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P44/WDR77 restricts the sensitivity of proliferating cells to TGFβ signaling

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ABSTRACT

We previously reported that a novel WD-40 domain-containing protein, p44/WDR77, drives quiescent epithelial cells to re-enter the cell cycle and plays an essential role for growth of lung and prostate cancer cells. Transforming growth factor beta (TGFβ) signaling is important in the maintenance of non-transformed cells in the quiescent or slowly cycling stage. However, both non-transformed proliferating cells and human cancer cells are non-responsive to endogenous TGFβ signaling. The mechanism by which proliferating cells become refractory to TGFβ inhibition is not well established. Here, we found that silencing p44/WDR77 increased cellular sensitivity to TGFβ signaling and that this was inversely correlated with decreased cell proliferation. Smad2 or 3 phosphorylation, TGFβ-mediated transcription, and TGFβ2 and TGFβ receptor type II (TβRII) expression were dramatically induced by silencing of p44/WDR77. These data support the hypothesis that p44/WDR77 down-regulates the expression of the TGFβ ligand and its receptor, thereby leading to a cellular non-response to TGFβ signaling. Finally, we found that p44/WDR77 expression was correlated with cell proliferation and decreased TGFβ signaling during lung tumorigenesis. Together, these results suggest that p44/WDR77 expression causes the non-sensitivity of proliferating cells to TGFβ signaling, thereby contributing to cellular proliferation during lung tumorigenesis.

1. Introduction

Transforming growth factor β (TGFβ) binds to and activates the cell surface receptor complex, which is composed of receptor types I (TβRI) and II (TβRII), to phosphorylate receptor-bound Smad (Smad2/3) transcription factors [1,2]. The phosphorylated Smad proteins translocate into the nucleus and regulate TGFβ-responsive genes. In cultured epithelial cells, TGFβs act as potent inhibitors of proliferation due to repression of c-myc transcription and up-regulation of cyclin-dependent kinase inhibitors $p15^{Ink4B}$ and $p21^{Cip}$ to prevent retinoblastoma protein (Rb) inactivation [3–5]. There is compelling evidence that TGFβ signaling plays an important role in the maintenance of non-transformed cells in the generally quiescent or slowly cycling stage [6]. Malignant progression is often associated with resistance to the growth-inhibitory effects of TGFβ [3,7]. Components of the TGFβ signaling pathway are commonly mutated or transcriptionally suppressed in many types of human epithelial tumors [8–10], which results in non-responsiveness of tumor cells to TGFβ signaling-mediated inhibition. Mutations in and functional inactivation of the TGFβ signaling pathway are not enough to explain the broad spectrum of TGFβ non-responsiveness in non-transformed proliferating cells and cancer cells; instead, other mechanisms may exist by which proliferating cells become refractory to the inhibitory functions of endogenous TGFβ.

We purified and cloned a novel androgen receptor-interacting protein, designated p44 [11,12]. P44 is composed of 342 amino acid residues and 7 putative WD-40 repeats; it is named WD Repeat Domain 77 (WDR77) in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/gene/79084). P44 localizes in the cytoplasm of epithelial cells and is required for cell proliferation at the growth stage of prostate development [12–14]. In contrast, in the adult prostate, p44 establishes and maintains luminal epithelia in a growth-arrested fully differentiated state (the G1/G0 cell cycle phase) in the nucleus. P44 translocation from the nucleus to

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the cytoplasm is associated with age-related prostatic intraepithelial hyperplasia and prostate tumorigenesis [12,14]. Cytoplasmic p44 is also required for the proliferation of prostate cancer cells [12,14,15]. Therefore, p44 cytoplasm translocation is a critical event that leads to the proliferation of prostate epithelial cells and prostate tumorigenesis. More recently, we found that p44 was highly expressed in mouse lung epithelial cells at the early stages of development, when cells are proliferating; however, its expression was diminished in adult lung cells [16]. P44 expression was also essential and sufficient for the proliferation of lung epithelial cells. P44 expression is re-activated in lung cancer, and silencing it abolished the growth of lung cancer cells in tissue cultures and lung tumor xenografts in nude mice. The results of these studies demonstrate that p44 plays an essential role in driving cell proliferation.

In the present study, we found that p44 inhibited TGFβ signaling; loss of p44 expression decreased cell growth and greatly enhanced the sensitivity of prostate and lung cancer cells to TGFβ signaling through the up-regulation of TGFβ2 and TβRII expression. We also observed p44 expression during lung tumorigenesis, which was correlated with down-regulation of TGFβ signaling. Given the opposing effects of p44 and TGFβ signaling in cell proliferation, our findings indicate that p44 drives cell proliferation while causing proliferating cells to become non-responsive to TGFβ signaling.

