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## Expression of mutant human epidermal receptor 3 attenuates lung fibrosis and improves survival in mice

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**Nethery, David E., Bethany B. Moore, George Minowada, James Carroll, Jihane A. Faress, and Jeffrey A. Kern.** Expression of mutant human epidermal receptor 3 attenuates lung fibrosis and improves survival in mice. *J Appl Physiol* 99: 298–307, 2005. First published February 24, 2005; doi:10.1152/jappphysiol.01360.2004.—Neuregulin-1 (NRG-1), binding to the human epidermal growth factor receptor HER2/HER3, plays a role in pulmonary epithelial cell proliferation and recovery from injury in vitro. We hypothesized that activation of HER2/HER3 by NRG-1 would also play a role in recovery from in vivo lung injury. We tested this hypothesis using bleomycin lung injury of transgenic mice incapable of signaling through HER2/HER3 due to lung-specific dominant-negative HER3 (DNHER3) expression. In animals expressing DNHER3, protein leak, cell infiltration, and NRG-1 levels in bronchoalveolar lavage fluid increased after injury, similar to that in nontransgenic littermate control animals. However, HER2/HER3 was not activated, and DNHER3 animals displayed fewer lung morphological changes at 10 and 21 days after injury ( $P = 0.01$ ). In addition, they contained 51% less collagen in injured lungs ( $P = 0.04$ ). Transforming growth factor- $\beta_1$  did not increase in bronchoalveolar lavage fluid from DNHER3 mice compared with nontransgenic littermate mice ( $P = 0.001$ ), suggesting that a mechanism for the decreased fibrosis was lack of transforming growth factor- $\beta_1$  induction in DNHER3 mice. Severe lung injury (0.08 units bleomycin) resulted in 80% mortality of nontransgenic mice, but only 35% mortality of DNHER3 transgenic mice ( $P = 0.04$ ). Thus inhibition of HER2/HER3 signaling protects against pulmonary fibrosis and improves survival.

lung injury; bleomycin; growth factors; receptor tyrosine kinases; neuregulin

ACUTE LUNG INJURY ELICITS a variety of epithelial cell responses, including the upregulation of growth factors (20, 32), suggesting a role for this class of proteins in recovery from lung injury. In rodent lungs, increased levels of epidermal growth factor (EGF) and transforming growth factor (TGF)- $\alpha$  are found in airways and alveolar epithelia following intratracheal instillation of asbestos, naphthalene, or bleomycin (19, 20, 22, 35). In addition, EGF receptor (EGFR) expression is increased after bleomycin-induced lung injury (20). A direct role of epithelium-derived growth factors in lung injury was shown by Madtes et al. (21), who found that TGF- $\alpha$  knockout mice developed less pulmonary fibrosis than wild-type control mice after bleomycin-induced injury. Growth factors also impact lung fibrosis in noninjury models. Korfhagen and colleagues (7, 14) con-

structed transgenic mouse strains that expressed human TGF- $\alpha$  in type II pneumocytes conditionally or constitutively by placing a TGF- $\alpha$  cDNA under the control of the human surfactant protein C (SP-C) promoter. Adult transgenic mice spontaneously developed severe pulmonary fibrosis that was directly proportional to the level of TGF- $\alpha$  expression (8, 14). When TGF- $\alpha$ -overexpressing mice were bred to a transgenic mouse strain expressing a dominant-negative EGFR in the pulmonary epithelium, TGF- $\alpha$ -induced pulmonary fibrosis was decreased (6). In contrast, other studies have shown that inhibition of EGFR activation augments pulmonary fibrosis. Suzuki et al. (33) demonstrated that blocking EGFR phosphorylation with the EGFR-specific tyrosine kinase inhibitor ZD1839 increased bleomycin-induced fibrosis. In their study, blockade of EGFR activation reduced regenerative epithelial cell proliferation, but had no effect on proliferation of lung fibroblasts. Taken together, these data suggested that pulmonary fibrosis occurred, in part, through growth factor receptor and ligand interaction in the pulmonary epithelium.

Along with EGFR (HER1), human EGFR (HER2), HER3, and HER4 comprise the EGFR receptor tyrosine kinase family. In the lung, HER2, HER3, and the ligand neuregulin-1 (NRG-1) are also important mediators of pulmonary epithelial cell proliferation (18, 34, 36). Upon ligand binding, HER3 heterodimerizes with HER2, forming a high-affinity receptor for NRG-1. Subsequent phosphorylation of receptor intracellular tyrosines initiates intracellular signaling cascades. Our laboratory's recent in vitro studies have shown that, in transformed pulmonary epithelial cell lines, NRG-1/HER2/HER3 interaction is an autocrine process (5). In addition, HER2 is activated on disruption of a pulmonary epithelial cell monolayer, and receptor activation reduces the time to monolayer reformation, suggesting a role for this autocrine process in recovery of the injured pulmonary epithelium (36). In human tissue, our laboratory has also shown that NRG-1 induces pulmonary epithelial cell proliferation (25). Based on these studies, we hypothesized that activation of the HER2/HER3 receptor would influence recovery from lung injury in vivo. To test this hypothesis, our strategy was to study the lung's response to injury in transgenic mice with lung-specific expression of a dominant-negative HER3 receptor (DNHER3) that selectively blocks HER2/HER3 signaling. The DNHER3 receptor was

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expressed specifically in the pulmonary epithelium under the control of the human SP-C promoter and completely inhibited NRG-1-induced HER2 signaling.

Our studies found that, in the normal lung, bleomycin injury induced NRG-1 production, HER2 activation [demonstrated by phosphorylated HER2 (pHER2)], protein leak, inflammatory cell infiltration, and collagen deposition. In DNHER3 mice, HER2 receptor activation did not occur after lung injury, although NRG-1 production was induced similar to nontransgenic littermates. The lungs of DNHER3 mice had decreased collagen deposition 10 and 21 days after injury compared with nontransgenic littermate control mice. Finally, when challenged with a high dose of bleomycin, mice expressing DNHER3 had a clear survival advantage compared with nontransgenic littermate mice.

## MATERIALS AND METHODS

**Mice.** All experimental procedures were approved by the Case Western Reserve University (CWRU) Institutional Animal Care and Use Committee. C57BL/6J transgenic mice expressing DNHER3 in the lung and nontransgenic littermates were housed in the CWRU Animal Resource Center under specific pathogen-free conditions in enclosed filter-topped cages. Food and water were allowed ad libitum. All mice were observed daily by veterinarians and were maintained and handled using microisolator techniques.

**Construction of a DNHER3 receptor.** Using PCR techniques, a DNHER3 cDNA was constructed from a full-length HER3 cDNA template. The HER3 extracellular and transmembrane domains were left intact, whereas the intracellular domain was deleted, starting 20 amino acids beyond the transmembrane domain (F = 5'-AAAAGTCGACGGAGTCATGAGGGCGAACGACGCTCTG-3', R = 5'-TTTTTGTGCGACTTATTTGTCGTCGTCGTCCTTTGTAGTCAGCCCTTTTATTCTGAAT-3'). The carboxy terminus was epitope tagged with a FLAG sequence for Western blot identification. DNHER3 cDNA produced from PCR reactions was separated on a 1% agarose/Tris-acetate-EDTA gel, extracted (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA), and ligated into pcDNA3 (Invitrogen, Carlsbad, CA). *Escherichia coli* (HB101 competent cells, Invitrogen) were transformed with DNHER3/pcDNA3 and selected for ampicillin resistance. DNA from individual colonies was analyzed by *Eco*R I digestion, and clones that yielded restriction fragments predicted for DNHER3 were expanded and isolated (QIAfilter Plasmid Maxi Kit, Qiagen). For in vitro characterization, COS-7 and A549 cells were transfected with the DNHER3/pcDNA3 construct using lipofectamine (Invitrogen), according to the manufacturer's instructions.

