TISSUE ENGINEERING: Part A
Volume 00, Number 00, 2013

DOI: 10.1089/ten.tea.2012.0066

Original Article

Keratinocyte Growth Factor and Dexamethasone Plus Elevated cAMP Levels Synergistically Support Pluripotent Stem Cell Differentiation into Alveolar Epithelial Type II Cells

Sabrina Schmeckebier,[1,2,3,*] Christina Mauritz, DVM,[1,2,3,*] Katherina Katsirntaki,[1,2,3] Malte Sgodda, PhD,[2,4] Verena Puppe, MD,[2,4] Julia Duerr, PhD,[3,5] Susanne C. Schubert, PhD,[5] Andreas Schmiedl, MD, PhD,[2,6] Qiong Lin,[7] Jiri Paleček, PhD,[2,8] Gerald Draeger, PhD,[2,8] Matthias Ochs, MD,[2,3,6] Martin Zenke, PhD,[7] Tobias Cantz, MD,[2,4] Marcus A. Mall, MD,[3,5,9] and Ulrich Martin, PhD[1,2,3]

Alveolar epithelial type II (ATII)-like cells can be generated from murine embryonic stem cells (ESCs), although to date, no robust protocols applying specific differentiation factors are established. We hypothesized that the keratinocyte growth factor (KGF), an important mediator of lung organogenesis and primary ATII cell maturation and proliferation, together with dexamethasone, 8-bromoadenosine-cAMP, and isobutylmethylxanthine (DCI), which induce maturation of primary fetal ATII cells, also support the alveolar differentiation of murine ESCs. Here we demonstrate that the above stimuli synergistically potentiate the alveolar differentiation of ESCs as indicated by increased expression of the surfactant proteins (SP-) C and SP-B. This effect is most profound if KGF is supplied not only in the late stage, but at least also during the intermediate stage of differentiation. Our results indicate that KGF most likely does not enhance the generation of (mes)endodermal or NK2 homeobox 1 (Nkx2.1) expressing progenitor cells but rather, supported by DCI, accelerates further differentiation/maturation of respiratory progeny in the intermediate phase and maturation/proliferation of emerging ATII cells in the late stage of differentiation. Ultrastructural analyses confirmed the presence of ATII-like cells with intracellular composite and lamellar bodies. Finally, induced pluripotent stem cells (iPSCs) were generated from transgenic mice with ATII cell-specific lacZ reporter expression. Again, KGF and DCI synergistically increased SP-C and SP-B expression in iPSC cultures, and lacZ expressing ATII-like cells developed. In conclusion, ATII cell-specific reporter expression enabled the first reliable proof for the generation of murine iPSC-derived ATII cells. In addition, we have shown KGF and DCI to synergistically support the generation of ATII-like cells from ESCs and iPSCs. Combined application of these factors will facilitate more efficient generation of stem cell-derived ATII cells for future basic research and potential therapeutic application.

Introduction

Pluripotent stem cells (PSCs), like embryonic stem cells (ESCs) or induced PSCs (iPSCs), have the potential to differentiate into cell types of all three germ layers, including lung epithelial cells.[1–3] This makes them a potential source for novel cellular therapies targeting hereditary or acquired diseases of the airways and parenchyma of the lung.

Very recently, stepwise protocols for targeted differentiation of PSCs into definitive endoderm-derived lung

[1]Leibniz Research Laboratories for Biotechnology and Artificial Organs (LEBAO), Department for Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany.
[2]REBIRTH Cluster of Excellence.
[3]Member of the German Center for Lung Research (DZL).
[4]Junior Research Group Stem Cell Biology, Hannover Medical School, Hannover, Germany.
[5]Division of Pediatric Pulmonology & Allergy and Cystic Fibrosis Center, Department of Pediatrics III, University of Heidelberg, Heidelberg, Germany.
[6]Institute of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany.
[7]Institute for Biomedical Engineering-Cell Biology, Aachen University Medical School, RWTH Aachen, Aachen, Germany.
[8]Institute of Organic Chemistry and Centre of Biomolecular Drug Research (BMWZ), Leibniz University of Hannover, Hannover, Germany.
[9]Department of Translational Pulmonology, Translational Lung Research Center (TLRC-H), University of Heidelberg, Heidelberg, Germany.
*These authors contributed equally to this work.

progenitors were developed.[4,5] However, no robust protocols for subsequent specific differentiation into more mature respiratory lineages are available yet. The underlying reasons are manifold and include the lack of reliable approaches for detection and enrichment of specific cell types, such as alveolar epithelial type II (ATII) cells, as well as fragmentary knowledge concerning the factors and mechanisms involved in induction and control of differentiation into specific pulmonary cell lineages.[6] Accordingly, detection and quantification of respiratory PSC derivatives is sophisticated, barely reliable, and the differentiation protocols reported so far typically yield low proportions of putative lung epithelial-like cells.

So far, a very limited number of reports, only, addressed the targeted differentiation of ESCs into different respiratory epithelial cell lineages, with most of them focusing on the differentiation into ATII cells.[3,7–20] ATII cells are cuboidal cells of the alveoli and play a crucial role in lung homeostasis. They produce and secrete surfactant, a mixture of lipids and proteins, which is essential for the regulation of the alveolar surface tension and involved in host defense. Surfactant is assembled and stored in specific organelles, called lamellar bodies.[21,22] Whereas, the surfactant proteins (SP-) A, B, and D are not exclusively expressed in ATII cells, SP-C is considered to be ATII cell-specific.[23] Furthermore, ATII cells act as progenitor cells for ATI cells, thus being able to renew the alveolar epithelium.[24] Therapeutically, ATII cells generated from gene-corrected autologous iPSCs might be applicable, for instance, to replace the functionally compromised alveolar epithelium of patients with hereditary surfactant deficiencies.[25]

