

Transforming Growth Factor- β -Induced RBFOX3 Inhibition Promotes Epithelial-Mesenchymal Transition of Lung Cancer Cells

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The RNA-binding protein Rbfox3 is a well-known splicing regulator that is used as a marker for post-mitotic neurons in various vertebrate species. Although recent studies indicate a variable expression of Rbfox3 in non-neuronal tissues, including lung tissue, its cellular function in lung cancer remains largely unknown. Here, we report that the number of RBFOX3-positive cells in tumorous lung tissue is lower than that in normal lung tissue. As the transforming growth factor- β (TGF- β) signaling pathway is important in cancer progression, we investigated its role in RBFOX3 expression in A549 lung adenocarcinoma cells. TGF- β 1 treatment inhibited RBFOX3 expression at the transcriptional level. Further, RBFOX3 depletion led to a change in the expression levels of a subset of proteins related to epithelial-mesenchymal transition (EMT), such as E-cadherin and Claudin-1, during TGF- β 1-induced EMT. In immunofluorescence microscopic analysis, mesenchymal morphology was more prominent in RBFOX3-depleted cells than in control cells. These findings show that TGF- β -induced RBFOX3 inhibition plays an important role in EMT and propose a novel role for RBFOX3 in cancer progression.

INTRODUCTION

RNA-binding proteins (RBPs) play functionally important roles in cellular homeostasis by controlling the expression of genes at various post-transcriptional regulatory checkpoints (Glisovic et al., 2008). The RNA-binding Fox (RBFOX) family of proteins is known to play prominent roles in the regulation of alternative

splicing (AS) of pre-mRNA (Arya et al., 2014; Kuroyanagi, 2009). Each member of the RBFOX family shows divergent expression patterns. RBFOX1 is expressed in the central nervous system (CNS), heart, and skeletal muscle, while RBFOX3 is exclusively expressed in post-mitotic neurons of the CNS, and RBFOX2 is extensively expressed in various cell and tissue types (Jin et al., 2003; Kim et al., 2009; 2011). Studies on molecular and cellular functions of RBFOX3 have revealed that it is important for neural tissue development and physiological function of the adult brain (Kim et al., 2013; 2014; Wang et al., 2015). Moreover, recent genetic studies have implicated RBFOX3 in numerous neurological dysfunctions in humans, such as development of autistic features, epilepsy, cognitive impairments, and neurodevelopmental delay (Amin et al., 2016; Lal et al., 2013; Wang et al., 2015). Our discovery that RBFOX3 is a neuronal nuclear antigen (NeuN) is significant (Kim et al., 2009). NeuN has been widely used to detect post-mitotic neurons in the CNS of a variety of mammalian species, with a few exceptions such as Purkinje cells in the cerebellum and mitral cells in the olfactory bulb. Furthermore, RBFOX3 has also been detected in non-neuronal cells such as the lung cancer cell lines and non-neural tissues (Langenfeld et al., 2013; Shuangshoti et al., 2005), but its influence on cellular homeostasis in non-neuronal cell types is largely unknown.

Epithelial-mesenchymal transition (EMT) is the process by which cells gain the ability to migrate into neighboring tissue and lose their epithelial phenotype (Huber et al., 2005; Lee et al., 2006; Tarin et al., 2005; Thiery, 2002; Tse and Kalluri, 2007). EMT is thought to be a critical step in metastasis of cancer cells. Loss of E-cadherin has been identified as a characteristic feature of epithelial cells undergoing EMT. Transforming growth factor- β (TGF- β) is a multi-functional cytokine that induces EMT in human epithelial cells through stepwise stimulation of multiple feedback loops (Xu et al., 2009). Additionally, TGF- β signaling regulates proliferation and differentiation of cells depending on cell types and cellular contexts. TGF- β signaling is critically involved in cancer progression in various tissues (de Caestecker et al., 2000; Jakowlew, 2006). Deregulation of TGF- β -signaling pathway can result in tumor development. Although previous studies have shown that EMT-associated AS is regulated by several RBPs including RBFOX2 (Braeutigam et al., 2014; Shapiro et al., 2011), the roles of RBFOX in TGF- β -induced EMT have not been investigated comprehensively.