**Production of transgenic mice expressing lung-specific DNHER3.** DNHER3 cDNA was cloned into an expression vector under the control of the human 3.7-kb SP-C promoter (a gift from Drs. Jeffrey Whitsett and Stephan Glasser, University of Cincinnati). Following cloning and expansion, the backbone of the SP-C plasmid was removed by endonuclease digestion to yield a transgene consisting of the SP-C promoter, DNHER3 cDNA, FLAG tag, SV40 small T intron, and a poly(A) tail. Pronuclear injection of purified DNA was performed by the CWRU Transgenic Core Facility to produce transgenic C57BL/6J mice. Founders with lung-specific expression of DNHER3 were used to develop DNHER3 strains. Genotyping for DNHER3 in the hemizygous F<sub>1</sub> generation was performed on extracted tail DNA using primers directed against HER3 and the FLAG sequence (F = 5'-TGGGCCCCACTGTGTGAGCAGCTGCCCCCA-3', R = 5'-GGATCCTCTAGAGTCGACTTA TTTGTCGTCGTCGTCCTTTGTAGTC-3').

**Bleomycin lung injury.** Intratracheal bleomycin injection was used as a model of lung injury and fibrosis (15–17). DNHER3 transgenic mice or nontransgenic littermates were anesthetized with an intraperi-

toneal injection of a mixture of ketamine HCl, xylazine HCl, and acepromazine in sterile PBS. A single incision was made in the neck, and the trachea was exposed. A 30- $\mu$ l injection containing 0.025 or 0.08 units of bleomycin (Sigma, St. Louis, MO) diluted in sterile PBS was injected into the trachea using a Tridak stepper (Indicon, Brookfield, CT) and a 30-gauge needle. The skin was then closed with surgical glue, and the animals were allowed to recover.

**Bronchoalveolar lavage fluid and lung collection.** At specific time points postsurgery (0, 3, 7, 10, 14, or 21 days), mice were killed by CO<sub>2</sub> asphyxiation. A midline thorax to neck incision was made, the ribs were removed, the trachea was exposed, and a blunt-end 21-gauge needle was inserted and tied into the trachea. Blood was flushed from the pulmonary capillary bed by injecting PBS into the right ventricle and letting it drain out of a needle inserted into the left ventricle. The lungs were lavaged with sterile PBS ( $\times 3$ , 0.5 ml each), the collected bronchoalveolar lavage fluid (BALF) was centrifuged (1,500 rpm, 10 min, 4°C), and the supernatant was stored for analysis. Cells in the BALF were applied to slides using a Cytospin centrifuge (ThermoShandon, Pittsburgh, PA). Slides were dried overnight and stained with modified Wright stain (Diff-Quik, Dade Behring, Deerfield, IL), and cell count was performed on 150–200 nucleated cells. After BALF collection, the lungs were removed, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for subsequent biochemical analyses.

**Western blot analysis.** Lungs were homogenized in cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100) containing protease inhibitors (50  $\mu\text{g}/\text{ml}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin, 50  $\mu\text{g}/\text{ml}$  pepstatin, 0.4 mM EDTA, 0.4 mM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate) using a Polytron homogenizer. Protein concentrations of the lung homogenates and BALF were determined by the Bradford method (Bio-Rad Laboratory, Hercules, CA). Equal protein amounts were added to Laemmli sample buffer and boiled for 5 min. Proteins were separated by electrophoresis on 7.5 or 10% SDS-PAGE gels (Bio-Rad) and electroblotted onto polyvinylidene difluoride membranes. Nonspecific binding was blocked by incubating blots in 5% nonfat dry milk or 3% BSA in PBS/Tween 20 at 4°C. Membranes were probed with NRG-1 polyclonal antibody (C-20), HER2 polyclonal antibody (C-18), phosphorylated-HER2 polyclonal antibody (Tyr 1248), HER3 polyclonal antibody (C-17; all from Santa Cruz Biotechnology, Santa Cruz, CA), or FLAG polyclonal antibody (Affinity Bioreagents, Golden, CO). Following incubation with secondary antibodies conjugated to horseradish peroxidase (1:5,000 dilution; Santa Cruz Biotechnology), specific protein bands were detected by enhanced chemiluminescence autoradiography (Amersham, Piscataway, NJ). Relative amounts of individual protein bands were quantified by analysis of digitized images using National Institutes of Health Image software.

For immunoprecipitation studies, cell lysates containing 200  $\mu\text{g}$  of protein were incubated with appropriate antibody (2  $\mu\text{g}$  for 1 h at 4°C). The antibody-protein complexes were then incubated with protein A beads for 1 h at 4°C with mixing. The beads were washed three times in PBS and resuspended in Laemmli sample buffer. Western blot analysis was conducted as described above.

**Histological preparation.** Lungs from transgenic DNHER3 mice and nontransgenic littermate controls were prepared for histology by inflation fixation. Following death, the animal's chest was opened, and the ribs were removed to allow uncompromised lung inflation. The trachea was cannulated, and the lungs were inflated and deflated three times with 10% formalin (1 ml) by means of a 3-ml syringe. The cannula was then attached to a reservoir containing 10% Formalin, and the lungs were fixed for 1 h at 10-cm pressure. At the conclusion of fixation, lungs were removed en bloc and transferred to a cassette for paraffin embedding. Following embedding, 5- $\mu\text{m}$  sections were cut, transferred to slides, and stained with hematoxylin and eosin (H&E).

**Pathological scoring of H&E-stained lung sections.** Lung sections stained with H&E were scored to determine the relative volume densities of epithelia, mesenchyme, and air space (25, 37). Following

staining, random fields from all sections were digitally photographed at  $\times 200$  magnification using a SPOT camera (Diagnostic Instruments, Sterling Heights, MI) mounted to a Nikon Optiphot-2 microscope (Nikon, Melville, NY). Images of two random fields from each animal (3 nontransgenic littermates and 3 DNHER3 mice for each of 0-, 10-, and 21-day bleomycin time points) were overlaid with an  $11 \times 11$  grid. The area underlying each grid intersection was classified as mesenchyme, epithelia, or air space. Cells directly bordering air space were classified as epithelia, whereas cells separated from the air space by intervening cell layers were classified as mesenchyme. A total of 121 intersection points was counted for each field, and 242 intersection points were counted for each animal. The total number of mesenchyme, epithelia, and air space points was divided by 242 to determine the volume density of each category for each animal at each time point. Data were averaged to determine volume densities for nontransgenic and DNHER3 mice for each condition.

