ERJ Express. Published on July 22, 2010 as doi: 10.1183/09031936.00142409

Dickkopf proteins influence lung epithelial cell proliferation in idiopathic pulmonary fibrosis

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Keywords: Dickkopf proteins, lung epithelial cells, pulmonary fibrosis, Wnt signaling

Abstract

Idiopathic pulmonary fibrosis (IPF) is a fatal interstitial lung disease with unknown pathogenesis. The WNT/β-catenin pathway has recently been reported to be operative in epithelial cells in IPF. Dickkopf (DKK) proteins are known to regulate WNT signaling via interaction with Kremen (KRM) receptors, yet their expression and role in the adult lung and in IPF has not been addressed.

We analyzed the expression, localization, and function of DKK and KRM proteins in IPF lungs using Western blotting, quantitative RT-PCR, immunohistochemistry, ELISA, and functional *in vitro* studies.

Enhanced expression of DKK1 and 4 and KRM1 was detected in lung homogenates of IPF patients compared with transplant donors. Immunohistochemistry revealed that DKK1 was predominantly localized in basal bronchial epithelial cells. Furthermore, prominent expression of all proteins was observed in hyperplastic alveolar epithelial cells in IPF. Quantitative measurement of DKK1 revealed enhanced protein expression in the bronchoalveolar lumen of IPF patients. Finally, functional studies using human bronchial and alveolar epithelial cell lines demonstrated that WNT-induced epithelial cell proliferation is regulated by DKK1 in a dose-dependent fashion.

In sum, DKK proteins are expressed in the lung epithelium in IPF. DKK proteins influence epithelial cell proliferation and may therefore be suitable therapeutic targets for IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal interstitial lung disease with unknown pathogenesis and limited responsiveness to current therapies [1-3]. It is the most common form of idiopathic interstitial pneumonias, which are characterized by destruction of lung architecture and loss of respiratory function [1, 4, 5]. The histological pattern of IPF is usual interstitial pneumonia (UIP) [6, 7], and aggregates of activated myofibroblasts, so-called fibroblast foci, are hallmark lesions of IPF/UIP. It has been proposed that repetitive alveolar injury leads to initial alveolar epithelial cell death, subsequent hyperplasia, and aberrant activation of the alveolar epithelium [8, 9]. The subepithelial localization of fibroblast foci in these areas suggest that impaired epithelial-mesenchymal crosstalk contributes to the pathobiology of IPF [8, 10, 11].

The WNT family of proteins is highly conserved secreted growth factors and known to control key events during lung development [12, 13]. WNT signaling is regulated via binding of extracellular WNT ligands to receptors of the Frizzled (FZD) family or low density lipoprotein receptor-related proteins (LRP). The best characterized WNT signaling pathway is the β-catenin-dependent, or canonical, WNT signaling pathway: In unstimulated cells, β-catenin, the main signaling intermediate of canonical WNT signaling, is bound to the scaffold proteins Axin and adenomatosis polyposis coli (APC), and constitutively phosphorylated by its interaction with casein kinase I (CKI) and glycogen synthase kinase (GSK)-3β, and degraded. Upon WNT stimulation, the LRP6 receptor gets phosphorylated, which leads to the recruitment of Dishevelled proteins and Axin, thereby preventing phosphorylation of β-catenin. As a result, hypophosphorylated β-catenin accumulates in the cytoplasm, translocates to the nucleus, interacts with the T-cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors, and regulates target gene expression. The reactivation of this pathway has been reported in several different diseases,

mainly cancer [13]. Importantly, recent studies have linked increased WNT/β-catenin signaling to impaired epithelial function in the pathogenesis of IPF [4, 14-17].

The WNT/β-catenin pathway is tightly controlled in a spatio-temporal manner. WNT regulators, such as proteins of the Dickkopf (DKK) family, are expressed in response to active WNT/β-catenin signaling. Four different DKK proteins (DKK1 - 4) have been discovered, sharing conserved cysteine-rich domains. DKK proteins bind to the LRP receptors and bind also to a second class of transmembrane receptors, called Kremen (KRM) [18], which potentiate the ability of DKK to regulate WNT signaling [19-21].

In this respect, we hypothesized that the WNT regulators DKK and KRM are differentially expressed in IPF, possibly affecting impaired epithelial injury and repair processes.

Material and Methods

Human lung tissue. Lung tissue biopsies were obtained from 15 IPF patients with histological usual interstitial pneumonia (UIP) pattern (4 females, 11 males; mean age = 58 ± 8 years; mean VC = 48% ± 7%; Mean TLC = 50% ± 5%; mean DL_{CO}/VA = 23% ± 3%; O_2 = 2-4 l/min; Pa_{O2} = 49-71 mmHg, Pa_{CO2} = 33-65 mmHg) and 9 control subjects (organ donors; 4 females, 5 males; mean age 42 ± 10 years). Individual patient characteristics have been described before [4]. Samples were immediately snap frozen or placed in 4% (w/v) paraformaldehyde after explantation. The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93). Informed consent was obtained in written form from each subject for the study protocol.

Human bronchial lavage fluids. Patients were recruited at the Department of Medicine of the Justus-Liebig-University in Giessen, Germany in 2006 and 2007. The study protocol was approved by the local ethics committee, and informed consent was obtained from the patients. Flexible fiberoptic bronchoscopy was performed in patients and controls by one physician in a standardized manner as previously described [22]. Individual patient characteristics are shown in table 1. The control group consisted of 4 spontaneously breathing healthy non-smoking volunteers, with normal pulmonary function, clinical blood tests without pathological findings, and without any history of cardiac or lung disease (medical students from the Medical School of the Justus-Liebig University Giessen, Germany).