In this study, we focused on the role of RBFOX3 in non-

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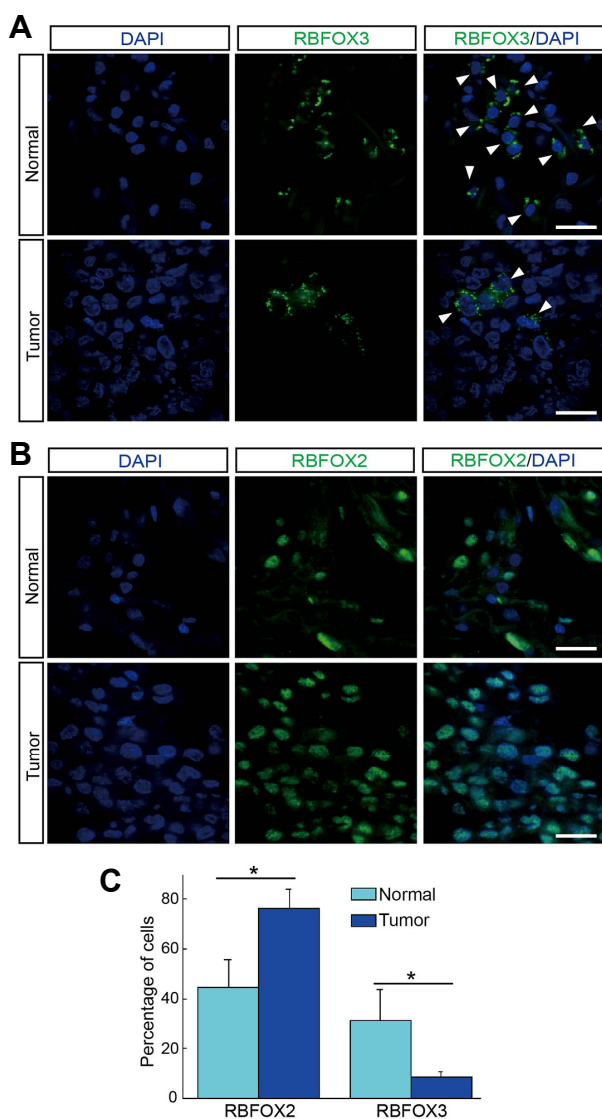


Fig. 1. Differential expression of RBFOX3 in human lung tissue. (A) Representative immunofluorescence images showing RBFOX3 immunoreactivity (green) in samples from normal human lung and lung tumor. Arrowheads indicate RBFOX3-positive cells. (B) Representative immunofluorescence images showing RBFOX2 immunoreactivity (green) in samples from normal human lung and lung tumor. DAPI (blue) was used to stain nuclei. Scale bars indicate 50 μ m. (C) Quantification of Rbfox-positive cells in samples from normal human lung and lung tumor (mean values \pm SD, 3 images/section). * P < 0.05 (two-sided t test).

neuronal cell types. We detected cells possessing cytoplasmic RBFOX3 in normal lung tissue and found that the number of RBFOX3-positive cells in tumorous lung tissue was lower than that in normal lung tissue. Further, we also report that RBFOX3 expression is inhibited during TGF- β -induced EMT, thereby promoting EMT. Our results demonstrate a novel mechanism by which RBFOX3 plays an important role in TGF- β -induced EMT through post-transcriptional regulation of a subset of EMT-related genes.

MATERIALS AND METHODS

Reagents and culturing of cells

A549 human lung adenocarcinoma cells were purchased from American Tissue Culture Collection (USA). They were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA) at 37°C in a humid 5% CO₂ atmosphere. Neutralizing anti-TGF- β antibody (1D11) and human TGF- β 1 (R&D Systems, USA) were supplemented into the culture medium at concentrations of 25 μ g/ml and 5 ng/ml, respectively. Transfection was performed using the PolyMAG Magnetofection kit (Chemiceil GmbH, Germany) according to the manufacturer's instructions.

