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USF1 prompt melanoma through upregulating TGF-β signaling pathway

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Abstract. – OBJECTIVE: The activation of TGF-β signaling contributes to abnormal EMT process and upstream stimulatory family1 (USF1) was recently found to activate the expression of TGF-β. However, the specific role of USF1 in melanoma has never been explored.

MATERIALS AND METHODS: The expression of USF1 was analyzed using real-time PCR and Western blot. The changes of cell morphology were observed under a microscope. Cell migration was determined using *in vitro* scratch test. A specific siRNA was applied to knockdown of USF1.

RESULTS: The mRNA and protein levels of USF1 were significantly enhanced in melanoma cell lines, 1205-Lu, DO4, WM3211, and WM278, compared with normal human melanocytes PIG1. Overexpression of USF1 induced demonstrated an elongated and spindle-shaped morphology in the 1205-Lu cells. Meanwhile, USF1 induced the expression of α-SMA, Vimentin and Fibronectin, while the epithelial marker, E-cadherin (E-cad), was significantly decreased. Furthermore, in vitro scratch test demonstrated that overexpression of USF1 markedly enhanced 1205-Lu cell migration capacity at 24 h and 48 h. More importantly, knockdown of USF1 could partially reverse TGF-β1-treatment-induced changes of EMT markers as well as cell morphological changes.

CONCLUSIONS: We first demonstrate that overexpression of USF1 prompts the EMT process through the accumulation of TGF-β1 production in melanoma cells.

Key Words:
Melanoma, TGF-β signaling.

Introduction

Melanoma is one of the most life-threatening malignancies, which is characterized by a poor prognosis in its advanced stages[1]. It is estimated that melanoma accounts for 80% of skin cancer deaths[2]. However, the specific mechanism underlying melanoma development and progression has not been fully elucidated.

The major cause of melanoma mortality belongs to the fact that the tumor cells can experience widespread dissemination to distant sites[3,4]. But the process of metastasis remains controversial. Studies[5] have suggested that epithelial-mesenchymal transition (EMT) is a necessary prerequisite for tumor metastasis. At present, many investigations[6,7] have been performed to explore the mechanisms of EMT due to the clinical importance of metastasis. Accumulating evidence has shown that TGF-β signaling pathway plays a key role in promoting EMT, thereby contributing to increased tumor invasion[8,9].

The upstream stimulatory family (USF) of transcription factors belongs to the basic helix-loop-helix leucine zipper family[10,11]. A highly conserved COOH-terminal domain is identified on the structure of USF, which is responsible for dimerization and DNA binding[12]. In the livers, USFs have been found to widely modulate gene expression, including L-PK, S-14, fatty acid lipase, and osteopontin[13]. In breast cancer cell lines, the loss of USF was found to favor rapid cell proliferation[14]. Moreover, in the kidney and livers, an E-box motif (CANNTG or CACGTG) was identified in the promoter region of the TGF-β1 gene, which significantly contributes to the fibrosis of both organs[13,14]. However, the specific role of USF in melanoma has not been explored. In the present study, we explored the expression pattern of USF in samples of melanoma patients, to investigate the role of USF on the development and progression of melanoma. We hypothesized that upregulation of USF1 would contribute to TGF-β1-induced EMT process in the transition of melanoma.

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Materials and Methods

Cell Culture

1205-Lu melanoma cells were purchased from Coriell Institute of Medical Research (Camden, NJ, USA). The DO4, WM3211, and WM278 melanoma cell lines and normal human melanocytes were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium. Normal human melanocytes PIG1 were purchased from Lifeline Cell Technology and grown in LL-0027 medium (Lifeline Cell Technology, Walkersville, MD, USA). All cultures were supplemented with 10 % fetal bovine serum (FBS), streptomycin (100 mg/ml) and penicillin (100 IU/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

Transient Transfection

Cells were seeded at 10^6 cells/well in the 6-well plates. Meanwhile, the siRNA or a non-specific siRNA (NC) were mixed with HiperFect transfection reagent (Qiagen, Hilden, Germany) and incubated at room temperature for 10 min. Then, the complex was added into the culture medium for 48 h.

