Loss of Transforming Growth Factor Beta Type II Receptor Increases Aggressive Tumor Behavior and Reduces Survival in Lung Adenocarcinoma and Squamous Cell Carcinoma

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Abstract

Purpose: Lung adenocarcinoma and lung squamous cell carcinoma (SCC) are the most common non–small cell lung cancer (NSCLC) subtypes. This study was designed to determine whether reduced expression of TGFβ type II receptor (TGFβRII) promotes lung adenocarcinoma and SCC carcinogenesis.

Experimental Design: We examined TGFβRII expression at the protein and mRNA levels in human NSCLC samples and assessed the relationship between TGFβRII expression and clinicopathologic parameters. To determine whether sporadic TGFβRII deletion in airway epithelial cells induces NSCLC formation, we targeted TGFβRII deletion alone and in combination with oncogenic KrasG12D to murine airways using a keratin 5 (K5) promoter and inducible Cre recombinase.

Results: Reduced TGFβRII expression in human NSCLC is associated with male gender, smoking, SCC histology, reduced differentiation, increased tumor stage, increased nodal metastasis, and reduced survival. Homozygous or heterozygous TGFβRII deletion in mouse airway epithelia increases the size and number of KrasG12D-initiated adenocarcinoma and SCC. TGFβRII deletion increases proliferation, local inflammation, and TGFβ ligand elaboration; TGFβRII knockdown in airway epithelial cells increases migration and invasion.

Conclusions: Reduced TGFβRII expression in human NSCLC is associated with more aggressive tumor behavior and inflammation that is, at least partially, mediated by increased TGFβI expression. TGFβRII deletion in mouse airway epithelial cells promotes adenocarcinoma and SCC formation, indicating that TGFβRII loss plays a causal role in lung carcinogenesis. That TGFβRII shows haploid insufficiency suggests that a 50% TGFβRII protein reduction would negatively impact lung cancer prognosis. Clin Cancer Res; 18(8); 1–11. ©2012 AACR.

Introduction

Although more than 175,000 cases of non–small cell lung cancer (NSCLC) are diagnosed annually in the United States, the 5-year survival rate remains less than 20% (1). Lung adenocarcinoma and lung squamous cell carcinoma (SCC) are the most common histologic subtypes and although most (~85%) lung cancer is smoking-related, lung SCC is more strongly associated with smoking exposure (2). It is hypothesized that lung adenocarcinoma arises from the distal airway epithelial progenitor (3), whereas lung SCC arises from the keratin-positive basal cell population that contains the upper airway epithelial progenitor (4). TGFβ is a multifunctional cytokine that promotes epithelial differentiation and inhibits cell growth (5) and defective TGFβ signaling is often associated with more aggressive tumor behavior (6). TGFβ binds a heterodimer of TGFβ type I and type II receptors (TGFβRI and TGFβRII) that signal by phosphorylating Smad family transcription factors (5).

In previous small studies with poorly defined patient populations, reduced TGFβRII expression was reported in 40% to 80% of NSCLC at the protein and/or mRNA level (7–9). Although TGFβRII mutations are uncommon (10),...
Translational Relevance

Although lung cancer is a common malignancy, the 5-year survival remains low, illustrating a need for an improved understanding of basic molecular mechanisms that can subsequently be translated into novel therapeutic strategies. In this study, we found that reduced TGFβ type II receptor (TGFβRII) expression in human non–small cell lung cancer (NSCLC) samples is associated with more aggressive tumor behavior and reduced patient survival. Our mouse model shows that TGFβRII deletion in sporadic airway epithelial cells promotes formation of both adenocarcinomas and squamous carcinomas, supporting a causal role of TGFβRII loss in lung carcinogenesis and suggesting that reduced TGFβRII expression is a negative prognostic marker in lung cancer. Our mouse model will be a useful resource for testing novel therapeutic approaches directed toward NSCLC with reduced TGFβRII expression.

