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Nerve Growth Factor Enhances Clara Cell Proliferation after Lung Injury

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Abstract

The lung epithelia facilitate wound closure by secretion of various cytokines and growth factors. Nerve growth factor (NGF) has been well described in airway inflammation; however its likely role in lung repair has not been examined thus far.

To investigate the repair function of NGF, experiments were performed *in vitro* using cultured alveolar epithelial cells and *in vivo* using naphthalene induced model of Clara epithelial cell injury.

Both *in vitro* and *in vivo* experiments revealed airway epithelial cell proliferation following injury to be dependent on NGF and the expression of its receptor, tropomyosin-receptor-kinase A. Additionally, NGF also augmented *in vitro* migration of alveolar type II cells. *In vivo*, transgenic mice over-expressing NGF in Clara cells (NGFtg) did not reveal any proliferation or alteration in Clara cell phenotype. However, following Clara cell specific injury, proliferation was increased in NGFtg and impaired upon inhibition of NGF. Furthermore, NGF also promoted the expression of collagen I and fibronectin *in vitro* and *in vivo* during repair, where significantly higher levels were measured in re-epithelializing NGFtg mice.

Our study demonstrates that NGF promotes the proliferation of lung epithelium *in vitro* and the renewal of Clara cells following lung injury *in vivo*.

Keywords: Alveolar type II cells, airway epithelium, Clara cells, lung injury and repair, naphthalene, nerve growth factor.

Introduction

The mature healthy airway is a complex, yet fragile structure that is constantly exposed to inhaled environmental agents. Due to this close connection with the outside environment the lung is easily susceptible to damage; thus, injury and epithelial shedding are commonly described events in respiratory infections, asthma, chronic bronchitis and interstitial lung disease (1). The lung has a remarkable capacity to facilitate repair through the use of specialized compartments with several distinctly functioning cell types. These cell types participate in a series of overlapping events to restore the respiratory epithelium. In the alveolar compartment of the lung, alveolar epithelial type II cells (AECII) are the putative progenitors of the alveolar epithelium and play an important role in the re-epithelialization of alveolar walls after lung injury (2;3). Among the cell types lining the airway, the non-ciliated bronchiolar cells (Clara cells) comprise the principal epithelial cell type of many species, including mice. Clara cells, which act as progenitors for goblet and ciliated epithelial cells are major contributors to the airway repair process (4;5). In humans, Clara cells substantially contribute to the proliferation of tracheobronchial epithelium and have been described as an important cell population for the maintenance of the normal epithelium especially in the distal conducting airways (6).

Depending on the wound size and the degree of epithelial denudation, renewal of the damaged airway epithelium involves migration and proliferation of the residual epithelial cells from the adjoining, undamaged areas (7). Expression of extracellular matrix (ECM) proteins in the provisional matrix are increased, and for Clara cells, factors like fibronectin and collagen I have been described to attract and provide adherence points for migration (8).

The Clara cell is uniquely susceptible to injury due to its capability to transform chemicals like naphthalene (NA) into toxic intermediates. NA is a polycyclic aromatic

hydrocarbon commonly found in cigarette smoke and diesel exhaust and is capable of inducing a highly dose-dependent and cell type- and site-selective toxicity (9;10). The toxicity of NA is due to the metabolism of this compound by cytochrome P450 monooxygenases to a toxic intermediate that causes Clara cell swelling, vacuolization, and exfoliation into the lumen of the airways 24 h after injury is initiated (11). The use of NA in this study enabled us to investigate the ongoing repair processes following Clara cell specific injury *in vivo*.

Following injury, the mediators that drive airway epithelial repair may be derived from many sources. In addition to factors released from activated local immune cells (12), epithelial cells are also likely sources of autocrine and/or paracrine growth factors. One such mediator actively secreted by the airway epithelium is nerve growth factor (NGF), an eminent member of the neurotrophin growth factor family initially described to promote neuronal growth and survival (13). Recently, the importance of NGF and its high affinity receptor, the tropomyosin-receptor-kinase A (TrkA) has been described in inflammatory diseases related to airway dysfunction (14;15). Examples of this include NGF over-expression in the activated allergic bronchial (14), nasal (15) and airway epithelium as well as elevated epithelial NGF mediating eosinophil infiltration (16) and survival (17). NGF is also expressed in the epithelia of other tissues, especially after activation via inflammation or during regenerative conditions (18-21). Despite a few reports suggesting a role for NGF in wound healing elsewhere (22-24), its involvement in lung epithelial repair processes has not been investigated thus far.

The objective of this study was to examine the role of NGF in the repair of injured airway epithelia and our results highlight its contribution towards the proliferation of Clara airway epithelial cells in the lung following injury.

Methods

Mice

6-8 week old female C57BL/6 mice were obtained from Harlan Winkelmann (Borchen, Germany). Age matched transgenic mice over-expressing NGF under the control of the lung-specific Clara-cell secretory protein promoter (NGFtg) were used (25). All animal experiments were performed according to "The guidelines for the care and use of experimental animals" prepared and published by the Society for Laboratory Animal Sciences (GV-SOLAS; Biberach a.d. Riss, 1988).

Wound assay for lung epithelial cells

LA4 murine lung alveolar epithelial cells (American Type Culture Collection, Rockville, USA) seeded at $1x10^6$ cells/well and supplemented with F12K Nutrient Mixture containing 15 % FCS (Gibco, Carlsbad, USA) were cultured until confluence onto 12-well plates. After overnight (o/n) serum starvation, monolayers were scratched from top to bottom using a 200 µl pipette tip then incubated with either medium, medium containing recombinant human NGF (β-NGF, 100ng/ml, R&D systems, MN, USA), polyclonal blocking antibodies to NGF (anti-NGF, 625 ng/ml, Peprotech, NJ, USA), or IgG control (IgG, 625ng/ml, Sigma, Missouri, USA. All groups were incubated in medium containing 1% FCS. Medium with the accompanying supplements was changed every 24 h. Wound size was measured in µm by light microscopy (Olympus, Hamburg, Germany) after wounding and every 24 h for 72 h using the Cell^F imaging software program (Soft Imaging System GmbH, Münster, Germany). At least 3-4 wounds of n=6 LA4 cell monolayers were analysed.

Proliferation of cultured lung epithelial cells.

Primary alveolar epithelial type II cells (AECII) were isolated according to the protocol provided in the supplementary section**.** $1x10^5$ AECII or LA4 cells/well were grown in 96 well plates in triplicates. AECII were grown overnight in medium containing 10% FCS followed by medium containing 0.1%, 1% or 10% FCS for 24, 48 and 72 h. Proliferation of AECII cells was further assessed with 0.1% FCS containing medium, NGF, anti-NGF or control IgG for 72h in concentrations described above for wound assay. For LA4 cells, 20 pmol/µl of NGF, TrkA or control siRNA (Qiagen, Hilden, Germany) were incubated with cells according to the manufacturer's instructions. Cell proliferation assays were performed using a BrdU (5-bromo-2'-deoxy-uridine) labelling kit according to the manufacturer's instructions (Roche, Mannheim, Germany). For further details including primer sequences please see the *online supplement*.