Plasmid constructs

The expression construct encoding myc-tagged human RBFOX3 in the pCMV6 plasmid was obtained from Origene (RC224826, USA). For cloning and expression of clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas9 (CRISPR-Cas9) of *RBFOX3*, the human *RBFOX3* mRNA sequence (NM_001082575.2) was used to design the sgRNA sequence using the CRISPR design tool (<http://crispr.mit.edu>). The designed sgRNA sequence was 5'-CGA ACA TTT GCC GCA AGT CG-3'. For expression of the CRISPR/Cas9 components, a modified one-vector system pX330 (Addgene, USA) with Sleeping Beauty inverted terminal repeats flanking sgRNA and Cas9 expression cassette was used (CRISPR-SB) (PMID26508638). The sgRNA oligonucleotides 5'-CAC CCG AAC ATT TGC CGC AAG TCG-3' and 5'-AAA CCG ACT TGC GGC AAA TGT TCG-3' were annealed for cloning into BbsI-digested CRISPR-SB vector (PMID 24157548).

RNA preparation and RT-PCR

Total RNA was isolated using TRIzol reagent (ThermoFisher Scientific) and reverse-transcribed using SuperScript III First-Strand Synthesis System (ThermoFisher Scientific) using random hexamers. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using FastStart Universal SYBR green Master Premix (Roche, USA) on the AriaMx Real-Time PCR system (Agilent Technologies, USA). The PCR primers used in this study were 5'-CCA TGG AGA AGG CTG GGG-3' and 5'-CAA AGT TGT CAT GGA TGA CC-3' for *GAPDH* and 5'-CCA GGC TCC GAG GCC AGC ACA C-3' and 5'-TGT AGG GTC GGA GGG GTG GAG-3' for *RBFOX3* mRNA. The specificity of products generated by each set of primers was examined by agarose gel electrophoresis and was further confirmed by sequence analysis.

Preparation of extracts and immunoblot analysis

A549 cells were washed in cold phosphate-buffered saline (PBS) and proteins were extracted using M-PER protein extraction reagent (ThermoFisher Scientific), supplemented with a protease inhibitor mixture (Roche). Protein samples (25 μ g per well), denatured and reduced by sodium dodecyl sulfate (SDS) and β -mercaptoethanol, respectively, were separated on 10% (Fig. 3) or 4-12% (Fig. 2) SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk (NFD) prepared in PBS. Further, they were incubated with primary antibodies diluted in the blocking solution overnight at 4°C. The membranes were washed thrice with PBS. The signal obtained by the binding of horseradish peroxidase-conjugated secondary antibodies (Abcam, USA) was detected by the SuperSignal system

