Modulation of NFκB Activity and E-cadherin by the Type III Transforming Growth Factor β Receptor Regulates Cell Growth and Motility*

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Transforming growth factor β is growth-inhibitory in non-transformed epithelial cells but becomes growth-promoting during tumorigenesis. The role of the type I and II receptors in tumorigenesis has been extensively studied, but the role of the ubiquitously expressed type III receptor (TβRIII) remains elusive. We developed short hairpin RNAs directed against TβRIII to investigate the role of this receptor in breast cancer tumorigenesis. Nontumorigenic NMuMG mouse cells stably expressing short hairpin RNA specific to mouse TβRIII (NM-kd) demonstrated increased cell growth, motility, and invasion as compared with control cells expressing shRNA to human TβRIII (NM-con). Reconstitution of TβRIII expression with rat TβRIII abrogated the increased growth and motility seen in the NM-kd cells. In addition, the NM-kd cells exhibited marked reduction in the expression of the adherens junction protein, E-cadherin. This loss of E-cadherin was due to increased NFκB activity that, in turn, resulted in increased expression of the pleitropic cytokines that are involved in cell growth and pro-motility.*

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The transforming growth factor (TGF) βs belong to a family of pleitropic cytokines that are involved in cell growth and proliferation, differentiation, deposition of extracellular matrix, and motility (1). The three TGFβ isoforms (β1, β2, and β3) bind to transmembrane receptor serine/threonine kinases. TGFβ1 and TGFβ3 can bind with high affinity to the TGFβ type II receptor, resulting in activation of Smad 2/3 and downstream target genes. In contrast, TGFβ2 binds with low affinity to the type II receptor. The type III receptor (TβRIII), or betaglycan, binds with high affinity to all three TGFβ isoforms and is required for presenting TGFβ2 to the type II receptor (2).

TβRIII has been shown to play an essential role in the formation of the atrioventricular cushion in the development of the heart (3). Consistent with this observation, the TβRIII null mouse is embryonically lethal because of heart and liver defects (4). The role for TβRIII in cancer is less clear. Increased expression of TGFβ1 and all three TGFβ receptors was found in higher grade lymphomas (5). Conversely, reduced expression of TβRIII was found associated with advanced stage neuroblastomas and ovarian carcinomas (6, 7). Similarly, a recent report using cDNA microarrays demonstrated that low TβRIII expression correlates with higher grade among a cohort of breast cancers (8). Additionally, overexpression of TβRIII in MDA-231 human breast cancer cells and DU145 prostate cancer cells resulted in decreased tumor invasion in vitro and in vivo (9, 10). The reasons for this apparent contradictory role for TβRIII in these different tumor types have not been elucidated.

Epithelial to mesenchymal transition (EMT) is a process by which TGFβ can promote tumorigenesis. EMT is characterized by a decrease in epithelial cell markers, such as E-cadherin and ZO-1, and an increase in mesenchymal markers including N-cadherin, vimentin, and fibronectin. This is associated with a decrease in cell-cell adhesion and changes in the actin cytoskeleton. Loss of E-cadherin expression, either through genetic or epigenetic alterations, is the hallmark of EMT in epithelial cells. Several proteins (i.e. Snail, Slug, Twist, and Sip1) have been identified as transcriptional repressors of E-cadherin (11–13).

NFκB is a family of hetero- or homodimeric transcription factors involved in cell survival and regulation of the immune response (14). The NFκB signaling pathway appears to be a critical mediator of EMT (15, 16). Additionally, it has been reported that NFκB is required for EMT during breast cancer progression (17). NFκB also appears to be a mediator of Snail expression (15, 18). A recent report demonstrated that expression of E-cadherin can down-regulate NFκB activity in melanoma cells, suggesting a direct link between these two pathways (19).