2. Materials and methods

2.1. Lung tumor samples and immunohistochemical analysis of p44, TβRII, and Smad3 expression

Lung tumor samples and immunostaining with anti-p44 (1:1000) [11], -TβRII (Santa Cruz Biotechnology, 1:200), and -Smad3 (Cell Signaling Technology, 1:200) antibodies were described previously [16].

2.2. Cell culture and cell growth assay

A549 cells were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS), 2% vitamins, 1% L-glutamine, 1% non-essential amino acids, and 1% sodium pyruvate. PC3 cells were cultured in RPMI 1640 medium with 10% FBS. For the cell growth assay, cells were plated on 24-well plates (2000 cells/well) and counted every day for 7 days. Human recombinant TGFβ2 was purchased from BioLegend (cat. #583301). Silencing p44 expressing via lentivirus expressing non-target and p44 shRNA were performed as described previously [16].

2.3. Luciferase assay

The luciferase reporter plasmid (pGL3-4xSBE-E4-luc) contains four tandem copies of the Smad binding-element (SBE: GTCTAGAC) upstream of the minimal adenovirus E4 promoter (−38 to +12). Luciferase report assay was performed with PC3 cells as described previously [17–20,12]. Three independent experiments were performed for each transient transfection assay, and the results are presented as the mean ± SD ($n = 3$).

2.4. Western blot and immunohistochemical analysis

Primary antibodies were used at dilutions of 1:1000 (anti-p44), 1:1000 (anti-HSP90β, anti-Myc, anti-c-Jun, anti-actin [Santa Cruz Biotechnology]), or 1:1000 (anti-Smad3 and anti-Smad3-pS425, anti-TGFβ2, anti-TβRII [Cell Signaling Technology]). Western blot and immunostaining of cultured cells were performed as described previously [16]. The relative intensities were quantified using the ImageJ software program (ImageJ64, National Institutes of Health).

2.5. Gene expression profiling

A gene expression profiling analysis was performed on A549 cells expressing NT or p44 shRNA (GSE56757) and reported previously [16]. Gene set enrichment analysis (GSEA) was performed as described [21].

2.6. Statistical analysis

Data are presented as the means of three or more independent experiments ± the standard error of the mean (SEM). A 2-tailed unpaired Student *t*-test was used to determine whether differences between control and experiment samples were statistically significant. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Silencing p44 activates the TGFβ signaling pathway

P44 silencing dramatically inhibited growth of lung (A549 and PC14) and prostate (LNCaP and PC3) cancer cells by decreasing cellular proliferation [14,16,22]. The results of our Western blot analysis indicated that p44 silencing did not significantly affect the protein levels of c-Myc and c-Jun in the nuclei of A549 cells (Fig. 1A), although it slightly increased Smad3 protein expression levels. More significantly, phosphorylated Smad3 (pS425-Smad3) levels were dramatically increased in the nuclei of A549 cells expressing p44 shRNA (Fig. 1A). Smad2 and Smad3 were predominantly located in the cytoplasm in A549 cells (sFigs. 1A and 2A). Silencing p44 enhanced the nuclear translocation of Smad3 (sFig. 1A), Smad2 (sFig. 2A), and Smad4 (sFig. 3). More dramatically, p44 silencing promoted the nuclear translocation of phosphorylated Smad3 (Fig. 1B and sFig. 1B) and Smad2 (sFig. 2B). The subcellular localization of Smad1, Smad5, Smad6, and phosphorylated Smad1/5 was not affected by p44 silencing (sFig. 3). Thus, silencing p44 expression activates TGFβ signaling.

Consistent with these observations, we found that silencing of p44 statistically enriches a set of genes up-regulated by TGFβ (www.broadinstitute.org/gsea/msigdb/cards/TGFB_UP.V1_UP.html) (Fig. 1C). To further confirm the activation of TGFβ signaling, we constructed a Smad luciferase reporter (pGL3-4xSBE-E4-luc). This reporter was transiently transfected into PC3 cells that expressed NT or p44 shRNA and the promoter activity was determined by measuring the luciferase activity. Silencing p44 increased the Smad-reporter activity in a dosage-dependent manner (Fig. 1D). Thus, p44 blocks TGFβ signaling transcriptional responses.

