Loss of KLF4 and Consequential Downregulation of Smad7 Exacerbate Oncogenic TGF-β Signaling in and Promote Progression of Hepatocellular Carcinoma

Hongcheng Sun[1,2], **Zhihai Peng**[2], **Huamei Tang**[3], **Dacheng Xie**[4], **Zhiliang Jia**[1], **Lin Zhong**[2], **Senlin Zhao**[2], **Zhengyu Ma**[5], **Yong Gao**[4], **Lixian Zeng**[6], **Rongcheng Luo**[6], and **Keping Xie**[1]

[1]Department of Gastroenterology, Hepatology and Nutrition, The University of Texas MD Anderson Cancer Center, Houston, TX

[2]Department of General Surgery, Shanghai Jiaotong University Affiliated Shanghai General Hospital, Shanghai, People's Republic of China

[3]Department of Pathology, Shanghai Jiaotong University Affiliated Shanghai General Hospital, Shanghai, People's Republic of China

[4]Department of Oncology and Tumor Institute, Shanghai East Hospital, Tongji University School of Medicine, Shanghai, People's Republic of China

[5]Liver Transplantation Section, Center for Organ Transplantation, Fudan University, Shanghai 200040, People's Republic of China

[6]Tumor Hospital, Southern Medical University, Guangzhou, Guangdong, People's Republic of China

Abstract

Hyperactivation of TGF-β signaling pathway is a common feature of hepatocellular carcinoma (HCC) progression. However, the driver factors leading to enhanced TGF-β activity are not well characterized. Here, we explore the mechanisms that loss of Krüppel-like factor 4 (KLF4) exacerbates oncogenic TGF-β signaling in human HCC. The expression of KLF4 and TGF-β signaling components in primary HCC and their clinicopathologic relevance and significance was evaluated by using tissue microarray and immunohistochemistry. Cellular and molecular impacts of altered KLF4 expression and TGF-β signaling were determined using immunofluorescence, Western blot, reverse-transcriptase polymerase chain reaction, chromatin immunoprecipitation, and promoter reporter assays. Loss of KLF4 expression in primary HCC closely correlated with decreased Smad7 expression, increased p-Smad2/3 expression, and independently predicts reduced

Address requests for reprints to: Keping Xie, MD, PhD, Department of Gastroenterology, Hepatology and Nutrition, Unit 1644, the University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030. Telephone: 713-794-5073; fax: 713-745-3654; kepxie@mdanderson.org; Rongcheng Luo, MD, PhD, Tumor Hosptial, Southern Medical University, Guangzhou, Guangdong, People's Republic of China. Phone: 86-20-61650001; Fax: 86-20-61650009; luorc02@vip.163.com; or Zhihai Peng, MD, PhD, Department of General Surgery, Shanghai General Hospital, 85 Wujin Road, Shanghai, 200080, People's Republic of China. Phone: 86-21-63240090-3132; Fax: 86-21-63241377; xczmd@sjtu.edu.cn.

overall and relapse-free survival after surgery. TGF-β signaling components were expressed in most HCC cells, and activation of TGF-β signaling promoted cell migration and invasion. Enforced KLF4 expression blocked TGF-β signal transduction and inhibited cell migration and invasion via activation of Smad7 transcription, whereas deletion of its C-terminal zinc-finger domain diminished this effect. KLF4 protein physically interacts with the Smad7 promoter. Promoter deletion and point mutation analyses revealed that a region between nucleotides −15 bp and −9 bp of the Smad7 promoter was required for the induction of Smad7 promoter activity by KLF4. Our data indicate that KLF4 suppresses oncogenic TGF-β signaling by activation of Smad7 transcription, and that loss of KLF4 expression in primary HCC may contribute to activation of oncogenic TGF-β signaling and subsequent tumor progression.

Keywords

HCC; Prognosis; Progression; KLF4; TGF-β

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with an estimated over half a million new cases and an almost equal number of deaths each year.[1] Surgical treatment remains the mainstay of curative therapy for patients with HCC. However, despite treatment with curative intent, later recurrence and/or metastatic spread are common and negatively affect survival. The overall prognosis is still unsatisfactory, and little progress has been made in finding new treatment options. As such, it is urgent to elucidate the molecular pathogenesis and identify molecular targets responsible for the biological behavior of HCC so that effective therapy for this fatal disease can be developed.

The transforming growth factor-β (TGF-β) signaling pathway, which contributes to liver fibrosis, cirrhosis, and subsequent progression to HCC, plays a central role in hepatocarcinogenesis.[2,3] In response to TGF-β, receptor-activated Smads (R-Smads) are phosphorylated in their C-terminal SXS motif by type I receptors. Phosphorylated R-Smads form a complex with Smad4 and are transported into the nucleus where they regulate target genes. TGF-β signaling is tightly controlled by many factors, including Smad7, which is the principal negative feedback regulator.[4] Smad7 represses TGF-β signaling by stably binding to the cytoplasmic domain of activated type I receptors and blocking Smad2/3 phosphorylation.[4] It was initially thought that TGF-β exerted tumor suppressive functions at early stages of HCC because it triggers senescence and growth inhibition in some hepatoma cell lines.[5,6] However, it has become more apparent that during carcinogenesis, tumor cells tend to down-regulate the growth-inhibitory responses associated with TGF-β, and, instead, respond preferentially to the growth-promoting cues elicited by TGF-β signaling.[3,7,8] Moreover, inhibition of TGF-β signaling has been shown to suppress HCC growth, vascular invasion, and neo-angiogenesis by regulating critical factors involved in these processes.[9–11] Increased serum levels of TGF-β1 are associated with poor prognosis of HCC patients and application of TGF-β inhibitors showed antitumor activity and improved clinical outcome;[12,13] these findings suggest that a subset of human HCC tumors rely on TGF-β

signaling for progression. Nevertheless, the underlying mechanisms responsible for hyperactivation of oncogenic TGF-β signaling in HCC are poorly understood.

Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor that is expressed in terminally differentiated epithelial cells and has diverse functions in cell differentiation, proliferation, embryogenesis, and pluripotency.[14] Recent studies demonstrated that KLF4 transcriptionally upregulates TGF-β1 and contributes to cardiac myofibroblast differentiation.[15] Conversely, KLF4 can inhibit TGF-β1/Smad3 function by competing with Smad3 for the C terminus of the coactivator p300/CBP. As such, KLF4 promotes inflammatory responses in macrophages[16], suggesting that KLF4 interacts with TGF-β pathway under non-cancerous conditions as well. Research conducted over the past decade has highlighted the significance of KLF4 deregulation in cancer development and progression.[17] However, little is known about the impact of deregulated KLF4 on TGF-β signaling in the context of tumor development. We and others have recently characterized KLF4 as a potent tumor suppressor in HCC, demonstrating that loss of KLF4 promotes tumorigenesis and epithelial to mesenchymal transition (EMT) of HCC cells. Because active TGF-β signaling is a key inducer of EMT in HCC, we hypothesized that decreased KLF4 expression contributes to hyperactivation of TGF-β signaling and subsequent EMT and HCC progression.

