Enolase 1 (ENO1) and protein disulfide-isomerase associated 3 (PDIA3) regulate Wnt/β-catenin-driven trans-differentiation of murine alveolar epithelial cells

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ABSTRACT
The alveolar epithelium represents a major site of tissue destruction during lung injury. It consists of alveolar epithelial type I (ATI) and type II (ATII) cells. ATII cells are capable of self-renewal and exert progenitor function for ATI cells upon alveolar epithelial injury. Cell differentiation pathways enabling this plasticity and allowing for proper repair, however, are poorly understood. Here, we applied proteomics, expression analysis and functional studies in primary murine ATII cells to identify proteins and molecular mechanisms involved in alveolar epithelial plasticity. Mass spectrometry of cultured ATII cells revealed a reduction of carbonyl reductase 2 (CBR2) and an increase in enolase 1 (ENO1) and protein disulfide-isomerase-associated 3 (PDIA3) protein expression during ATII-to-ATI cell trans-differentiation. This was accompanied by increased Wnt/β-catenin signaling, as analyzed by qRT-PCR and immunoblotting. Notably, ENO1 and PDIA3, along with T1α (podoplanin; an ATI cell marker), exhibited decreased protein expression upon pharmacological and molecular Wnt/β-catenin inhibition in cultured ATII cells, whereas CBR2 levels were stabilized. Moreover, we analyzed primary ATI cells from mice with bleomycin-induced lung injury, a model exhibiting activated Wnt/β-catenin signaling in vivo. We observed reduced CBR2 significantly correlating with surfactant protein C (SFTPC), whereas ENO1 and PDIA3 along with T1α were increased in injured ATI cells. Finally, siRNA-mediated knockdown of ENO1, as well as PDIA3, in primary ATII cells led to reduced T1α expression, indicating diminished cell trans-differentiation. Our data thus identified proteins involved in ATII-to-ATI cell trans-differentiation and suggest a Wnt/β-catenin-driven functional role of ENO1 and PDIA3 in alveolar epithelial plasticity in lung injury and repair.

KEY WORDS: Alveolar epithelial cells, Differentiation, Lung injury and repair, Beta-catenin, Wnt pathway, Fibrosis

INTRODUCTION
Chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF), represent a significant health burden worldwide owing to their progressive nature and the current lack of curative treatments. Therapeutic options improving disease pathology or attenuating disease progression are limited (Barnes, 2014; Fernandez and Eickelberg, 2012).

The alveolar epithelium constitutes a major site of injury and tissue destruction in chronic lung diseases and thus represents an area of intensive research (Tsuji et al., 2006; Camelo et al., 2014; Selman and Pardo, 2006). In the adult lung, the alveolar epithelium consists largely of two morphologically distinct epithelial cell types, which are crucial to maintain lung homeostasis. Alveolar epithelial type I (ATI) cells are elongated squamous cells with a large cell surface, which, owing to their close proximity to endothelial cells of the alveolar capillaries, facilitate gas exchange in the lung. Furthermore, ATI cells are highly water permeable, enabling and facilitating ion transport and maintenance of lung fluid balance (Dobs et al., 2010; Johnson et al., 2002). Although ATI cells are not the most abundant cell type, they cover the largest surface area of the distal lung (Stone et al., 1992; Weibel, 2015). Alveolar epithelial type II (ATII) cells, which exhibit a cuboidal cell morphology, account for a much larger number of cells in the distal lung while covering a significant lower surface area (Stone et al., 1992). ATII cells are involved in ion transport and liquid homeostasis (Fehrenbach, 2001) but, most importantly, ATII cells are responsible for the production, storage, secretion and recycling of pulmonary surfactant, a complex mixture of lipids and proteins, lining the alveolar epithelium. Surfactant lowers the surface tension at the tissue-air barrier to allow proper inflation and deflation of the lung during breathing (Halliday, 2008; Lher et al., 2007). Pulmonary surfactant also contributes to host defense in the lung (Strunk et al., 1988). Although the general steady-state cellular turnover of the adult lung is rather low in comparison to other organs (Hogan et al., 2014), recent studies have demonstrated repair capacity of the lung in response to injury (Butler et al., 2012). In a variety of different lung injury models, such as bleomycin-induced fibrosis, hyperoxia or viral infection, ATII cells have been described to serve as progenitor cells for ATI cells (Rock et al., 2011; Desai et al., 2014; Liu et al., 2011). Furthermore, recent studies utilizing lineage tracing technology have established that ATII cells are capable of long-term self-renewal, indicating that these cells represent a major stem-cell population in the adult alveolar epithelium (Barkauskas et al., 2013; Desai et al., 2014).

Pathways enabling the activation and plasticity of this cell population in response to injury and allowing for proper repair, however, are poorly understood. Established markers to define ATII and ATI cell phenotypes do exist, such as surfactant proteins and T1α (podoplanin), respectively. Markers that accurately reflect the differentiation status of alveolar epithelial cells especially during injury and repair processes, however, are not well characterized. Here, we applied the model of ATII cell cultivation in vitro, in which ATII cells trans-differentiate into an ATI-cell-like phenotype during...
primary culture (Borok et al., 1994; DeMaio et al., 2009; Bhaskaran et al., 2007), to mimic differentiation and repair processes. Recent studies have reported that the Wnt/β-catenin pathway, an essential developmental pathway, is activated during alveolar epithelial cell injury and repair in general (Selman et al., 2008; Königshoff et al., 2009; Beers and Morrisey, 2011; Ulsamer et al., 2012; Tanjore et al., 2013), and in ATII-to-ATI cell trans-differentiation in particular (Flozak et al., 2010; Marconett et al., 2013). The detailed mechanism, however, of how Wnt/β-catenin signaling mediates its cellular effects on ATII-to-ATI cell trans-differentiation, remains elusive.

The aim of the presented study was to identify proteins controlling ATII-to-ATI cell trans-differentiation using expression analysis, 2D gel electrophoresis and mass spectrometry of cultured primary murine ATII cells in vitro. Secondly, we sought to define molecular programs and markers for differentiation associated with the Wnt/β-catenin pathway and to investigate their relevance for injury and repair processes in the alveolar lung epithelium. We present for the first time evidence for β-catenin-dependent ENO1 and PDIA3 expression in alveolar epithelial injury and repair processes.