***In vitro* migration assay using AECII**

AECII were assayed for NGF induced migration using 24 well modified Boyden chamber (Falcon, BD, NJ, USA) using transwell membranes (8-µm pore size). The upper chamber contained 10^5 AECII in 500 µl medium and the lower chamber contained 500 µl of cell-free medium containing NGF (100 ng/ml). Cells were incubated for 6 h at 37°C and 5% CO_2 after which inserts were removed and cells on the lower surface were counterstained with Diff- Quick (Dade, Behring, Germany) for 5 min. An average of five ×100 fields per insert was counted of 5 samples per group using light microscopy (Olympus, Hamburg, Germany). Migration is expressed as migrated cells per ×100 field.

***In vivo* model of Clara cell injury and denudation using naphthalene (NA).**

Wildytpe (WT) or NGFtg control mice received intraperitoneal (i.p) corn oil (Sigma Aldrich Munich, Germany), WT or NGFtg mice undergoing Clara cell exfoliation received i.p NA (250 mg/kg, Sigma Aldrich Munich, Germany) dissolved in corn oil at day 0 (*online supplement, Figure S1, A and B*). In additional groups, WT control mice and WT mice treated with NA also received either intranasal anti-NGF (anti-NGF, 2.5S, N 6655, Sigma-Aldrich, Munich, Germany) or intranasal control IgG (IgG, Sigma-Aldrich, Munich, Germany) at a concentration of 24 µg at day 0, 3, 6 and 9 (*online supplement, Figure S1, A and B*). A new set of mice also received BrdU in drinking water at a concentration of 1 mg/ml (water containing 1 % glucose) from day 0 to day 10 (*online supplement Figure S1, A*). On days 1, 10 or 29 after treatment, bronchoalveolar lavage (BAL) was performed twice with 800 µl of ice-cold PBS plus protease inhibitor mix (Roth, Karlsruhe, Germany) and whole lungs were removed for histological, mRNA and protein analysis.

NGF ELISA

NGF ELISA (Promega, Madison, USA) was performed according to the manufacturer's instructions with cell free culture supernatants of n=6 LA4 cell monolayers (with and without wound at 6, 12, 24, 48 and 72 h) and BALF of WT and NGFtg animals (control and NA treated at days 10 and 29). Plates were read in a microplate autoreader (Tecan, Salzburg, Austria) at 405 nm.

Immunoblot analysis

Total protein was extracted from LA4 cell monolayers, AECII and lung tissues of WT and NGFtg control and NA treated mice using the M-PER mammalian protein extraction kit (Thermo scientific Pierce, Bonn, Germany). Immunoblotting was performed as previously

described (26) with NGF, TrkA (1:500, Santa Cruz Heidelberg, Germany), anti-beta (β)-actin monoclonal antibody (1:40,000, Sigma, Munich, Germany) and secondary peroxidase conjugated antibodies (1:5000, Sigma, Munich, Germany). Immunoreactive protein bands were visualized with a chemiluminiscent substrate (SuperSignal, Thermo scientific Pierce, Bonn, Germany). Bands were scanned with the Kodak Image Station CF 450 and analysed using the Kodak 1D 3.5 imaging software. See *online supplement* for further details.

RNA isolation, realtime PCR and analysis

$5x10^5$ AECII /ml were incubated in 24 well plates with either anti-NGF (625 ng/ml), control IgG (625 ng/ml), or recombinant human NGF (100 ng/ml) for 24 hrs. Total RNA was isolated from AECII or lung tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using the QuantiTect SYBR® Green PCR Kit (Qiagen, Hilden, Germany). Primers specific for collagen I, and fibronectin were used for realtime PCR. The mRNA copy numbers were compared and expressed relative to mouse ribosomal L32 as the housekeeping gene. See *online supplement* for further details.

Immunohistochemistry

Immunohistochemistry was performed on sectioned paraffin embedded lung tissues as described previously (27). Primary antibody concentrations were; anti-CCSP (diluted 1:3000, courtesy of Jörg Klug, Giessen); anti-fibronectin (diluted 1:1000, Biozol, Eching, Germany), anti-collagen I (diluted 1:500, Biozol, Eching, Germany), sheep anti-BrdU (diluted 1:10, Biozol, Eching, Germany). Secondary biotinylated antibodies (Vector Laboratories, Calif, USA) were diluted 1:400. Sections were analysed using light microscopy (Olympus Europa GmbH, Hamburg, Germany).

Morphometric analysis of Clara cells and BrdU incorporating proliferating cells.

Mouse lungs were fixed by airway instillation using 6 % phosphate buffered paraformaldehyde at a pressure of 20 cm fluid column. Lung volume was determined by fluid displacement, and systemic uniform random (SUR) samples of lung tissue were taken and processed according to standard methods (25). The morphometric analysis for Clara cell specific protein (CCSP) and BrdU positive cells was performed using a modified version of a previously described method (28). Proximal and distal airways were counted in a blinded manner. CCSP positive cells containing nuclear profiles and BrdU positive nuclear profiles were counted. *See* online supplement for further details.

Statistical analysis

Statistical significance for normally distributed samples was analyzed by using an unpaired *t* test. Comparisons between more than 2 groups were made by one way analysis of variances (ANOVA). p values of less than 0.05 were considered statistically significant . The stars signify: * = $p< 0.05$, ** = $p< 0.01$, *** = $p< 0.001$. All numerical data are expressed as mean ± SEM. Calculations were performed using Graph Pad prism software, version 3.02.

Results

NGF production and TrkA expression is increased in wounded lung epithelial cells *in vitro*

The expression of NGF in lung epithelial cells was first examined in an *in vitro* wound assay model using murine LA4 cells. Wounding confluent monolayers of LA4 cells led to an increase in the expression of both NGF and its receptor, TrkA (Figure 1). NGF protein levels in wounded epithelial cells were significantly higher compared to non-wounded LA4 cells at all time points measured (Figure 1, A). A progressive increase in NGF after wounding was observed until 72 h with elevated levels observed as early as 6 h. NGF production also increased with time in the non-wounded cells correlating with normal cell proliferation. Correspondingly, TrkA was upregulated at 24 h and 48 h post wounding and was present in significantly higher amounts compared to non-wounded monolayers (Figure 1, B). The increase in TrkA expression coincided with the increase of NGF in the supernatant (Figure 1, A). These results indicate that autocrine NGF and TrkA are induced upon lung epithelial cell injury.

NGF promotes wound closure and proliferation of epithelial cells *in vitro*

To determine if the observed upregulation of NGF and its receptor has an effect on the closure of wounds, wounded monolayers of LA4 cells were incubated with either blocking antibodies to NGF (anti-NGF) or with exogenous NGF. Inhibition of NGF markedly delayed closure of wounds, whereas exogenous NGF protein (NGF) significantly accelerated wound closure (Figure 2, A and B). Control wounded monolayers incubated with control IgG antibodies or medium only had wounds of similar size (Figure 2, A and B). In control monolayers, wound closure was achieved at 72 h, whereas wounds failed to close in the anti-NGF treated groups even at 72 h, (Figure 2, A). In contrast, wounds incubated with NGF

closed at an earlier time point of 48 h. Since the proliferative effect of NGF is reported to be mediated by its high affinity receptor TrkA, the effect of NGF and TrkA on the regulation of lung epithelial cell proliferation was also investigated *in vitro*. siRNA inhibition of NGF or TrkA knocked down the mRNA and protein levels to significantly lower levels compared to the medium control (*online supplement, Figure S2*). Inhibition of TrkA and NGF with these siRNAs significantly decreased proliferation of LA4 epithelial cells by ~ 75% and 50% of medium control respectively (Figure 2, C). These results indicate that NGF and TrkA are important for the proliferation and repair of wounded lung epithelia *in vitro*.

