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Keratinocyte growth factor protects against Clara cell injury induced by naphthalene

Ali Önder Yildirim[1§], Martina Veith[1§], Tanja Rausch[1], Bernd Müller[1], Patrick Kilb[1], Laura S. Van Winkle[2], Heinz Fehrenbach[1]

[1]*Clinical Research Group "Chronic Airway Diseases", Medical Faculty, Philipps-University Marburg, Germany,*

[2]*Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine and Center for Health and the Environment, University of California, Davis, California, 95615, USA*

§ **These authors contributed equally to this work.**

To whom correspondence should be addressed:

Ali Önder Yildirim, DVM
Clinical Research Group "Chronic Airway Diseases", Medical Faculty,
Philipps-University Marburg, Germany
Hans-Meerwein Str.1
35043 Marburg, Germany
Tel: 0049 6421 28 66046, Fax: 0049 6421 28 66095
E mail: ayildiri@med.uni-marburg.de

Short title: KGF protects against acute airway injury

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ABSTRACT

Airway epithelial cells are exposed to environmental toxicants that result in airway injury. Naphthalene (NA) causes site selective damage to Clara cells in mouse distal airways. ΔN23-KGF protects against acute lung injury. This study investigated whether ΔN23-KGF protects against NA-induced acute Clara cell damage by measuring airway responses specifically and to identify underlying molecular mechanisms.

Mice were treated with ΔN23-KGF or PBS 33h prior to injection of 200 mg/kg NA. Lung function was analysed by head-out body plethysmography. Distal airways isolated by microdissection were assessed by ethidium homodimer-1 for cell permeability. Immunohistochemistry for Clara cell specific protein in conjunction with a physical disector was used to quantify Clara cell numbers. RNA was isolated from frozen airways to analyse gene expression by quantitative RT-PCR.

ΔN23-KGF prevented NA-induced airflow limitation and Clara cell permeabilty, and resulted in twice as many Clara cells compared to PBS pre-treatment. ΔN23-KGF pre-treated mice exhibited increased mRNA expression for proliferating cell nuclear antigen. Cytochrome P450 isoform 2F2 (CYP2F2), which converts NA into its toxic metabolite, was reduced by about 50%.

Our results demonstrate that pre-treatment with ΔN23-KGF protects against NA-induced injury. We suggest that ΔN23-KGF exerts its beneficial effect through a decrease in the expression of CYP2F2.

Keywords: Airway epithelial injury, cytochrome P450, keratinocyte growth factor, naphthalene, secretoglobin

INTRODUCTION

Airway injury, repair, and remodeling are implicated in the pathogenesis of diverse lung diseases such as asthma, chronic obstructive pulmonary disease, and lung cancer [1]. Among the many agents that cause lung injury, hazardous air pollutants have chronic, adverse effects on lung function [2], and are likely to contribute significantly to morbidity and mortality in humans [3]. The airway epithelium is composed of a few major cell types: ciliated epithelial cells, Clara cells, neuroepithelial cells, basal cells, mucous cells, and serous cells [4]. It provides a selectively permeable barrier between the internal tissues and the potentially hazardous agents entering the body via the airways. In particular the distal airways are exposed at high levels to various environmental oxidants, which may lead to epithelial injury in this specific region [5].

Naphthalene (NA), a harmful environmental toxin, can be found in ambient air, ground water, cigarette smoke, and is produced by combustion [6]. NA is used as feedstock in various chemical industrial processes and was suggested to pose a problem in the workplaces of various industries [7]. NA, which was found to be the most abundant polycyclic aromatic hydrocarbon in sidestream cigarette smoke [8], selectively injures nonciliated Clara cells of the conducting airways. The toxicity of NA requires metabolic activation, which is catalyzed by cytochrome P450 monooxygenases [9]. In addition to their general secretory role [10], Clara cells are the primary cellular site of cytochrome P450 monooxygenase isoform 2F2 (CYP2F2) in mice [11], an enzyme with both high catalytic activity for NA and great abundance in the mouse airway [12]. Therefore, murine Clara cells are more susceptible to NA-induced cytotoxic injury than the other types of airway epithelial cells [13].

Keratinocyte growth factor (KGF), also known as fibroblast growth factor-7 (FGF-7), has been identified as an important paracrine mediator of proliferation, migration, and differentiation of alveolar epithelial type II cells [14]. KGF specifically binds to the KGF receptor, a splice variant of FGF receptor 2 (FGFR2-IIIb), which is expressed by epithelial cells [15]. In animal studies, KGF reduces alveolar injury and mortality in rat and/or mouse models of acute lung injury to the gas exchange area [14]. Our knowledge is much more limited with regard to the role of KGF in acute airway epithelial injury and repair. KGF was demonstrated to be important for the detoxification of reactive oxygen species [16], and to enhance proliferation [17] in airway cells. Administration of KGF was shown to protect against radiation-induced increases in airway epithelial barrier permeability [18], to exert protective effects against oxidant injury [19], and to limit allergen-induced alterations of the airway epithelial integrity [20]. Further, KGF accelerated epithelial wound closure in bronchial epithelial cells cultured *in vitro* [21], and studies in the heterotopic tracheal transplant model revealed that KGF enhanced airway epithelial repair involving both local resident progenitor epithelial cells and the mobilization and engraftment of circulating epithelial progenitor cells [22].

Despite the important function of KGF in the lung, the role of KGF in the airways is not well-understood. Microarray analyses of human primary bronchial epithelial cells incubated with KGF *in vitro* revealed a decrease in the mRNA expression of CYP2F1 [23]. Human CYP2F1 exhibits 80% homology to mouse CYP2F2 and is also expressed in the respiratory tract [24]. Therefore, we hypothesized that pre-treatment of lungs with N-terminally truncated recombinant human KGF (ΔN23-KGF, palifermin), which is characterized by enhanced protein stability [25], protects against

NA-induced acute Clara cells damage of murine distal airways. To address this question we used four experimental approaches: 1) distal airways from mice pre-treated with ΔN23-KGF or PBS were isolated by microdissection [26] and analyzed for cell membrane integrity, cell proliferation, and Clara cell numbers after NA-induced injury; 2) serial non-invasive lung function analysis was performed in unanaesthetized mice by means of head-out body plethysmography [27] to assess the functional relevance of NA-induced acute airway injury and of a potential protective effect of ΔN23-KGF on the organ level; 3) the effect of KGF-treatment on mRNA expression of CYP2F2, which catalyzes the conversion of NA into the cytotoxic 1R, 2S-oxide [12], was analyzed in microdissected distal airways as well as in Clara cell-enriched pulmonary epithelial cells isolated from mouse lungs; and 4) mice were therapeutically treated with ΔN23-KGF at different time-points after NA-injection to test the hypothesis that the protective effect of KGF-treatment was dependent on the presence of Clara cells.

MATERIALS AND METHODS

Animals

Pathogen-free male C57BL/6 mice (Harlan Winkelmann, Hannover, Germany), weighing 25–29 g and 8-10 weeks of age, were used in all experiments. The animals were allowed free access to food and water. All animals were euthanized by an over-dose of sodium pentobarbital (i.p., 100 mg/kg) followed by exsanguination. All animal experiments were approved by the local animal care committee.

