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Neurotrophins, airway smooth muscle and the fetal breathing-like movements

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Summary. Central nervous system and skeletal muscles secrete a group of polypeptide hormones called neurotrophins (NTs). More recent studies show that NTs and their receptors are also expressed in the lung, suggesting a role for NTs in lung development. To examine the role of NTs during normal and diseased lung organogenesis, we employed wild-type and amyogenic mouse embryos (designated as *Myf5-/-:MyoD-/-*). Amyogenic embryos completely lacked skeletal muscles and were not viable after birth due to the respiratory failure secondary to lung hypoplasia. To examine the importance of lung-secreted NTs during normal and hypoplastic lung organogenesis, immunohistochemistry was employed. Distribution of NTs and their receptors was indistinguishable between normal and hypoplastic lungs. To further examine the importance of non-lung-secreted NTs (e.g., from the skeletal muscle and CNS) in lung organogenesis, *in utero* injections of two NTs were performed. The exogenously introduced NTs (i.e., non-lung-secreted) did not appear to improve development of the lung in amyogenic embryos. Moreover, immunohistochemistry showed significantly reduced number of airway smooth muscle cells (ASMCs) in hypoplastic lungs of amyogenic embryos, suggesting that the number of ASMCs is primarily regulated by the fetal breathing-like movements (i.e., mechanical factors).

Key words: Lung hypoplasia, Mouse embryo, Myf5 and MyoD, Neurotrophins, Smooth muscle cells

Introduction

Neurotrophins (NTs) are a group of polypeptide hormones that control growth, differentiation and survival of neurons in the central and peripheral nervous system (Lewin and Barde, 1996). The first protein discovered in this family was nerve growth factor (NGF) (Klein, 1994). Additional members of the NT family are neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and brain derived neurotrophic factor (BDNF) which are all structurally homologous to NGF (Klein, 1994). NTs modulate their activity through different high and low affinity receptors (Barbacid, 1995). The high affinity receptors include Trk A, Trk B, and Trk C which are all part of the Trk tyrosine kinase family (Barbacid, 1995). NGF binds Trk A, BDNF and NT-4/5 bind Trk B, NT-3 binds Trk C and to a lesser degree Trk A and Trk B receptors (Barbacid, 1995). The low affinity NT receptor p75 belongs to the tumor necrosis family and binds all NTs to date (Barbacid, 1994).

Even though NTs are mainly derived from the central nervous system (CNS) and skeletal muscle (Ernfors et al., 1992), they are also expressed in many non-neuronal tissues and seem to be essential for the development of various organs (Lomen-Hoerth and Shooter, 1995). More recently, NTs and their receptors have been reported to be expressed in the lung of various species including: adult human (Shibayama and Koizumi, 1996; Ricci et al., 2000, 2004), adult rat (Lomen-Hoerth and Shooter, 1995), adult mouse (Hikawa, 2002) and embryonic mouse (Tessarollo et al., 1993). Several hypotheses are proposed for the role of NTs in the lung including: regulation of immune function (Ricci et al., 2000; Hikawa et al., 2002), participation in pathophysiology of pulmonary disorders such as asthma (Ricci et al., 2004) and regulation of visceral sensory and motor neurons (Lommatzsch et al., 1999).

During the last third of gestation, mechanical forces are produced by the intermittent contractile activity of the main respiratory muscles, called fetal breathing-like movements (FBMs) (Kitterman, 1996). Lately, our laboratory has been studying the role of the respiratory muscles and FBMs in lung organogenesis. We established several mouse models of pulmonary hypoplasia (e.g., *mdx:MyoD-/-*^{9th} embryos lack most of the diaphragm; *Myf5-/-* embryos lack the intercostals and *Myf5-/-:MyoD-/-* embryos lack all skeletal muscles) (Inanlou et al., 2003; Inanlou and Kablar, 2003;

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2005a,b). Since our mouse models of pulmonary hypoplasia lack various subpopulations of muscles (e.g., *mdx:MyoD-/-*^{9th} and *Myf5-/-* embryos) or the entire skeletal muscle lineage (*Myf5-/-:MyoD-/-* embryos), we hypothesized that, in addition to FBMs, developing lung of our mouse models may also lack skeletal muscle-secreted NTs. As stated previously, various aspects of lung development (and even of remaining internal organs) may depend on NTs that are secreted either endogenously (i.e., by the lung) or by the skeletal muscle and CNS. Therefore, in the current report, we hypothesize that the endogenous (or lung-secreted) NTs or/and the exogenous (or skeletal muscle-secreted) NTs contribute to the pathogenesis of the observed pulmonary hypoplasia in our embryonic mouse models. In this report we are not considering CNS-secreted NTs, because it is the skeletal muscle and not the CNS that is entirely ablated in *Myf5-/-:MyoD-/-* embryos (Rudnicki et al., 1992).

Lung is composed of two main systems: bronchial and respiratory (Ten Have-Opbroek, 1991). Air is conducted through the bronchial system whereas the respiratory system is responsible for the gas exchange (Ten Have-Opbroek, 1991). In the bronchial system, airways are surrounded by smooth muscle cells whose function is still unknown. However, spontaneous contractions observed in the airway smooth muscles (Sorokin, 1961) are suggested to be important for normal development of airspaces by changing intraluminal pressure and movement of the lung fluid in the fetal airways (McCray, 1993). In addition, *in vitro* studies have shown that mechanical forces play a role in smooth muscle cell proliferation (Buck, 1983; Dartsch et al., 1986; Tschumperlin and Drazen, 2005). In the second part of the current study, we used amyogenic embryos to investigate the role of FBMs (i.e., as an *in vivo* mechanical force) in airway smooth muscle cell (ASMC) proliferation. We hypothesized that the amount of ASMCs may contribute to the pathogenesis of lung hypoplasia.