Reverse transcription and quantitative RT-PCR. RNA extraction and quantitative (q)RT-PCR was performed using fluorogenic SYBR Green and the Sequence Detection System Fast 7500 (PE Applied Biosystems), as previously described [4]. HPRT1, an ubiquitously and equally expressed gene free of pseudogenes, was used as a reference gene in all human qRT-PCR reactions. PCR primers are listed in table 2. Relative transcript abundance of a gene is

expressed in ΔCt values ($\Delta Ct = Ct^{reference} - Ct^{target}$). Relative changes in transcript levels compared to donors are ΔΔCt values ($\Delta\Delta Ct = \Delta Ct^{IPF} - \Delta Ct^{donor}$). All ΔΔCt values correspond approximately to the binary logarithm of the fold change (log-fold change) as mentioned in the text. When relative transcript abundance is of information, expression levels are given in ΔCt levels.

Western blot analysis. Human lung tissue was homogenized in extraction buffer and whole proteins were extracted by centrifugation (12.000 × g) for 10 min at 4°C, as described previously [4]. The following antibodies were used: DKK1 (sc-25516, Santa Cruz Biotechnology, Santa Cruz, CA), DKK2 and DKK4 (ab38594 and ab38589, both from Abcam, Cambridge, UK), KRM1 (AF2127, R&D Systems, Minneapolis, MN), KRM2 (HP A003223, Sigma-Aldrich, Saint Louis, MO). Densitometric analysis of autoradiographies was performed using a GS-800TM Calibrated Densitometer and the 1-D analysis software Quantity One (both from Bio-Rad Laboratories, Hercules, CA). Changes in expression levels are expressed as fold-change (mean ± sem).

Immunohistochemistry. Human lungs were placed in 4% (w/v) PFA after explantation, and processed for paraffin embedding. Sections (3 μm) were cut, mounted on slides, subjected to antigen retrieval, and quenching of endogenous peroxidase activity using 3 % (v/v) H_2O_2 for 20 min. The following antibodies were used: DKK1 and DKK4 (sc-25516 and sc-25519, Santa Cruz Biotechnology), DKK2 (ab38594, Abcam), KRM1 (AF2127, R&D Systems), KRM2 (HP A003223, Sigma-Aldrich). Immune complexes were visualized using peroxidase-coupled secondary antibodies, according to the manufacturer's protocol (Histostain *Plus* Kit; Invitrogen).

ELISA. A human DKK1 enzyme-linked immunosorbent assay (DY1906, R&D Systems) was performed on BALF following the manufacturers instructions. 100µl of BALF was used, each sample was measured twice. A seven point standard curve with a high standard of 4000pg/ml and 2-fold serial dilutions to a low standard (75 pg/ml) was performed at the same plate, two measurements for each dilution. Double values of samples and standards were averaged.

Cell culture. The human bronchial epithelial cell line BEAS-2B (European Collection of Cell Cultures (ECACC)) was maintained in LHC-9 medium (Invitrogen). The human lung epithelial cell line A549 (ATCC CCL-185; American Type Culture Collection, Manassas, VA, USA) was maintained in DMEM (GIBCO/Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, from PAA Laboratories, Pasching, Austria). Cells were plated in 24-well plates, serumstarved for 20 h in 0.1% FCS medium. Stimulation for 24 h was done with recombinant WNT3a or DKK1 (both from R&D Systems), as indicated. Cell counting was performed using a hemacytometer according to standard protocols.

Statistical analysis. All ΔCt values obtained from real-time RT-PCR were analyzed for normal distribution using the Shapiro-Wilk test, using assignment of a normal distribution with $p > 0.05$. Normality of data was confirmed using quantile-quantile plots. The means of indicated groups were compared using two-tailed Student's *t*-test, or a one-way analysis of variance (ANOVA) with Tukey HSD post hoc test for studies with more than 2 groups. Results were considered statistically significant when $p < 0.05$.

Results

Initially, we quantified the mRNA expression of Dickkopf (DKK) 1 – 4 and Kremen (KRM) 1 and 2 in homogenized lung tissue specimen of transplant donors and IPF patients (n = 10 each) using quantitative (q)RT-PCR. As demonstrated in figure 1a, all Dickkopf proteins were expressed in donor and IPF lungs, but exhibited variable basal expression levels. DKK3 was highly expressed in both, donor and IPF tissue, whereas DKK4 showed the lowest mRNA level in both conditions. DKK1 and DKK4 presented significantly increased mRNA expression in fibrotic tissue (log-fold change (mean ± sem): 0.85 ± 0.29 and 2.09 ± 0.70, respectively), while DKK2 mRNA was decreased (log-fold change: -0.94 ± 0.27). The receptors KRM1 and KRM2 were also expressed in lung tissue, with lower expression of KRM2 compared with KRM1 in donor and IPF tissue. In IPF, enhanced KRM1 mRNA levels were detected (log-fold change: 1.31 ± 0.21) (figure 1b).