3.2. Silencing p44 enhanced the sensitivity of proliferating cells to TGFβ signaling

In serum-free medium or TGFβ2 concentrations less than 0.01 ng/ml, less amount (gray values <10) of Smad3 localized in the nuclei of A549 cells (Fig. 2, left, 1st to 3rd panels; sFig. 4). We observed significant increase in nuclear Smad3 levels in A549 cells at the high TGFβ2 concentrations (gray values: 39 at 0.1 ng/ml TGFβ2; gray values: 60 at 1 ng/ml TGFβ2) (Fig. 3, left, 4th and 5th panels; sFig. 4). However, significant amount (gray values >30) of Smad3 localized in the nucleus in the A549 cell that expressed p44 shRNA even at low TGFβ2 concentrations (0.001 and 0.01 ng/ml) (Fig. 3, right, 2nd and 3rd panels; sFig. 4). Thus,

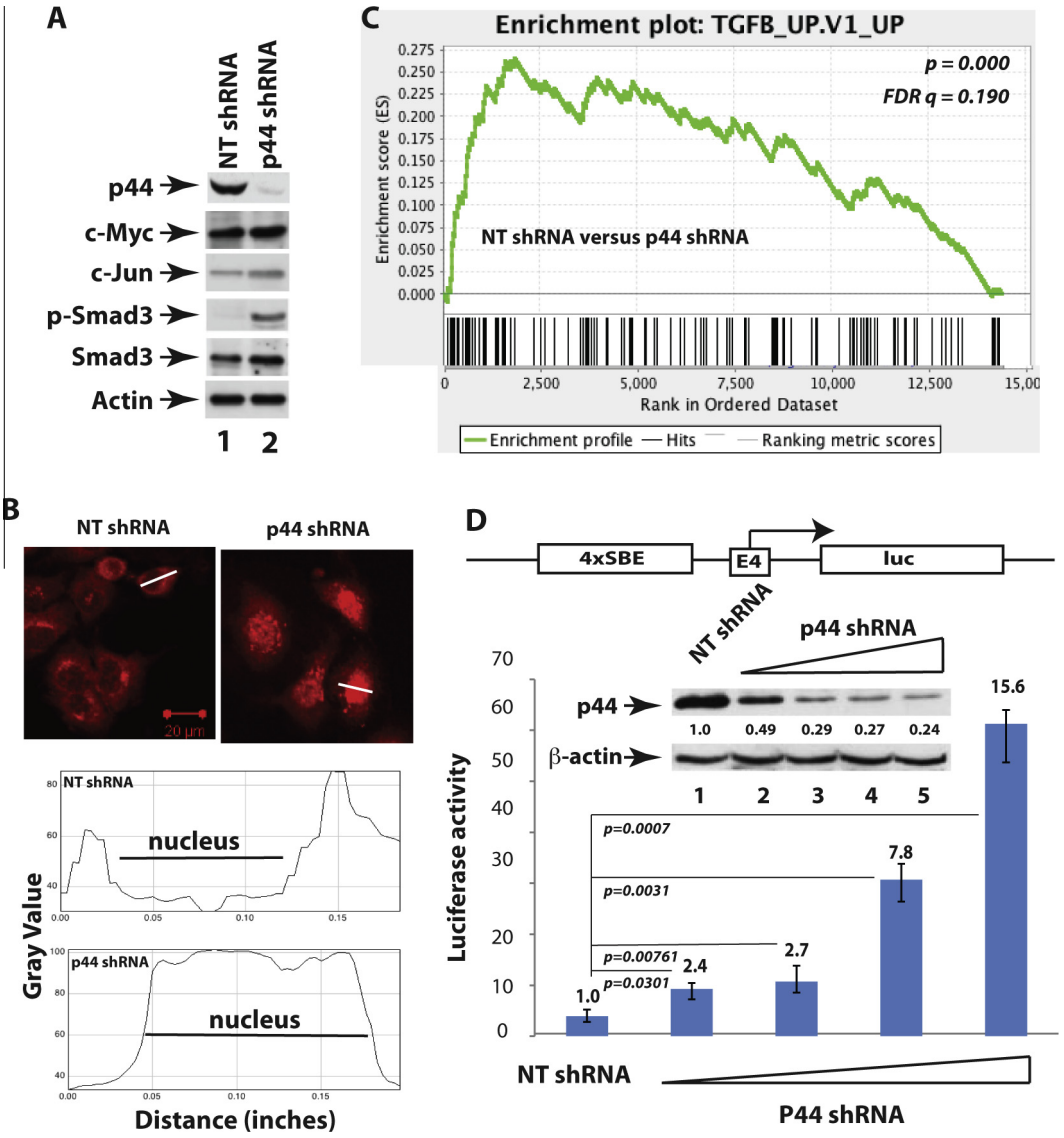

Fig. 1. Silencing p44 expression activated the TGFβ signaling pathway. (A) Western blot analysis of nuclear extracts derived from A549 cells expressing NT shRNA (lane 1) or p44 shRNA (lane 2) with the indicated antibodies. (B) Silencing p44 expression activated Smad3. Immunohistochemical analysis of phosphorylated Smad3 (red) in A549 cells expressing NT or p44 shRNA. The samples were observed under a confocal microscope. The fluorescence intensity (gray value) changes across the white lines were plotted as the line intensities in the histograms (ImageJ64, NIH) on the bottom. (C) GSEA enrichment plot shows that silencing p44 expression increased expression of TGFβ target genes. (D) Silencing p44 expression enhanced the activity of the TGFβ reporter. PC3 cells expressing NT or p44 shRNA were transfected with 200 ng of the reporter plasmid pGL3-4xSBE-E4-luc. The transfected cells were allowed to grow for 48 h and then harvested for the luciferase assay. The values represent the mean ± SD ($n = 3$). Insert, a Western blot analysis of whole cell lysates derived from the transfected cells expressing NT shRNA (lane 1) or p44 shRNA (lanes 2–5) with the indicated antibodies. The relative p44 protein levels in the transfected cells are indicated by the numbers in the middle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