To further optimize current protocols for generation of PSC-derived ATII cells, novel detection tools, such as transgenic PSC lines with lineage-specific reporter expression, are urgently required. In addition, a better understanding of the underlying mechanisms of lung development is necessary, and mimicking the fetal lung environment *in vitro* might help to improve the differentiation efficiency. Known differentiation factors in lung development include matrix components as well as various soluble proteins, such as members of the fibroblast growth factor (FGF) family.[26] Among them, the keratinocyte growth factor (KGF, FGF-7), which is an important mediator of mesenchymal–epithelial interactions during lung development and injury, represents a promising key factor for alveolar *in vitro* differentiation and maturation. KGF is expressed in lung mesenchymal cells and interacts with its high-affinity receptor FGFR2-IIIb (KGFR) on lung epithelial cells during lung organogenesis.[27–32] Disruption of the KGF/KGFR signaling inhibits lung branching in fetal mouse and rat lungs.[29,31] Moreover, it has been shown that KGF has a mitogenic and maturational effect on isolated fetal ATII cells leading to increased synthesis of surfactant components.[33,34] In the adult lung, KGF instillation stimulates ATII cell proliferation and prevents bleomycin-induced lung injury.[35–37] Furthermore, the application of KGF maintains the phenotype of isolated adult ATII cells in culture by preventing and reversing their differentiation toward ATI cells.[35,38]

Other factors that are known to support maturation of fetal lungs and cultured primary pulmonary epithelial cells comprise glucocorticoids and cAMP. Already in 1969, it has been noticed that glucocorticoids not only cause premature delivery of fetuses, but also accelerate fetal lung maturation and the appearance of surfactant.[39] To date, glucocorticoids are routinely administered antenatal to reduce the incidence of respiratory distress syndrome in prematurely born infants due to their maturational effect on fetal lungs.[40] The effect of elevated cAMP levels on surfactant synthesis has already been recognized in 1987.[41,42] cAMP derivatives as well as compounds that elevate intracellular cAMP levels, such as isobutylmethylxanthine (a phosphodiesterase inhibitor), are now known to accelerate maturation of primary ATII cells.[43] Finally, the combination of all three factors, glucocorticoids plus cAMP and isobutylmethylxanthine has been shown to most effectively support maturation and maintenance of primary fetal and adult ATII cells.[44,45]

Based on the finding that the effect of glucocorticoids plus cAMP elevating agents on primary ATII cells can even be enhanced by the simultaneous application of KGF,[46] we hypothesized that KGF and a three-factor combination of the synthetic glucocorticoid dexamethasone plus 8-bromoadenosine-cAMP and isobutylmethylxanthine might also support the differentiation of PSCs into ATII cells. It was therefore the aim of the present study to determine the effect of KGF, DCI, or a combination of both on the differentiation of ATII-like cells from murine ESCs and iPSCs. Moreover, transgenic iPSC lines with ATII cell-specific lacZ expression have been established that enable reliable detection of iPSC-derived ATII cells and represent an important tool for identification of further key regulators of alveolar differentiation and for optimization of *in vitro* differentiation protocols.

Materials and Methods

Generation of iPSC clones from transgenic ATII cell reporter mice

iPSC clones, Alv6 and Alv11, were generated from a double-transgenic Clara cell secretory protein (CCSP)-rtTA2^s-M2/GFP-tetO$_7$-lacZ mouse. Transgenic mice were obtained by crossbreeding of the CCSP-rtTA2^s-M2 activator mouse line 38, which drives transgene expression predominantly in ATII cells,[47] with GFP-tetO$_7$-lacZ reporter mice.[48] For details see supplementary data (Supplementary data are available online at www.liebertpub.com/tea).

In vitro *differentiation of ESCs and iPSCs into ATII-like cells*

In brief, the murine ESC clone E14.1, 129/Ola with a GFP transgene targeted to the *brachyury* locus[49] was differentiated via embryoid body (EB) formation. The basal differentiation medium (BM) was supplemented at different time points with the following growth factors: 20 ng/mL recombinant human KGF (kindly provided by Amgen Inc., CA) and/or a three-factor combination of 10 nM dexamethasone (Sigma-Aldrich, Taufkirchen, Germany) plus 0.1 mM 8-bromoadenosine 3′:5′-cyclic monophosphate sodium salt (Sigma-Aldrich) and 0.1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), abbreviated as DCI. The medium was replaced every second to third day. An overview of the differentiation approaches is given in Figure 1. For direct comparison of ESCs with iPSCs (Alv6), both cell types were differentiated via EBs and either cultivated in BM without factors or in the presence of

20 ng/mL or 100 ng/mL KGF (d0–24) with or without DCI (d14–24) or DCI (d14–24) alone. For induction of lacZ expression, differentiating iPSCs (Alv6 and Alv11) were cultivated for additional 2 days (d24–26) and supplemented with 1 μg/mL doxycycline hyclate (Dox; Sigma-Aldrich). The medium was replaced every second to third day. An overview of the differentiation approaches is given in Figure 4A. For details see supplementary data.

Analysis of mRNA expression in ESC and iPSC differentiation cultures by quantitative reverse transcriptase polymerase chain reaction

Isolation of RNA and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were essentially performed as previously described.[50] For details see supplementary data.

Affimetrix microarrays of ESC differentiation cultures

Total RNA from ESC differentiation cultures either nontreated (BM) or treated with KGF (d0–24), DCI (d14–24), or the combination of KGF (d0–24) and DCI (d14–24) was isolated at three time points (d8, 17, and 24) as described in the supplementary data. For each differentiation setting, RNA from each of the three independent experiments was pooled ($n=3$) and subjected to microarray analysis. For details see supplementary data.