Application of Growth Factor

Mice were anaesthetized by short term inhalation of isofloren (Forene, Abbot, Wiesbaden, Germany). Each mouse received a single bolus of 10 mg ΔN23-KGF (Amgen, CA, USA) per kg body weight by oropharyngeal aspiration or an equivalent volume of PBS (80 µl). This dose was found to induce the maximal proliferative response of alveolar epithelial type II cells in mice [28]. Treatment was performed 33 h prior to i.p. injection of NA or corn oil.

Application of Naphthalene (NA)

NA was purchased from Fisher (Aschaffenburg, Germany) and dissolved in corn oil. C57BL/6 mice were injected with 200 mg NA per kg body weight or corn oil alone as vehicle control and were sacrificed 12 h later. This time point was chosen because experiments studying the kinetics of NA-induced acute airway injury revealed that after 12 h Clara cells were already damaged but exfoliation was still incomplete whereas complete exfoliation was observed 24 h after NA-injection (see online supplemental data). Distal airways were microdissected and processed for histological analysis. To evaluate the protective effect of ΔN23-KGF, mice were pre-

treated with ΔN23-KGF or PBS. Mice were sacrificed 12 h after NA treatment and distal airways were isolated by microdissection. To exclude effects of circadian rhythm, all applications were performed between 8 am and 10 am.

In order to evaluate if the effect of ΔN23-KGF is primarily related to Clara cells, mice were treated with 200 mg NA per kg body weight 2 or 24 hours prior to oropharyngeal aspiration of 10 mg ΔN23-KGF per kg body weight. Animals were sacrificed 5 days later.

Tissue Preparation and Microdissection

Procedures for microdissecting defined airway generations have been described in detail previously [26]. Briefly, after sacrifice by cervical dislocation the trachea was exposed and cannulated. The lungs were removed from the thorax and inflated with 1% low melting point agarose (Sigma-Aldrich, Munich, Germany) dissolved in 2x Waymouth´s medium (Sigma-Aldrich) and incubated in Ham's F-12 nutrient mixture (Sigma-Aldrich) on ice for 30 min to allow the agarose to solidify. Distal airways were isolated from the left lung of each mouse.

Assessment of Cell Permeability

For cell permeability studies, microdissected airways were incubated at +37°C with ethidium homodimer-1 (EH-1) (Sigma-Aldrich) in Ham's F-12 nutrient mixture for 20 min to label the nuclei of any membrane-permeable cells as described earlier [29]. Airways were washed three times at +37°C with F-12 medium to remove unincorporated EH-1 and fixed with 330 mOsm Karnovsky's fixative. Airways were stored in fixative in the dark until embedment into glycol methacrylate (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany). Embedded airways were cut into 1 µm thick

sections. A water-soluble, nonfluorescing mounting medium Fluoromount-G™ (Southern Biotechnology, Birmingham, USA) was used.

For imaging, an epifluorescent microscope (Olympus, Tokyo, Japan) equipped with a wide-band ultraviolet (UV) fluorescence excitation-emission filter set was used. Because of a phase shift in the wide-band UV excitation emission filter set, the tissue autofluoresces green and EH-1 positive nuclei fluoresce pinkish-white.

Airway Fixation

For quantitative morphological analysis, at least four microdissected distal airways from each mouse (n=3) were fixed in 1% phosphate buffered formalin, orientated so that the resulting sections were normal to the longitudinal axis of the airways, and embedded into paraffin. For RNA isolation and subsequent gene expression analyses, three microdissected airways from each mouse (n=3) were snap frozen in liquid-nitrogen and stored at -80°C.

Immunohistochemistry

As described earlier [17], cross sections of airways 2 µm in thickness were deparaf-finized in xylene, and rehydrated in ethanol and PBS. Endogenous peroxidase activity was inactivated with 1% H_2O_2 in Methanol (Roth, Karlsruhe, Germany), pH 7.2, for 30 min. Antigen retrieval was performed by microwave treatment in 3% citrate buffer at pH 6.0 (Roth, Karlsruhe, Germany). After washing in PBS, the sections were incubated for 30 min in PBS with 1% bovine serum albumin (Serva, Heidelberg, Germany) followed by incubation with a polyclonal rabbit antibody (courtesy of Jörg Klug, Marburg) against Clara cell-specific protein 10 kD (CC10) diluted 1:3000 in the same solution at +37 C for 1 h. Sections were then incubated at room temperature for 30 min with anti-rabbit secondary antibody diluted 1:10, which was visualized

using 3,3′-diaminobenzidine as chromogen according to the ABC method (Vectastain Elite ABC Kit; Vector Laboratories, USA) following the manufacturer's instructions. To identify ciliated cells and cell proliferation, immunostaining was performed using mouse monoclonal antibodies against β-Tubulin-IV (Bio Genex, San Ramon,CA, USA; diluted 1:150) and against proliferating cell nuclear antigen (PCNA) (Dako Cytomation, Hamburg, Germany; diluted 1:600), respectively was performed. Antibodies were detected using a mouse-on-mouse immunodetection kit (Vector MOM kit, Vector Laboratories). All sections were counterstained with hematoxylin.

Quantitative-Morphological Analysis

The number of Clara cells was determined by design-based stereology using a physical disector [30]. Clara cell numbers were normalized to airway epithelial basal lamina area. In addition, the arithmetic mean thickness of Clara cells was estimated by point and intersection counting. Four to six paraffin embedded airways were studied per mouse. From each airway two serial cross-sections were cut on a motorized microtome with block cooling device (H 355 S, Microm, Walldorf, Germany), which was calibrated for block advance. Sections 2 µm in thickness were stained for CC10 and analyzed by means of a computer-assisted Olympus light microscope BX 51, which was equipped with a CAST-Grid System (Olympus, Denmark).

The arithmetic mean thickness of Clara cells was calculated using the following formula (1):

$$\overline{\tau_{cc}} = \frac{2 \bullet P_{cc} \bullet l_c}{\pi \bullet I_{bl}} \qquad (1)$$

where P_{cc} is the sum of points that hit the Clara cells, l_c is the length per point on a cycloid grid, and I_{bl} is the sum of intersections of cycloid test lines with the epithelial basal lamina.

The number of Clara (N_{CC}) was calculated according to formula (2) from their numerical density ($Nv_{cc,epi}$), which was determined using formula (3), and normalized to the surface area of the basal lamina in the same reference volume estimated by using formula (4):

$$Ns_{cc,bl} = \frac{Nv_{cc,epi}}{Sv_{bl,epi}} \qquad (2)$$

$$Nv_{cc,epi} = \frac{0.5 \bullet cell\ count}{h \bullet a_{fr} \bullet P_{epi}} \qquad (3)$$

$$Sv_{bl,epi} = \frac{\pi \bullet I_{bl}}{2 \bullet l_c \bullet P_{epi}} \qquad (4)$$

where *cell count* is the number of nuclear profiles of CC10 immuno reactive cells in the look-up section of the disector, which are not seen in the reference section, *h* is the height of the disector (i.e. the calibrated mean block advance of 2 µm), a_{fr} is the area of the unbiased counting frame, and P_{epi} is the sum of points hitting airway epithelium. As counting was performed in both directions (double disector), the cell count was multiplied by 0.5 in formula (2).