In this study, we have evaluated the potential inhibitory effect of KLF4 in regulating TGF-β/Smad signaling in human HCC tissue and cell lines. We show that KLF4 blocks TGF-β signal transduction by transcriptionally activating Smad7; deletion of the zinc finger domain (ZFD) of KLF4 abrogated this effect. Taken together, our study demonstrates that loss of KLF4 transcriptionally downregulates Smad7 in human HCC. This, in turn, increases TGF-β signaling and tumor progression of HCC.

Results

Expression of KLF4, p-Smad2, p-Smad3, and Smad7 and their correlation with prognostic significance in HCC patients

Using a TMA, we first investigated the expression of KLF4 and related TGF-β signaling molecules in 148 pairs of primary HCC and matched adjacent non-tumor tissue obtained from patients who underwent liver resection. We found that reduced KLF4 expression was accompanied by decreased Smad7 expression but increased expression of p-Smad2 and p-Smad3 in primary HCC tissues when compared with those in the matched non-tumor tissues (Fig. 1A & 1B; Supplementary Table S2 and S3). These results were verified by Western blot analysis (Fig. 1C). Moreover, KLF4 positively correlated with Smad7, and negatively with p-Smad2 and p-Smad3 in primary HCC specimens (Fig. 1D and 1E). Smad7 expression in HCC inversely correlated with p-Smad2 and p-Smad3 expression (Fig. 1D, Supplementary Fig. S1). In addition, decreased expression of KLF4 and Smad7 was associated with malignant cilinicopathological features of HCC, including younger age, higher AFP level, larger tumor size, macrovascular invasion, and advanced tumor stage (Supplementary Table S4 and S5). Univariate survival analysis demonstrated that reduced KLF4 and Smad7 expression and increased p-Smad2 and p-Smad3 levels in primary HCCs were significantly associated with reduced OS and RFS after surgery (Supplementary Fig.

S2; Supplementary Table S6). Multivariate analysis further revealed that, along with well-established prognostic factors, such as macrovascular invasion, reduced expression of KLF4 was an independent prognostic factor for unfavorable clinical outcome of HCC patients (Supplementary Table S6).

Activation of TGF-β/Smad signaling promotes migration and invasion of human HCC cells

We investigated the integrity of TGF-β/Smad signaling and the response of this pathway to TGF-β1 in human HCC cell lines. As shown in Fig. 2A, all components of the TGF-β signaling pathway were detected in most of the HCC cell lines used in this study. The only exceptions were loss of TGF-β type II receptor (TβRII) in SNU475 cells and deficiency of Smad3 in SNU398 cells. Because phosphorylation of R-Smads at two serines in their C-terminal SXS motif is a crucial step in TGF-β signal transduction, the activity of TGF-β in HCC cells was measured by immunoblot using specific p-Smad2 (Ser465/467) and p-Smad3 (Ser423/425) antibodies in the presence or absence of TGF-β1. HCC cells showed very low levels of basal p-Smad2 and p-Smad3 expression. In contrast, TGF-β1 stimulation of most HCC cell lines resulted in upregulation of p-Smad2 and/or p-Smad3 (Fig. 2B; Supplementary Fig. S3). These data indicate that the TGF-β signaling pathway is intact and functional in most of HCC cell lines.

We next determined the effect of TGF-β signaling on the biological behaviors of HCC cells. As shown in Fig. 2C and 2D, stimulation of SNU387 and SNU423 cells with TGF-β1 (3 ng/ml) promotes cell migration and invasion, whereas inhibition of TGF-β signaling by SB-431542 (0.3 μM), a potent and specific inhibitor of TGF-β type I receptor kinases, completely abrogated this effect. Nevertheless, cell proliferation was not affected by TGF-β1 or TGF-β1+SB-431542 treatment (Fig. 2E).

KLF4 overexpression attenuates TGF-β signal transduction upon TGF-β1 stimulation in HCC cells

Given that nuclear accumulation of Smad2/Smad3 is a key feature of TGF-β signal activation, we tested whether KLF4 overexpression affects the nuclear localization of Smad2/Smad3 in HCC cells. By performing immunofluorescent staining in SNU387 cells, we found that TGF-β1 treatment significantly increased the nuclear translocation of Smad2/Smad3. In contrast, KLF4 overexpression significantly suppressed nuclear translocation of Smad2/Smad3 upon exposure to TGF-β1 (Fig. 3A). To determine whether the transcriptional activity of KLF4 is involved in this process, we constructed a KLF4 vector (KLF4 ZFD) lacking the zinc finger domain (ZFD) (Supplementary Fig. S4A). Similar to full-length KLF4, KLF4 ZFD protein was mainly distributed in the nucleus (Supplementary Fig. S4B). However, in contrast to the full-length protein, KLF4 ZFD lost both its transcriptional activity (Supplementary Fig. S4C) and its inhibitory effect on Smad2/Smad3 nuclear translocation upon TGF-β1 stimulation (Fig. 3). To confirm these findings, cytoplasmic and nuclear protein extracts from SNU387 cells were subject to Western blot analysis. As shown in Supplementary Fig. S5, KLF4 overexpression inhibited nuclear localization of total and phosphorylated Smad2, Smad3, and the Co-Smad Smad4 in the presence of TGF-β1, whereas deletion of its ZFD abrogated this effect. These findings

suggest that KLF4 inhibits nuclear localization of Smad2/Smad3 in HCC cells upon TGF-β1 stimulation via its transcriptional activity.

Because phosphorylated Smad2/Smad3 mainly localized in the nucleus, we hypothesized that KLF4 suppresses nuclear retention of Smad2/3 through inhibiting their phosphorylation. As shown in Fig. 4A, KLF4 overexpression in SNU387 and SNU423 cells, which showed low levels of basal KLF4 expression (Supplementary Fig. S6A), significantly inhibited phosphorylation of Smad2/Smad3 and expression of plasminogen activator inhibitor type 1 (PAI-1), a prototypic TGF-β target gene, upon exposure to TGF-β1. However, KLF4 overexpression did not change the expression levels of total Smad2/Smad3 or Smad4. Moreover, deletion of the KLF4 ZFD significantly abrogated the inhibitory effect of KLF4 on Smad2/Smad3 phosphorylation and PAI-1 expression upon TGF-β1 treatment (Fig. 4B, Supplementary Fig. S6B). These results suggested that KLF4 suppresses phosphorylation of Smad2/Smad3 via its transcriptional activity in the HCC cells.