Immunohistochemical staining of ESC differentiation cultures

In brief, d24 ESC differentiation cultures were pelleted, fixed in 4% paraformaldehyde (Merck Chemicals, Darmstadt, Germany), embedded in Tissue-Tek (Sakura Finetek, Heppenheim, Germany), and cut into 8-μm cryosections. Immunohistochemical staining was then performed with a rabbit polyclonal immunoglobulin G anti-proSP-C antibody (AB28744, whole antiserum; Abcam, Cambridge, UK), and for control staining, adjacent cryosections were incubated with nonimmunized rabbit serum. For details see supplementary data.

Transmission electron microscopy of ESC and iPSC differentiation cultures

Transmission electron microscopy (TEM) was essentially performed as previously described.[3] For details see supplementary data.

β-galactosidase staining of mouse lungs and iPSC differentiation cultures

To induce reporter expression, 8-week-old CCSP-rtTA2^s-M2/GFP-tetO$_7$-lacZ mice were exposed to 2 mg/mL Dox dissolved in 5% sucrose and supplied as drinking water for 7 days. Subsequently, native lungs (no fixation, no preservation of normal lung architecture due to atelectasis) were explanted and stored at −80°C. Lungs were embedded in Tissue-Tek and cut into 8-μm cryosections. In iPSC differentiation cultures, ATII cell-specific lacZ expression was induced by the application of 1 μg/mL Dox from d24–26. Lung cryosections and d26 iPSCs differentiation cultures were then stained for *Escherichia coli* β-galactosidase activity with X-gal (see supplementary data).

Statistical analysis

GraphPad Prism 5 was used for statistical analysis. The data of each treatment were analyzed versus the respective nontreated (BM) control group with the nonparametric Mann–Whitney U test or in case of more than two groups with the nonparametric Kruskal–Wallis test followed by the Dunn's multiple comparison test. Values are given as mean ± SEM. Differences versus the BM control were considered significant at $p<0.05$.

Results

KGF and DCI synergistically enhance the expression of ATII cell marker genes during differentiation of ESCs

D24 ESC differentiation cultures were analyzed by qRT-PCR to assess the effect of KGF and DCI (experimental setup depicted in Fig. 1) on the expression of the most relevant distal lung epithelial markers (Fig. 2). The application of KGF only, to differentiating ESCs starting either at differentiation d0 (9.2 ± 1.9-fold increase vs. BM; $n=16$), d5 (6.5 ± 1.5-fold increase vs. BM; $n=9$), or d17 (4.2 ± 1.2-fold increase vs. BM; $n=9$) enhanced mRNA expression of the ATII cell-specific marker SP-C versus the BM control (Fig. 2A). The addition of the three-factor combination DCI from d5–24 (9.3 ± 4.1-fold increase vs. BM; $n=4$), d10–24 (9.0 ± 5.0-fold increase vs. BM; $n=3$), and d14–24 (4.6 ± 1.1-fold increase vs. BM; $n=6$) of differentiation also resulted in accelerated SP-C expression versus BM. The combined treatment with both, an early KGF supplementation (d0–24) and an application of DCI starting either at d5, 10, or 14, induced a greater than additive stimulating effect on SP-C expression. The significantly highest SP-C expression was synergistically induced by the combination of KGF applied during the whole differentiation and DCI supplementation either from d10–24 (48.5 ± 19.2-fold increase vs. BM; $n=13$) or d14–24 (42.8 ± 6.7-fold increase vs. BM; $n=16$) of differentiation. In combination with DCI, a similar range of SP-C expression was obtained in case of a later KGF application starting on d5 of differentiation (KGF d5–24 + DCI d14–24; 47.0 ± 18.9-fold increase vs. BM; $n=4$). Notably, KGF application restricted to the final stage of differentiation (d17–24) together with DCI (d14–24) still resulted in a synergistically enhanced SP-C expression. However, the expression level (20.9 ± 2.6-fold increase vs. BM; $n=4$) was lower as compared to an earlier KGF supplementation, indicating an additional KGF effect during the intermediate phase of differentiation. Interestingly, the synergistic interaction of KGF and DCI resulted in SP-C expression levels similar to untreated MLE12 cells, a murine ATII-like cell line.[51]

Comparable results were obtained for the other ATII cell marker SP-B (Fig. 2B). Already, the single treatment with either KGF or DCI increased SP-B mRNA expression versus BM. However, similar to SP-C, the highest SP-B expression levels were induced by the combined treatment with KGF and DCI. KGF (d0–24) with the addition of DCI from d10–24 (13.8 ± 3.8-fold increase vs. BM; $n=13$) or d14–24 (14.0 ± 2.5-fold increase vs. BM; $n=16$) of differentiation significantly augmented SP-B expression. KGF application starting on d5 of differentiation resulted in a comparable range of SP-B expression (KGF d5–24 + DCI d14–24; 14.8 ± 5.9-fold increase vs. BM; $n=4$). Again, KGF application restricted to