RNA Isolation and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from microdissected airways using SV Total RNA Isolation System (Promega, Germany), and cDNA was synthesized with Omniscript Reverse Transcription Kit (Quiagen, Hilden, Germany). Real-time RT-PCR was performed

using QuantiTect® SYBR® Green Master Mix (Abgene, Germany) and BioRad ICycler (BioRad Laboratories, Hercules, Germany). 10 µl QuantiTect® SYBR® Green Master Mix, 1 µl of each primer at a concentration of 50 pmol/µl, and 20 µl water were added to 3 µl of cDNA, standard or water (negative control). Primer sequences were generated from the respective mRNA sequences obtained from the European Molecular Biology Laboratory (EMBL) gene bank (for primer pair sequences, see online supplemental Table E1). Primers were synthesized by MWG Biotech (Ebersberg, Germany). ΔC_T analysis was used to calculate expression in comparison to glyceraldehyde phosphate dehydrogenase (GAPDH). In additional, because NA caused Clara cell exfoliation and CYP2F2 isozyme is predominantly expressed in Clara cells, mRNA expression of CYP2F2 isozyme was determined relative to mRNA expression of CC10.

***Effects of ΔN23-KGF on Clara Cells cultured* in vitro**

Mouse Clara cells were isolated from C57BL/6 mice using the protocol described by Elizur and co-workers [31], by which a purity of 70% of Clara cells is obtained. Briefly, the lung was perfused free of blood with PBS via the right ventricle, 1 ml of 1% low-melting agarose in PBS was instilled through the trachea followed by 0.5 ml of 0.25% bovine pancreatic trypsin (Sigma-Aldrich). After incubation on ice, the lungs were excised and incubated at +37°C for 10 min. The tissues were diced in medium (DMEM + 250 µg/ml DNase + 2x antibiotic/antimycotic) filtered sequentially through 100- and 40-µm filters. The final pellet was resuspended in 10 ml of medium. For experiments, cells were placed into 35 mm cell culture plates. Cells received fresh medium supplemented with either ΔN23-KGF (50 ng/ml) or an equivalent amount of PBS as a control. After 1 and 4 h of in vitro culture at +37°C, RNA Lysis buffer (SV Total RNA Isolation System, Promega) covering the cells was collected, and cells

were harvested for RNA isolation and subsequent real-time RT-PCR analysis as described above.

Non-Invasive Measurement of Lung Function

Immediately prior to sacrifice, mid-expiratory airflow (EF_{50} in ml/s), a parameter suitable to monitor bronchoobstruction [27], was measured using head-out body plethysmography as described previously [32]. Briefly, the system consisted of a glass body to which four head-out body plethysmograph chambers were attached (Forschungswerkstätten, Hannover Medical School, Hannover, Germany). A calibrated pneumotachograph (PTM 378/1.2, Hugo Sachs Electronics, March-Hugstetten, Germany) and a differential pressure transducer (8T-2, Gaeltec, Dunvegan, UK) coupled to an amplifier (Gould Universal Amplifier, Dietzenbach, Germany) were attached to the top port of each plethysmograph chamber. The amplified analog signal from the pressure transducer was digitized via an A/D converter (DAS-16, Keithley, Germering, Germany) at a sampling rate of 2000/s. For airflow measurements, one mouse was placed into each chamber with the head protruded through a flexible neck collar so that the chamber was sealed airtight against the main glass body. Airflow was measured during a 15 min period after the animals had resumed normal breathing. EF_{50} values measured after treatment with ΔN23-KGF and/or NA were determined as percent changes of the mean baseline value of the respective control group.

Statistical Analysis

Results are presented as mean values ± SD unless stated otherwise. One way analysis of variance (ANOVA) was used to determine the level of significance of differences observed between groups if normality and equal variance were given at $p>0.1$. Otherwise, Kruskal-Wallis ANOVA on ranks was used. All analyses were

performed by means of GraphPad Prizm 4 software program (El Camino Real, San Diego, CA USA). Differences were considered statistically significant at $P<0.05$.

RESULTS

Pre-treatment with ΔN23-KGF protects Clara cells against NA-induced Necrosis

Failure of cell membrane integrity was assessed by EH-1, a sensitive indicator of irreversible cell injury [29]. EH-1 positive cells, which were identified as Clara cells based on morphological characteristics, were abundant in distal airways of mice at 12 h after injection of 200 mg NA/kg (Fig. 1B) whereas EH-1 positive cells were completely absent in control mice (Fig. 1A) or mice treated with ΔN23-KGF (Fig. 1C). Pre-treatment of lungs with ΔN23-KGF prior to NA-injection markedly reduced the appearance of EH-1 positive cells in distal airway epithelium (Fig. 1D).

ΔN23-KGF induces PCNA Activation and Airway Epithelial Proliferation

PCNA is a proliferation marker because its gene is expressed from early G1-phase to S-phase of the cell cycle with the protein confined to the nucleus. Epithelial cells with nuclear staining for PCNA were rarely seen in untreated airways (Fig. 2A). Immuno-reactivity for PCNA was regularly observed in airway epithelial cells of mice 12 h after injection of NA (Fig. 2B). However, PCNA positive cells were abundant in distal airways of lungs pre-treated with ΔN23-KGF (Fig. 2C and D).

Quantitative real time RT-PCR revealed that in mice pre-treated with ΔN23-KGF 33 h prior to NA-injection mRNA expression of PCNA was significantly increased as compared with NA-injected mice pre-treated with PBS (Fig. 2E).

Pre-treatment with ΔN23-KGF inhibits Clara Cell Loss

Clara cells were identified using immunohistochemistry for Clara cell-specific protein 10 kD (CC10), which is also known as CC16, uteroglobin or secretoglobin [33]. Clara cell numbers were analyzed using design-based stereology with a physical disector approach. Quantitative morphological analysis revealed that 12 h after NA-injection the number of CC10-positive Clara cells (5,047 ± 829) was reduced to 50% of the Clara cell number characteristic of distal airways of control mice (10,157 ± 229). Pre-treatment of lungs with ΔN23-KGF caused a slight increase in Clara cell numbers after 33 h (12,142 ± 399) and significantly restricted the loss of Clara cells 12 h after NA-treatment (9,404 ± 528) to only 23% of lungs treated with ΔN23-KGF alone (Fig. 3).

Consistent with the physical disector counts of Clara cell numbers, real time RT-PCR of the mRNA expression for CC10 relative to GAPDH was significantly reduced in distal airways of NA-treated mice (4.4% of PBS control), whereas the CC10 mRNA level in airways of mice pre-treated with ΔN23-KGF prior to NA-injection was approximately 4-fold higher (Fig. 4). KGF was previously shown to decrease CC10 mRNA expression levels in rat Clara cells [34]. Consequently, pre-treatment with KGF alone resulted in a reduction in CC10 mRNA expression as compared with PBS-treated control mice despite the increase in proliferation and Clara cell numbers.

