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### Title

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### Permalink

<https://escholarship.org/uc/item/8wg5p6m5>

### Journal

Glycobiology, 24(8)

### ISSN

0959-6658

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### Publication Date

2014-08-01

### DOI

10.1093/glycob/cwu035

Peer reviewed

# Endothelial and leukocyte heparan sulfates regulate the development of allergen-induced airway remodeling in a mouse model

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Received on January 12, 2014; revised on April 24, 2014; accepted on April 24, 2014

Heparan sulfate (HS) proteoglycans (HSPGs) participate in several aspects of inflammation because of their ability to bind to growth factors, chemokines, interleukins and extracellular matrix proteins as well as promote inflammatory cell trafficking and migration. We investigated whether HSPGs play a role in the development of airway remodeling during chronic allergic asthma using mice deficient in endothelial- and leukocyte-expressed *N*-deacetylase/*N*-sulfotransferase-1 (Ndst1), an enzyme involved in modification reactions during HS biosynthesis. Ndst1-deficient and wild-type (WT) mice exposed to repetitive allergen (ovalbumin [OVA]) challenge were evaluated for the development of airway remodeling. Chronic OVA-challenged WT mice exhibited increased HS expression in the lungs along with airway eosinophilia, mucus hypersecretion, peribronchial fibrosis, increased airway epithelial thickness and smooth muscle mass. In OVA-challenged Ndst1-deficient mice, lung eosinophil and macrophage infiltration as well as airway mucus accumulation, peribronchial fibrosis and airway epithelial thickness were significantly lower than in allergen-challenged WT mice along with a trend toward decreased airway smooth muscle mass. Leukocyte and endothelial Ndst1 deficiency also resulted in significantly decreased expression of IL-13 as well as remodeling-associated mediators such as VEGF, FGF-2 and TGF- $\beta$ 1 in the lung tissue. At a cellular level, exposure to eotaxin-1 failed to induce TGF- $\beta$ 1 expression by Ndst1-deficient eosinophils relative to WT eosinophils. These studies suggest that leukocyte and endothelial Ndst1-modified HS contribute to the development of allergen-induced airway remodeling by promoting recruitment of inflammatory cells as well as regulating expression of pro-remodeling factors such as IL-13, VEGF, TGF- $\beta$ 1 and FGF-2 in the lung.

**Keywords:** allergen-induced airway remodeling / FGF-2 / heparan sulfates / *N*-Deacetylase/*N*-Sulfotransferase-1 / TGF- $\beta$

## Introduction

Heparan sulfates (HS) are linear glycosaminoglycans (GAGs) covalently attached to a core protein occurring as HS proteoglycans (HSPGs). HSPGs are the most abundant sulfated GAGs in the lung parenchyma and are expressed by all cells in the body (Papakonstantinou and Karakiulakis 2009). Because of their ability to bind to multiple growth factors and their receptor tyrosine kinases as well as chemokines, interleukins, enzymes, enzyme inhibitors, lipases and apolipoproteins, extracellular matrix and plasma proteins (Bishop et al. 2007), HSPGs play a critical role in various physiological aspects including normal development and growth control, cell signaling and morphogenesis, cellular crosstalk, organization of basement membrane barriers, nutritional metabolism, injury and wound repair (Bishop et al. 2007), inflammatory responses (Parish 2006) and even microbial adhesion to host cells (Rostand and Esko 1997). The role of sulfotransferases in generating critical binding sites or niches for proteins to interact with GAGs is well recognized (Kusche-Gullberg and Kjellen 2003). The modification reaction involving *N*-sulfation which is unique to HS and does not occur in other sulfated GAGs is initiated with *N*-deacetylation followed by *N*-sulfation of selected *N*-acetylglucosamine residues and is carried out by a family of four bifunctional glucosaminyl *N*-deacetylase/*N*-sulfotransferase isoenzymes (Ndst1–4) (Kusche-Gullberg and Kjellen 2003).

The extent of sulfation and distribution of the sulfate substitutions on the HS backbone appear to play a vital role in regulating normal physiology. A systemic deletion of Ndst1 is lethal for mice due to forebrain defects (Ringvall et al. 2000) and various other defects are associated with targeted disruption of the Ndst1 gene (Ringvall and Kjellen 2010) which include skeletal malformation (Grobe et al. 2005; Pallerla et al. 2007), anophthalmia (Pan et al. 2006), lung hypoplasia with respiratory distress (Fan et al. 2000) and abnormal functioning of bone morphogenetic protein (Hu et al. 2009). In addition, HSPGs play an important role during conditions of inflammation. Endothelial HSPGs were found to promote neutrophilic inflammation in several acute inflammatory models (Wang et al. 2005). This is further supported by studies demonstrating that endothelial HSPGs promote recruitment of leukocytes to inflammatory sites by acting as ligands for

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adhesion molecules such as L-selectin as well as for chemokines and thus sequestering chemotactic gradients (Celie et al. 2007; Massena et al. 2010). Recent studies have shown that inactivation of the enzyme uronyl 2-O-sulfotransferase downstream from Ndst1 in endothelial cells resulting in a change in HS structure that is caused by increased N-sulfation and 6-O-sulfation of glucosamine units in response to the decreased 2-O-sulfation of uronic acid residues enhances neutrophil infiltration during acute inflammation in mice (Axelsson et al. 2012). This underscores the importance of the frequency and distribution of sulfate residues to the differential role of HSPGs during inflammation.

Using mice deficient in endothelial and leukocyte Ndst1 (*Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>*), we have previously demonstrated that endothelial but not leukocyte HSPGs play an important role in the development of airway inflammation including airway hyperresponsiveness (AHR) by supporting recruitment of inflammatory cells, specifically eosinophils, to the airways in a murine model of acute allergic airway inflammation (Zuberi et al. 2009). Allergic asthma, on the other hand, is a chronic disease caused by repetitive allergen exposure and is distinguished by several structural changes in the airways, including airway epithelial alterations, subepithelial fibrosis, increased airway smooth muscle mass and increased airway vascularization that are collectively termed as airway remodeling (Hamid and Tulic 2009). Given the previously identified role for Ndst1-modified HSPGs in supporting recruitment of inflammatory cells to allergic airways (Zuberi et al. 2009) and the ability of mediators such as cytokines, chemokines and growth factors released by these cells to drive airway remodeling (Al-Muhsen et al. 2011), we have examined whether these HSPGs contribute to the development of airway inflammation with features of remodeling in a murine model of chronic allergen-induced airway inflammation.

## Results

### *HS expression in lungs of allergen-challenged WT and Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup> mice*

Previous studies have demonstrated altered metabolism of proteoglycans, including small HSPGs, by bronchial fibroblasts from asthmatics that may result in increased proteoglycan deposition in the bronchial mucosa and contribute to AHR as well as airway remodeling (Westergren-Thorsson et al. 2002). Increased HSPG expression has also been shown in the airways of allergen-exposed rats as well as of asthmatic subjects (Venkatesan et al. 2012). We examined HSPG expression in the lungs of chronic allergen-challenged mice using a previously characterized phage display anti-HS antibody (Kurup et al. 2007) that interacts with saccharides containing N-, 2-O- and 6-O-sulfate as well as internal 2-O-sulfate groups. An increase in these sulfated proteoglycans was noted in the lungs of allergen-challenged WT mice relative to control mice. While HSPG expression was noted by endothelial cells and airway epithelial cells in the saline and allergen-exposed mice, inflammatory cells recruited to the airways also stained positive for HSPG in the allergen-exposed group (Figure 1A, left panels and B, center panel). In the lungs of allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice, HSPG expression was observed largely by airway epithelial cells and there was an overall reduction in HSPG expression consistent with the

phenotype of these mice, i.e. endothelial and leukocyte-specific deficiency of Ndst1 (Figure 1A, right panels and B, bottom panel). Sub-epithelial basement membrane and smooth muscle cells were positive for expression of these sulfated proteoglycans in WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice.

### *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup> mice exhibit attenuated cellular infiltration of the airways after chronic allergen challenge*

We next compared inflammatory cellular responses in WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. After chronic allergen challenge, WT mice demonstrated a significantly larger number of inflammatory cells in the lung tissue and bronchoalveolar lavage fluid (BALF) in comparison with *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice (Figure 2A and B). A marked increase was noted in the number of eosinophils, neutrophils and lymphocytes in the allergen-challenged WT mice relative to the saline-exposed (control) group with a tendency for macrophages also to be higher (Figure 2B and C). *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice also showed a similar trend after allergen exposure. However, compared with allergen-exposed WT mice, the number of eosinophils and macrophages in the BALF of allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* was significantly reduced with a trend toward a decrease in the number of neutrophils and lymphocytes as well albeit not statistically significant. In addition, allergen-exposed WT mice exhibited significantly higher lung tissue infiltration by eosinophils and macrophages relative to corresponding *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice as assessed by eosinophil-specific major basic protein (MBP) (Figure 3A and B) and macrophage-specific F4/80 (Figure 3C and D) immunostaining, respectively. The number of lung tissue eosinophils and macrophages in control WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice were negligible.