**Collagen assay.** Total soluble collagen content was determined by using the Sircol Collagen Assay (Biocolor, Newtownabbey, UK). Lungs were homogenized in 1 ml of complete lysis buffer and centrifuged for 10 min (10,000 g, 4°C). Fifty microliters of supernatant were added to 50  $\mu$ l of 0.5 M acetic acid and 1 ml of Sircol dye reagent. Samples were mixed for 30 min at room temperature to allow the formation of dye-collagen complexes. Samples were centrifuged at 10,000 g to pellet the complexes. Bound dye was then solubilized in 1 ml of 0.5 M NaOH and analyzed spectrophotometrically at 540 nm. Collagen concentration was determined by comparison to a standard curve constructed using known amounts of type I collagen.

**TGF- $\beta_1$  bioassay.** TGF- $\beta_1$  content was measured in BALF by using a reporter gene bioassay (19). Mink lung epithelial cells permanently transfected with a construct containing the TGF- $\beta_1$  responsive human plasminogen activator inhibitor-1 promoter fused to a luciferase reporter gene (a gift from Daniel Rifkin, NYU Medical Center, New York, NY) were used. These cells were seeded into individual wells of 12-well tissue culture plates ( $10^5$  cells/well) in DMEM with 10% FCS and allowed to adhere overnight, followed by 24 h of serum starvation. Cells were then stimulated with 500  $\mu$ l of BALF collected from DNHER3 or nontransgenic littermate mice. Cells were lysed 24 h after BALF exposure, and luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI). TGF- $\beta_1$  content was quantified from luciferase activity using a standard curve constructed from reporter cells exposed to known amounts of TGF- $\beta_1$ .

**Survival analysis.** Survival following lung injury was evaluated by increasing the intratracheal bleomycin dose from 0.025 to 0.08 units. Mice were injected with bleomycin, as described above, allowed to recover, and then monitored twice a day until death.

**Statistics.** An unpaired *t*-test was used to compare single variables across animal groups to determine statistical differences. All data are presented as means (SD). Data from survival studies were plotted using the Kaplan-Meier method and analyzed by log rank test. A *P* value of  $<0.05$  was taken to indicate statistical significance.

## RESULTS

**Deletion of the HER3 cytoplasmic domain produces a dominant-negative receptor.** Our *in vitro* studies identified that HER2 is activated on disruption of a pulmonary epithelial cell monolayer, receptor activation reduces the time to monolayer reformation, and NRG-1 treatment induces pulmonary epithelial cell proliferation in human tissue. These findings led us to hypothesize that the NRG-1/HER2/HER3 ligand-receptor axis was involved in recovery from lung injury *in vivo*. To test this hypothesis, we chose to inhibit HER2/HER3 activation following injury by using a dominant-negative receptor strategy with a mutant HER3 (DNHER3). Mutation of HER3 was chosen over HER2 because previous studies had shown that HER2

activation required HER3's intracellular domain (27, 28). By deleting the HER3 intracellular domain, HER2/HER3 receptor complex formation could occur, but HER2 activation (i.e., phosphorylation) would be eliminated (26). In addition, HER3 is expressed at levels 10-fold lower than HER2 and is rate limiting in receptor formation. Thus high-level expression of a DNHER3 would strongly favor HER2/DNHER3 heterodimer formation. Using standard PCR techniques, a DNHER3 was developed from a full-length human HER3 cDNA template. The salient features of the DNHER3 cDNA are intact extracellular and transmembrane domains, a deleted intracytoplasmic domain, and an epitope tag (FLAG sequence) at the carboxy terminus (Fig. 1A).

The DNHER3 cDNA was cloned into the pcDNA3 expression vector under the control of a cytomegalovirus promoter. The pcDNA3/DNHER3 construct was transiently transfected into COS-7 and A549 cells for *in vitro* characterization. These initial studies verified that 1) the expressed DNHER3 protein contained an extracellular HER3 epitope and the FLAG epitope (Fig. 1B); 2) DNHER3 correctly localized to the cell membrane; and 3) in A549 cells, which express HER2 and HER3 and are NRG-1 responsive, transfection with DNHER3 abrogated the response to NRG-1, as evidenced by decreasing HER2 tyrosine phosphorylation with increasing DNHER3 expression (Fig. 1C, pHER2).

**Construction and characterization of transgenic mice expressing DNHER3 in the lung.** With verification that the mutant HER3 acted in a dominant-negative fashion, we cloned the DNHER3 cDNA into an expression vector under the control of the human SP-C promoter, thus enabling lung-specific expression of DNHER3 in SP-C-expressing cells. The DNHER3 transgene, consisting of the SP-C promoter, DNHER3 cDNA, FLAG, SV40 small T intron, and a poly(A) tail, was recovered by restriction endonuclease digestion, purified, and sequenced. Transgenic mice were then produced by standard pronuclear injection techniques using this DNA. Three different founder mice with germ line integration of DNHER3 DNA were identified. The lines generated from these founders were designated 3, 15, and 23. Western blot analyses for the FLAG epitope in lung homogenates from F<sub>1</sub> generation mice from each of these lines showed different levels of DNHER3 expression. *Line 15* exhibited the highest level of DNHER3 expression in the lung, and all subsequent experimentation was conducted using hemizygous mice from this line.

A human HER3 cDNA was used to generate the DNHER3 transgene. As in humans, both HER2 and HER3 are normally expressed in rodent lungs. The amino acid sequences of murine and human HER2 and HER3 are highly conserved; murine HER2 is 87.5% homologous to human HER2, and rodent HER3 is 85–90% homologous to human HER3 (10, 13, 31). No known functional differences exist between rodent and human HER2/HER3.

To verify that DNHER3 expression was restricted to the lung, protein samples from a transgenic F<sub>1</sub> animal were analyzed. Only lung tissue expressed FLAG protein (Fig. 2A). In addition, the FLAG-containing protein had a mass corresponding to the predicted molecular weight of DNHER3, identifying it as DNHER3. DNHER3 retained its ability to dimerize with HER2. Homogenates of total lung proteins were immunoprecipitated with a HER2 antibody and Western blotting per-