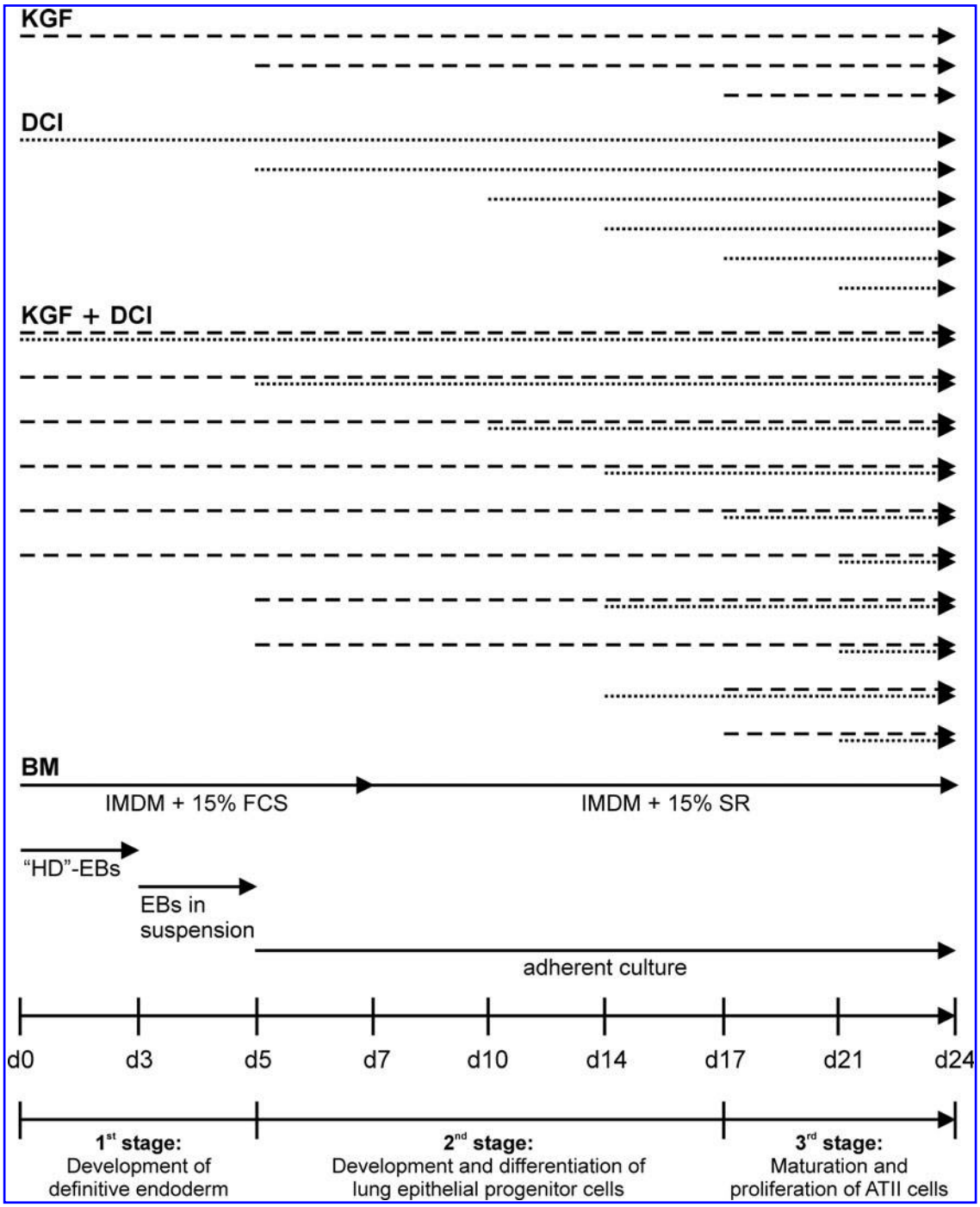

FIG. 1. Experimental setup for induction of alveolar differentiation of embryonic stem cells (ESCs). Differentiation of ESCs was initiated via hanging drop (HD)-based formation of embryoid bodies (EBs; d0), followed by transfer of the EBs to low-adherent culture dishes on d3, and thereafter, to adherent cultures dishes on d5. The basal differentiation medium (BM) was supplemented with keratinocyte growth factor (KGF) and/or dexamethasone plus 8-bromoadenosine 3′:5′-cyclic monophosphate sodium salt and 3-isobutyl-1-methylxanthine (DCI) starting at different time points, as indicated in the scheme. ESCs were differentiated for 24 days. SR, serum replacement.

the final stage of differentiation (d17–24) in combination with DCI (d14–24) was less effective than earlier KGF supplementation. Like SP-C, the SP-B expression levels were higher than the sum of the respective single treatments indicating a synergistic stimulating effect also on SP-B expression by the combined treatment with KGF and DCI.

Another marker for distal lung epithelium expressed in bronchial/bronchiolar epithelial Clara cells is CCSP. In contrast to SP-C and SP-B, the single treatment with KGF had obviously no inductive effect on CCSP mRNA expression (Fig. 2C). The highest CCSP expression levels, although not significant, were measured following the single application

of DCI starting at d10 (5.8 ± 1.6-fold increase vs. BM; $n = 3$), d14 (2.9 ± 0.9-fold increase vs. BM; $n = 6$), or d17 (5.0 ± 2.8-fold increase vs. BM; $n = 6$) of differentiation. The combined treatment with DCI and KGF (d0–24) resulted either in comparable or lower expression levels following DCI from d10–24 (1.4 ± 0.5-fold increase vs. BM; $n = 13$), d14–24 (3.2 ± 0.9-fold increase vs. BM; $n = 16$), or d17–24 (2.5 ± 1.0-fold increase vs. BM; $n = 12$) in relation to the respective single treatment with DCI. CCSP expression levels were even more reduced down to levels similar or below the BM, when DCI treatment (d14–24) was combined with later KGF supplementation (d5/17–24).

Although not significant in this experiment, qRT-PCR analyses also revealed a slightly enhanced expression of the NK2 homeobox 1 (Nkx2.1) transcription factor, which is expressed in lung epithelial progenitor cells and in both, mature ATII cells and Clara cells,[52] at day 24 of differentiation following application of KGF from d0–24 in combination with DCI from d14–24 of differentiation (2.1 ± 0.5-fold increase vs. BM; $n = 16$; Fig. 2D).