To investigate the hypothesized role of NTs produced by the lung compared to those produced by the skeletal muscle/CNS in lung organogenesis, the immunohistochemical analysis was followed by the inta-amniotic injections of NTs, in both control and diseased mouse embryos. In addition, to study the role of ASMCs in the pathogenesis of pulmonary hypoplasia, we employed three different mouse models of lung hypoplasia lacking either various subpopulations of muscles (e.g., *mdx:MyoD-/-*^{9th} and *Myf5-/-* embryos) or the entire skeletal muscle lineage (*Myf5-/-:MyoD-/-* embryos). Summarized, our data show the following: a) retained expression of NTs and their receptors in the hypoplastic lung suggests that the endogenous or lung-secreted NTs do not play a role in the pathogenesis of pulmonary hypoplasia; b) development of the hypoplastic lung is not rescued by intra-amniotic injections of either BDNF or NT-3, suggesting that the lack of exogenous NTs is an unlikely contributor to the pathogenesis of lung hypoplasia; and c) significantly reduced number of ASMCs in hypoplastic lungs of only amyogenic embryos (and not in other embryos that also die from lung hypoplasia) suggests that the amount of ASMCs does not play a role in the development of lung hypoplasia. Instead, the number of ASMCs appears to be greatly affected by the complete absence of FBMs, since it is observed only in amyogenic embryos.

Materials and methods

Animal care and interbreeding

To produce amyogenic mouse embryos, homozygous *MyoD-/-* mice were bred with *Myf5+/-* knock-out heterozygous mice, as previously described (Rudnicki et al., 1992). The resulting heterozygous offspring for both Myf5 and MyoD (i.e., *Myf5+/-*: *MyoD+/-*) from this cross were interbred to obtain embryos of nine different genotypes including *Myf5-/-:MyoD-/-* with a frequency of 1:16.

To generate *Myf5-/-* mouse embryos, heterozygous males and females were bred with each other to obtain embryos of three different genotypes including *Myf5-/-*.

mdx mice (i.e., mice that carry a loss-of-function point mutation in the X-linked *dystrophin* gene and are an animal model for human Duchenne and Becker muscular dystrophy) (Sicinski et al., 1994) were bred with *MyoD-/-* mice to generate *mdx:MyoD+/-* and *mdx:MyoD-/-* mice. These mice were subsequently backcrossed (i.e., the tenth generation of *mdx:MyoD+/-* mice was bred with the first generation of *mdx:MyoD-/-* mice) until no viable *mdx:MyoD-/-* newborn was detectable. The embryos generated in the described manner were designated as *mdx:MyoD-/-*^{9th} to indicate that they were double-mutant products after nine generations of backcrossing (Inanlou and Kablar, 2003).

Pregnant mice were sacrificed by cervical dislocation at E17.5 or E18.5 and subsequently Caesarean section was done to collect the embryos and fetal portion of the placenta. Genomic DNA was isolated from the fetal portion of the placenta in accordance to the procedure of Laird et al. (1991). The embryos were genotyped by PCR (Henneberger et al., 2000). Animal care was in accordance with the institutional guidelines.

Tissue preparation

Wild-type and mutant embryos were delivered via Caesarean section and subsequently decapitated. The intrathoracic cavities were exposed and the lungs were removed under the stereomicroscope. Lungs were fixed in 4% paraformaldehyde for 2 hours, dehydrated in alcohol and then embedded in paraffin. Serial sections were cut with a rotary microtome to a thickness of 4 μm and left on a slide drier at 37°C overnight. The sections were subsequently used for immunohistochemistry and tissue staining [i.e., hematoxylin-eosin (H&E)].

Immunohistochemistry

Sections were prepared for immunohistochemical staining with various antibodies by using xylene to deparaffinize, alcohol to dehydrate and 5% H_2O_2 to neutralize endogenous peroxidase activity. Antigen retrieval for the Trk C antibody was done using 1 mM ethylene diamine tetra acetic acid (EDTA) pH 8.0, whereas 10 mM citric acid buffer, pH 6.0 was employed for BDNF, NT-3, NT-4/5, Trk B and alpha-smooth muscle actin (SMA). Nonspecific binding of antibody was prevented by using appropriate blocking serums specific to each experiment. Lung sections were examined for the expression of BDNF, NT-4/5, Trk B and Trk C using rabbit polyclonal antibodies (Santa Cruz) diluted 1:25 and goat polyclonal antibody (Santa Cruz) diluted 1:10 for detecting NT-3 expression. The slides were left overnight at 4°C. Mouse monoclonal SMA (Sigma) diluted 1:100 was used for staining smooth muscle cells. Finally, mouse monoclonal antibody (Dako) diluted 1:500 was used against PCNA (Proliferating cell nuclear antibody).