RESULTS
Dynamic changes of alveolar epithelial markers during ATII-to-ATI cell trans-differentiation
Isolated primary murine (pm) ATII cells exhibited a robustly high purity and viability, and expressed specific ATII cell-marker and tight-junction proteins as previously published (Königshoff et al., 2009; Aumiller et al., 2013). Cytoplasmic expression of the ATII cell marker pro surfactant protein C (proSFTPC) and the tight-junction protein occludin (OCLN) is shown in Fig. 1A. The pmATII cells were cultured on cell culture dishes over a period of 5 days to induce ATII-to-ATI cell trans-differentiation and were further analyzed for their expression pattern of ATII cell (Sftpc and Sftpa) and ATI cell (T1α) markers, as well as for tight-junction proteins. As displayed in Fig. 1, ATII cells are characterized by a high mRNA and protein expression level of ATII cell markers Sftpc and Sftpa, which decreased significantly over the culture period. By contrast, whereas we observed rather stable expression of tight-junction proteins, the expression of the ATI cell marker T1α [podoplanin (Pdpn)] significantly increased over time, accompanied by a more flattened cell morphology (Fig. 1B,C). Analysis of additional markers involved in ATII-to-ATI cell trans-differentiation, such as forkhead box M1 (FoxM1), a transcription factor described to be essential for ATII-to-ATI cell trans-differentiation in an influenza lung injury model (Liu et al., 2011), as well as advanced glycosylation end product-specific receptor (Ager), further supported a phenotypical switch at day 3 of culture (supplementary material Fig. S1). These data demonstrate a differentiation of pmATII cells towards an ATI-cell-like phenotype under the applied culture conditions.

Proteomic analysis of trans-differentiating alveolar epithelial cells revealed CBR2, ENO1 and PDIA3 to be differentially expressed
To identify proteins involved in alveolar epithelial differentiation and wound repair, we applied a proteomics approach by using 2D gel electrophoresis (2DE) and subsequent mass spectrometry (MS) analysis of pmATII cells cultured for 1, 3 or 5 days. A representative image of 2D gels at day 1, day 3 and day 5 is shown in Fig. 2A. The analysis revealed several proteins to be differentially expressed in this process (Table 1 and supplementary material Table S1). We verified and confirmed differentially expressed proteins identified by 2DE and MS, using qPCR and immunoblotting. Most proteins, including carbonyl reductase 2 (CBR2), enolase 1 (ENO1; also known as α-enolase) and protein disulfide-isomerase associated 3 (PDIA3), were differentially expressed at the mRNA level and protein level over time of culture (Fig. 2B,C).

Interestingly, CBR2, also known as ‘mouse lung carbonyl reductase’ (MLCR) was one of the most downregulated proteins over the culture period, with very similar kinetics compared to proSFTPC expression. Moreover, ENO1, a protein involved in glycolytic processes, and PDIA3 [also named ERp57 and GRP58 (Turano et al., 2011)], a protein disulfide isomerase, were significantly induced and PDIA3 protein localized to T1α-positive cultured alveolar epithelial cells at day 3 of culture (Fig. 2C and supplementary material Fig. S2). The described role of CBR2 and ENO1 in cellular differentiation in other organs (Wenz et al., 1992; Lopez-Alemany et al., 2003; Ryu et al., 2012) prompted us to further investigate the relevance of these proteins in the context of alveolar epithelial differentiation. Furthermore, PDIA3 is involved in the

Clinical issue
Chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF), represent a major health burden worldwide, with no curative treatment options currently available. These disease entities are characterized by reduced repair capacity of the alveolar compartment, in particular by impaired trans-differentiation of alveolar epithelial type II (ATII) to alveolar type I (ATI) cells. Thus, advances in our understanding of alveolar epithelial plasticity during lung injury and repair are of utmost importance. The characterization of relevant in vitro systems is required to underpin their validity and suitability for mechanistic studies and for identifying targets for future clinical intervention in human chronic lung diseases. In this study, the authors aimed to identify proteins involved in alveolar epithelial cell injury and repair processes.

Results
Using a proteomic approach, the authors reported for the first time carbonyl reductase 2 (CBR2), enolase 1 (ENO1) and protein disulfide isomerase associated 3 (PDIA3) as functional alveolar epithelial cell proteins. These proteins are altered during ATII-to-ATI cell trans-differentiation in vitro. Reduced CBR2 expression was accompanied by reduced expression of pro surfactant protein C (proSFTPC; an ATII cell marker). Moreover, ENO1 and PDIA3 were increased along with the ATI cell marker T1α. Notably, expression of ENO1, PDIA3 and T1α decreased upon inhibition of Wnt/β-catenin signaling (a pathway that is involved in impaired alveolar epithelial cell repair in vitro and in vivo and is suggested as a potential therapeutic target for pulmonary fibrosis) during ATII-to-ATI trans-differentiation, whereas CBR2 levels were stabilized.

Moreover, in primary ATI cells from bleomycin-induced lung injury – a model exhibiting activated Wnt/β-catenin signaling and pulmonary fibrosis in vivo – CBR2 expression was reduced, significantly correlating with reduced pro-SFTPC, whereas ENO1, PDIA3 and T1α were increased. Finally, loss of ENO1 and PDIA3 function in primary ATI cells led to reduced T1α expression, indicating their functional role in alveolar epithelial cell plasticity.

Implications and future directions
In summary, these data validate the ATII-to-ATI cell trans-differentiation in vitro system as a suitable model of alveolar epithelial cell injury and wound repair in vivo. In addition, this study implies CBR2, ENO1 and PDIA3 as newly identified alveolar epithelial cell proteins involved in β-catenin-driven alveolar epithelial cell plasticity. Therefore, these proteins might represent potential drug targets in chronic lung disease.
quality control of newly synthesized glycoproteins, suggesting a possible connection to aberrantly activated Wnt/β-catenin signaling in lung injury and repair (Chilosi et al., 2003; Königshoff et al., 2008, 2009).

**Fig. 1. Dynamic mRNA and protein expression changes during ATII-to-ATI cell trans-differentiation in vitro.** (A) Immunofluorescence staining of pmATII cells on coverslips for epithelial cell marker expression at day 2 after isolation. Fluorescent images represent a 400× magnification. The scale bar represents 50 µm. (B) mRNA expressions of epithelial cell markers during the culture of pmATII cells over a period of 5 days. mRNA levels were measured by quantitative RT-PCR (qRT-PCR) and normalized to Hprt as housekeeping gene. Data represent means of ΔCt values±s.e.m. of at least three independent experiments. (C) Protein expression of epithelial markers in cultured pmATII cells. Cells were lysed at the indicated time points and 15 µg of total protein per sample was subjected to immunoblot analysis. β-actin expression served as loading control. A representative experiment and a densitometric analysis of at least three independent experiments are shown. Means at indicated time points were compared to day 1 (d1) using one-way ANOVA, followed by Dunnett’s post-hoc test. Significance: *P<0.05; **P<0.01; ***P<0.001.