As phosphorylated Smad2 and Smad3 are major mediators of TGF-β signaling, we further tested the role of KLF4 in Smad2/Smad3-mediated transcriptional activity. As shown in Fig. 4C, overexpression of KLF4 in SNU387 and SNU423 cells resulted in a dramatic decrease in the activity of pSBE4-Luc, a Smad3-specific luciferase reporter, and pARE-Luc, a Smad2-specific luciferase reporter, following treatment with TGF-β1. Nevertheless, deletion of the ZFD of KLF4 significantly abrogated its negative impact on the activity of both SBE4-Luc and ARE-Luc upon TGF-β1 stimulation.

Because TGF-β signaling promotes metastatic potential of HCC cells, we determined whether KLF4 inhibits TGF-β-induced migration or invasion of HCC cells. As demonstrated in Fig. 4D and 4E, KLF4 inhibits migration and invasion of SNU387 cells, and this inhibitory effect was much more significant upon TGF-β1 stimulation. Furthermore, TGF-β-mediated cell migration was not substantially altered in the cells lacking Smad7 when KLF4 was overexpressed (Supplementary Fig. S6C).

KLF4 Inhibits TGF-β Signaling via Activation of Smad7 Transcription

Because KLF4 has been reported to interact with Smad3 in myofibroblasts,[22] we examined whether KLF4 interacts with Smad2 and/or Smad3 in HCC cells and whether this interaction could influence the phosphorylation status of Smad2/Smad3. We found that KLF4 interacts with both Smad2 and Smad3 independent of its ZFD (Supplementary Fig. S7). As such, the interaction between KLF4 and Smad2/Smad3 may not correlate with phosphorylation of Smad2/Smad3. Given that the suppressive function of KLF4 on TGF-β signaling depends on its ZFD, we investigated whether KLF4 regulates the key components of TGF-β signaling, including Smad7, at a transcriptional level. As shown in Fig. 5, enforced KLF4 expression in SNU387 and SNU423 cells increased levels Smad7 mRNA and protein in a dose-dependent manner, whereas deletion of its ZFD eliminated this effect. In contrast, but consistent with these data, KLF4 knockdown in PLC/PRF/5 cells using siRNA led to a dose-dependent decrease in the expression of Smad7 mRNA and protein (Fig. 5). Altered KLF4 expression in these HCC cells had no impact on the expression levels of TβRII, TβRI, Smad2, Smad3, Smad4, Smad6, Smurf1, or Smurf2 (Fig. 5). These results suggest that KLF4 specifically regulates Smad7 expression at the transcriptional level.

To further characterize the regulatory role of KLF4 on Smad7 expression, we analyzed the Smad7 promoter sequence for the presence of potential KLF4 binding sites using the KLF4 consensus sequence 5′-G/AG/AGGC/TGC/T-3′.[23] We identified three putative KLF4-binding elements (referred to as sites #1, #2, and #3) in the Smad7 promoter region and constructed several mutant reporter constructs: pS7Pro+40 (lacks all binding sites), pS7Pro +14 (contains binding site #1), pS7Pro−56 (contains sites #1 and #2), and pS7Pro−127 (contains sites #1, #2, and #3) (Fig. 6A). We then co-transfected the deletion mutant reporters with KLF4, KLF ZFD, or control pcDNA3.1 into SNU387 cells; pGL3-basic and pFLAG-KLF4 were co-transfected as negative controls. Overexpression of wild-type KLF4 but not KLF4 ZFD enhanced the promoter activity of pS7Pro−56 and pS7Pro−127. In contrast, overexpression of either KLF4 or KLF4 ZFD had no impact on the promoter activity of pS7Pro+40 and pS7Pro+14 (Fig. 6B). Moreover, mutations of the conserved nucleotides in the KLF4 binding site #2 (pS7Pro−127Mut) significantly decreased the promoter activity of pS7Pro−127 (Fig. 6A and 6C). These findings indicate that KLF4 binding site #2 is essential for the transcriptional regulation of Smad7 by KLF4. To provide direct proof that KLF4 is recruited to the endogenous Smad7 promoter during transcription *in vivo*, we performed chromatin immunoprecipitation assays using chromatin prepared from SNU387, SNU423, and PLC/PRF/5 cells and a primer set flanking the 302-bp (−117 to +185) region of the Smad7 promoter. The 302-bp DNA fragment was amplified from the precipitates by anti-KLF4 antibody but not by control IgG (Fig. 6D). Collectively, these results clearly suggested that KLF4 positively regulates Smad7 transcription via direct binding to the KLF4 binding site #2.

We next examined whether upregulation of Smad7 by KLF4 is sufficient to inhibit TGF-β signaling. As shown in Fig. 6E, KLF4 overexpression upregulated Smad7 expression and suppressed Smad2/Smad3 phosphorylation and PAI-1 expression in HCC cells when treated with TGF-β1. In contrast, Smad7 knockdown using siRNA rescued Smad2/Smad3 phosphorylation and PAI-1 expression upon TGF-β1 stimulation.

Discussion

In this report, we provided many lines of evidence that KLF4 in human HCC transcriptionally activates Smad7 expression, whereas loss of KLF4 leads to downregulation of Smad7 and thereby hyperactivation of oncogenic TGF-β signaling and subsequent tumor progression (Fig. 7). First, the expression levels of KLF4, p-Smad2, p-Smad3, and Smad7 correlate with prognostic significance in HCC patients. Second, activation of TGF-β/Smad signaling promotes migration and invasion of human HCC cells, and KLF4 inhibits those effects. Third, KLF4 overexpression attenuates TGF-β signal transduction upon TGF-β1 stimulation in HCC cells. Finally, KLF4 inhibits TGF-β signaling via activation of Smad7 transcription. Our clinical and mechanistic studies strongly suggest the important role of this KLF4-Smad7-TGF-β signaling axis in HCC development and progression and a potential target for designing effective therapy against this deadly disease.