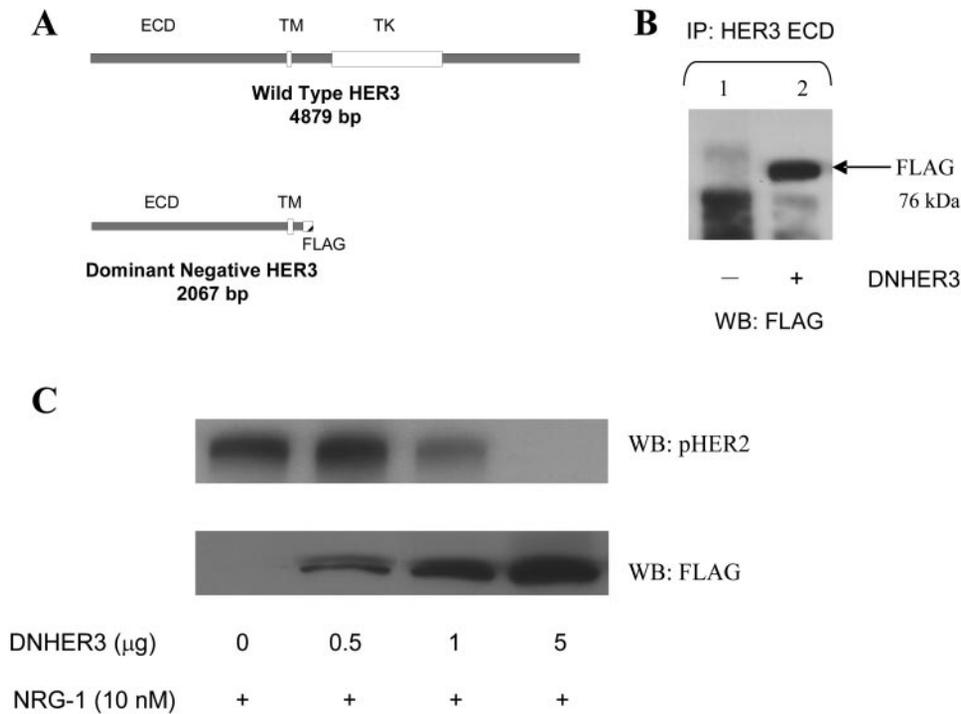


Fig. 1. In vitro characterization of a dominant-negative HER3 (DNHER3) construct. **A:** schematic representation of the DNHER3. Wild-type HER3 cDNA composed of a ligand-binding extracellular domain (ECD), transmembrane (TM) domain, and cytoplasmic domain containing the tyrosine kinase (TK) region was used as a template for the construction of DNHER3. DNHER3 consists of intact ECD and TM domains, but with the majority of the cytoplasmic domain deleted. A FLAG epitope sequence was added to the carboxy terminus of the intracellular domain. **B:** protein lysates from COS-7 cells, with and without transfection, were immunoprecipitated (IP) with an antibody recognizing HER3 extracellular epitope and Western blotted (WB) for FLAG. **C:** HER2 and HER3 expressing A549 cells were transfected with increasing concentrations of DNHER3, stimulated with 10 nM neuregulin-1 (NRG-1), lysed, and Western blotted for phosphorylated HER2 (pHER2) to demonstrate DNHER3 function. DNHER3 expression was confirmed by Western blotting for FLAG.

formed with a FLAG antibody. The presence of a positive FLAG-staining protein band indicated that DNHER3 still associated with HER2 (Fig. 2B).

As a test of DNHER3 function, freshly dissected lung from DNHER3-expressing mice was minced and incubated in NRG-1 (10 nM) to determine the effect on receptor activation. After various incubation times, lung tissue was homogenized and subjected to Western blot analysis for pHER2. In lung tissue from DNHER3 mice, HER2 was not activated by

NRG-1 exposure at any time point studied (Fig. 2C). However, NRG-1 activated HER2 in lung tissue from nontransgenic F<sub>1</sub> littermates (no DNHER3 expression), as evidenced by increased levels of pHER2 [2.07 (SD 0.11) and 1.76-fold (SD 0.13) increase at 10 and 20 min of activation, respectively].

*NRG-1 is produced and HER2 is activated in the lung following bleomycin injury.* Our initial in vivo study defined the kinetics of NRG-1 production and HER2 activation after injury of the normal lung. To induce acute lung injury,

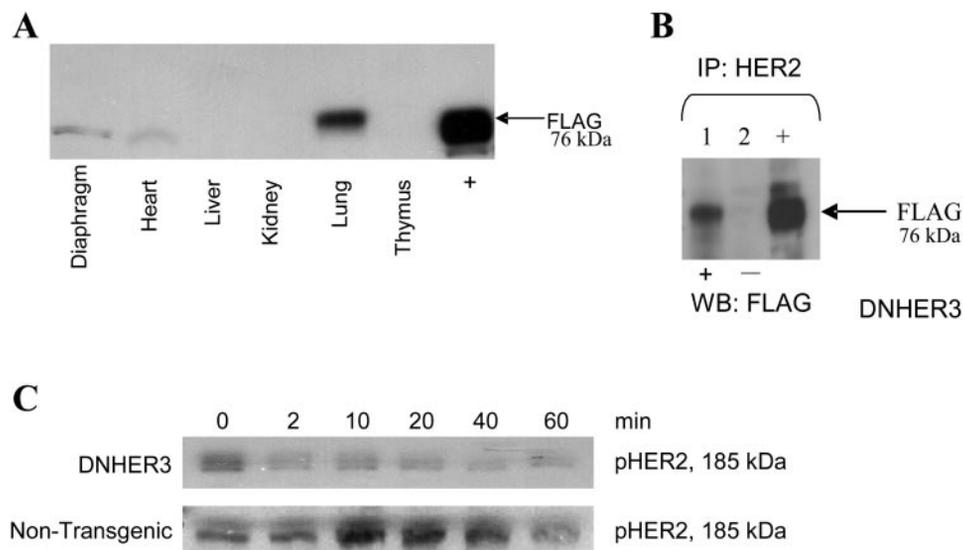


Fig. 2. Characterization of DNHER3 in transgenic mice. **A:** Western blot of tissues from F<sub>1</sub> offspring of a transgenic male founder. Protein homogenates from diaphragm, heart, liver, kidney, lung, and thymus were subjected to Western blot analysis for FLAG epitope. FLAG immunoreactivity was only detected in lung tissue and was of the predicted molecular mass for DNHER3 (76 kDa). **B:** lung homogenates from a DNHER3 mouse (lane 1) and a nontransgenic littermate (lane 2) were immunoprecipitated with a HER2 antibody and subjected to Western blot analysis for the FLAG epitope. The positive FLAG staining protein band in lane 1 indicates dimerization between HER2 receptor and FLAG-expressing DNHER3. **C:** freshly dissected lungs from DNHER3 transgenic mice or nontransgenic littermates were incubated in 10 nM NRG-1 for 0, 2, 10, 20, 40, or 60 min and then homogenized in lysis buffer. Homogenates were subjected to Western blot analysis for pHER2 ( $n = 3$ ). +, Positive FLAG control consisting of cell lysates from COS-7 cells transfected with DNHER3.

Table 1. Protein leak in nontransgenic and DNHER3 mice

Bleomycin Injury Time	BALF Protein Concentration, $\mu\text{g}/\mu\text{l}$	
	Nontransgenic	DNHER3
0 Days	0.07 (SD 0.05)	0.06 (SD 0.04)
3 Days	0.80 (SD 0.27)	0.20 (SD 0.15)*
7 Days	1.19 (SD 0.28)	0.95 (SD 0.70)
10 Days	1.74 (SD 0.44)	1.26 (SD 0.56)
14 Days	1.68 (SD 0.22)	1.34 (SD 0.23)
21 Days	1.62 (SD 0.38)	1.73 (SD 0.89)

Values are means (SD);  $n = 4$ . BALF, bronchoalveolar lavage fluid; DNHER3, dominant-negative HER3. \* $P = 0.008$  for the comparison of DNHER3 to nontransgenic littermates.

nontransgenic C57BL6/J littermates were injected intratracheally with 0.025 units of bleomycin. Mice were killed at 0, 3, 7, 10, 14, or 21 days postinjection (3 per time point), BALF was collected, and lung tissue was homogenized for protein analysis (*day 0* mice were not exposed to bleomycin).

