Targeted disruption of NDST-1 gene leads to pulmonary hypoplasia and neonatal respiratory distress in mice

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Abstract In order to address the biological function of GlcNAc *N*-deacetylase/*N*-sulfotransferase-1 (NDST-1), we disrupted the NDST-1 gene by homologous recombination in mouse embryonic stem cells. The NDST-1 null mice developed respiratory distress and atelectasis that subsequently caused neonatal death. Morphological examination revealed type II pneumocyte immaturity, which was characterized by an increased glycogen content and a reduced number of lamellar bodies and microvilli. Biochemical analysis further indicated that both total phospholipids and disaturated phosphatidylcholine were reduced in the mutant lung. Our data revealed that NDST-1 was essential for the maturation of type II pneumocytes and its inactivation led to a neonatal respiratory distress syndrome.

Key words: NDST-1; Disruption; Atelectasis; Type II pneumocyte; Immaturity; Respiratory distress syndrome

1. Introduction

Heparan sulfate proteoglycans (HSPGs) are macromolecules associated with the cell surface and the extracellular matrix where they regulate cell adhesion, growth, migration, differentiation and gene expression. They are composed of a core protein and the negatively charged heparan sulfate glycosaminoglycan side chain, which is covalently bound to the core protein (reviewed in [1]). The modifications of the glycosaminoglycan chains are catalyzed by various enzymes while GlcNAc *N*-deacetylation/*N*-sulfation are prerequisites for all of the other modifications and are catalyzed by a single-chain polypeptide GlcNAc *N*-deacetylase/*N*-sulfotransferase (NDST) [2]. Three different isozymes with high sequence similarity have been identified and named NDST-1, NDST-2, and NDST-3 [2–5]. The NDST-2-deficient mice are unable to synthesize sulfated heparin and show abnormal mast cells [6,7].

2. Materials and methods

2.1. Construction of the targeting vector

A murine NDST-1 genomic DNA fragment was acquired by screening a 129/sv genomic library (kindly provided by Dr. En Li). A *Hin*dIII fragment and an *Eco*RI fragment were subcloned into plasmid pBluescript II KS$^+$. The precise structure of the first exon was determined by sequencing. The pgk-neo-polyA cassette was released from vector with *Eco*RI and *Hin*dIII, filled in with Klenow enzyme, and cloned into the blunt-ended *Bsp*EI site of the NDST-1 gene's first exon at 19 bp downstream of the ATG site and an *Eco*RI site was re-created.

2.2. Transfection and selection of embryonic stem (ES) cells

The linearized targeting vector was electroporated into J1 ES cells (a kind gift from Dr. En Li) and clones that had survived selection in G418 were Southern-blotted using a *Hin*dIII fragment flanked the targeting vector (Fig. 1) according to Li et al. [8].

2.3. Generation of chimeras and NDST-1 null mutant

Chimeras were generated as described by Bradley [9]. Two ES cell clones that had undergone homologous recombination were injected into C57BL/6 blastocysts. Resultant chimeras were crossed with C57BL/6 and *agouti* F1 progeny were screened by tail biopsy. By intercrossing heterozygous mice, NDST-1-deficient mice were acquired. F2 progeny were genotyped by PCR. Amplification was performed for 35 cycles at 94°C, 30 s, 65°C, 30 s and 72°C, 5 min. The regions of the primers (p1: GGTCT TGGAA TTGTG CAGTG TC; p2: GTGCT TTACG GTATC GCCGC TC; p3: GCCAT TAGCT GAACA TCCTG ACC) are indicated in Fig. 1A.

2.4. Histology and transmission electron microscopy

Newborn mice were prefixed transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Tissues were dehydrated in a series of alcohol steps, transferred into xylene and embedded in paraffin. Hematoxylin-eosin staining was performed. For electron microscopy (EM), the lungs were fixed overnight in 2% glutaraldehyde, washed in phosphate-buffered saline, dehydrated in a series of ethanol steps, and processed for EM using routine procedures.

2.5. Alveolar surfactant analysis

Lung tissues were homogenized in 0.85% saline and the amount of disaturated phosphatidylcholine (DSPC) was measured by extracting an aliquot with chloroform/methanol (2:1), followed by treatment of the lipid extract with OsO_4 in carbon tetrachloride and alumina column chromatography according to Mason et al. [10]. Total phospholipids (TPL) were determined as described by Bartlett [11]. The concentration was expressed as mg/g wet lung.