NGF mediates proliferation, migration and ECM expression by AECII

After determining the important role of NGF in a lung epithelial cell line, we were interested to examine the effect of NGF and TrkA in primary AECII. NGF and TrkA were found to be expressed in primary AECII (Figure 3, A and B). In order to test the role of NGF on primary AECII cell proliferation, we established an *in vitro* assay. A time and dose response for AECII proliferation *in vitro* at 24 h, 48 h and 72 h in 0.1%, 1% and 10% FCS concentration *(online supplement, Figure S3, C)* revealed 72 h as the optimal time point to examine proliferation. NGF was seen to significantly influence proliferation at 0.1% FCS concentration. In line with our previous *in vitro* results using LA4 cells, NGF increased the proliferative capacity of AECII, whereas anti-NGF significantly downregulated proliferation compared to the medium and IgG control (Figure 3, C). We also investigated the role of NGF in mediating migration, which is a critical event in wound repair. NGF was seen to strongly induce migration of AECII upon incubation with exogenous NGF (Figure 3, D). Furthermore the expression of ECM factors such as collagen I and fibronectin were also investigated *in vitro* in primary cell cultures of AECII. The mRNA expression of fibronectin and collagen I were significantly increased upon incubation with exogenous NGF (Figure 3, E and F) and the inhibition of NGF led to a significant decrease in fibronectin mRNA expression (Figure 3,

E). These results imply that NGF actively promotes proliferation and migration of AECII, which are critical requisites for wound repair.

NGF and TrkA are increased during repair of airway epithelia *in vivo*

To examine the role of NGF in airway epithelium repair *in vivo*, we employed a NA induced model of Clara epithelial cell injury in the lung. Expression of NGF and TrkA was determined at 10 and 29 days post injury with NA in the BAL fluid and total lung protein of WT and NGFtg animals (Figure 4). In untreated NGFtg control mice, the baseline level of NGF was significantly higher due to the constitutive over-expression of NGF under the Clara cell specific-promoter compared to WT control animals (Figure 4, A). Following injury with NA, WT animals showed a progressive upregulation of NGF which lasted until day 29 (Figure 4, A). Conversely, NGF levels in the NGFtg animals declined after NA treatments at all time points compared to untreated NGFtg animals due to the NA induced loss of NGF over-expressing Clara cells. However, the amount of NGF in the NA treated NGFtg animals was significantly higher at all time points compared to NA treated WT animals. The over-expression of NGF in Clara cells had no effect on the total number of Clara cells lining the airways (data not shown) and the denudation of Clara cells 24 h after NA injury was found to be similar in NA treated WT (WT-NA) and NGFtg (NGFtg-NA) animals (*online supplement, Figure S4, A*). Additionally, the number of exfoliated cells quantified in the BALF of both groups of mice was comparable (*online supplement, Figure S4, B).*

To investigate the role of TrkA as a downstream target of NGF during the repair processes, its levels were quantified concomitantly in lung tissues. TrkA was found to be significantly upregulated at days 10 and 29 post Clara cell injury in the NGFtg animals compared to WT animals (Figure 4, B).

These results suggest that NGF and its receptor, TrkA are upregulated upon airway epithelial damage and their induction is maintained throughout the ongoing repair process.

NGF promotes renewal of Clara cells

We next tested the role of NGF in the renewal of Clara cells upon injury. Clara cells comprise a major epithelial cell population lining the murine bronchial airways. Following NA treatment, Clara cells were denuded by day 1 (Figure 5, A). At day 10, Clara cells in the airways had started reappearing around the airways in all groups treated with NA, as quantified by CCSP staining (Figure 5, B). Mice treated with NA or NA and control IgG showed similar Clara cell numbers at day 10, with considerable portions of the airway already lined by Clara cells. In contrast, animals treated with NA and neutralizing anti-NGF antibodies, had significantly lower counts of CCSP positive cells lining the airway at day 10, reappearing only sporadically around the airway epithelium (Figure 5). These results indicate that NGF facilitates Clara cell renewal *in vivo* upon NA induced injury.

NGF induces collagen I and fibronectin mRNA expression upon NA induced Clara cell injury

Cellular migration and proliferation critically depend on the adhesive interactions between cells and the underlying matrix. Also, considering that Clara cells show preferential adherence to collagen I and fibronectin, we utilized the NA model to examine the effect of NGF on the expression of these matrix molecules (Figure 6). Indeed, collagen I and fibronectin mRNA levels were significantly increased in lung homogenates of NGFtg animals at day 10 after NA exposure compared to untreated NGFtg control animals (Figure 6, A and B). Moreover, the application of anti-NGF reduced the expression of collagen I in NA treated WT animals (Figure 6, A). Immunohistochemical staining for collagen I and fibronectin corroborated the qPCR data and more intense collagen I and fibronectin staining underlying

the epithelial layer was observed in the control and NA treated NGFtg animals compared to their respective controls (Figure 6, C and D). The application of anti-NGF revealed relatively reduced staining for collagen I in NA treated WT animals. These results suggest that NGF actively augments the expression of the ECM factors collagen I and fibronectin during repair processes in the lung.

NGF promotes proliferation of Clara cells in the lung

After observing that NGF promotes Clara cell renewal as well as the expression of collagen I and fibronectin expression, we investigated the direct effect of NGF on epithelial cell proliferation by administration of BrdU *in vivo* (*online supplement, Figure S1, B*). Quantification of BrdU positive proliferating cells revealed lack of proliferation in the airways of untreated WT and NGFtg control animals, whereas all NA treated groups contained BrdU positive nuclei (Figure 7). Staining serial sections in parallel with BrdU and CCSP revealed proliferating Clara cells (Figure 7, A). Injury and cell denudation was therefore shown to induce re-epithelialization via increased Clara cell proliferation in the lung. Upon inhibition of NGF with anti-NGF antibody, significantly lower numbers of proliferating epithelial cells were observed, while application of a control IgG antibody showed no effect. In line with these results, NGFtg mice treated with NA exhibited the highest numbers of proliferating epithelial cells (Figure 7, B). Together, these results show strong effects of NGF on Clara cell proliferation during repair of injured lung.

Discussion

NGF and other members of the neurotrophin family were initially identified as neuronal growth and survival factors but are currently recognized to have biological activities in tissues outside the nervous system (29). Various visceral epithelia are known to produce neurotrophins which were initially speculated to regulate tissue innervation (30) and autocrine functions of neurotrophins are increasingly being described. For example, NGF production is increased during inflammatory processes that are accompanied by epithelium damage in asthma (17;31), infections (32) and sarcoidosis (33).

Previous reports have suggested the involvement of NGF in epithelial wound healing in other tissues. Topical application of NGF has been shown to accelerate wound closure and re-epithelialization in surgical wounds of the skin (34) and the cornea (23). Moreover, NGF is strongly produced at the site of the wounded skin and is increased in newly formed epithelial cells at the edge of the wound but not in the surrounding epidermal keratinocytes (35). In the cornea, an increase in NGF was observed after epithelial injury and corneal epithelial cell expression of NGF and its receptor TrkA was observed (33). When epithelial surfaces are injured, the normal response is to upregulate receptors for growth factors that drive proliferation and repair (36). The purpose of the present study was to determine the function of NGF in facilitating proliferation of lung epithelial cells, especially Clara cells that comprise the predominant airway epithelial cell population lining murine airways.

