ErbB4 deletion leads to changes in lung function and structure similar to bronchopulmonary dysplasia


ErbB4 deletion leads to changes in lung function and structure similar to bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 294: L516–L522, 2008. First published January 18, 2008; doi:10.1152/ajplung.00423.2007.—Neuregulin is an important growth factor in fetal surfactant synthesis, and downregulation of its receptor, ErbB4, impairs fetal surfactant synthesis. We hypothesized that pulmonary ErbB4 deletion will affect the developing lung leading to an abnormal postnatal lung function. ErbB4-deleted lungs of 11- to 14-wk-old adult HER4<sup>heart</sup> mice, rescued from their lethal cardiac defects, were studied for the effect on lung function, alveolarization, and the surfactant system. ErbB4 deletion impairs lung function and structure in HER4<sup>heart</sup> mice resulting in a hyperreactive airway system and alveolar simplification, as seen in preterm infants with bronchopulmonary dysplasia. It also leads to a downregulation of surfactant protein D expression and an underlying chronic inflammation in these lungs. Our findings suggest that this animal model could be used to further study the pathogenesis of bronchopulmonary dysplasia and might help design protective interventions.

**MATERIALS AND METHODS**

Rabbit anti-human surfactant protein (SP)-A, rabbit anti-sheep SP-B, rabbit anti-human pro-SP-C, and rabbit anti-mouse SP-D antibody were obtained from Chemicon (Hoffheim, Germany); EcoRI restriction enzyme was obtained from New England Biolabs (Frankfurt am Main, Germany); digoxigenin (DIG) labeling kit (SP6/T7), T7 RNA polymerase, anti-DIG alkaline phosphatase, proteinase K, blocking reagent, and BM Purple were from Roche Diagnostics (Mannheim, Germany); SuperFrost Plus microscopic slides were from Menzel-Gläser (Braunschweig, Germany); RNAeasy MiniElute Cleanup Kit was from Qiagen (Hilden, Germany); goat monoclonal anti-actin clone AC-40 was obtained from Sigma (St. Louis, MO); goat anti-rabbit IgG (horseradish peroxidase-labeled) and goat anti-mouse IgG (horseradish peroxidase-labeled) were from Zymed Laboratories (Invitrogen, Carlsbad, CA); Precision Plus Protein Dual Color Standards were from Bio-Rad (Hercules, CA); Protran nitrocellulose transfer membrane was from Schleicher & Schuell BioScience (Keene, NH); and Western Lightning Chemiluminescence Reagent Plus (ECL) was from PerkinElmer Life Sciences (Boston, MA). Osmium tetroxide and glycolic ether were obtained from Paesel & Lorei (Frankfurt, Germany), uranyl acetate dihydrate was from Merck (Darmstadt, Germany), and glycid ether 100 (epon) was obtained from Serva (Heidelberg, Germany). Transgenic ErbB4<sup>-</sup>deleted mice, rescued from their lethal cardiac defects by expressing human ErbB4<sup>HER4</sup> cDNA under the cardiac-specific α-myosin heavy chain promoter (34), were kindly provided by Dr. Carmen Birchmeier in agreement with Dr. Martin Gassmann. Animal experiments were performed on adult HER4<sup>heart</sup> mice (11–14 wk old), and heterozygote siblings were used as controls since heterozygote breeding revealed a low number of wild-type animals. All animals were housed in a pathogen-free animal facility at Hannover Medical School, and the protocols were approved by the appropriate governmental and institutional authorities at Hannover Medical School.