3. Results

To investigate the function of NDST-1, we used homologous recombination to disrupt the NDST-1 gene in mice. The targeting construct is illustrated in Fig. 1. Two ES cell clones which underwent homologous recombination were injected into C57BL/6 blastocysts, From these NDST-1(+/−) and NDST-1(−/−) mice were generated. The phenotypes were analyzed using F2 littermates on outbred genetic background.

Homozygote F2 offspring mice with homozygous null alleles for NDST-1 ($n = 41$) derived from the intercross of heterozygous animals died in the neonatal period (range 5 min–

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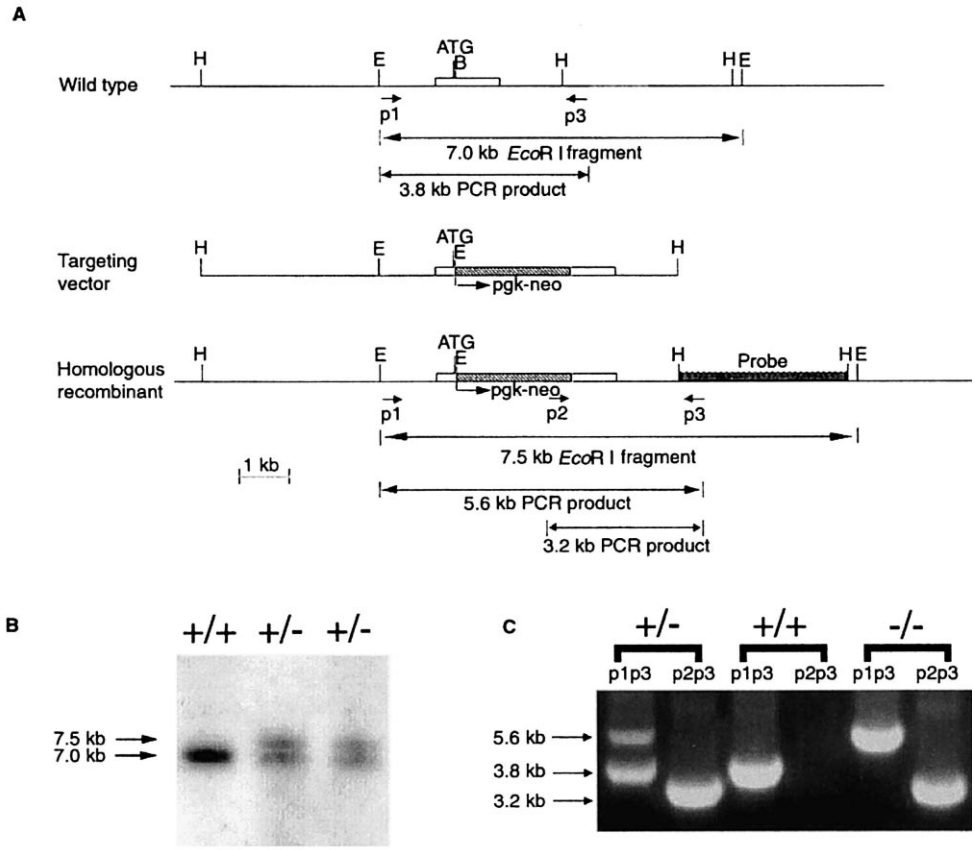

Fig. 1. Targeted disruption of the mouse NDST-1 gene. A: Structure of the wild type NDST-1 gene, the targeting vector and the mutated locus. Open box: first translated exon. B, *Bsp*EI; E, *Eco*RI; H, *Hin*dIII. B: Genotyping of ES cells and F1 offspring mice by Southern blot. The NDST-1 genotype is indicated. C: Genotyping of F2 progeny by PCR. The PCR products are indicated in A.