silencing p44 dramatically enhanced the sensitivity of A549 cells to the TGFβ signaling to its ligand.

We previously demonstrated that p44 is required for cell growth and that silencing p44 expression significantly decreased cell proliferation [15,16]. The p44 protein levels in PC3 cells were gradually decreased by infecting cells with increased amounts of lentivirus that expressed p44 shRNA (Fig. 3A). By doing so, we obtained PC3 cells with average growth rates that ranged from 572 to 230 cells per hour (Fig. 3B). These cells were grown in the absence or presence of 0.001, 0.01, 0.1, or 1 ng/ml of recombinant TGFβ2. The sensitivity of PC3 cells to TGFβ2 inhibition was greatly enhanced (up to 105-fold at a concentration of 0.001 ng/ml TGFβ2) by decreasing the cell growth rate (Fig. 3C). Similarly, the decreased growth rate of A549 cells caused by silencing p44 expression (Fig. 3D and E) also resulted in a dramatic increase in cellular sensitivity to TGFβ2 inhibition (Fig. 3F). These experiments directly demonstrated that the growth rate controlled by p44 influences cellular sensitivity to TGFβ signaling.

3.3. Silencing of p44 increased expression of TGFβ2 and TβRII

On DNA microarray analysis (GSE56757) [16], we found enhanced expression of TGFβ2 and TβRII in A549 cells that expressed p44 shRNA (data not shown). An RT-PCR analysis confirmed this observation (sFig. 5). Silencing of p44 expression enhanced the mRNA expression of TGFβ2 and TβRIIV1 by 3.7- and 4.0-fold, respectively. In contrast, TGFβ1 and TβRI expression was not significantly affected by p44 silencing. A Western blot analysis revealed that silencing p44 increased the protein levels of TGFβ2 (1.6-fold for the dimer and 2.4-fold for the monomer) and TβRII (1.4-fold) (Fig. 4A, lane 2 versus lane 1). Thus, p44 regulates the expression of TGFβ2 and TβRII to affect TGFβ signaling.