**Alveolar epithelial cell trans-differentiation is accompanied by an activation of the Wnt/β-catenin pathway**

Because the Wnt/β-catenin pathway has been described to be involved in relevant developmental and regenerative processes in
the lung in general (Beers and Morrisey, 2011), as well as in lung injury and repair processes in particular (Flozak et al., 2010; Aumiller et al., 2013; Königshoff et al., 2009), we asked the question, are the newly identified proteins linked to Wnt/β-catenin signaling? First, we investigated Wnt/β-catenin activity in our model. Importantly, we found a considerably increased level of active β-catenin (ABC) at day 3 and day 5 accompanied by increased T1α expression (Figs 3A and 1C), which is in accordance to previous findings in rat alveolar epithelial cells (Flozak et al., 2010). In order to determine whether the activation of β-catenin is...
mediated by a Wnt-ligand-dependent signaling process, we
determined the expression and phosphorylation status of dishevelled
segment polarity protein 3 (DVL3), a cytoplasmic protein, which is
phosphorylated and therefore activated upon binding of a Wnt ligand
to a Wnt receptor (frizzled) and co-receptor (low-density lipoprotein
receptor-related protein 5 or 6). As displayed in Fig. 3B, we observed
expression of the canonical Wnt ligands Wnt3a and Dickkopf-related protein 2 (Dkk2), detected
during the trans-differentiation of ATI cells [Fig. 3C; Axin2: ΔCt day
1 (d1) −4.56± 0.34 s.e.m., ΔCt day 5 (d5) −0.72±0.37 s.e.m.,
P<0.001; Dkk2: ΔCt d1 −6.47±0.06 s.e.m., ΔCt d5 −0.99±0.31
s.e.m., P<0.001].

Next, we investigated the expression of the canonical Wnt ligands
Wnt3a, Wnt10a and Wnt10b (Baarsma et al., 2013) to further clarify
which Wnt ligands might induce active Wnt signaling in this
process. Notably, we found that Wnt10a and Wnt10b, but not
Wnt3a, exhibited a remarkable increased expression as early as
day 2, linking these Wnt ligands to ATI-to-ATI cell trans-
differentiation. By using another β-catenin inhibitor, which has already been applied in an experimental lung fibrosis model in vivo (ICG-001) (Henderson et al., 2010) (supplementary material Fig. S3). Furthermore, we
utilized an independent approach to inhibit β-catenin signaling using siRNA-mediated downregulation of Ctnnb1 (β-catenin).
Importantly, β-catenin knockdown also led to decreased expression
of the ATI marker T1α as well as reduced ENO1 and PDIA3 expression in cultured AT cells, whereas CBR2 expression was
restored, thus further corroborating the previous findings achieved
by pharmacological inhibition (Fig. 4C,D). In a complementary
approach, we evaluated whether further activation of Wnt/β-catenin
signaling leads to enhanced trans-differentiation of pmATII cells as
well as PDIA3 and ENO1 expression. To this end, we applied the
glycogen synthase kinase-3 (GSK3) inhibitor CT99021, which is a
well-known activator of β-catenin (Uhl et al., 2015). Indeed, we
observed an induction of T1α, ENO1 and PDIA3; however, this did
not reach statistical significance, indicating that intrinsic activated
β-catenin signaling might already have reached maximal induction
(supplementary material Fig. S4).

Taken together, our data strongly support the notion that active
β-catenin signaling regulates ENO1, PDIA3 and CBR2 protein
eexpression in alveolar epithelial cells in vitro.

Bleomycin-induced lung injury regulates CBR2, ENO1 and
PDIA3 levels in pmATII cells

Next, we aimed to investigate whether the newly identified proteins
are involved in alveolar epithelial cell injury and repair processes in vivo.
Therefore, we utilized the murine bleomycin-induced lung
injury model, in which active Wnt/β-catenin signaling has been
demonstrated in alveolar epithelial cells (Königshoff et al., 2009;
Flozak et al., 2010). We isolated pmATII cells at day 7 and day 14
after instillation and subsequently analyzed the freshly isolated cell

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n.d., not determined. Values indicate normalized fluorescence in arbitrary units.
population (d0) on mRNA (Fig. 5A-C) and protein (Fig. 5D) level. The analysis revealed a significant reduction of Cbr2 mRNA expression in pmATII cells derived from bleomycin-instilled mice compared to phosphate-buffered saline (PBS)-treated mice with a concurrent reduction in ATII-cell-marker Sftpc expression (Fig. 5A; Cbr2: ΔCt d7 PBS 7.48±0.06 s.e.m.; ΔCt d7 BLEO 5.02±0.46, P<0.001; ΔCt d14 PBS 7.18±0.15 s.e.m.; ΔCt d14 BLEO 5.98±0.18 s.e.m., P<0.01; Sftpc: ΔCt d7 PBS 10.96±0.45 s.e.m.; ΔCt d7 BLEO 8.57±0.59 s.e.m., P=ns; ΔCt d14 PBS 11.36±0.52 s.e.m.; ΔCt d14 BLEO 8.92±0.78 s.e.m., P<0.05). Importantly, combined analysis of Cbr2 and Sftpc expression using a linear regression model revealed a significant correlation of the expression of both proteins (r²: 0.3697; P-value: 0.0162) (Fig. 5B), suggesting that similar regulatory pathways are involved in their expression. Similarly, we found increased levels of Eno1 and T1α at day 7 as well as at day 14 (Fig. 5C; Eno1: ΔCt d14 PBS −0.38±0.17 s.e.m.; ΔCt d14 BLEO 1.43±0.29 s.e.m., P<0.05; T1α: ΔCt d14 PBS −1.67±0.43 s.e.m.; ΔCt d14 BLEO 0.85±0.35, P=0.001), whereas Pdia3 levels were decreased at day 7 but increased at day 14 upon bleomycin-induced lung injury (Fig. 5C; Pdia3: ΔCt d7 PBS 2.11±0.08 s.e.m.; ΔCt d7 BLEO 1.69±0.06, P<0.01; ΔCt d14 PBS 1.9±0.03 s.e.m.; ΔCt d14 BLEO 2.18±0.08, P<0.001). These findings were further confirmed on protein level (Fig. 5D). We determined increased ENO1, PDIA3 and T1α protein expression accompanied by decreased CBR2 and proSFTPC protein expression in injured ATII cells isolated 14 days after bleomycin-induced lung injury compared to PBS-treated lungs (Fig. 5D). These data suggest that there is an ongoing epithelial cell wound repair attempt in vivo as early as 7 days after induction of injury and that this response is characterized by increased expression of ENO1, PDIA3 and T1α.

**siRNA-mediated knockdown of ENO1 and PDIA3 reduces differentiation of pmATII cells**

Finally, we asked whether modulation of ENO1 or PDIA3 expression impacts the trans-differentiation process from pmATII cells towards a more ATI-cell-like phenotype. Therefore, we induced siRNA-mediated knockdown of ENO1 and PDIA3, respectively, in pmATII cells. As shown in Fig. 6A, the treatment of pmATII cells using an ENO1-targeting siRNA pool significantly reduced ENO1 expression until day 5 [scrambled control siRNA (siScr) 78.4±9.31

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**Fig. 3. Wnt/β-catenin pathway is activated during ATII-to-ATI cell trans-differentiation.** pmATII cells were isolated and cultured for the indicated time points (days). (A,B) Cells were lysed using T-Per lysis buffer containing protease inhibitors and 15 µg of total protein per sample was subjected to immunoblot analysis. β-actin expression served as loading control. A representative experiment and a densitometric analysis of at least three independent experiments is shown. ABC, active β-catenin. (C) mRNA expression of Wnt/β-catenin target genes. (D) mRNA expression of canonical Wnt ligands. mRNA expression was measured by qRT-PCR and normalized to Hprt. Data represent means of ΔCt values±s.e.m. of at least three independent experiments. Means at indicated time points were compared to day 1 (d1) using one-way ANOVA, followed by the Dunnett’s post-hoc test. Significance: *P<0.05; **P<0.01; ***P<0.001.
Notably, ENO1 knockdown significantly inhibited T1α induction compared to pmATII cells treated with the scrambled control (siScr 61.39±4.19 s.e.m. % of control, \(P<0.001\)). Importantly, knockdown of either ENO1 or PDIA3 did not affect cell viability in T1α-expressing cells, as shown by WST-1 analysis (Fig. 6C). Taken together, these data suggest a functional role of ENO1 and PDIA3 for the process of β-catenin-driven ATII-to-ATI cell transdifferentiation, and injury and repair, \textit{in vitro} and \textit{in vivo}.