both microsatellite instability and promoter methylation have been associated with reduced TGFβRII expression (8, 11). From these studies, it is unknown whether reduced TGFβRII expression in human NSCLC is associated with specific histologic subtypes, more aggressive tumor behavior, or reduced patient survival. In lung cancer cell lines, TGFβRII restoration reduces proliferation, anchorage-independent growth, and xenograft growth (7) whereas TGFβRII knockdown increases Transwell migration (12). In mouse models, TGFβRII deletion promotes the development of oncogene-initiated malignancies in the pancreas, colon, breast, and oral epithelium (13–16), and a recent study shows that TGFβRII loss increases the invasiveness of Kras-initiated lung adenocarcinomas (17). However, because most lung cancer mouse models produce predominantly, if not exclusively, adenomas and adenocarcinomas (18), it is unknown whether TGFβ signaling disruption affects development of other NSCLC subtypes.

In this study, we evaluated TGFβRII expression at the protein and mRNA level in a large number of human NSCLC samples and show that reduced TGFβRII expression is associated with more aggressive tumor behavior, including reduced tumor differentiation, higher tumor stage, increased nodal metastases, and reduced survival. To generate a model that can produce lung SCCs and to determine whether TGFβRII loss initiates or promotes lung carcinogenesis, we targeted TGFβRII deletion to the conducting airway epithelium with the keratin-5 Cre PR transgene (K5CrePR; ref. 19). The K5 promoter targets the multipotent basal cell progenitor of the upper airway (20). CrePR is a humanized Cre recombinase progesterone receptor (CrePR) fusion protein that can be activated by R1486 but has low ligand-independent activity (21). Tracheal R1486 activates CrePR in sporadic airway epithelial cells (21), hence oncogenic alterations driven by this construct allow for the clonal expansion of cells with somatic mutations, analogous to what occurs in human cancer. Using this system, we show that TGFβRII deletion markedly promotes the development of Kras-initiated lung tumors, indicating that TGFβRII loss plays a causal role during lung carcinogenesis. Our studies in human samples show that reduced TGFβRII expression is a negative prognostic marker in lung cancer.

Materials and Methods

NSCLC samples

After Institutional Review Board (IRB) approval, 38 NSCLC samples were obtained from the Oregon Health & Sciences University (OHSU; Portland, OR) Department of Pathology Tumor Bank. Demographic and pathologic data were recorded when banked; histology and grade were confirmed by a second pathologist (S. White). RNA was extracted from 13 corresponding frozen samples. We also purchased a tissue microarray (T8235724, lot A606098; BioChain) composed of 64 NSCLC sections with accompanying demographic and pathologic data. With IRB approval, 85 NSCLC samples were obtained from the Colorado Lung SPORE Tissue Bank Core. Pathologic diagnosis of these samples was confirmed by a second pathologist (W. Franklin). Additional clinical and pathologic data were extracted from pathology reports and medical records; survival data were obtained by searching the social security death index (SSDI) database. Of these samples, 77 had paraffin blocks available for immunostaining and 37 had matched tumor and nonmalignant lung RNA available for analysis.

Immunostaining and quantitative reverse transcriptase PCR of human NSCLC samples

Immunostaining was conducted as previously described (14) with anti-TGFβRII antibody (1:200; Santa Cruz C6) and staining intensity was graded as 3+ (intensity greater than normal airway), 2+ (equal to normal airway), 1+ (reduced compared with normal airway), or 0 (absent) by 2 independent observers (S.P. Malkoski and S.M. Haeger). Because of the low frequency of samples with increased (3+) or absent (0) staining, samples were grouped as having preserved (2+ or 3+) or reduced (0 or 1+) staining for analysis. Immunostaining for lymphocytes and macrophages was conducted with anti-CDD antibody (Abcam #5690 at 1:250) and anti-MAC387 (Abcam #22506 at 1:200). Staining was quantified by counting the number of positive cells on 4 random 10× fields in at least 24 independent human NSCLC samples. RNA was harvested by homogenization in RNAzol (Invitrogen) and then purified with RNeasy columns (Qiagen). Quantitative reverse transcriptase PCR (qRT-PCR) was carried out with the Brilliant II Kit (Stratagene) using a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) internal control and TaqMan probe Hs00559657 (Applied Biosystems) on an ABI 7900 thermal cycler. Data were analyzed by the ΔΔCt method and expressed as fold reduction compared with paired nonmalignant lung. When paired nonmalignant...
lung was unavailable, expression in tumor samples was compared with grouped nonmalignant samples run on the same plate. Differences in immunostaining frequency between groups were analyzed by a χ² or Fisher exact test, depending on the expected frequency values. Differences in mRNA expression between groups were analyzed by non-parametric methods (Mann–Whitney or Kruskal–Wallis). Survival curves were obtained using the Kaplan–Meier method, and comparisons across groups were made with log-rank and stratified log-rank tests. Analyses were conducted with Prism5 (GraphPad) and SAS version 9.2 (SAS Institute, Inc.).