The combination of KGF (d0–24) and DCI (d14–24) was used for all further experiments concerning alveolar differentiation of PSCs. Analyzing the time course of SP-C mRNA expression during ESC differentiation (either depicted in

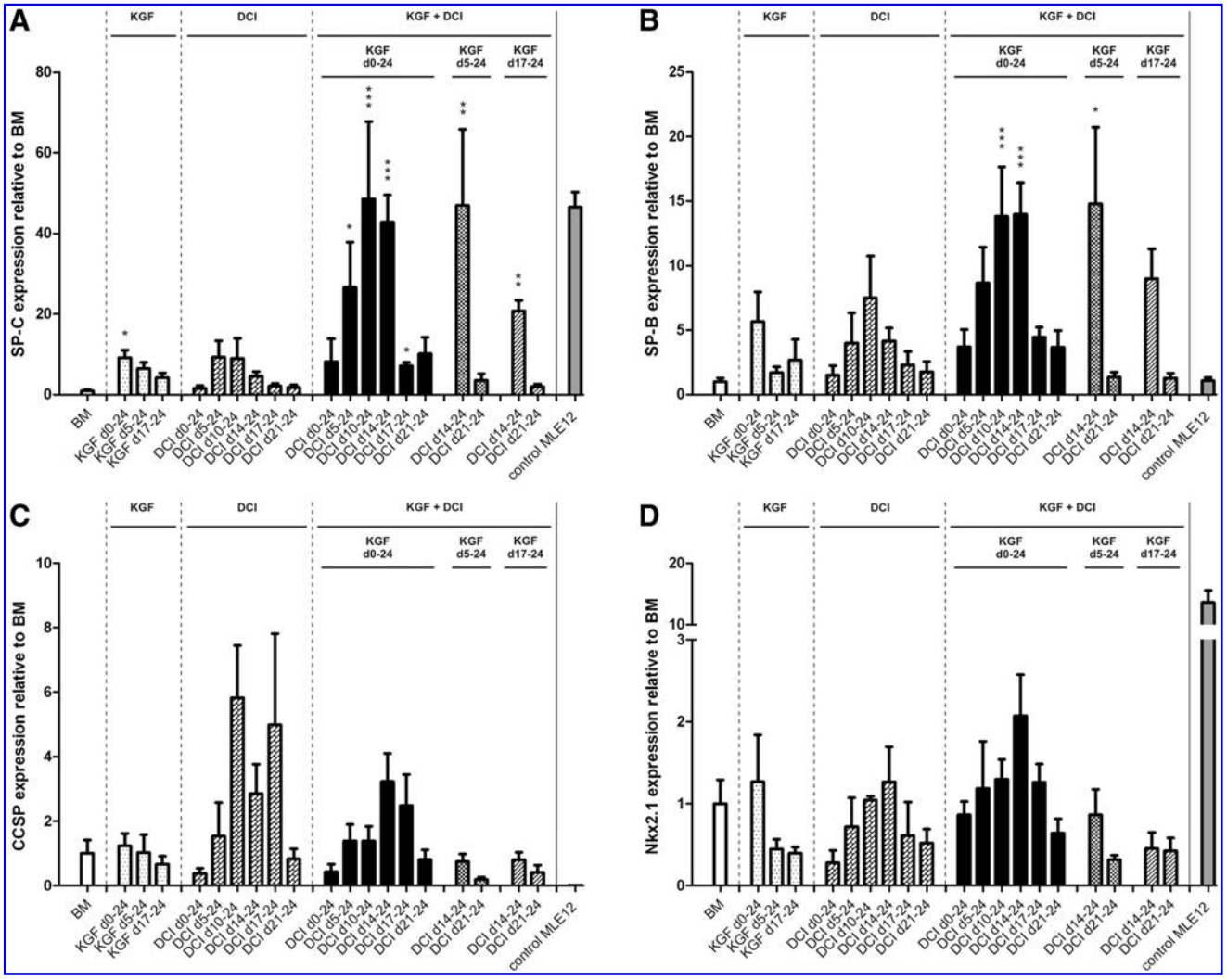

FIG. 2. KGF and DCI synergistically enhance alveolar epithelial type II (ATII) cell marker expression in ESC differentiation cultures. **(A–D)** D24 ESC differentiation cultures were analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). In contrast to surfactant proteins (SP)-C and SP-B, the highest Clara cell secretory protein (CCSP) mRNA expression was induced following DCI treatment without KGF. Depicted is the expression (mean ± SEM) of lung-specific markers following treatment in relation to BM, (mean set to level 1). Untreated ATII-like MLE12 cells served as reference for marker expression levels. **(A)** SP-C, **(B)** SP-B, **(C)** CCSP, and **(D)** NK2 homeobox 1 (Nkx2.1). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus BM. **(E)** Time course of SP-C mRNA expression (mean ± SEM, $n = 3$) during differentiation of ESCs treated with KGF (d0–24) and/or DCI (d14–24). Depicted is the fold-change expression over undifferentiated ESCs (fold-change of undifferentiated ESCs = 1). Untreated MLE12 cells served as reference for marker expression levels. Undiff., undifferentiated ESCs. $^{*}p < 0.05$ versus BM. **(F–I)** Depicted are cryosections of d24 ESC differentiation cultures treated with KGF (d0–24) and DCI (d14–24; **F,G**) and of adult mouse lung (**H,I**). **(F,H)** Anti-proSP-C rabbit serum. **(G,I)** Nonimmunized rabbit control serum. ProSP-C expressing cells (ATII cells) are colored in red. Nuclei are stained with hemalum solution. The same structure either stained with the proSP-C rabbit serum **(F)** or with the control serum on the adjacent cryosection **(G)** is marked by red arrows. Scale bars: 50 μm. Color images are available online at www.liebertpub.com/tea