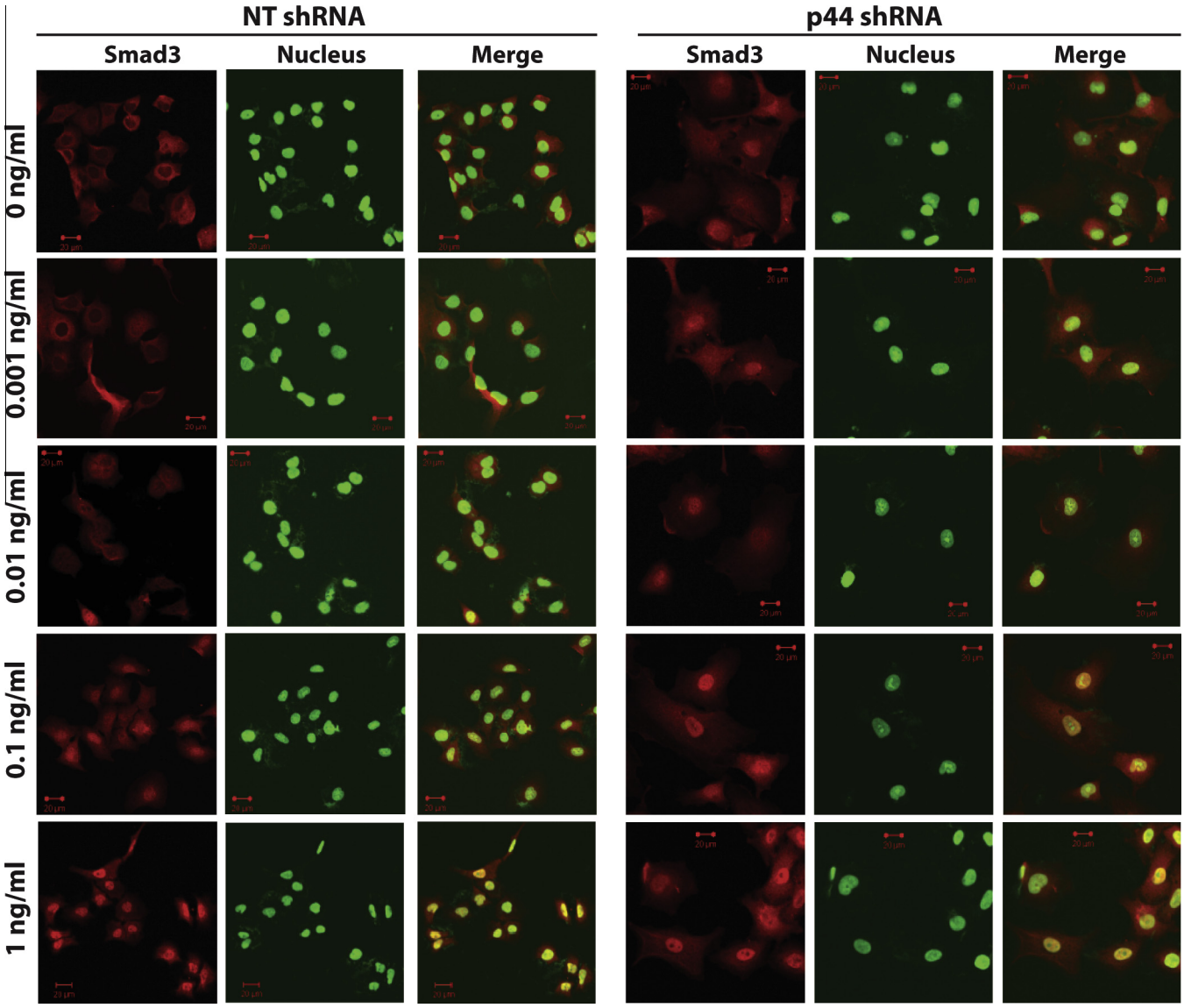

Fig. 2. Silencing p44 expression sensitized A549 cells to TGFβ signaling. NT (left) and p44 (right) shRNA-expressing A549 cells were grown in the absence of FBS for 24 h. Recombinant TGFβ2 was added to the medium at a final concentration of 0, 0.001, 0.01, 0.1, or 1 ng/ml and cultured at 37 °C for 45 min. Cells were evaluated by immunohistochemical analysis of Smad3 (red). The nuclei were contrastained with SYTOX Green (green). The Smad3 staining was merged with the nuclear staining. The samples were observed under a confocal microscope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Inverse correlation between p44 expression and TGFβ signaling during lung tumorigenesis

We found that p44 is expressed during the early stage of lung development, when lung cells are proliferating; its expression is silenced in adult lung tissue [16]. In lung cancer, its expression is re-activated and is required for the growth for lung cancer cells. Consistent with these observations, we detected high p44 expression in the hyperplastic region but no expression in the benign tissue region (Fig. 4B, panel a). TβRII expression was low in lung hyperplastic cells but high in benign cells (Fig. 4B, panel b). Some cells (indicated by red arrows) in the hyperplastic region still expressed high levels of TβRII but they more like benign cells from the appearance of their small sizes of nuclei. We observed Smad3 expression and nuclear localization in benign cells and absent nuclear expression in hyperplastic cells (Fig. 4B, panel c). Thus, p44-mediated down-regulation of TGFβ signaling may play an important role in lung tumorigenesis.