RNA Extraction and Real-time PCR

The total RNA from cultured cells was isolated with TriZol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA was reverse transcribed into complementary DNA (cDNA) with TaqMan RNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). A quantitative real-time PCR assay was performed using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a BIO-RAD iCycleriQ real-time PCR detection system as previously described[15]. The primers used were listed as follows:

Protein Extraction and Western Blot Analysis

Proteins samples were extracted in radioimmunoprecipitation assay (RIPA) buffer (1% TritonX-100, 15 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, China) supplemented with a protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and, then, separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to a PVDF membrane. After soaking with 8% milk in phosphate buffered saline and Tween (PBST; pH 7.5) for 2 h at room temperature, the membranes were incubated with the following primary antibodies: anti-Smad2, anti-E-cadherin (E-cad), α-SMA, Fibronectin (FN), Vimentin (Vi), and anti-GAPDH (Cell Signaling, Danvers, MA, USA). Immunodetection was performed by enhanced chemiluminescence detection system (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The house-keeping gene GAPDH was used as the internal control.

Migration Assay

Cell migration was identified using in vitro scratch assays. Firstly, cells were cultured at 10^5 cells/well in 12-well plates for 24 h. Then, a pipette tip was applied to create an artificial gap in the confluent cell monolayer. After transfection with ad-USF1 or ad-NC for 48 h, the cells were washed with pre-warmed PBS for three times to remove the debris. The initial images of the scratch (0 h) and final images of the scratch (48 h) were taken with Olympus 1X51 inverted microscope (Olympus, Tokyo, Japan). The distance of migration and area covered by migrating cells were analyzed quantitatively from the acquired images by using Olympus cellS-ens® digital imaging software and ImageJ software, respectively.

Statistical Analysis

Data were presented as mean ± SD from 3 independent experiments or 5 mice. Statistical analysis was carried out with Student's *t*-test. $p < 0.05$ was considered as statistically significant.

Results

Upregulation of USF1 in Melanoma Cells

First, we explored the expression of USF1 in melanoma cells. Real-time PCR analysis demonstrated that the mRNA and protein levels of USF1 were significantly enhanced in melanoma cell lines, 1205-Lu, DO4, WM3211, and WM278, compared with normal human melanocytes PIG1 (Figure 1A and 1B).

USF1 Enhances Epithelial-Mesenchymal Transition (EMT) in 1205-Lu Cells

Then, we explored the possible effect of USF1 on the process of EMT in 1205-Lu cells, which

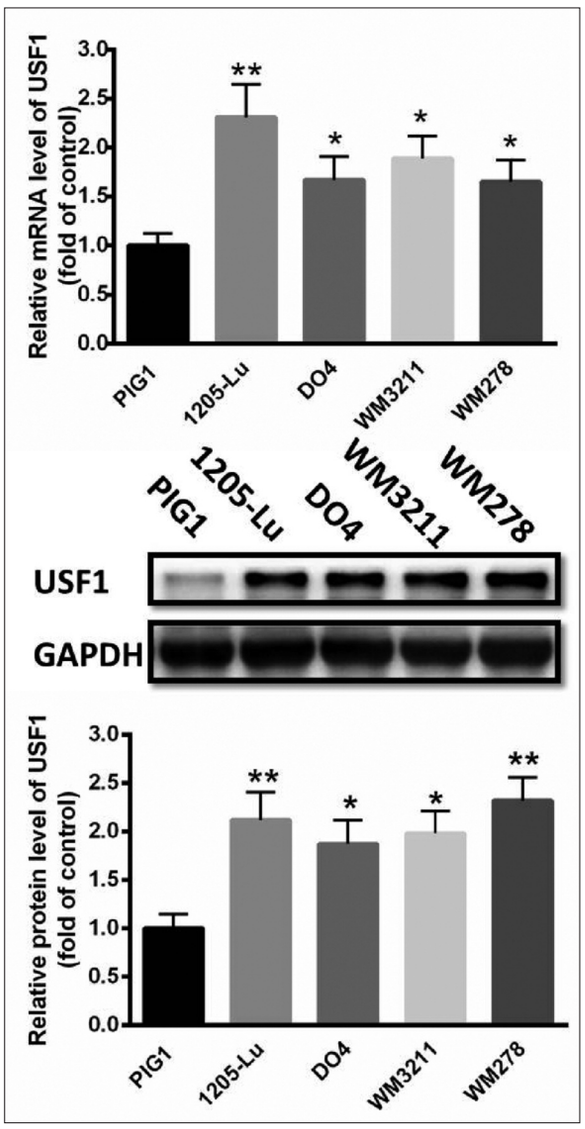

Figure 1. The level of USF1 was increased in melanoma cells compared with normal human melanocytes. ***A,*** Real time PCR analysis of USF1 in melanoma cells. ***B,*** Western blot analysis of USF1 expression in melanoma cells compared with normal human melanocytes. n = three independent experiments, *$p < 0.05$, **$p < 0.01$, *vs*. PIG1.