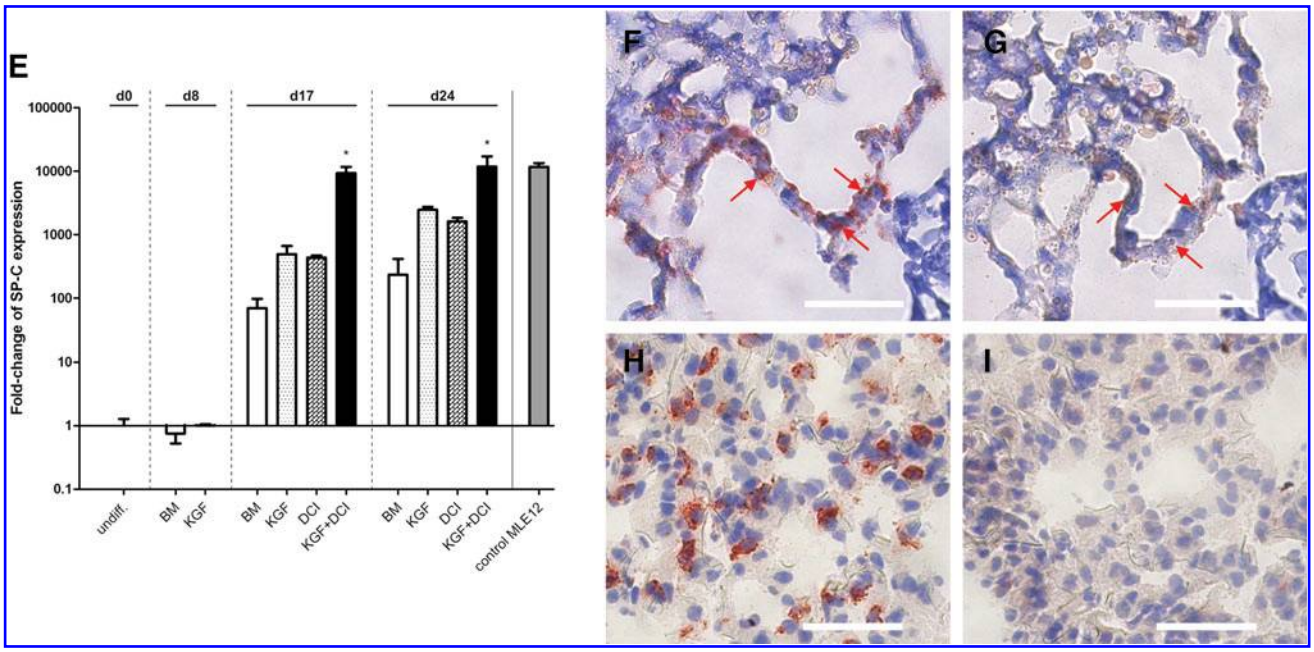

FIG. 2. (Continued).

Fig. 2E as fold-change over undifferentiated ESCs or in Supplementary Fig. S3A as relative expression normalized to β-actin), the treatment with KGF and DCI revealed a 11,876 ± 5,240-fold induction of SP-C expression on d24 of differentiation over undifferentiated ESCs (d0).

Gene array analyses demonstrate a disproportional high number of differentially expressed lung-associated genes in differentiating ESCs after KGF and DCI treatment

In support of the qRT-PCR data, microarray analyses, performed on d8, 17, and 24 ESC differentiation cultures treated either by the single factors KGF (d0–24) or DCI (d14–24) or by the combination of KGF (d0–24) and DCI (d14–24) versus the untreated (BM) control, underlined the stimulating effect of KGF plus DCI on the pulmonary differentiation of PSCs (Supplementary Figs. S4–7, Supplementary Tables S2–S16).

KGF supplementation starting at d0 of differentiation induced the differential expression of 74 probe sets representing lung-associated genes (selected from a lung developmental study of Kho *et al.*,[53] for more details please see supplementary data) on d8 of differentiation. Notably, KGF did not increase Nkx2.1 mRNA expression at day 8 of differentiation versus the BM control (Supplementary Table S2). This finding was in line with flow cytometrical analyses of differentiation cultures of Nkx2.1-GFP knockin reporter ESCs (Supplementary Fig. S3B).

On differentiation d17 and 24, the combined treatment with KGF and DCI resulted in the highest number of differentially expressed lung-associated probe sets, including those for SP-C (Supplementary Fig. S4A). Interestingly, the proportion of differentially expressed lung-associated genes among all differentially expressed genes was clearly higher than the proportion of all analyzed lung-associated genes among the entirety of genes evaluated (3,290 lung-associated probe sets among a total of 45,101 probe sets = 7.3%), especially following the single treatment with DCI (d17: 18.2%; d24: 17.4%) or in combination with KGF (d17: 17.1%; d24: 16.2%; Supplementary Fig. S4A).

A detailed list of all differentially up- and downregulated genes versus BM with corresponding GO terms is provided in Supplementary Tables S2–S16.

Immunohistochemical evidence of ATII-like cells in ESC differentiation cultures

A reliable detection of SP-C expressing cells via commercially available rabbit polyclonal anti-proSP-C sera in the heterogenic PSC-derived differentiation cultures was challenged by similar staining patterns frequently occurring following incubation with nonimmunized rabbit control sera also (Supplementary Fig. S8). Moreover, PSC differentiation cultures typically show high levels of autofluorescence. To obtain more reliable results, the differentiation cultures of interest were pelleted for cryosectioning to enable control staining of the same structure on adjacent cryosections. The anti-proSP-C staining was performed on every second cryosection, while nonimmunized rabbit control serum was applied to the corresponding adjacent cryosections. Furthermore, we used immunohistochemical instead of immunofluorescence staining to overcome the problem of autofluorescence.

Using this method, proSP-C^{pos} epithelial-like structures (red color, marked by arrows, Fig. 2F) were detected in d24 ESC differentiation cultures treated with KGF (d0–24) and DCI (d14–24). In contrast, the same structure in the adjacent cryosection stained negatively with the control serum (red arrows, Fig. 2G) underlining the specificity of the proSP-C staining. Slices of the adult mouse lung were stained for proSP-C as a positive control (Fig. 2H) or with the nonimmunized rabbit control serum (Fig. 2I).