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Invasive measurement of pulmonary function. Mice were anesthetized with 1.5% halothane-30% oxygen by inhalation and orotracheally intubated. The technique for invasive and repetitive lung function measurement used in this study has been described recently (11, 14). Briefly, two intubated, spontaneously breathing animals were placed in supine position in temperature-controlled body plethysmographs (type 871; HSE-Harvard Apparatus, March-Hugstetten, Germany). The orotracheal tube was directly attached to a pneumotachograph (HSE-Harvard Apparatus) connected to a differential pressure transducer (Validyne DP 45-14, HSE-Harvard Apparatus) to determine tidal flow. Transpulmonary pressure (P_{pl}) was measured via a water-filled esophagus catheter coupled to a pressure transducer (P75, HSE-Harvard Apparatus). Pulmonary resistance (RL), dynamic lung compliance, tidal midexpiratory flow, tidal volume, and respiratory frequency were calculated from P_{pl} and tidal flow signals over an entire breath cycle using HEM 3.5 software (Notocord, Croissy, France). Airway responsiveness was assessed as an increase in RL in response to aerosolized methacholine (MCh). Dried MCh aerosols were generated by an aerosol generator system (particle size 2.5 μm mass median aerodynamic diameter; Bronchii III, Fraunhofer ITEM; licensed by Buxco, Troy, NY). MCh doses of 0.0625–4.0 μg were applied to the animals.

Tissue processing. After intratracheal instillation with 4% paraformaldehyde (PFA) and 0.1% glutaraldehyde in 0.2 M HEPES buffer (pH 7.35) using an instillation device (pressure of 20 cmH2O) and 4 h postfixation in the same fixation solution, two blocks from the right lung were embedded in paraffin and used for in situ hybridization. The left lung was cryoprotected in glucose solution and cryofixed for 10-m-thick sections from the left lung of each animal. Maximal diameter of counted alveoli was 258.95 μm (H9262), the more pronounced increase of RL in HER4<sup>heart</sup>−/− mice compared with control animals at MCh doses of 0.125, 0.5, and 1.0 μg, increasing from 10.3% ± 4.7%, 25.8% ± 8.1%, and 44.5% ± 11.8% in control animals to 31.9% ± 8.2% (P = 0.034), 49.1% ± 5.8% (P = 0.018), and 69.6% ± 7.9% (P = 0.047), respectively, above baseline values in HER4<sup>heart</sup>−/− mice. At MCh doses of 0.0625, 0.25, 2.0, and 4.0 μg, the more pronounced increase of RL in HER4<sup>heart</sup>−/− mice compared with control animals fell short of statistical significance (Fig. 1). ANOVA testing revealed sig-

Table 1. Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
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<tbody>
<tr>
<td>β-Actin</td>
<td>5′-AGTCTACATCATATTGGCAAGGA-3′</td>
<td>5′-CAAGCTACACTGCTAGATGGA-3′</td>
<td>5′-(FAM)-AGCTTCTTCATTTGGATAGACTTGT-(TAMRA) 3′</td>
</tr>
<tr>
<td>SP-A</td>
<td>5′-AGCTGACTGATCGCCGATGACGGCG-3′</td>
<td>5′-ACTCTAGAGATCGACGTACCCTG-3′</td>
<td>5′-(FAM)-TGGAAAGCTGAGACTCCATATGGT-(TAMRA) 3′</td>
</tr>
<tr>
<td>SP-B</td>
<td>5′-AGCTGACCCATCTGAGATGCG-3′</td>
<td>5′-CCAGGCTAGTCATACCTGAG-3′</td>
<td>5′-(FAM)-TGACGCTAGTCATACCTGAG-(TAMRA) 3′</td>
</tr>
<tr>
<td>SP-C</td>
<td>5′-GCAACTTCTGCGGCACTTGGTG-3′</td>
<td>5′-GCTAGGTCTCTCGAGGTGGCAATG-3′</td>
<td>5′-(FAM)-CTACAGGGCCCTCTGGACGCGG-(TAMRA) 3′</td>
</tr>
<tr>
<td>SP-D</td>
<td>5′-GGCTCTGTCACCTGCTGCT-3′</td>
<td>5′-GGAGACAGAGGAGATGCAAAAGG-3′</td>
<td>5′-(FAM)-AGCAGACCTGTGUTGGAGCCCAGGACC-(TAMRA) 3′</td>
</tr>
</tbody>
</table>