plays a key role in cancer cell migration. As shown in Figure 2A, the 1205-Lu cells demonstrated an elongated and spindle-shaped morphology after transfection with ad-USF1 for 48 h. Meanwhile, Western blot analysis showed that overexpression of USF1 induced the expression of mesenchymal markers, including α-SMA, Vimentin and Fibronectin, while the epithelial marker, E-cadherin (E-cad), was significantly decreased (Figure 2B). Furthermore, *in vitro* scratch test demonstrated that overexpression of USF1 markedly enhanced 1205-Lu cell migration capacity at 24 h and 48 h, respectively (Figure 2C).

USF1 Stimulates the Expression of TGF-β1 in 1205-Lu Cells

The previous study has shown that USF1 transcriptional activates the expression of USF1 in the livers and kidney. Then, ad-USF1 was transfected into 1205-Lu cells for 48 h. Western blot analysis showed that overexpression of USF1 significantly enhanced the expression TGF-β1 (Figure 3A). Meanwhile, the downstream effectors, including Smad2 andα-SMA, were also upregulated (Figure 3A). In contrast, the silence of USF1 with a specific siRNA significantly suppressed the expression of TGF-β1 as well as the downstream effectors, Smad2 andα-SMA (Figure 3B). These data suggested that USF1 could stimulate the expression of TGF-β1 and the downstream signaling in 1205-Lu cells.

Silence of USF1 Partially Abolishes TGF-β1-Induced EMT Process in 1205-Lu cells

To further determine whether USF1 prompts the EMT process in 1205-Lu cells through stimulating TGF-β1 expression, we treated 1205-Lu cells with si-USF1, TGF-β1 alone or both. As shown previously, the silence of USF1 significantly suppressed the TGF-β1 signaling pathway. In comparison, treatment with TGF-β1 markedly activated the TGF-β1 signaling pathway (Figure 4A). Interestingly, knockdown of USF1 could partially reverse TGF-β1-treatment-induced changes of EMT markers (Figure 4A). Moreover, we also determined the morphological changes of 1205-Lu cells. As shown in Figure 4B, TGF-β1-induced cell morphological changes could be partially reversed by knockdown of USF1. These data indicated that USF1 prompted 1205-Lu cell malignancies mainly by stimulating the expression of TGF-β1.

Discussion

Skin cancer is one of the most common human cancers. Although it can be early detected and treated, the incidence is still increasing[16]. TGF-β signaling plays a key role in tissue homeostasis and cancer progression[17]. It was reported that activation of TGF-β signaling