Bleomycin exposure resulted in protein leak, as evidenced by increased BALF protein (Table 1). Nontransgenic mice studied 21 days after bleomycin injury had BALF protein concentrations  $\sim 23$  times higher than uninjured mice ( $P = 0.001$ ). Total white blood cell count increased steadily over time following bleomycin, becoming eightfold higher at 21 days than at *day 0* (Table 2). Neutrophil recruitment peaked on *day 3* postinjury, and lymphocyte recruitment peaked on *day 10*.

Bleomycin lung injury induced NRG-1 secretion into BALF. As shown in Fig. 3A, only trace amounts of NRG-1 were detected in BALF collected from uninjured mice. Bleomycin injury induced a 7.3-fold (SD 1.61) increase in NRG-1 levels present in BALF by *day 3* ( $P = 0.0009$ ). This increase was sustained even 21 days postinjury with NRG-1 concentration 9.6-fold (SD 0.21) higher ( $P = 0.001$ ).

Bleomycin lung injury also resulted in HER2 activation (Fig. 3B, left). Low levels of pHER2 were detected in lungs not exposed to bleomycin (*day 0*). Bleomycin injection produced a 1.3-fold (SD 0.28) increase in HER2 phosphorylation at 3 days postinstillation ( $P = 0.03$ ). HER2 activation continued to increase, with maximum activation at *day 10*, 2.4 (SD 0.78) times higher than at *day 0* ( $P = 0.01$ ). Receptor activation decreased thereafter, but remained greater than baseline. Total receptor mass, as defined by levels of HER2 and its dimerization partner HER3, increased slightly on *day 3* [40% (SD 15)], but then was unchanged throughout the course of bleomycin exposure.

*Injury induces NRG-1 production but not HER2 activation in DNHER3 mouse lungs.* The response of DNHER3 transgenic mice to bleomycin injury was studied next. We first examined the production of NRG-1 by DNHER3 mice to ensure that any changes in response to injury were due to the presence of DNHER3 and not confounded by alterations in ligand production. As observed in wild-type mice, bleomycin lung injury resulted in an increase in soluble protein detected in BALF [Table 1; 29-fold (SD 9) at 21 days;  $P = 0.01$ ], and an influx of white blood cells into BALF [Table 2; 5.1-fold (SD 0.8) increase at 21 days]. These changes over time paralleled those observed in nontransgenic littermate mice and indicated

that the level of injury induced by bleomycin was similar in wild-type and DNHER3 mice.

NRG-1 in BALF also increased in response to bleomycin treatment, with NRG-1 levels 11-fold (SD 4.1) higher than baseline at 10 days postinjection (Fig. 3A), similar to the response in nontransgenic littermates. Low-level basal phosphorylation of HER2 was present, similar to levels seen in nontransgenic littermate animals. However, bleomycin injury did not induce receptor activation at any time point examined (Fig. 3B, right). Therefore, expression of DNHER3 effectively blocked in vivo signaling through the HER2/HER3 receptor complex.

*DNHER3 expression prevents injury-induced inflammation and collagen deposition.* Over time, bleomycin-induced lung injury produces fibrosis (9, 11, 29). To determine the role of HER2 receptor activation in the fibrotic response, lungs from bleomycin-injured nontransgenic littermate and DNHER3 transgenic mice were used to determine collagen content and fixed for histological examination. Figure 4 displays representative H&E stains of lung sections from nontransgenic littermates and transgenic DNHER3 mice killed at 0, 10, or 21 days postbleomycin injury. Lungs from both littermate controls and DNHER3 mice showed no cellular infiltration or morphological abnormalities before bleomycin exposure. By 10 days following bleomycin injury, nontransgenic lungs had significant inflammatory cell and fibroblast infiltration into alveolar spaces. By 21 days, the vast majority of alveolar space was taken up by the cellular infiltrate. Epithelia and air space volume density decreased in response to bleomycin injury (Table 3;  $P = 0.048$  and  $P = 0.0018$ , respectively), while mesenchyme volume density increased dramatically ( $P = 0.0001$ ).

DNHER3-expressing mouse lungs showed a very different response to injury. The cellular infiltration was markedly reduced at both 10 and 21 days postbleomycin injury compared with wild-type mice, with large areas of normal-appearing lung present at 21 days (Fig. 4). Epithelia volume density was decreased, but not significantly, while air space volume density decreased ( $P = 0.015$ ) and mesenchyme volume density increased ( $P < 0.01$ ; Table 3). However, at 21 days postbleomycin injury, mesenchymal cell expansion was less in DNHER3 than in nontransgenic littermate control mice: 35.2 (SD 2.90) vs. 61.6% (SD 0.21) ( $P = 0.01$ ). In addition, air

Table 2. Analysis of BALF from bleomycin-treated nontransgenic and DNHER3 mice: cell counts

	Total BALF WBC	% of Total			
		Macrophages	Lymphocytes	Neutrophils	Monocytes
Nontransgenic					
0 Days	38,000	82.1	8.5	1.0	0.6
3 Days	76,000	71.5	11.3	5.4	
10 Days	228,000	50.4	39.9	3.2	1.8
21 Days	314,000	66.0	28.1	2.9	0.8
DNHER3					
0 Days	48,000	82.3	7.2	0.9	
3 Days	82,000	53.5	18.7	15.9	
10 Days	220,000	62.7	31.2	2.4	0.8
21 Days	242,000	67.9	27.3	2.0	1.0

WBC, white blood cells.

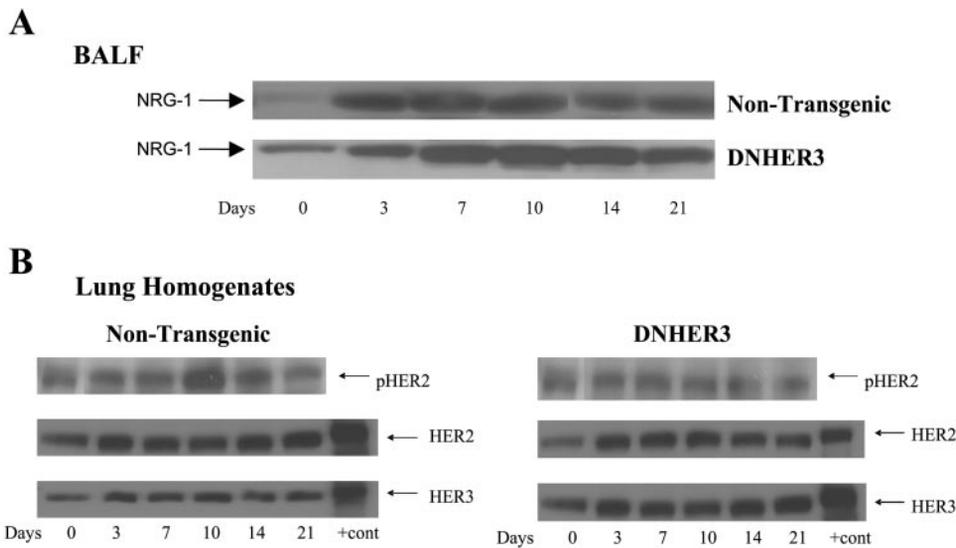


Fig. 3. NRG-1 secretion and HER2 receptor activation after injury. *A*: bronchoalveolar lavage fluid (BALF) from nontransgenic littermates (*top*) and DNHER3 mice (*bottom*) collected 0, 3, 7, 10, 14, or 21 days following bleomycin instillation was subjected to Western blot analysis for NRG-1. Blots shown are representative of 3 experiments. *B*: lungs taken from bleomycin-injured nontransgenic littermates and DNHER3 mice were homogenized in lysis buffer containing protease inhibitors and subjected to Western blot analysis for HER2 activation, as defined by tyrosine pHER2. Lung proteins were also analyzed for total HER2 and HER3 to verify stability of total receptor mass. Blots are representative of 3 experiments.

space volume density decreased to a lesser extent in DNHER3 mice than in wild type: 47.1 (SD 0.42) vs. 23.0% (SD 2.55) ( $P = 0.004$ ).