The contribution of epithelial NGF in wound repair was first investigated *in vitro* using both a clonal murine lung alveolar type II epithelial cell line (LA4) and primary murine AECII. *In vitro* experiments with wounded LA4 cell monolayers revealed increased protein expression of NGF and its receptor TrkA upon injury. Furthermore, wound closure was accelerated with NGF supplementation and suppressed upon NGF inhibition. Investigation of

primary AECII cells, which also express NGF and TrkA, revealed a chemotactic response of AECII to NGF. AECII cells also responded to NGF by expressing increased collagen I and fibronectin mRNA. Most importantly, in both cell types, NGF was seen to strongly mediate proliferation. We then investigated the pro-proliferative function of NGF *in vivo* by employing a NA model of Clara cell injury.

We investigated the functional effects of NGF on airway epithelium repair in the lung *in vivo* by utilizing a Clara cell specific injury model. The complete renewal of Clara cells after NA mediated injury takes between 1 to 3 months. The NA model of epithelial injury was investigated for a 29 day period to assess the role of NGF in lung epithelia repair. For this we included NGFtg animals that over-express NGF constitutively under the CCSP promoter in Clara cells. The NGFtg animals express around 155 fold higher NGF levels compared to WT mice. The presence of increased NGF levels under normal conditions caused no alteration in the total numbers of Clara cells or any change in cell phenotype. Furthermore, WT and NGFtg animals revealed similar levels of injury at day 1 demonstrated by comparable numbers of exfoliated Clara cells in both mouse strains (*see online supplement, Figure S4*). The NGFtg animals lost most of the NGF over-expressing Clara cells upon exfoliation; however, the remaining cells produced significantly higher levels of NGF compared to WT animals, which may be associated with the accelerated epithelial repair seen in the NGFtg animals. Following injury of Clara cells, lung NGF levels were increased at day 10 and remained significantly elevated until day 29 post injury in both WT and NGFtg mice, emphasizing the importance of NGF in the continuing lung repair processes. This is supported by *in vitro* findings with wounded lung epithelial cells, where NGF synthesis was observed to be upregulated as early as 6 h after injury and increased progressively with time. Interestingly, the lack of proliferation observed in uninjured lungs of NGFtg animals indicates that the pro-proliferative function of NGF is limited to regenerative situations. Therefore, Clara cell

renewal was, at least in part, dependent on the pro-proliferative function of NGF post injury, where BrdU incorporation *in vivo* was enhanced in NGFtg animals and could be inhibited by anti-NGF antibodies.

The over-expression of NGF in the lungs of NGFtg mice has been previously reported to modulate lung innervation and regulate airway hyperresponsiveness (25). NGF has been suggested as a potential candidate for airway remodelling in asthma and also reported to induce smooth muscle cell proliferation (40;41), migration and differentiation of fibroblasts (42). In this study, NGF also appeared to modulate collagen I and fibronectin expression, supported by the observations *in vitro* in AECII and *in vivo* in the NA model of Clara cell injury. Collagen I and fibronectin mRNA expression were significantly increased upon incubation of primary AECII with exogenous NGF and similar results were seen *in vivo* under higher NGF levels in lungs of transgenic animals after injury. Based on these findings we suggest that NGF could potentially modulate ECM expression in other processes involving injury and repair such as airway remodelling in diseases like asthma, where NGF has been previously implicated. Future investigation of the role of NGF in remodelling and fibrosis may shed further light on mechanisms that turn reparative healing processes to pathological events.

In summary, our findings provide evidence that NGF orchestrates proliferative events during repair of the lung epithelia, promoting the renewal of Clara cells *in vivo*. Therefore, NGF may be considered as an important autocrine epithelial growth factor governing repair in the bronchial airway. While neurotrophins were originally described as molecules that maintain neuronal outgrowth and survival, there is now growing evidence that they belong to a class of multifunctional signalling molecules, which can also mediate repair and proliferation of lung epithelia.

Acknowledgments

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Legends

Figure 1. **Production of NGF and expression of TrkA *in vitro* after LA4 cell monolayer wounding.** (A) NGF production was measured by ELISA in unwounded control (white bars), and wounded (black bars) LA4 cells at 6, 12, 24, 48 and 72 h. (B) The expression of TrkA was examined by quantifying intensity of bands normalized to β-actin by western blotting in control (white bars) and LA4 monolayers with wound (black bars) at 24, 48 and 72 h. A representative blot showing expression of TrkA (140 kDa) and β-actin (42 kDa) in wounded and control LA4 monolayers at 24, 48 and 72 h from the same experiment is shown below the graph. Figures represent n= 6 samples ± SEM for each time point. ** = $p< 0.01$, *** = $p< 0.001$

