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Dickkopf proteins influence lung epithelial cell proliferation in idiopathic pulmonary fibrosis

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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 signalling *via* interaction with Kremen (KRM) receptors, yet their expression and role in the adult lung and in IPF has not been addressed.

We analysed the expression, localisation 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 DKK4 and KRM1 was detected in lung homogenates of IPF patients compared with transplant donors. Immunohistochemistry revealed that DKK1 was predominantly localised 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 summary, 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.

KEYWORDS: Dickkopf proteins, lung epithelial cells, pulmonary fibrosis, WNT signalling

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 characterised 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 localisation 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, highly conserved secreted growth factors are known to control key events during lung development [12, 13]. WNT signalling is regulated *via* binding of extracellular WNT ligands to receptors of the frizzled family or low density lipoprotein receptor-related proteins (LRP). The best characterised WNT signalling pathway is the β-catenin-dependent, or canonical, WNT signalling pathway. In unstimulated cells, β-catenin, the main signalling intermediate of canonical WNT signalling, is bound to the scaffold proteins axin and adenomatosis polyposis coli, and constitutively phosphorylated by its interaction with casein kinase I and glycogen synthase kinase-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 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

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have linked increased WNT/β-catenin signalling 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 signalling. Four different DKK proteins (DKK1–4) have been discovered, sharing conserved cysteine-rich domains. DKK proteins bind to the LRP receptors and also bind to a second class of transmembrane receptors, called Kremen (KRM) [18], which potentiate the ability of DKK to regulate WNT signalling [19–21].

In this respect, we hypothesised 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 UIP pattern (four females, 11 males: mean age 58±8 yrs; mean vital capacity 48±7%; mean total lung capacity 50±5%; mean diffusing capacity of the lung for carbon monoxide per unit of alveolar volume 23±3%; O_2 2–4 L·min^{-1}; arterial oxygen tension 49–71 mmHg; arterial carbon dioxide tension 33–65 mmHg) and nine control subjects (organ donors, four females, five males; mean age 42±10 yrs). Individual patient characteristics have been described previously [4]. Samples were immediately snap frozen or placed in 4% (weight/volume) paraformaldehyde after explantation. The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine, Giessen, Germany (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 Dept of Medicine at the Justus-Liebig-University in 2006 and 2007. The study protocol was approved by the local ethics committee, and informed consent was obtained from the patients. Flexible fibreoptic bronchoscopy was performed in patients and controls by one physician in a standardised manner, as previously described [22]. Individual patient characteristics are shown in table 1. The control group consisted of four spontaneously breathing healthy nonsmoking 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).

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, Carlsbad, CA, USA), 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 article. When relative transcript abundance is given, expression levels are presented in ΔCt levels.

Western blot analysis

Human lung tissue was homogenised 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, USA), DKK2 and DKK4 (ab38594 and ab38589; Abcam, Cambridge, UK), KRM1 (AF2127; R&D Systems, Minneapolis, MN, USA), and KRM2 (HP A003223; Sigma-Aldrich, Saint Louis, MO, USA). 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, USA). Changes in expression levels are expressed as fold change (mean±SEM).

TABLE 1 Characteristics of idiopathic pulmonary fibrosis (IPF) patients

No.	Diagnosis	Sex	Age yrs	VC % pred	$D_{L,CO}/V_A$ % pred	TLC % pred	O_2 L·min^{-1}	P_{a,O_2} mmHg	P_{a,CO_2} 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

VC: vital capacity; % pred: % predicted; $D_{L,CO}/V_A$: diffusing capacity of the lung for carbon monoxide per unit of alveolar volume; TLC: total lung capacity; P_{a,O_2}: arterial oxygen tension; P_{a,CO_2}: arterial carbon dioxide tension; UIP: usual interstitial pneumonia; NA: not applicable.