Elevated serum levels of TGF-β1 in patients with HCC correlate with worse prognosis, suggesting that hyperactive TGF-β signaling may represent a hallmark of this tumor type.[12] In this study, we detected increased phosphorylation of Smad2/3 in primary HCC compared

to adjacent non-tumorigenic tissue. This provided us with the first direct evidence for activation of TGF-β signaling in human HCC cells *in vivo*. Our data also revealed significant correlation between increased expression of phosphorylated Smad2/3 and both reduced patient survival and enhanced metastatic potential of HCC cells when exposed to TGF-β1 *in vitro*, indicating a pro-metastatic role of TGF-β/Smad signaling in HCC. Previous work has described dual roles for TGF-β signaling—as a tumor suppressor in premalignant lesions and early HCC and as a cancer promoter in developed hepatoma.[3] Functional switching of this pathway in human gastrointestinal cancers, such as colorectal and pancreatic cancers, commonly involves deletion or mutation of the TGF-β signal components.[24,25] However, alterations in these genes are rare in HCC. Consistent with earlier reports, we observed intact and functional TGF-β signaling in most of the investigated HCC cell lines.[26] Therefore, in these cell lines, it is likely that the oncogenic shift of TGF-β signaling may be determined by some other factors, for example, CD44 overexpression[27] or p53 loss.[28]

Loss of KLF4 likely contributes to the enhanced invasive and metastatic phenotypes of tumors.[14,18,21] Here, we report that KLF4 is a potent tumor suppressor in HCC. We also show that loss of KLF4 transcriptionally downregulates Smad7 expression in HCC, which leads to hyperactivation of TGF-β signaling and subsequent tumor progression. Mechanistically, this effect is not attributed to the protein-protein interaction between KLF4 and Smad2/3. Instead, it is dependent on the DNA binding activity of KLF4, as deletion of KLF4 ZFD completely abrogated its inhibitory effect on TGF-β signal transduction. Previous studies have shown that KLF4 suppresses TGF-β1 signaling, thereby promoting an inflammatory response in macrophages; KLF4 functions in this manner by competing with Smad3 for the coactivator p300/CBP, independent of its DNA binding activity.[16] To the best of our knowledge, this is the first study to explore the impact and mechanism of KLF4 on TGF-β signaling in the context of cancer. The present study not only expands our knowledge of KLF4's function as a tumor suppressor, but also provides an experimental rationale for designing novel and tumor specific therapy for HCC.

Smad7 is a well-established key negative regulator of TGF-β signaling and an important cross-talk mediator between the TGF-β/Smad pathway and other signaling networks.[29] Previous work has indicated that Smad7 may exert pro- or anti-tumorigenic effects depending on the given tumor type.[30] In human HCC, the expression profile of Smad7 remains controversial. Park *et al.* reported a much higher Smad7-expression rate in advanced tumors than in dysplastic nodules and early HCCs.[31] In contrast, Xia *et al.* identified decreased expression of Smad7 in HCC samples, particularly in patients with early recurrence and poor prognosis.[32] Our data demonstrated reduced Smad7 expression in HCC compared to matched non-tumorigenic tissue, which was associated with malignant clinicopathological features of HCC and unfavorable clinical outcome of patients after surgery. Using a mouse model of HCC induced by diethylnitrosamine, Wang *et al.* showed that Smad7 knockout (KO) mice displayed higher tumor incidence and multiplicity than wild-type mice.[33] Additionally, tumor cells from Smad7 KO mice demonstrated increased proliferation, diminished apoptosis, and higher colony formation compared with those from wild-type littermates.[33] These findings support the notion that Smad7 is a critical tumor suppressor in HCC. As such, in the present study, we have focused on how KLF4 directly

regulates transcription of the Smad7 gene. To avoid possible interference from other transcription factors, *e.g.*, Smad2/3, Sp1, we cloned a very short proximal region of the Smad7 promoter (ranging from −127bp to +287bp), which included three putative KLF4 binding sites. This region has not been well studied because of significant change of the initiation site in comparison to a previous report;[34] the physiologic role of this region in the regulation of Smad7 expression remains to be defined. Our study provides novel insight into the functional role of the proximal region of the Smad7 promoter in the regulation of Smad7 gene expression. Nevertheless, KLF4 may activate Smad7 expression at the transcriptional level independent of TGF-β/Smads signaling and Smad7 may execute its multiple functions though various mechanisms. Future studies are clearly warranted to dissect in great details the molecular basis underlying the transcriptional activation of Smad7 expression by KLF4 and the detailed mechanisms that Smad7 inhibits R-Smads phosphorylation in HCC.

In summary, our data show that loss of KLF4 in human HCC transcriptionally downregulates Smad7 expression, thereby leading to hyperactivation of oncogenic TGF-β signaling and subsequent tumor progression. The present study not only expands our current knowledge on the tumor suppressive role of KLF4 in HCC, but also provides novel insight into the mechanism of TGF-β signaling in human HCC. Our results strongly suggest a routine evaluation of KLF4 by immunohistochemistry in primary HCC after surgical resection, which may offer a novel pathological biomarker for forecasting patient prognosis and improve our insight into patient selection for TGF-β targeted therapy.

Materials and methods

The Supplementary Methods section contains details about the materials and methods regarding immunohistochemistry and tissue microarray (TMA) analysis,[18,20] Western blot analysis,[18] cell proliferation assay,[18,21] scratch assay,[18] cell invasion assay,[18] RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR) analysis (Supplementary Table S1), immunofluorescent cell staining, construction of mutant KLF4 with deletion of zinc finger domain (KLF4 ZFD)-expressing vectors, construction of Smad7 promoter reporter plasmids and mutagenesis, and chromatin immunoprecipitation (ChIP) assay.[21]

Patients, Clinicopathological Analysis, and Tissue Specimens

Patient information and extraction of follow-up and clinicopathological data were previously described.[20] Matched pairs of HCC and formalin-fixed, paraffin-embedded non-tumor tissue blocks for TMA and paired fresh HCC and non-tumor tissue specimens were collected as previously described.[20] The study protocol was approved by the Shanghai Jiaotong University Institutional Review Board, and informed written consent for use of the tissue specimens was obtained from each patient or his or her guardian.

TMA Construction and Immunohistochemistry

TMA construction was previously described in detail.[20] Standard immunohistochemical procedures were performed with human HCC TMA specimens using anti-KLF4 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-Smad2 (Ser465/467) (1:2000, AB3849,

Millipore), p-Smad3 (Ser423/425) (1:50, ab51451, Abcam), and Smad7 (1:50 dilution, MAB2029; R&D Systems) antibodies. The staining results were scored by two pathologists blinded to the clinical data as described previously[18,20] and in Supplementary Methods. Use of archived tissue specimens was approved by the Shanghai Jiaotong University Institutional Review Board.