4. Discussion

Cell growth is controlled by negative and positive regulatory signals. TGFβ is a negative regulatory signal and functions as a major growth constraint for non-transformed quiescent cells. P44 represents a positive regulatory signal and is essential and sufficient to drive the growth of quiescent epithelial cells. The results presented here demonstrate that p44 restricts the sensitivity of proliferating cells to TGFβ signaling through down-regulating TβRII and TβR2 expression. Furthermore, we demonstrated that the regulation of TGFβ signaling by p44 might play a critical role in lung tumorigenesis.

Most normal adult cells are fully differentiated and generally quiescent. TGFβ acts as a key physiological factor that ensures the maintenance of cell quiescence [23,24]. Somatic stem cells are also largely quiescent in spite of their considerable proliferative potential [25,26]. In single cell culture assays, it was shown that low, physiological concentrations of TGFβ specifically maintain

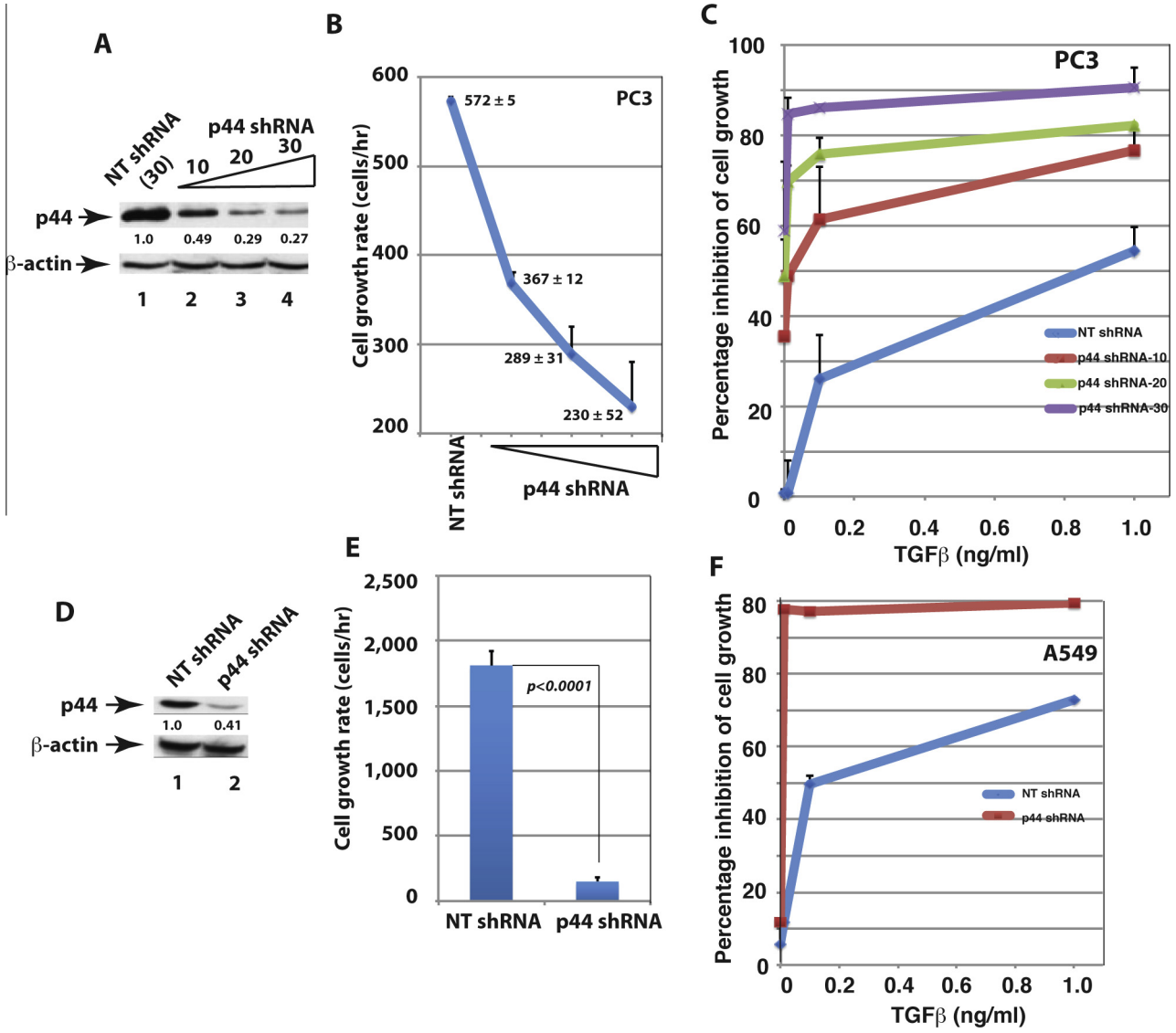

Fig. 3. The cell growth rate is inversely correlated with cellular sensitivity to TGFβ. (A) A Western blot analysis of whole cell lysates from PC3 cells infected with a lentivirus expressing NT shRNA (30 μl) (lane 1) or p44 shRNAs (10, 20, or 30 μl) (lanes 2–4) with anti-p44 (top) or anti-actin (bottom) antibody. The relative levels of p44 protein were indicated between two panels. (B) Silencing p44 expression decreases the cell growth rate. The average growth rate of PC3 cells infected with NT shRNA or p44 shRNA lentivirus in 7 days. (C) The growth rate of PC3 cells was inversely correlated with cellular sensitivity to TGFβ signaling. PC3 cells infected with NT shRNA or p44 shRNA lentivirus were grown in the absence or presence of 0.001, 0.01, 0.1, or 1 ng/ml recombinant TGFβ2 for 7 days. The percentage inhibition of cell growth was plotted against the concentration of TGFβ2. The percentage inhibition of cell growth (=1 – growth rate in the presence of TGFβ2/growth rate in the absence of TGFβ2) was plotted against the concentration of TGFβ2. (D) A Western blot analysis of whole cell lysates from A549 cells infected with NT shRNA (lane 1) or p44 shRNAs (lane 2) lentivirus