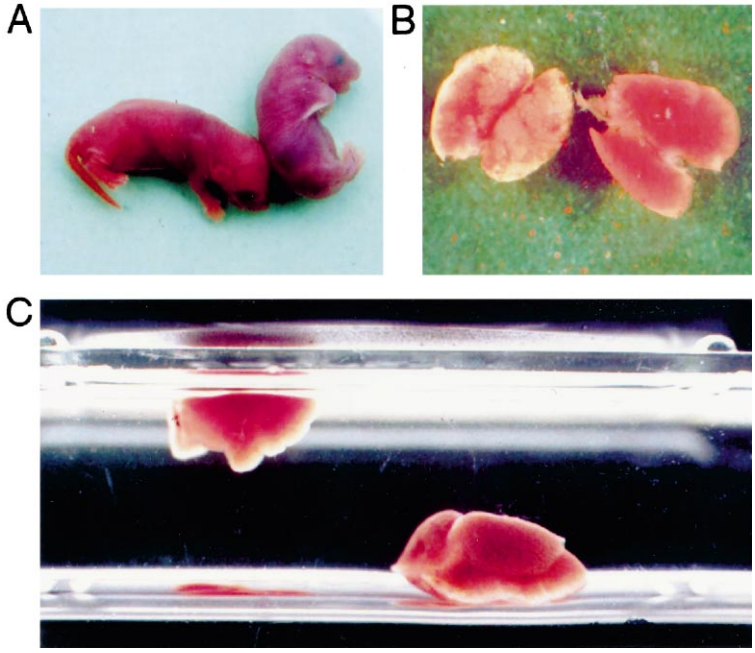

Fig. 2. Appearance of newborn mice and their lungs. A: Shortly after birth (about 5 min) a control mouse (left) appears pink while a −/− mouse (right) appears cyanotic with breathing difficulty. B: Neonatal lungs of a control mouse (left) and a NDST-1(−/−) mouse (right). Lungs of control mice appear pink and those of −/− mice appear red and liver-like. C: NDST-1(−/−) lungs (the lower one) have sunk while the control lungs are floating in the water.