ΔN23-KGF downregulates Cytochrome P450 2F2 mRNA Expression

CYP2F2 is important in the metabolization of NA because it converts NA into a cytotoxic epoxide intermediate. Administration of ΔN23-KGF with or without subsequent NA-injection resulted in a significant down-regulation of CYP2F2 mRNA expression (Fig. 5). This was corroborated by real time RT-PCR analyses of Clara cell-enriched lung epithelial cells isolated from mouse lungs. After 4 h of incubation of isolated Clara cells with ΔN23-KGF *in vitro*, the level of mRNA expression of CYP2F2 was significantly decreased (Fig. 6). Because NA resulted in a significant loss of Clara cells 12 h after injection, normalization of CYP2F2 to GAPDH mRNA expression would bear the problem that a reduction in CYP2F2 might simply reflect the loss of Clara cells. Therefore, mRNA expression of CYP2F2 was normalized to the mRNA expression level of CC10 instead of GAPDH. Because treatment with ΔN23-KGF alone reduces CC10 expression, this procedure is likely to result in an underestimation of the effect of ΔN23-KGF on CYP2F2 rather than in an overestimation.

Pre-treatment with ΔN23-KGF prevents NA-induced Impairment of Lung Function

To assess the functional relevance of NA-induced acute airway injury and of the effect of ΔN23-KGF, airflow was investigated by means of head-out body plethysmography. At 33 h following administration, ΔN23-KGF or PBS alone had no significant effect on mid-expiratory flow (EF_{50}) as compared to EF_{50} values recorded immediately prior to pre-treatment (0 h), which were 0.74 ± 0.18 ml/s in PBS treated mice and 0.78 ± 0.43 ml/s in mice treated with ΔN23-KGF. However, 12 h after injection of NA there was a significant reduction in EF_{50} recorded from mice pre-treated with

PBS, whereas EF_{50} values did not change in mice pre-treated with ΔN23-KGF (Fig. 7).

Therapeutic effect of ΔN23-KGF after NA-induced Airway Epithelial Injury depends on the Presence of Clara Cells

To test the hypothesis that the protective effect of ΔN23-KGF is primarily related to Clara cells, treatment with ΔN23-KGF was performed at 2 hours after NA-injection, when Clara cells are already injured but still present, or at 24 hours after NA-injection, a time-point at which Clara cells are almost completely lost from the distal airway epithelium (Fig. E1). In mice treated with ΔN23-KGF 2 hours after NA-injection, Clara cell numbers were significantly higher than in NA-injected mice and only slightly but insignificantly lower than in control mice injected with corn oil (Fig. 8). In contrast, mice receiving ΔN23-KGF 24 hours after NA-injection, Clara cell numbers were similar to mice injected with NA and significantly lower than in corn oil-injected control mice.

DISCUSSION

Keratinocyte growth factor (KGF) is a potent mitogen for epithelial cells and has been demonstrated to protect the lung against acute injuries of the pulmonary parenchyma in various animal models [14]. However, its potential to protect against airway epithelial injury has not been well characterized. Therefore, the aim of this study was to investigate whether pre-treatment with N-terminally truncated recombinant human KGF (ΔN23-KGF, palifermin), which results in enhanced KGF

protein stability [25], protects the airway epithelium against naphthalene-induced acute airway injury *in vivo*. We used three experimental approaches to address this question. These approaches defined different aspects of the cell biology of the early injury process, the effect of treatment on pulmonary function and the effect of KGF treatment on the biology of the airway epithelium. We found that ΔN23-KGF prevented the NA-induced loss of cell membrane integrity, increased airway epithelial cell proliferation, and largely reduced the loss of Clara cells as well as the impairment of lung function due to NA-induced acute airway injury. The decrease in mRNA expression of CYP2F2 in microdissected distal airways as well as in isolated Clara cells indicates that ΔN23-KGF exerted its protective effects at least in part by the downregulation of this NA-metabolizing xenobiotic enzyme. The failure of ΔN23-KGF to ameliorate NA-induced injury when applied 24 hours after NA-injection, i.e. at a time-point when Clara cells are almost completely lost from distal airway epithelium, demonstrates that the protective effect of ΔN23-KGF is primarily related to Clara cells.

The NA-model of airway epithelial cell injury has been well established in the mouse [13, 35]. The high susceptibility to NA-cytotoxicity revealed to be associated with the expression of CYP2F2 in Clara cells of distal airway epithelium of the mouse [12]. Whereas the cytotoxic effect of NA was extensively investigated in Swiss Webster [9, 36] and FVB mice [37-40], little was known about NA-induced injury to the distal airway epithelium of C57BL/6 mice [41, 42], which was particularly true for the early time course. In order to define the optimal time-point for the analysis of potential protective effects of ΔN23-KGF against NA-induced Clara cell injury, we first investigated the kinetics of NA-induced effects on distal airway epithelium. The sequence of events in C57BL/6 mice following injection of 200 mg NA per kg body

weight was similar to that seen in Swiss Webster mice. By 3 h after NA-injection Clara cells exhibited slight swelling and onset of vacuolization with exfoliation being almost complete by 24 h, which is in line with previous findings in C57BL/6 mice [41, 42]. After 12 h, most of the Clara cells were swollen and contained vacuoles, and only half of the Clara cells were lost by exfoliation. Therefore, this time-point was chosen to reveal the potential protective effects of ΔN23-KGF on NA-induced Clara cell injury.

Previous studies have adapted ethidium homodimer-1 (EH-1) for use on microdissected airways and demonstrated EH-1 to be a reliable probe for cells undergoing necrosis [43]. Intraperitoneal injection of NA resulted in a marked increase of EH-1 permeable Clara cells by 6 h post injection in Swiss Webster mice [29], and at 12 h post injection in C57BL/6 mice (this study) indicating that NA-induced exfoliation of Clara cells was due to necrosis. Pre-treatment of C57BL/6 mice with ΔN23-KGF largely abolished the incorporation of EH-1 into Clara cell nuclei, which suggests that ΔN23-KGF protected Clara cells of distal airways against NA-induced necrotic cell death. As a consequence, in mice pre-treated with ΔN23-KGF only 23% of the Clara cells were lost at 12 h after NA-injection, whereas 50% of the Clara cells were lost in mice pre-treated with control vehicle as demonstrated by design-based quantitative morphology [30]. Similarly, the mRNA expression of Clara cell-specific protein CC10 was reduced after NA-injection in mice pre-treated with PBS to a level of less than 5% of control mice, whereas in mice pre-treated with ΔN23-KGF expression of CC10 mRNA was 38% of the level recorded in controls treated with ΔN23-KGF only. The reduction in CC10 mRNA expression in KGF-treated animals is probably a consequence of the decrease of CC10 transcripts per

cell, which was demonstrated by *in situ* hybridization studies [34], rather than being a result of Clara cell exfoliation. The marked loss of Clara cells after NA-injection is likely to affect the barrier function of the airway epithelium and KGF can be suspected to maintain airway epithelial integrity, which is essential e.g., for lung fluid balance, host defense, and clearance or metabolization of inhaled agents [10]. This is in line with *in vitro* studies that demonstrated KGF to protect monolayers of bronchial epithelial cells against a loss of barrier function induced by hydrogen peroxide [19], or irradiation [18].

Head-out bodyplethysmography was used to assess the effects of airborne chemicals on respiratory function [44]. This study demonstrates for the first time that acute airway epithelial damage caused by NA was associated with a significant airway dysfunction indicating that the highly specific injury to Clara cells resulted in a significant functional impairment at the organ level. Whether this is the result of changes in airway permeability, inflammation, a direct effect of NA on airway smooth muscle or an indirect effect due to the alterations in the airway epithelium remains to be elucidated. Pre-treatment of mice with ΔN23-KGF 33 h prior to NA-induced airway injury almost completely prevented the impairment of midexpiratory airflow.