**NSCLC mouse model**

All animal studies were Institutional Animal Care and Use Committee (IACUC)-approved. The TGFβRII conditional allele, lox-stop-lox-KrasG12D conditional allele, and K5Cre transgene have been previously described (16, 19, 22, 23). Mice were treated with tracheal RU486 (500 μg in 25 μL 10% acetone/90% sesame oil) between 4 and 6 weeks of age as previously described (21). Mice were monitored weekly and euthanized at 1 year of age or if they lost more than 15% of their body weight. When euthanized, a blood sample was collected by cardiac puncture, tumors were enumerated and measured, and a bronchoalveolar lavage (BAL) sample was collected by instillation of two 1-mL aliquots of sterile PBS. The left lung was inflated with 10% formalin and then paraffin-embedded. Blood was centrifuged (5,900 g, 15', 25°C) and BAL supernatant stored at −80°C. BAL cells were separated by centrifugation (5,900 × g, 15', 25°C) and BAL supernatant stored at −80°C. BAL cells in four 40× objectives were scored by morphologic criteria, and differences between genotypes compared by unpaired t tests.

**Analysis of mouse NSCLC**

Lung tumors were separated from grossly normal lung and DNA extracted with the DNeasy Kit (Qiagen); PCR for the recombinant TGFβRII allele was carried out as previously described (16). RNA extraction, qRT-PCR, and TGFβRII immunohistochemistry were conducted as described above. Total TGFβ1 in BAL and serum was quantified by ELISA after acid activation per the manufacturer’s instructions (R&D Systems), and differences between groups were compared by one-way ANOVA or unpaired t test. Lesions were classified as atypical adenomatous hyperplasia (AAH), adenoma, adenocarcinoma, or squamous carcinoma on hematoxylin and eosin sections by 2 investigators (S.P. Malkoski and S.M. Haeger) using previously described criteria (24) with independent confirmation by a pathologist (D. Merrick) on a subset (~10%) of lesions. Tumors were immunostained with antibodies against K5 (1:1,000; Abcam #53121) and thyroid transcription factor (TTF; 1:100; Abcam #40880) to further confirm histologic subtype. Images were acquired on a Nikon Eclipse 80i and analyzed with Nikon Elements Software. Lesions were quantified per lung section and per mm² lung and differences compared by one-way ANOVA or unpaired t test. Immunostaining for proliferating cell nuclear antigen (PCNA, 1:100; Santa Cruz SC-56) and leukocytes (1:500 anti-CD3; Abcam #5690) was conducted as described above and quantified by counting the number of positive cells per tumor area.