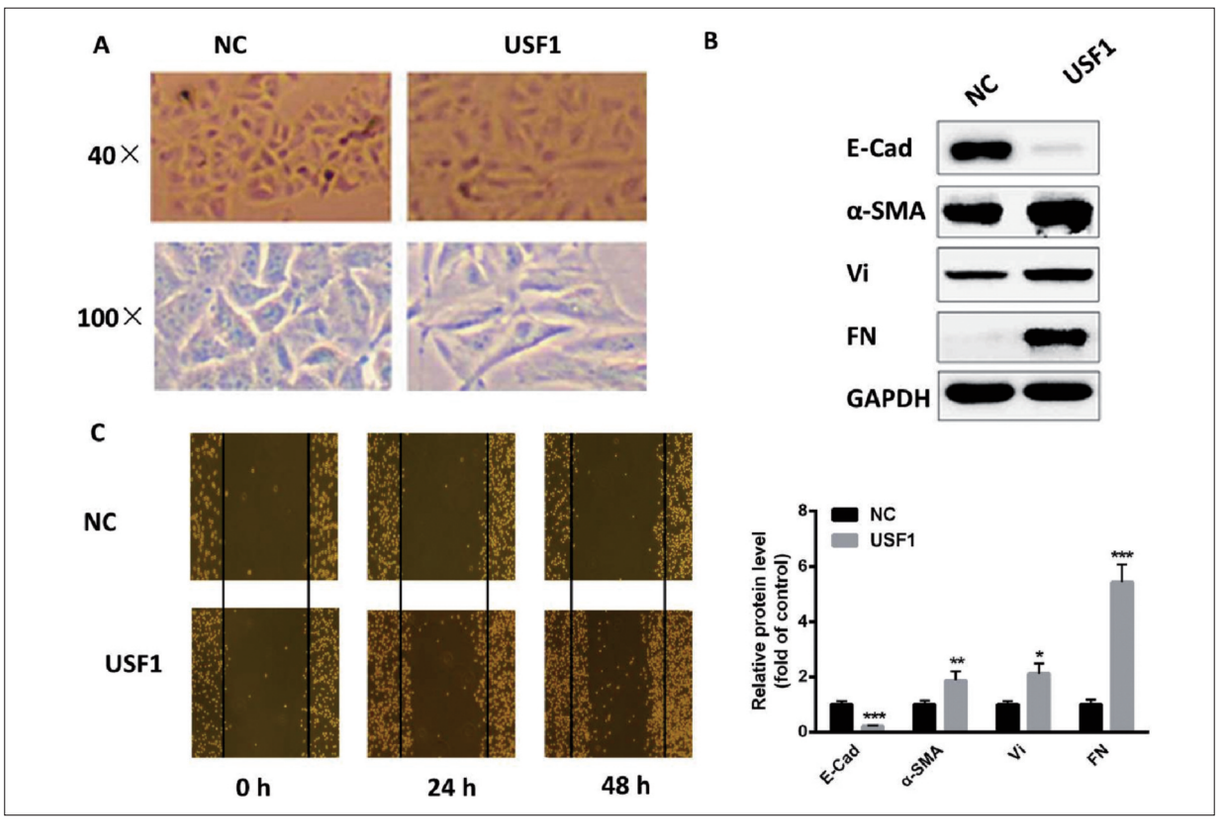

Figure 2. USF1 enhances EMT in 1205-Lu cells. ***A,*** The 1205-Lu cells demonstrated an elongated and spindle-shaped morphology after transfection with ad-USF1 for 48 h. ***B,*** The changes of EMT markers after transfection with ad-USF1 were analyzed using western blot analysis. ***A,*** In vitro scratch test demonstrated that overexpression of USF1 markedly enhanced 1205-Lu cell migration capacity at 24 h and 48 h, respectively. n = three independent experiments, $*p < 0.05$, $**p < 0.01$, *vs*. PIG1.

contributes to abnormal EMT process[8]. Recently, it was reported that a ubiquitously expressed transcription factor, USF1, was found to bind an E-box motif (CANNTG or CACGTG) in the promoter region of the TGF-β gene[18,19]. However, the specific role of USF1 in melanoma has never been explored.

In the present work, we first demonstrated that USF1 was significantly upregulated in melanoma cells compared with normal human melanocytes. Further study reveals that in human melanoma cells, overexpression of USF1 enhanced the expression of TGF-β1, which was associated with cell migration, loss of E-cad and activation of Smad2 and α-SMA. Correlated with these observations, transfection with ad-USF1 induced the morphological changes of human melanoma cells. TGF-β1 is considered to be the primary driver of EMT process and tumor progression. Interestingly, current studies have demonstrated that the TGF-β1 production could be markedly induced by USF1[20,21]. Here, our data was in line with previous researches and first determined that USF1 could activate TGF-β1 production and the downstream signaling.

The TGF-β-SMAD pathway enhances melanoma progression by controlling different stages in the process of cancer cell metastasis, including epithelial-to-mesenchymal transition (EMT)[22,23]. Abnormal EMT transformation enhances tumor cells migration from the primary site into circulation[24,25]. Several key regulators are implicated in the process of EMT, including Smad2, Snail, Slug, ZEB2 and FOXC2[26-28]. The previous study has shown that overactivation of TGF-β-SMAD2 signaling suppresses the expression of E-cadherin via controlling DNA methylation[25,27]. Furthermore, enhanced TGF-β expression was reported to induce vessel invasion, metastasis, advanced tumor stages and shorter survival times in patient with melanoma[6,29]. In contrast, the suppression of TGF-β signaling was effective for the suppression of cell migration in the mouse model[30-32]. Here, we showed that up-