Ultrastructural evidence of ATII-like cells in ESC differentiation cultures

To confirm the generation of ATII-like cells on an ultrastructural level, TEM was performed. D24 ESC differentiation cultures treated with the combination of KGF (d0–24) and DCI (d14–24) were processed for and analyzed by TEM. These differentiation cultures contained epithelial-like structures with a single layer of cuboidal nonciliated epithelial cells that might represent cells of the distal lung epithelium (Fig. 3A). These epithelial cells showed a clear basal to apical orientation with microvilli at their apical side. They had lateral cell–cell contacts, including tight junctions and resided on a basal lamina, which was adjoined to the subepithelial connective tissue. Figure 3B–F shows ATII-like cells with electron-dense, tightly packed organelles most likely representing lamellar bodies. Furthermore, some cells also showed composite body-like structures, the precursors of mature lamellar bodies (Fig. 3C,D). The ATII-like cell depicted in Figure 3E contained lipid droplets with one of those droplets forming an intermediate form with a lamellar body-like structure.

Alveolar differentiation of ATII cell reporter iPSCs

Finally, the established protocol was applied to reporter iPSCs (Fig. 4). These iPSCs were generated from mice with inducible lacZ expression under the control of a 2.3-kb rat CCSP promoter element. Interestingly, the cell type specificity of transgene expression from this promoter element appears to critically depend on the integration site, and different mouse lines showed transgene expression restricted to Clara cells, to ATII cells, or to both cell types.[54] The clonal CCSP-rtTA2^{s}-M2 activator mouse line 38, crossed with bi-transgenic reporter mice expressing luciferase and Cre recombinase, was previously shown to express luciferase after Dox induction predominantly in ATII cells.[47]

For the present study, the activator mouse line 38 was crossed with GFP-tetO$_7$-lacZ expressing reporter mice.[48] In these double-transgenic CCSP-rtTA2^{s}-M2/GFP-tetO$_7$-lacZ mice, reporter gene expression was induced in ~20% of ATII cells following Dox administration (Fig. 5A). We used a double-transgenic mouse to establish iPSC clones with an ATII cell-specific lacZ expression (Supplementary Fig. S1). Two generated iPSC clones, Alv6 and Alv11, were analyzed