Considering previously identified difficulties with the neurotrophin immunohistochemistry (Rush and Zhou, 2001), we included the following control experiments (data shown in Fig. 1 and 2 and data not shown): negative controls (i.e., the omission of primary antibody or the employment of an irrelevant antibody resulted in no staining of the tissue of interest; performed for all antibodies), immunoabsorption (i.e., with the increasing amount of specific blocking peptide against the antibody, the intensity of immunohistochemical staining gradually decreased and eventually was removed; performed for NT-3 and BDNF) and finally knock-out embryonic tissues kindly provided by Dr. Tessarollo (Tessarollo et al., 1994) (i.e., NT-3-/- and BDNF-/- lungs were negative for NT-3 and BDNF respectively).

TUNEL in situ detection

To identify the apoptotic cells terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) method was used (R&D Apoptosis Detection Kit). In brief, after employing protein kinase (15 minutes) and quenching steps, the sections were labelled by biotinylated nucleotides using Terminal deoxynucleotidyl Transferase (TdT) enzyme for 1 hour in a humidity chamber at 37°C. Some samples were incubated without TdT as negative controls. The incorporated nucleotides were detected using streptavidin solution. The sections were counterstained with hematoxylin.

Injection of NTs

It has been clearly demonstrated that administration of a single injection of different putative neurotrophic factor to mouse embryos *in utero* on E14 is sufficient to significantly reduce the normal death of spinal motor neurons when assessed on E18 (Houenou et al., 1994). To study the role of exogenous NTs in lung organogenesis, saline, BDNF or NT-3 was injected into the amniotic sac of embryos at E13.5 (before this stage, the viability of embryos was extremely low due to the amniotic puncture and this issue was extensively examined in the past) (MacIntyre et al., 1995). Nonetheless, we believe that the choice of the embryonic day for the intra-amniotic injections was appropriate, because it preceded the first appearance of FBMs at E14.5 (Kitterman, 1996) by one day. In brief, anesthetized pregnant mice were administered a subcutaneous injection of Ketoprofen (i.e., analgesic) 5 µg/g of body weight and Baytril (i.e., antibiotic) 2.5 µg/g of body weight diluted in saline. After exposing the abdominal cavity by a midline incision, one horn of the uterus containing the embryos was pulled out. A modified Hamilton syringe and a needle with outer diameter of 0.15 mm was used for injections. The embryos were injected intra-amniotically with 10 µl of saline (i.e., sham-injected group), 10 µl of BDNF (1 µg/µl) (i.e., BDNF-injected group) or 10 µl of NT3 (1 µg/µl) (i.e., NT-3-injected group). The concentration of the injected NTs was determined according to the previously performed experiments, where a neuronal rescue was successfully obtained (Houenou et al., 1994). The horn of the uterus was then returned to the abdominal cavity and the same technique was used for the other horn. The female was sutured and allowed to recover. The injected embryos were harvested at E17.5.

Morphometry and statistical analysis

The smooth muscle cells with the positive reaction against SMA, PCNA and TUNEL (i.e., proliferation and apoptosis index respectively), were counted among 500 randomly selected cells in the airways of corresponding size (N.B., the size of the airways was determined by the characteristics of the respiratory epithelium in the particular section through the airway) in serial sections at the magnification of 400x, as previously described (Inanlou and Kablar, 2003, 2005a,b). The number of SMA stained cells was counted under the magnification of 400 times in 10 randomly chosen microscopic fields (up to 500 cells) with the area of 9 mm^2 per field. An effort was made to distinguish between SMA-positive cells not associated with an airway from those cells that are clearly polarized around an airway. At E18.5, the arterial and airway smooth muscle cells were distinguished based on the epithelial morphology (i.e., the blood vessels endothelium was simple squamous, while the epithelium of the airways was at least cuboidal). The number of independent experiments was three for the control (n=3) and three for the mutant (n=3) embryos in all experiments. The statistical analysis was done using SPSS (version 11) software. Data are presented as means ± standard deviation (sd). Statistical significance was considered at $P<0.05$ in all

experiments. The values were compared by ANOVA followed by Bonferoni post hoc correction for multiple or Student's t test for paired comparisons.

Results

The expression pattern of the endogenous (i.e., pulmonary secreted) NTs and their receptors is similar between control and hypoplastic lung

The first step in understanding whether NTs and their receptors play a role during lung development, and in particular in the pathogenesis of pulmonary hypoplasia, was to compare the expression pattern of NTs and the receptors between control and hypoplastic lung employing immunohistochemistry. The expression of pulmonary-secreted BDNF, NT-3 and NT-4/5 was examined in both wild-type (i.e., control) and amyogenic (i.e., *Myf5-/-:MyoD-/-*) embryos. Careful examination of lung mesenchyme revealed that cells in the mesenchymal lung compartment expressed the NTs similarly in both normal and hypoplastic lungs. In