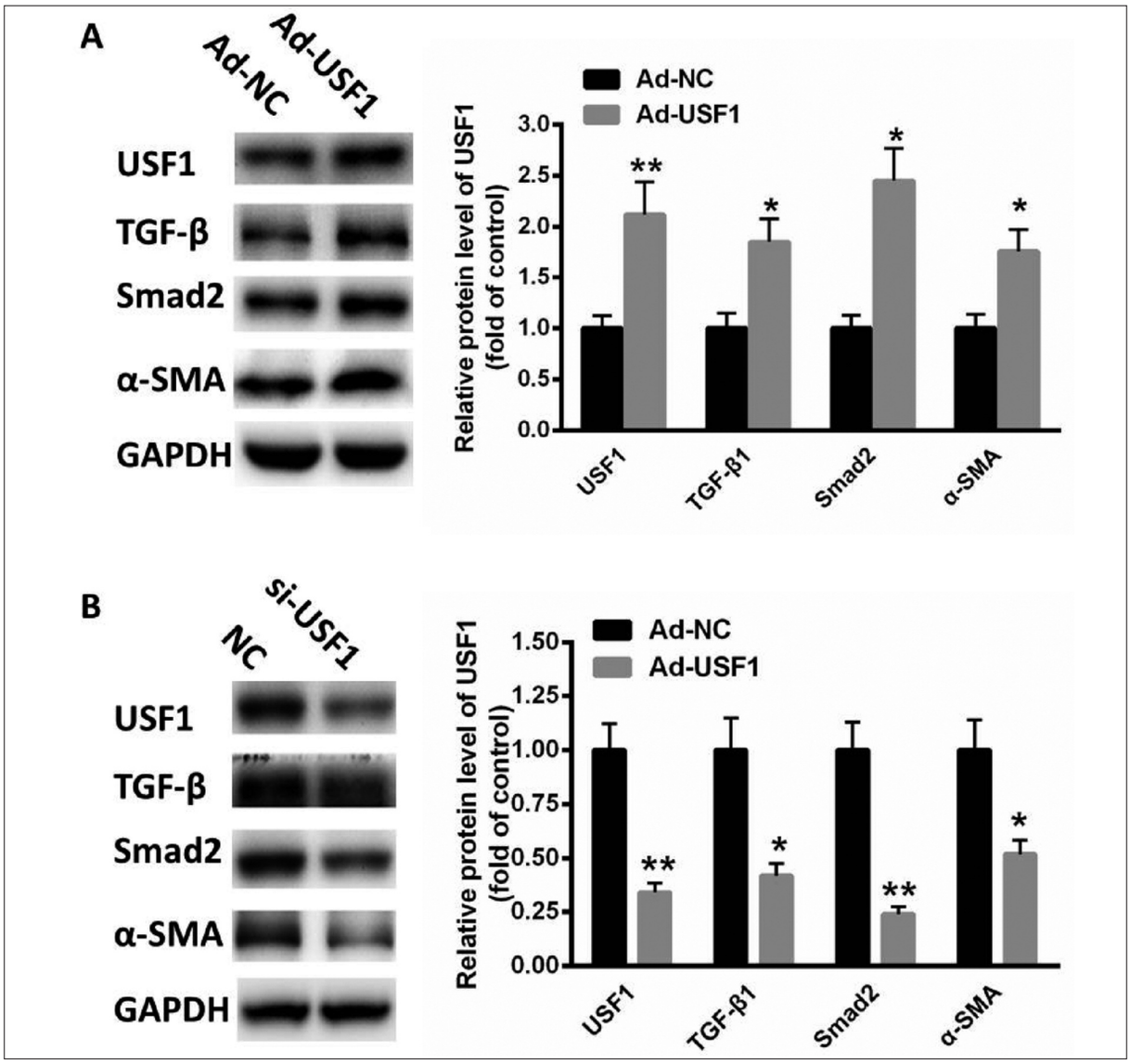

Figure 3. USF1 stimulates the expression of TGF-β1 in 1205-Lu cells. ***A,*** Western blot analysis showed that overexpression of USF1 significantly enhanced the expression TGF-β1 and the downstream signaling pathway. ***B,*** Silence of USF1 with a specific siRNA significantly suppressed the expression of TGF-β1 as well as the downstream effectors, Smad2 andα-SMA. n=three independent experiments, *$p < 0.05$, **$p < 0.01$, *vs*. PIG1.

regulation of USF1 enhanced melanoma cell migration through increasing EMT process mainly by stimulating TGF-β1 expression.

Conclusions

In summary, we first demonstrate that USF1 is of major importance in mediating the stimulation of TGF-β1 in melanoma cells. Overexpression of USF1 prompts the EMT process through the accumulation of TGF-β1 production.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Hintsala HR, Haapasaari KM, Soini Y, Karihtala P. An immunohistochemical study of NFE2L2, KEAP1 and 8-hydroxy-2'-deoxyguanosine and the EMT markers SNAI2, ZEB1 and TWIST1 in metastatic melanoma. Histol Histopathol 2016: 11778.