Next, we analyzed the protein expression pattern of Dickkopf (DKK) and Kremen (KRM) proteins in lung homogenates of donors and IPF patients (n = 5 each). As depicted in figure 2, Western blotting of DKK proteins showed enhanced levels of all investigated DKK proteins in IPF. Quantification of immunoblots demonstrated significantly increased intensity for DKK1 and DKK4 (figure 2a and b) (increases in optical density *versus* β-actin, DKK1: 0.32 ± 0.06 and DKK4: 0.62 ± 0.08), which is in accordance to elevated transcript levels depicted in figure 1. Protein expression of DKK2 was also significantly enhanced (0.52 ± 0.1) in IPF compared with lung tissue from transplant donors, however, transcript levels were decreased in IPF lung specimen (figure 1a). Both receptors KRM1 and 2 were expressed in the lung (figure 2c), with significantly increased expression of KRM1 (0.45 ± 0.04) (figure 2d).

We then sought to identify the cells capable of expressing DKK ligands and KRM receptors. Therefore, we performed immunohistochemical stainings on IPF and donor lung sections. As demonstrated in figure 3, DKK1 was mainly localized in bronchial epithelial

cells in donor (figure 3a, upper panel) and IPF lungs (figure 3a, lower panel). Interestingly, we observed a pronounced and distinct accumulation of DKK1 in basal bronchial epithelial cells (figure 3a, arrows). In IPF lungs, DKK1 was particularly localized in hyperplastic alveolar epithelial cells (figure 3b, lower panel, arrows). In addition, granulocytes (figure 3b, upper panel) presented staining of DKK1 protein. DKK4 protein expression was largely localized to bronchial epithelial cells and interstitial cells, in donor as well as IPF tissues. Of note, DKK4 expression exhibited an equal basal-apical intensity in bronchial epithelial cells (figure 4a, upper and lower panel) in donor and IPF tissue. As depicted in figure 4b, DKK4 was strongly expressed in hyperplastic alveolar epithelial cells and areas of bronchiolization in IPF (figure 4b, lower panel, arrows).

We went on to localize the expression of the DKK-binding receptors KRM1 and KRM2. KRM1 protein exhibited expression in bronchial epithelial (figure 5a), smooth muscle cells (figure 5a, lower panel, arrows) and endothelial cells (figure 5a, upper panel) in donor lung tissue. In IPF, a heterogeneous staining of the bronchial epithelium (figure 5a, lower panel) and in hyperplastic alveolar cell regions was dominant (figure 5b, lower panel, arrows). KRM1 was also detected in alveolar macrophages in donor lung tissue (figure 5a and 5b, **upper panels). Similarly, scattered protein expression of KRM2 was localized to bronchial epithelial (figure 6a) in donor and IPF lungs. In IPF, hyperplastic alveolar epithelial cells expressed KRM2 (figure 6b, lower panel, arrow).**

Taken together, these results demonstrated increased expression of the DKK ligands and their receptors in IPF. All proteins largely localized to the lung epithelium, suggesting that epithelial cells respond to secreted DKK ligands in an autocrine fashion. To further elucidate this, we next determined the DKK1 concentration in the bronchial lumen. DKK1 protein was quantified in bronchoalveolar lavage fluids (BALF) of healthy volunteers (n = 4), patients with chronic bronchitis (n = 3), or IPF (n = 9) using an enzyme-linked immunosorbent assay (ELISA). As depicted in figure 7, DKK1 was expressed in all

investigated samples, with a significantly increased amount of DKK1 in BALF of IPF patients (mean ± sem: 456 ± 44 pg/ml), compared with healthy controls (266 ± 8 pg/ml), or patients with chronic bronchitis (223 ± 34 pg/ml).

Finally, we wanted to explore the effects of DKK1 on lung epithelial cell function. To this end, we stimulated the human bronchial epithelial cell line BEAS-2B or the human lung epithelial cell line A549 with recombinant WNT3a, DKK1, or a combination thereof, and analyzed the effects on epithelial cell proliferation. As presented in figure 8a, stimulation of BEAS-2B with WNT3a induced a significant increase in cell proliferation compared with controls (relative proliferation (mean ± sem: 1.39 ± 0.06)). Interestingly, low concentrations of DKK1 (100 ng/ml) alone also led to a significant increase in bronchial epithelial cell proliferation and failed to inhibit WNT3a-induced effects significantly (1.6 ± 0.14 versus 1.41 ±0.13, respectively). Higher concentrations of DKK1 led to a reduction of WNT3a-induced effects, while no significant effect on cell proliferation was observed after stimulation with DKK1 alone. Similarly to bronchial epithelial cells, WNT3a increased the proliferative capacity of alveolar epithelial cells (figure 8b) (relative proliferation (mean ± sem: 1.42 ± 0.08)), which was attenuated by high concentrations of DKK1 (DKK1 500ng/ml: 1.07 ± 0.05 and DKK1 1000ng/ml: 1.11 ± 0.08). The effect of lower concentration of DKK1, however, was different, as DKK1 treatment alone did not lead to an increase in alveolar epithelial cells proliferation.

Discussion

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal lung disease with limited responsiveness to current therapies [2, 3]. The molecular mechanisms involved in IPF are still poorly understood. The WNT/β-catenin pathway, known to be critical during lung morphogenesis and associated with the development of lung carcinoma [13, 23], has recently been demonstrated to be expressed and active in IPF, modulating epithelial cell injury and repair [4, 14, 15]. Here, we performed a comprehensive analysis of the expression and localization of the WNT modulators Dickkopf (DKK) and their Kremen (KRM) receptors demonstrating that both DKK and KRM proteins are enhanced in lung tissue specimen of donors and IPF patients. Importantly, the expression analysis was performed in lung homogenate samples, which implies that the expression profiles are subject to the cellular composition of the samples used. Immunohistochemical analysis revealed that DKK and KRM proteins largely localize to lung epithelial cells. Of note, DKK1 exhibited strong expression in basal bronchial and hyperplastic alveolar epithelial cells in IPF. Analysis of bronchoalveolar lavage fluids (BALF) revealed increased DKK1 expression in the bronchoalveolar lumen in IPF. Furthermore, *in vitro* studies demonstrated that DKK1 alter WNT-induced epithelial cell proliferation in a dose-dependent fashion.