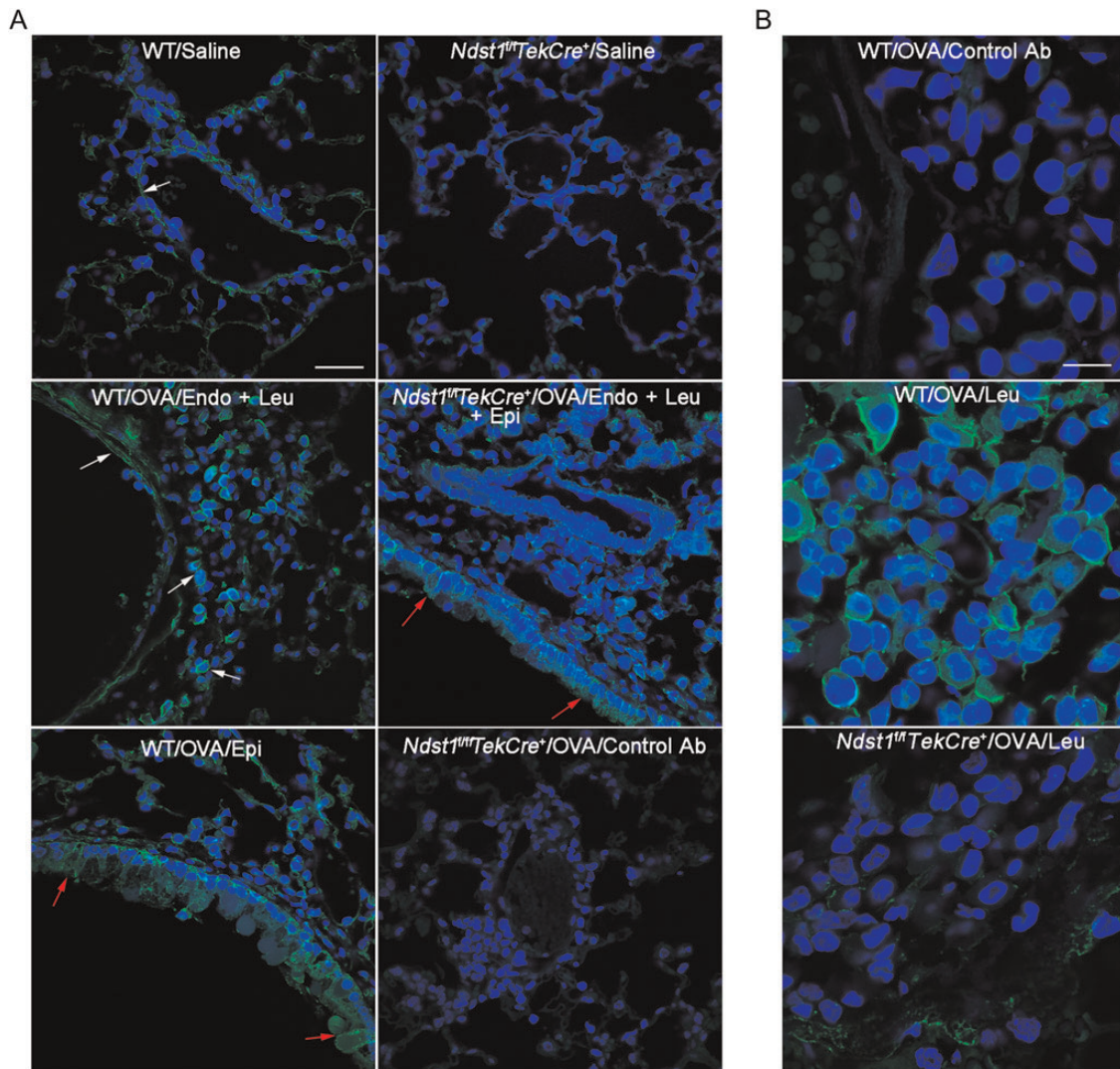
### *Allergen-challenged Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup> mice display lower levels of IFN- $\gamma$ and IL-13*

Chronic allergic inflammation including asthma is a complex disease involving many inflammatory cytokines that are central to almost every aspect of the immune response to an allergen. Th2 (IL-4, IL-5 and IL-13) and Th1 (IL-2 and IFN- $\gamma$ ) cytokine as well as TNF- $\alpha$  levels in the lung tissue of control and allergen-challenged WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice were determined (Table I). In addition, eotaxin-1 in the BALF of these mice was measured. In WT mice, repetitive allergen challenge induced a significant increase in levels of IL-5, IL-13, IFN- $\gamma$  and eotaxin-1. Compared with WT mice, allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice showed significantly lower levels of lung IL-13 and IFN- $\gamma$ . No change was noted in IL-4 levels in either group after allergen exposure. Allergen-induced IL-5 and eotaxin-1 expression in *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was similar to that observed in WT counterparts. IL-2 levels decreased to a similar extent in both groups after chronic allergen exposure while no significant differences were observed in TNF- $\alpha$  levels.

### *Allergen-challenged Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup> mice exhibit reduced airway remodeling*

Since HSPGs are involved in biological functions such as activation of chemokines, enzymes and growth factors to modulate inflammatory responses, we examined the impact of altered endothelial and leukocyte HSPG expression caused by Ndst1

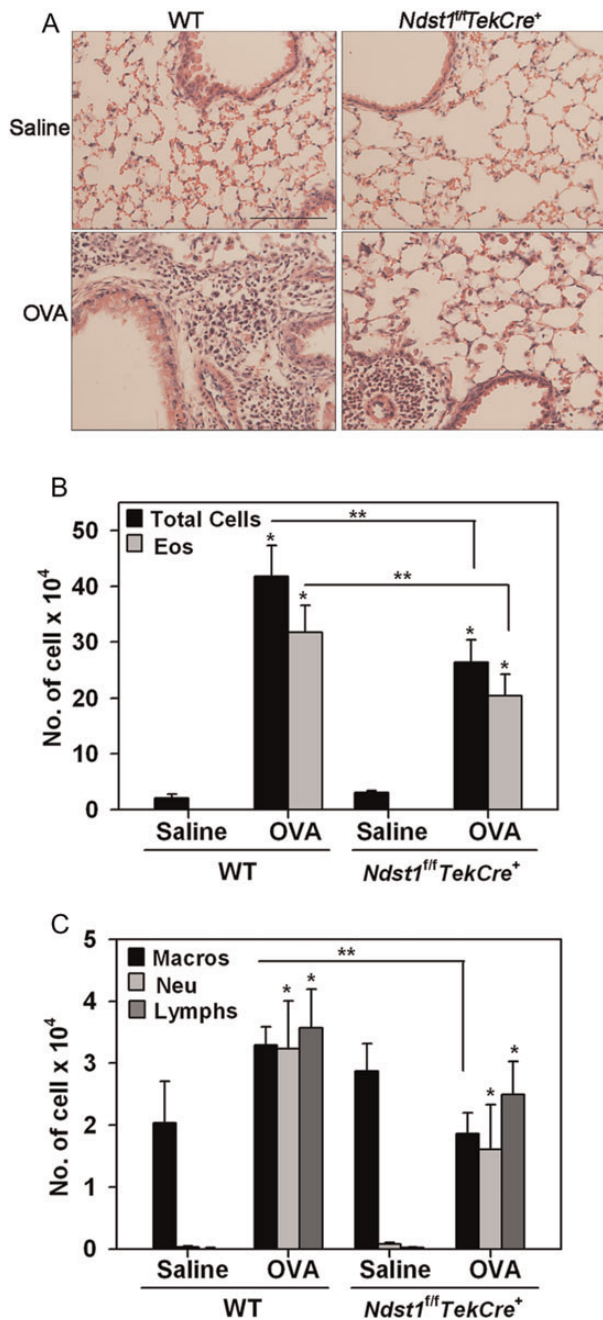




**Fig. 1.** Lung HS expression after allergen exposure. (A) Expression of sulfated PG in lungs of control and repetitively OVA-challenged WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice examined by immunofluorescence staining with VSV-tagged phage display-derived single chain antibody AO4B08 that interacts with the *N*-, 2-*O*-, and 6-*O*-sulfated saccharide motif as well as an internal 2-*O*-sulfate group. A non-GAG binding antibody MPB49 was used as a control. White arrows indicate positive (bright green) staining in the endothelium (Endo) and leukocytes (Leu). Red arrows indicate positive staining in the airway epithelium (Epi). Staining with a control antibody MPB49 in OVA-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice is shown. Scale bar represents 30  $\mu$ m. (B) Expression of sulfated PG by leukocytes recruited to the lungs of OVA-challenged WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice by immunofluorescence staining with antibody AO4B08. Staining with antibody MPB49 is shown as the control. Scale bar represents 10  $\mu$ m. Representative data of  $n = 3$  mice/group is shown.