SP, surfactant protein.
Taken together with the stereological results, pulmonary doses of 0.125, 0.5, and 1.0 \( \text{HER4heart} \) caused a dose-related increase of RL in \( \text{HER4heart} \) resistance (RL) above baseline in control \( \text{HER4heart} \) measured in 14-wk-old mice. MCh doses of 0.0625–4.0 \( \mu \text{g} \) mice were applied by \( \text{HER4heart} \) mice. (zygote \( \text{HER4heart} \) lungs, \( n \) 5–8; * \( P < 0.05 \)). In \( \text{HER4heart} \) mice, the volume density of the ductal space was significantly decreased to 91.06% (\( P = 0.02 \)) and 120.22% (\( P = 0.01 \)) compared with \( \text{HER4heart} \) control animals (Table 3).

**Alveolar count.** The number of alveoli in \( \text{HER4heart} \) lungs (\( n = 9 \)) was significantly decreased by 20% \( \pm \) 5% (\( P = 0.018 \)) compared with \( \text{HER4heart} \) control lungs (\( n = 6 \)). Taken together with the stereological results, pulmonary \( \text{HER4heart} \) deletion also leads to an alveolar simplification.

**Electron microscopy.** Differences in lamellar body structure were not detected by electron microscopy in \( \text{HER4heart} \) lungs. However, the number of test fields containing granulocytes was significantly increased (\( P = 0.016 \)) in \( \text{HER4heart} \) lungs (\( n = 4 \)) to 7.65% \( \pm \) 0.56% compared with 4.52% \( \pm \) 0.66% in control lungs (\( n = 5 \)) (Fig. 3), suggesting a chronic inflammatory process in these \( \text{HER4heart} \)-deleted lungs.

**Surfactant protein expression.** SP-A, SP-B, and SP-C protein expression were similar for both genotypes studied, revealing 105% \( \pm \) 9.4% (\( P = 0.5 \)) for SP-A, 101% \( \pm \) 2.3% (\( P = 0.74 \)) for SP-B, and 99% \( \pm \) 2.1% (\( P = 0.47 \)) for SP-C in \( \text{HER4heart} \) lungs (\( n = 11 \)) compared with lungs of control animals (\( n = 13 \)). However, there was a significant decrease in expression of SP-D to 91% \( \pm \) 3% (\( P = 0.017 \)) in \( \text{HER4heart} \) lungs (Fig. 4B). Immunohistochemical SP-B protein expression confirmed the Western blot results.

**Surfactant protein mRNA expression.** SP-B mRNA expression studied by in situ hybridization confirmed similar expression of this protein independent of the genotype (data not shown). Real-time PCR measurements for SP-A (the difference of the samples DCT and the baseline, DDCt \( = 0.3 \pm 0.4 \); \( P = 0.5 \)), SP-B (DDCt \( = 0.23 \pm 0.3 \); \( P = 0.6 \)), and SP-C (DDCt \( = 0.7 \pm 0.3 \); \( P = 0.2 \)) did not show significant differences between \( \text{HER4heart} \) and \( \text{HER4heart} \) control lungs, whereas SP-D mRNA expression was significantly decreased in \( \text{HER4heart} \) control lungs (DDCt \( = 0.6 \pm 0.3 \); \( P = 0.045 \)) compared with \( \text{HER4heart} \) control lungs (Fig. 5), confirming our protein evaluations.

## DISCUSSION

Neuregulin and its receptor, ErbB4, play prominent roles in fetal type II cell maturation (8, 40), underlining the strong association of ErbB4 and fetal tissue differentiation (31). Therefore, we hypothesized that ErbB4 is involved in the regulation of normal lung development. We used a transgenic animal model where the embryonic lethality of ErbB4 knockout mice is rescued by expressing the ErbB4 receptor under a cardiac-specific promoter (34). Here, we show for the first time that pulmonary ErbB4 deletion leads to a hyperreactive airway system and to alveolar simplification, a phenotype similar to bronchopulmonary dysplasia (BPD) in preterm infants. Interestingly, SP-D expression is downregulated, and there are signs of an underlying chronic inflammation in these lungs.