TABLE 2 Primer sequences and amplicon sizes for human tissues[#]

Gene	Accession	Sequences (5′ → 3′)	Length bp	Amplicon bp
DKK1	NM012242	For: CGCCGAAAACGCTGCAT	17	109
		Rev: TTTCCTCAATTTCTCCTCGGAA	22	
DKK2	NM014421	For: TCAGGCCGCCAATCGA	16	85
		Rev: GTAGGCCTGCCCCAGGTT	18	
DKK3	NM015881	For: GCTTCTGGACCTCATCACCTG	21	119
		Rev: TCGGCTTGCACACATACACC	20	
DKK4	NM014420	For: GAAGGGCTCACAGTGCCTGT	20	131
		Rev: AGCACATGGCATCTCGCTG	19	
KRM1	NM001039570	For: TGGAAGCCACAGAGTTGAAGG	21	146
		Rev: GACAATCCCTAAGGTCCCCTG	21	
KRM2	NM172229	For: CTGGCGCTACTGCGACATC	19	62
		Rev: AGTCCACAAAGCATCCCAGGTA	22	
HPRT1	NM000194	For: AAGGACCCCACGAAGTGTTG	20	157
		Rev: GGCTTTGTATTTTGCTTTTCCA	22	

DKK: Dickkopf; KRM: Kremen; HPRT1: hypoxanthine guanine phosphoribosyl transferase 1; for: forward; rev: reverse. [#]: 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.

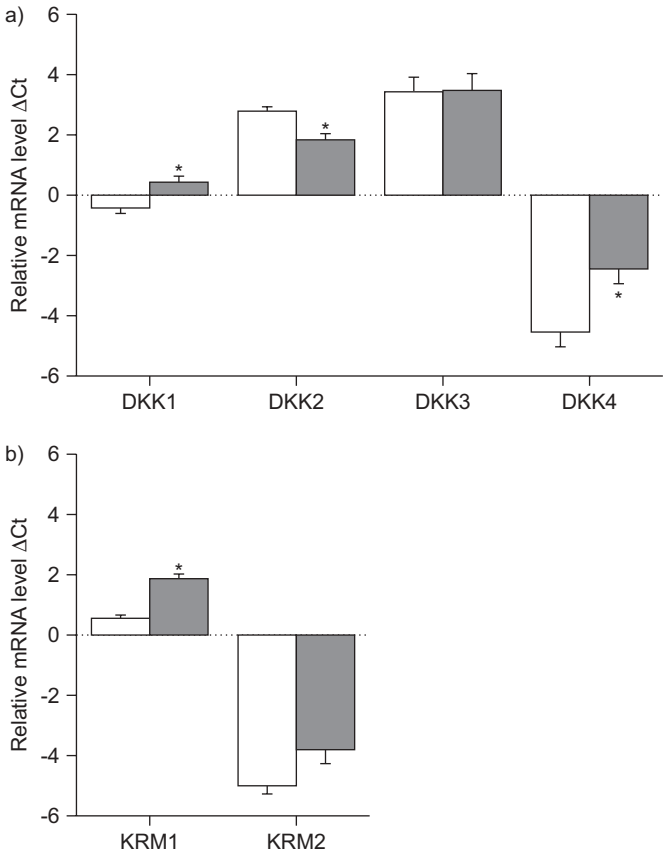

FIGURE 1. The mRNA expression of Dickkopf (DKK) and Kremen (KRM) proteins in idiopathic pulmonary fibrosis (IPF) and donor tissue. The mRNA expression of a) DKK1–4 and b) the receptors KRM1 and 2 was analysed in total lung homogenates from donor (□) and IPF lung specimen (■) by quantitative RT-PCR. Results from 10 donors and 10 IPF patients are shown as relative mRNA expression compared to the reference gene (ΔCt), and presented as mean±SEM. *: $p<0.05$.

Immunohistochemistry

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

ELISA

A human DKK1 ELISA (DY1906; R&D Systems) was performed on bronchoalveolar lavage fluid (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 4,000 $pg \cdot mL^{-1}$ and two-fold serial dilutions to a low standard (75 $pg \cdot mL^{-1}$) 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) 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 Dulbecco's modified Eagle medium (GIBCO; Invitrogen), supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Austria). Cells were plated in 24-well plates, serum starved for 20 h in 0.1% fetal calf serum medium. Stimulation for 24 h was performed with recombinant WNT3a or DKK1 (both from R&D Systems), as indicated. Cell counting was performed using a haemacytometer according to standard protocols.