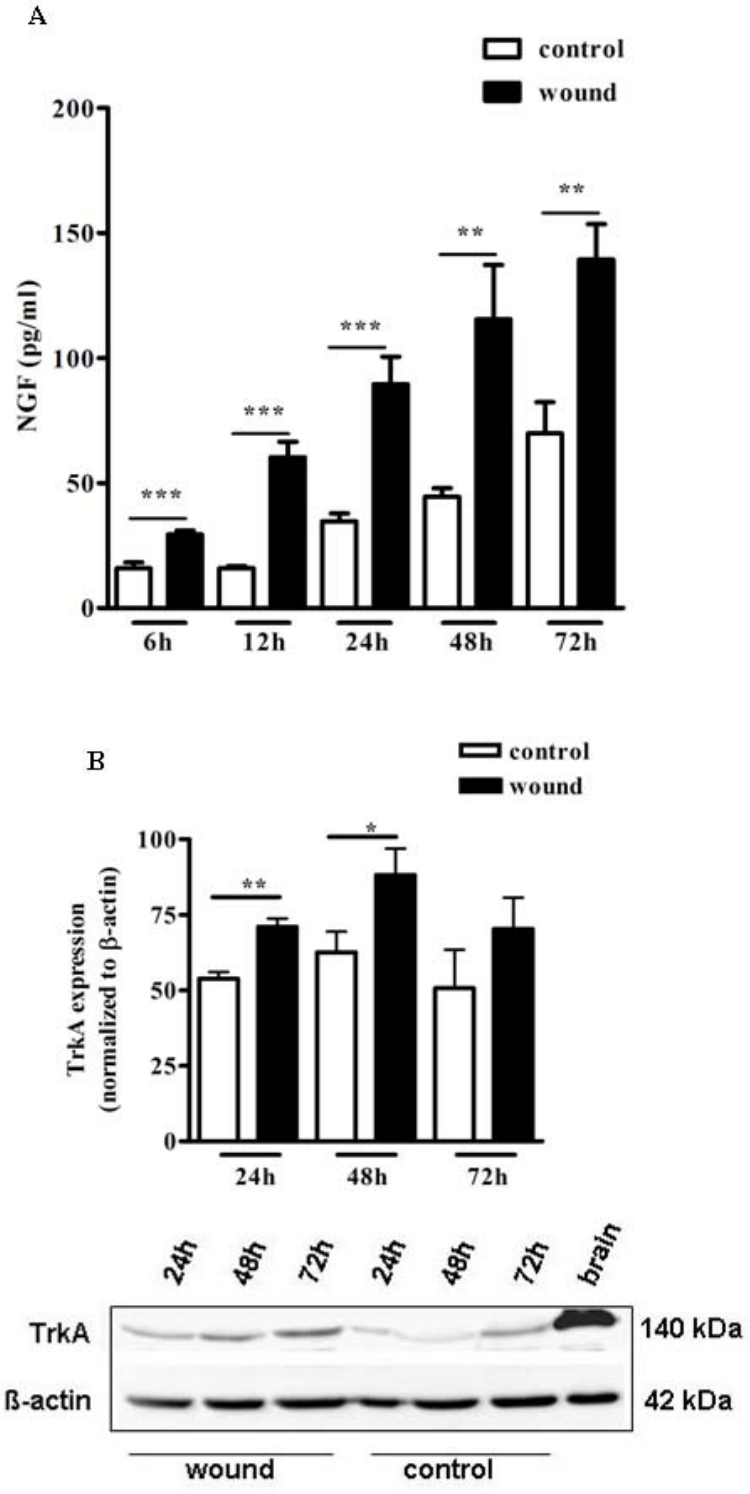
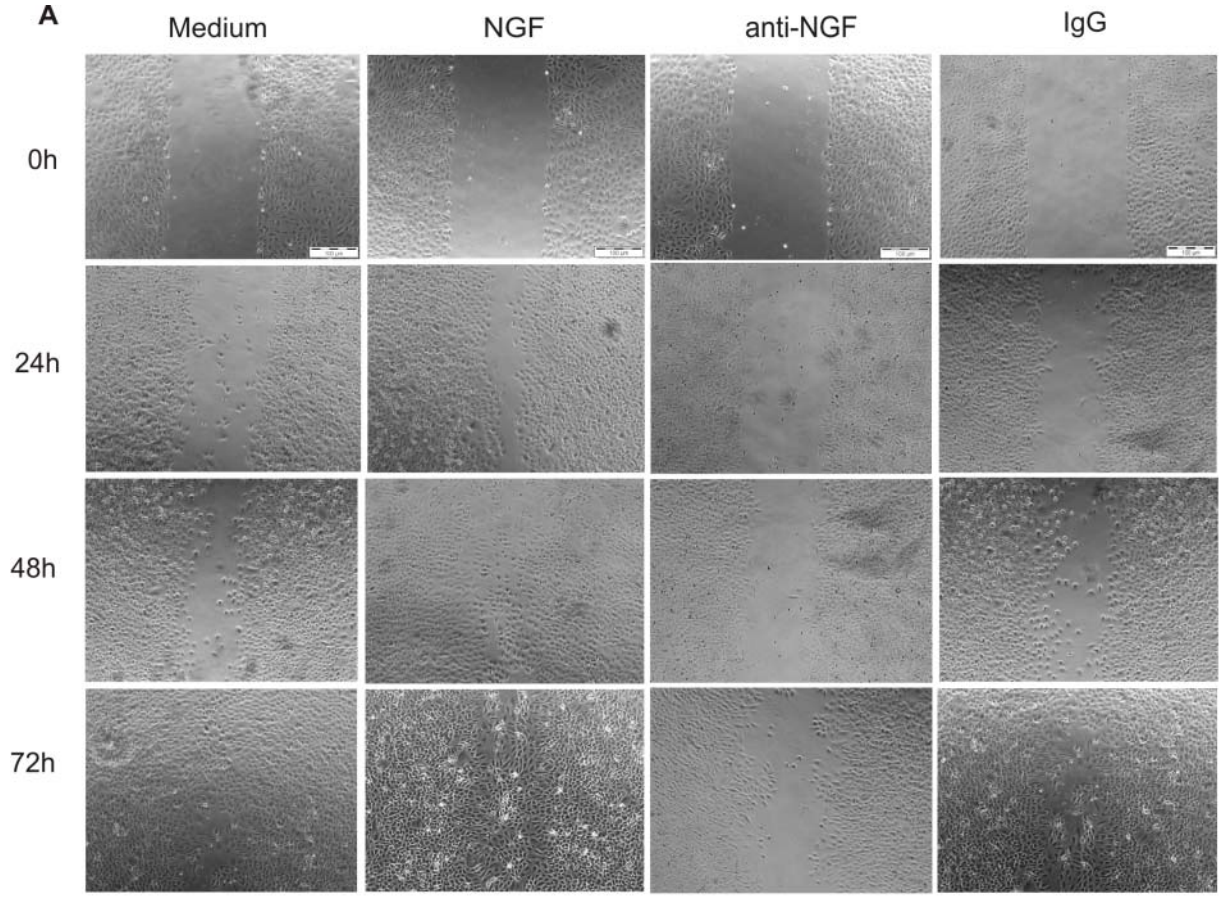


Figure 2. NGF regulates wound closure of LA4 cells *in vitro*. (A, B) Confluent monolayers of LA4 cells were wounded and the kinetics of wound closure was monitored in monolayers grown in medium only (Medium, ■), medium with recombinant NGF (NGF, o), IgG control

(IgG, •), or with anti-NGF (anti-NGF, □) at 0, 24, 48, and 72 h. Wound closure was quantified by measuring wound length in μm across the wound. Statistical comparisons for NGF stimulated and anti-NGF treated groups are made to medium only group. (C) Cell proliferation was determined by measuring incorporation of BrdU in DNA of proliferating LA4 cells. The rate of proliferation is represented relative to basal proliferation in the medium control where BrdU incorporation was taken as 100% (dotted line). TrkA and NGF were blocked by specific siRNA (dark grey and black bars respectively) and compared to cells grown in the transfection assay medium (white bar) and a non-specific siRNA (light grey bar). Figure represents n = 6 samples ± SEM for each time point. *** = $p < 0.001$.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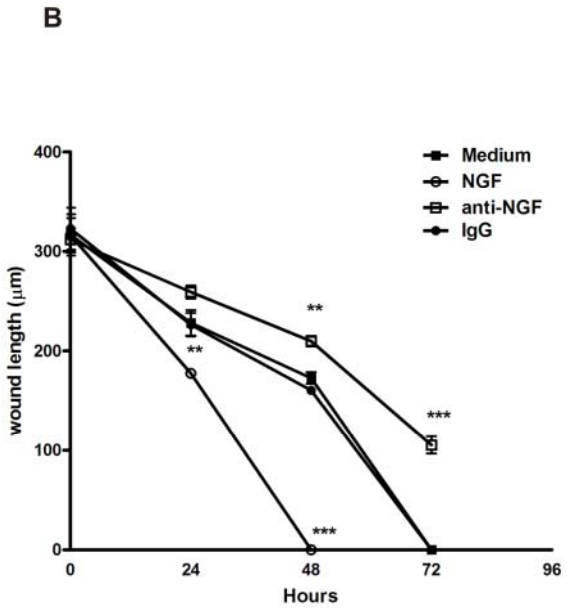
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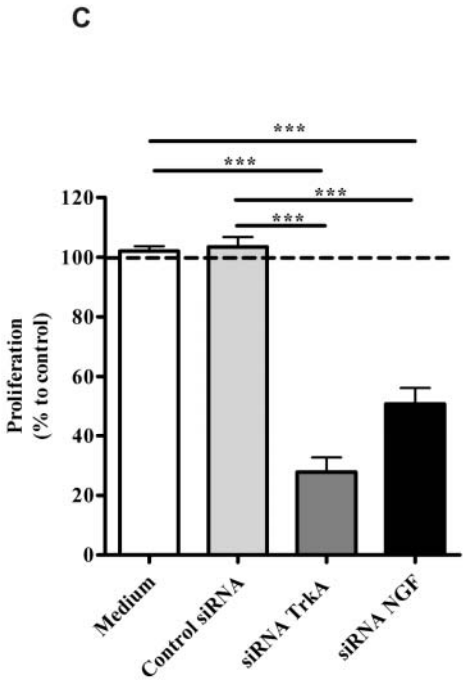
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Figure 3. NGF regulates proliferation, migration and ECM mRNA expression of AECII. (A, B) NGF and TrkA protein expression by AECII (C) AECII proliferation analysed after 72 h incubation with either medium only, medium containing NGF, anti-NGF or control IgG. (D) AECII migration analysed after 6 h incubation with medium or recombinant NGF. Migration is expressed as migrated cells per ×100 field of view. Relative mRNA levels of (E) fibronectin (F) collagen I in AECII cells incubated for 24 h in either medium only, medium containing NGF, anti-NGF or control IgG. Values are expressed as copy number target gene per copy L32 (rel. mRNA). Figure represents n=6 samples ± SEM for each group. * = $p< 0.05$, *** = $p< 0.001$.