deficiency on airway remodeling, a distinctive feature of chronic airway inflammation associated with repetitive allergen exposure. As expected, WT mice demonstrated significantly increased airway mucus metaplasia, peribronchial fibrosis (based on tissue collagen), airway epithelial thickness, another feature characteristic of remodeled airways (Cohen et al. 2007) and airway smooth muscle mass after repetitive allergen exposure (Figures 4 and 5). While some degree of airway mucus metaplasia (Figure 4A) and peribronchial fibrosis (Figure 4C) was visible even in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice relative to corresponding control mice, levels were significantly lower than in WT allergen-exposed mice (Figure 4B and D). Further, allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice exhibited significantly attenuated epithelial thickness compared with corresponding WT mice (Figure 5A

and B). A similar trend was noted with respect to airway smooth muscle hypertrophy/hyperplasia, although the decrease in smooth muscle mass in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* was not significantly lower than in WT mice (Figure 5C and D). Chronic airway inflammation is also associated with vascular remodeling that is largely mediated by vascular endothelial growth factor (VEGF) which is expressed and secreted by structural cells as well as inflammatory cells recruited to the airways (Meyer and Akdis 2013). We examined VEGF expression by inflammatory cells, predominantly macrophages (based on cell morphology), in the lung tissue of chronic allergen-challenged WT vs. *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice by immunohistochemistry. Although an increased number of VEGF-positive inflammatory cells were present in the alveolar spaces of allergen-challenged



**Fig. 2.** Reduced cellular infiltration of the airways in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. Lung tissue and BALF was collected from control and allergen-challenged WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice 24 h after the last challenge. (A) Cellular infiltration was evaluated by H&E staining of paraformaldehyde-fixed paraffin embedded lung tissue sections. Images representative of each group are shown. Scale bar represents 100  $\mu$ m. (B and C) BALF was evaluated for total as well as differential cell counts by microscopic evaluation of cytocentrifuged slides based on morphological criteria after Hema 3 staining. Total and eosinophil [Eos] counts are shown in B; macrophage [Macros], neutrophil [Neu], and lymphocyte [Lymphs] counts are shown in C. Data represent mean  $\pm$  SEM. Combined data of  $n = 5$  mice for control groups and 7–8 mice for allergen-challenged groups is shown in B and C. \* $P < 0.01$  in B and  $< 0.04$  in C for comparison of control vs. allergen-exposed mice. \*\* $P < 0.05$  in B and C for comparison of allergen-challenged groups.

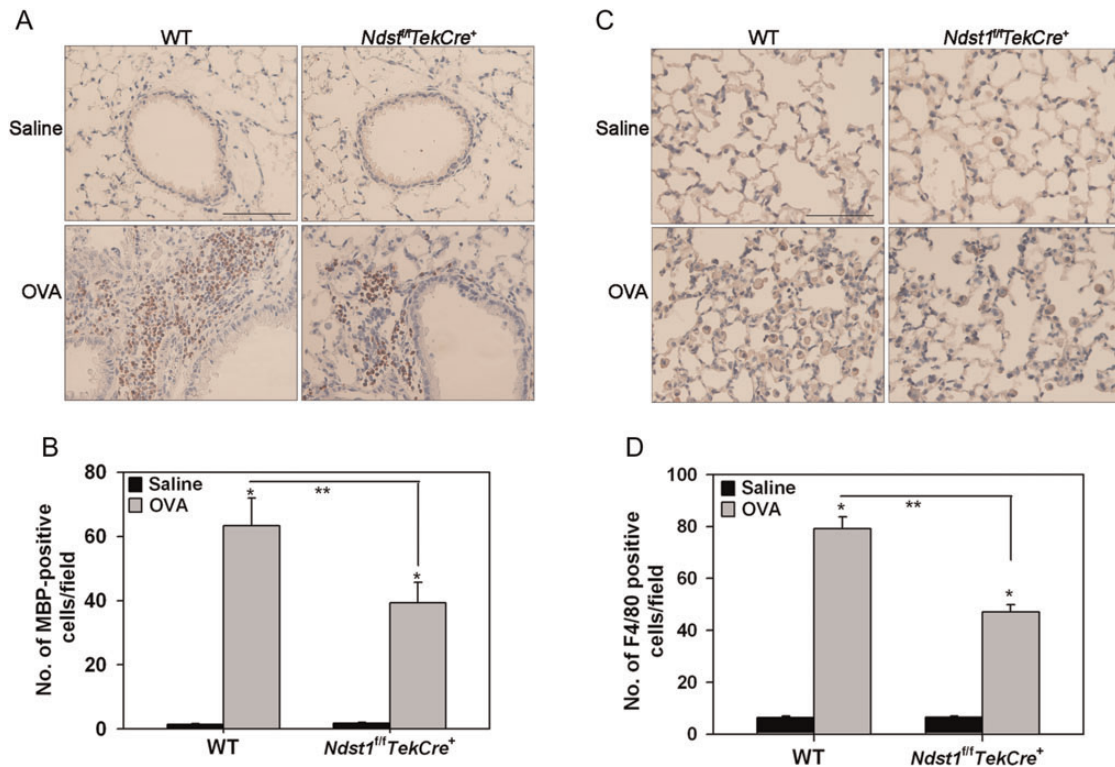
WT as well as *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice relative to respective control mice (Figure 5E and F), the number of VEGF-positive cells in the *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* allergen-challenged group was significantly lower compared with corresponding WT counterparts.

*Allergen-challenged Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup> mice exhibit decreased levels of lung TGF- $\beta$ 1 and FGF-2*

Given that airway remodeling was attenuated in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice relative to WT mice, we investigated the level of expression of TGF- $\beta$ 1 and FGF-2 in the lungs of these mice as their role in promoting events such as cell proliferation and differentiation that contribute to airway remodeling is well recognized (Bosse and Rola-Pleszczynski 2008; Halwani et al. 2011). Consistent with increased airway remodeling, allergen exposure significantly induced total TGF- $\beta$ 1 levels in BALF of WT mice. While BALF TGF- $\beta$ 1 levels in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice were also higher than in corresponding control mice, they were significantly lower than in the allergen-exposed WT mice (Figure 6A). Further, evaluation of lung sections for total TGF- $\beta$ 1 expression by immunohistochemistry correlated positively with these findings (Figure 6B). An increased number of TGF- $\beta$ 1-positive inflammatory cells, predominantly macrophages (based on cell morphology), were present in the alveolar spaces of allergen-challenged WT mice relative to control mice (Figure 6B and C). In allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice, there were significantly fewer TGF- $\beta$ 1-positive inflammatory cells in the alveolar spaces compared with WT counterparts. Further, intensity of TGF- $\beta$ 1-positive staining of the inflammatory cells was lower in the allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice compared with WT mice (Figure 6B). In the case of TGF- $\beta$ 1 and also VEGF (described above), the number of cells positive for expression of these growth factors correlated with the reduced number of F4/80-positive macrophages present in the lung tissue of these mice after allergen challenge (Figure 3C and D).