The WNT signaling system is tightly controlled by different secreted WNT regulators, such as the secreted frizzled receptor (sFRP) or Dickkopf (DKK) proteins [21, 24]. Both protein families use a fundamentally different mechanism to modulate WNT signaling: While sFRP bind directly to WNT ligands and inhibit their interaction with the membrane receptors frizzled (FZD) or low density lipoprotein receptor-related proteins (LRP), DKK modulate the WNT/β-catenin pathway by binding directly to LRP receptors and KRM receptors. The formation of a ternary complex of DKK, KRM, and LRP6 is thought to lead to the internalization of the whole complex from the cell surface, thereby inhibiting WNT signaling [21].

The potential of sFRP to modulate organ fibrosis has been demonstrated in the kidney *in vivo* and *in vitro* [25, 26], and sFRPs have been reported to be differentially expressed in pulmonary fibrosis [15]. With respect to the DKK family, most studies focused on DKK1 thus far. Inhibition of WNT/β-catenin signaling by DKK1 has been demonstrated in mouse lung organ cultures *in vivo* [27], however, the potential of DKK1 to modulate a fibrotic response via inhibition of WNT signaling has been demonstrated only in hepatic stellate cells [28], as well as in irradiated fibroblasts *in vitro* [29]. Recently, DKK1 has also been implicated in the development of rheumatoid arthritis [30]. Here, we report for the first time that proteins from the DKK family are differentially regulated in IPF.

DKK1 is the founding member of the DKK family and originally identified as embryonic head inducer and WNT inhibitor in *Xenopus* [21]. In contrast, DKK2 has been described to act as a WNT antagonist as well as a WNT agonist, depending on the cellular context and the availability of WNT- and co-receptors [21]. In addition, DKK1 is known to be a direct target gene upregulated after WNT stimulation [31], whereas for DKK2 this has not been demonstrated yet. Our study also suggests that the transcriptional (feedback) control or protein stability due to posttranslational processing may differ between DKK proteins.

The availability of DKK receptors in the lung is a basic requirement for secreted DKK proteins to exert their effects on WNT/β-catenin signaling. Expression of LRP5 and 6 in lung homogenates of donors and IPF patients has been demonstrated in a recent study [4]. Here, we focused on the receptors KRM1 and 2. Immunohistochemical staining of KRM1 and KRM2 revealed that the bronchial and hyperplastic alveolar epithelium, in particular in areas of bronchiolization of IPF specimen, are major sources in donor and IPF lung tissue specimen, indicating autocrine effects on epithelial cells as a main signaling mechanism for the WNT/β-catenin pathway. The receptors KRM1 and 2, however, demonstrated a heterogeneous expression pattern in the epithelium, which highlights the importance of the microenvironment influencing WNT signaling *in vivo*. In addition, it has to be pointed out

that fibroblast have been recently reported to be capable of WNT signal transduction [4, 32], and also take part in the fibrotic process induced by WNT/β-catenin signaling.

Importantly, DKK1 concentrations were only increased in BALF of IPF patients compared with healthy volunteers, but not in patients with chronic bronchitis, suggesting that WNT/β-catenin activation and regulation does not primarily reflect an advanced inflammatory response.

Notably, we observed a distinct expression pattern for DKK1 with strong staining in basal bronchial epithelial cells in donor as well as in IPF lungs. Basal cells exhibit a proliferative activity and are thought to be important progenitors for the maintenance of the bronchial epithelium in general, and after lung injury in particular [33, 34]. In addition, basal bronchial epithelial cells are also known to be involved in the development of lung cancer. Squamous cell carcinoma accounts for 20% of all human lung cancers and basal cell metaplasia is a premalignant finding in the bronchial epithelium [35]. Importantly, bronchial epithelial cell metaplasia is also a common feature in IPF lung tissue specimen [36]. In addition, Chilosi *et al.* reported abnormal proliferation of bronchial epithelial cells in IPF, but not other interstitial pneumonias, such as acute or non-specific pneumonias [37]. It has been suggested that patients with bronchial epithelial cell metaplasia tend to develop lung carcinomas [38]. The WNT/β-catenin pathway has been implicated in epithelial proliferation and it has been demonstrated that primary bronchial epithelial cells exhibit the potential to response to WNT signaling [39].

Here, we analysed the proliferative capacity of bronchial and alveolar epithelial cells revealing that only high concentrations of DKK1 inhibited the WNT-induced proliferative effect. Notably, low concentrations of DKK1 alone led to increased bronchial cell proliferation, but not alveolar epithelial cell proliferation. These results allow the assumption that DKK proteins modulate bronchial epithelial cell maintenance, and may be involved in an increased bronchial cell metaplasia, possibly leading to an increased lung cancer

development. Further, our data suggest that DKK1, although expressed and secreted by the alveolar epithelium, is not able to fulfill an effective negative-feedback-loop on WNT-induced aberrant alveolar epithelial cell proliferation in IPF *in vivo*. The effects of DKK on WNT signaling crucially depend on the concentration of the respective WNT or DKK ligands, as well as on the sensitivity of the effector cells due to receptor availability. It has to be pointed out, however, that the mere use of cell lines is a significant limitation of the current study, the results of which need to be analyzed in more detail using primary alveolar and bronchial epithelial cells in future studies.