Lungs from nontransgenic littermate and DNHER3 transgenic mice were next homogenized and assayed for total collagen content (Fig. 5). In nontransgenic mice, lung collagen content rose over the entire 21-day study period to 148.03  $\mu\text{g}/\text{mg}$  lung protein (SD 38.71). In DNHER3 mice, lung collagen content was the same as in control mice at *day 10*. However, 21 days after bleomycin injury, DNHER3 lung collagen was 72.47  $\mu\text{g}/\text{mg}$  lung protein (SD 20.42), one-half that of wild-type mice ( $P = 0.04$ ).

*Injury-induced TGF- $\beta_1$  production is ablated in DNHER3 transgenic mice.* We hypothesized that HER2 signaling during injury regulated profibrotic cascades. Therefore, loss of signaling in the DNHER3 strain would explain the decrease in fibrosis. Principal among fibrotic mediators is TGF- $\beta_1$  (9, 11, 30). To determine whether the loss of HER2 signaling affected the TGF- $\beta_1$  response, BALF from DNHER3 transgenic mice and nontransgenic littermates was assayed for total TGF- $\beta_1$  content using a luciferase reporter gene bioassay (Fig. 6A). In nontransgenic mice, bleomycin injury induced TGF- $\beta_1$  in BALF. Ten days after bleomycin instillation, TGF- $\beta_1$  level was 2.8-fold (SD 0.61) higher than that of uninjured mice [2.47

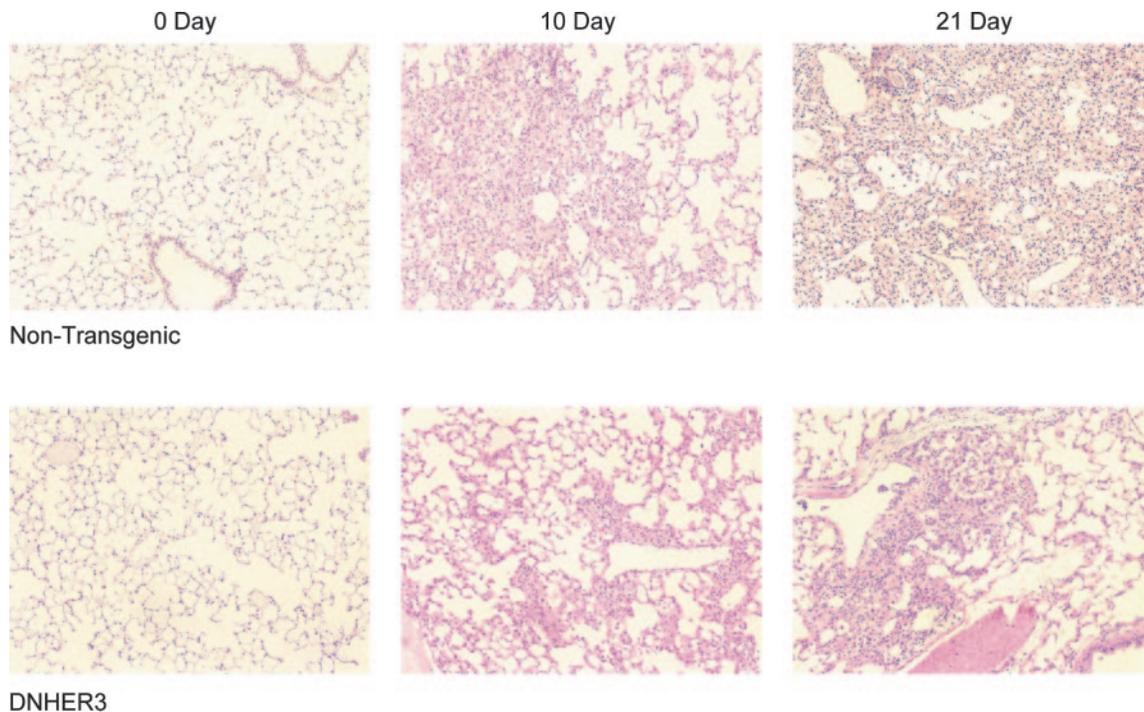


Fig. 4. Hematoxylin and eosin staining of bleomycin-injured lungs. Lungs from wild-type and DNHER3 mice were fixed at 10 cmH<sub>2</sub>O with 10% formalin at 0, 10, or 21 days post-bleomycin injury. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Stained sections from wild-type mice are shown in the *top* panels, and sections from DNHER3 mice are shown in the *bottom* panels.

Table 3. Volume density in nontransgenic and DNHER3 mice

Bleomycin Treatment Time	Fraction	Volume Density, %	
		Nontransgenic	DNHER3
0 Days	Epithelia	24.2 (SD 0.35)	26.4 (SD 1.56)
	Mesenchyme	4.5 (SD 0.92)	4.5 (SD 0.64)
	Air space	71.2 (SD 1.41)	69.0 (SD 2.19)
21 Days	Epithelia	15.4 (SD 2.83) <sup>†</sup>	17.8 (SD 2.55)
	Mesenchyme	61.6 (SD 0.21) <sup>†</sup>	35.2 (SD 2.90) <sup>†*</sup>
	Air space	23.0 (SD 2.55) <sup>†</sup>	47.1 (SD 0.42) <sup>†*</sup>

Values are means (SD);  $n = 4$ . <sup>†</sup> $P < 0.05$  compared with 0 days; \* $P < 0.01$  compared with nontransgenic littermates.

(SD 0.31) and 0.89 ng/ml (SD 0.13), respectively;  $P = 0.001$ , and, by 21 days, TGF- $\beta_1$  was 2.5-fold (SD 0.25) higher [2.21 ng/ml (SD 0.13);  $P = 0.001$  compared with uninjured mice]. DNHER3 mice showed a very different response. Injury did not increase TGF- $\beta_1$  in BALF. Although absolute levels of TGF- $\beta_1$  were higher at *day 0* in DNHER3 mice than in nontransgenic littermate controls [2.93 (SD 0.35) and 0.89 ng/ml (SD 0.13), respectively], they did not increase with injury. The level of TGF- $\beta_1$  in BALF from DNHER3 mice 21 days after bleomycin injection was the same as on *day 0*.

Lung homogenates from nontransgenic littermate and DNHER3 mice were analyzed for TGF- $\beta$  receptor levels by Western blotting to determine whether the lack of response to TGF- $\beta_1$  in DNHER3 animals was due to changes in TGF- $\beta$  receptor expression. Figure 6B shows that TGF- $\beta$  receptor I and II levels were the same in nontransgenic littermate and DNHER3 mice before injury (*day 0*). Following injury, no differences were observed between groups in either TGF- $\beta$  receptor.

