGCGCGTCAGAAG-5′ |
| Rif1 F                   | 5′-ACTGTCACACTTCAGCATATA-3′ |
| Rif1 R                   | 3′-GACACTCCACTTCAGCATATA-5′ |
| Lefty1 F                 | 5′-CAGCGAGATTTTCAAGAGGCT-3′ |
| Lefty1 R                 | 3′-ATCCCGCGTTATGCTGCTCT-5′ |
| Lefty2 F                 | 5′-ACGCTGCAAGAAGCTACGGCG-3′ |
| Lefty2 R                 | 3′-GACACTCCACTTCAGCATATA-5′ |
| Rif1 shRNA1 F            | 5′-GAGAGTTGACATCGTGGCCTGAGAACAGAG-3′ |
| Rif1 shRNA1 R            | 3′-GGTGGGAAGTGGGATCAAG-5′ |
| Rif1 shRNA2 F            | 5′-CCTGGTGGTGTTTTGTTGTG-3′ |
| Rif1 shRNA2 R            | 3′-AGCATGACGGACAAGTACC-5′ |
| Lefty2 R                 | 3′-GACACTCCACTTCAGCATATA-5′ |
| Rif1-1 F                 | 5′-GCGGAAATCTTTCCCTGCCT-3′ |
| Rif1-1 R                 | 3′-GATGGTCACGCGTTGCTGCGTCC-5′ |
| Rif1-2 F                 | 5′-GATGGTCACGCGTTGCTGCGTCC-3′ |
| Rif1-2 R                 | 3′-GATGGTCACGCGTTGCTGCGTCC-5′ |
| Oct4 F                   | 5′-AGCCAGAAGCTACGGCG-3′ |
| Oct4 R                   | 3′-GACACTCCACTTCAGCATATA-5′ |

ChIP-qPCR primers

| Primers used in the study | ChIP-qPCR primers |
|--------------------------|------------------|
| Actin F                  | 5′-GTTACCGCGGATAGCTGACT-3′ |
| Actin R                  | 3′-GACACTCCACTTCATC-5′ |
| Lefty1 F                 | 5′-GATGCCAGACAGAAAGCACC-3′ |
| Lefty1 R                 | 3′-GACACTCCACTTCATC-5′ |
| Lefty2 F                 | 5′-GATGATAGGAGCTCCACAC-3′ |
| Lefty2 R                 | 3′-GACACTCCACTTCATC-5′ |
| Mmp2 F                   | 5′-ATGTGACGATACGGGGAT-3′ |
| Mmp2 R                   | 3′-GACACTCCACTTCATC-5′ |
| Mmp9 F                   | 5′-GGATGACCTAAGACGCCTC-3′ |
| Mmp9 R                   | 3′-GACACTCCACTTCATC-5′ |
| Eomes F                  | 5′-CCTGCGTGTTTTTGGTGCTG-3′ |
| Eomes R                  | 3′-GACACTCCACTTCATC-5′ |
| Elf5 F                   | 5′-CCTGTTCGCTTACCTCCACAC-3′ |
| Elf5 R                   | 3′-GACACTCCACTTCATC-5′ |
| Cgta F                   | 5′-GAGGGCTGTTTCTCTGCTGCTT-3′ |
| Cgta R                   | 3′-GACACTCCACTTCATC-5′ |
| Cga F                    | 5′-GGGCAAGTGGGAGCATC-3′ |
| Cga R                    | 3′-GACACTCCACTTCATC-5′ |

shRNA sequences

| Primers used in the study | shRNA sequences |
|--------------------------|-----------------|
| Rif1 shRNA F             | 5′-GATCCCACCAGATCTGACTGACT-3′ |
| Rif1 shRNA R             | 3′-GACACTCCACTTCATC-5′ |
| Rif1 shRNA2 F            | 5′-GATCCCACCAGATCTGACTGACT-3′ |
| Rif1 shRNA2 R            | 3′-GACACTCCACTTCATC-5′ |
| Rif1 shRNA3 F            | 5′-GATCCCACCAGATCTGACTGACT-3′ |
| Rif1 shRNA3 R            | 3′-GACACTCCACTTCATC-5′ |
| Rif1 shRNA4 F            | 5′-GATCCCACCAGATCTGACTGACT-3′ |
| Rif1 shRNA4 R            | 3′-GACACTCCACTTCATC-5′ |

Rif1 promoter primers

| Primers used in the study | Rif1 promoter primers |
|--------------------------|-----------------------|
| Rif1 F                   | 5′-GGCTGCAGCTGGCTGCTCTCG-3′ |
| Rif1 R                   | 3′-GACACTCCACTTCATC-5′ |

cDNA clone primers

| Primers used in the study | cDNA clone primers |
|--------------------------|--------------------|
| Gfp F                    | 5′-ATCCACGAGATCTGAGGAGGACAGG-3′ |
| Gfp R                    | 3′-GACACTCCACTTCATC-5′ |
| Smad3 F                  | 5′-GTGTCACATCTGACTGTCCCTCGCCT-3′ |
| Smad3 R                  | 3′-GACACTCCACTTCATC-5′ |
| Pou5f1 F                 | 5′-GTGTCACATCTGACTGTCCCTCGCCT-3′ |
| Pou5f1 R                 | 3′-GACACTCCACTTCATC-5′ |

Genotyping primers

| Primers used in the study | Genotyping primers |
|--------------------------|--------------------|
| pLh-Rif1 shRNA F         | 5′-GCTGACGTCATCATCAACCCTGCCTGAAAGAG-3′ |
| pLh-Rif1 shRNA R         | 5′-CTGCAATGCTGACGGGTGGGAGGAGGAG-5′ |
Author contributions
PL performed most experiments. XM helped on teratoma assay. IRA prepared Rif1 antibody and revised the manuscript. PY designed the project and wrote the manuscript.

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The role of Rif1 in mouse ES cell stability
P Li et al.

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