Since TGF- $\beta$ 1 levels in the BALF was higher in allergen-challenged WT mice relative to *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice and eosinophils constitute the major inflammatory cell type in allergic airways, expression of TGF- $\beta$ 1 at a cellular level was examined in bone marrow (BM)-cultured eosinophils from naïve (unstimulated) WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice by quantitative real-time RT-PCR (qPCR). In cells that were unstimulated but cultured in medium which contains IL-5 as part of the routine culture medium, expression of TGF- $\beta$ 1 mRNA was similar in WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice (Figure 7A). However, since activated eosinophils are a major source of TGF- $\beta$ 1 in asthmatic airways that can contribute to fibrotic changes in the lungs (Minshall et al. 1997), TGF- $\beta$ 1 expression by WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* eosinophils with and without exposure to eotaxin-1 was examined. Although exposure to eotaxin-1 did not have any effect on *Ndst1* expression in WT eosinophils (Figure 7B), it induced a near 2-fold increase in TGF- $\beta$ 1 expression in WT eosinophils, but had no effect on *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* eosinophils (Figure 7C), suggesting that *Ndst1*-modified HS may play a role in regulating TGF- $\beta$ 1 expression in activated eosinophils. Baseline expression of FGF-2 was similar in WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice and no





**Fig. 3.** Reduced tissue eosinophils and macrophages in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. Lung tissue from control and allergen-exposed WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was evaluated for eosinophils and macrophages by immunohistochemical staining. (A) Eosinophils were identified with eosinophil-specific MBP using rat mAb against murine MBP. A representative image of lung sections from each group is shown. (B) MBP-positive cells in randomly selected non-overlapping microscopic fields of lung tissue (5 fields/mouse for control group and  $17 \pm 2$  [Mean  $\pm$  STD] fields/mouse for allergen-exposed groups) were counted at a magnification of  $\times 400$ . (C) Lung tissue macrophages were identified with rat anti-mouse F4/80. A representative image of lung sections from each group is shown. (D) The number of F4/80-positive cells in randomly selected non-overlapping microscopic fields (10 fields/mouse) of the alveolar tissue was counted at a magnification of  $\times 400$ . Scale bar in A and C represents 100  $\mu$ m. Combined data (Mean  $\pm$  SEM) of  $n = 5-6$  mice/group is shown in B and D. \* $P < 0.01$  in for comparison of control vs. allergen-exposed mice and \*\* $P < 0.02$  for comparison of allergen-challenged groups.

**Table I.** Lung cytokine and eotaxin-1 levels in chronic allergen-exposed WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice

Cytokine	WT		<i>Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup></i>	
	Saline	OVA	Saline	OVA
IFN $\gamma^a$	33.75 $\pm$ 5.02	99.84 $\pm$ 18.86*	38.87 $\pm$ 5.24	25.61 $\pm$ 9.44**
IL-2 <sup>a</sup>	10.10 $\pm$ 2.5	6.3 $\pm$ 0.49	10.43 $\pm$ 1.73	5.81 $\pm$ 0.37
IL-4 <sup>a</sup>	9.58 $\pm$ 0.66	11.67 $\pm$ 1.23	9.29 $\pm$ 0.4	8.45 $\pm$ 0.47
IL-5 <sup>a</sup>	0.25 $\pm$ 0.84	84.11 $\pm$ 19.81*	1.73 $\pm$ 1.12	81.73 $\pm$ 21.90*
IL-13 <sup>a</sup>	25.0 $\pm$ 4.07	39.41 $\pm$ 5.17*	26.30 $\pm$ 2.66	27.05 $\pm$ 2.91**
TNF- $\alpha^a$	106.57 $\pm$ 30.24	127.66 $\pm$ 38.85	106.65 $\pm$ 15.42	75.88 $\pm$ 11.71
Eotaxin-1 <sup>b</sup>	12.9 $\pm$ 1.67	24.71 $\pm$ 5.47*	13.46 $\pm$ 0.82	30.13 $\pm$ 8.6

<sup>a</sup>pg/100 mg protein in lung tissue.

<sup>b</sup>pg/mL BALF.

\* $P < 0.05$  for comparison of control vs. allergen-exposed mice and \*\* $P < 0.05$  for comparison of allergen-challenged groups.  $n = 5-8$  mice/group.

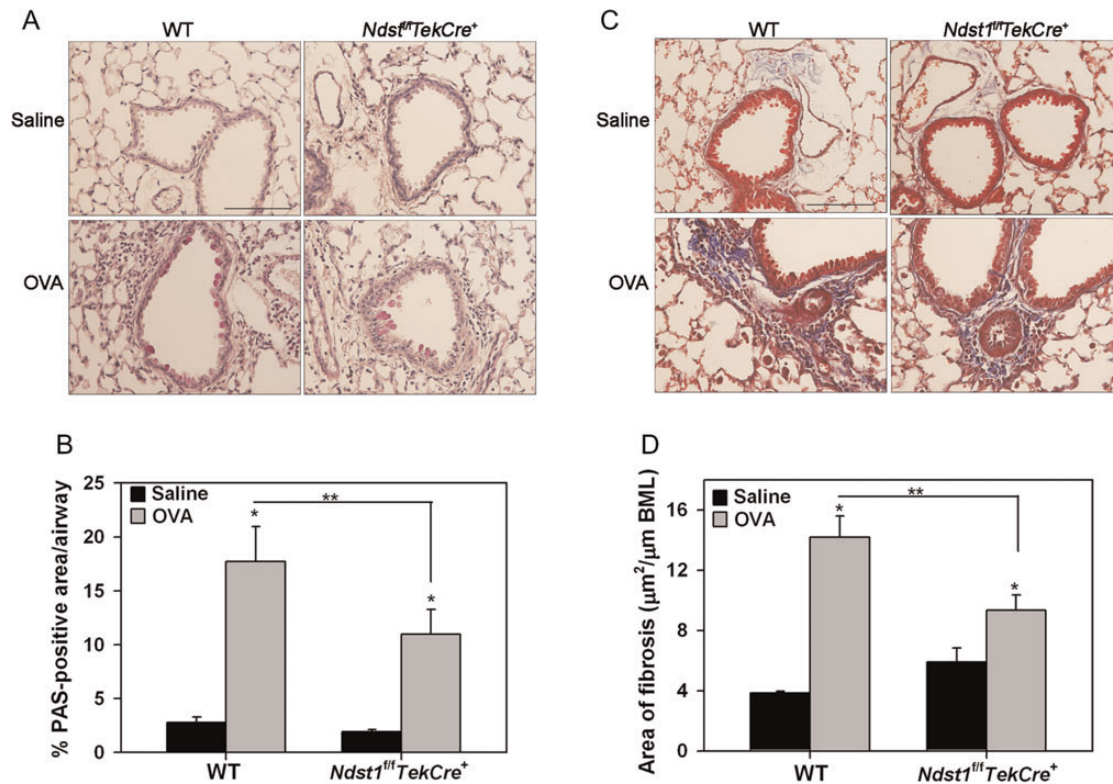
effect was noted in WT eosinophils after exposure to eotaxin-1 (data not shown).

Evaluation of lung tissue FGF-2 expression by immunohistochemistry demonstrated that allergen-exposure results in a dramatic increase in FGF-2 expression mostly associated with

macrophages (based on morphology) in the alveolar spaces of WT mice (Figure 8A and B). Negligible FGF-2 staining was noted in control mice of both groups. FGF-2 expression in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was only marginally higher than in corresponding control mice. Additionally, quantitative assessment of cellular FGF-2 expression based on intensity of staining demonstrated that FGF-2 expression by lung tissue macrophages in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice is significantly lower compared with WT counterparts (Figure 8C).

## Discussion

HSPGs have multiple roles in inflammatory responses such as supporting leukocyte recruitment and transmigration as well as mediating cell proliferation and differentiation during establishment of acute and chronic inflammation (Parish 2006). We have previously demonstrated that endothelial HSPGs play an important role during the establishment of acute allergic airway inflammation by facilitating recruitment of inflammatory cells, particularly eosinophils, to the airways (Zuberi et al. 2009). Persistent exposure to allergens causes chronic airway inflammation including asthma that is characterized by lung tissue remodeling in addition to all the inflammatory responses seen