with anti-p44 or anti-actin antibody. (E) Silencing p44 expression decreases the cell growth rate. (F) The average growth rate of A549 cells in 7 days was inversely correlated with cellular sensitivity to TGFβ signaling. A549 cells infected with NT shRNA or p44 shRNA lentivirus were grown in the absence or presence of 0.001, 0.01, 0.1, or 1 ng/ml recombinant TGFβ2 for 7 days.

primitive human hemopoietic stem or progenitor cells in quiescence [6]. In our previous studies, we demonstrated that cytoplasmic p44 is expressed only in the proliferating epithelial cells, not in quiescent epithelial cells [15,16]. Furthermore, its expression is sufficient to drive the proliferation of quiescent epithelial cells. Here, we demonstrated that p44 expression drives cell growth while dramatically decreasing cells' sensitivity to TGFβ signaling. P44 restricts the sensitivity of TGFβ signaling by suppressing the expression of TGFβ2 and TβRII. These results provide a novel mechanism for the non-responsiveness of proliferating cells to endogenous TGFβ signaling.

Tumorigenesis is also involved in the loss of cellular sensitivity to TGFβ signaling, and most human cancers are resistant to TGFβ-mediated growth inhibition through a variety of mechanisms, including mutations in or functional inactivation of TβRII, decreased expression of TβR1 or TβRII, and inactivating mutations in components of the TGFβ signaling pathway [1]. We observed that p44 expression was associated with lung initiation (hyperplasia) and the loss of expression of TβRII and inactivation of TGFβ signaling. Therefore, abrogation of TGFβ-induced growth inhibition by p44 may provide a mechanism by which human tumors become non-responsive to TGFβ signaling.

TGFβ signaling inhibits the growth of cancer cells, although some cells can escape this inhibition through a mutation of a component of the TGFβ signaling pathway. Consistent with the published observation [27,28], we observed that TGFβ2 inhibited the growth of A549 and PC3 cells by 2.7-fold at a concentration of 1 ng/ml (sFig. 6). Increasing the TGFβ2 concentration to 10 ng/ml did not lead to further inhibition and at concentrations lower than 0.01 ng/ml, TGFβ2 had little effect. Given that plasma active TGFβ