In sum, our study demonstrated altered expression of the WNT regulators DKK and KRM, which may be crucial for lung epithelial cell injury and repair mechanism in IPF, Further studies are needed to elucidate the effects of DKK proteins on different cell types to reveal the potential therapeutic capability in IPF.

Acknowledgements

We are grateful to Walter Klepetko and Robert Voswinckel for providing human lung tissues and Sabine Heinemann for excellent help with patient sampling.

Funding: The Clinical Research Group 118 “Lung Fibrosis”, European 6th Framework Programme (PULMOTENSION), and the Career Development Grant (“Anschubfinanzierung”) from the School of Medicine, Justus-Liebig-University Giessen, Germany.

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Figure legends

Figure 1. The mRNA expression of Dickkopf (DKK) and Kremen (KRM) proteins in IPF and donor tissue. The mRNA expression of DKK 1 - 4 (**a**) and the receptors KRM 1 and 2 (**b**) was analyzed in total lung homogenates from donor (open bars) and IPF lung specimen (black bars) by quantitative (q)RT-PCR. Results from 10 donors and 10 IPF patients are shown as relative mRNA expression compared to the reference gene (ΔCt), and presented as mean ± s.e.m., * = $p < 0.05$.

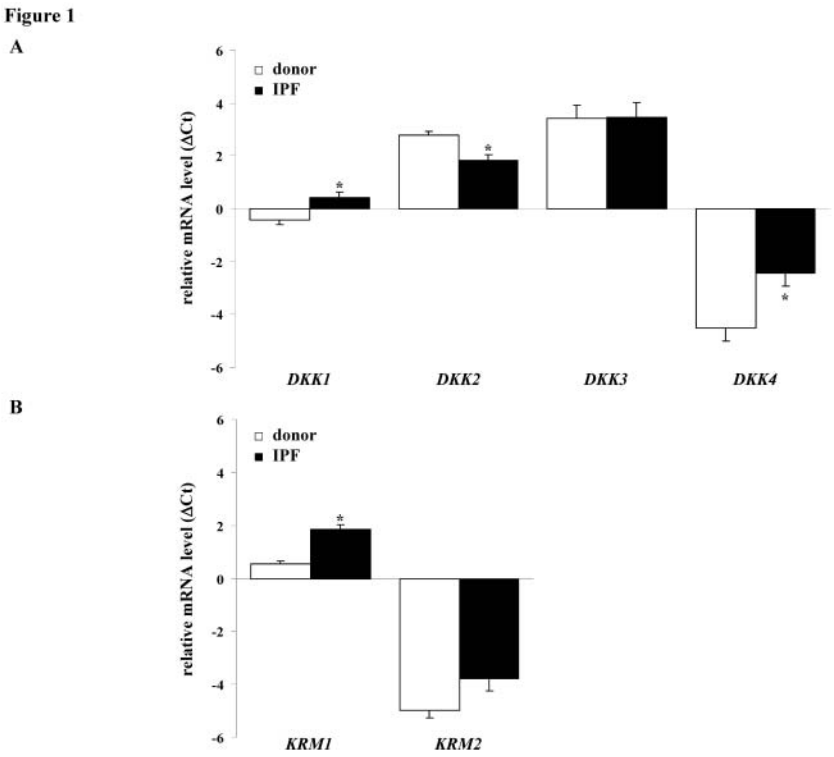

Figure 2. Protein expression of DKK and KRM in lung homogenates of donors and IPF patients. Expression of DKK1, 2 and 4 (**a,b**) and KRM1 and 2 (**c,d**) in total protein lysates of donor and IPF lung homogenates was determined by Western Blot analysis. Antibodies were used as indicated, β-actin served as a loading control. Immunoblots were carried out for at least two times, a representative Blot is shown (**a,c**). Densitometry is shown in 2b and 2d,

respectively. Ratio of optical density (optical density of indicated protein/optical density of β-actin) is presented for donor and IPF tissues as mean ± s.e.m., * = $p < 0.05$.