Statistical analysis

All ΔCt values obtained from real-time RT-PCR were analysed 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 a two-tailed unpaired t-test, or a ANOVA with Tukey HSD *post hoc* test for studies with more than two groups. Results were considered statistically significant when $p<0.05$.

RESULTS

Initially, we quantified the mRNA expression of DKK1–4 and KRM1 and 2 in homogenised lung tissue specimens of transplant donors and IPF patients (n=10 each) using (q)RT-PCR. As demonstrated in figure 1a, all DKK 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) (fig. 1b).

Next, we analysed the protein expression pattern of DKK and 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 (fig. 2a and b) (increases in optical density *versus* β-actin, DKK1 0.24±0.08 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 specimens (fig. 1a). Both receptors KRM1 and 2 were expressed in the lung (fig. 2c), with significantly increased expression of KRM1 (0.45±0.04) (fig. 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 localised in bronchial epithelial cells in donor and IPF lungs (fig. 3a). Interestingly, we observed a pronounced and distinct accumulation of DKK1 in basal bronchial epithelial cells (fig. 3a, arrows). In IPF lungs, DKK1 was particularly localised in hyperplastic alveolar epithelial cells (fig. 3b, arrows). In addition, granulocytes (fig. 3b) presented staining of DKK1 protein. DKK4 protein expression was largely localised to bronchial