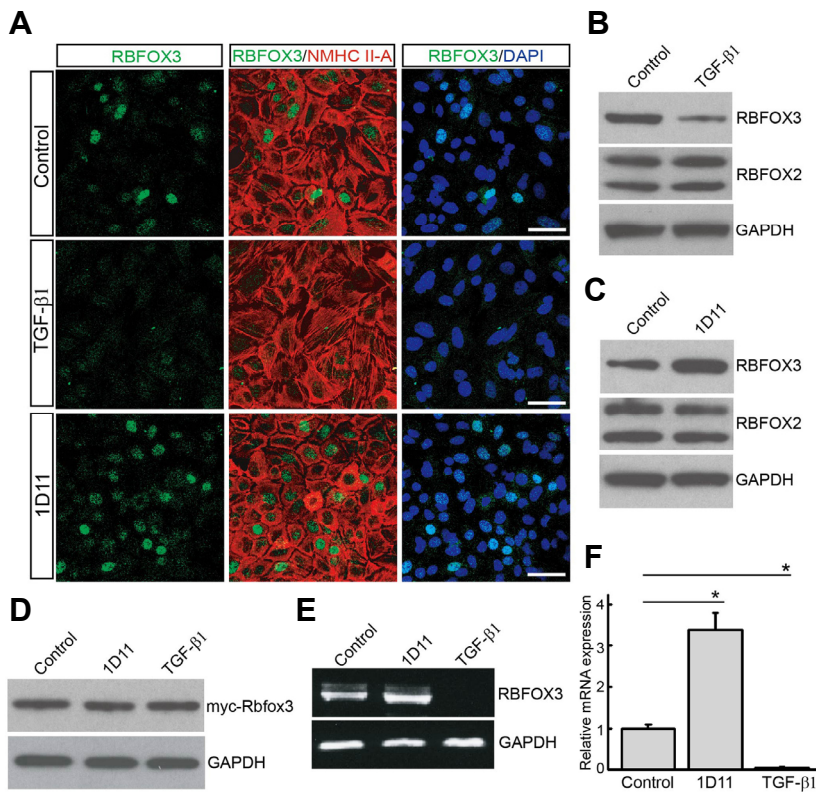


Fig. 2. Transcriptional repression of RBFOX3 by TGF- β 1 treatment. (A) TGF- β 1 treatment decreased RBFOX3 expression. A549 cells were treated with TGF- β 1 (5 ng/ml) and 1D11 (25 μ g/ml) for 48 h. The fixed and permeabilized cells were immunostained with anti-RBFOX3 (green) or anti-NMHC II-A (red). DAPI (blue) was used to stain nuclei. Scale bars indicate 50 μ m. (B, C) Immunoblot analysis of A549 cell lysates for determining expression of RBFOX2 and RBFOX3 after treatment with TGF- β 1 (5 ng/ml) and 1D11 (25 μ g/ml) for 48 h. (D) Immunoblot analysis of lysates of Myc-tagged RBFOX3-expressing A549 cells after treatment with TGF- β 1 (5 ng/ml) and 1D11 (25 μ g/ml) for 48 h. (E-F) TGF- β 1 treatment decreased the expression of RBFOX3 at the transcriptional level. A549 cells were treated with TGF- β 1 (5 ng/ml) and 1D11 (25 μ g/ml) for 48 h. Total RNA was analyzed by quantitative real-time PCR to determine RBFOX3 mRNA level, which was then normalized to that of GAPDH. Mean values \pm SD (error bars) are shown for three independent experiments. * P < 0.05 (two-sided t -test).

(ThermoFisher Scientific). Band intensities were quantified using NIH ImageJ software. The primary antibodies recognized MYC (ThermoFisher Scientific), EMT-related proteins (Cell Signaling, USA), RBFOX3 (EMD Millipore, Germany), RBFOX2 (Bethyl Laboratories, USA), NMHC II-A (Covance, USA), and GAPDH (Meridian Life Science, USA).

Immunofluorescence microscopy

A549 cells were fixed using 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 prepared in PBS for 15 min. Frozen sections of normal and tumorous lung tissues were purchased from Biochain (USA). The sections were stained with the following primary antibodies: NMHC II-A (Covance), Vimentin (Cell Signaling), RBFOX2 (Bethyl Laboratories), and RBFOX3 (EMD Millipore). Alexa Fluor 488- and 532-conjugated goat antibodies against mouse and rabbit IgG (ThermoFisher Scientific), respectively, were used as secondary antibodies. Nuclear DNA was stained with 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Images were captured by an LSM 510 Live Configuration Vario Two VRGB confocal laser-scanning microscope (Carl Zeiss).

RESULTS AND DISCUSSION

Number of RBFOX3-positive cells was lower in tumorous lung tissue than in normal lung tissue

We have previously reported the neural tissue-specific expression patterns of Rbfox3 in mouse tissues (Kim et al., 2011). However, several groups have detected RBFOX3-positive cells in non-neural tissues, including human lung tissue (Langenfeld