The reported conflicting roles of TβRIII in tumorigenesis lead us to investigate its role in mammary cell transformation.
**TβRIII Regulates NFκB and E-cadherin**

For this purpose, we used a loss of function approach with short hairpin RNAs (shRNAs) specific to TβRIII in nontumorigenic NMuMG mammary epithelial cells. In this study we show that knock-down of TβRIII expression in NMuMG cells results in increased growth, motility, invasion, and tumor formation in vivo using a xenograft mouse model. In addition, we demonstrate that these changes are due to an increase in NFκB signaling that, in turn, results in transcriptional repression of E-cadherin. These results were not limited to NMuMG cells because we also observed a similar phenotype in EMT6 mouse mammary tumor cells in which TβRIII was knocked down by stable RNA interference.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Viral Infections, and shRNA**—All of the cells were purchased from American Type Culture Collection (Manassas, VA). NMuMG cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Cambrex) supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml insulin. EMT-6, Phoenix-Ampho, and 293A cells were grown in DMEM containing 10% FBS in a humidified 5% CO2 incubator at 37 °C. To generate retroviruses expressing short hairpin RNAs specific to mouse TβRIII, the complimentary oligonucleotides 5’-GAAA-UUGACAUUCUUCCUAC and 5’-UGGAAGGGAGUAUCUUG were annealed and ligated into the BglII/HindIII site of the pSuper vector. Retrovirus expressing shRNA specific to human TβRIII (5’-GAGAUGCAUUCUUCCUAC and 5’-GUUGAAGGAUGUCAUC) was used as a control. The resulting pSuper plasmids were transfected into Phoenix-Ampho cells using Superfect transfection reagent (Qiagen) per the manufacturer’s directions. Supernatant containing viral particles was collected 72 h after transfection and filtered through a 20-μm syringe filter. Retrovirus containing mouse or human short hairpin RNA specific to the type III receptor were used to infect NMuMG cells in the presence of 4 μg/ml polybrene (Sigma). Stably expressing cells were selected with 1 μg/ml puromycin. The pGEM-4Z rat TβRIII plasmid was a gift from Dr. John Massague (Memorial Sloan Kettering Cancer Center, New York, NY). Rat TβRIII was digested out of pGEM-4Z using EcoRI and cloned into the EcoRI site of the LZRS-MS-neo retroviral plasmid. NM-kd cells were infected with retrovirus containing rat TβRIII and selected using 600 μg/ml g/ml polybrene (Sigma). Stably expressing cells were selected with 1 μg/ml puromycin. The pGEM-4Z rat TβRIII plasmid was a gift from Dr. John Massague (Memorial Sloan Kettering Cancer Center, New York, NY). Rat TβRIII was digested out of pGEM-4Z using EcoRI and cloned into the EcoRI site of the LZRS-MS-neo retroviral plasmid. NM-kd cells were infected with retrovirus containing rat TβRIII and selected using 600 μg/ml G418 (Invitrogen). Adenovirus containing constitutively active IKK2 (CA-IKK2) or dominant negative IkBo (dn IkBo) were provided by Dr. Timothy Blackwell (Vanderbilt University, Nashville, TN). Adenovirus containing GFP was used as a vector control. 293A cells were infected with adenovirus to produce a concentrated stock of virions. For adenoviral infection, NMuMG cells were plated to ~70% confluency in 100-mm dishes. Adenovirus was added to the cells in 3 ml of serum-free medium for 3 h, at which point 7 ml of medium containing 10% FBS was added. The cells were allowed to grow for 48 h before being subjected to further treatment.

**Antibodies and Reagents**—TGFβ1 and TGFβ2 were purchased from R & D Systems (Minneapolis, MN). Growth factor-reduced Matrigel was purchased from Clontech (Mountainview, CA). Antibodies to E-cadherin and Smad 2/3 were from Transduction Laboratories (Lexington, KY); p-Smad 2 was from Cell Signaling (Danvers, MA); anti was from Sigma. Phalloidin-fluoresceine isothiocyanate (actin) and goat anti mouse Oregon Green 488 antibodies were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated secondary antibodies used for immunoblots were from Promega (Madison, WI).

**125I-TGFβ1 Affinity Cross-linking Assays**—125I-TGFβ1 affinity cross-linking assays were performed as described (20). Cross-linked cell lysates were separated on a 3–12% gradient SDS-PAGE and visualized by autoradiography.