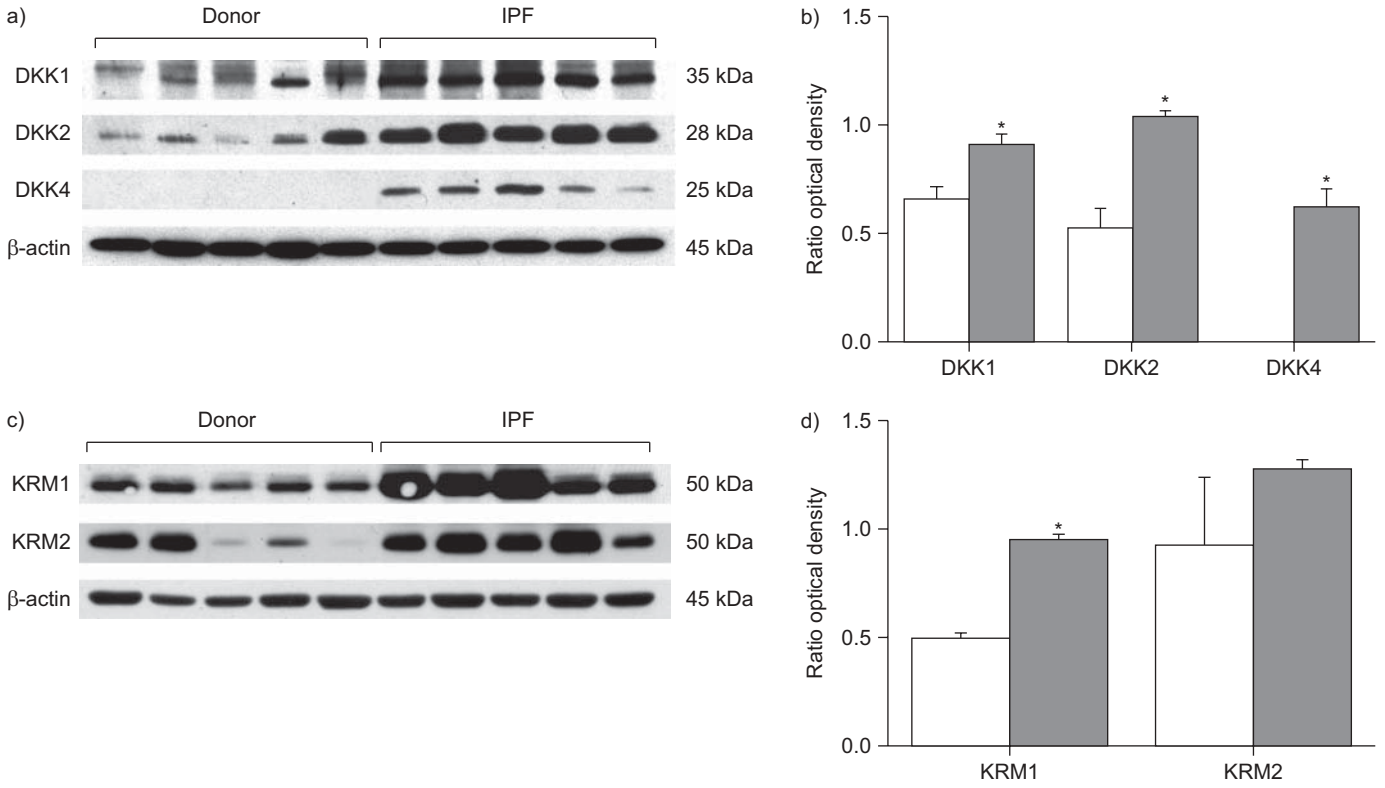

FIGURE 2. Protein expression of Dickkopf (DKK) and Kremen (KRM) in lung homogenates of donors (□) and idiopathic pulmonary fibrosis (IPF; ■) patients. a, b) Expression of DKK1, 2 and 4 and c, d) KRM1 and 2 in total protein lysates of donor and IPF lung homogenates was determined by Western blot analysis. Antibodies were used as indicated, β-actin served as a loading control. Immunoblots were carried out at least twice, a representative blot is shown (a and c). Densitometry is shown in b and d, respectively. Ratio of optical density (optical density of indicated protein/optical density of β-actin) is presented for donor and IPF tissues as mean±SEM. The ratio optical density in donors for DKK4 was not available. *: $p<0.05$.

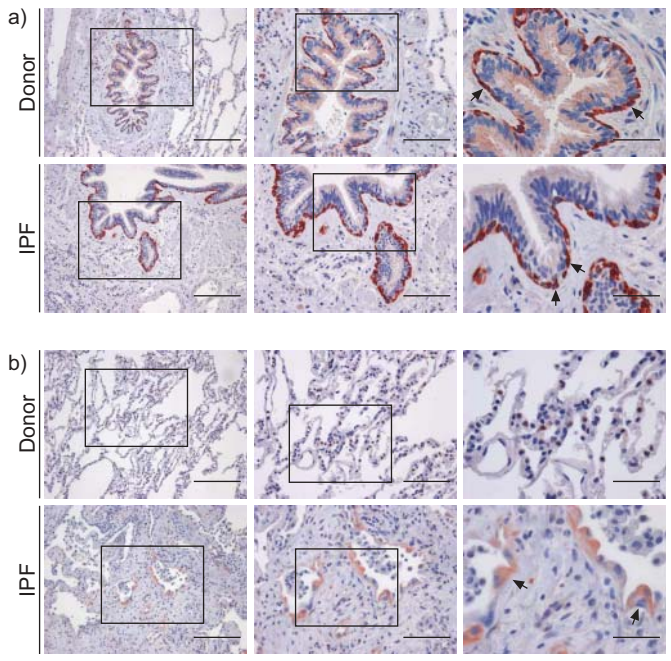

FIGURE 3. Expression and localisation of Dickkopf (DKK)1 in lung tissue of donors and idiopathic pulmonary fibrosis (IPF) patients. Immunohistochemistry for DKK1 was performed at lung tissue sections of donors and IPF patients. Stainings were performed at least twice using three different donor and IPF lungs. Representative a) bronchiolar and b) alveolar regions for donor and IPF lungs are shown in three magnifications (200, 100 and 50 μm from left to right). Arrows indicate basal bronchial epithelial cells (a) and hyperplastic alveolar epithelial cells (b).