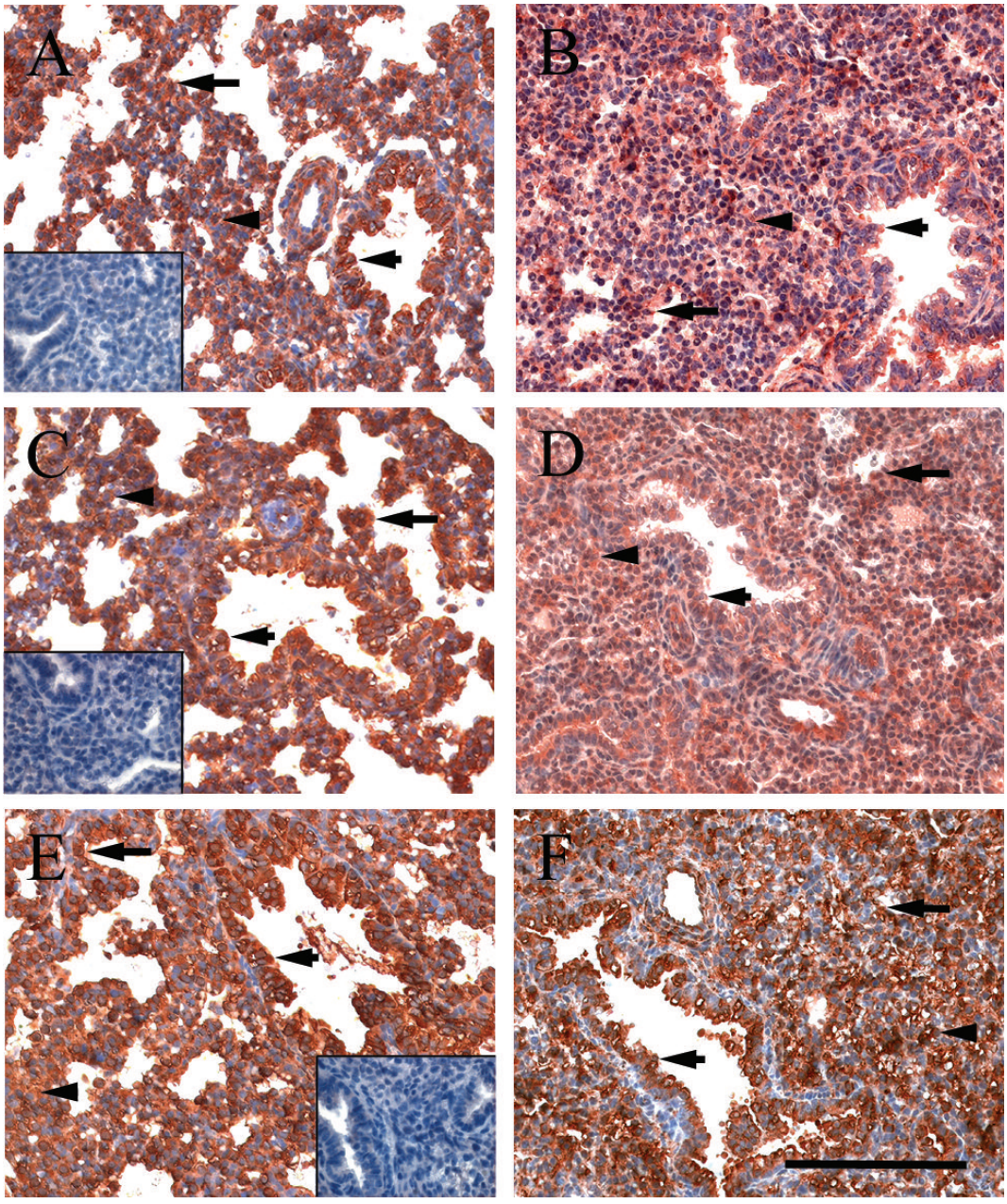

Fig. 1. Expression pattern of NTs is indistinguishable between wild-type and amyogenic embryos. In paraffin sections of E18.5 wild-type (**A, C, E**) and hypoplastic (**B, D, F**) lungs, immunostained against NT-3 (**A, B**), BDNF (**C, D**) and NT-4/5 (**E, F**), it is revealed that the expression pattern of all NTs (i.e., the red-brown immunohistochemical signal) is indistinguishable between the wild-type and amyogenic embryos in the: a) epithelium of the conductive system (short arrows), b) epithelium of the respiratory system (long arrows), and c) mesenchymal compartment of the lung (arrowheads). Note that while normally developing lung of wild-type embryos is composed of widened saccules, the hypoplastic lung of amyogenic embryos is composed of acinar or dilated acinar tubules (see Figure 3 for more details). Negative controls are represented in the A, C and E insets. Scale bar: 50 μm.

addition, the epithelial cells of the conductive and respiratory systems showed no difference in the expression of NTs in wild-type compared to amyogenic embryos (Fig. 1A-F). Similarly, the distribution of high-affinity neurotrophin receptors Trk B and Trk C seemed to be indistinguishable in the mesenchymal and epithelial compartments of both normal and hypoplastic lungs (Fig. 2A-D). Even though the abundant expression of BDNF, NT-3 and their receptors was observed in the lung tissue, a very precise expression pattern of the same antibodies was observed in other adjacent tissues of the same section (i.e., the positive control tissues). For example, somatic motor neurons in the thoracic spinal cord expressed all four proteins in a very specific manner, suggesting that the staining in the lung tissue was also specific. In addition, the insets in Fig. 1 and Fig. 2 show no immunohistochemical signal when the primary antibody is omitted (i.e., the negative controls). Finally, Western analysis on the control and hypoplastic lung tissues using anti-BDNF and anti-NT-3 confirm our immunohistochemical findings (data not shown).