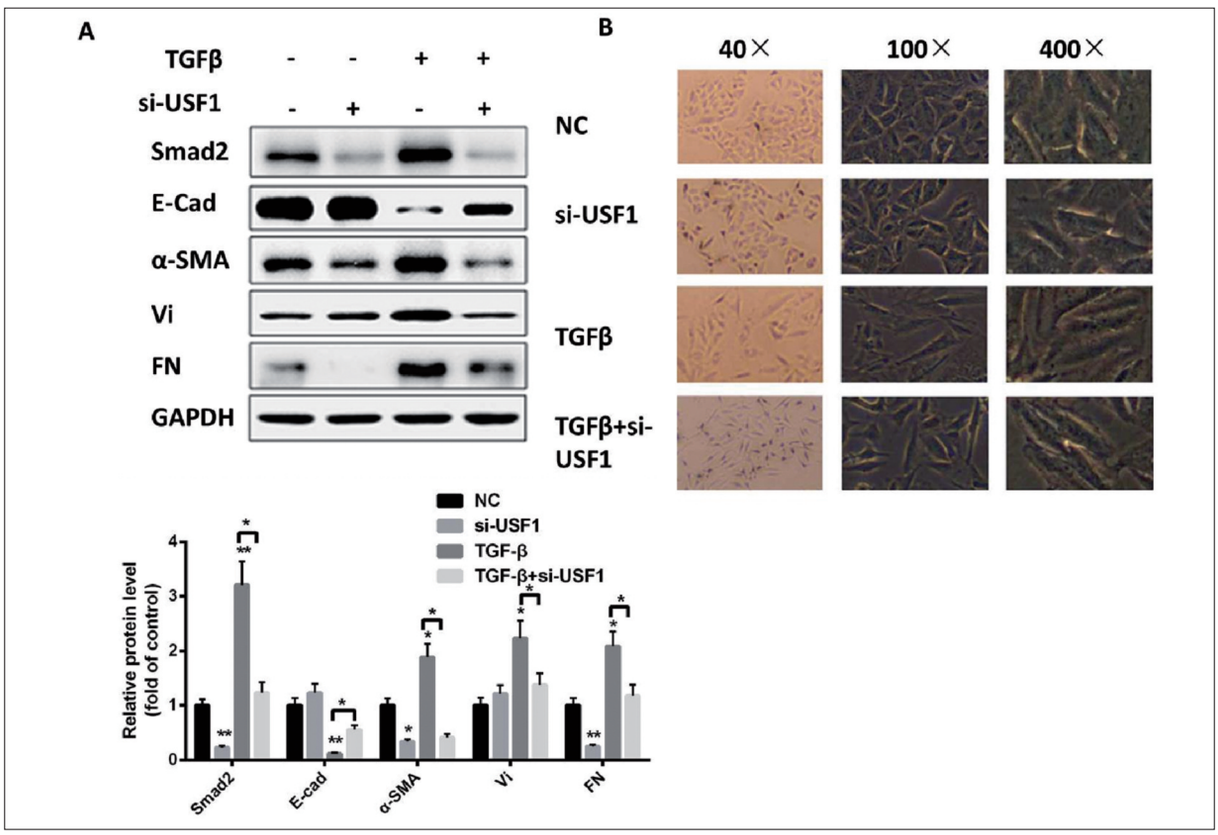

Figure 4. Silence of USF1 partially abolishes TGF-β1-induced EMT process in 1205-Lu cells. ***A,*** Western blot analysis of TGF-β1 signaling pathway when 1205-Lu cells were treated with si-USF1, TGF-β1 alone or both. ***B,*** The morphological changes of 1205-Lu cells were determined in 1205-Lu cells were treated with si-USF1, TGF-β1 alone or both. n = three independent experiments, $*p < 0.05$, $**p < 0.01$, *vs*. PIG1.

2) Asnaghi L, Gezgin G, Tripathy A, Handa JT, Merbs SL, van der Velden PA, Jager MJ, Harbour JW, Eberhart CG. EMT-associated factors promote invasive properties of uveal melanoma cells. Mol Vis 2015; 21: 919-929.

3) Caramel J, Papadogeorgakis E, Hill L, Browne GJ, Richard G, Wierinckx A, Saldanha G, Osborne J, Hutchinson P, Tse G, Lachuer J, Puisieux A, Pringle JH, Ansieau S, Tulchinsky E. A switch in the expression of embryonic EMT-inducers drives the development of malignant melanoma. Cancer Cell 2013; 24: 466-480.