**Migration and invasion assays in Beas2b cells with TGFβRII knockdown**

Beas2B human bronchial epithelial cells (American Type Culture Collection) were cultured in BEGM basal media supplemented with BEGM SingleQuot growth factors (Lonza) under standard conditions. Experiments were carried out between the 50th and 65th passages. A stable TGFβRII knockdown line was produced by transfecting the pcPUR+U6 plasmid containing the short hairpin RNA (shRNA) against TGFβRII (25) with the pcPUR+U6 cassette as a control (iGene Therapeutics). Cells were transfected at 60% confluence with 16 μg/mL plasmid DNA and 40 μL/mL Lipofectamine 2000 (Invitrogen); 48 hours post-transfection, puromycin (1.0 μg/mL; Invivogen) selection was initiated. TGFβRII knockdown was confirmed by quantitative PCR (qPCR) and Western blotting against TGFβRII (1:200; SC400 Santa Cruz Biotechnology) with GAPDH (1:40,000; Abcam #8245) control. Scratch assays were conducted by seeding 4 × 10⁵ cells into 6-well plates, allowing cells to grow to confluence and then wounding with a P200 pipette tip. Wounds were photographed at 0 and 24 hours and wound closure quantified as a percentage of the original wound area (26). Transwell assays were conducted using 200 μL Blindwell Boyden chambers (50-mm² area) and polyvinyl pyrrolidone–free polycarbonate membranes with 12-μm pores (Neuro Probe; ref. 27). Migration membranes were coated with 0.01% porcine gelatin; invasion membranes were coated with 5% growth factor–reduced Matrigel (BD Biosciences). Media conditioned for 24 hours with 3T3 cells were used as a chemoattractant in the lower chamber and 3.5 × 10⁴ cells were seeded into the upper chamber. After 24 hours, nonmigrating cells were removed, membranes were fixed, stained with Diff-Quik (Siemens Healthcare Diagnostics Inc.), and photographed at 100×. Cells from 5 fields of view per insert were counted, experiments were repeated 5 times, and differences between groups were compared by unpaired t tests.

**Results**

Reduced TGFβRII expression is more common in males, smokers, and lung SCC

Reduced TGFβRII expression has been previously reported in a small study with limited demographic data (7). We analyzed TGFβRII expression in 187 NSCLC samples (full demographic data shown in Supplementary Table S1) using a combination of immunostaining and qRT-PCR. Immunostaining was conducted on 172 of 187 samples and each sample was scored as having either preserved (intensity
equal to or greater than normal airway) or reduced (intensity less than 50% of normal airway in at least 50% of cells) immunostaining. mRNA expression was analyzed in 48 of 187 samples and was compared with TGFβRII expression in either paired nonmalignant lung (37 of 48 samples) or grouped nonmalignant lung (11 of 48 samples). In Figs. 1 and 2, immunostaining data are expressed as the fraction (%) of NSCLC samples with reduced staining and mRNA expression data are expressed as fold reduction of TGFβRII expression compared with nonmalignant lung. We found that 45% of NSCLC samples had reduced TGFβRII immunostaining and that samples with reduced TGFβRII immunostaining also had reduced TGFβRII mRNA expression (Fig. 1A). Although reduced TGFβRII immunostaining was somewhat more common in males and smokers, this did not reach statistical significance (Fig. 1B and C, top); in contrast, both males and smokers had significantly reduced TGFβRII mRNA expression (Fig. 1B and C, bottom) and the degree of reduced TGFβRII expression was weakly correlated with smoking exposure (Supplementary Fig. S1). Interestingly, reduced TGFβRII immunostaining and mRNA expression were markedly more common in SCCs compared with adenocarcinomas (Fig. 1D).

Figure 1. Reduced TGFβRII immunostaining and mRNA expression in human NSCLC is more common in males, smokers, and SCC histology. A, example of reduced TGFβRII immunostaining in human NSCLCs. Staining intensity (compared with normal airway) was characterized as preserved (equal to or greater than normal airway) or reduced. Reduced TGFβRII immunostaining was observed in 45% of NSCLC and correlated with reduced expression by qPCR (bottom). B and C, reduced TGFβRII immunostaining and mRNA expression are more common in males and current smokers, although only reduced mRNA expression reached significance in these comparisons. Immunostaining data are expressed as the fraction (%) of samples with reduced TGFβRII immunostaining. D, reduced TGFβRII immunostaining and mRNA expression are both more common in SCC than adenocarcinoma (AdC).
Reduced TGFβRII expression is associated with more aggressive NSCLC behavior