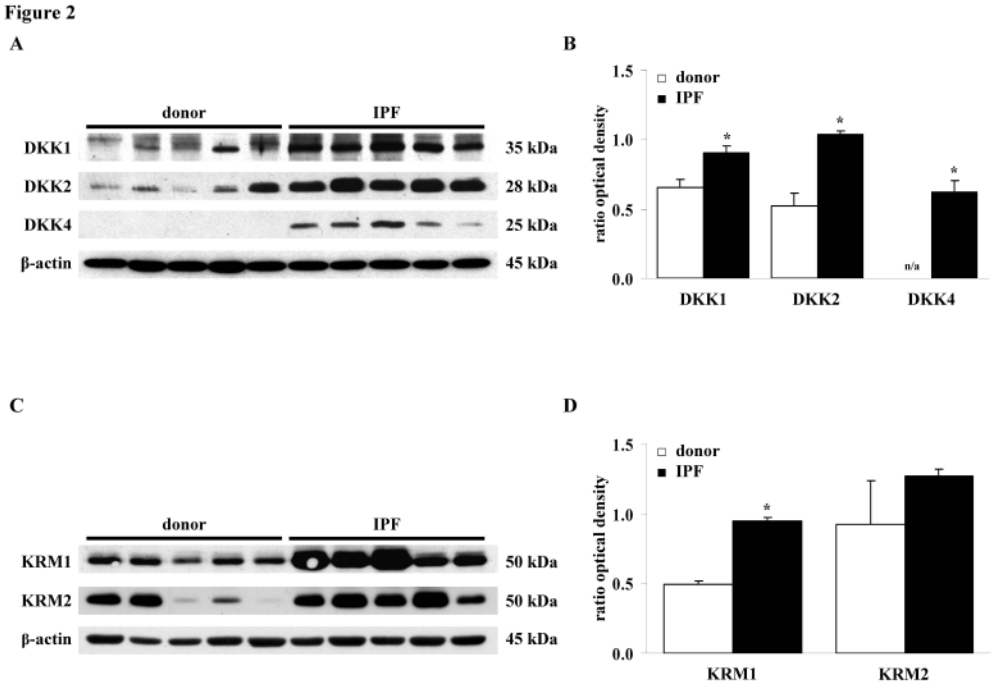

Figure 3. Expression and localization of DKK1 in lung tissue of donors and IPF patients. Immunohistochemistry for DKK1 was performed at lung tissue sections of donors and IPF patients. Stainings were performed for at least two times using three different donor and IPF lungs. Representative bronchiolar (**a**) and alveolar (**b**) regions for donor (**upper panels**) and IPF (**lower panels**) lungs are shown in three magnifications, as indicated. Arrows indicate basal bronchial epithelial cells (**a**) and hyperplastic alveolar epithelial cells (**b**, **lower panel**).

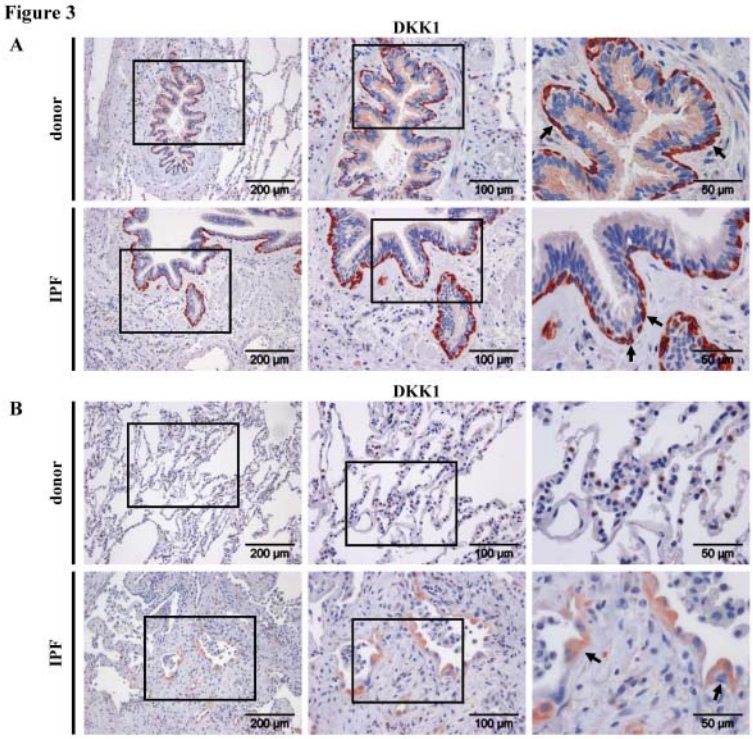

Figure 4. Expression and localization of DKK4 in lung tissue of donors and IPF patients. Immunohistochemistry for DKK4 was performed at lung tissue sections of donors and IPF patients. Stainings were performed for at least two times using three different donor and IPF lungs. Representative bronchiolar (**a**) and alveolar (**b**) regions for donor (**upper panels**) and IPF (**lower panels**) lungs are shown in three magnifications, as indicated. Arrows indicate positive hyperplastic alveolar epithelial cells (**b**, **lower panel**).

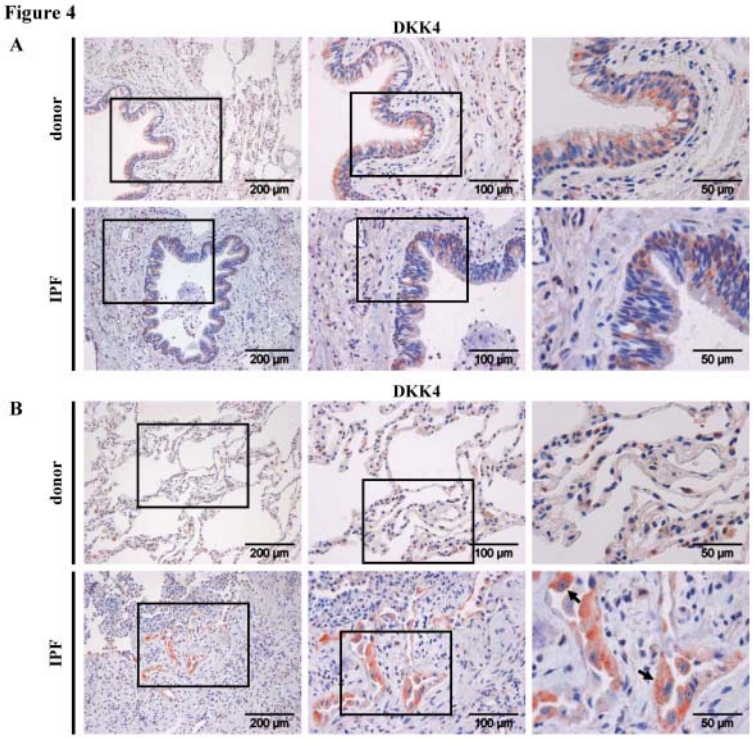

Figure 5. Expression and localization of KRM1 in lung tissue of donors and IPF patients. Immunohistochemistry for KRM1 was performed at lung tissue sections of donors and IPF patients. Stainings were performed for at least two times using three different donor **and IPF lungs. Representative bronchiolar (a) and alveolar (b) regions for donor (upper panels) and IPF (lower panels) lungs are shown in three magnifications as indicated. Arrows point out positive smooth muscle cells (a, lower panel) and hyperplastic alveolar epithelium (b, lower panel).**