**RNA Isolation and Quantitative PCR**—Total RNA was extracted using the RNeasy Mini-kit (Qiagen) per the manufacturer’s directions. RNA (5 μg) was reverse transcribed in a 100-μl reaction. Quantitative PCR was carried out on 500 ng of cDNA using the iQ SYBR Green Supermix from Bio-Rad in a Bio-Rad iCycler IQ multicolor real time PCR detection system. Primers were designed using the Universal Probe Library from Roche Applied Science. Primer sequences are listed in Table 1. A standard curve was generated by amplifying known concentration of cDNA using actin primers. All of the reactions were performed in triplicate.

**Cell Growth and Motility Assays**—The cells (1 × 10⁴ cells/well) were seeded in 12-well plates in medium containing 10% FBS. The cells were harvested every other day for 7 days, and the cell numbers in each well were measured using a Coulter Counter. Three-dimensional growth assays were carried out in growth factor reduced Matrigel (BD Biosciences) as described (21). The cells were dissolved from the Matrigel using Cell Recovery Solution (BD Biosciences), and their numbers were measured in a Coulter Counter. For motility assays, confluent sheets of cells were “wounded” by scraping with a pipette tip at time of treatment. Wound closure in the presence of added ligand was assessed over time as described previously (22). Transwell assays were performed as previously described (23). In brief, the cells (1.5 × 10⁵/well) were plated in serum-free medium in the upper chamber of 8-μm transwells (Costar) and incubated with or without 2 ng/ml TGFβ. After 24 h, cells that had migrated to the underside of the transwell filters were fixed and stained utilizing Diff-Quick Stain Set from Dade Behring AG (Dudingen, Switzerland). The cells in five random fields at 200× magnification were counted.

**Immunoblot Analysis**—The cells were plated in 60-mm plates and allowed to grow overnight. The cells were then placed in low serum medium (1% FBS) for 16 h, after which the medium was replaced with low serum medium containing either 2 ng/ml TGFβ1 or TGFβ2 for 6 h. The cells were lysed in

| **Table 1** Quantitative PCR primer sequences |
|-----------------------------------------------|
| **Name** | **Sequence** |
| **Mouse E-cadherin** | 5’-AAAT GGC GCC AAT GCA ATC CCA AGA |
| **Mouse Snail** | 5’-TCC CCA AGA CCG ATG GTG GAG ATT |
| **Mouse Slug** | 5’-CAG ACT GCA ACC CAC ACA ATP CCT |
| **Mouse Sip1** | 5’-ATG GCA ACA CAT GGG TTT AGC GGG |
| **Mouse Twist** | 5’-ATG GAG GAA CTC TGA GCA GAT GAT GGT |
| **Actin** | 5’-AGA GGT TGG TPG AAG GTC TCA AA |
FIGURE 1. Knock-down of TβRIII in NMuMG impairs response to TGFβ2. A, NMuMG, NM-con, NM-kd, and NM-kd&RIII cells were affinity labeled with \(^{125}\)I-TGFβ1 and cross-linked with BS\(^{3}\) as described under “Experimental Procedures.” Labeling was competed with 100 pM cold TGFβ. Protein lysates were separated by 3–12% gradient SDS-PAGE and visualized by autoradiography. B, subconfluent NMuMG cell monolayers were incubated in DMEM, 1% FBS overnight. The following day, fresh DMEM, 1% FBS containing either 2 ng/ml TGFβ1 or TGFβ2 was added. The cells were lysed in Nonidet P-40 buffer, and protein was harvested 6 h after treatment. Total protein from whole cell lysates was separated by SDS-PAGE and subjected to immunoblot analysis with the indicated antibodies. Actin was used as a control. C, cells were transfected with the 3TP-Lux TGFβ-responsive promoter, serum-starved overnight in 1% FBS, and stimulated with 2 ng/ml TGFβ1 or TGFβ2 for 24 h. The cells were lysed and assayed for luciferase activity as described under “Experimental Procedures.” Relative luciferase units (RLU) represents the ratio of firefly to Renilla luciferase activities. Each data point represents the mean ± S.D. of three wells.

Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF) as previously described (22). Protein concentrations were determined using BCA protein assay reagent (Pierce); 50 μg of protein was separated by 9% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in TBST containing 5% bovine serum albumin for 1 h and then incubated with primary antibody overnight at 4°C. This was followed by incubation with secondary antibody for 1 h at room temperature. The membranes were washed three times in TBST, and the bands were visualized using ECL (Amersham Biosciences).

Transcriptional Reporter Assays—The cells were seeded in 60-mm plates and transfected with 5 μg of either 3TP-Lux (provided by Dr. Joan Massague, Memorial Sloan Kettering Cancer Center), NFκB-Luc (provided by Dr. Timothy Blackwell, Vanderbilt University), E-cad-Luc (provided by Dr. Amparo Cano, Universidad Autonoma de Madrid, Madrid, Spain), or 0.5 μg pCMV- Renilla (Promega) using Superfect transfection reagent (Qiagen) according to the manufacturer’s protocol. The next day, the cells were split equally into 48-well plates and incubated overnight in DMEM, 1% FBS, after which the medium was replaced with fresh DMEM, 1% FBS containing either 2 ng/ml TGFβ1 or TGFβ2 for 24 h. Firefly luciferase and Renilla reniformis luciferase activity was measured using a dual luciferase reporter system (Promega) according to the manufacturer’s published protocol in a Monolight 3010 luminometer (Analytical Luminescence Laboratory).

ELISAs—Serum-free conditioned medium was removed from growing cells after 72 h and tested using the TGFβ1 or the TGFβ2 Quantikine ELISA kit (R & D Systems) following acid activation as indicated in the manufacturer’s protocol. A standard curve using 31.5–2,000 pg/ml human recombinant TGFβ was generated using the kit reagents and used to calculate the TGFβ equivalents in the conditioned medium. Each sample was examined in triplicate for a total of three times as described (24). All of the ELISA data are corrected for cell number (pg/ml/cell number).

Immunofluorescent Microscopy—Immunofluorescent microscopy was performed as previously described (23). Briefly, the cells were grown in 8-well chamber slides for 48 h, fixed in 3% paraformaldehyde, incubated with either phalloidin (1:40 dilution) or E-cadherin (1:500 dilution) primary antibodies for 1 h, and then incubated with fluorescent secondary antibodies for 30 min. Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axio phot upright microscope.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed using the Promega Gel Shift Assay Kit (Promega) according to the manufacturer’s protocol. NM-con and NM-kd cells were grown in DMEM, 1% FBS in the presence or absence of 2 ng/ml TGFβ2 for 1 h. Nuclear extracts were harvested as described previously (25). Nuclear extracts (10 μg) were incubated with \(^{32}\)P-labeled NFκB oligonucleotides, separated by 6% SDS-PAGE, and visualized by autoradiography. Unlabeled NFκB oligonucleotides (cold NFκB) were used as a competitive inhibitor, and unlabeled Oct-1 oligonucleotides were used as a negative control.

Xenograft Assays—The cells (1 × 10\(^6\) cells) were resuspended in 200 μl of phosphate-buffered saline and injected with a 22-gauge needle into the right inguinal mammary gland (number 4) of anesthetized 6-week old athymic nude mice (Harlan Sprague-Dawley, Indianapolis, IN) and allowed to grow for 10 weeks. Fat pads, tumors, and lungs were collected, fixed in 10% formalin, and embedded in paraffin.