Although reduced TGFβRII immunostaining or expression has been associated with increased invasion in human lung adenocarcinoma (12, 17) and more aggressive tumor behavior in other human malignancies (28–30), the relationship between reduced TGFβRII expression and clinical tumor behavior in human NSCLC has not been defined. We found that reduced TGFβRII immunostaining and mRNA expression were more common in more poorly differentiated tumors (Fig. 2A), tumors with higher tumor (T) stage (Fig. 2B), and tumors with nodal metastases (Fig. 2C). Interestingly, there was no association between the degree of reduced TGFβRII mRNA expression and tumor size as a continuous variable (not shown), suggesting that reduced TGFβRII expression with higher tumor stage may be driven more by the invasive parameters that dictate clinical tumor stage (e.g., invasion) as opposed to size. Because tumor stage and nodal status dictate overall clinical stage, reduced TGFβRII immunostaining was also more common with increasing clinical stage (not shown) and reduced TGFβRII immunostaining was associated with reduced overall survival in NSCLC (Fig. 2D). After adjusting for stage in a Cox proportional hazards survival model, preserved TGFβRII immunostaining remained associated with improved survival with an HR of 0.49 [95% confidence interval (CI), 0.24–0.98; P = 0.04].

TGFβRII deletion promotes growth and multiplicity of Kras-induced NSCLC

TGFβRII deletion promotes development and malignant conversion of Kras-initiated tumors in both oral
epithelium (14) and pancreas (13). To address the role of TGFβRII loss in lung cancer development in vivo, we developed a mouse model combining TGFβRII deletion with oncogenic KrasG12D activation. We used the K5CrePR transgene that contains an RU486-inducible Cre recombinase (19) and tracheal RU486 to direct TGFβRII deletion (16) and oncogenic KrasG12D (22) activation to the airways (21). As expected, the recombinant TGFβRII allele could be detected in tumors but not in adjacent grossly normal lung (Fig. 3A) and TGFβRII immunostaining was reduced in tumors with TGFβRII deletion (hereafter referred to as Kras.TGFβRII+/−/C0 and Kras.TGFβRII+/−/C0) compared with Kras tumors (Fig. 3B). In addition, TGFβRII mRNA expression in whole lung homogenate negatively correlated with increasing tumor burden (hereafter referred to as Kras.TGFβRII+/−/C0; Fig. 3C). We only observed one lung tumor in 30 K5CrePR.TGFβRII−/− animals (hereafter referred to as Kras.TGFβRII−/−) compared with Kras tumors (Fig. 3B). In addition, TGFβRII mRNA expression in whole lung homogenate negatively correlated with increasing tumor burden (Fig. 3C).

We only observed one lung tumor in 30 K5CrePR.TGFβRII−/− animals (hereafter referred to as Kras.TGFβRII−/−); however, approximately 65% of animals harboring both the K5CrePR transgene and a lox-stop-lox KrasG12D allele (K5CrePR.LSL-Kras; hereafter referred to as Kras) developed lung tumors after tracheal RU486 (Supplementary Table S2). Kras animals typically developed 2 to 3 tumors per animal; deletion of one or both TGFβRII alleles increased tumor multiplicity by approximately 10-fold (Fig. 4A) and also increased overall tumor size (Supplementary Fig. S2). Because of the increased number of small tumors with TGFβRII deletion, we analyzed the largest single tumor in each animal to assess the effect of TGFβRII deletion on established tumor growth (Fig. 4B); this analysis showed that TGFβRII deletion increased the growth of established Kras-initiated lung tumors. Although there was a low level of tumor formation in vehicle-treated Kras.TGFβRII−/− animals, there was a clear, dose-dependent increase in tumor formation with increasing tracheal RU486 dose (Supplementary Table S2). These data show that the K5CrePR transgene can be used for lung targeting and that TGFβRII deletion greatly increases both the number and size of Kras-initiated lung tumors.