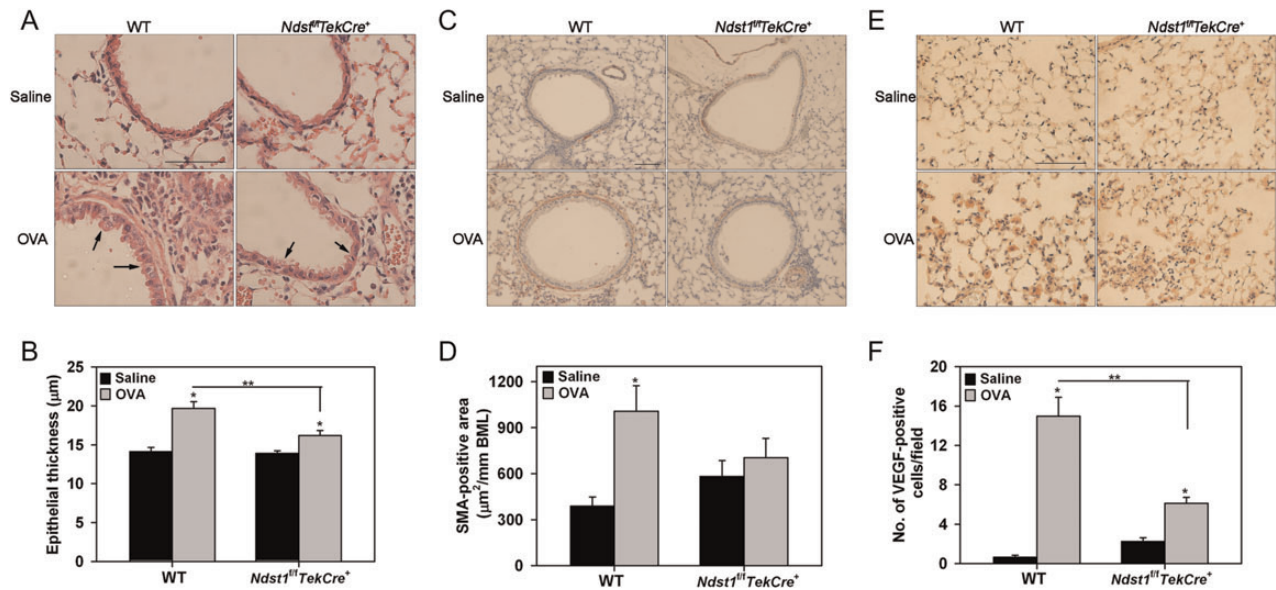


**Fig. 4.** Decreased airway mucus secretion and peribronchial fibrosis in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. (A and B) Airway mucus secretion in control and allergen-challenged WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was examined by PAS staining of lung sections. Images representative of each group are shown. PAS-positive area in airways (5–8 airways/mouse) was quantitated by ImageJ analysis of captured images. (C and D) Peribronchial fibrosis around airways was examined by staining lung sections with Masson's trichrome stain for collagen deposition. Images representative of each group are shown. Fibrotic area was quantitated (2–13 airways/mouse with similar basement membrane length of  $660 \pm 20 \mu\text{m}$ ) by image analysis using ImageJ. Scale bar in A and C represents 100  $\mu\text{m}$ . Combined data (Mean  $\pm$  SEM) of  $n = 5$ –6 mice/group is shown. \* $P < 0.01$  for comparison of control vs. allergen-exposed mice in B and D and \*\* $P < 0.05$  in B and  $< 0.01$  in D for comparison of allergen-challenged groups.

with acute allergic inflammation (Hamid and Tulic 2009). Although pathological changes associated with airway remodeling are described extensively, mechanisms underlying chronic inflammation-associated airway remodeling are not fully identified. Here we have extended our previous observations with *Ndst1*-modified HSPGs and allergic airway inflammation to demonstrate that chronic allergen exposure results in increased HSPG expression in the lungs and that HSPGs contribute to the development of airway remodeling associated with sustained allergen exposure.

Inflammation caused by persistent recruitment of eosinophils, lymphocytes, macrophages, neutrophils and mast cells to airway tissues is assumed to be the initiating event for airway remodeling (Tagaya and Tamaoki 2007). In the present study, mice deficient in endothelial and leukocyte *Ndst1* demonstrated significantly decreased recruitment of eosinophils and macrophages to the airways and lung tissue after exposure to chronic allergen challenge compared with corresponding WT mice. However, the reduction in neutrophil and lymphocyte numbers was only slightly lower than that in the WT mice. While the overall reduction in leukocyte recruitment to the airways of chronic allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice is consistent with our previous findings in acute allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice (Zuberi et al. 2009), the reduction in the number of each

cell type recruited relative to allergen-exposed WT mice was not as striking as in the previous study. This could be a consequence of the repetitive allergen exposure resulting in sustained cellular recruitment and inflammation. Previous studies by us and others have revealed an important role for HSPGs in promoting leukocyte trafficking and recruitment in other models of acute inflammation as well such as neutrophilic inflammation (Wang et al. 2005), neuroinflammation (Floris et al. 2003; Zhang et al. 2012) and nephritis (Rops et al. 2008) by functioning as ligands for leukocyte-expressed receptors like L-selectin and Mac-1 as well as by facilitating chemokine gradients for transmigration (Diamond et al. 1995; Celie et al. 2005, 2007; Wang et al. 2005; Parish 2006). Further, our previous studies showed that trafficking of circulating leukocytes in lung microvessels of allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was significantly lower than that observed in corresponding WT mice and that endothelial-expressed HSPGs, but not eosinophil-expressed HSPGs, are essential for efficient eosinophil rolling under conditions of flow (Zuberi et al. 2009). In the current study, eotaxin-1 levels in chronic allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice were similar to that in WT mice and are not likely to be a contributing factor for the reduced airway eosinophilia in these mice. However, cell surface HSPGs are also known to interact with and/or regulate activity of other eosinophil- and/or macrophage-selective



**Fig. 5.** Epithelial thickness, smooth muscle mass and expression of VEGF is decreased in allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. (A and B) Epithelial thickness in all intact airways was measured in H&E-stained lung sections from control (25–27 airways) and allergen-exposed (32–42 airways) WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice using ImageJ. Images representative of each group are shown. Arrows indicate the airway epithelium in OVA-challenged WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. Scale bar represents 50 µm. (C and D) Airway smooth muscle mass in lung sections from control and allergen-exposed WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was examined by immunohistochemical staining for  $\alpha$ -SMA. Images representative of each group are shown. Peribronchial smooth muscle mass was quantitated (2–14 airways/mouse with similar basement membrane length of  $714 \pm 20$  µm) by image analysis using ImageJ. Scale bar represents 100 µm. (E and F) VEGF expression by inflammatory cells in lung tissue from control and OVA-challenged WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was detected by immunohistochemistry with polyclonal antibodies against VEGF. The number of VEGF-positive cells in randomly selected non-overlapping microscopic fields (5 fields/mouse) of the alveolar tissue in lung sections was counted at a magnification of  $\times 400$ . Representative images are shown for each group. Scale bar represents 100 µm. Combined data (Mean  $\pm$  SEM) of  $n = 5$ –6 mice/group is shown. \* $P < 0.01$  for comparison of control vs. allergen-exposed mice in B, D and F, and \*\* $P < 0.01$  for comparison of allergen-challenged groups in B and F.

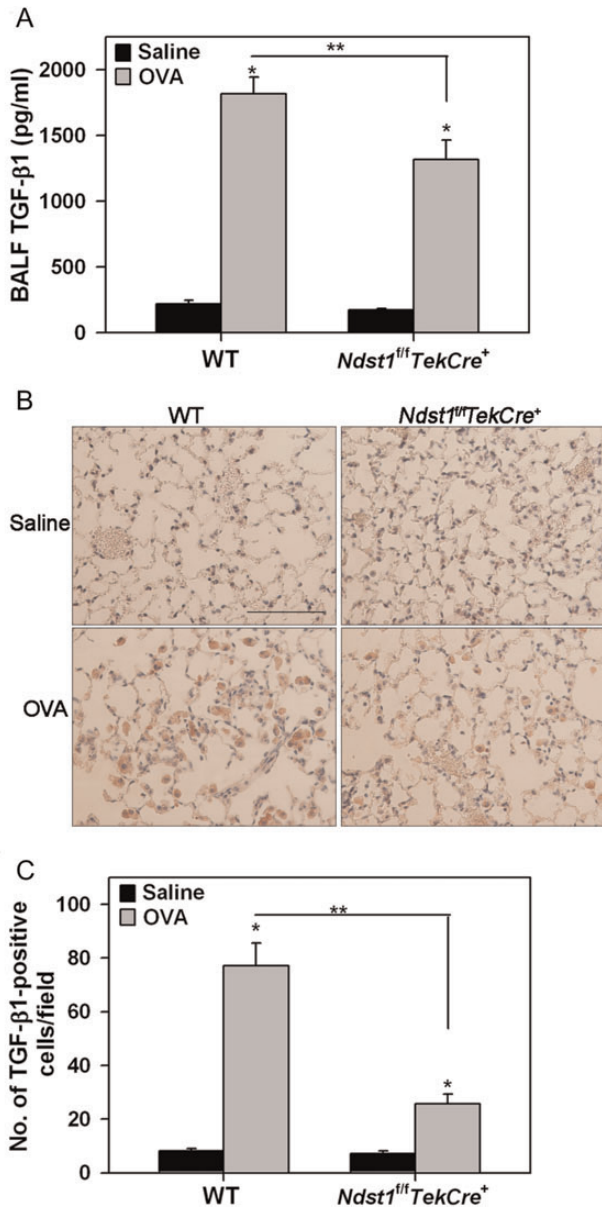
chemokines which are important in the context of allergic asthma such as RANTES and MCP-1 (Zimmermann et al. 2003; Pease and Williams 2006) to assist cellular recruitment (Hoogewerf et al. 1997; Ali et al. 2002; Hardy et al. 2004). While levels of these cytokines were not measured in the current study, altered endothelial and leukocyte HSPG expression in the *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice may impact their retention or biological activity at sites of inflammation resulting in decreased eosinophil and macrophage recruitment after allergen-exposure.