RESULTS

Knock-down of TβRIII in NMuMG Cells Impairs Response to TGFβ—We developed shRNAs specific to TβRIII to determine the role of this receptor in mammary epithelial cells. Nontumorigenic NMuMG mouse epithelial cells were infected with a virus containing the shRNA specific to human (NM-con) or mouse (NM-kd) TβRIII, and individual clones were isolated through serial dilution. The clones were initially screened using semi-quantitative reverse transcription PCR with primers designed to amplify TβRIII and actin (data not shown). Positive clones were confirmed by receptor cross-linking with \(^{125}\)I-
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As a control, mouse NMuMG cells were also infected with shRNA to human TβRIII. Affinity cross-linking with 125I-TGFβ1 showed loss of the type III receptor protein only in NM-kd cells but not in cells transduced with the control shRNA specific to human TβRIII (Fig. 1A). To determine the effect of TβRIII knock-down on TGFβ signaling, we examined p-Smad2 by Western blot after TGFβ1 and TGFβ2 treatment. NM-kd cells had significantly reduced p-Smad2 levels after treatment with 2 ng/ml TGFβ2 compared with control cells (Fig. 1B). Additionally, the ability of the NM-kd cells to activate the 3TP-Lux TGFβ-responsive promoter after TGFβ treatment was markedly inhibited, whereas reconstitution of the receptor with a retrovirus encoding rat TβRIII (NM-kd&RIII) restored ligand-induced 3TP-Lux reporter activity (Fig. 1C). The transduced rat TβRIII was also detectable by affinity cross-linking with 125I-TGFβ1 (Fig. 1A).

Knock-down of TβRIII in NMuMG Cells Results in Increased Growth, Motility, and Invasiveness—TGFβ1 is an inhibitor of epithelial cell growth (26). Thus, we examined proliferation and migration of cells in which TβRIII has been reduced by RNA interference. NM-kd cells grew significantly faster than NM-con cells, and re-expression of rat TβRIII in the NM-kd cells reduced their growth rate (Fig. 2A). Additionally, the NM-kd cells were able to migrate and close a wound in a wound closure assay under reduced serum conditions, whereas control cells and NM-kd cells reconstituted with TβRIII did not close the wound (Fig. 2B). The NM-kd cells were also more invasive than the NM-con cells as determined by their ability to migrate through transwell filters in the presence of low serum. TGFβ1 and TGFβ2 inhibited transwell migration of control cells but not of the NM-kd cells (Fig. 2C). This result is consistent with the dampened transcriptional response to added ligands observed in NM-kd cells (Fig. 1C).

NMuMG cells are nontumorigenic and form small rounded acini when grown in Matrigel. In contrast, the NM-kd cells form invasive branching structures in three-dimensional Matrigel (Fig. 2D). Reconstitution of TβRIII abrogated the ability of the NM-kd cells to form these structures in Matrigel. To determine whether the changes in observed cell behavior could involve the production of autocrine ligands, we investigated the secretion of TGFβ ligands by NMuMG cells. ELISAs were used to determine the amount of total TGFβ1 and TGFβ2 secreted from these cells. The NM-kd cells secret significantly less TGFβ1 and TGFβ2 compared with control cells (Fig. 2E).
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**Loss of TβRIII Results in Down-regulation of E-cadherin—**
The increased motility and invasiveness of the NM-kd cells was suggestive of EMT. Changes in the content and localization of E-cadherin at adherent cell junctions is a hallmark of EMT (27). Western blot analyses, real time quantitative PCR, and immunocytochemistry demonstrated that the NM-kd cells express greatly reduced levels of E-cadherin (Fig. 3, A–C). The ability of the NM-kd cells to transcriptionally activate an E-cadherin promoter luciferase reporter was also diminished as compared with NM-con cells (Fig. 3D). E-cadherin promoter activity is restored in the NM-kd&RIII cells, where the type III receptor had been re-expressed.

The E-cadherin gene can be regulated by a variety of mechanisms including epigenetic changes, such as hypermethylation, as well as transcriptional repression. Therefore, we examined the mRNA levels of the E-cadherin transcriptional repressors Snail, Slug, Sip1, and Twist by quantitative PCR. mRNA levels of Snail, Slug, Sip1, and Twist were significantly increased in NM-kd cells compared with NM-con cells (Fig. 4). This increase correlated with the reduced expression of E-cadherin (Fig. 3). Further, E-cadherin expression was restored with a corresponding decrease in Snail, Slug, Twist, and Sip1 in NM-kd&RIII cells. These data suggest a role for TβRIII in maintaining the epithelial phenotype through control of the transcriptional repressors of E-cadherin.