4) Fenouille N, Tichet M, Dufies M, Pottier A, Mogha A, Soo JK, Rocchi S, Mallavialle A, Galibert MD, Khammari A, Lacour JP, Ballotti R, Deckert M, Tartare-Deckert S. The epithelial-mesenchymal transition (EMT) regulatory factor SLUG (SNAI2) is a downstream target of SPARC and AKT in promoting melanoma cell invasion. PLoS One 2012; 7: e40378.

5) Jiang GM, Xie WY, Wang HS, Du J, Wu BP, Xu W, Liu HF, Xiao P, Liu ZG, Li HY, Liu SQ, Yin WJ, Zhang QG, Liang JP, Huang HJ. Curcumin combined with FAPalphac vaccine elicits effective antitumor response by targeting indolamine-2,3-dioxygenase and inhibiting EMT induced by TNF-alpha in melanoma. Oncotarget 2015; 6: 25932-25942.

6) Laurenzana A, Biagioni A, Bianchini F, Peppicelli S, Chillà A, Margheri F, Luciani C, Pimpinelli N, Del Rosso M, Calorini L, Fibbi G. Inhibition of uPAR-TGFbeta crosstalk blocks MSC-dependent EMT in melanoma cells. J Mol Med (Berl) 2015; 93: 783-794.

7) Lin K, Baritaki S, Militello L, Malaponte G, Bevelacqua Y, Bonavida B. The role of B-RAF mutations in melanoma and the induction of EMT via dysregulation of the NF-κB/Snail/RKIP/PTEN circuit. Genes Cancer 2010; 1: 409-420.

8) Schlegel NC, von Planta A, Widmer DS, Dummer R, Christofori G. PI3K signalling is required for a TGFbeta-induced epithelial-mesenchymal-like transition (EMT-like) in human melanoma cells. Exp Dermatol 2015; 24: 22-28.

9) Tulchinsky E, Pringle JH, Caramel J, Ansieau S. Plasticity of melanoma and EMT-TF reprogramming. Oncotarget 2014; 5: 1-2.

10) Laurila PP, Soronen J, Kooijman S, Forsström S, Boon MR, Surakka I, Kaiharju E, Coomans CP, Van Den Berg SA, Autio A, Sarin AP, Kettunen J, Tikkanen

E, Manninen T, Metso J, Silvennoinen R, Merikanto K, Ruuth M, Perttilä J, Mäkelä A, Isomi A, Tuomainen AM, Tikka A, Ramadan UA, Seppälä I, Lehtimäki T, Eriksson J, Havulinna A, Jula A, Karhunen PJ, Salomaa V, Perola M, Ehnholm C, Lee-Rueckert M, Van Eck M, Roivainen A, Taskinen MR, Peltonen L, Mervaala E, Jalanko A, Hohtola E, Olkkonen VM, Ripatti S, Kovanen PT, Rensen PC, Suomalainen A, Jauhiainen M. USF1 deficiency activates brown adipose tissue and improves cardiometabolic health. Sci Transl Med 2016; 8: 323ra313.

11) Hung CC, Kuo CW, Wang WH, Chang TH, Chang PJ, Chang LK, Liu ST. Transcriptional activation of Epstein-Barr virus BRLF1 by USF1 and Rta. J Gen Virol 2015; 96: 2855-2866.

12) Fujimi TJ, Aruga J. Upstream stimulatory factors, USF1 and USF2 are differentially expressed during Xenopus embryonic development. Gene Expr Patterns 2008; 8: 376-381.

13) Chen H, Lu S, Zhou J, Bai Z, Fu H, Xu X, Yang S, Jiao B, Sun Y. An integrated approach for the identification of USF1-centered transcriptional regulatory networks during liver regeneration. Biochim Biophys Acta 2014; 1839: 415-423.

14) Ismail PM, Lu T, Sawadogo M. Loss of USF transcriptional activity in breast cancer cell lines. Oncogene 1999; 18: 5582-5591.

15) Guo J, Li M, Meng X, Sui J, Dou L, Tang W, Huang X, Man Y, Wang S, Li J. MiR-291b-3p induces apoptosis in liver cell line NCTC1469 by reducing the level of RNA-binding protein HuR. Cell Physiol Biochem 2014; 33: 810-822.

16) Tang MR, Wang YX, Guo S, Han SY, Li HH, Jin SF. Prognostic significance of in situ and plasma levels of transforming growth factor beta1, -2 and -3 in cutaneous melanoma. Mol Med Rep 2015; 11: 4508-4512.