In previous studies, comparing non-invasive measurements of midexpiratory airflow (EF50) with invasive measurements of pulmonary conductance (GL) and dynamic compliance, very good agreements between EF50, GL and dynamic compliance values were observed [45, 46]. During airway constriction the main changes in the tidal flow signal, recorded by head-out body plethysmography, occur during the midexpiratory phase. Therefore, EF50 (ml/s) was defined as the tidal flow at the midpoint (50%) of expiratory tidal volume [46]. Consequently, EF50 is closely linked to tidal volume and expiration time. However,

EF50 was clearly demonstrated to be independent of breathing frequency [45]. Only a slight but insignificant decrease in mid-expiratory airflow was observed 33 h after instillation of ΔN23-KGF, whereas modest but significant changes in lung resistance and forced expiratory airflow were seen by invasive lung function analysis 3 days after treatment with KGF in rats [47]. These differences may relate to the fact that non-invasive assessment of lung function is less sensitive than invasive measurements of respiratory function parameters in anaesthetized and intubated animals [27], although we can not rule out that there may be quantitative differences of mice and rats in their response to ΔN23-KGF.

Clara cells are the preferred progenitor cell for regeneration of the distal airway epithelium following injury [9, 37, 38]. Transdifferentation of ciliated cells has been suggested to significantly contribute to epithelial repair following NA-induced injury [40]. Recently, however, Rawlins and co-workers suggested that a small population of ´variant´ Clara cells ($Clara^{V}$), which survive after NA injury [42], are most likely the progenitors for Clara cells after NA injury. Their data suggest that ciliated cells do not transdifferentiate into Clara cells. Human recombinant KGF was shown to stimulate DNA synthesis and proliferation of human bronchial epithelial cells *in vitro* and of rat and mouse airway epithelium *in vivo*. We have previously demonstrated that the KGF-induced stimulation of distal airway epithelial proliferation was restricted to Clara cells, and peaked between days 1 and 2 after intratracheal instillation of KGF into rat lungs *in vivo* [17]. In this study, we observed a similar strong induction of mouse Clara cell proliferation in response to exogenous ΔN23-KGF as evidenced both by immuno histochemistry for PCNA protein and quantitative real time RT-PCR for PCNA mRNA expression. Consequently, Clara cells numbers were increased by approximately 20% in mice at 33 h after treatment with ΔN23-KGF

as compared with control treated mice. At 12 h after NA-treatment, we did not observe significant proliferation of airway epithelium in C57BL/6 mice treated with NA alone. However, proliferation of distal airway epithelium was demonstrated at later time-points and was shown to peak at day 2 after NA-injection into Swiss Webster mice [9, 37]. Notably, the induction of this endogenous repair process was recently suggested to be related to the up-regulation of airway epithelial expression of KGF receptor at day 1 after NA-induced injury [48].

In rats, Northern blot analysis of whole lung homogenate revealed that administration of KGF *in vivo* lead to a significant decrease in CC10 mRNA expression on days 2 and 3 when compared with vehicle treated control lungs, and then gradually recovered to normal values [34]. In our study, treatment with ΔN23-KGF resulted in a slight albeit insignificant decrease in CC10 mRNA expression in distal airways of C57BL/6 mice, which might relate to the earlier time-point examined in our study. In rats, the decrease in mRNA for CC10 appeared to be due to a decreased expression per cell as assessed by *in situ* hybridization [34]. Correspondingly, CC10 protein expression as assessed by means of quantitative immuno histochemistry exhibited decreased immuno reactivity per cell in distal rat airway epithelium [17]. In response to intratracheal administration of exogenous KGF *in vivo*, a quantitative shift from Clara cells exhibiting normal strong staining for CC10 to Clara cells exhibiting weak immuno reactivity for CC10 was observed. The findings of these studies suggested that the reduction in CC10 mRNA and protein expression was not the result of a decrease in the number of CC10-expressing Clara cells, but reflected a decreased expression per cell. This is supported by our findings that Clara cell numbers were increased by 20%, whereas CC10 mRNA expression was concomitantly decreased

down to approximately 60% in mice treated with ΔN23-KGF compared with vehicle controls.

The species-specific and region-selective cytotoxicity of NA has been directly linked to its metabolism to naphthalene 1R,2S-oxide, which is catalyzed by the xenobiotic enzyme CYP2F2 [12]. In adult mice, expression of CYP2F2 protein appeared to be strictly limited to non-ciliated airway epithelial cells [11]. Because both ΔN23-KGF and NA had significant effects on the amount of Clara cells per distal airway, as shown above, we had to take into account that a decrease in Clara cell numbers per airway could simulate a decrease in CYP2F2 mRNA expression when related to GAPDH. Therefore, we related the expression of CYP2F2 mRNA to the expression of CC10, which is highly specific for Clara cells [49]. Despite the slight reduction of CC10 mRNA expression in lungs pre-treated with ΔN23-KGF, ΔN23-KGF reduced the mRNA expression of CYP2F2 to at least 25% of the expression level of vehicle controls. Correspondingly, our *in vitro* study of Clara cell-enriched lung epithelial cells isolated from C57BL/6 mice revealed a significant reduction of CYP2F2 mRNA expression in cells cultured for 4 h in the presence of ΔN23-KGF. Notably, a population of CC10-positive variant Clara cells, which are maintained within neuroepithelial bodies, was demonstrated to be resistant to NA-induced injury due to the low expression level of CYP2F2 [39]. This sub-population of Clara cells with low CYP2F2 expression was suggested to be critical for the renewal of proximal airway epithelium after NA-induced injury [50]. A similar NA-resistant Clara cell sub-population was located to the bronchiolar-alveolar duct junction and exhibited a number of features characteristic of region-specific endogenous stem cells [39]. These data suggest that a reduction in the expression of CYP2F2 mediated by ΔN23-KGF pre-treatment is able to significantly limit the Clara cell cytotoxicity of NA.

The failure of ΔN23-KGF to ameliorate NA-induced injury when applied 24 hours after NA-injection, i.e. at a time-point when Clara cells are almost completely lost from distal airway epithelium [41, 42], whereas at 2 hours after NA-injection Clara cell loss was significantly reduced by treatment with ΔN23-KGF, demonstrates that the protective effect of ΔN23-KGF is primarily related to Clara cells. This is in line with previous findings that the proliferative effect of KGF is restricted to Clara cells in rat distal airway epithelium [17].

As was reviewed recently, NA is used as feedstock in various chemical industrial processes and was suggested to pose a problem in the workplaces of various industries [6, 7]. Hence, it is conceivable that therapeutic application of ΔN23-KGF immediately after an accidential occupational exposure to high levels of NA may help to limit the development of severe airway epithelial injury although additional studies in humans are required before such an approach can be translated into clinics.

In summary, we have demonstrated that pre-treatment of lungs with ΔN23-KGF significantly reduced the NA-induced loss of Clara cells by necrosis and prevented the deterioration of respiratory function associated with airway injury. Our findings *in vivo* and *in vitro* suggest that the protective effect of ΔN23-KGF is mediated at least in part by the marked down-regulation of the expression of CYP2F2, the xenobiotic enzyme responsible for the metabolization of NA into its cytotoxic intermediate. The success of a therapeutic application of ΔN23-KGF depends on the presence of Clara cells.