**EMT-6 Cells, with Reduced Expression of TβRIII, Demonstrated an Increased Growth Rate and Decreased E-cadherin Expression—**To confirm that these results were specific to loss of TβRIII expression and not specific to NMuMG cells or caused by clonal variation, we knocked down TβRIII expression in EMT-6 mouse mammary cancer cells. EMT6-con and EMT6-kd cell lines were generated as described for the NMuMG cells (Fig. 1). The clones were screened by semi-quantitative PCR (Fig. 5A), and EMT6-kd clone C5 was used in the remaining experiments. The EMT6-kd cells demonstrated a muted response to TGFβ2, but not TGFβ1, as determined by 3TP-Lux reporter assays (Fig. 5B). Similar to NMuMG cells, EMT6-kd cells grew better in Matrigel compared with EMT6-con cells (Fig. 5C). In addition, these cells showed decreased E-cadherin protein expression and E-cadherin luciferase activity (Fig. 5, D and E) with a corresponding increase in Snail mRNA (Fig. 5E). These data demonstrate that the effect of TβRIII loss on growth and E-cadherin expression is not limited to NMuMG cells.

**NFκB Activity Is Higher in TβRIII Knock-down Cells—**NFκB is a known regulator of Snail (28), and NFκB activity has been shown to be regulated (positively and negatively) by TGFβ2 (29,
Therefore, we examined NFκB activity using a NFκB-responsive luciferase reporter in transiently transfected NMuMG cells. The NM-kd cells showed high basal levels of NFκB reporter activity as compared with NM-con cells. Re-expression of the type III receptor in NM-kd cells reduced NFκB transcription to the same levels as those seen in NM-con cells (Fig. 6A). The ability of NFκB to bind to DNA was confirmed by electrophoretic mobility shift assays using 32P-radiolabeled oligonucleotides containing the putative NFκB-binding site (Fig. 6B). Interestingly, TGFβ2 blocked DNA binding in both cell lines. To examine the role of NFκB in the invasive phenotype seen in the NM-kd cells, we used an adenovirus containing either dominant negative IκBα (dn IκBα S32A/S36A) to abrogate NFκB function or a constitutively active IKK2 (CA-IKK2 D177E/D181E) to induce NFκB signaling. NM-kd and NM-con cells were transduced with adenoviruses, plated in growth factor reduced Matrigel 24 h later, and allowed to grow for 8 days. NM-kd cells infected with dn IκBα containing adenovirus showed a significant reduction of growth in Matrigel (Fig. 6, C and D). Transduction of adenoviruses encoding dn IκBα into NM-kd cells significantly diminished NFκB promoter activity. On the other hand, NM-kd cells infected with CA-IKK2 showed a significant increase in NFκB reporter activity that was not seen in NM-control cells (Fig. 6, E and F). This result implies that the endogenous type III TGFβ receptor may counteract the NFκB-inducing effect of CA-IKK2. It is significant to note that the reduction in NFκB activity did not reduce the ability of the NM-kd cells to grow in Matrigel down to that of the NM-con cells and that these cells still retain their invasive phenotype, suggesting the involvement of additional signaling pathways on the cells invasive behavior.

The decreased ability to grow in Matrigel corresponded to a significant increase in base-line E-cadherin promoter activity in...
NM-con versus NM-kd cells. Infection with an adenovirus containing dn IκBα up-regulated the low basal E-cadherin promoter activity in NM-kd but not in NM-con cells (Fig. 7, A and C). Additionally, NM-con cells infected with adenovirus containing constitutively active IKK2 showed a decrease in E-cadherin promoter reporter activity (Fig. 7, A and B), consistent with the role of NFκB signaling on EMT. In accordance with these results, E-cadherin mRNA, as measured by quantitative PCR, is significantly increased in NM-kd cells transduced with dn IκBα (Fig. 7C) with a corresponding decrease in Snail (Fig. 7D). These changes were not observed in the control cells (Fig. 7, C and D).