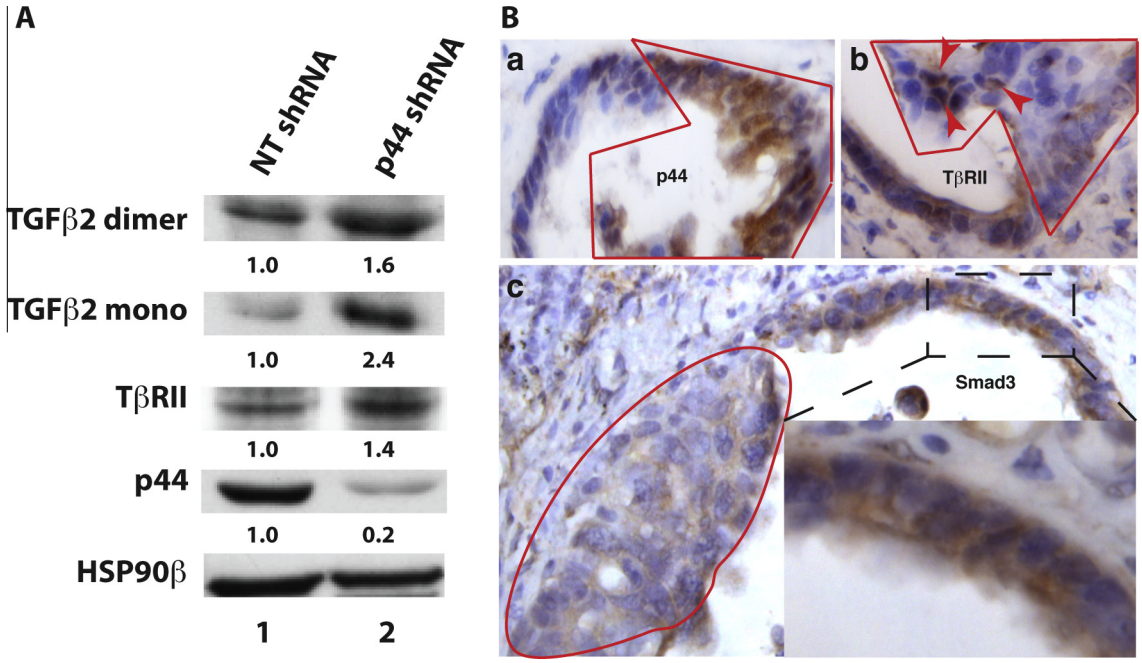

Fig. 4. (A) Silencing p44 expression up-regulated the expression of TGFβ2 and TβRII. (A) Western blot analysis of whole cell lysates (10 μg protein per sample) derived from A549 cells expressing NT (lane 1) or p44 (lane 2) shRNA with the indicated antibodies. The relative protein levels are indicated by the numbers underneath. (B) TGFβ signaling is inactivated in lung hyperplasia. Sections of human lung tissue were immunostained for p44 (a), TβRII (b), and Smad3 (c). The hyperplastic regions are surrounded by red lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

levels are very low (<0.01 ng/ml) [29], TGFβ signaling may not significantly inhibit the growth of prostate and lung cancers *in vivo*. TGFβ induced expression of the $p21^{Cip1}$ gene through the TGFβ-responsive element in the $p21^{Cip1}$ promoter [30] and arrested the cell cycle of epithelial cells at the G1 phase [1]. Silencing p44 expression resulted in the up-regulation of cyclin-dependent kinase inhibitor $p21^{Cip1}$, Rb hypophosphorylation, and G1 cell cycle arrest [12,31,32]. On the basis of these observations, one would expect that p44 regulates cell growth largely through TGFβ signaling. SB431542, a potent and specific inhibitor of the TGFβ signaling pathway, completely abolished p44 silencing-induced TGFβ signaling activation (phosphorylation of Smad3) in the nuclei of A549 cells (sFig. 7A, lane 3 versus lane 2). Inhibition of TGFβ signaling slightly inhibited the growth of A549 cells (sFig. 7B), consistent with the documented role of TGFβ in cell growth through Smad1, 5, or 8. In contrast, blocking TGFβ signaling only slightly but statistically significantly relieved the A549 cell growth inhibition that had been induced by p44 silencing (sFig. 7B). We previously found that the $p21^{Cip1}$ and Rb pathways are also only partially responsible for p44-mediated cell growth regulation [16]. These results indicate that p44 controls cell growth only partially through TGFβ signaling and other signals might contribute to more dramatic down-regulation of cell growth by p44 silencing. Indeed, the expression of multiple oncogenes and tumor suppressors is also regulated by p44 in prostate and lung cancer cells (unpublished observations), which may mainly mediate the p44-driven cell growth. It should be noticed that these conclusions were based on studies using prostate and lung cancer cell lines; p44-regulated TGFβ signaling might contribute more significantly to proliferation of normal epithelial cells *in vivo*.

Multiple lines of evidence suggest that human cancers and non-transformed proliferating cells are generally functionally resistant to TGFβ-induced growth suppression. In the present study, we described how TGFβ growth suppression is abrogated by p44 in proliferating cells. One important question, however, remains unanswered: how does p44 regulate TGFβ2 and TβRII expression? The results of the present study pave the way for the further investigation of the importance of p44 and TGFβ signaling as a regulatory mechanism that controls cell proliferation and tumorigenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.125.

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