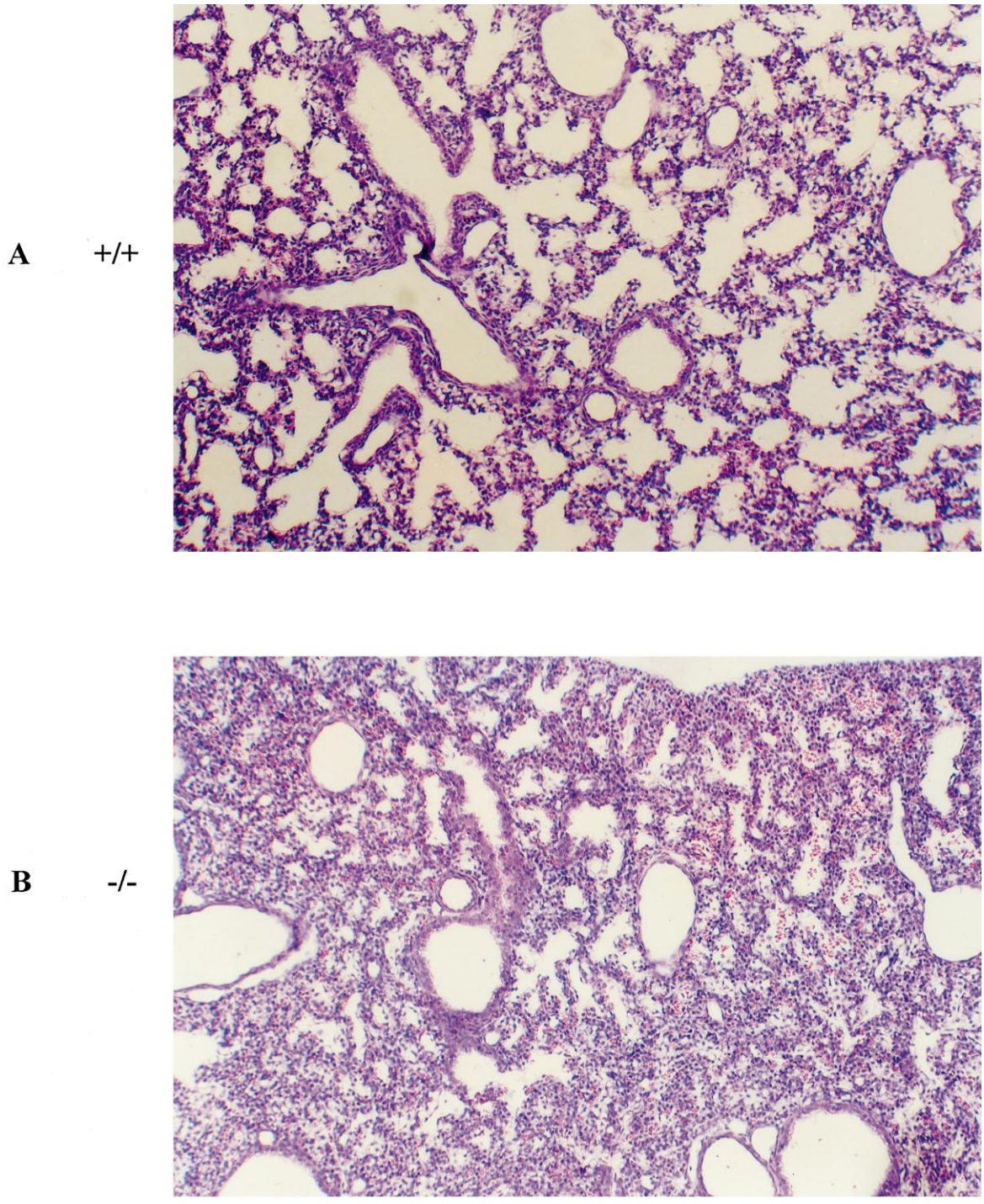

Fig. 3. Histology of the neonatal lungs. After 10 min spontaneous breathing, control lungs (A) aerated alveolar spaces, while NDST-1(−/−) lungs (B) showed severe atelectasis of the alveolar spaces. Scale bar: 140 μm.

10 h). The null mice experienced severe respiratory difficulties as judged from their gasping breath and cyanotic skin color (Fig. 2A). Autopsy of NDST-1(−/−) mice revealed no visible developmental defects other than abnormal development of the lung that appeared red in color, liver-like (Fig. 2B) and airless (Fig. 2C). Histological examination demonstrated that NDST-1(−/−) lungs have normal branching morphology but exhibit atelectasis and thicker alveolar septa compared with those of control [NDST-1(+/+) and NDST-1(+/−)] littermates (Fig. 3).

Morphological examination by EM revealed that the type II pneumocytes of NDST-1(−/−) lungs had glycogen accumulations, reduced numbers of lamellar bodies and attenuated microvilli compared with control mice (Fig. 4). These pheno-

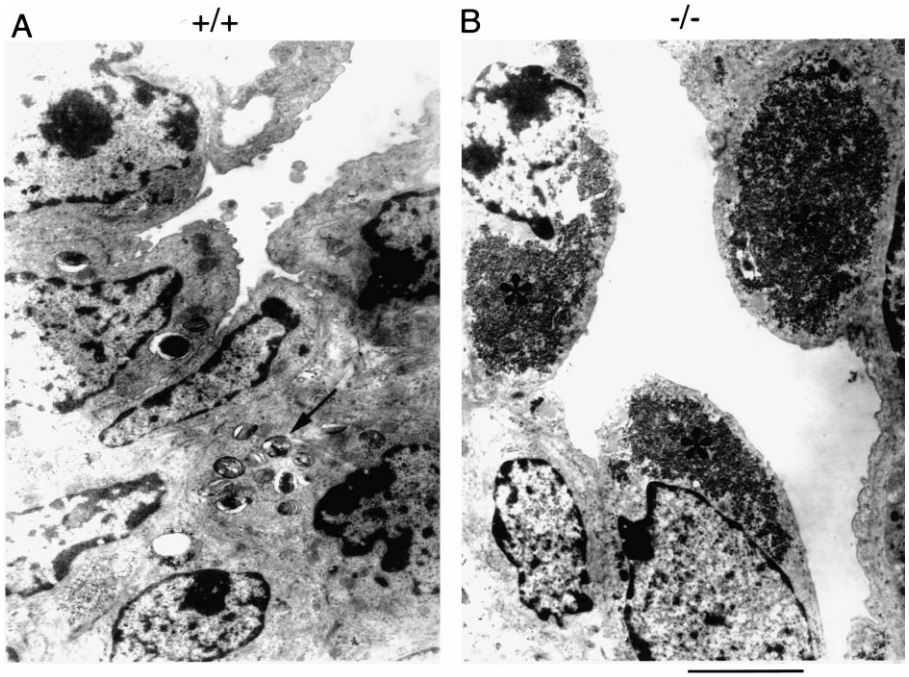

Fig. 4. Electron microscopy analysis. NDST-1(−/−) alveolar epithelial cells (B) contained more glycogen (*) and fewer lamellar bodies (arrowhead) than cells of control (A). Scale bar: 3.5 μm.

types are representative for immaturity of the type II pneumocytes in the homozygous null-allele mice. It is well known that at a late stage of fetal development, mammalian alveolar epithelia differentiate abruptly for postnatal adaptation. Some of the most significant changes occur in type II pneumocytes, such as the disappearance of glycogen and the generation of microvilli and lamellated inclusion granules, which contain pulmonary surfactant. Premature birth before adequate type II cell maturation could be associated with neonatal respiratory distress syndrome (RDS), which is often fatal [12]. In our observation, neonatal NDST-1(−/−) mice experienced developmental arrest of the type II pneumocytes, which was consistent with clinical and pathological features of RDS [12].