NM-kd Cells Form Invasive Tumors in Vivo—NMuMG cells are not able to grow and form tumors in athymic nude mice. Because of the apparent gain-of-function effects as a result of knock-down of TβRIII, we hypothesized that these cells would be able to form tumors in vivo. The cells were injected subcutaneously into the number 4 right inguinal mammary fat pad of nude mice. The mice were sacrificed 12 weeks later, and fat pads were harvested for further pathology. None of the mice injected with NM-con cells developed tumors. However, five of five mice injected with NM-kd cells formed high grade, poorly differentiated carcinomas with mesenchymal and fibroblastic features (Fig. 8). Macroscopic and microscopic examination of mouse lungs did not reveal any metastases.

**DISCUSSION**

It has recently been reported that the loss of TβRIII correlates with higher grade breast cancer, suggesting that this receptor acts as a tumor suppressor (8). Additionally, the loss of TβRIII has been associated with the development of renal cell carcinoma (31). Two recent reports have also suggested a tumor-suppressor role for TβRIII in prostate cancer (9, 32). The mechanisms by which TβRIII may exert an antitumor effect are not known. In contrast, TGFβ1, as well as all three TGFβ receptors, were found to be expressed at higher levels in high grade lymphomas compared with patients diagnosed with low grade lymphomas (5), suggesting a potential oncogenic role for TβRIII.

TGFβ signaling can act as a tumor suppressor or a tumor promoter depending on the cellular context. The molecular actions that result in the switch from suppressor to promoter are under intense investigation. The pro-oncogenic properties of TGFβ include increased motility and invasiveness and enhancement of EMT (33). Excess production of TGFβ in this
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**FIGURE 7. NFκB modulates E-cadherin expression in the NM-kd cells.**

A. Cells were infected with adenovirus containing either GFP alone (ad-GFP), ad-dn IκBα, or ad-CA-IKK2 and transfected with E-cadherin promoter luciferase and allowed to grow for 72 h. The cells were lysed and assayed for luciferase activity as described under “Experimental Procedures.” Each bar indicates the mean of three wells ± S.D. Western blot analysis was used to demonstrate infection efficiency of the adenovirus (B). Quantitative PCR was used to determine the mRNA level of E-cadherin (C) or Snail (D) in cells infected with adenovirus containing GFP or dn IκBα as described under “Experimental Procedures.” Each bar indicates the mean of three wells ± S.D.

**FIGURE 8. NM-kd cells form invasive tumors in vivo.**

A. Cell Line | No. of Tumors
---|---
NM-con | 0/5
NM-kd | 5/5
NM-kd & RIII | 3/5

B. NM-con and NM-kd tumors were examined by light microscopy. The cells were harvested, fixed in formalin, and embedded in paraffin followed by staining with hematoxylin and eosin. Representative low and high power hematoxylin and eosin-stained sections depicting tumor-free mammary gland from mice inoculated with control cells and a high grade carcinoma in a mouse injected with NM-kd cells.

The ability of TGFβ to induce EMT has been well documented, although the molecular mechanisms have still to be elucidated. It has been clearly demonstrated that TGFβ can induce the expression of Snail and Slug (35, 36). TGFβ1 treatment of epithelial cells results in a mitogen-activated protein kinase-dependent induction of Snail (35). A recent report demonstrates that Snail is required for TGFβ-induced EMT through activation of the phosphatidylinositol 3-kinase and Akt pathway (37). TGFβ2, specifically, has been shown to activate Slug during EMT required for the development of the chick heart (36). TGFβ2- and Slug-mediated EMT are dependent on the expression of the TGFβ type III receptor because blocking this receptor inhibits the initial stages of EMT (36). TGFβ can also directly modulate NFκB signaling. TGFβ inhibits NFκB activity in B cells (38), and NFκB has been reported to inhibit TGFβ signaling via up-regulation of the inhibitory Smad 7 (39), suggesting that there is cross-talk between these two pathways. Furthermore, secretion of FGF5 and TGFβ2 can induce NFκB activity, and this autocrine induction results in the constitutive activation of the NFκB pathway observed in many tumors (29).