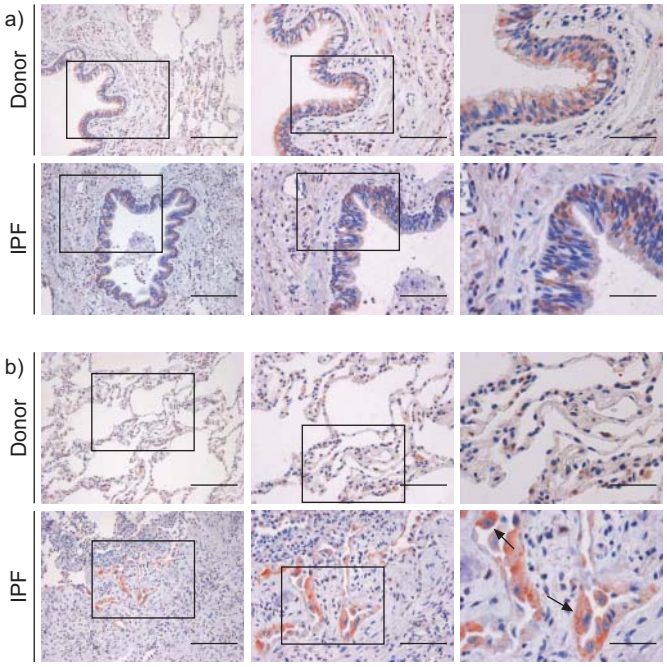

FIGURE 4. Expression and localisation of Dickkopf (DKK)4 in lung tissue of donors and idiopathic pulmonary fibrosis (IPF) patients. Immunohistochemistry for DKK4 was performed on lung tissue sections of donor and IPF patients. Stainings were performed at least twice using three different donor and IPF lungs. Representative a) bronchiolar and b) alveolar regions for donor and IPF lungs are shown in three magnifications (200, 100 and 50 μm from left to right). Arrows indicate positive hyperplastic alveolar epithelial cells.

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 (fig. 4a) in donor and IPF tissue. As depicted in figure 4b, DKK4 was strongly expressed in hyperplastic alveolar epithelial cells and areas of bronchiolisation in IPF (fig. 4b, arrows).

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

Taken together, these results demonstrated increased expression of the DKK ligands and their receptors in IPF. All proteins largely localised 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 BALF of healthy volunteers (n=4), patients with chronic bronchitis (n=3) or IPF (n=9) using an 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^{-1}), compared with healthy controls (266±8 pg·mL^{-1}) or patients with chronic bronchitis (223±34 pg·mL^{-1}).

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 analysed 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^{-1}) 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. Similar to bronchial epithelial cells, WNT3a increased the proliferative capacity of alveolar epithelial cells (fig. 8b) (relative proliferation (mean±SEM) 1.42±0.08), which was attenuated by high concentrations of DKK1 (DKK1 500 ng·mL^{-1} 1.07±0.05, and DKK1 1,000 ng·mL^{-1} 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.

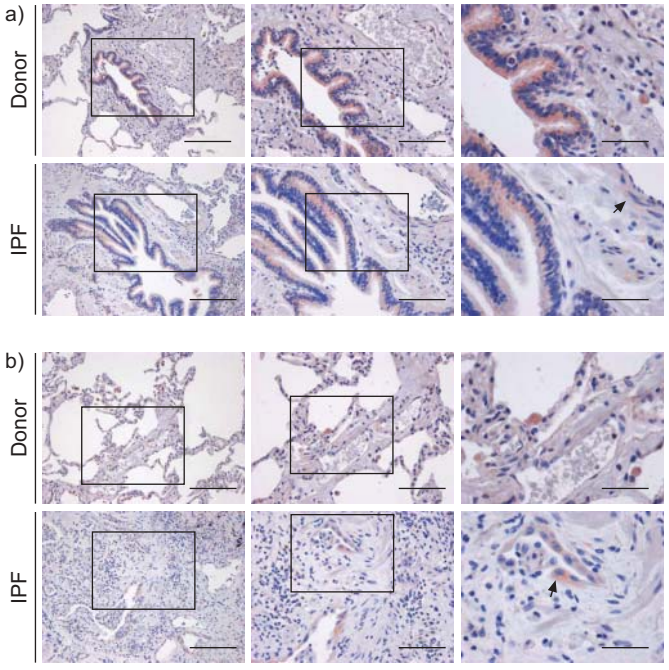

FIGURE 5. Expression and localisation of Kremen (KRM)1 in lung tissue of donors and idiopathic pulmonary fibrosis (IPF) patients. Immunohistochemistry for KRM1 was performed on lung tissue sections of donor and IPF patients. Stainings were performed at least twice using three different donor and IPF lungs. Representative a) bronchiolar and b) alveolar regions for donor and IPF lungs are shown in three magnifications (200, 100 and 50 μm from left to right). Arrows indicate positive smooth muscle cells (a) and hyperplastic alveolar epithelium (b).