Several pro-inflammatory Th2 type cytokines (e.g. IL-5, IL-13) released by leukocytes recruited to the airways are key players contributing to airway remodeling in chronic asthma (Tagaya and Tamaoki 2007; Al-Muhsen et al. 2011). In the present study, levels of IL-4, -5 and -13 were measured as representative Th2 cytokines in the lungs of allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* and WT mice. Allergen-induced IL-13 levels were significantly lower in *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice relative to corresponding WT mice while IL-5 levels remained similar to those in WT mice. T cells are the predominant producers of both these cytokines and the number of lymphocytes in allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was not significantly lower than in WT counterparts to account for the decreased IL-13. Studies have shown that expression and release of IL-13 is induced by airway epithelial cells when they undergo injury and repair as in airway remodeling (Allahverdian et al. 2008) and can thus be an additional source of IL-13 in asthmatic airways. Since allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice demonstrate an overall reduction in airway remodeling, IL-13 expression and release by the airway

epithelium in these mice may be lower than in corresponding WT mice and thus account for the lower IL-13 levels in the lung tissue. Human eosinophils (Spencer et al. 2009) and macrophages express IL-13 (Hancock et al. 1998) and decreased recruitment of the murine counterparts of these two cell types in the BALF as well as the lung tissue of chronic allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice in the current study could in part contribute to the reduced IL-13 levels. Another possibility is an indirect effect of eosinophil- and/or macrophage-released factor(s) that may regulate IL-13 expression and release by T cells. Reduced eosinophil and macrophage recruitment in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice would thus result in decreased IL-13 release by recruited T cells. However, additional studies are needed to further examine this. Consistent with the reduced lung cellular inflammation and IL-13 levels, airway epithelial thickness, mucus hypersecretion and peribronchial fibrosis were significantly attenuated in allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice relative to corresponding WT mice. Likewise, airway smooth muscle mass in these mice was reduced to levels observed in saline-exposed control *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice, although the reduction compared with allergen-exposed WT was not statistically significant.

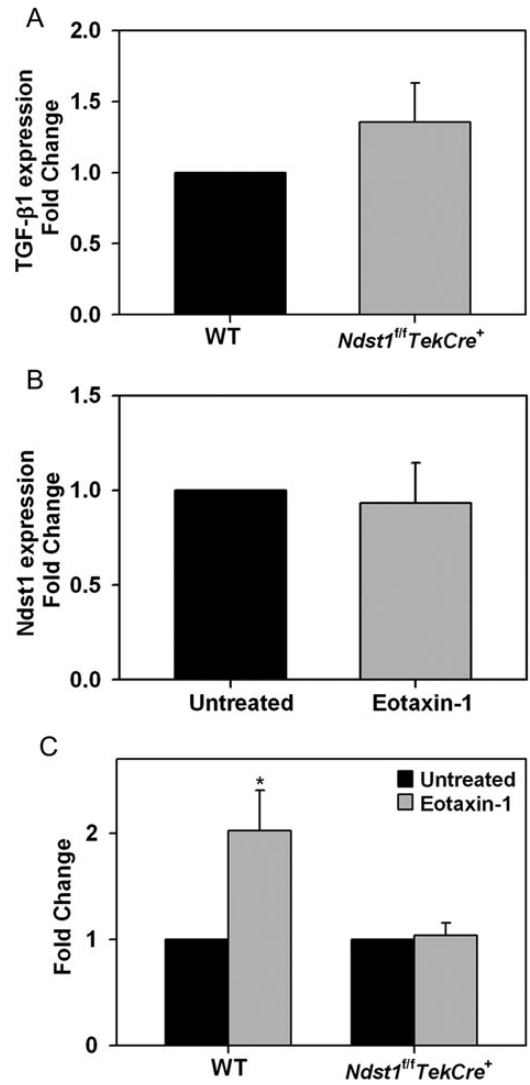
Another allergen-induced cytokine that was significantly reduced in *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was the Th1 cytokine IFN- $\gamma$ . IFN- $\gamma$  binds to basement membrane as well as cell surface HSPGs which protects IFN- $\gamma$  from proteolytic cleavage and promotes increased stability (Lortat-Jacob 2006). Altered expression of HSPG by the endothelium and leukocytes in *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice may affect the binding and thus the stability of IFN- $\gamma$





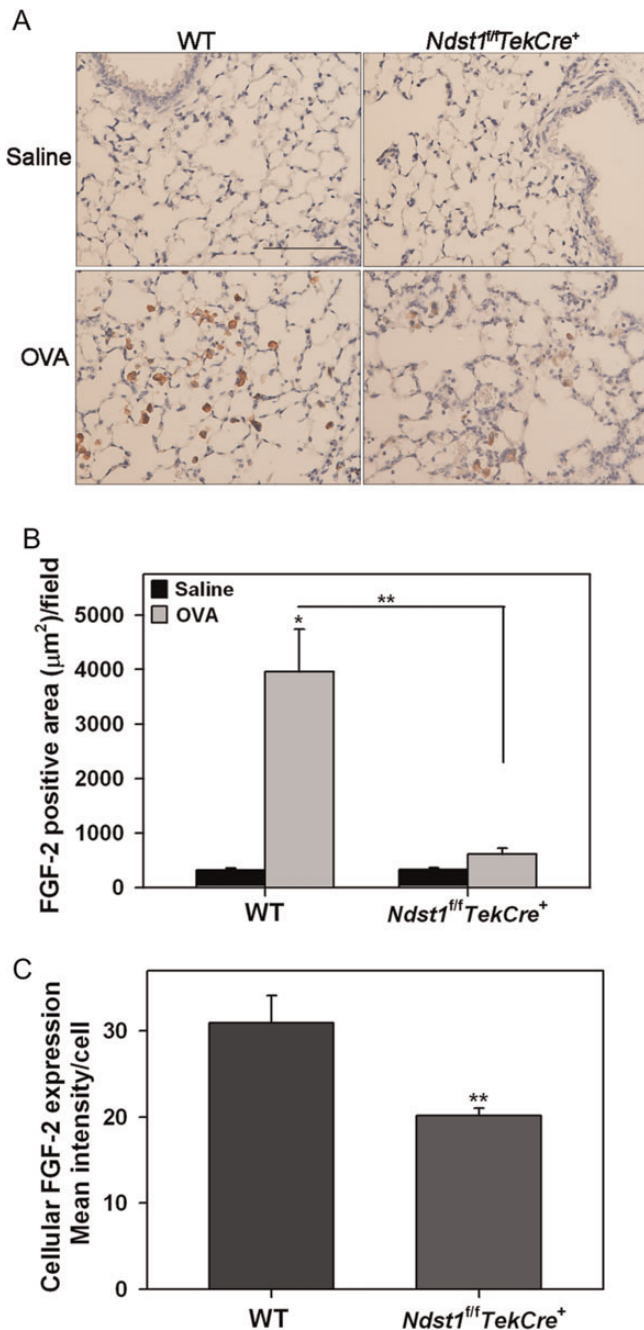
**Fig. 6.** Lung TGF-β1 expression is reduced in allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. (A) TGF-β1 in BALF from control (5–6 mice/group) and allergen-exposed (7–8 mice/group) WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was determined by ELISA. (B) TGF-β1 expression in lung tissue of these mice was detected by immunohistochemistry with polyclonal antibodies against TGF-β1. Images representative of each group are shown. Scale bar represents 100 μm. (C) The number of TGF-β1-positive cells in randomly selected non-overlapping microscopic fields (5 fields/mouse) of the alveolar tissue in lung sections from control and allergen-exposed mice (*n* = 5–6 mice/group) were counted at a magnification of ×400. Data represents mean ± SEM. \**P* < 0.01 in A and C for comparison of control vs. allergen-exposed mice. \*\**P* < 0.02 in A and C for comparison of allergen-challenged groups.

resulting in decreased levels of this cytokine in the lung. While allergic asthma is largely a Th2-driven inflammatory disease, IFN-γ has also been shown to play a proinflammatory role. Elevated serum IFN-γ in atopic asthma correlates with impaired lung function (ten Hacken et al. 1998) and a co-operative role for