At birth, mouse lungs are at the saccular stage of the pulmonary development, and alveoli formation occurs mainly after birth (3). The pulmonary sacs are divided through serial septation processes into alveoli, increasing the gas exchange surface of the lung. Our morphological observations in the lung tissue, specifically the decrease of alveolar number, increase in thickness and volume density of alveolar septae, and the decrease of the alveolar ductal space, indicate lung immaturity with inhibited formation of secondary alveolar septae and can
be summarized as alveolar simplification. However, a combined effect of decreased formation of secondary septa and progressive destruction of parenchyma might be present in these animals. These morphological changes are similar to the changes observed in the lungs of the newborn mice after prolonged exposure to 85% oxygen. Hyperoxia leads to a similar picture seen in BPD lungs, showing decreased alveolar septation, an increase in terminal air space size, and lung fibrosis. It also leads to an increased number of inflammatory cells in lung tissue and in bronchoalveolar lavage fluid (36). Although hyperoxia and barotrauma induce BPD (4), signs of impaired alveolarization can be seen in the lungs of very immature baboons even in the absence of marked hyperoxia and high ventilation settings (5). In the ErbB4-deleted lungs, the size of alveoli was not increased, but all other morphometric parameters resemble those of premature infants suffering from an interruption of normal lung development after the exposure to cytokines or glucocorticoids (24, 39) ultimately leading to the clinical picture of BPD (16). Similarly, exposure of fetal sheep to intra-amniotic endotoxin or IL-1β leads to a decreased alveolarization and microvascular injury (15). VEGF blockade in newborn rats decreases lung angiogenesis and impairs alveolar development, mimicking BPD (33).

Young adults who have been born preterm have a higher prevalence of asthma and respiratory symptoms (35). Airway hyperresponsiveness in preterm born infants is mostly seen without any inheritance of allergy (13), and it remains unclear whether the anatomic changes are the pathological correlate for these symptoms. In agreement with these observations in humans, we found an association of airway hyperresponsive-

![Fig. 2. Histological photomicrographs of control HER4heart+/− and HER4heart−/− lungs. Representative light microscopic images from cryosections stained with hematoxylin-eosin. Photomicrographs show an increased thickness and volume density of alveolar septa in HER4heart−/− lungs (B) compared with control HER4heart+/− lungs (A). Bar = 5 μm.](image)

![Fig. 3. Electron photomicrographs of control HER4heart+/− and HER4heart−/− lungs. Representative electron microscopic images from 70-nm sections stained with lead citrate and uranyl acetate. Photomicrographs show an increase in number of granulocytes in HER4heart−/− lungs (B) compared with control HER4heart+/− lungs (A). Arrows indicate neutrophils and eosinophils. Bar = 5 μm.](image)

<table>
<thead>
<tr>
<th>Morphometric Parameters</th>
<th>% of Family-Specific Controls</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Volume density of parenchyma</td>
<td>100.5±0.58</td>
<td>0.12</td>
</tr>
<tr>
<td>Volume density of nonparenchyma</td>
<td>93.51±9.3</td>
<td>0.44</td>
</tr>
<tr>
<td>Thickness of alveolar septa</td>
<td>120.22±4.82</td>
<td>0.01†</td>
</tr>
<tr>
<td>Volume density of alveolar septa</td>
<td>120.13±4.99</td>
<td>0.02†</td>
</tr>
<tr>
<td>Volume density of alveolar space</td>
<td>106.83±4.70</td>
<td>0.45</td>
</tr>
<tr>
<td>Surface density of alveoli</td>
<td>96.16±2.41</td>
<td>0.12</td>
</tr>
<tr>
<td>Size of alveoli</td>
<td>107.44±3.60</td>
<td>0.08</td>
</tr>
<tr>
<td>Volume density of alveolar ductal space</td>
<td>91.06±7.54</td>
<td>0.03†</td>
</tr>
<tr>
<td>Size of alveolar ducts</td>
<td>96.25±3.53</td>
<td>0.68†</td>
</tr>
<tr>
<td>Volume density of alveolar ductal septa</td>
<td>96.24±6.17</td>
<td>0.34</td>
</tr>
<tr>
<td>Thickness of alveolar ductal septa</td>
<td>106.34±4.93</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05; †P value of Student’s t-test (otherwise, Mann-Whitney U-test).
leads us to the speculation that the minimal SP-D deficiency in normally developed alveoli in these animals. This observation of enlargement of the air spaces (2, 17), implying remodeling of lungs at birth, and postnatal macrophage influx leads to an foamy macrophages (17). Also, SP-D null mice have normal combined with giant lamellar bodies and the occurrence of ational described hypertrophy and hyperplasia of type II cells HER4heart