Statistical Analysis

Statistical analyses were performed using the SPSS software program (version 17.0; IBM Corporation, Armonk, NY). For continuous variables, data were expressed as medians in the interquartile range and compared using the Kruskal-Wallis test. For categorical variables, data were expressed as numeral counts and percentages and compared using the Pearson chi-square test or Fisher exact test. Differences in protein expression between the matched specimens were examined using the marginal homogeneity test or Mann-Whitney U test. Survival rates were calculated and survival curves were plotted using the Kaplan-Meier method, and differences were compared using the log-rank test. Univariate Cox regression was used to analyze differences in survival among patient groups. The significant factors in the univariate analyses were included in multivariate Cox proportional hazards models. The significance of the *in vitro* data in the groups was determined using a two-tailed Student *t*-test. Statistical significance was indicated by a conventional *P* value less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used in this paper

ChIP	chromatin immunoprecipitation
FFPE	formalin-fixed paraffin-embedded
KLF4	Krüppel-Like Factor 4
EMT	epithelial to mesenchymal transition
ZDF	zinc finger domain
PBS	phosphate-buffered saline
PBS-T	PBS containing 0.1% Triton X-100
PCR	polymerase chain reaction
qPCR	quantitative PCR

RT	reverse transcription
siRNA	small interfering RNA
TGF	transforming growth factor
TMA	tissue microarray
UTR	untranslated region

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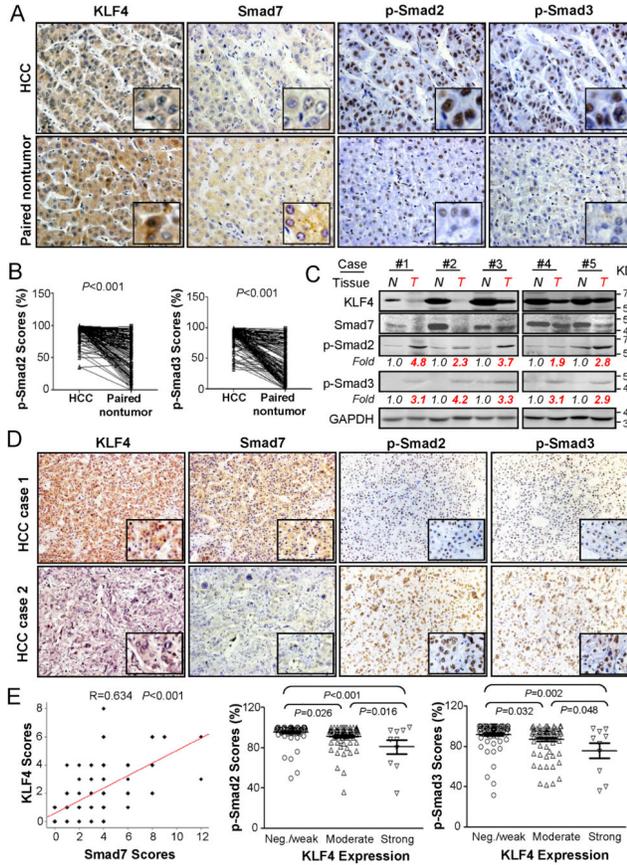
Figure 1. Correlation between expression of KLF4, Smad7, p-Smad2, and p-Smad3 and prognosis in human HCC specimens