Exogenous NTs alone cannot rescue lung hypoplasia in amyogenic embryos

To examine the role of non-lung-secreted NTs in the pathogenesis of lung hypoplasia, the embryos were injected intra-amniotically with saline (i.e., sham or control injections) or BDNF or NT-3 (i.e., experimental injections) at E13.5 and histological evaluation was performed at E17.5 as a criterion for detecting pulmonary hypoplasia (Porter, 1998). Since the skeletal muscle precursor cells and the CNS does not secrete adequate amounts of NTs in the amyogenic embryos (Kablar and Belliveau, 2005; Stephens et al.,, 2005), there is a possibility that the lung hypoplasia is caused by the lack of non-lung secreted NTs. Our findings showed that, compared to the lung of wild-type embryos composed of well-expanded saccules at E17.5 (Fig. 3A, C,E), the amyogenic lung consisted of acinar or dilated acinar tubules (Fig. 3B,D,F) and did not have visible saccules in either of the two experimental groups (i.e., BDNF- or NT-3-injected mouse embryos). Therefore, development of the hypoplastic lung was not rescued, based on the histological examination, after the canalicular stage by either BDNF or NT-3. At this point, we did not perform further analysis of the lung phenotype of NT-injected embryos. In the same experiment, the injected BDNF or NT-3 rescued spinal cord motor neurons (Geddes and Kablar; Angka and Kablar, unpublished data), indicating that the injected NTs actually reached the target. This is an expected result, since it was clearly demonstrated in the past, that the administration of a single injection of different putative neurotrophic factor to mouse embryos *in utero* on E14 is sufficient to significantly reduce the normal

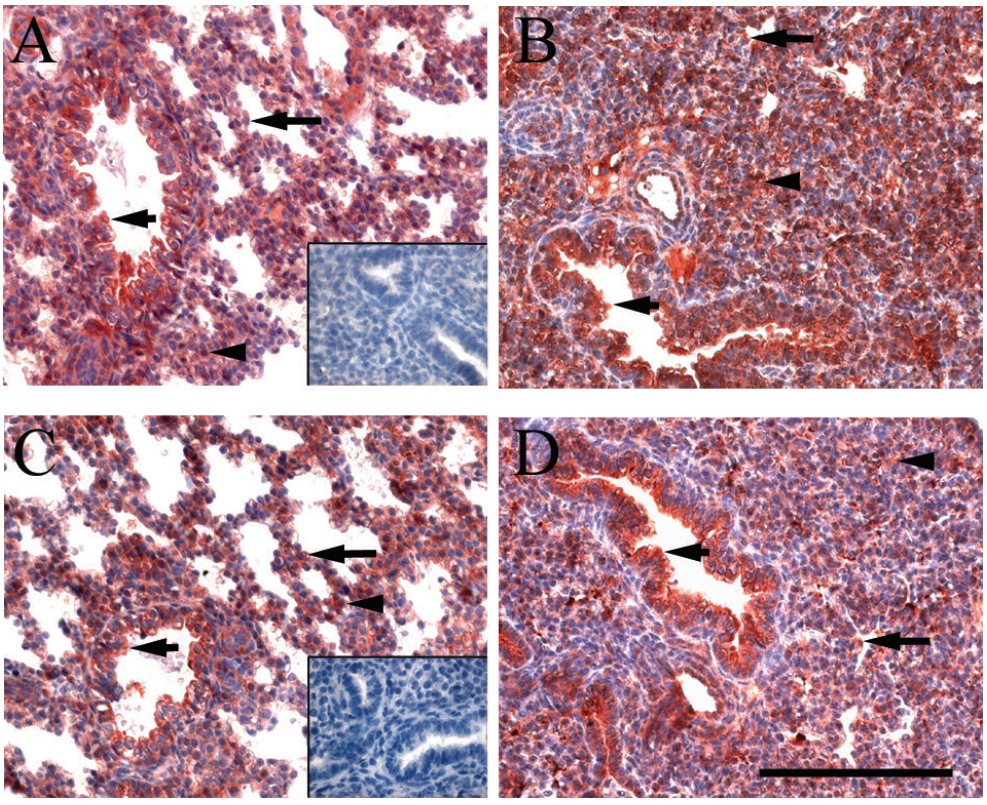

Fig. 2. Expression pattern of high affinity neurotrophin receptors is similar between wild-type and amyogenic embryos. In paraffin sections of E18.5 control (**A, C**) and hypoplastic (**B, D**) lungs, immunostained against Trk B (**A, B**) and Trk C (**C, D**), the expression pattern of the receptors is similar between the: a) epithelium of the conductive system (short arrows), b) epithelium of the respiratory system (long arrows), and c) mesenchymal compartment of the lung (arrowheads). Note that while normally developing lung of wild-type embryos is composed of widened saccules, the hypoplastic lung of amyogenic embryos is composed of acinar or dilated acinar tubules (see Figure 3 for more details). Negative controls are represented in the A and C insets. Scale bar: 50 μm.

death of spinal motor neurons when assessed on E18 (Houenou et al., 1994). Moreover, since Western analysis on the control and hypoplastic lung tissues using anti-BDNF and anti-NT-3 cannot discriminate between the endogenously present NTs versus the exogenously introduced NTs, the sham injections and the motor neuron rescue remain the only possible controls for this experiment.