*DNHER3 expression improves survival following lung injury.* With decreased fibrosis, we postulated that DNHER3 expression may also decrease mortality following severe lung injury. To test this hypothesis, we induced lung injury with a

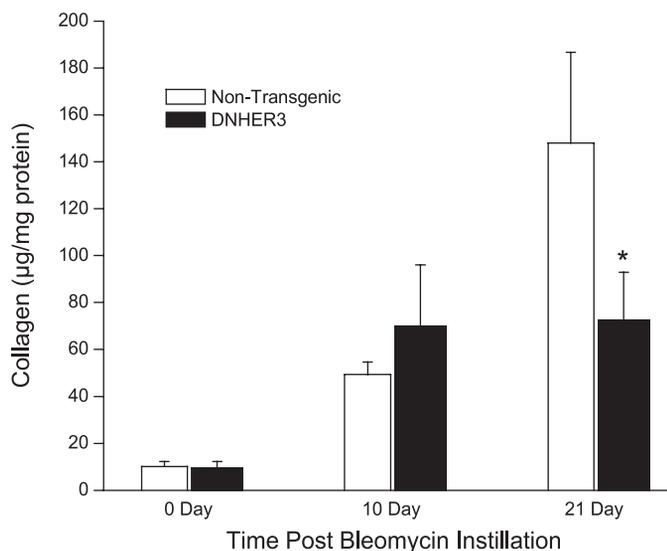


Fig. 5. Collagen deposition is decreased in DNHER3 mice. Total collagen formation is shown in lungs from nontransgenic littermates and DNHER3 mice killed at 10 or 21 days following bleomycin injection. Values are means (SD);  $n = 3$  for each group. \* $P = 0.04$ .

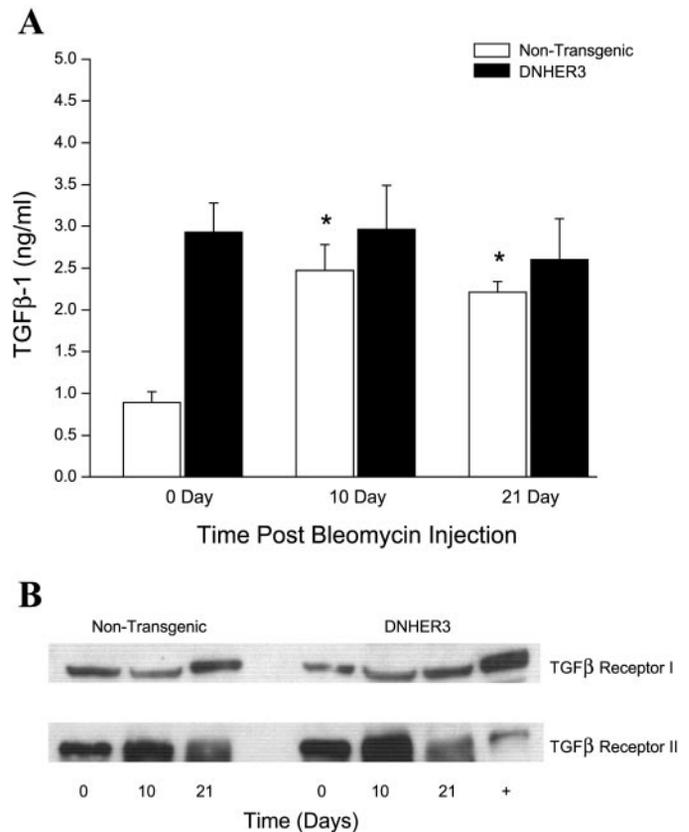


Fig. 6. Transforming growth factor (TGF)- $\beta_1$  induction does not occur in DNHER3 mice while TGF- $\beta$  receptor levels are unchanged. A: BALF collected from nontransgenic littermates and DNHER3 mice 0, 10, or 21 days following bleomycin injury was analyzed for total TGF- $\beta_1$  content by luciferase reporter gene assay. Values are means (SD). \* $P = 0.001$  compared with *day 0* value. B: lung homogenates from nontransgenic littermates and DNHER3 mice collected 0, 10, or 21 days following bleomycin injury were subjected to Western blot analysis for TGF- $\beta$  receptors I and II. Blots are representative of 2 experiments.

threefold higher level of bleomycin (0.08 units). Nontransgenic littermate mice began to die as soon as 8 days postinjection (Fig. 7) with 50% mortality at 14 days and 80% at 29 days. In contrast, 50% mortality was not reached in DNHER3-expressing mice, and no animal died before 10 days. In the DNHER3 strain, bleomycin lung injury resulted in 35% mortality, whereas 65% of the mice survived to 30 days. Thus survival following bleomycin injection was significantly improved in DNHER3 mice compared with wild-type mice ( $P = 0.04$ ).

## DISCUSSION

The present study examined our hypothesis that signaling through the HER2/HER3 receptor complex is important in the recovery of the lung from injury. Using a bleomycin model of injury, NRG-1 production and HER2 receptor activation were increased in the lungs of C57BL/6J nontransgenic littermate mice. These changes were associated with protein leak, inflammatory cell infiltration, mesenchyme volume expansion, increased BALF TGF- $\beta_1$ , and collagen deposition in the lung, all of which increased over time. Transgenic mice incapable of signaling through the HER2 receptor due to the expression of a lung-specific DNHER3 also increased NRG-1 production, but receptor activation did not occur. Expansion of the mes-

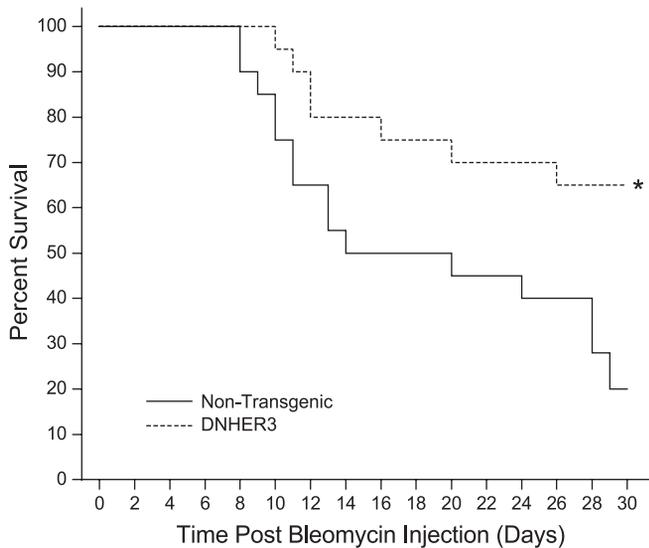


Fig. 7. Inhibition of HER2/HER3 receptor function improves survival following lung injury. Lung injury in nontransgenic littermates and DNHER3-expressing mice ( $n = 17$  each) was induced with 0.08 units of bleomycin and monitored for survival. Data are expressed as a Kaplan-Meier plot.  $*P = 0.04$  for the comparison of DNHER3 to nontransgenic littermates.

enchyme volume and collagen deposition in the lungs of these mice was reduced compared with that in nontransgenic littermate control mice. BALF TGF- $\beta_1$ , while at a higher basal level, did not increase in DNHER3 mice. No differences were seen in TGF- $\beta$  receptor I or TGF- $\beta$  receptor II expression between transgenic and nontransgenic mice. Finally, when challenged with an increased dose of bleomycin, DNHER3 mice displayed a survival advantage compared with nontransgenic littermates. These data support our hypothesis that the HER2/HER3 heterodimer plays an important role in the lung's recovery from injury.