**DISCUSSION**

Alveolar epithelial injury and remodeling is strongly associated with chronic lung diseases. To date, several studies have demonstrated that the population of ATII cells possesses properties of stem cells within the adult lung alveolar epithelium \textit{in vivo} (Adamson and Bowden, 1974; Chapman et al., 2011; Barkauskas et al., 2013;...
Treutlein et al., 2014; Desai et al., 2014; Vaughan et al., 2015). In-depth characterization of alveolar epithelial subpopulations and their function during homeostasis and disease is hampered by a limited number of marker proteins. Here, we aimed to identify previously unknown alveolar epithelial cell proteins and thus get deeper insight into the molecular mechanism and programs of alveolar epithelial cell differentiation, which is a prerequisite for proper repair induction. To this end, we initially utilized the model of murine ATII-to-ATI cell trans-differentiation in primary culture in vitro. We report, for the first time, that CBR2, ENO1 and PDIA3 are functional alveolar epithelial cell proteins. We further investigated the relevance of these proteins for alveolar epithelial cell trans-differentiation and cellular plasticity functionally in vitro as well as in the bleomycin-induced lung injury mouse model in vivo, demonstrating their involvement in alveolar epithelial injury and repair mechanisms in a β-catenin-dependent manner.

The trans-differentiation of primary ATII cells into ATI cells in vitro is a widely applied model to investigate alveolar epithelial

Fig. 5. ENO1, PDIA3 and CBR2 expression is altered in injured pmATII cells. Mice were instilled with either PBS or bleomycin (BLEO) (5 U/kg body weight). At day 7 or day 14 after instillation, mice were sacrificed and lungs of four PBS- and four bleomycin-treated mice were pooled for pmATII cell isolation. (A) Freshly isolated pmATII cells (day 0) were analyzed for mRNA expression of Cbr2 and Sftpc. (B) Correlation analysis of mRNA expression of Cbr2 and Sftpc using a linear regression model. Data points represent ΔCt values for the respective genes. The corresponding regression line is indicated in red. 95% confidence intervals are depicted by a gray scattered line. r² and P-value is given in the graph for the compared variables. (C) Eno1, Pdia3 and T1α mRNA expression is shown using qRT-PCR. Means of the indicated groups were compared using one-way ANOVA, followed by Bonferroni multiple-comparison test. Significance: *P<0.05; **P<0.01; ***P<0.001; ns, not significant. (D) Protein expression of the indicated proteins in freshly isolated pmATII cells from PBS-treated or BLEO-treated mice at day 14 after isolation. β-actin expression served as loading control.
cell phenotypes and function, and primary ATII cells from various species have been used (Flozak et al., 2010; Zhao et al., 2013; Ghosh et al., 2013; Marconett et al., 2013). In the study presented here, we detect a strong decrease of surfactant protein expression on mRNA level as well as on protein level, whereas levels of the ATI-cell-specific protein T1\(\alpha\) increased and structural epithelial markers were stably expressed. Taken together, this suggests a change in epithelial characteristics in the direction of an ATI-like phenotype (Fig. 1B,C). These data, gained from murine ATII cells, are in line with studies largely using primary rat ATII cells that also demonstrated the loss of ATII-cell-specific proteins accompanied by an increase of ATI cell genes and stable expression of structural epithelial cell markers, such as cytokeratin (Flozak et al., 2010; Wang et al., 2013; DeMaio et al., 2009; Marconett et al., 2013). Here, we mainly based our ATI-cell-like definition on the expression of T1\(\alpha\) along with typical morphological changes in the AT phenotype over culture. Although other ATI cell markers have been described, including aquaporin 5, HOP homeobox (Hopx1) or receptor for advanced glycosylation end products (RAGE), T1\(\alpha\) has been established as a robust ATI cell marker conserved over species (Flozak et al., 2010; Zhao et al., 2013; Marconett et al., 2013; Barkauskas et al., 2013; Treutlein et al., 2014), has been associated with an ATI cell phenotype in vivo (Yee et al., 2006), and, most importantly, this has been confirmed in human lung tissue (Fujino et al., 2012; Ghaedi et al., 2014; Barkauskas et al., 2013; Marconett et al., 2013).

Although ATII cell plasticity and progenitor cell function is well-described in vitro and in vivo, data on ATI cell function and plasticity remain sparse. ATI cells are thought to be terminally differentiated cells (Crosby and Waters, 2010; Weibel, 2015). It has been reported, however, that ATI-like cells derived from in vitro culture are capable of switching and even in part reversing their phenotype (Borok et al., 1998; Gonzalez et al., 2009). Moreover, recent in vivo lineage-tracing data by Jain et al. demonstrate that ATI cells (labeled by Hopx1) can give rise to SFTPC-positive ATII cells after pneumonectomy in vivo as well as in lung organoid cultures.

**Fig. 6. The siRNA-mediated knockdown of ENO1 and PDIA3 inhibits ATII-to-ATI cell trans-differentiation.** pmATII cells were transfected at day 2 using an siRNA pool targeting *Eno1*, *Pdia3* and a scrambled (siScr) control sequence, respectively. Non-transfected cells served as additional control. At day 5 cells were lysed and subjected to immunoblot analysis. Knockdown efficiency at day 5 was determined by detection of ENO1 and PDIA3 protein, respectively. A representative experiment and a quantification of three independent experiments of T1\(\alpha\) expression upon (A) ENO1 and (B) PDIA3 knockdown are shown. Means were compared to time-matched transfection control (siScr) using one-way ANOVA followed by Bonferroni multiple-comparison test. Significance: *\(P<0.05\); ***\(P<0.001\). (C) Viability of pmATII cells transfected with siScr, siEno1 or siPdia3, respectively. Analysis was performed at day 3 and day 5 using the WST-1 assay. Data were measured in triplicates and were normalized to untreated control cells at the respective time points.
Involved in is not only a marker for ATI cells in the adult lung but has also been studied for its differentiation as well as induced injury is intriguing, because T1 α is crucial for organ injury and repair responses (Zhang et al., 2013). Furthermore, the co-regulation of ENO1 with T1 α is required for muscle regeneration after injury in a murine model (Lopez-Alemany et al., 2013). ENO1 was described as a serum biomarker in mice displaying bleomycin-induced lung injury, localizing to mitochondria of non-ciliated club cells and ciliated cells of the bronchioles, as well as ATII cells (Matsuura et al., 1994). Functionally, CBR2 is proposed to be involved in the metabolism of endogenous or inhaled carbonyl compounds (Ebert et al., 2015) and, interestingly, has also been described to be involved in cellular differentiation, such as adipocyte differentiation (Wenz et al., 1992). ENO1 has also been linked to migration and invasion of glioma cells (Song et al., 2014). ENO1 has also been described to exert its migratory function as a plasminogen receptor (Díaz-Ramos et al., 2012). Whether its functions in differentiation, such as adipocyte differentiation (Wenz et al., 1992), are limited and future studies addressing the role of CBR2 in this context might benefit from novel 3D tissue culture models, mimicking the natural special environment in the alveolus as well as providing potential for cell-cell interactions.