(A–C) KLF4, Smad7, p-Smad2, and p-Smad3 expression in HCC and paired nontumor tissue samples. *A*, immunostaining of HCC and matched adjacent nontumor tissue specimens in consecutive TMAs was conducted using a specific anti-KLF4, anti-Smad7, anti-p-Smad2, or anti-p-Smad3 antibody, representative photos (magnification 200×) were shown; *B*, p-Smad2 and p-Smad3 staining in HCC and paired nontumor tissues was quantified and compared with Mann-Whitney u test (*P*<0.001); *C*, Western blot analysis of KLF4, Smad7, p-Smad2, and p-Smad3 protein in HCC (*T*) and paired nontumor tissue (*N*) specimens. (D & E) Correlation of KLF4 expression with Smad7, p-Smad2, and p-Smad3 expression in primary HCC. *D*, representative immunostaining of KLF4, Smad7, p-Smad2, and p-Smad3 in 2 cases of HCC (magnification 200× for the inserts, 100× for all others); *E*, KLF4 expression positively correlated with Smad7 expression (*P*<0.001 [Spearman Rank Correlation test]) but inversely associated with p-Smad2 and p-Smad3 staining (*P*<0.05 [Mann-Whitney u test]).
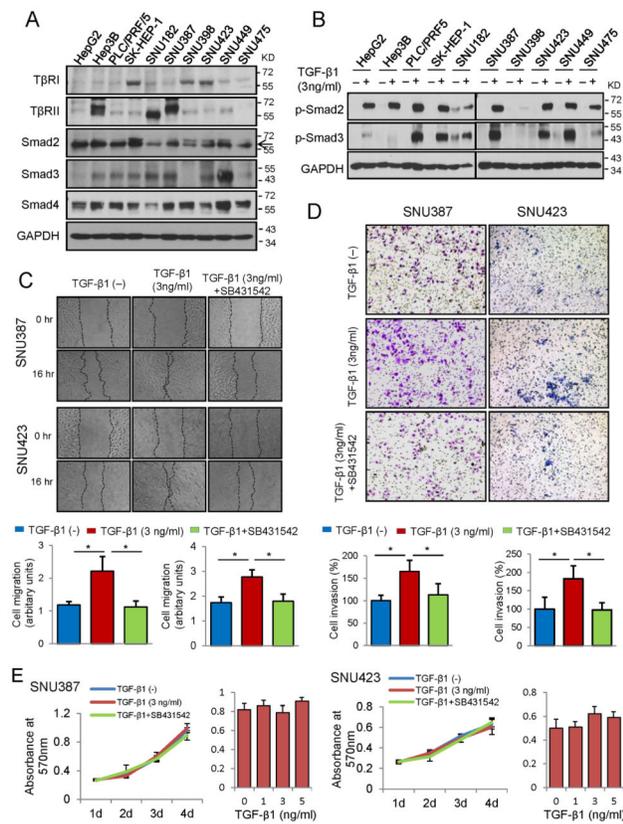
Figure 2. Integrity of TGF-β/Smad signaling in HCC cells and its impact on cellular behavior
(A) Western blot analysis of the components of TGF-β signaling in HCC cell lines. (B) Western blot analysis of p-Smad2 and p-Smad3 expression in HCC cells with TGF-β1 stimulation. (C) SNU387 and SNU423 cells were cultured with or without TGF-β1 (3 ng/ml), or with TGF-β1 (3 ng/ml) + SB431542 (0.3 μM) as indicated, and cell migration was assessed by scratch assay. Shown are photos taken at the indicated time points after the cultures were wounded by scratching. Quantification of cell migration is shown on the right measured by ImageJ2x software, *P<0.05; data are expressed as Mean ± SEM of 3 independent experiments. (D) SNU387 and SNU423 cells were treated as described above and subjected to cell invasion assay. Representative photos are shown (left panels). Control cell groups (Ctrl) were given an arbitrary invasion percentage of 100% (right panels); data are expressed as Mean ± SEM of 3 independent experiments, *P<0.05. (E) SNU387 and SNU423 cells were treated as described above and subjected to cell proliferation assay; data are expressed as Mean ± SEM of 3 independent experiments.
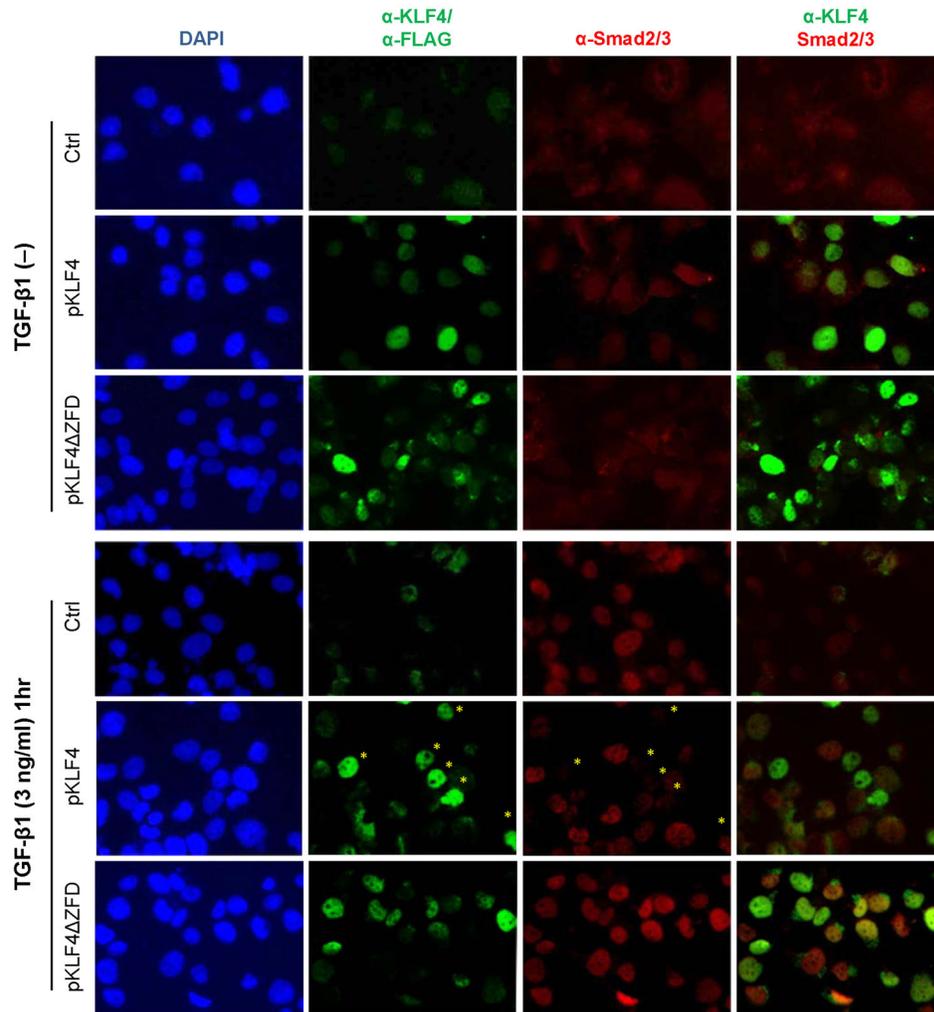
Figure 3. Influence of KLF4 expression on nuclear translocation of Smad2/Smad3 in HCC cells
SNU387 cells were transfected with control vector pcDNA3.1 (Ctrl), pFLAG-KLF4 vector (pKLF4), or pFLAG-KLF4 ZFD vector (pKLF4 ZFD) as indicated. Forty-eight hours after transfection, cells were treated with or without TGF-β1 (3 ng/ml) for 1 hr and subjected to cell immunofluorescence assay; anti-FLAG antibody was used instead of anti-KLF4 antibody in the "pKLF4 ZFD" group. Shown are photos taken under magnification 400×. Yellow asterisks indicate that an increased expression of nuclear KLF4 caused a decreased expression of nuclear Smad2/3.
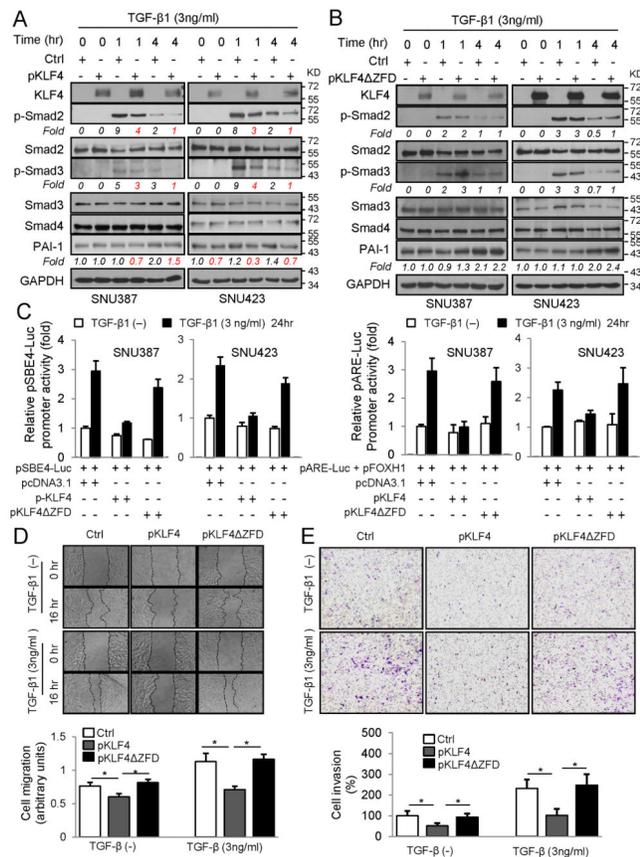
Figure 4. Influence of KLF4 expression on phosphorylation of Smad2/Smad3, Smad2/Smad3-mediated transcriptional activity, and biological behavior of HCC cells