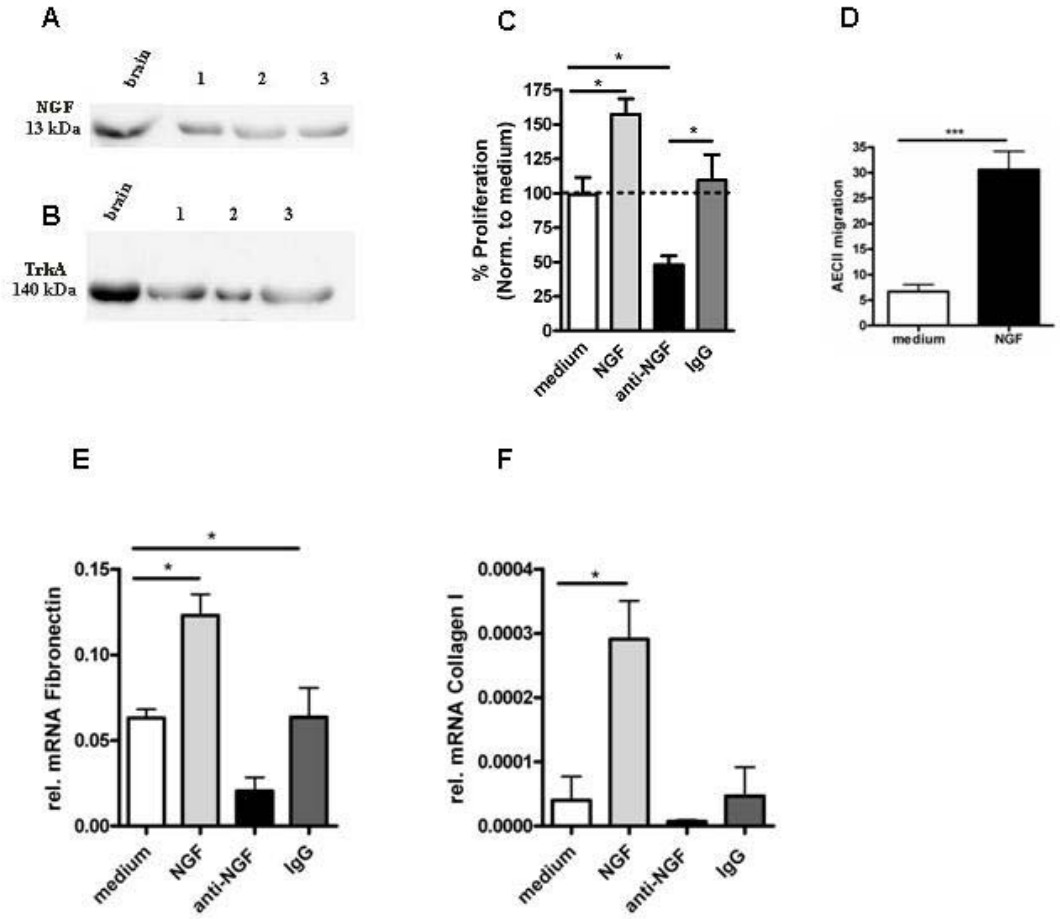


Figure 4. Expression of NGF and TrkA *in vivo* following Clara cell lung injury. (A) NGF levels in NGFtg (black bars) and WT (white bars) animals without NA treatment (control) and after NA treatment (NA) at day 10 and 29 (d10, d29). NGF expression was measured by

ELISA in BALF. (B) The expression of TrkA is quantified and represented as band intensity normalized to the housekeeping gene, β-actin. A representative blot showing expression of TrkA (140 kDa) and β-actin (40 kDa) in control NGFtg and WT animals (con) and after NA treatment at day 10 and 29 (d10, d29) from the experiment is shown below the graph. Figures represent n=6 samples ± SEM for each group of mice. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

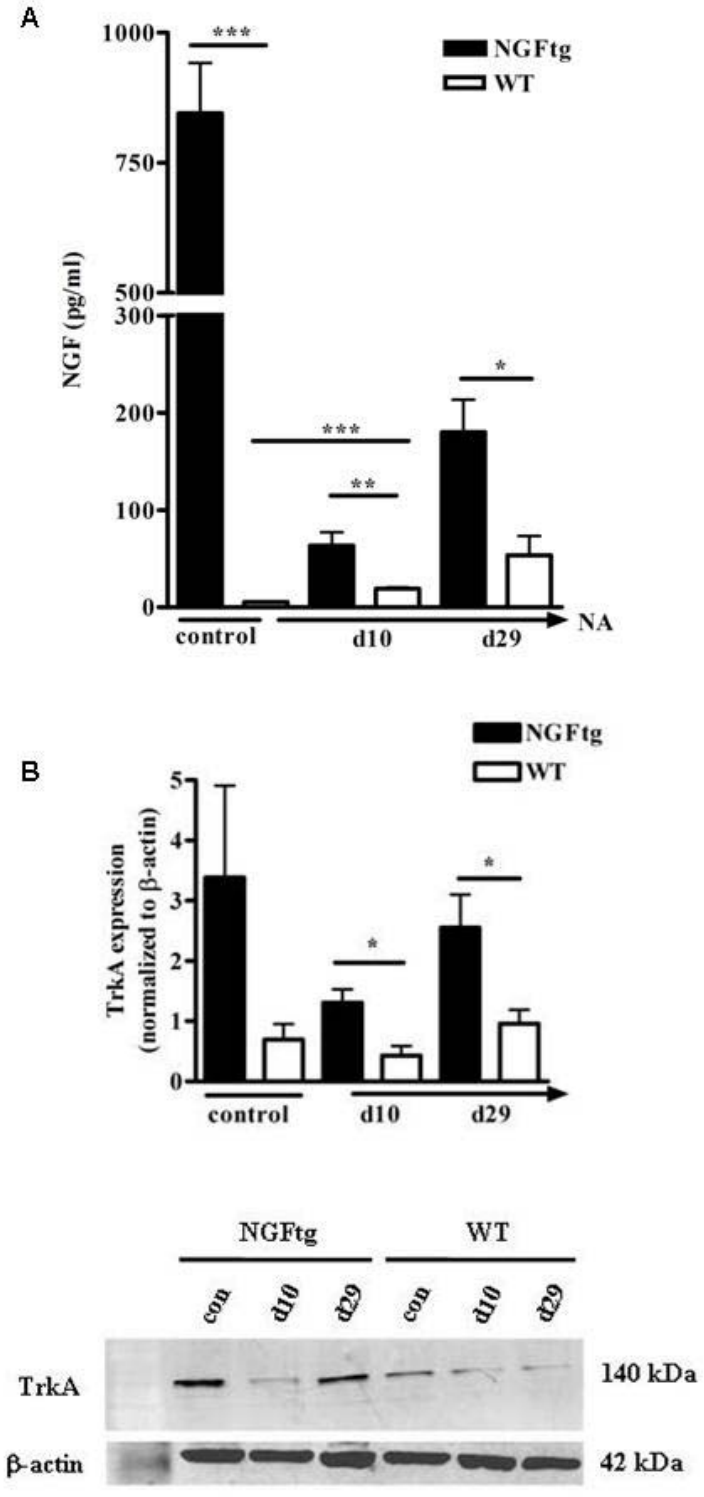


Figure 5. Clara cell renewal. (A) Intact Clara cells in corn oil treated WT control animals at day 1 and day 10 (WT-control) and after NA treatment (WT-NA) with anti-NGF (WT-anti-NGF-NA) or control IgG (WT-IgG-NA) antibodies. Clara cells are stained for Clara cell