et al., 2013; Shuangshoti et al., 2005). To investigate whether human lung tissue contains RBFOX3-positive cells, we examined normal and tumorous lung tissue samples using anti-RBFOX3 antibody and DAPI, a DNA-binding stain. About 30% of cells observed in the defined field showed RBFOX3 expression in the normal lung tissue (Figs. 1A and 1C). Moreover, significantly fewer RBFOX3-positive cells were observed in the tumorous lung tissue (Figs. 1A and 1C). In contrast to the above result, RBFOX2-positive cells were significantly more in the tumorous tissue than in the normal lung tissue (Figs. 1B and 1C). Interestingly, while RBFOX2 showed strong nuclear localization (Fig. 1B), RBFOX3 showed strong cytoplasmic localization in both normal and tumorous lung tissues, suggesting that it is involved in cytoplasmic gene regulation in the lung tissue (Fig. 1A). Recent studies report that the function of the Rbfox family members is not restricted to regulating AS in the nucleus, but they also perform roles in the cytoplasm (Carreira-Rosario et al., 2016; Lee et al., 2016). For instance, cytoplasmic Rbfox has been reported to regulate mRNA stability and translation by binding to the 3'-UTRs of target RNAs, in some cases, by competing with miRNAs (Lee et al., 2016). Therefore, it will be interesting to examine the importance of the cytoplasmic function of RBFOX3 in gene regulation in the lung tissue.

RBFOX3 transcription is down-regulated by TGF- β 1 treatment

The observation that the number RBFOX3-positive cells is lower in tumorous lung tissue prompted us to investigate the molecular mechanisms involved in RBFOX3 downregulation. First, the RBFOX3 expression in A549 human lung adenocarc

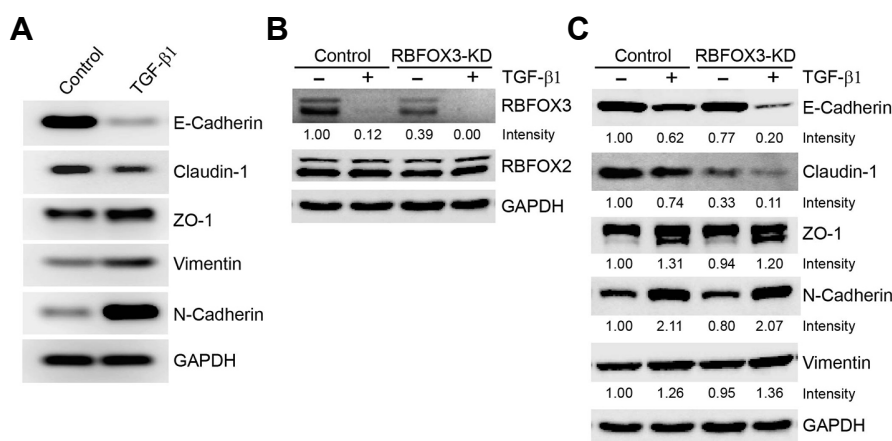


Fig. 3. Changes in the expression of EMT-related proteins following RBFOX3-depletion. (A) TGF- β 1 treatment induced EMT in A549 cells. A549 cells were treated with TGF- β 1 (5 ng/ml) for 48 h, and then cell lysates were subjected to immunoblot analysis for the indicated proteins. (B, C) Immunoblot analysis of EMT-related proteins. Construct of CRISPR-Cas9 for RBFOX3-KD were transfected into A549 cells. At 24 h after transfection, cells were treated with TGF- β 1 (5 ng/ml) for 48 h. The cell lysates were subjected to immunoblot analysis for the indicated proteins. The values under the gels denote relative intensities of the protein bands.