Amyogenic embryos have less smooth muscle cells in their airways

It has been shown *in vitro* that mechanical forces are important for proliferation of smooth muscle cells (Buck, 1983; Dartsch et al., 1986). In addition, pulmonary development is reported to be influenced by mechanical forces due to the spontaneous contractions of ASMCs (McCray, 1993). To study the amount, proliferation and cell death of smooth muscle cells in the wall of airways *in vivo*, we performed immunostaining against smooth muscle actin (i.e., SMA), proliferating cell nuclear antigen (PCNA) and TUNEL labeling of apoptotic cells in the lung of control, *mdx:MyoD-/-*^{9th}, *Myf5-/-* and amyogenic embryos. Even though, a few other markers, such as the smooth muscle myosin heavy chain, h-caldesmon, caplonin and SM22, would be more

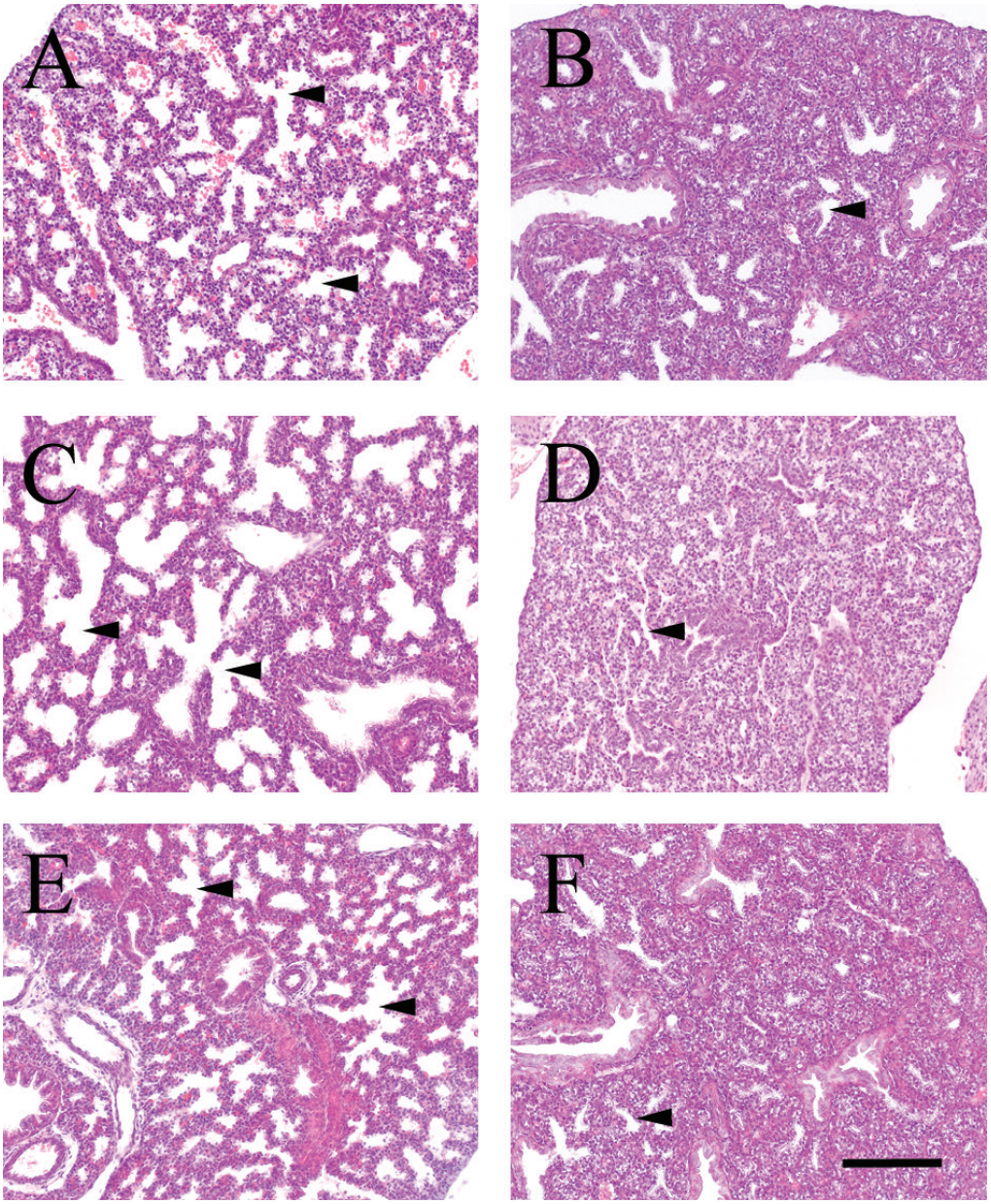

Fig. 3. Intra-amniotic injections of NTs do not rescue the hypoplastic lung phenotype. H&E stained cross sections of the control (i.e., sham-injected embryos) (**A, B**) and the experimental lung tissues (i.e., BDNF-injected embryos in **C, D** and NT-3-injetced embryos in **E, F**) show normal lung developed in wild-type embryos (**A, C, E**) and consistently hypoplastic lung development in amyogenic (**B, D, F**) embryos. Intra-amniotic injections of BDNF (**C, D**) and NT-3 (**E, F**) do not improve the histo-pathology of the hypoplastic lung in amyogenic embryos (**D, F**); while normally developing lung of wild-type embryos is composed of widened saccules (arrowheads in **A, C, E**), the hypoplastic lung of amyogenic embryos is composed of acinar or dilated acinar tubules (arrowhead in **B, D, F**), suggesting that it is arrested in the canalicular stage. Scale bar: 50 μm.

specific for the ASMCs, we employed anti-SMA because it is also expressed in myofibroblasts, myoepithelial cells and myoendothelial cells, i.e., the cell sub-populations that may be the only possible phenotype in the double-compound lung.