secretory protein (CCSP) as brown coloured non ciliated columnar cells against the blue counter stain. Scale bar represents 50µm. (B) Quantification of CCSP positive cells per mm of the basement membrane at day 10 after NA treatment. Figure represents n= 6 animals ± SEM for each group of mice. * = p< 0.05, ** = p< 0.01, *** = p< 0.001

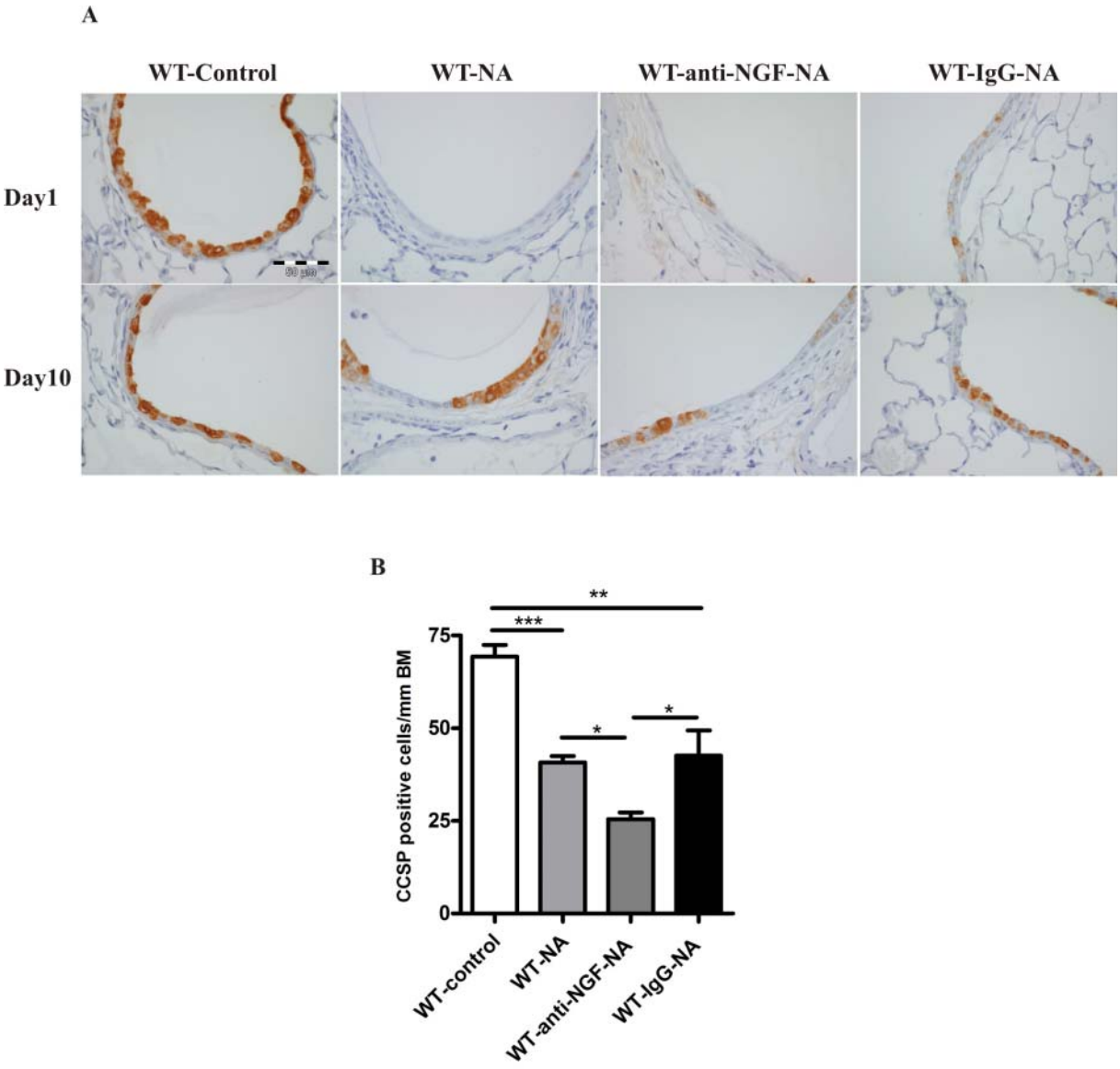


Figure 6. Modulation of collagen I and fibronectin expression by NGF. Relative mRNA levels of collagen I (A) and fibronectin (B) are shown, values are expressed as copy number target gene per copy L32 (rel. mRNA). mRNA was analysed in WT (white bars), NGFtg (black bars), WT animals treated with IgG or NA + IgG (light grey bars) or anti-NGF or NA + anti-NGF (dark grey bars). Immunohistochemical staining of (C) collagen I and (D)

fibronectin in corn oil treated WT (WT-control) and NGFtg (NGFtg-control) animals and NA treated WT (WT-NA), with anti-NGF (WT-anti-NGF-NA) or with control IgG (WT-IgG-NA) and NA treated NGFtg (NGFtg-NA) animals analysed at day 10. Scale bar represents 50 μm. Figure represents n=6 samples ± SEM for each group of mice. * = p< 0.05, *** = p< 0.001