These results are in agreement with our findings in the knockout mice show signs of postnatal inflammation (2, 17), a reduced number but an increased size of alveoli, which is combined with a decreased surface area of the alveoli (26, 38). These results are in agreement with our findings in the HER4heart−/− mice. However, we have not detected the additional described hypertrophy and hyperplasia of type II cells combined with giant lamellar bodies and the occurrence of foamy macrophages (17). Also, SP-D null mice have normal lungs at birth, and postnatal macrophage influx leads to an enlargement of the air spaces (2, 17), implying remodeling of normally developed alveoli in these animals. This observation leads us to the speculation that the minimal SP-D deficiency in

The increased number of granulocytes and decrease in SP-D expression in ErbB4-deleted lungs confirm the presence of a chronic ongoing inflammatory process in these lungs. SP-D knockout mice show signs of postnatal inflammation (2, 17), a reduced number but an increased size of alveoli, which is combined with a decreased surface area of the alveoli (26, 38). These results are in agreement with our findings in the HER4heart−/− mice. However, we have not detected the additional described hypertrophy and hyperplasia of type II cells combined with giant lamellar bodies and the occurrence of foamy macrophages (17). Also, SP-D null mice have normal lungs at birth, and postnatal macrophage influx leads to an enlargement of the air spaces (2, 17), implying remodeling of normally developed alveoli in these animals. This observation leads us to the speculation that the minimal SP-D deficiency in

the ErbB4 null mice cannot account for the anatomic alterations seen in these animals. We speculate that the relatively minor downregulation of SP-D leaves enough residual function of this protein in the animals to support baseline surfactant homeostasis. It is difficult to draw any major clinical implication from the small decrease in SP-D expression, but it can be speculated that it affects the lung function and is involved in the etiology of the mild signs of chronic inflammation.

The other surfactant proteins, SP-A, SP-B, and SP-C, are affected to an even lesser extent by ErbB4 deletion in these adult lungs. Since we have demonstrated that in vitro down-regulation of the ErbB4 receptor using siRNA downregulates surfactant synthesis in fetal type II epithelial cells (40, 41), we assume that compensatory mechanisms through other ErbB receptors must have taken place in vivo leading to the normal surfactant protein expression of SP-A, SP-B, and SP-C in these adult mice. Redirection of ErbB signaling toward formation of other ErbB receptor hetero- and homodimers may compensate parts of the missing function of ErbB4 in the developing surfactant system. It can be speculated that ErbB4 deletion leads to a shifting in dimer formation resulting in alveolar simplification seen in HER4heart−/− lungs. Indeed, growth factor stimulation leads to different biological results dependent on which ErbB receptor dimer partners are involved (32). Ligand binding by ErbB receptor stimulates homo-and/or heterodimer formation leading to their extensive signaling potential and signal diversification (25, 27). Redirection of ErbB1/ErbB4 signaling seen in type II cells under normal conditions (40) toward ErbB2/ErbB4 dimer formation in dexamethasone-exposed type II cells might play an important role in dexamethasone induced changes in the surfactant system of the type II epithelial cells (7).