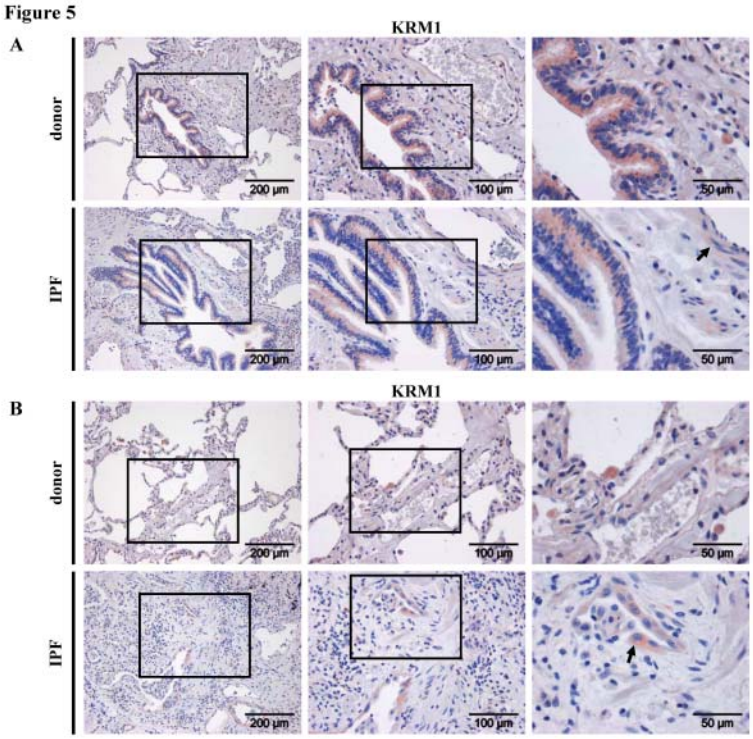

Figure 6. Expression and localization of KRM2 in lung tissue of donors and IPF patients. Immunohistochemistry for KRM2 was performed at lung tissue sections of donors and IPF patients. Stainings were performed for at least two times using three different donor **and IPF lungs. Representative bronchiolar (a) and alveolar (b) regions for donor (upper panels) and IPF (lower panels) lungs are shown in three magnifications as indicated. Arrows indicate hyperplastic alveolar epithelial cells (b, lower panel).**

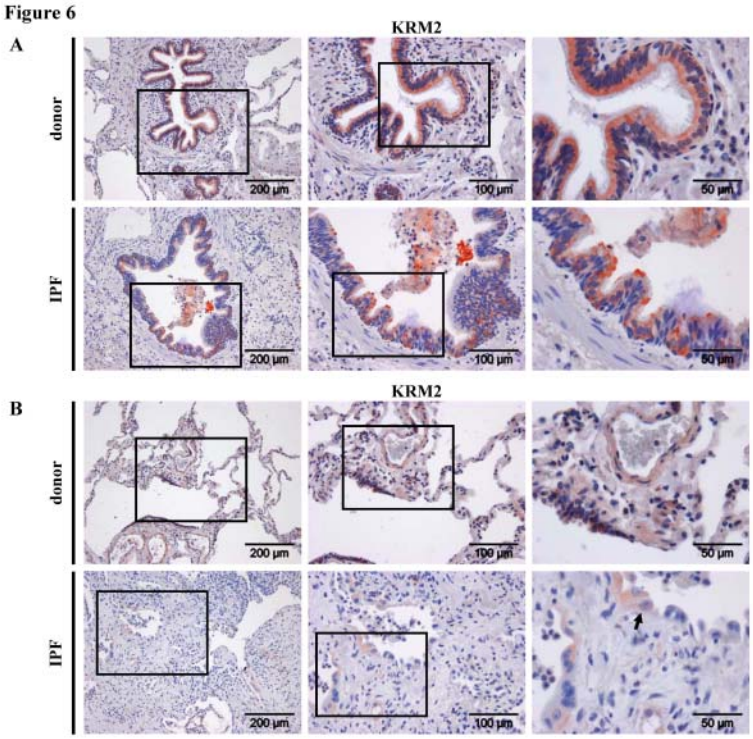

Figure 7. Quantification of DKK1 in bronchoalveolar lavage fluid (BALF) from healthy volunteers, patients with chronic bronchitis and IPF patients. DKK1 protein concentration in bronchoalveolar lavage fluid (BALF) of healthy volunteers, patients with chronic bronchitis, and IPF patients was quantified using enzyme-linked immunosorbent assay (ELISA). Results are derived from four healthy volunteers, three patients with chronic bronchitis, and 9 IPF patients. * = $p < 0.05$ compared with healthy volunteers, # = $p < 0.05$ compared with chronic bronchitis.