The differential effects of TGFβ on epithelial cells may be dependent on the activation or inhibition of other oncoproteins or tumor suppressor genes. Oncogenic Ras has been shown to overcome the growth-inhibitory effects of TGFβ (40), and high expression of Ras and active Smad2 are required for metastasis (41). Additionally, the cells may be refractory to TGFβ growth inhibition but may still rely on TGFβ signaling for invasion. For example, it has been reported that the tumorigenic 4T1 cell line, which is not growth inhibited by treatment with TGFβ, requires functional TGFβ signaling for its ability to metastasize (42). Another report demonstrated that treatment of tumor cells with a truncated soluble TGFβ type II receptor had no effect on tumor cell growth but was able to block tumor metastases (43). In endothelial cells, TGFβ can signal through two distinct type I receptors, ALK1 and ALK5. ALK1 signaling through Smads 1/5 results in cell proliferation and migration, whereas ALK5 signaling through Smads 2/3 is growth-inhibitory (44). Goumans et al. (45) demonstrate that ALK5 signaling is required for ALK1 activation and that ALK1 signaling is in turn inhibitory of ALK5. In addition, endoglin, an endothelial cell-specific type III TGFβ receptor, is required to mediate the activation of ALK1 in these cells (46). In this report we present data to support a tumor suppressor role of TFBRIII in NMuMG setting results in an increase in extracellular matrix production, angiogenesis, and an increased production of matrix metalloproteinases (34).
nontumorigenic mammary cells. Decreased expression of TβRIII resulted in an increased growth rate both in monolayer and in three-dimensional Matrigel and increased motility and invasion through transwell filters. All of these effects on growth and motility were abrogated by expression of exogenous rat TβRIII in the NM-kd cells. Additionally, NM-kd cells were able to form invasive tumors in immunodeficient mice.

Our data are consistent with previous reports suggesting a tumor suppressor role for TβRIII in breast cancer. In this study, we report for the first time a mechanism by which this receptor inhibits cell growth and motility. We demonstrate that TβRIII inhibits growth and motility of NMuMG cells by repressing NfκB. The loss of TβRIII results in increased NfκB signaling and, in turn, transcriptional repression of E-cadherin resulting enhanced invasiveness in vitro and tumorigenesis in vivo. How TβRIII is modulating NfκB activity is less clear. Lu et al. (29) demonstrated that secreted TGFβ2 could activate NfκB. The loss of TβRIII results in diminished TGFβ2 expression (Fig. 2) and may therefore inhibit further the ability of TGFβ2 to activate NfκB in autocrine fashion.

Other signaling pathways may also be involved. It has recently been reported that TβRIII can activate p38 signaling in the absence of TGFβ ligand (47), suggesting that this receptor may interact with signaling pathways independent of TGFβ.

This is an intriguing speculation because TβRIII has no known signaling motif, although its C terminus has been shown to be necessary for TGFβ2-induced signaling (48). TβRIII may also modulate NfκB indirectly through interactions with other proteins. The cytoplasmic tail of TβRIII has been shown to bind the GTPase-activated protein Goi subunits interacting protein, C terminus, which stabilizes the expression of TβRIII at the cell surface (48), and β-arrestin 2, which is involved in the degradation of TβRIII (49), resulting in another layer of regulation of TGFβ signaling. There may be additional, yet to be identified, protein-binding partners for this receptor that may regulate signaling. An interaction of TβRIII with signaling pathways independent of TGFβ would also explain the incomplete reversal of the ability of NM-kd cells to grow in Matrigel when NfκB activity is diminished (Fig. 5).

In summary, the data presented herein suggest a role for TβRIII in regulating cell growth and motility. In nontumorigenic NMuMG mouse mammary epithelial cells, the loss of TβRIII resulted in an increased ability to grow and invade both in vitro and in vivo because of up-regulated NfκB activity and the loss of E-cadherin expression.

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