(A) SNU387 and SNU423 cells were transfected with pcDNA3.1 (Ctrl) or vector pFLAG-KLF4 (pKLF4) as indicated. Forty-eight hours after transfection cells were treated with or without TGF-β1 (3 ng/ml) as indicated and total protein lysates were prepared for Western blot analysis. (B) SNU387 and SNU423 cells were transfected with pcDNA3.1 (Ctrl) or vector pFLAG-KLF4 ΔZFD (pKLF4 ΔZFD) as indicated. Cells were treated and subjected to Western blot analysis as described in A. (C) The Smad3-specific luciferase reporter pSBE4-Luc (left panel) or Smad2-specific luciferase reporter pARE-Luc (right panel) was transfected in triplicate with pFLAG-KLF4, pFLAG-KLF4 ΔZFD, or control vector pcDNA3.1 into SNU387 and SNU423 cells. Forty-eight hours after transfection cells were treated with or without TGF-β1 (3 ng/ml) for 24 hours, the activity of the promoter reporters was measured and expressed as the fold activity in their respective control groups without TGF-β1 treatment; data are expressed as Mean ± SEM of 3 independent experiments. (D) SNU387 cells were transfected with pcDNA3.1 (Ctrl), pFLAG-KLF4 (pKLF4), or pFLAG-KLF4 ΔZFD (pKLF4 ΔZFD) as indicated. Forty-eight hours after transfection, cells were cultured with or without TGF-β1 (3 ng/ml), and cell migration was assessed by scratch assay. Shown are photos taken at the indicated time points after the cultures were wounded by scratching (left panels). Quantification of cell migration is shown below measured by ImageJ2x software; data are expressed as Mean ± SEM of 3 independent experiments, *P<0.05. (E) SNU387 cells were treated as described above and subjected to cell invasion

assay. Representative photos are shown (right panels). Quantification of cell invasion is shown below, control cell group without TGF-β1 treatment was given an arbitrary invasion percentage of 100% (right panel); data are expressed as Mean ± SEM of 3 independent experiments, *P<0.05.
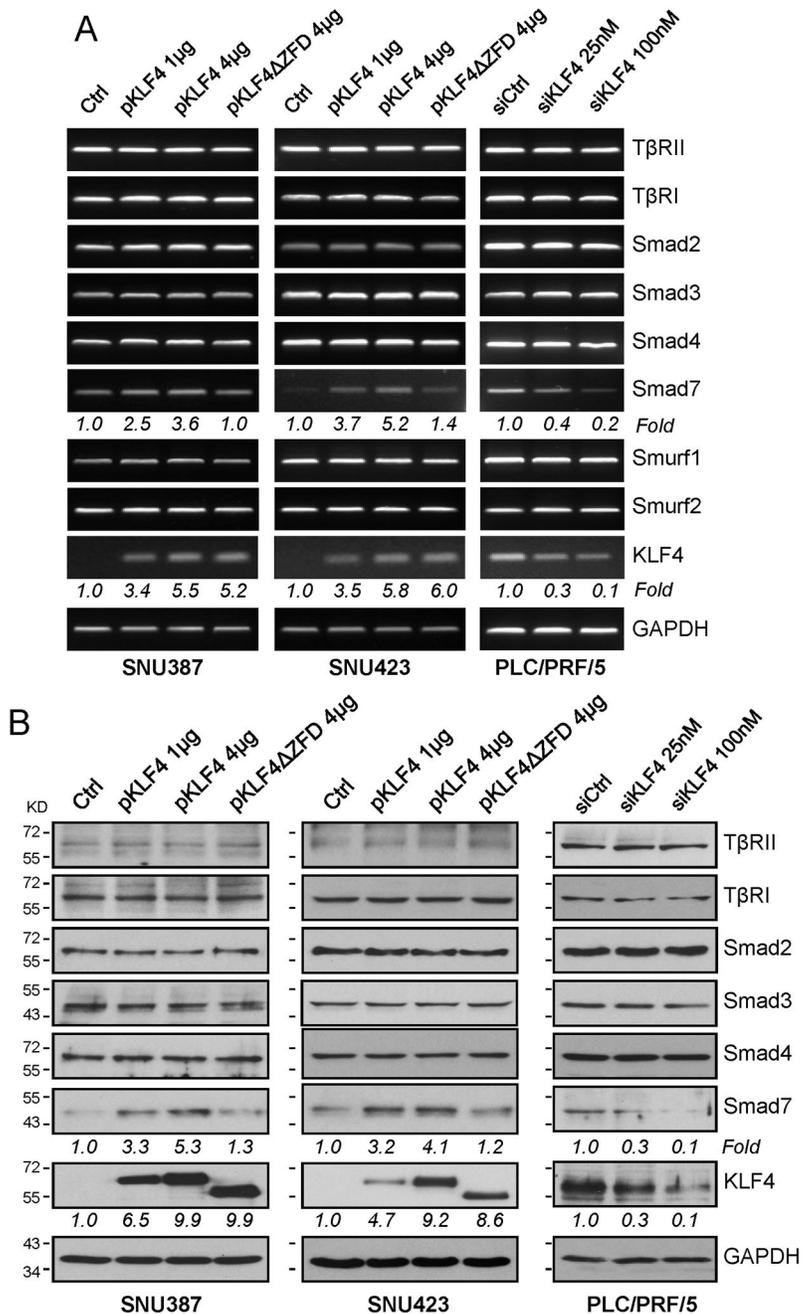
Figure 5. Influence of altered KLF4 expression on the components of TGF-β/Smad signaling pathway

(A) SNU387 and SNU423 cells were transfected with 4 μg control pcDNA3.1 vector (Ctrl), 1 μg pFLAG-KLF4 vector plus 3 μg pcDNA3.1 vector (pKLF4 1μg), 4μg pFLAG-KLF4 vector (pKLF4 4 μg), and 4 μg pFLAG-KLF4 ZFD vector (pKLF4 ZFD 4 μg) in six-well plates. PLC/PRF/5 cells were transfected with control siRNA (siCtrl) or KLF4 siRNA (siKLF4) in six-well plates at the indicated doses. Total RNA was harvested from the cultures 48 hr after transfection and reverse-transcribed into cDNA. The expression of KLF4 and TGF-β signal components was measured using RT-PCR. (B) SNU387, SNU423, and

PLC/PRF/5 cells were transfected as in A, total protein lysates were prepared 48 hr after transfection and subjected to Western blot analysis.
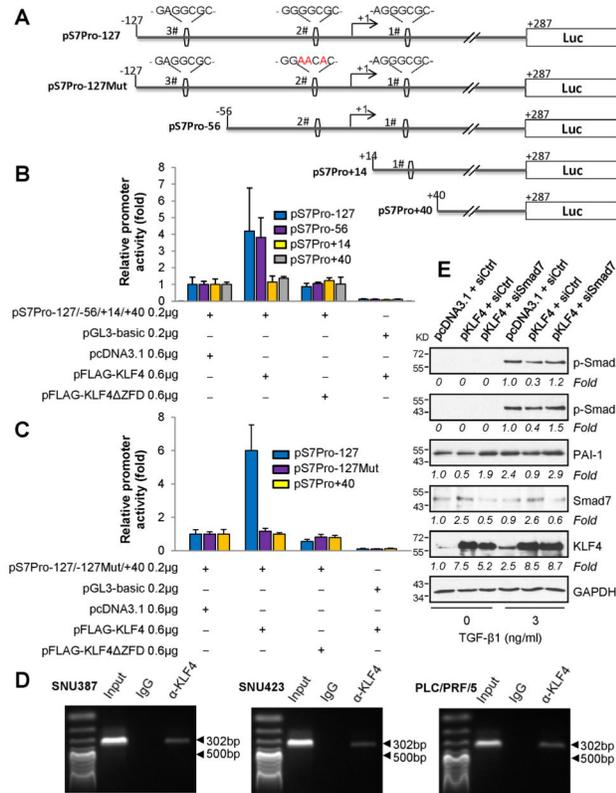
Figure 6. Regulation and direct binding of the Smad7 promoter by KLF4 and the impact of KLF4-Smad7 pathway on TGF-β signaling