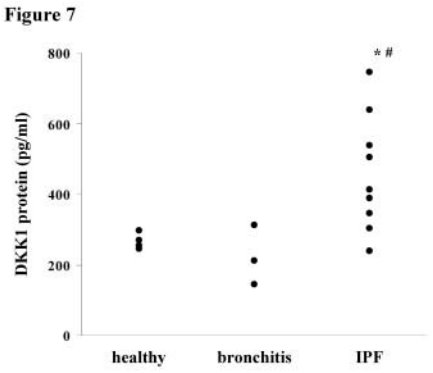

Figure 8. Effects of DKK1 and WNT3a on epithelial cell proliferation. BEAS-2B cells (**a**) or A549 cells (**b**) were stimulated with WNT3a, DKK1, or a combination thereof, as indicated. Proliferation was assessed by cell counting using a hemacytometer according to standard protocols. Data is shown as relative counts compared with control. Results are derived from four independent experiments and presented as mean ± s.e.m., ***** = $p < 0.05$ compared with control and # = $p < 0.05$ compared with WNT3a stimulated cells.

Figure 8

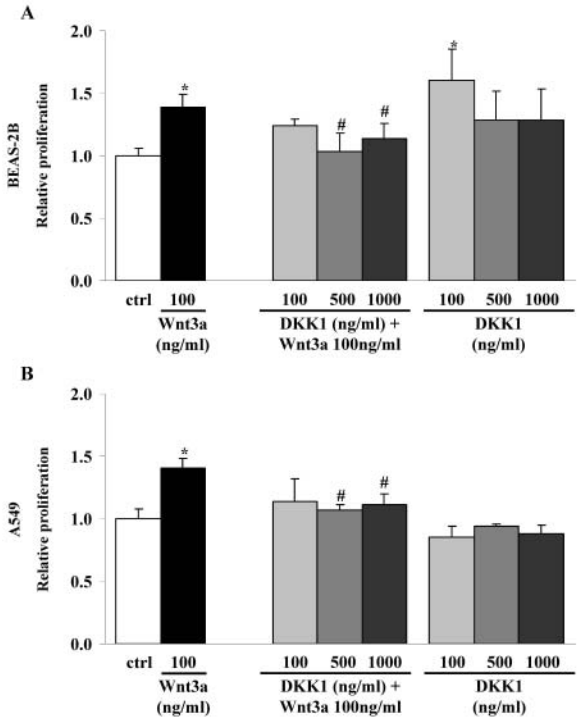
A
BEAS-2B
Relative proliferation
2.0
1.5
1.0
0.5
0.0
*
#
#
#
ctrl
100
100
500
1000
100
500
1000
Wnt3a
(ng/ml)
DKK1 (ng/ml) +
Wnt3a 100ng/ml
DKK1
(ng/ml)
B
A549
Relative proliferation
2.0
1.5
1.0
0.5
0.0
*
#
#
ctrl
100
100
500
1000
100
500
1000
Wnt3a
(ng/ml)
DKK1 (ng/ml) +
Wnt3a 100ng/ml
DKK1
(ng/ml)

Table 1 Characteristics of IPF patients. VC = vital capacity, TLC = total lung capacity, DL_{CO}/VA = diffusing capacity of the lung for CO per unit of alveolar volume (all in % predicted), $Pa_{O2/CO2}$ = partial pressure of O_2 /CO_2 in the arterial blood

no.	diagnosis	gender	age (yr)	VC (%)	DL_{CO}/VA (%)	TLC (%)	O_2 (l/min)	Pa_{O2} (mmHg)	Pa_{CO2}(mmHg)
1	IPF (UIP)	male	66	86	56	78	2	90	41
2	IPF (UIP)	male	76	41	73	47	-	79	38
3	IPF (UIP)	male	68	57	37	55	-	51	34
4	IPF (UIP)	male	60	33	na	42	5	69	41
5	IPF (UIP)	male	64	69	54	71	-	70	35
6	IPF (UIP)	male	79	81	42	75	-	45	37
7	IPF (UIP)	male	65	60	48	62	-	61	34
8	IPF (UIP)	male	65	64	75	58	-	78	35
9	IPF (UIP)	male	69	36	na	41	na	71	46

Table 2 Primer sequences and amplicon sizes for human tissues. All primer sets worked under identical real-time PCR cycling conditions with similar efficiencies to obtain simultaneous amplification in the same run. Sequences were taken from GeneBank, all accession numbers are denoted.

gene	accession		sequences (5′ → 3′)	length	amplicon
DKK1	NM012242	for	CGCCGAAAACGCTGCAT	17bp	109bp
		rev	TTTCCTCAATTTCTCCTCGGAA	22bp	
DKK2	NM014421	for	TCAGGCCGCCAATCGA	16bp	85bp
		rev	GTAGGCCTGCCCCAGGTT	18bp	
DKK3	NM015881	for	GCTTCTGGACCTCATCACCTG	21bp	119bp
		rev	TCGGCTTGCACACATACACC	20bp	
DKK4	NM014420	for	GAAGGGCTCACAGTGCCTGT	20bp	131bp
		rev	AGCACATGGCATCTCGCTG	19bp	
KRM1	NM001039570	for	TGGAAGCCACAGAGTTGAAGG	21bp	146bp
		rev	GACAATCCCTAAGGTCCCCTG	21bp	
KRM2	NM172229	for	CTGGCGCTACTGCGACATC	19bp	62bp
		rev	AGTCCACAAAGCATCCCAGGTA	22bp	
HPRT1	NM000194	for	AAGGACCCCACGAAGTGTTG	20bp	157bp
		rev	GGCTTTGTATTTTGCTTTTCCA	22bp	