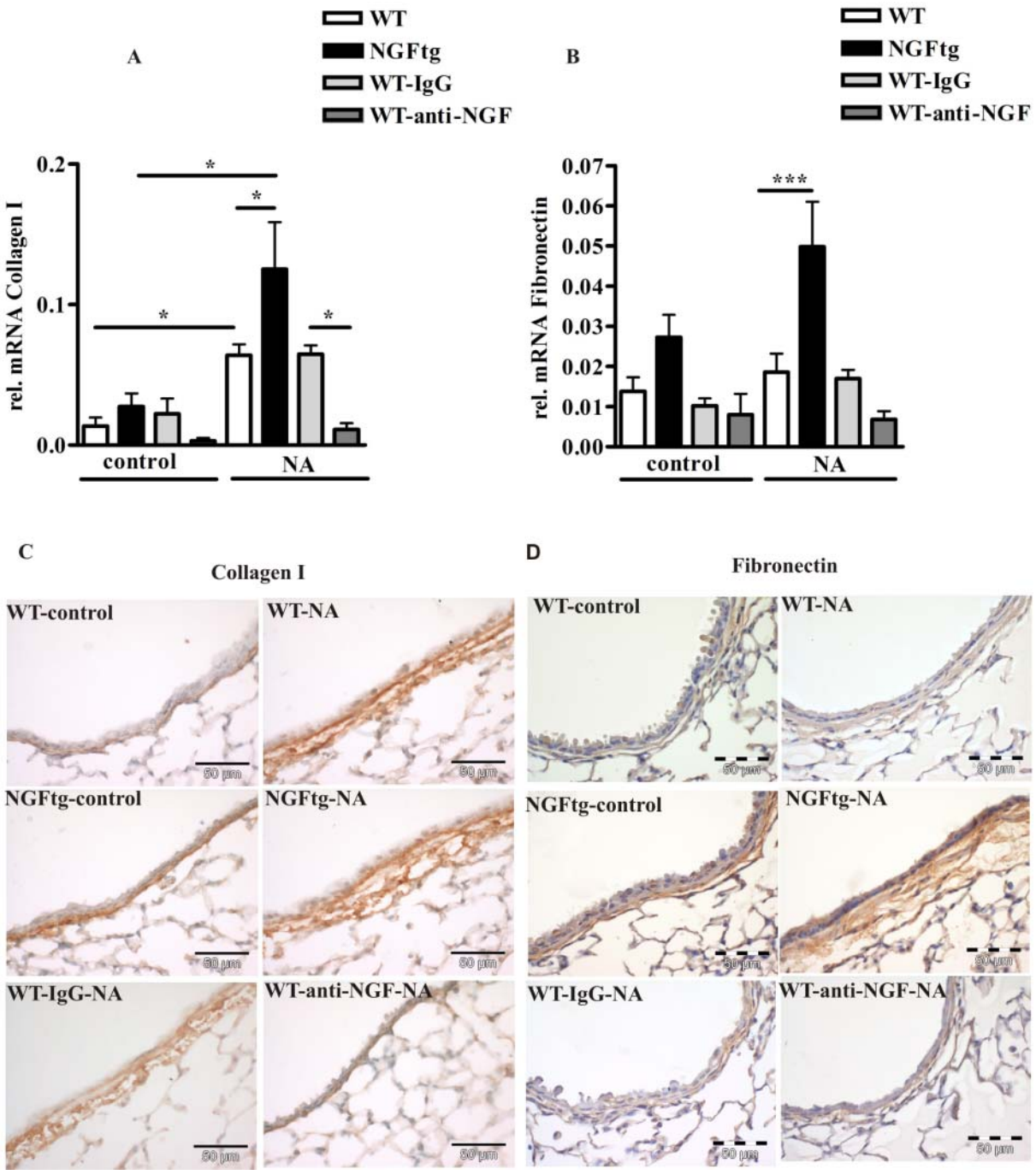
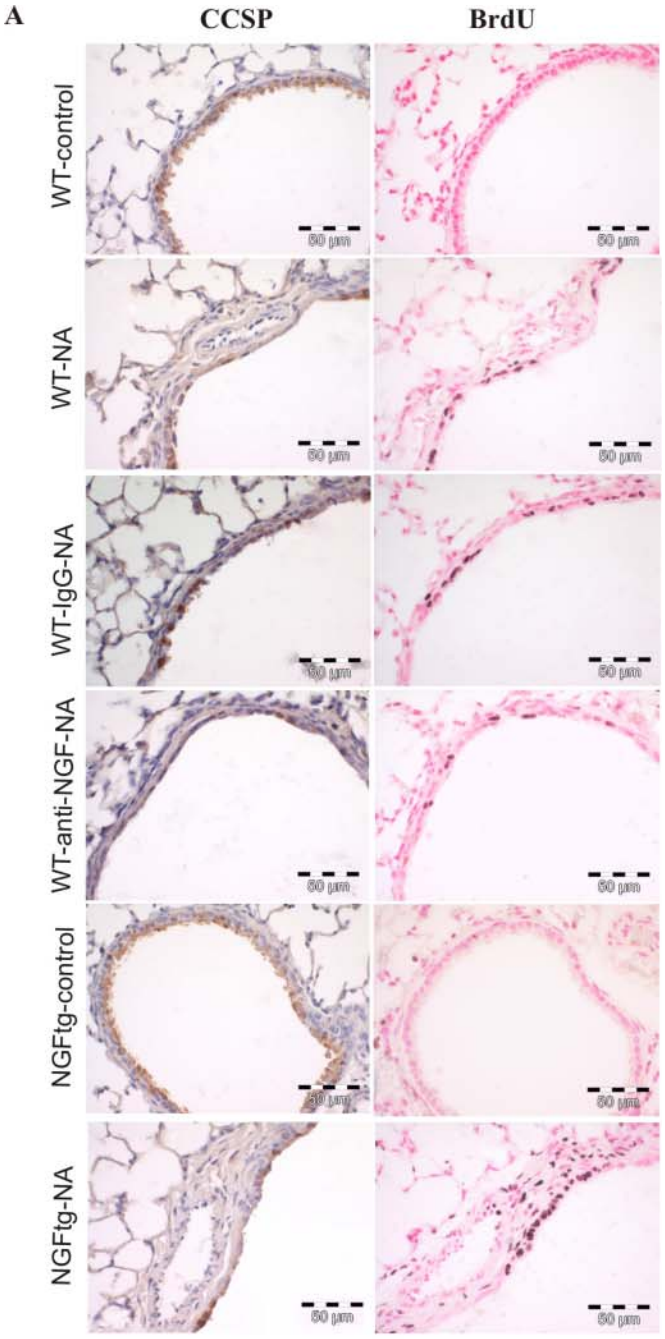
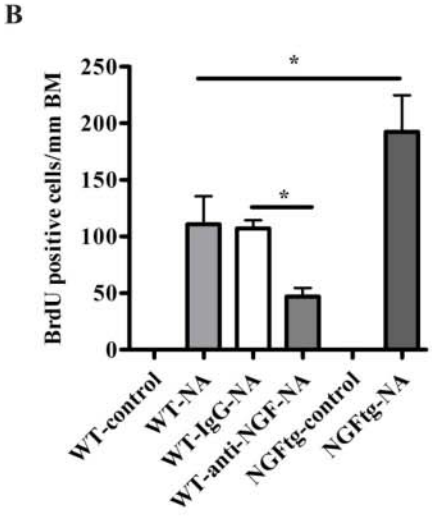


Figure 7. Analysis of proliferation by BrdU incorporation in bronchial epithelium. (A) Serial sections of WT control (WT-control), NA treated WT (WT-NA) with control IgG (WT-

IgG-NA) or with anti-NGF (WT-anti-NGF-NA) and NGFtg control (NGFtg-control) and NA treated NGFtg (NGFtg-NA) animals stained for CCSP and BrdU are shown. The CCSP positive Clara cells are stained brown against the blue counter stain and the BrdU positive cells exhibit a dark brown nucleus against the nuclear fast red counter stain. All animals treated with NA show positive nuclear staining for BrdU. Scale bar represents 50 μm. (B) Quantification of BrdU positive nuclear profiles per mm of the basement membrane at day 10 after NA treatment. Figure represents n= 6 animals ± SEM for each group of mice. * = $p < 0.05$.


A
CCSP
BrdU
WT-control
WT-NA
WT-IgG-NA
WT-anti-NGF-NA
NGFtg-control
NGFtg-NA
50 μm


B
BrdU positive cells/mm BM
250
200
150
100
50
0
*
*
WT-control
WT-NA
WT-IgG-NA
WT-anti-NGF-NA
NGFtg-control
NGFtg-NA

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