cinoma cells was examined. Consistent with a previous report, immunofluorescence microscopy using anti-RBFOX3 revealed that RBFOX3 is expressed in A549 cells and localizes to the nucleus (Fig. 2A). However, as revealed from the staining pattern, RBFOX3 showed nuclear localization in A549 cells, whereas it showed cytoplasmic localization in human lung tissue. This discrepancy could be due to the differences in AS isoforms or cellular environments. Because the TGF- β signaling pathway plays a pivotal role in diverse cellular processes, including cancer progression, its effect on RBFOX3 expression was examined. TGF- β 1 treatment led to a significant reduction in RBFOX3-positive cells, whereas inhibition of TGF- β 1 by 1D11 treatment resulted in an increase in RBFOX3-positive cells (Fig. 2A). In addition, TGF- β 1 treatment induced morphological changes in the cells, which were observed by staining the cytoskeletal protein NMHC II-A. Further, immunoblot analyses with anti-RBFOX3 antibody demonstrated that RBFOX3 is inhibited or activated by TGF- β 1 treatment or 1D11 treatment, respectively (Figs. 2B and 2C). However, the expression level of RBFOX2 remained unaffected. The stability of RBFOX3 was examined by forced expression of myc-tagged RBFOX3 after treatment with TGF- β 1 or 1D11. There was no change in stability of the myc-tagged RBFOX3 protein (Fig. 2D). Further, to understand the mechanism by which TGF- β 1 inhibits RBFOX3 expression, the mRNA expression pattern of RBFOX3 after treatment with TGF- β 1 or 1D11 was determined. The results were similar to those obtained for RBFOX3 protein regulation by TGF- β signaling; the mRNA levels decreased or increased following treatment with TGF- β 1 or 1D11, respectively (Figs. 2E and 2F). These results suggest that TGF- β signaling regulates RBFOX3 expression at the transcriptional level. How does TGF- β signaling regulate RBFOX3 expression at the transcriptional level? Since Smad proteins lie at the core of the TGF- β signaling pathway (Massague et al., 2005), further studies are required to confirm which Smads are involved in TGF- β -mediated RBFOX3 inhibition. Because RBFOX3 is preferentially expressed in neural tissues, future studies should focus on elucidating the mechanism by which TGF- β signaling regulates RBFOX3 expression in neurons.

EMT is stimulated in RBFOX3-depleted cells

TGF- β signaling is known to play an important role in EMT, and treatment with TGF- β is a convenient way to induce EMT in many epithelial cells (Xu et al., 2009). To study the induction of

EMT by TGF- β 1 treatment, the expression levels of EMT-related proteins in A549 cells were examined. TGF- β 1 treatment decreased the expression of E-cadherin and Claudin-1, whereas it increased the expression of ZO-1, Vimentin, and N-Cadherin (Fig. 3A). To investigate the involvement of RBFOX3 in EMT, endogenous RBFOX3 protein was depleted using the CRISPR-Cas9 system. Transient CRISPR-Cas9 application showed 60% efficiency for the knockdown of endogenous RBFOX3 (Fig. 3B). TGF- β 1 decreased RBFOX3 expression in both control and RBFOX3 knockdown (RBFOX3-KD) cells, whereas no significant effect was observed on RBFOX2 expression. The expression of EMT-related proteins was examined using immunoblot analysis. The expression of epithelium-specific E-cadherin and Claudin-1 was downregulated in RBFOX3-KD cells (Fig. 3C). Moreover, the reduction was more prominent in RBFOX3-KD after TGF- β 1 treatment. However, there were no significant changes in the levels of proteins encoded by mesenchyme-specific genes in RBFOX3-KD cells (Fig. 3C). These results suggest that RBFOX3-depletion downregulates epithelium-specific genes in EMT.

RBFOX3-depletion stimulates morphological changes in EMT phenotype

The observation that RBFOX3-KD promotes downregulation of epithelium-specific genes in EMT prompted us to investigate the effect of RBFOX3-KD on morphological changes induced in EMT phenotype. Immunofluorescence microscopy using anti-NMHC II-A antibody revealed that TGF- β 1 treatment induces cytoskeletal rearrangement. We observed that the thicker cytoskeletal fibers running parallel to the long axis of the RBFOX3-KD cells were more prominent than those running parallel to the long axis of the control cells (Figs. 4A and 4B). In addition, increase in the formation of Vimentin-specific filaments in the absence of TGF- β 1 was clearly observed at the 24 h time point. This increase was further stimulated by TGF- β 1 treatment, suggesting that TGF- β -induced RBFOX3 inhibition promotes EMT (Figs. 4A and 4C). Cells are considered to have undergone EMT following the loss of epithelial marker proteins. We have shown that RBFOX3-depletion downregulates epithelium-specific genes in EMT process. Thus the promotion of EMT by RBFOX3-depletion might be originated from the inhibition of epithelium-specific gene expression. However, future studies will endeavor to understand the mechanism of RBFOX3-mediated regulation of epithelium-specific genes at the molecular