At earlier stages of lung development, the expression pattern of all markers in the layer of smooth muscle cells seemed to be indistinguishable between normal and hypoplastic lungs (data not shown). However, at later stages, the expression pattern of SMA appeared to be decreased and the number of SMA-expressing cells was 52% lower ($P<0.05$) in amyogenic embryos (i.e., 115±6 SMA-positive cells per 500 nuclei counted) in comparison to the control (i.e., 241±9 SMA-positive cells per 500 nuclei counted) and Myf5-/- or mdx:MyoD-/-9th embryos (Fig. 4 and data not shown), indicating that airways are surrounded by lower amounts of SMA-expressing cells in the hypoplastic lungs of only amyogenic embryos. In all experimental groups it was not possible to observe any TUNEL-positive cells in the place of SMA-expressing smooth muscle cells and only a few smooth muscle cells were positive for PCNA (i.e., 2±1 PCNA-positive cells per 500 nuclei counted in the control and all experimental groups; $P>0.05$). These data indicate that cell death and cell proliferation are not likely mechanisms leading to a difference in the number of ASMCs of amyogenic embryos.

Discussion

It has been shown that mechanical factors (e.g., FBMs) are required for normal lung growth (Kitterman, 1996; Inanlou and Kablar, 2003, 2005a,b). Surprisingly, recent studies show that NTs and their receptors are also expressed in the lung, suggesting a role of NTs in lung development (Shibayama and Koizumi, 1996; Lommatzsch et al., 1999; Ricci et al., 2000, 2004; Hikawa et al., 2002). Finally, the CNS and the skeletal musculature is an important source of certain NTs (Ernfors et al., 1992), such as BDNF and NT-3. Taken together, the purpose of this study was to investigate the potential role of lung- and non-lung-secreted NTs as hormonal factors in lung organogenesis and pathogenesis of the lung hypoplasia in vivo. Employing genetically engineered mouse embryos which lack skeletal muscles (i.e., *Myf5-/-:MyoD-/-*) provided us with the opportunity to study lung growth in the absence of NTs secreted by the skeletal musculature. In addition, to study the role of ASMCs in the pathogenesis of pulmonary hypoplasia, we employed three different mouse models lacking either various subpopulations of muscles (e.g., *mdx:MyoD-/-*^{9th} and *Myf5-/-* embryos) or the entire skeletal muscle lineage (*Myf5-/-:MyoD-/-* embryos). Our data show that neither endogenous (found in the lung) nor exogenous (from CNS/muscle) BDNF and NT-3 are likely contributors to the lung organogenesis and pulmonary hypoplasia. In addition, the amount of ASMCs does not seem to play a role in the development of lung hypoplasia, but ASMC number appears to be greatly affected in the complete absence of FBMs.

In adult mice, BDNF was observed in the respiratory epithelium (Lommatzsch et al., 1999), while the expression of all three muscle-secreted neurotrophins (i.e., BDNF, NT-3 and NT-4/5) was detected in certain types of pulmonary cells (e.g., macrophages and type I pneumocytes) (Hikawa et al., 2002). Moreover, in adult mice, both Trk B and Trk C were found to be expressed (Hikawa et al., 2002). Very recently, in the adult human lung, bronchial epithelium was found immunoreactive for all muscle-secreted NTs, with a higher intensity of BDNF, but it did not express NT receptors (Ricci et al., 2004). On the other hand, alveolar epithelium was immunoreactive for Trk C, but did not express NTs or other receptors (Ricci et al., 2004). In our experiments, immunohistochemistry against BDNF, NT-3, NT-4/5 and their high affinity receptors (i.e., Trk B and Trk C) was performed to elucidate the expression pattern of

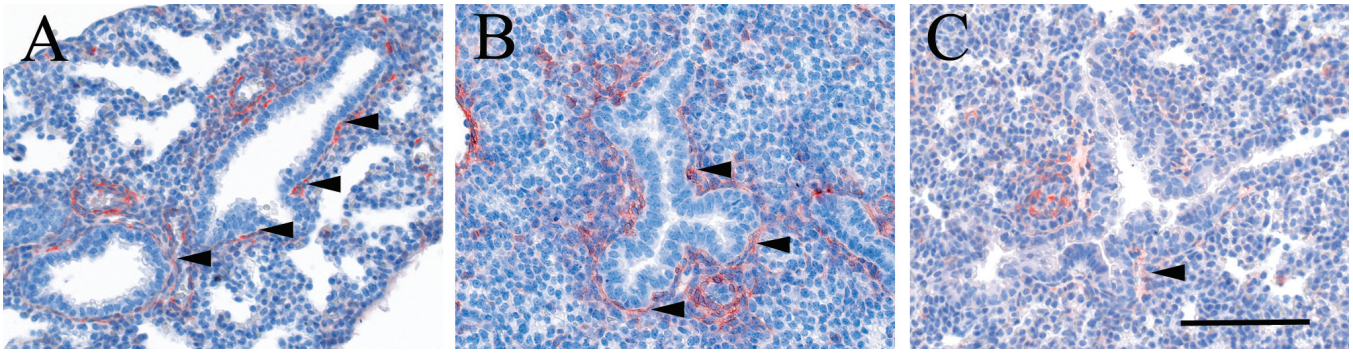

Fig. 4. The number of smooth muscle cells is significantly decreased in the airways of the lung in amyogenic embryos. Immunohistochemistry against SMA reveals a weaker and less defined immunostaining (i.e., the red-brown signal; arrowheads in all pictures) around the airways in the lung of amyogenic embryos (arrowhead in **C**), compared to wild-type embryos (arrowheads in **A**) or Myf5-/- embryos (arrowheads in **B**), at later stages of lung development (i.e., E17.5-E18.5). Scale bar: 50 μm.