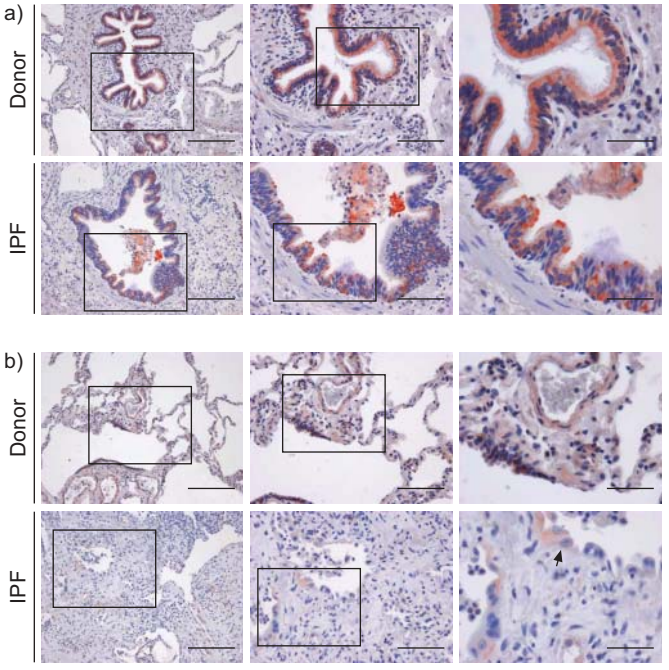

FIGURE 6. Expression and localisation of Kremen (KRM)2 in lung tissue of donor and idiopathic pulmonary fibrosis (IPF) patients. Immunohistochemistry for KRM2 was performed on lung tissue sections of donor and IPF patients. Stainings were performed at least twice using three different donor and IPF lungs. Representative a) bronchiolar and b) alveolar regions for donor and IPF lungs are shown in three magnifications (200, 100 and 50 μm from left to right). Arrows indicate hyperplastic alveolar epithelial cells.

DISCUSSION

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]. Herein, we performed a comprehensive analysis of the expression and localisation of the WNT modulators DKK and their KRM receptors, demonstrating that both DKK and KRM proteins are enhanced in lung tissue specimens 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 localise to lung epithelial cells. Of note, DKK1 exhibited strong expression in basal bronchial and hyperplastic alveolar epithelial cells in IPF. Analysis of 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 signalling system is tightly controlled by different secreted WNT regulators, such as the secreted frizzled receptor (sFRP) or DKK proteins [21, 24]. Both protein families use a fundamentally different mechanism to modulate WNT signalling. While sFRP bind directly to WNT ligands and inhibit their interaction with the membrane receptors frizzled or low-density 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 internalisation of the whole complex from the cell surface, thereby inhibiting WNT signalling [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 to date have focused on DKK1. Inhibition of WNT/β-catenin signalling 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 signalling has only been demonstrated 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]. Herein, 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

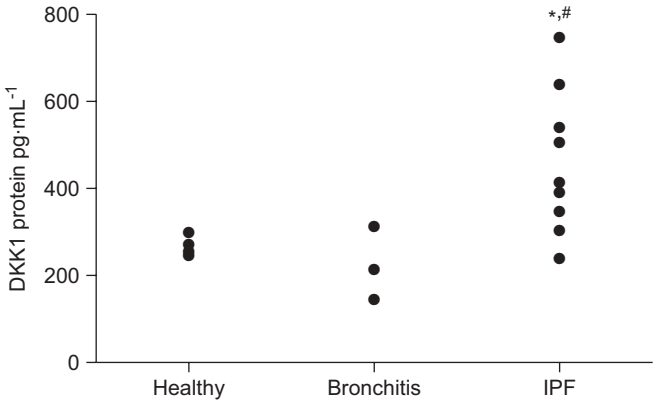

FIGURE 7. Quantification of Dickkopf (DKK)1 in bronchoalveolar lavage fluid (BALF) from healthy volunteers, patients with chronic bronchitis and idiopathic pulmonary fibrosis (IPF) patients. DKK1 protein concentration in BALF of healthy volunteers, patients with chronic bronchitis and IPF patients was quantified using an ELISA. Results are derived from four healthy volunteers, three patients with chronic bronchitis and nine IPF patients. *: $p<0.05$ compared with healthy volunteers; #: $p<0.05$ compared with chronic bronchitis.