**Fig. 7.** Expression of TGF-β1 by eosinophils from WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. (A) Expression of TGF-β1 in unstimulated eosinophils cultured from BM of WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice by qPCR. (B) Expression of *Ndst1* mRNA by eosinophils from WT mice with and without activation (100 nM eotaxin-1 for ~16 h). (C) Expression of TGF-β1 mRNA by eosinophils from WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice with and without activation with eotaxin-1. Combined data of three independent experiments with eosinophils from 2 different mice/experiment is shown (*n* = 6 mice/group). Results are expressed as fold change in expression relative to expression in WT cells in A and untreated cells in B and C that is set as 1. Data represent mean ± SEM. \**P* < 0.025 in C for comparison of untreated vs. eotaxin-1-treated eosinophils.

this cytokine with Th2 cytokines in allergen-induced AHR has been identified more so in a chronic setting (Kumar et al. 2006). More recently, a role for IFN-γ was shown in the development of allergen-induced AHR, eosinophilic inflammation and airway remodeling, specifically subepithelial fibrosis and hyperplasia of mucus-secreting goblet cells, via mast cell IFN-γ receptor (Yu et al. 2011). Consistent with these findings, diminished levels of lung IFN-γ could be an added explanation for the attenuated airway remodeling in allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice.



**Fig. 8.** Allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice exhibit decreased lung FGF-2 expression. (A) FGF-2 expression in lung tissue of control and allergen-exposed WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice was detected by immunohistochemistry with polyclonal antibodies against FGF-2. Images representative of each group are shown. Scale bar represents 100 μm. (B) FGF-2 expression in the alveolar tissue was quantitated by image analysis using ImageJ. Five randomly selected non-overlapping fields were analyzed/mouse ( $n = 6$  mice/group). (C) FGF-2 expression by individual inflammatory cells in the alveolar spaces of allergen-challenged WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice ( $n = 6$  mice/group) was quantitated using ImageJ. Images of 5 non-overlapping microscopic fields were captured at a magnification of  $\times 200$  and the intensity of FGF-2 expression by 10 randomly selected cells (predominantly macrophages based on cell morphology) in each field was measured. Data represents mean  $\pm$  SEM. \* $P < 0.01$  in B for comparison of control vs. allergen-exposed WT mice. \*\* $P < 0.01$  in B and C for comparison of allergen-challenged groups.

In addition to IL-13 and IFN $\gamma$ , expression of VEGF, total TGF- $\beta$ 1 and FGF-2 in the lungs of allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice were significantly lower than in corresponding WT mice. HSPGs are known to bind to and regulate the biological activity of VEGF (Robinson et al. 2006; Xu et al. 2011), TGF- $\beta$ 1 (Lyon et al. 1997) and FGF-2 (Maccarana et al. 1993). VEGF, a known pro-angiogenic growth factor (Meyer and Akdis 2013), is elevated in asthmatic patients (Hoshino et al. 2001) as well as chronic allergen-exposed mice (Lee et al. 2006). Recent studies indicate that alveolar macrophage-derived VEGF is necessary for allergic airway inflammation in asthmatic mice (Song et al. 2012). IL-13, which is elevated in allergen-exposed mice, is known to induce release of specific isoforms of VEGF (Corne et al. 2000; Wen et al. 2003). In the current study, increased recruitment of inflammatory cells that express VEGF to the lungs along with elevated IL-13 levels and retention of secreted VEGF by binding to HSPG are all likely to contribute to vascular remodeling in the airways of allergen-challenged WT mice. In contrast, decreased cellular inflammation along with lower IL-13 levels and less binding and retention due to altered HSPGs expression caused by endothelial and leukocyte *Ndst1* deficiency may lead to reduced vascular remodeling in the lungs of allergen-challenged *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice. Recent studies have shown that HSPG isolated from endothelial cells treated with micro RNA to suppress *Ndst1* have reduced affinity for VEGF and the treated cells are less responsive to VEGF (Kasza et al. 2013). FGF-2 and TGF- $\beta$ 1 promote bronchial smooth muscle cell and fibroblast proliferation and differentiation and thus contribute to airway remodeling (Bosse and Rola-Pleszczynski 2008; Halwani et al. 2011). While the effect of FGF-2 on differentiation of smooth muscle cells appears to be controversial (Bosse and Rola-Pleszczynski 2008; Schuliga et al. 2013), its proliferative effects on human (Hetzl et al. 2005) and mouse (Xiao et al. 2012) fibroblasts as well as endothelial cells (Sahni and Francis 2004) is well established. Besides endothelial cells, epithelial cells, fibroblasts and airway smooth muscle cells, eosinophils and macrophages are producers of FGF-2 (Cordon-Cardo et al. 1990; Kranenburg et al. 2002; Stenfelt and Wennerås 2004; Yum et al. 2011) and TGF- $\beta$ 1 (Halwani et al. 2011) and expression of both these growth factors is induced after repetitive allergen exposure in mice, especially by macrophages (Yum et al. 2011). FGF-2 levels are known to be elevated in the BALF and sputum of patients with asthma (Redington et al. 2001; Bissonnette et al. 2014). Thus, elevated FGF-2 levels in chronic allergen-challenged airways can exert proinflammatory effects on various cell types in the lungs.

Decreased expression of VEGF, TGF- $\beta$ 1 and FGF-2 in the lungs of allergen-exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice, especially by macrophages in the alveolar tissue (based on cell morphology), could be attributed to a reduced number of tissue macrophages in these mice as well as a reduced expression of these growth factors at a cellular level. Binding of these growth factors to HSPGs allows creation of a local reservoir and protection from degradation. Altered expression of HSPGs by endothelial cells and inflammatory leukocytes in *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice may lead to decreased binding and retention of these growth factors released by eosinophils and macrophages themselves or other cell types in response to sustained allergen exposure, thus resulting in reduced cell activation in these mice. Further,

intracellular Ndst1-modified HSPG may also play a role in regulating expression of these growth factors. Intracellular HSPG in general have received less attention relative to those in the extracellular matrix or on the cell surface, although they have been reported to have biological functions in storage granules, the nucleus and other intracellular organelles (Kolset et al. 2004). Based on previous studies indicating that eotaxin does not interact with HS or a range of GAG (Ellyard et al. 2007), our data demonstrating induction of TGF- $\beta$ 1 expression by eotaxin-1 in WT but not *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* eosinophils suggest a potential role for intracellular Ndst1-modified HSPG in regulating expression of TGF- $\beta$ 1 by these cells. Although no effect was noted on FGF-2 expression in eosinophils after eotaxin-1 exposure, it is possible that this chemokine may just not be an inducer of FGF-2 while other inflammatory mediators of chronic airway inflammation may have an alternate effect. Overall, combined with reduced recruitment of inflammatory cells, release of decreased levels of VEGF, TGF- $\beta$ 1 and FGF-2 during inflammatory conditions or in response to inflammatory mediators, as exemplified in the case of TGF- $\beta$ 1 by eosinophils, may result in attenuated airway remodeling in chronic allergen exposed *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice.

In summary, our studies demonstrate a role for HSPGs in promoting airway remodeling associated with chronic allergic airway inflammation and suggest that targeting HSPG in the lungs to modulate inflammatory cell recruitment and pro-inflammatory activity of mediators may serve as a mechanism for potential pharmacological intervention to inhibit airway remodeling associated with chronic allergic asthma.

## Materials and methods

### *Animal model of chronic allergic airway inflammation*

*Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice on C57Bl/6 background and deficient in endothelial and leukocyte Ndst1 were generated as described previously (Fuster et al. 2007; Zuberi et al. 2009). Offspring were genotyped using genomic DNA isolated from tail clippings as described (Wang et al. 2005; Fuster et al. 2007). Wild-type (WT) C57Bl/6 and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice (8–12 weeks) were exposed to chronic allergen challenge with ovalbumin (OVA) (Grade V, Sigma Chemical Co., St Louis, MO) as described previously (Cho et al. 2010, 2011). Briefly, mice were sensitized with 50  $\mu$ g OVA in 0.5 mg aluminum hydroxide by subcutaneous injection on days 0, 7, 14 and 21 and then challenged intranasally with OVA (20  $\mu$ g/mouse) on days 23, 25, 28. This was followed by biweekly intranasal challenges with OVA for an additional 4 weeks. Age and gender matched WT and *Ndst1<sup>fl/fl</sup>TekCre<sup>+</sup>* mice sensitized and challenged with saline instead of OVA were used as controls. All studies involving mice were performed following procedures and standards approved by the local Institutional Animal Care and Use Committee.