Kras.TGFβRII−/− mice develop both adenocarcinomas and SCCs

In our mouse model that uses a K5 promoter and tracheal RU486 to target our genetic manipulations (19, 21), we observed the formation of both adenocarcinomas and SCCs. Adenocarcinomas displayed typical glandular morphology and stained negative for K5 and positive for TTF, whereas SCCs displayed keratinization and intercellular bridges and were K5-positive and TTF-negative (Supplementary Fig. S3). To analyze the effect of TGFβRII deletion on the formation of specific tumor types, we classified individual lesions according to consensus recommendations (24) and found that TGFβRII deletion increased the number of both benign (AAH and adenomas) and malignant (adenocarcinoma and SCC) lesions (Fig. 4C).
and D). In sum, TGFβRII deletion increases KrasG12D-dependent tumor initiation and allows for the formation of a spectrum of malignant lesions that includes the 2 most common human NSCLC subtypes.

**TGFβRII deletion increases proliferation and inflammation in vivo**

Because TGFβ signaling inhibits epithelial proliferation (5), we compared proliferation in Kras and Kras*TGFβRII*−/− tumors and found that Kras.TGFβRII*−/− tumors had a 5-fold increase in staining for the proliferation marker PCNA (Fig. 5A and immunostaining example shown in Supplementary Fig. S4A). Loss of functional TGFβ signaling can cause a compensatory increase in TGFβ1 ligand production (14, 31) which can then promote tumor development through increased angiogenesis and inflammation in the tumor microenvironment (14, 32, 33). We found that TGFβRII deletion increased TGFβ1 ligand in the BAL fluid (Fig. 5B) and that increasing tumor burden correlated with higher BAL TGFβ1 (Supplementary Fig. S4B). TGFβRII deletion was also associated with increased serum TGFβ1 ligand levels (not shown) similar to the increased serum TGFβ1 levels reported in humans with NSCLC (34). Similarly, TGFβRII knockdown in human Beas2B cells increased TGFβ1 ligand production (Supplementary Fig. S5C). We did not find increased angiogenesis in TGFβRII*−/− lung tumors (not shown) but observed that TGFβRII deletion increased the number of macrophages, lymphocytes, and neutrophils in the BAL (Fig. 5C) and that the BAL macrophage count correlated with increasing tumor burden (Supplementary Fig. S4C). In addition, compared with Kras tumors, Kras.TGFβRII*−/− tumors had increased infiltration of CD3+ lymphocytes (Fig. 5D, immunostaining example shown in Supplementary Fig. S4D). To determine whether a similar relationship between reduced TGFβRII expression and increased inflammation exists in human lung cancer, we immunostained human NSCLC samples for both lymphocytes and

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Figure 4. TGFβRII deletion in mouse airway epithelia increases formation of both adenocarcinomas and SCCs. A, deletion of one or both TGFβRII alleles increases the number of Kras-initiated lung tumors. Each data point represents the number of tumors observed in an individual animal; the mean overlies individual tumor data points. Kras animals had 3.2 ± 1.0 tumors per animal whereas Kras.TGFβRII+/− animals had 25.4 ± 4.7 tumors per animal and Kras.TGFβRII−/− animals had 34.4 ± 5.9 tumors per animal (both significant compared with Kras). B, deletion of one or both TGFβRII alleles increases the size of established Kras-initiated lung tumors. Each data point represents the single largest tumor from an individual animal; Kras tumors were 1.15 ± 0.20 mm whereas Kras.TGFβRII+/− tumors were 2.28 ± 0.19 mm and Kras.TGFβRII−/− tumors were 2.64 ± 0.20 mm (both significant compared with Kras). A separate analysis that includes all tumors is shown in Supplementary Fig. S2. C and D, TGFβRII deletion increased the number of benign and malignant lung lesions per tumor section and the number of malignant lesions per mm² lung. Because SCCs were not observed in Kras animals, P values could not be calculated for these lesions. Additional data about the age, number of animals, tracheal RU486 dosage, and tumor penetrance are presented in Supplementary Table S2. AdC, adenocarcinoma. SCC, squamous cell carcinoma; AAH, atypical adenomatous hyperplasia.
macrophages and found that human lung cancers with reduced TGFβRII expression had increased numbers of infiltrating CD3⁺ lymphocytes but not macrophages (Supplementary Fig. S6). In sum, these data show that TGFβRII deletion in a murine lung cancer model increases both proliferation and local inflammation and human lung cancer with reduced TGFβRII expression also have increased inflammation.