The epithelial cell HER2/HER3 axis has not been previously shown to play a role in lung injury or fibrosis, yet expression of other growth factors, such as TGF- $\alpha$ , clearly plays a critical role in the development of pulmonary fibrosis (14). Our current results support and extend these studies. These experiments suggest that autocrine activation of epithelial cell growth factor receptors (EGFR, HER2) causes fibrosis, and fibrosis can be prevented by inhibition of growth factor receptor signaling.

These results differ from our laboratory's recently published *in vitro* work (36), where HER2 activation improved epithelial cell monolayer reformation following injury. Significant differences in study conditions most likely explain the differences in results. In our *in vitro* study, only one cell type was present. *In vivo*, an integrated system made up of multiple interacting cell types (epithelial cells, mesenchymal cells, inflammatory cells), growth factors, and cellular stimuli is present. The injury stimulus used *in vitro* was direct mechanical wounding, whereas a more complex injury stimulus was used in this *in vivo* study. Bleomycin administration in our *in vivo* studies results in oxidant injury, upregulating a variety of growth factors and activating intracellular signal pathways. Differences in *in vitro* vs. *in vivo* results have also been seen in other examples of pulmonary epithelia wound healing (3, 23). Using airway epithelial cells lacking CC chemokine receptor 2 (CCR2), Christensen et al. (3) reported the loss of monocyte

chemoattractant protein-1-induced signaling through CCR2-delayed closure of a mechanical wound *in vitro*. However, *in vivo*, CCR2(-/-) mice are protected against bleomycin-induced fibrosis (23).

Previous studies have demonstrated that HER2 inactivation has profound effects during development. HER2 null mice die before embryonic *day 11* due to cardiac trabeculation defects. When mice that specifically express rat HER2 in the heart were crossed into the null HER2 background, the cardiac phenotype was rescued (24). Similarly, when HER2 was conditionally deleted in the heart, adult mice displayed chamber dilation, cardiac wall thinning, and decreased contractility (24). HER2 activation has also been shown to be critically important for central nervous system development. In transgenic mice expressing a kinase-inactive HER2, sympathetic chain ganglia formation was not initiated (1). Although HER2 knockout mice die at embryonic *day 10.5*, these mice exhibited generalized effects on the developing neural crest, characterized by severe cranial ganglia defects, reduction in Schwann cells, enteric ganglia, and adrenal chromaffin cells (4).

HER2/HER3 activation has also been reported to be damaging. Zanazzi et al. (38) have shown that addition of glial growth factor, a NRG-1 isoform, to cultured Schwann cells inhibited myelination in unmyelinated cells and caused demyelination in mature myelinated cultures. In neural tissue of Alzheimer disease patients and transgenic mice expressing mutations of  $\beta$ -amyloid precursor protein, NRG-1 and its receptors are upregulated in reactive astrocytes surrounding neuritic plaques (2). In the epidermal response to ultraviolet radiation and in breast cancers overexpressing HER2, activation of HER receptors by NRG-1 can lead to cell cycle arrest and apoptosis (16, 17). In human keratinocyte cell lines, inhibition of HER receptors by either pharmaceutical or immunological means prevents ultraviolet-B-induced apoptosis (17). In SKBr3 breast cancer cell lines overexpressing HER2, NRG-1 induces apoptosis, as evidenced by caspase-9 and caspase-7 activation and poly(ADP-ribose)polymerase cleavage (15). In light of these results, the loss of HER2 signaling in the DNHER3 strain may protect from NRG-1/HER2/HER3-dependent apoptosis induced by bleomycin.

Our present results are specific to HER2 and HER3. It is not clear, however, which is the actual mediator of the effect. We speculate that the loss of HER2 signaling is the important event, as HER3's kinase domain is catalytically impaired *in vivo*. However, HER3 can be transphosphorylated by HER2 and has unique intracellular docking motifs to activate intracellular signal pathways. Specific inhibition of HER2 will be necessary to understand the role of HER2 vs. HER3. In addition, the response does not seem to involve HER4. HER4 can homodimerize or heterodimerize with HER2 to form a functional NRG-1 receptor (12), but we have been unable to detect HER4 in the lungs of mice by RT-PCR or Western blotting. Therefore, HER4 either is not present or is below our detection limits. Regardless of expression level, if HER4 is present, it does not affect the interpretation of our data, as the DNHER3 receptor would not impact HER4 activation.

The mechanism resulting in decreased fibrosis and mortality in the DNHER3 strain is not clear. No differences were noted in cellular infiltration, and only the *day 3* BALF protein content differed between DNHER3 and nontransgenic animals, so loss of HER2/HER3 signaling did not decrease lung injury. Further

defining any role of HER2/HER3 in injury will require a comparison to receptor inhibition after bleomycin exposure. Another mechanism behind the decreased fibrosis and possibly decreased mortality appears to be, in part, a lack of TGF- $\beta$ <sub>1</sub> induction, directly or indirectly, by HER2/HER3 signaling. Surprisingly, TGF- $\beta$ <sub>1</sub> levels were higher in DNHER3 mice than nontransgenic littermate controls at baseline, yet this had little effect on basal collagen or bleomycin-induced fibrosis. The small increases that were observed in bleomycin-induced collagen formation may have been due to other factors known to play a part in the profibrotic cascade, such as TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$ , IL-4, and IL-13. The lack of TGF- $\beta$ <sub>1</sub> induction in DNHER3 mice suggests that HER2/HER3 signaling may modulate the fibrotic environment through regulation of this profibrotic cytokine. The lack of increased basal collagen levels in the DNHER3 strain with its elevated basal expression of TGF- $\beta$ <sub>1</sub> further suggests that TGF- $\beta$ <sub>1</sub> alone is not sufficient to induce fibrosis and requires HER2/HER3 signaling. No changes in TGF- $\beta$  receptor expression in DNHER3 animals were seen to account for reduced fibrosis, both at baseline and following injury, in the face of increased TGF- $\beta$ <sub>1</sub> levels. Lung homogenates from both DNHER3 and nontransgenic littermate mice had similar basal levels of TGF- $\beta$  receptors I and II. Additionally, the expression pattern of TGF- $\beta$  receptor I and II was identical with injury in both groups of mice. Reduced fibrosis observed in DNHER3 mice, therefore, cannot be explained by changes in TGF- $\beta$  receptor expression. Not yet determined, however, is whether NRG-1 mediates the deleterious effects of HER2/HER3 signaling or whether other ligands are responsible for receptor activation. In addition, it is unknown whether the observed changes are due to a direct epithelial cell effect or an indirect effect, such as altered epithelial cell/mesenchymal cell interaction.

These findings suggest a novel strategy for the treatment of human acute lung injury. We speculate that blocking HER2/HER3 receptor activation may inhibit the fibrotic process and improve recovery following lung injury.

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