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REFERENCES

1. Puchelle E, Zahm JM, Tournier JM, Coraux C. Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006; 3: 726-733.

2. Gauderman WJ, Avol E, Gilliland F, Vora H, Thomas D, Berhane K, McConnell R, Kuenzli N, Lurmann F, Rappaport E, Margolis H, Bates D, Peters J. The effect of air pollution on lung development from 10 to 18 years of age. *N Engl J Med* 2004; 351: 1057-1067.

3. Leikauf GD. Hazardous air pollutants and asthma. *Environ Health Perspect* 2002; 110: 505-526.

4. Plopper CG, Hyde DM. Epithelial cells of bronchioles. *In*: Parent RA, ed. Comparative Biology of the Normal Lung. Boca Raton, CRC Press, 1992; pp. 85-92.

5. Plopper CG, Van Winkle LS, Fanucchi MV, Malburg SR, Nishio SJ, Chang A, Buckpitt AR. Early events in naphthalene-induced acute Clara cell toxicity. II. Comparison of glutathione depletion and histopathology by airway location. *Am J Respir Cell Mol Biol* 2001; 24: 272-81.

6. Preuss R, Angerer J, Drexler H. Naphthalene--an environmental and occupational toxicant. *Int Arch Occup Environ Health* 2003; 76: 556-576.

7. Preuss R, Drexler H, Bottcher M, Wilhelm M, Bruning T, Angerer J. Current external and internal exposure to naphthalene of workers occupationally exposed to polycyclic aromatic hydrocarbons in different industries. *Int Arch Occup Environ Health* 2005; 78: 355-362.

8. Witschi H, Espiritu I, Maronpot RR, Pinkerton KE, Jones AD. The carcinogenic potential of the gas phase of environmental tobacco smoke. *Carcinogenesis* 1997; 18: 2035-2042.

9. Van Winkle LS, Buckpitt AR, Nishio SJ, Isaac JM, Plopper CG. Cellular response in naphthalene-induced Clara cell injury and bronchiolar epithelial repair in mice. *Am J Physiol* 1995; 269: 800-18.

10. Massaro GD, Singh G, Mason R, Plopper CG, Malkinson AM, Gail DB. Biology of the Clara cell. *Am J Physiol* 1994; 266: L101-L106.

11. Fanucchi MV, Murphy ME, Buckpitt AR, Philpot RM, Plopper CG. Pulmonary cytochrome P450 monooxygenase and Clara cell differentiation in mice. *Am J Respir Cell Mol Biol* 1997; 17: 302-314.

12. Shultz MA, Choudary PV, Buckpitt AR. Role of murine cytochrome P-450 2F2 in metabolic activation of naphthalene and metabolism of other xenobiotics. *J Pharmacol Exp Ther* 1999; 290: 281-288.

13. Plopper CG, Macklin J, Nishio SJ, Hyde DM, Buckpitt AR. Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. III. Morphometric comparison of changes in the epithelial populations of terminal bronchioles and lobar bronchi in mice, hamsters, and rats after parenteral administration of naphthalene. *Lab Invest* 1992; 67: 553-65.

14. Ware LB, Matthay MA. Keratinocyte and hepatocyte growth factors in the lung: roles in lung development, inflammation, and repair. *Am J Physiol Lung Cell Mol Physiol* 2002; 282: 924-940.

15. Finch PW, Cunha GR, Rubin JS, Wong J, Ron D. Pattern of keratinocyte growth factor and keratinocyte growth factor receptor expression during mouse fetal development suggests a role in mediating morphogenetic mesenchymal-epithelial interactions. *Dev Dyn* 1995; 203: 223-40.

16. Boardman KC, Aryal AM, Miller WM, Waters CM. Actin re-distribution in response to hydrogen peroxide in airway epithelial cells. *J Cell Physiol* 2004; 199: 57-66.

17. Fehrenbach H, Fehrenbach A, Pan T, Kasper M, Mason RJ. Keratinocyte growth factor-induced proliferation of rat airway epithelium is restricted to Clara cells in vivo. *Eur Respir J* 2002; 20: 1185-97.

18. Savla U, Waters CM. Barrier function of airway epithelium: effects of radiation and protection by keratinocyte growth factor. *Radiat Res* 1998; 150: 195-203.

19. Chapman KE, Waters CM, Miller WM. Continuous exposure of airway epithelial cells to hydrogen peroxide: protection by KGF. *J Cell Physiol* 2002; 192: 71-80.

20. Tillie-Leblond I, Gosset P, Le BR, Janin A, Prangere T, Tonnel AB, Guery BP. Keratinocyte growth factor improves alterations of lung permeability and bronchial epithelium in allergic rats. *Eur Respir J* 2007; 30: 31-39.

21. Waters CM, Savla U. Keratinocyte growth factor accelerates wound closure in airway epithelium during cyclic mechanical strain. *J Cell Physiol* 1999; 181: 424-32.

22. Gomperts BN, Belperio JA, Fishbein MC, Keane MP, Burdick MD, Strieter RM. Keratinocyte growth factor improves repair in the injured tracheal epithelium. *Am J Respir Cell Mol Biol* 2007; 37: 48-56.

23. Prince LS, Karp PH, Moninger TO, Welsh MJ. KGF alters gene expression in human airway epithelia: potential regulation of the inflammatory response. *Physiol Genomics* 2001; 6: 81-9.

24. Ding X, Kaminsky LS. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol* 2003; 43: 149-173.

25. Hsu E, Osslund T, Nybo R, Chen BL, Kenney WC, Morris CF, Arakawa T, Narhi LO. Enhanced stability of recombinant keratinocyte growth factor by mutagenesis. *Protein Eng Des Sel* 2006; 19: 147-153.

26. Van Winkle LS, Buckpitt AR, Plopper CG. Maintenance of differentiated murine Clara cells in microdissected airway cultures. *Am J Respir Cell Mol Biol* 1996; 14: 586-98.

27. Hoymann HG. Invasive and noninvasive lung function measurements in rodents. *J Pharmacol Toxicol Methods* 2007; 55: 16-26.

28. Ulrich K, Stern M, Goddard ME, Williams J, Zhu J, Dewar A, Painter HA, Jeffery PK, Gill DR, Hyde SC, Geddes DM, Takata M, Alton EW. Keratinocyte growth factor therapy in murine oleic acid-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2005; 288: L1179-L1192.

29. Van Winkle LS, Gunderson AD, Shimizu JA, Baker GL, Brown CD. Gender differences in naphthalene metabolism and naphthalene-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2002; 282: L1122-L1134.

30. Hyde DM, Harkema JR, Tyler NK, Plopper CG. Design-based sampling and quantitation of the respiratory airways. *Toxicol Pathol* 2006; 34: 286-295.

31. Elizur A, Adair-Kirk TL, Kelley DG, Griffin GL, deMello DE, Senior RM. TNF-{alpha} from macrophages enhances LPS-induced Clara cell expression of KC. *Am J Respir Cell Mol Biol* 2007; in press (doi:10.1165/rcmb.2007-0203OC).

32. Wegmann M, Fehrenbach A, Heimann S, Fehrenbach H, Renz H, Garn H, Herz U. NO2-induced airway inflammation is associated with progressive airflow limitation and development of emphysema-like lesions in C57BL/6 mice. *Exp Toxicol Pathol* 2005; 56: 341-350.