**TGFβRII knockdown in bronchial epithelial cells increases migration and invasion**

That reduced TGFβRII expression was associated with both increased aggressiveness of human NSCLC and progression of murine lung tumors prompted us to assess whether TGFβRII knockdown promotes cell migration and invasion in vitro. We stably knocked down TGFβRII in a human bronchial epithelial cell line (Beas2B) using an shRNA approach (25) and achieved reduced TGFβRII expression at both the RNA and protein level (Supplementary Fig. S5A). Interestingly, TGFβRII knockdown did not affect proliferation of Beas2B cells (Supplementary Fig. S5D), potentially because these cells are SV40 T antigen immortalized and hence not sensitive to TGFβ-mediated growth inhibition. In a scratch assay, TGFβRII knockdown increased in vitro wound closure by almost 3-fold (Fig. 6A and B). In a Matrigel Transwell invasion assay, TGFβRII knockdown increased migration by approximately 2-fold and invasion by approximately 3-fold (Fig. 6C and D). These data suggest that increased lung tumor aggressiveness in tumors with reduced TGFβRII expression is, at least, partially mediated through increased invasion and migration of tumor cells.

**Discussion**

**Reduced TGFβRII expression in human NSCLC is associated with more aggressive tumor behavior**

In this study, we found that reduced TGFβRII expression is common in NSCLC and occurs more frequently in males, smokers, and tumors with SCC histology; this grouping of associations is not surprising given that lung SCCs are more common in males and are more strongly associated with tobacco exposure (35); however, this is the first description clearly linking reduced TGFβRII expression with smoking exposure. Consistent with previous reports that TGFβRII expression in NSCLC can be reduced through promoter methylation or microsatellite instability (8, 11), we found both reduced TGFβRII at both the mRNA and protein levels, suggesting that reduced TGFβRII expression occurs pretranslationally. We also show that reduced TGFβRII expression is associated with more aggressive NSCLC behavior including reduced differentiation, higher T stage, nodal metastases, and reduced patient survival. This is consistent with TGFβRII functioning as an NSCLC...
tumor suppressor, although other molecules clearly also contribute to malignant progression. These associations suggest a causal role of TGFβRII reduction in NSCLC progression, which is further supported by our animal study in which TGFβRII deletion increases NSCLC development in vivo.

**Targeting TGFβRII deletion to K5-positive airway cells promotes formation of multiple NSCLC subtypes**

We targeted TGFβRII deletion to the conducting airway epithelium using a K5-driven, RU486-inducible Cre recombinase and tracheal RU486 (19, 21). Because keratin expression in the lung is limited to basal cells which are thought to contain the SCC progenitor cell (4, 36), we expected these animals to develop exclusively SCCs. To our surprise, while these animals did develop some SCCs, they predominantly developed adenomas and adenocarcinomas. In the murine airway, K5/K14-positive basal cells function as stem cells or facultative progenitors capable of giving rise to multiple cell types (20, 37); our data suggest that K5-positive cells contain both adenocarcinoma and SCC progenitors.