### *BALF and lung tissue collection*

Mice were sacrificed 24 h after the last allergen challenge and lungs were lavaged with 1 mL of saline. Total cell counts in the BALF were determined using a hemocytometer. BALF was centrifuged and the supernatant was stored at  $-80^{\circ}\text{C}$  for further evaluation. Differential cell counts were determined based on morphological criteria from cytopsin slides of BALF cells

stained with Hema 3 staining system (Fisher Diagnostics, Middletown, VA). Right lungs were snap-frozen and stored at  $-80^{\circ}\text{C}$  while left lungs were perfused with 4% paraformaldehyde to preserve pulmonary structure, fixed in 4% paraformaldehyde for 48 h at  $4^{\circ}\text{C}$  and paraffin-embedded.

### *Measurement of lung Th1/Th2 cytokines*

Protein concentration in supernatants of lung tissue homogenates prepared in lysis buffer (PBS containing 1% Triton X-100 and protease inhibitors) was measured (BCA Protein Assay Kit, Pierce Biotechnology, Rockford, IL). Th1 (IL-2 and IFN- $\gamma$ )/Th2 (IL-4, IL-5, IL-13) cytokine and TNF- $\alpha$  levels in the lung tissue supernatants was determined using cytometric bead array Flex Set kits (BD Biosciences, San Diego, CA) according to the manufacturer with a FACScan flow cytometer equipped with CellQuest Pro™ Software (BD Biosciences) for data acquisition and FlowJo Software (Tree Star, Inc., Ashland, OR) for analysis or a FACSCanto II flow cytometer (for IL-13) with FACSDiva™ Software for data acquisition and analysis. Level of each cytokine was expressed as pg cytokine/100 mg protein.

### *Measurement of eotaxin-1 and TGF- $\beta$ 1 by ELISA*

Eotaxin-1 and total TGF- $\beta$ 1 in the BALF was measured using ELISA kits (R & D Systems, Minneapolis, MN) according to the manufacturers' recommendations. Optical density of the color developed was measured at 450/570 nm with a microplate reader (FLUOstar OPTIMA, BMG LABTECH, Durham, NC) and the concentration of eotaxin-1 or TGF- $\beta$ 1 in the sample was determined against a standard curve generated with a detection limit of 15.6 pg/mL.

### *Lung histology*

To examine HS expression in lung tissue, immunofluorescence studies were performed using a VSV-tagged phage display-derived single chain antibody AO4B08, that interacts with the *N*-, 2-*O*-, and 6-*O*-sulfated saccharide motif, as well as an internal 2-*O*-sulfate group (Kurup et al. 2007) and a non-GAG binding antibody MPB49 as the control (both antibodies kindly provided by Dr. T. van Kuppevelt, Radboud University Nijmegen Medical Center, The Netherlands). Bound antibodies were detected with monoclonal anti-VSV glycoprotein (Sigma-Aldrich, St. Louis, MO) followed by FITC-conjugated goat anti-mouse IgG. To examine cellular infiltration of lungs, tissue sections were stained with Harris Modified Hematoxylin and Shandon instant eosin (H&E, Thermo Fisher Scientific Co., Pittsburgh, PA). Lung tissue eosinophils and macrophages were detected by immunohistochemical staining of lung sections of mice from two independent allergen challenge studies for eosinophil-specific MBP with rat mAb against mouse MBP (1  $\mu$ g/mL) or with rat anti-mouse F4/80 (10  $\mu$ g/mL, Abcam, Cambridge, MA) (Zuberi et al. 2009), respectively. MBP-positive or F4/80-positive cells in randomly selected non-overlapping microscopic fields were counted at a magnification of  $\times 400$  and results were expressed as average number of MBP-positive or F4/80-positive cells per field. For MBP, 5 fields for saline groups and an average of  $17 \pm 2$  (mean  $\pm$  STD) fields for allergen-challenged groups were counted per slide and for F4/80, 10 fields were counted per slide. For expression of VEGF, TGF- $\beta$ 1 and FGF-2, lung tissue sections were



stained with polyclonal antibodies against VEGF (4 µg/mL), total TGF-β1 (1 µg/mL) or FGF-2 (0.2 µg/mL) (all from Santa Cruz Biotechnology, Dallas, TX). VEGF or TGF-β1-positive cells (predominantly macrophages based on cell morphology) were counted in 5 randomly selected non-overlapping fields of the alveolar tissue per slide at a magnification of ×400. FGF-2 expression was quantitated in images of 5 non-overlapping fields of the alveolar tissue per slide at a magnification of ×100 using ImageJ image analysis system (Abramoff et al. 2004). The results were presented as FGF-2 positive area (µm<sup>2</sup>) per field. FGF-2 expression by individual inflammatory cells in the alveolar spaces was also quantitated using ImageJ. Images of 5 non-overlapping microscopic fields were captured at a magnification of ×200 and the intensity of FGF-2 expression by 10 randomly selected cells (predominantly macrophages based on cell morphology) in each field was measured. Results were expressed as mean intensity/cell.

#### Airway remodeling analysis

Lungs of representative mice from two independent allergen challenge studies were analyzed for airway mucus accumulation, peribronchial fibrosis, epithelial thickness and peribronchial smooth muscle hypertrophy/hyperplasia. Both large and small airways were examined in all analyses. To determine airway mucus production, lung sections were stained with periodic acid-Schiff's (PAS) reagent (Sigma-Aldrich®, St. Louis, MO). PAS-positive area was quantitated using ImageJ and expressed as percent PAS-positive area per airway (Kang et al. 2012). Subepithelial fibrosis was assessed using Masson's trichrome stain (Sigma-Aldrich®) and quantitated by ImageJ. Results were expressed as area of fibrosis (µm<sup>2</sup>) per µm basement membrane length (Ge et al. 2010). Airway epithelial thickness in H&E stained lung sections was measured as described previously (Doherty et al. 2012). Thickness of the epithelium from the bottom of the basement membrane to the mucosal surface of the airway epithelium was measured using ImageJ. All intact airways (up to 10) were analyzed and four different areas were measured per airway. To determine airway smooth muscle hypertrophy/hyperplasia, expression of α-smooth muscle actin (SMA) was evaluated by immunostaining using mAb against murine SMA (0.4 µg/mL, Sigma-Aldrich®). Peribronchial smooth muscle mass was quantitated by ImageJ and expressed as SMA positive area (µm<sup>2</sup>) per mm basement membrane length (Ge et al. 2010).

#### Murine eosinophils

Eosinophils were cultured from BM of naïve WT and *Ndst1*<sup>fl/fl</sup> *TekCre*<sup>+</sup> mice as described previously (Dyer et al. 2008) but with minor modifications as stated in our earlier studies (Bahaie et al. 2012). Cells between 12–14 days of culture differentiated based on Hema 3 staining and verified for expression of both MBP and Siglec-F (>95% eosinophils) (Bahaie et al. 2012) were used in studies.

#### qPCR

Eosinophils from WT and *Ndst1*<sup>fl/fl</sup> *TekCre*<sup>+</sup> mice were activated by overnight culture (~16 h) in medium containing eotaxin-1 (100 nM) as described in our previous study (Ha et al. 2013)

and then evaluated for expression of TGF-β1 or FGF-2 by qPCR using previously published primers specific for each molecule (Teshima-Kondo et al. 2004; Sullivan et al. 2009). qPCR was carried out in a iQ<sup>TM</sup>5 multicolor real-time PCR detection System (Bio-Rad, Hercules, CA) as described in our previous studies (Ha et al. 2013). The amount of TGF-β1 or FGF-2 mRNA in each sample was calculated based on its threshold cycle, Ct, suggested by the software (IQ<sup>TM</sup>5 Optical System software) after subtraction of the Ct of the housekeeping gene β-actin. Results are expressed as fold change in expression relative to expression in untreated cells.

#### Statistical analysis

The results are expressed as the mean ± SE. Statistical significance was determined by unpaired Student's *t*-test. A *P* value of <0.05 was considered as significant.

#### Funding

This work was supported by National Institutes of Health [U19-AI70535 and AI35796 to P.S. and HL107150 to J.D.E.].

#### Conflict of interest

None declared.

#### Abbreviations

HS, heparan sulfate; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; *Ndst1*, glucosaminyl *N*-deacetylase/*N*-sulfotransferase-1; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; FGF-2, fibroblast growth factor-2; MBP, major basic protein; OVA, ovalbumin; PAS, periodic acid-Schiff; SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor.

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