We further demonstrated the induction of ENO1 in the context of differentiation and alveolar epithelial injury in ATII cells. ENO1 is a key glycolytic enzyme, displaying various functions in eukaryotic as well as prokaryotic cells. ENO1 has been linked to migration and invasion of glioma cells (Song et al., 2014). ENO1 has also been described to exert its migratory function as a plasminogen receptor at the cellular surface, supporting the degradation of extracellular matrix molecules by plasmin (Díaz-Ramos et al., 2012). Whether its upregulation during alveolar epithelial cell differentiation is linked to a migratory phenotype associated with wound repair still remains elusive. Remarkably, ENO1 seems to be required for muscle regeneration after injury in a murine model (Lopez-Alemany et al., 2003), linking this protein to repair, which supports our observation of impaired trans-differentiation and possibly wound repair of ATII cells in the presence of siRNA-mediated knockdown of ENO1 (Fig. 6A). Moreover, ENO1 was described as a serum biomarker in hepatic fibrosis, which provides additional evidence that this protein is crucial for organ injury and repair responses (Zhang et al., 2013). Furthermore, the co-regulation of ENO1 with T1α in the context of differentiation as well as induced injury is intriguing, because T1α is not only a marker for ATII cells in the adult lung but has also been implicated in migration and wound repair in the skin (Krishnan et al., 2013; Honma et al., 2012).

Similar to ENO1, we found increased expression of PDIA3 involved in β-catenin-driven ATII-to-ATI cell trans-differentiation. PDIA3 exerts diverse functions based on its expression in different subcellular compartments (Turano et al., 2011). PDIA3 exhibits a major role as molecular chaperone involved in the quality control process for newly synthesized glycoproteins in the endoplasmic reticulum (ER), interacting with calnexin or calreticulin (Oliver et al., 1999). Interestingly, we also found increased calreticulin in our in vitro screen as well as in injured ATII cells in vivo (data not shown), which might suggest that both proteins are involved in cellular stress responses in vitro and in vivo. PDIA3 has previously been associated with ER stress following lung injury (Robertson et al., 2002), as well as enhanced apoptosis and dysregulated repair (Anathy et al., 2012). In renal fibrosis, PDIA3 has been shown to contribute to extracellular matrix accumulation (DiDazi et al., 2013), which supports our observation of increased PDIA3 expression in injured and fibrotic ATII cells from bleomycin-treated lungs 14 days after injury. Taken together, these data suggest that induction of PDIA3 during ATII-to-ATI cell trans-differentiation reflects an initial (impaired) repair response, which might ultimately result in fibrosis development.

The Wnt/β-catenin pathway represents a crucial component of the attempted repair response in lung injury and fibrosis. Active signaling of developmental pathways, including the Wnt/β-catenin pathway, has been demonstrated to be involved in lung tissue development (Beers and Morrisey, 2011) and in repair and regenerative processes (Königshoff and Eickelberg, 2010; Liu et al., 2011). In particular, Wnt/β-catenin signaling has been linked to insufficient or dysregulated repair in chronic lung disease, including IPF and COPD, as well as experimental models thereof (Selman et al., 2008; Kneidinger et al., 2011; Königshoff et al., 2009; Wang et al., 2011; Uhl et al., 2015). Here, we demonstrate activation of Wnt-ligand-dependent β-catenin signaling by increased ABC and phosphorylated DVL3 levels, as well as target gene induction, implying a Wnt-ligand-dependent mechanism (Fig. 3). These results are in agreement with findings in rat ATII by Flozak et al. (2010). Here, we report that the canonical Wnt ligands Wnt10a and Wnt10b represent potential Wnt ligands driving this process and future studies will clarify the contribution of different Wnt ligands. Interestingly, overexpression of Wnt10b has been shown to contribute to the development of skin fibrosis (Akhtmetshina et al., 2012). Furthermore, Wang and colleagues link the activation of Wnt/β-catenin signaling to a decreased expression of microRNA miR-375, regulating the expression of Wnt receptor frizzled 8 (FZD8), a predicted and confirmed target of miR-375 (Wang et al., 2013). Wnt/β-catenin signaling has been further identified and confirmed by a comprehensive transcriptomic and epigenomic analysis describing the Wnt/β-catenin pathway as concomitantly regulated in rat and human trans-differentiating ATII cells (Marconnet et al., 2013).

Notably, expression of the newly identified proteins CBR2, ENO1 and PDIA3 was altered upon pharmacological as well as molecular β-catenin inhibition (Fig. 4, supplementary material Fig. S3), suggesting that the newly identified proteins function downstream of β-catenin signaling and thus further corroborating that β-catenin is a key regulator of alveolar epithelial cellular plasticity. Of note, we observed stabilized CBR2 level upon β-catenin inhibition, a marker that is closely linked to SFTPC expression, thereby supporting the notion that β-catenin inhibition might also promote ATII cell homeostasis and function. Protein levels of SFTPC, however, were not detectable in our cultured cells, which might be due to a limited sensitivity of the protein analysis by western blotting. Moreover, other essential factors, such as stretch and 3D structure, are missing in our culture model. Future studies using advanced ATII-to-ATI cell trans-differentiation models are needed to further investigate SFTPC metabolism upon β-catenin, ENO1 and/or PDIA3 modulation.
It has to be pointed out that the involvement of additional developmental pathways, such as transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) signaling, in alveolar epithelial ATI-to-ATII cell trans-differentiation (Bhaskaran et al., 2007; Zhao et al., 2013) has been demonstrated. This suggests a high complexity of interacting pathways with potential direct or indirect crosstalk at multiple levels. Along this line, inhibition of β-catenin signaling has been shown by several groups to attenuate bleomycin-induced lung fibrosis in vivo (Ulsamer et al., 2012; Henderson et al., 2010). Thus, although initial β-catenin signaling seems to be relevant to initiate wound repair, crosstalk of Wnt/β-catenin with other pathways might interfere with proper repair and result in fibrosis (Königshoff and Eickelberg, 2010; Ulsamer et al., 2012; Tanjore et al., 2013; Lam et al., 2014). Future studies are needed to further delineate signaling crosstalk, which also needs to be considered for the development of novel therapies and drugs aiming to modulate impaired lung injury and repair.