TGFβRII deletion alone in the airway epithelium results in a very low incidence of lung tumors; this is consistent with the observation that targeting TGFβRII deletion to a large fraction of lung epithelial cells by adenoviral-delivered Cre recombinase (AdCre) also fails to initiate tumor formation (17). In contrast, TGFβRII deletion markedly increases Kras-initiated lung tumor number and size, suggesting that intact TGFβ signaling inhibits lung tumor growth and that TGFβ signaling disruption increases both tumor initiation and progression. Increased proliferation and tumor size is likely driven by loss of TGFβ-mediated growth inhibition whereas increased tumor multiplicity may be a result of impaired immune surveillance from increased TGFβ. These data are consistent with TGFβRII function in other oncogene-initiated cancer models (13–16) as well as a recent report showing that invasive lung adenocarcinoma is modeled by Kras activation and TGFβRII deletion targeted by AdCre (17). Our data show that TGFβRII deletion in mice increases tumor number, tumor size, and the number of malignant lesions; these observations are consistent with our observation that reduced TGFβRII expression in human NSCLC is associated with more aggressive tumor behavior. Our study shows that in addition to abrogation of TGFβ-induced growth arrest, TGFβRII loss also promotes tumor cell migration and invasion. Although Kras.TGFβRII/C0/C0 tumors had little evidence of epithelial-to-mesenchymal transition (EMT), we have previously shown that TGFβRII loss causes an EMT-independent migratory and invasive phenotype in keratinocytes (38).

Interestingly, deleting one TGFβRII allele had an effect similar to deleting both TGFβRII alleles, indicating that TGFβRII exhibits haplode sufficiency, and that a 50% reduction (i.e., roughly the amount used to score human
NSCLCs) is sufficient to promote lung cancer growth. This is similar to what was observed in both a head and neck cancer model (14) and a pancreatic cancer model (13), where deletion of a single TGFβRII allele caused intermediate phenotypes in terms of tumor penetrance or survival, respectively. In another study, TGFβ1 haploid insufficiency also increased progression of Kras-initiated lung tumors and shortened survival (39), suggesting that reduced TGFβ signaling in tumor epithelial cells promotes lung tumor progression.

**TGFβRII deletion in airway epithelia increases inflammation that could promote malignant progression**

In our mouse model, TGFβRII deletion increased TGFβ1 ligand elaboration, BAL inflammation, and tumor-associated macrophages and lymphocytes. In human lung cancer samples, reduced TGFβRII expression was associated with increased lymphocyte infiltration but not with increased macrophage infiltration. Because macrophages are recruited to areas of tissue damage, it is possible that macrophage infiltration in human lung samples may predominantly be driven by environmental factors (e.g., smoking) as opposed to reduced TGFβRII expression. In contrast, increased TGFβ1 ligand produced by TGFβRII-negative tumors may recruit other inflammatory cells, for example, CD3-positive T lymphocytes, to the local environment where these cells could promote lung tumor progression (40). Supporting this notion, increased lymphocyte infiltration (of CD4 cells, CD8 cells, and B cells) was also reported with AdCre-mediated Kras.TGFβRII−/− lung tumorigenesis (17). In contrast to AdCre-mediated targeting that likely affects multiple cell types, targeting in our model was restricted to sporadic epithelial cells, thus inflammation was likely a result of secreted proinflammatory cytokines/chemokines, such as TGFβ1, by targeted epithelial cells. TGFβ1 can recruit myeloid cells (16) and induce development of regulatory T cells and T-helper (Th1) 17 lymphocytes (41), all of which can facilitate tumor growth. However, the paracrine effects of inflammatory cytokines in our model were insufficient to elicit the intense fibroelastic stromal response seen in AdCre-initiated KrasG12D.TGFβRII−/− tumors (17), presumably because AdCre targeting results in recombination in much higher proportion of epithelial cells than does K5Cre+PR targeting. Finally, although TGFβRII deletion and TGFβ1 overexpression have been associated with increased angiogenesis in other systems (14, 33), we did not observe increased angiogenesis in Kras.TGFβRII−/− lung tumors, potentially because the lung has a higher oxygen tension that limits the need for tumor neovascularization, particularly of small tumors.

In summary, our study shows that reduced TGFβRII expression in human NSCLC is a negative prognostic marker associated with more aggressive tumor behavior and worse clinical outcome. In addition, TGFβRII loss plays a causal role in promoting the development of multiple NSCLC subtypes. Our study should instigate investigation into mechanisms underlying the effects of reduced TGFβRII expression on both tumor epithelium and tumor stroma. This could potentially facilitate selection of patients for therapies targeting events downstream of TGFβRII loss.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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