ErbB receptors are known to be involved in lung development, injury, and remodeling. Deletion of ErbB1, also called EGFR, results in severe structural and functional changes in the lung, causing postnatal respiratory distress in these animals. The lungs of EGFR-deficient mice have impaired branching, a significant reduction of gas exchange surface, and surfactant deficiency (20, 29). EGFR forms heterodimers with ErbB4 and

\[ \begin{align*}
\text{SP-A (26kDa)} & \\
\text{SP-B (42kDa)} & \\
\text{SP-C (26kDa)} & \\
\text{SP-D (42kDa)} & \\
\text{actin (42kDa)} & \end{align*} \]

Fig. 4. Expression of surfactant proteins (SP) A, B, C, and D in control HER4heart+/− and HER4heart−/− lungs by Western blotting. A: mouse lung homogenates from control HER4heart+/− and HER4heart−/− animals were subjected to Western blotting to detect SP-A, SP-B, SP-C, and SP-D. α-Actin was used to control for equal protein loading. A representative blot for all surfactant proteins is shown. B: densitometric readings of blots from control HER4heart+/− and HER4heart−/− lungs. Intensity of surfactant protein bands was measured and normalized to the α-actin content of the sample. Expression of SP-D was downregulated in HER4heart−/− lungs. Data are shown as mean (%) ± SE; \( n = 11–12; * P < 0.05 \).

![Graph showing expression levels of surfactant proteins](http://example.com/surfactant_proteins_graph.png)

Fig. 5. Expression of SP-A, SP-B, SP-C, and SP-D in control HER4heart+/− and HER4heart−/− lungs (by real-time PCR). The differences in the cycle threshold (DCt) of SP-A, SP-B, SP-C, and SP-D and actin genes were determined in both genotypes. Actin was used as an internal control to normalize the surfactant protein cDNA levels. The DCt values of the HER4heart−/− lungs were compared with DCt values from lungs of HER4heart+/− control animals (DDCt). DDCt values are inversely proportional to the levels of surfactant protein mRNA. Data are shown as means ± SE; \( n = 7–11; * P < 0.05 \).
is the most prominent dimer partner in the fetal lung (40). Some of the effects seen in our animals might be due to altered EGFR signaling. Protein expression of the other ErbB receptors is not significantly altered in these lungs (data not shown), and further studies of ErbB receptor interactions need to be carried out in isolated cell types. On the other hand, a decreased concentration of EGF is found in the bronchoalveolar lavage fluid of infants who develop respiratory distress syndrome (RDS) or BPD, implying an involvement of EGF and its receptor in the development of BPD (6). Inhibition of pulmonary ErbB2/ErbB3 signaling by downregulation of ErbB3 expression protects from the development of pulmonary fibrosis (21).

ErbB receptors are also known for their central role in a wide variety of biological processes (19). They contribute to the development of many forms of human cancer where overexpression of the receptor, mutations in the receptor gene, or an autocrine ligand production plays a role in the aberrant activation of these receptors. Interfering with these aberrant pathways opens avenues for the treatment of the resulting diseases, which is already in wide use of many tumors. This approach might be a therapeutic option for ErbB receptor dysregulation in the development of other diseases.

The number of infants with significant BPD has actually increased, secondary to improvements in neonatal survival. This is due to the development of new therapeutic strategies in obstetric and neonatal care. BPD appears to be initiated by a primary exposure to hyperoxia and mechanical ventilation secondary to prenatal infection, subsequent chorioamnionitis, and obstetric and neonatal care. BPD may be one of the most prominent dimer partners in the fetal lung (40). Some of the effects seen in our animals might be due to altered EGFR signaling. Protein expression of the other ErbB receptors is not significantly altered in these lungs (data not shown), and further studies of ErbB receptor interactions need to be carried out in isolated cell types. On the other hand, a decreased concentration of EGF is found in the bronchoalveolar lavage fluid of infants who develop respiratory distress syndrome (RDS) or BPD, implying an involvement of EGF and its receptor in the development of BPD (6). Inhibition of pulmonary ErbB2/ErbB3 signaling by downregulation of ErbB3 expression protects from the development of pulmonary fibrosis (21).

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ACKNOWLEDGMENTS

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GRANTS

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DISCLOSURES

We received a PhD stipend for E. Purevordorj from Abbott.

REFERENCES


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