In summary, our data indicate that the model of ATIII-to-ATII cell trans-differentiation in vitro is a suitable alveolar epithelial cell injury and wound repair model that closely mimics the ongoing repair attempts as observed in lung injury and fibrosis in vivo. We demonstrate that CBR2, ENO1 and Pdia3 are newly identified alveolar epithelial cell proteins involved in β-catenin-driven ATIILto-ATII cell trans-differentiation, thus contributing to alveolar epithelial cell plasticity in lung injury and fibrosis.

MATERIALS AND METHODS

Animals

Eight- to ten-week-old, pathogen-free female C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were used for all experiments, which were conducted according to the Ethics Committee guidelines of the Helmholtz Zentrum München and Government of Bavaria. Mice had free access to water and rodent laboratory chow. For the induction of lung injury, mice were subjected to intratracheal bleomycin instillation. Bleomycin sulfate (Almirall, Barcelona, Spain) was dissolved in sterile PBS and applied using the Micro-Sprayer Aerosolizer, Model IA-1C (Pen-Century, Wyndmoor, PA), as a single dose of 0.08 mg in 200 µl solution per animal (5 U/kg body weight). Control mice were treated with 200 µl PBS. Mice were sacrificed at day 7 or day 14 after instillation for collection of ATIIL cells.

Primary murine ATIIL cell isolation and culture

Primary murine (pm) ATIIL cell isolation was performed as previously described (Corti et al., 1996; Königshoff et al., 2009). In brief, lungs of 8- to 10-week-old, pathogen-free female C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were lavaged with 500 µl of PBS twice. Lungs were flushed through the right heart using 0.9% NaCl solution (B. Braun Melsungen AG, Melsungen, Germany), inflated with 1.5 ml air and minced, filtered through 100 µm, 20 µm and 10 µm nylon meshes (Sefar, Heiden, Switzerland) and the cell suspension was centrifuged at 200 g for 10 min. The cell pellet was resuspended in DMEM cell culture medium (Sigma-Aldrich) and negative selection for fibroblasts was performed by adherence for 25 min on cell culture dishes. Cells adherent cells were collected and negative selection for fibroblasts was performed by incubation of the cell suspension on Petri dishes coated with antibodies against CD45 and CD16/32 (both BD Biosciences) for 30 min at 37°C. Non-adherent cells were collected and negative selection for fibroblasts was performed by adherence for 25 min on cell culture dishes. Cells were collected and cell viability was assessed by trypan blue exclusion. Cell purity was assessed by immunofluorescence staining of cells cultured cultured overnight on chamber slides for purity control

Cell culture treatments

PKI115-584-mediated inhibition of β-catenin activity in pmATII cells was performed by treatment with a concentration of 1 µM beginning at day 1 of culture until day 3 and day 5, respectively. Control cells were treated with the same concentration of DMSO. Treatment and control media were refreshed at day 3. ICG-001 treatment (7.5 µM) was applied in the same manner. CT99021 treatment (2 µM) of cells was performed from day 1 to day 3. DMSO-treated cells served as control. The pharmacological inhibitors were purchased from the following companies: PKI115-584 (Santa Cruz), ICG-001 (Biomol) CT99021 (Tocris). siRNA-mediated downregulation of Cbb1, Eno1 or Pdia3 (ERp57) was performed using an siRNA pool of three target-specific siRNAs (Cbb1 siRNA, α-Enolase siRNA, ERp57 siRNA, Santa Cruz Biotechnology, Dallas, TX). Cells were transdifferentiated at day 2 after isolation using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer’s instructions. Cells transfected with a scrambled control siRNA (siScr) (Santa Cruz Biotechnology, Dallas, TX) served as control. Non-transfected cells served as additional control. Cells were analyzed at day 5. siRNA efficiency was confirmed on mRNA level (data not shown) and protein level (Figs 4C and 6A,B).

Cell viability assay

Cell viability analysis following ENO1 or Pdia3 siRNA-mediated knockdown was performed using the WST-1 assay (Roche, Basel, Switzerland) according to the manufacturer’s instructions. The assay was performed at day 3 and day 5 and measured in triplicates.

Immunofluorescence staining

Immunofluorescence staining was performed either on pmATII cells cultured overnight on chamber slides (BD Biosciences) for purity control or on cells cultured for 48 h on poly-l-lysine-covered coverslips for epithelial characterization. Cells were fixed with acetone/methanol (1:1), and blocked with 5% (w/vol) bovine serum albumin (BSA; Sigma-Aldrich) for 30 min. Cells were subsequently incubated with the respective primary antibody at RT for 1 h in PBS containing 0.1% (w/vol) BSA, followed by incubation with the fluorescently labeled secondary antibody (goat anti-rabbit Alexa Fluor 555, Life Technologies) and DAPI staining (Roche) was used to visualize cell nuclei. Primary antibodies applied are the following: proSFTPC (Merck Millipore, Darmstadt, Germany), ICG-001 (Biomol) CT99021 (Tocris). siRNA knockdown was performed using an siRNA pool of three target-specific siRNAs (Cbb1 siRNA, α-Enolase siRNA, ERp57 siRNA, Santa Cruz Biotechnology, Dallas, TX). Cells were trans-differentiated at day 2 after isolation using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer’s instructions. Cells transfected with a scrambled control siRNA (siScr) (Santa Cruz Biotechnology, Dallas, TX) served as control. Non-transfected cells served as additional control. Cells were analyzed at day 5. siRNA efficiency was confirmed on mRNA level (data not shown) and protein level (Figs 4C and 6A,B).

Quantitative (q)RT-PCR

Total RNA from pmATII was extracted using the QIAGEN RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA of all samples was generated by reverse transcription using SuperScriptII (Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green and the LightCycler 480 System (both Roche). Hypoxanthine guanine phosphoribosyl transferase (Hprt) was utilized as a reference gene in all qRT-PCR reactions. The following primers were used in a final concentration of 200 nM: Ager (NM_0074253; NM_001271422; NM_001271423), Cbb1 (NM_0074253), Eno1 (NM_0074253), Fox3 (NM_0074253).

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5′-CCACAGGACTAAGACCTGTAAA-3′; Pdia3 (NM_007952.2), fw 5′-AGCAGAGGTGTATGACTC-3′, rv 5′-CCATCATCAAGACCCGGCTCTC-3′; Sfpna (NM_021314.4), fw 5′-GGAGGCTCGGAAGAGGGGCCGC-3′, rv 5′-ATCTCCTGAACTGAACTCCCTC-3′; Sfpnc (NM_011359.2), fw 5′-AGGAAAGAGTCTGCTGAGA-3′, rv 5′-GAGCAGACCCCTCATCATTACA-3′; T1a (NM_010329.2), fw 5′-ACAGTGTCATCTGGAGGCTT-3′, rv 5′-TCTCTCTAAGGGGGCTCGTCGTC-3′; Tpl1 (NM_009386.2; NM_001163574.1), fw 5′-ACAGAGAATGCTGGGCATTCT-3′, rv 5′-AACCGCATTTGGGCTCTACAT-3′; Wnt3a (NM_009522.2), fw 5′-GGCCACCGTCCGAACAACA-3′, rv 5′-GGGTGCGGTTGTTGACGACACA-3′; Wnt10a (NM_009518.2), fw 5′-GGCCCATCTTACGAGGACTGT-3′, rv 5′-CGTCCGAACCGAACTGCTTC-3′; Wnt10b (NM_011718.2), fw 5′-TGGACGGACCGAGGTGTTGTA-3′, rv 5′-CTCGACGTICCATGGACATTTG-3′. Relative transcript levels are expressed in ΔCt values (ΔCt = reference − Ctarget) or log-fold change (ΔΔCt values).