(A) Schematic of the Smad7 promoter reporters and their putative KLF4-binding sites. (B) The Smad7 promoter reporters (pS7Pro−127/−56/+14/+40) were transfected in triplicate with pFLAG-KLF4, pFLAG-KLF4 ZFD, or the control vector pcDNA3.1 into SNU387 cells. Co-transfection of pFLAG-KLF4 and pGL3-basic was used as a negative control. The activity of the promoter reporters was measured 36 hr after transfection, and the activities in the negative control and treated groups were expressed as the fold activity in their respective control groups; data are expressed as Mean ± SEM of 3 independent experiments. (C) The Smad7 promoter reporters (pS7Pro−127/−127Mut/+40) were transfected in triplicate as described in B. Co-transfection of pFLAG-KLF4 and pGL3-basic was used as a negative control. The promoter activity was measured and expressed as described in B. (D) Chromatin was extracted from SNU387, SNU423, and PLC/PRF/5 cells. ChIP assays were performed using a specific anti-KLF4 antibody or IgG as a negative control and oligonucleotides flanking the Smad7 promoter regions containing putative KLF4-binding sites. Chromatin fragments without IgG or the antibody were used as input controls. (E) SNU387 cells were transfected with 4 μg pcDNA3.1 vector plus 50nM control siRNA (pcDNA3.1+siCtrl), 4 μg pFLAG-KLF4 vector plus 50 nM control siRNA (pKLF4+siCtrl), 4 μg pFLAG-KLF4 vector plus 50 nM Smad7 siRNA (pKLF4+siSmad7) in six-well plates. Forty-eight hours after transfection cells were treated with or without TGF-β1 (3 ng/ml) for 3 hr as indicated and total protein lysates were prepared for Western blot analysis.
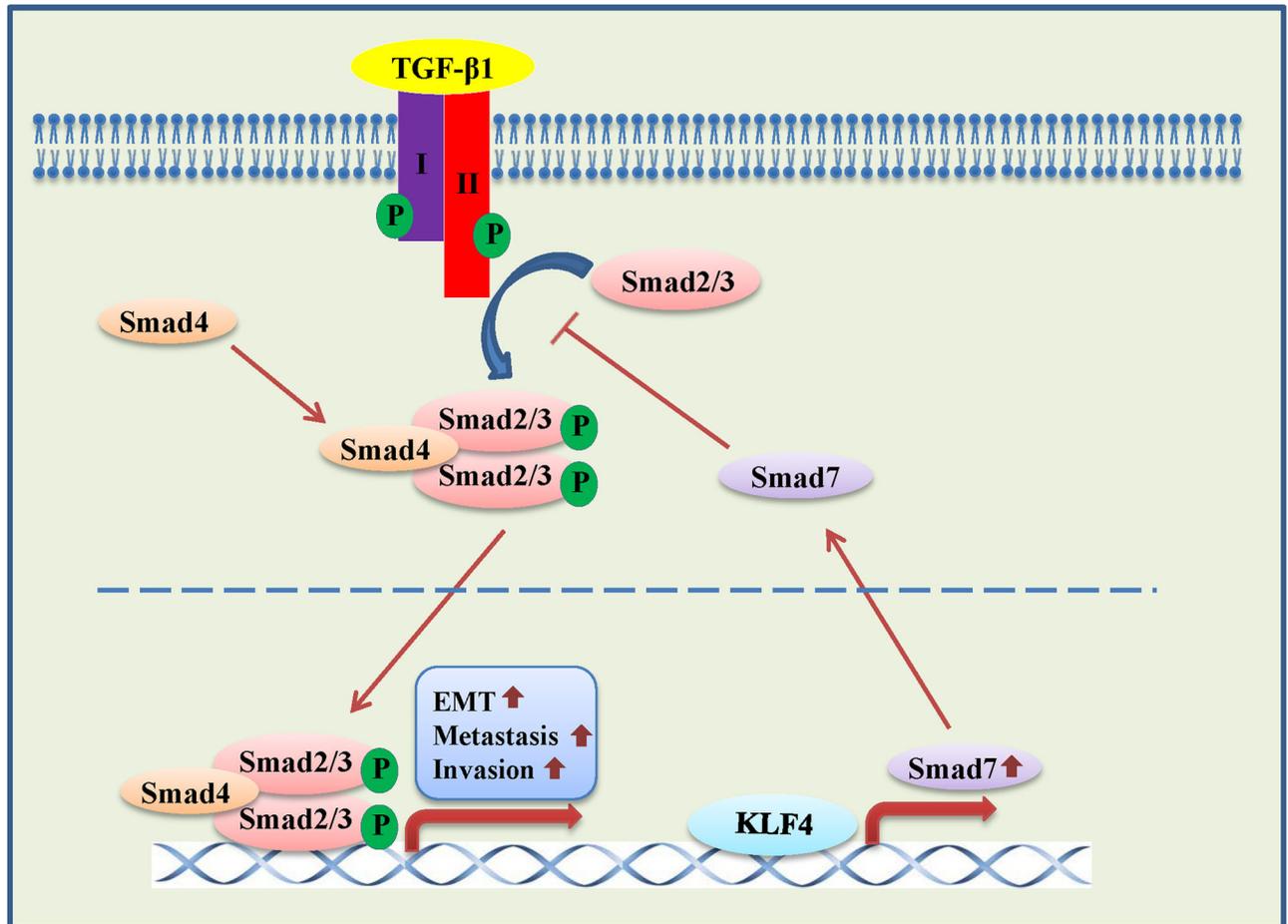
Figure 7. Loss of KLF4 expression mediates activation of TGF-β/Smad signaling in human HCC
In HCC cells with KLF4 expression, KLF4 protein could transcriptionally upregulate Smad7 expression, which subsequently attenuates phophorylation and nuclear translocation of Smad2/Smad3, attenuating oncogenic TGF-β signaling. In contrast, in HCC cells with KLF4 deficiency, loss of KLF4 results in lower Smad7 expression and subsequent enhanced phosphorylation and nuclear translocation of Smad2/Smad3 under TGF-β1 stimulation, leading to hyperactivation of TGF-β signaling and promotion of EMT, invasion, and metastasis of HCC cells.