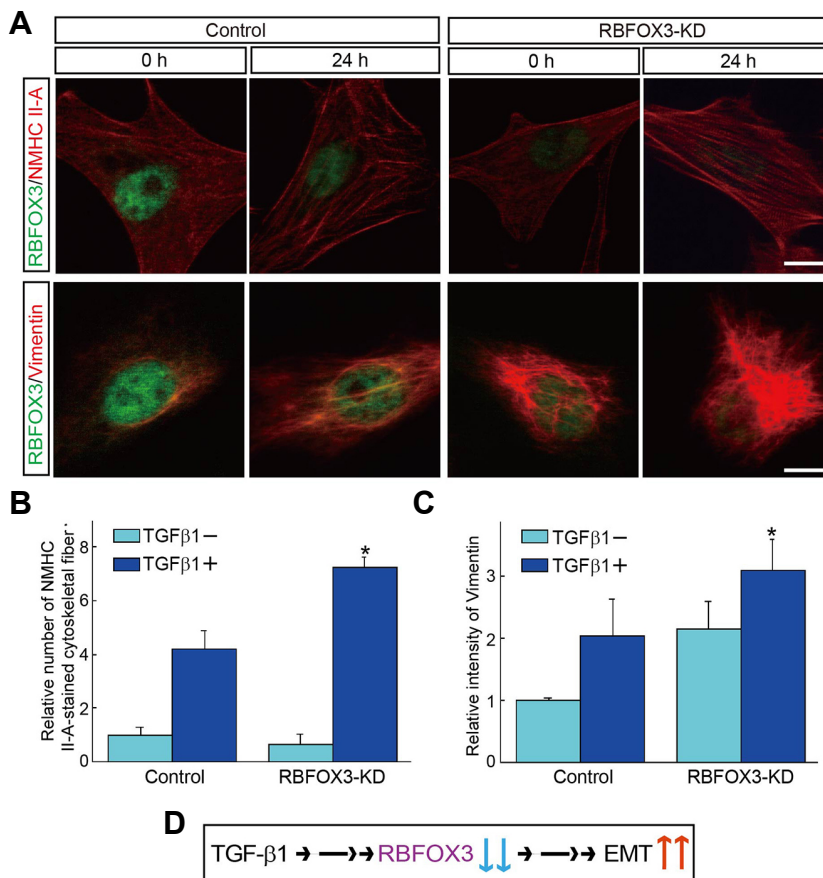


Fig. 4. RBFOX3 depletion-induced morphological changes in EMT phenotype. (A) RBFOX3-KD cells were treated with TGF- β 1 (5 ng/ml) for 24 h. The fixed and permeabilized cells were immunostained for the indicated proteins. Scale bars indicate 10 μ m. (B) Quantification of NMHC II-A-stained cytoskeletal fibers. Each bar represents the number of NMHC II-A-stained cytoskeletal fibers in the cytoplasm (mean values \pm SD, 10 cells). (C) Quantification of Vimentin-stained cytoskeletal fibers. Each bar represents the intensity of Vimentin-stained cytoskeletal fibers in the cytoplasm (mean values \pm SD, 10 cells). * P < 0.05 (two-sided t test) compared with control cells. (D) A model for the RBFOX3-mediated regulation of EMT processing. RBFOX3 expression is transcriptionally inhibited by TGF- β 1. RBFOX3-inhibition stimulates morphological EMT phenotype.

level.

In this study, we report that RBFOX3-positive cells are present in human lung tissue and that their number is decreased in tumorous lung tissue. Several studies report a marked increase in the expression of TGF- β in various cancers, including lung cancer (Gold, 1999). In addition, increased TGF- β expression is associated with more advanced stages of malignancy and metastasis and with decreased cell survival. Therefore, we speculate that the decrease in RBFOX3-positive cells in tumorous lung tissues is brought about by TGF- β signaling, and we confirmed the role of TGF- β signaling in the transcriptional repression of RBFOX3 (Fig. 4D). We also elucidated the biological relevance of TGF- β -induced RBFOX3 regulation in EMT.

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