**Immunoblotting**

Cells were washed twice with PBS (PAA Laboratories), lysed in T-PER lysis buffer (Thermo Fisher Scientific, Waltham, MA) supplemented with protease inhibitor cocktail tablets (Roche), and lysates were centrifuged at 5600 g at 4°C. Supernatant was collected and protein concentration was determined using the Quick Start Bradford Dye Reagent (Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat dry milk (Applichem) or 5% BSA (Sigma-Aldrich) in TRIS-buffered saline containing 0.05% (v/v) SDS, 20% (v/v) glycerol containing 2% (w/v) DTT and subsequently washed twice with 0.2% ampholytes and pH 3-10 (Bio-Rad Laboratories) together with 20 µg of sample for day 1, day 3 and day 5. IEF was performed under the following conditions: 100 V, 1 h; 250 V, 1 h; 750 V, 1 h; 1000 V, 1 h; 2500 V, 1 h; 12 kV, 1 h. The strips were maintained at ~80°C until further use. Prior to second dimension, the strips were equilibrated for 15 min in 10 ml equilibration buffer [6 M urea, 0.375 M Tris-HCl (pH 8.8), 2% (v/v) SDS, 20% (v/v) glycerol] containing 2% (w/v) DTT and subsequently for 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide. The separation in the second dimension was realized on SDS polyacrylamide gels (12.5%).

**Protein extraction for two-dimensional gel electrophoresis**

Primary ATII cells were washed twice with PBS (PAA Laboratories) and lysed in rehydration buffer (9 M urea, 4% CHAPS, 100 mM DTT) at RT. After centrifugation, the supernatant was collected and the protein concentrations were determined by the Total Protein Assay (Thermo Fisher Scientific, Waltham, MA) supplemented with 1% (v/v) SDS. Protein extracts were used. Prior to second dimension, the strips were equilibrated for 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide and digested with trypsin. The resulting peptides were concentrated on a Zip-Tip C18 column (Merck Millipore) and eluted onto an anchorpipet target for analysis on a Bruker Autoflex III MALDI TOF/TOF instrument (Billerica, MA). The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination and some of the peptides analyzed by MS/MS fragmentation for partial-peptide sequencing. For acquisition of peptide mass fingerprint spectra (PMF, MS), 3000 single-shot spectra were averaged and the peak finding was undertaken using the SNAP algorithm. Peptide fragmentation spectra (PFF, MS/MS) were acquired when possible. The MS and MS/MS spectra were combined and used for a MASCOT database search (MASCOT version 2.1.03) in the NCBI protein database. For PFF spectra, the mass tolerance was set at 60 ppm, allowing one missed cleavage site.

**Tryptic digestion and MALDI-TOF MS**

Proteins were identified using Pic’n’Post Protein identification service (Alphalyse, Odense, Denmark). Briefly, gel-extracted protein spots were reduced, alkylated with iodoacetamide, and digested with trypsin. The resulting peptides were concentrated on a Zip-Tip C18 column (Merck Millipore) and eluted onto an anchorpipet target for analysis on a Bruker Autoflex III MALDI TOF/TOF instrument (Billerica, MA). The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination and some of the peptides analyzed by MS/MS fragmentation for partial-peptide sequencing. For acquisition of peptide mass fingerprint spectra (PMF, MS), 3000 single-shot spectra were averaged and the peak finding was undertaken using the SNAP algorithm. Peptide fragmentation spectra (PFF, MS/MS) were acquired when possible. The MS and MS/MS spectra were combined and used for a MASCOT database search (MASCOT version 2.1.03) in the NCBI protein database. For PFF spectra, the mass tolerance was set at 60 ppm, allowing one missed cleavage site.

**Statistical analysis**

Results are presented as mean±s.e.m. and were considered statistically significant when P<0.05. Means of respective groups were compared using a one-way ANOVA, followed by the Dunnett’s post-hoc test or Bonferroni multiple-comparison test or two-tailed t-test as indicated in the figure legends. Linear regression analysis was used to determine correlation of mRNA expression of different genes. All statistical analysis was performed using GraphPad Prism5.

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The authors wish to thank all members of the Königshoff Laboratory for stimulating discussions, and particularly Cedric Thiel for experimental support and important discussions. Furthermore, we would like to especially thank Julia Kipp and Anastasia van den Berg for excellent technical assistance.

**Competition interests**

The authors declare no competing or financial interests.

**Author contributions**

K.M., M.K. and O.E. designed the experiments, analyzed the data and wrote the manuscript. K.M. performed the experiments. S.V. performed animal experiments. K.M., M.K. and O.E. designed the experiments, analyzed the data and wrote the manuscript. K.M. performed the experiments. S.V. performed animal experiments. K.M., M.K. and O.E. designed the experiments, analyzed the data and wrote the manuscript. K.M. performed the experiments. S.V. performed animal experiments.

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**Supplementary material**

Supplementary material available online at http://dmm.biologists.org/lookup/suppl doi:10.1242/dmm.019117/-/DC1

**References**


**Statistical analysis**

Results are presented as mean±s.e.m. and were considered statistically significant when P<0.05. Means of respective groups were compared using a one-way ANOVA, followed by the Dunnett’s post-hoc test or Bonferroni multiple-comparison test or two-tailed t-test as indicated in the figure legends. Linear regression analysis was used to determine correlation of mRNA expression of different genes. All statistical analysis was performed using GraphPad Prism5.

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The authors declare no competing or financial interests.

K.M., M.K. and O.E. designed the experiments, analyzed the data and wrote the manuscript. K.M. performed the experiments. S.V. performed animal experiments. J.M. performed 2DE analysis. K.M., M.K. and O.E. edited and revised the manuscript.