neurotrophins during normal and hypoplastic mouse lung development. Our data revealed a quite ubiquitous expression pattern of all five molecules in both, the epithelial and the mesenchymal compartments of the developing mouse lung. Surprisingly, the distribution pattern of any of the five molecules employed was indistinguishable between normal and hypoplastic lung in both compartments. In conclusion, these findings connoted that expression of NTs and their receptors by pulmonary cells was not disturbed in the hypoplastic lung, therefore suggesting that pulmonary-secreted NTs and their receptors do not play a role in the pathogenesis of pulmonary hypoplasia. However, by contrast, a down-regulation of NT-3 was reported in adult human chronic obstructive pulmonary disease (COPD) (Groneber et al., 2005), suggesting that in adult human COPD the requirements for NT-3 differ from those in the FBMs-dependent pulmonary hypoplasia in developing mouse embryos.

To study the possible role in lung organogenesis of exogenous NTs secreted by the skeletal muscles and CNS, wild-type and amyogenic embryos were injected intra-amniotically with either saline (sham), BDNF or NT3 at E13.5 and harvested at E17.5. Histological evaluation as a criterion for lung hypoplasia (Porter, 1998) showed no difference in the histology of the lung between sham- and NTs-injected embryos. These data indicate that during development non-lung-secreted NTs do not play a significant role in the pathogenesis of lung hypoplasia. On the other hand, it is hard to rule out the role for NTs in lung organogenesis by substituting only one NT at the time, since skeletal muscle and CNS are known to produce multiple factors that may be required for normal lung development. However, recent study that reports normal lung development in BDNF-/- mouse embryos (Lomatzsch et al., 1999) suggests that *BDNF* has no role in lung development, whereas to our knowledge no data are available on the lung development in NT-3-/- mice. Finally, considering that the vasculature of the developing lung is different from other tissues (and in the case of double-mutant embryos, it may even lack some smooth muscle cells), it could be that the pulmonary vasculature wall is not capable of transmitting the NTs to the correct target site.

During development, smooth muscle cells of the airways are subjected to intermittent mechanical forces produced by FBMs. Previous studies have shown that mechanical forces play an important role in modulating the growth of smooth muscles (Buck, 1983; Dartsch et al., 1986). In addition spontaneous contractions in the smooth muscle cells of the airways have been observed during development in many species including human (Sorokin, 1961; McCray, 1993). These contractions are suggested to cause distention and contraction within developing airways and pre-alveolar ducts by altering intra-luminal pressure and also to play a role in normal formation of airspaces through cell stretch. The fact that, in our experiments, the smooth muscle phenotype is only observable in amyogenic fetuses (and not for example in *Myf5-/-* or *mdx:MyoD-/-*^{9th} fetuses that also die from pulmonary hypoplasia) (Inanlou and Kablar, 2003, 2005a,b), leads to the conclusion that spontaneous contractions of airway smooth muscles should not have an impact on the pathogenesis of lung hypoplasia. Instead, it appears possible that in amyogenic embryos the absolute absence of mechanical forces from FBMs causes the decrease in the amount of smooth muscle cells via an unknown underlying mechanism. That mechanism probably does not involve disturbances in the cell cycle kinetics, according to our PCNA and TUNEL data. In fact, in spite of ASMCs hyperplasia in asthma, it was not possible to detect an increased rate of proliferation in the biopsies employing PCNA (Stewart, 2004). It has been argued that in chronic asthma the rate of cell proliferation may be too small to detect an increase over the baseline level of cell turnover. Alternatively, the hypertrophied ASMCs may be showing some changes in de-differentiation from the pre-existing fibroblasts (Gizycki et al., 1997) or a failure in migration into the appropriate layer of the airway wall (Vignola et al., 2001). However, the later possibility seems to be an unlikely mechanism in the hypoplastic embryonic lung, since the expression of SMA did not change in its localization within the airway wall and it was normal up to E18.5 even in amyogenic embryos. Instead, our data suggest that the achievement of normal amount of ASMCs could rely on myofibroblast de-differentiation (Gizycki et al., 1997) that seemingly failed in the absolute absence of FBMs. Finally, in the other two models (i.e., Myf5-/- or mdx:MyoD-/-9th embryos), the apparent presence of either diaphragmatic or intercostal muscle movements respectively is sufficient to maintain the normal number of ASMCs in spite of a concomitant down-regulation of growth factors (e.g., PDGFs, IGFs) (Inanlou and Kablar, 2003, 2005a,b), which are also suggested to influence proliferation of ASMCs (Noveral et al., 1994; Hirst et al., 1996). Clearly, the underlying mechanism of disturbance in the growth of smooth muscles of the airways has yet to be defined. In conclusion, it is tempting to speculate that the changes in cell cycle kinetics, in the expression of growth factors and in cell migration do not establish the amount of ASMCs, while FBMs emerge as the more important determinant of the ASMC numbers possibly via influencing the myofibroblast de-differentiation.

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