17) Westphal P, Mauch C, Florin A, Czerwitzki J, Olligschläger N, Wodtke C, Schüle R, Büttner R, Friedrichs N. Enhanced FHL2 and TGF-beta1 expression is associated with invasive growth and poor survival in malignant melanomas. Am J Clin Pathol 2015; 143: 248-256; quiz 307.

18) Sanchez AP, Sharma K. Transcription factors in the pathogenesis of diabetic nephropathy. Expert Rev Mol Med 2009; 11: e13.

19) Zhu Y, Casado M, Vaulont S, Sharma K. Role of upstream stimulatory factors in regulation of renal transforming growth factor-beta1. Diabetes 2005; 54: 1976-1984.

20) Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ, Natarajan R. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. Proc Natl Acad Sci U S A 2007; 104: 3432-3437.

21) Kim KS, Jung HS, Chung YJ, Jung TS, Jang HW, Lee MS, Kim KW, Chung JH. Overexpression of USF increases TGF-beta1 protein levels, but G1 phase arrest was not induced in FRTL-5 cells. J Korean Med Sci 2008; 23: 870-876.

22) Pelak MJ, Snietura M, Lange D, Nikiel B, Pecka KM. The prognostic significance of indoleamine-2,3-dioxygenase and the receptors for transforming growth factor beta and interferon gamma in metastatic lymph nodes in malignant melanoma. Pol J Pathol 2015; 66: 376-382.

23) Cantelli G, Orgaz JL, Rodriguez-Hernandez I, Karagiannis P, Maiques O, Matias-Guiu X, Nestle FO, Marti RM, Karagiannis SN, Sanz-Moreno V. TGF-beta-induced transcription sustains amoeboid melanoma migration and dissemination. Curr Biol 2015; 25: 2899-2914.

24) Xin Z, Zhang W, Xu A, Zhang L, Yan T, Li Z, Wu X, Zhu X, Ma J, Li K, Li H, Liu Y. Polymorphisms in the potential functional regions of the TGF-beta 1 and TGF-beta receptor genes and disease susceptibility in HBV-related hepatocellular carcinoma patients. Mol Carcinog 2012; 51 Suppl 1: E123-131.

25) Yang G, Liang Y, Zheng T, Song R, Wang J, Shi H, Sun B, Xie C, Li Y, Han J, Pan S, Lan Y, Liu X, Zhu M, Wang Y, Liu L. FCN2 inhibits epithelial-mesenchymal transition-induced metastasis of hepatocellular carcinoma via TGF-beta/Smad signaling. Cancer Lett 2016; 378: 80-86.

26) Serova M, Tijeras-Raballand A, Dos Santos C, Albuquerque M, Paradis V, Neuzillet C, Benhadji KA, Raymond E, Faivre S, de Gramont A. Effects of TGF-beta signalling inhibition with galunisertib (LY2157299) in hepatocellular carcinoma models and in ex vivo whole tumor tissue samples from patients. Oncotarget 2015; 6: 21614-21627.

27) Wu J, Han J, Hou B, Deng C, Wu H, Shen L. Sulforaphane inhibits TGF-beta-induced epithelial-mesenchymal transition of hepatocellular carcinoma cells via the reactive oxygen species-dependent pathway. Oncol Rep 2016; 35: 2977-2983.

28) Song S, Yuan P, Wu H, Chen J, Fu J, Li P, Lu J, Wei W. Dendritic cells with an increased PD-L1 by TGF-beta induce T cell anergy for the cytotoxicity of hepatocellular carcinoma cells. Int Immunopharmacol 2014; 20: 117-123.

29) Wang HX, Sharma C, Knoblich K, Granter SR, Hemler ME. EWI-2 negatively regulates TGF-beta signaling leading to altered melanoma growth and metastasis. Cell Res 2015; 25: 370-385.

30) Teraoka H, Sawada T, Yamashita Y, Nakata B, Ohira M, Ishikawa T, Nishino H, Hirakawa K. TGF-beta1 promotes liver metastasis of pancreatic cancer by modulating the capacity of cellular invasion. Int J Oncol 2001; 19: 709-715.

31) Li Y, Chen D, Hao FY, Zhang KJ. Targeting TGF-β1 and AKT signal on growth and metastasis of anaplastic thyroid cancer cell in vivo. Eur Rev Med Pharmacol Sci 2016; 20: 2581-2587.

32) Xu YB, Jiang W, Zhao FR, Li G, Du QH, Zhang MY, Guo XG. Propofol suppresses invasion and induces apoptosis of osteosarcoma cell in vitro via downregulation of TGF-β1 expression. Eur Rev Med Pharmacol Sci 2016; 20: 1430-1435.