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Fig. S1. Expression of FoxM1 and Ager in pmATII cells. mRNA expressions of FoxM1 and Ager during culture of pmATII cells. mRNA levels were measured by quantitative RT-PCR (qRT-PCR) and normalized to Hprt as housekeeping gene. Data represent means of ΔΔCt values (log-fold change) + s.e.m. of at least 3 independent experiments. Means at indicated time points were compared to day 1 using one-way ANOVA, followed by Dunnett’s post hoc test. Significance: * P<0.05
Fig. S2. Co-expression of T1α and PDIA3 in pmATII cells. Immunofluorescence staining of pmATII cells on cover slips for T1α and PDIA3 at day 3 after isolation. Fluorescent images represent a 400 x magnification. The scale bar represents 50 μm. A merged image of the secondary antibody control is shown.
Fig. S3. β-catenin inhibition using ICG-001 alters ATII to ATI cell trans-differentiation along with CBR2, ENO1 and PDIA3 expression. (A) pmATII were treated with ICG-001 or DMSO as control at d1 after isolation until day 3 and day 5, respectively. Treated cells were lysed and subjected to immunoblot analysis. β-actin expression served as loading control. (A) A representative experiment is shown. (B) Densitometric analysis of at least 3 independent experiments using ICG-001 treatment. Means of the indicated groups were compared to time matched treatment controls using one-way ANOVA, followed by Bonferroni multiple comparison test. Significance: * P<0.05; ** P<0.01; *** P<0.001. ns not significant.
**Fig. S4. Treatment of pmATII cells with β-catenin activator CT99021**

(A) pmATII were treated with CT99021 (2µM), or DMSO as control at d1 after isolation until day 3. Treated cells were lysed and subjected to immunoblot analysis. β-actin expression served as loading control. (A) 3 independent experiments are shown. (B) Densitometric analysis of 3 independent experiments using CT99021 treatment. Means of the indicated groups were compared using a two-tailed, paired t-test.
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<td>Caldesmon 1</td>
<td>18043856</td>
<td>24</td>
<td>357</td>
<td>40%</td>
<td>QKEPDPTITDOSLGGPSR129 GRYEVEETTVIK154</td>
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<tr>
<td>11</td>
<td>Enolase 1</td>
<td>54673814</td>
<td>12</td>
<td>277</td>
<td>34%</td>
<td>AAVPSGASTGYEAEALR50 LAMQFMILPGASSR2179 YDLDFKSPDPDSR209 YITPDQLADLYK281</td>
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<tr>
<td>12</td>
<td>Aldehyde dehydrogenase 2</td>
<td>6753036</td>
<td>19</td>
<td>363</td>
<td>38%</td>
<td>LADLIERDR118 TIPDDGFFSYTR174 327TFVQNYDEFVR340 VGPSNPSR437</td>
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<tr>
<td>13</td>
<td>Serine hydroxymethyl transferase 2</td>
<td>21312298</td>
<td>22</td>
<td>338</td>
<td>39%</td>
<td>GELIASENFCSR96 SYEGYPGKR208 LIAGTSAAY100 NAQAMADALLKR568</td>
</tr>
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**SUPPLEMENTARY TABLE S1**

Proteins identified on 2D gel by MALDI-TOF MS

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**Disease Models & Mechanisms 8: doi:10.1242/dmm.019117: Supplementary Material**
<table>
<thead>
<tr>
<th></th>
<th>Protein Name</th>
<th>Start Position</th>
<th>End Position</th>
<th>Sequence Coverage</th>
<th>Sequence ID</th>
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<tbody>
<tr>
<td>14</td>
<td>ATP synthase</td>
<td>6680748</td>
<td>19 327</td>
<td>37%</td>
<td>47SFLKDPETSQR81</td>
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<tr>
<td>15/16</td>
<td>Lamin A/C</td>
<td>1794160</td>
<td>44 728</td>
<td>55%</td>
<td>12SGAASSSTPLSPTR23 25LQKEKDLQELNDR41 51LSElenaGR60 20NSNLVGAHEElQQSR296 132RIDSLSAQiSOLQK320 579LLEGEEELLR305 440VAVEEVDDEEGFV555</td>
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<tr>
<td>17</td>
<td>Mitochondrial phosphoenolpyruvate carboxykinase 2</td>
<td>28077029</td>
<td>22 225</td>
<td>35%</td>
<td>14DTPVPLLAGGAR33 26TLiHGHPQd3R275 26FDSEQGQR299 69HGVFVGSAMR396 518RLEGEDSAQETPIGLVPK525 22DFWQEQVR535</td>
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<tr>
<td>18</td>
<td>Mitochondrial phosphoenolpyruvate carboxykinase 2</td>
<td>28077029</td>
<td>21 228</td>
<td>35%</td>
<td>14DTPVPLLAGGAR33 26TLiHGHPQd3R275 26FDSEQGQR299 69HGVFVGSAMR396 518RLEGEDSAQETPIGLVPK525 22DFWQEQVR535</td>
</tr>
<tr>
<td>19</td>
<td>Dihydrolipoyl dehydrogenase</td>
<td>6014973</td>
<td>12 154</td>
<td>23%</td>
<td>13ALTGGAIAHLFK43 53GRIPVNR542 141GKFPFAANSR548 40VCAHAPLASEAFR495</td>
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<tr>
<td>20</td>
<td>Glutamate dehydrogenase 1</td>
<td>6680027</td>
<td>8 150</td>
<td>16%</td>
<td>6MVGFFDR76 12DDGSEWIEGYR136 40HGGTIPVPTAEQDR196</td>
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<tr>
<td>21</td>
<td>Annexin A11</td>
<td>7304885</td>
<td>18 112</td>
<td>33%</td>
<td>35DVQELYAAAGENR593 387AHLYAVfNEYQR598</td>
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<tr>
<td>22</td>
<td>Heat shock 70kD protein 5</td>
<td>29748016</td>
<td>27 548</td>
<td>36%</td>
<td>65ITPSYVAFTEPGEK25 169VTAVVTVPAYFNDQAR522 138INEPTAAAIAYGLDKR275 150AKfeelnMDLR357 354KSDIDEIVLGSGSTR384</td>
</tr>
<tr>
<td>23</td>
<td>Electron transferring flavoprotein</td>
<td>38142460</td>
<td>13 241</td>
<td>49%</td>
<td>66EIIA5VSGPL7QTIR28 165EDGGLLT124 171LKLPAVvTADLR136 222VSVISeEPQQR253</td>
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<tr>
<td>24</td>
<td>Malate dehydrogenase 2</td>
<td>31982186</td>
<td>6 203</td>
<td>21%</td>
<td>52GCDVVVIPGVP104 165HGVTTLDIV178 216VDPDQGQLATLIGR239</td>
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<tr>
<td>25</td>
<td>Heterogeneous nuclear ribonucleoprotein A2/B1/B0</td>
<td>23266713</td>
<td>8 155</td>
<td>28%</td>
<td>13IDTIIEIADR147 206GENGFSSDR253 214GGGGFPGPGGPR228</td>
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<tr>
<td>26</td>
<td>Caldesmon</td>
<td>18043856</td>
<td>25 233</td>
<td>43%</td>
<td>148QKEPdPTTDDGSLGPSR129 242QtenAFSPSR251 342LKEEIER458</td>
</tr>
<tr>
<td>27</td>
<td>Ornithine aminotransferase</td>
<td>8393866</td>
<td>9 237</td>
<td>21%</td>
<td>12KTEGPPSSEYIFER48 30TEQGPPSEYIFER46 40LAPPLVIKEDEI426</td>
</tr>
</tbody>
</table>