study also suggests that the transcriptional (feedback) control or protein stability due to post-translational 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 signalling. Expression of LRP5 and 6 in lung homogenates of donors and IPF patients has been demonstrated in a recent study [4]. We focussed on the receptors KRM1 and 2. Immunohistochemical staining of KRM1 and 2 revealed that the bronchial and hyperplastic alveolar epithelium, in particular in areas of bronchiolisation of IPF specimen, are major sources in donor and IPF lung tissue specimens, indicating autocrine effects on epithelial cells as the main signalling 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 signalling *in vivo*. In addition, it has to be pointed out that fibroblasts 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 signalling.

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

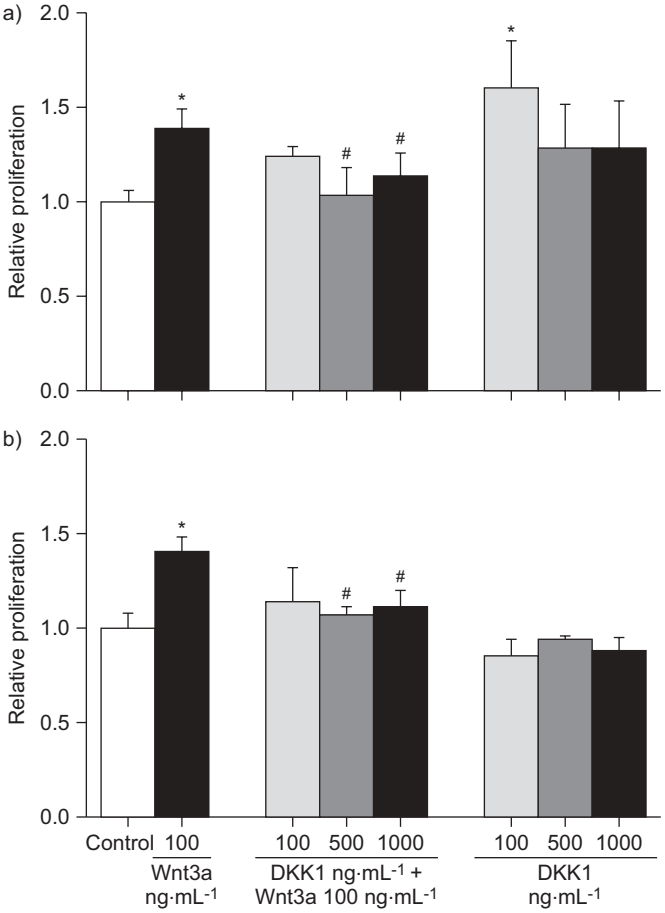

FIGURE 8. Effects of Dickkopf (DKK)1 and WNT3a on epithelial cell proliferation. a) BEAS-2B cells or b) A549 cells were stimulated with WNT3a, DKK1 or a combination thereof, as indicated. Proliferation was assessed by cell counting using a haemacytometer according to standard protocols. Data is shown as relative counts compared with control. Results are derived from four independent experiments and presented as mean±SEM. *: $p<0.05$ compared with control; #: $p<0.05$ compared with WNT3a stimulated cells.

epithelium [35]. Importantly, bronchial epithelial cell metaplasia is also a common feature in IPF lung tissue specimens [36]. In addition, CHILOSI *et al.* [37] reported abnormal proliferation of bronchial epithelial cells in IPF, but not other interstitial pneumonias, such as acute or nonspecific pneumonias. 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 respond to WNT signalling [39].

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 increased lung cancer development. Furthermore, our data suggest that DKK1, although expressed

and secreted by the alveolar epithelium, is not able to fulfil an effective negative feedback-loop on WNT-induced aberrant alveolar epithelial cell proliferation in IPF *in vivo*. The effects of DKK on WNT signalling 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 analysed in more detail using primary alveolar and bronchial epithelial cells in future studies.

In summary, our study demonstrated altered expression of the WNT regulators DKK and KRM, which may be crucial for lung epithelial cell injury and repair mechanisms 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.

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STATEMENT OF INTEREST

None declared.

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