33. Klug J, Beier HM, Bernard A, Chilton BS, Fleming TP, Lehrer RI, Miele L, Pattabiraman N, Singh G. Uteroglobin/Clara cell 10-kDa family of proteins: nomenclature committee report. *Ann N Y Acad Sci* 2000; 923: 348-354.

34. Yano T, Mason RJ, Pan T, Deterding RR, Nielsen LD, Shannon JM. KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in adult rat lung. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L1146-L1158.

35. Plopper CG, Suverkropp C, Morin D, Nishio S, Buckpitt A. Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. I. Histopathologic comparison of the respiratory tract of mice, rats and hamsters after parenteral administration of naphthalene. *J Pharmacol Exp Ther* 1992; 261: 353-363.

36. Van Winkle LS, Isaac JM, Plopper CG. Repair of naphthalene-injured microdissected airways in vitro. *Am J Respir Cell Mol Biol* 1996; 15: 1-8.

37. Stripp BR, Maxson K, Mera R, Singh G. Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. *Am J Physiol* 1995; 269: L791-L799.

38. Reynolds SD, Giangreco A, Power JH, Stripp BR. Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. *Am J Pathol* 2000; 156: 269-78.

39. Giangreco A, Reynolds SD, Stripp BR. Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. *Am J Pathol* 2002; 161: 173-82.

40. Park KS, Wells JM, Zorn AM, Wert SE, Laubach VE, Fernandez LG, Whitsett JA. Transdifferentiation of ciliated cells during repair of the respiratory epithelium. *Am J Respir Cell Mol Biol* 2006; 34: 151-157.

41. Lawson GW, Van Winkle LS, Toskala E, Senior RM, Parks WC, Plopper CG. Mouse strain modulates the role of the ciliated cell in acute tracheobronchial airway injury-distal airways. *Am J Pathol* 2002; 160: 315-327.

42. Rawlins EL, Ostrowski LE, Randell SH, Hogan BL. Lung development and repair: contribution of the ciliated lineage. *Proc Natl Acad Sci U S A* 2007; 104: 410-417.

43. Postlethwait EM, Joad JP, Hyde DM, Schelegle ES, Bric JM, Weir AJ, Putney LF, Wong VJ, Velsor LW, Plopper CG. Three-dimensional mapping of ozone-induced acute cytotoxicity in tracheobronchial airways of isolated perfused rat lung. *Am J Respir Cell Mol Biol* 2000; 22: 191-9.

44. Vijayaraghavan R, Schaper M, Thompson R, Stock MF, Boylstein LA, Luo JE, Alarie Y. Computer assisted recognition and quantitation of the effects of airborne chemicals acting at different areas of the respiratory tract in mice. *Arch Toxicol* 1994; 68: 490-499.

45. Glaab T, Hoymann HG, Hohlfeld JM, Korolewitz R, Hecht M, Alarie Y, Tschernig T, Braun A, Krug N, Fabel H. Noninvasive measurement of midexpiratory flow indicates bronchoconstriction in allergic rats. *J Appl Physiol* 2002; 93: 1208-14.

46. Glaab T, Ziegert M, Baelder R, Korolewitz R, Braun A, Hohlfeld JM, Mitzner W, Krug N, Hoymann HG. Invasive versus noninvasive measurement of allergic and cholinergic airway responsiveness in mice. *Respir Res* 2005; 6: 139.

47. Hohlfeld JM, Hoymann HG, Tschernig T, Fehrenbach A, Krug N, Fehrenbach H. Keratinocyte growth factor transiently alters pulmonary function in rats. *J Appl Physiol* 2004; 96: 704-710.

48. Atkinson JJ, Toennies HM, Holmbeck K, Senior RM. Membrane type 1 matrix metalloproteinase is necessary for distal airway epithelial repair and keratinocyte growth factor receptor expression after acute injury. *Am J Physiol Lung Cell Mol Physiol* 2007; 293: L600-L610.

49. Singh G, Katyal SL. Clara cells and Clara cell 10 kD protein (CC10). *Am J Respir Cell Mol Biol* 1997; 17: 141-3.

50. Reynolds SD, Hong KU, Giangreco A, Mango GW, Guron C, Morimoto Y, Stripp BR. Conditional clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. *Am J Physiol Lung Cell Mol Physiol* 2000; 278: 1256-63.

FIGURE LEGENDS

FIGURE 1. Pre-treatment with ΔN23-KGF protects Clara cells against NA-induced Necrosis. Ethidium homodimer-1 staining (red) of distal airways of mice. Mice were treated with corn oil (A) or naphthalene (200 mg/kg) (B). Alternatively, ΔN23-KGF (10 mg/kg) was administrated prior to injection of corn oil (C) or naphthalene (200 mg/kg) (D). Representative images of four distal airways per mouse, n=3 mice per group. Scale bar 50μm.

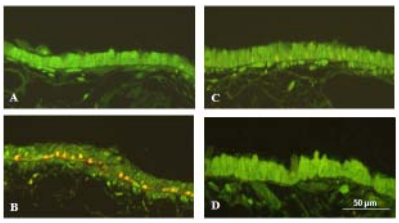

FIGURE 2. ΔN23-KGF induces PCNA Activation and Airway Epithelial Proliferation. Immuno histochemical staining for proliferating cell nuclear antigen (PCNA) in distal airways of mice treated with corn oil (A) or naphthalene (200 mg/kg) (B), and mice pre-treated with ΔN23-KGF prior to corn oil (C) or naphthalene (200 mg/kg) (D) injection. Representative images of four airways of n=3 mice per group. Scale bar 50μm. (E) Significantly increased PCNA mRNA expression levels (four distal airways per mouse, n=3 mice per group) were observed in mice pre-treated with ΔN23-KGF (KGF) versus PBS prior to injection of naphthalene (NA) or corn oil (CO). $^{**}P < 0.05$ s.