Since the type II pneumocytes of lungs are the site of synthesis, storage, secretion, and recycling of the pulmonary surfactant, which is essential for lowering surface tension at the air–liquid interface [13], it is likely that the immature lungs from the homozygous null-allele mice lack surfactant. We therefore measured the contents of DSPC and TPL in the whole lung tissue. We observed that in the null mice both DSPC and TPL decreased 33.2% ($n_1 = 8$, $n_2 = 4$, $P < 0.01$) and 24.4% ($n_1 = 8$, $n_2 = 4$, $P < 0.05$) respectively compared to the controls (Fig. 5). Taken together, our data indicated that the neonatal death of the NDST-1 null mice is due to immaturity of type II pneumocytes that further leads to insufficient production of surface tension-lowering pulmonary surfactant and NDST-1 is essential for the maturation of type II pneumocytes during pulmonary development.

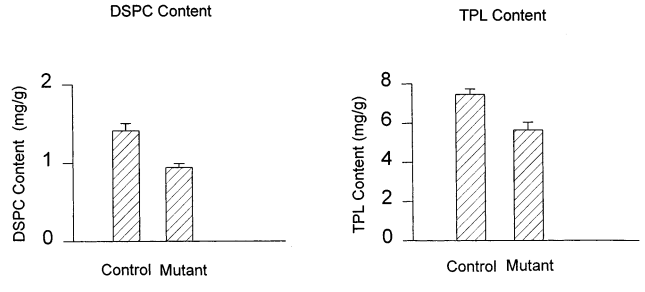

Fig. 5. Phospholipid analysis. DSPC decreased by 33.2% ($n_1 = 8$, $n_2 = 4$, $P < 0.01$) and TPL decreased by 24.4% ($n_1 = 8$, $n_2 = 4$, $P < 0.05$) compared to control. Values are mean ± S.E.M.

4. Discussion

We demonstrated that mice null for NDST-1 developed immaturity of type II pneumocytes. As a consequence they died shortly after birth. The development of other organs, however, showed no pathological defects suggesting that NDST-1 played a non-redundant role in the differentiation of type II pneumocytes. We propose two models to explain this phenotype. NDST-1 may be required for the modification of subtype heparin sulfate glycosaminoglycan (HS GAG) which is necessary for the differentiation of type II pneumocytes; alternatively, reduction of sulfation in NDST-1 null mice may quantitatively reduce the gross amount of HS GAG that is required for multiple signaling processes during lung development.

The development of the respiratory system requires epithelial–mesenchymal interaction mediated by specific fibroblast growth factor ligand–receptor signaling as well as modulation by other peptide growth factors including epidermal growth factor, platelet-derived growth factor A and transforming growth factor β and by extracellular matrix components (reviewed in [14]). HSPGs can function as coreceptors for all these growth factors through several mechanisms, including facilitating or stabilizing ligand–ligand dimerization and subsequent ligand–receptor interaction as well as altering their effective concentration [15–20]. The specificity of HSPG–ligand interactions resides, at least in part, in the structure of

the HS GAG side chains that vary in number, length, sequence composition, and sulfation pattern between different cell types and different developmental stages [21]. NDST-1 may be required for the modification of a lung-specific HS GAG that is necessary for signaling activity of a specific signaling molecule. Disruption of NDST-1 may result in defects of a lung-specific HS GAG necessary for signaling of a specific signaling molecule. This may further lead to immaturity of type II pneumocytes, or may quantitatively reduce the activities of several growth factors with the consequence of type II cell developmental arrest. Future experiments determining the signaling activity affected will be necessary to address this issue.

The specific lung defect observed in NDST-1 raised a very interesting issue about the tissue-specific function of individual members of the NDST protein family. Our results support the hypothesis that individual members of the NDST protein family may be required for the modification of subtype HS GAG that is required for specific cellular functions during development. Interestingly, and consistent with our hypothesis, mice with homozygous null alleles for NDST-2 develop only abnormal mast cells and are unable to synthesize sulfated heparin, suggesting that NDST-2 is crucial for heparin biosynthesis in mast cells as proposed by Forsberg et al. [6,7]. Considering the gene's widespread expression [2,5], it is interesting that other organs appear to develop normally in the mutant. Although it is possible that NDST-1 is only required in the developing lung, it is more likely that in other tissues and organs the function of NDST-1 can be compensated for by other members of the NDST family. Examination of mice null for both NDST-1 and other NDST family members will be able to address these issues.

Our results demonstrated that NDST-1 null mice develop lung defects that resemble neonatal RDS in humans [12], and thus provide a valuable genetic model for studying the disease.

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