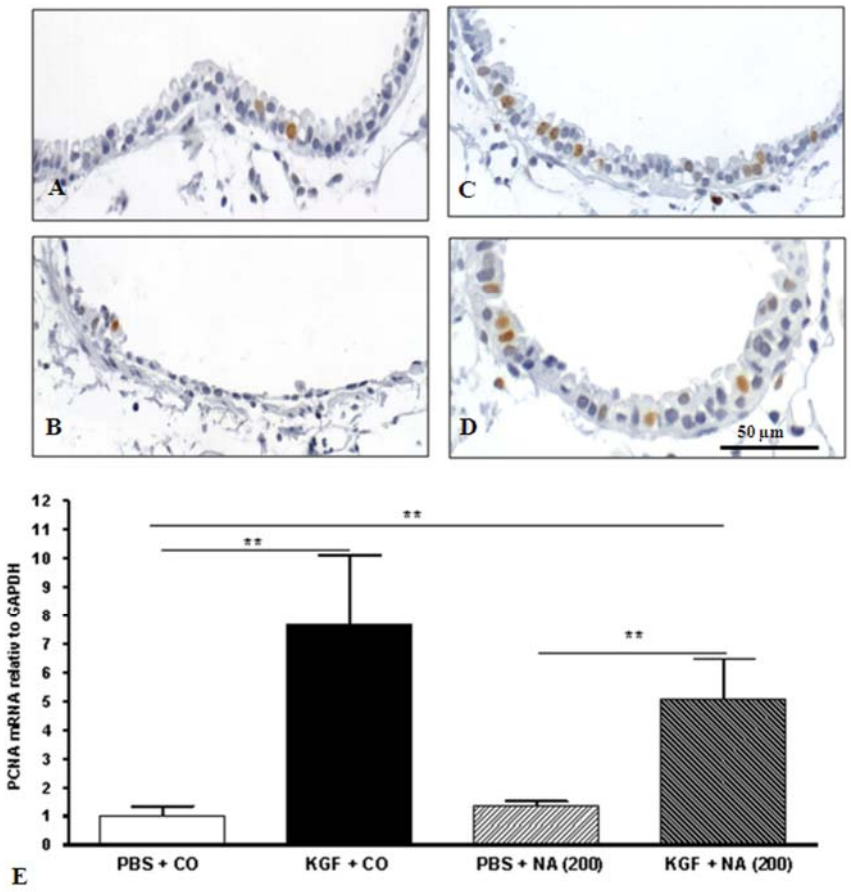

FIGURE 3. Pre-treatment with ΔN23-KGF inhibits Clara Cell Loss. Design-based stereological estimation of the number of CC10 positive Clara cells in distal airways obtained from mice (four distal airways per mouse, n=3 mice per group) pre-treated with ΔN23-KGF (KGF) or PBS prior to injection of naphthalene (NA) or corn oil (CO). ***P* < 0.05.

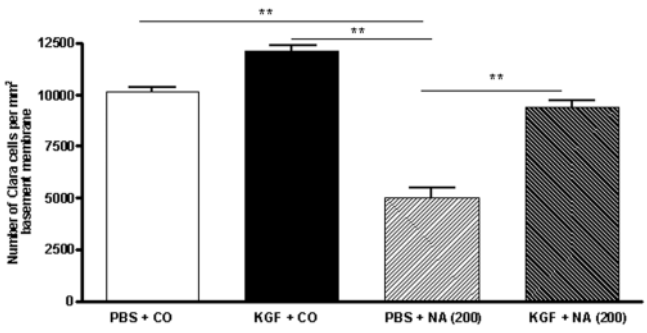

FIGURE 4. Quantitative real time RT-PCR analyzed with CC10 specific primer pairs. Total RNA was isolated (three distal airways per mouse, n=3 mice per groups) pre-treated with ΔN23-KGF (KGF) or PBS prior to injection of naphthalene (NA) or corn oil (CO). ***P* < 0.05.

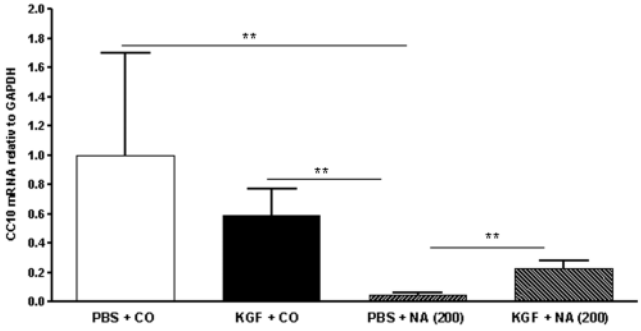

FIGURE 5. ΔN23-KGF downregulates Cytochrome P450 2F2 mRNA Expression in vivo. Quantitative real time RT-PCR analyzed with primer pairs specific for cytochrome P450 isozyme 2F2 (C2F2). Total RNA was isolated (three distal airways per mouse, n=3 mice per groups) pre-treated with ΔN23-KGF (KGF) or PBS prior to injection of naphthalene (NA) or corn oil (CO). mRNA expression of CYP2F2 was determined relative to mRNA expression of Clara cell specific protein, CC10. ***P* < 0.05.

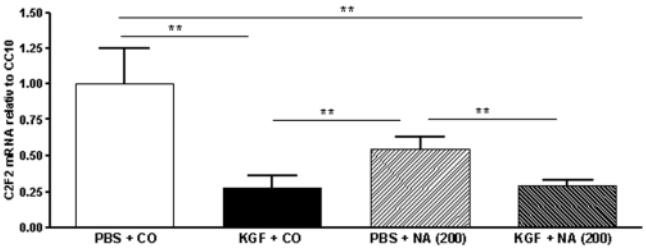

FIGURE 6. ΔN23-KGF downregulates Cytochrome P450 2F2 mRNA Expression in vitro. Quantitative real time RT-PCR analyzed with primer pairs specific for cytochrome P450 isozyme 2F2 (C2F2). Total RNA was obtained from Clara cell

enriched lung epithelial cells isolated from lungs of C57BL/6 mice (n=3). Isolated cells were harvested after 1h and 4h of incubation with cell culture medium which contained 50ng ΔN23-KGF per ml (KGF) or PBS. mRNA expression of CYP2F2 was determined relative to mRNA expression of Clara cell specific protein, CC10. $^{**}P < 0.05$.

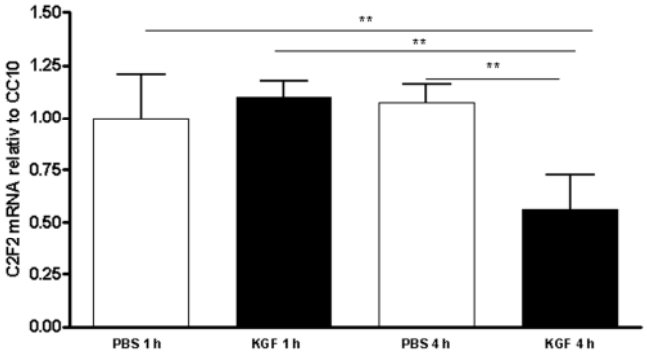

FIGURE 7. Pre-treatment with ΔN23-KGF prevents NA-induced Impairment of Lung Function. Non-invasive head-out body plethysmography of mice (n=6 per group) prior to (0 h) and 33 h after treatment with ΔN23-KGF (KGF) or PBS, and 12 h after injection of naphthalene (NA) following pre-treatment with ΔN23-KGF (KGF) or PBS. Data are given as percent change from the mean baseline level recorded at 0 h. $^{**}P < 0.05$.

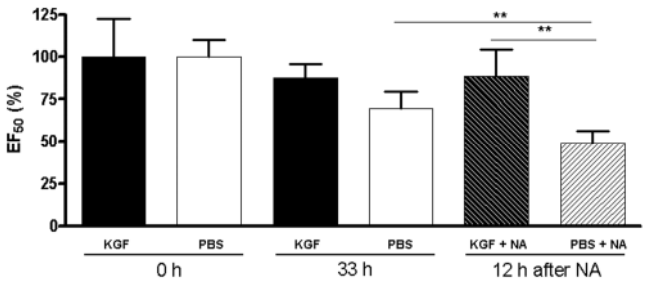

FIGURE 8. Therapeutic effect of ΔN23-KGF after NA-induced airway epithelial injury depends on the presence of Clara cells. Design-based stereological estimation of the number of CC10 positive Clara cells in distal airways obtained from mice (four distal

airways per mouse, n=3 mice per group) treated with PBS (PBS) or ΔN23-KGF (KGF) 2 hours (2h NA) or 24 hours (24h NA) after injection of naphthalene (NA) or corn oil (CO). Airways were obtained by microdissection at day 5 after injection of CO or NA. $^{**}P < 0.05$.