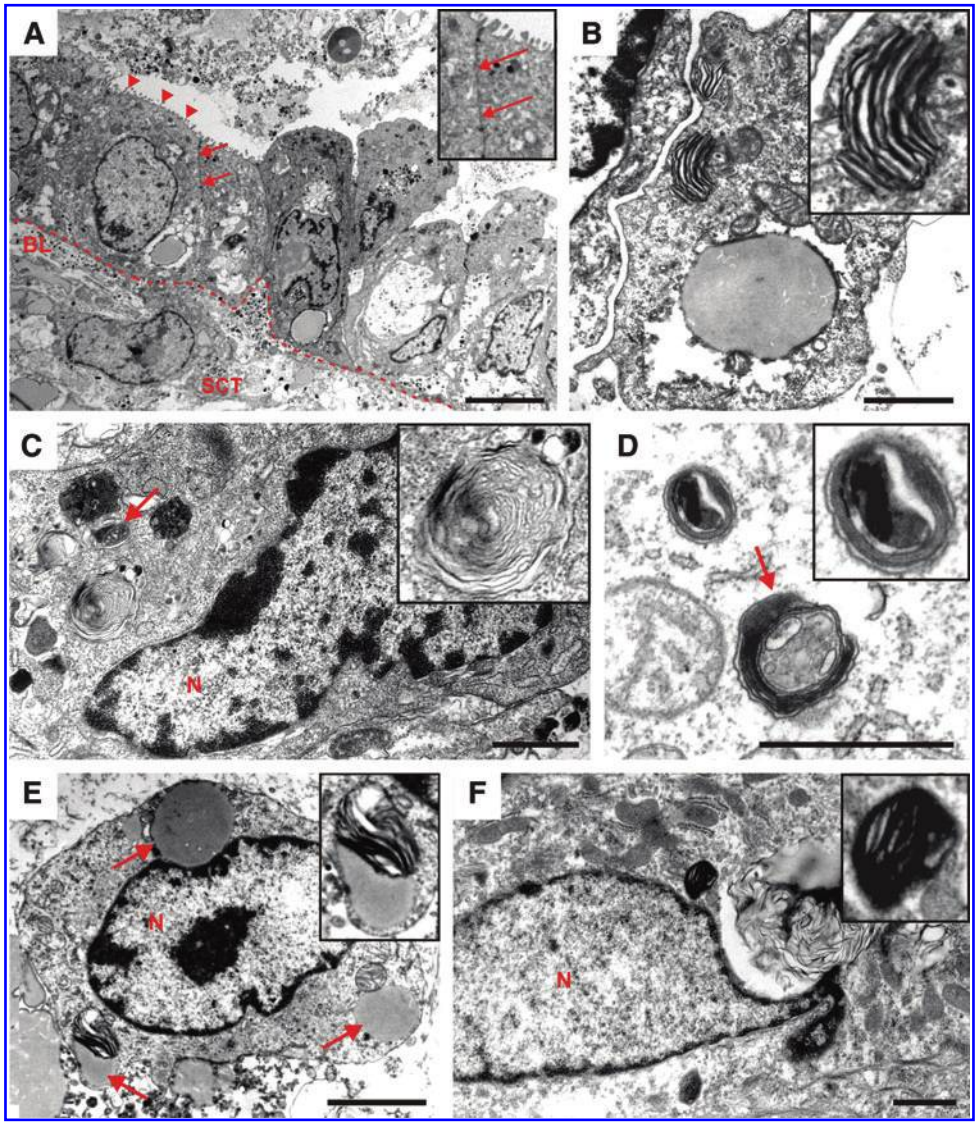

FIG. 3. Ultrastructure of ESC-derived ATII-like cells following differentiation with KGF and DCI. D24 ESC differentiation cultures were analyzed by Transmission electron microscopy (TEM). **(A)** Single layer of distal lung epithelium-like cuboidal nonciliated epithelial cells with microvilli (red arrowheads) at their apical side. The epithelial cells show lateral cell–cell contacts, including tight junctions (red arrows, inset in the upper right corner) and reside on a basal lamina (BL), which is adjoined to subepithelial connective tissue (SCT). Scale bar: 5 μm. **(B–F)** ATII-like cells with densely packed lamellar body-like structures (magnified in the upper right corner). Scale bars: 1 μm. **(C,D)** Composite body-like structures (red arrows) are additionally visible. **(E)** Lipid droplets (red arrows) and intermediate form of lipid droplet and lamellar body-like structure (magnified in the upper right corner). N, nucleus. Color images available online at www.liebertpub.com/tea

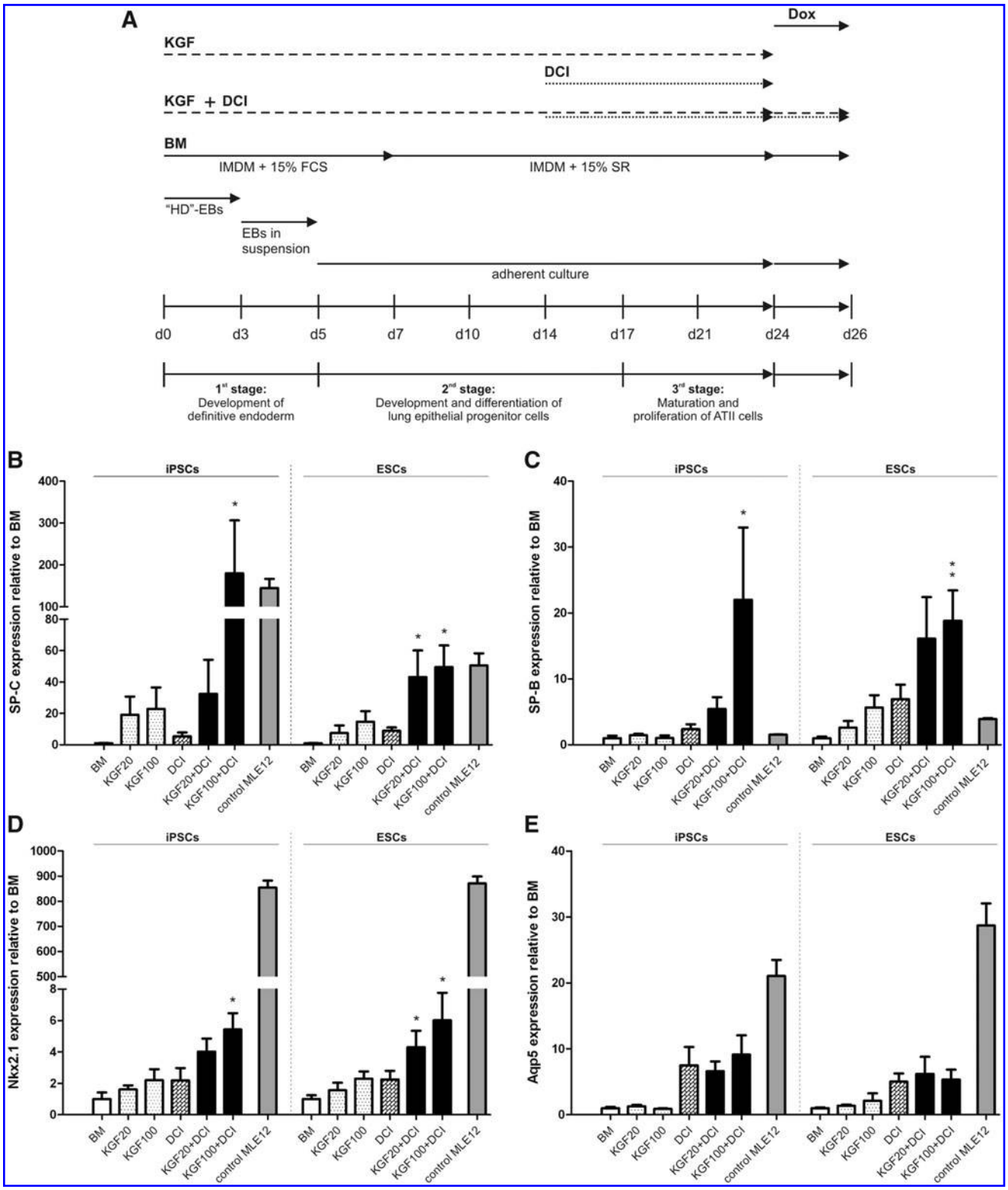

FIG. 4. KGF and DCI synergistically enhance ATII cell marker expression in induced pluripotent stem cell (iPSC) differentiation cultures. **(A)** iPSCs and ESCs, as control, were differentiated for 24 days. The BM was supplemented from d0–24 with 20 ng/mL (KGF20) or 100 ng/mL KGF (KGF100), and/or DCI (d14–24). To induce transgene expression, iPSCs were cultivated for additional 2 days in the presence of doxycycline hyclate (Dox) (d24–26). **(B–E)** KGF and DCI synergistically enhance ATII cell marker expression in d24 iPSC (Alv6) differentiation cultures as measured by qRT-PCR. Depicted is the expression (mean ± SEM) of lung-specific markers following treatment in relation to BM (mean set to level 1). Untreated ATII-like MLE12 cells served as reference for marker expression levels. **(B)** SP-C, **(C)** SP-B, **(D)** Nkx2.1, and **(E)** aquaporin 5 (Aqp5). $^{*}p < 0.05$, $^{**}p < 0.01$, versus BM.

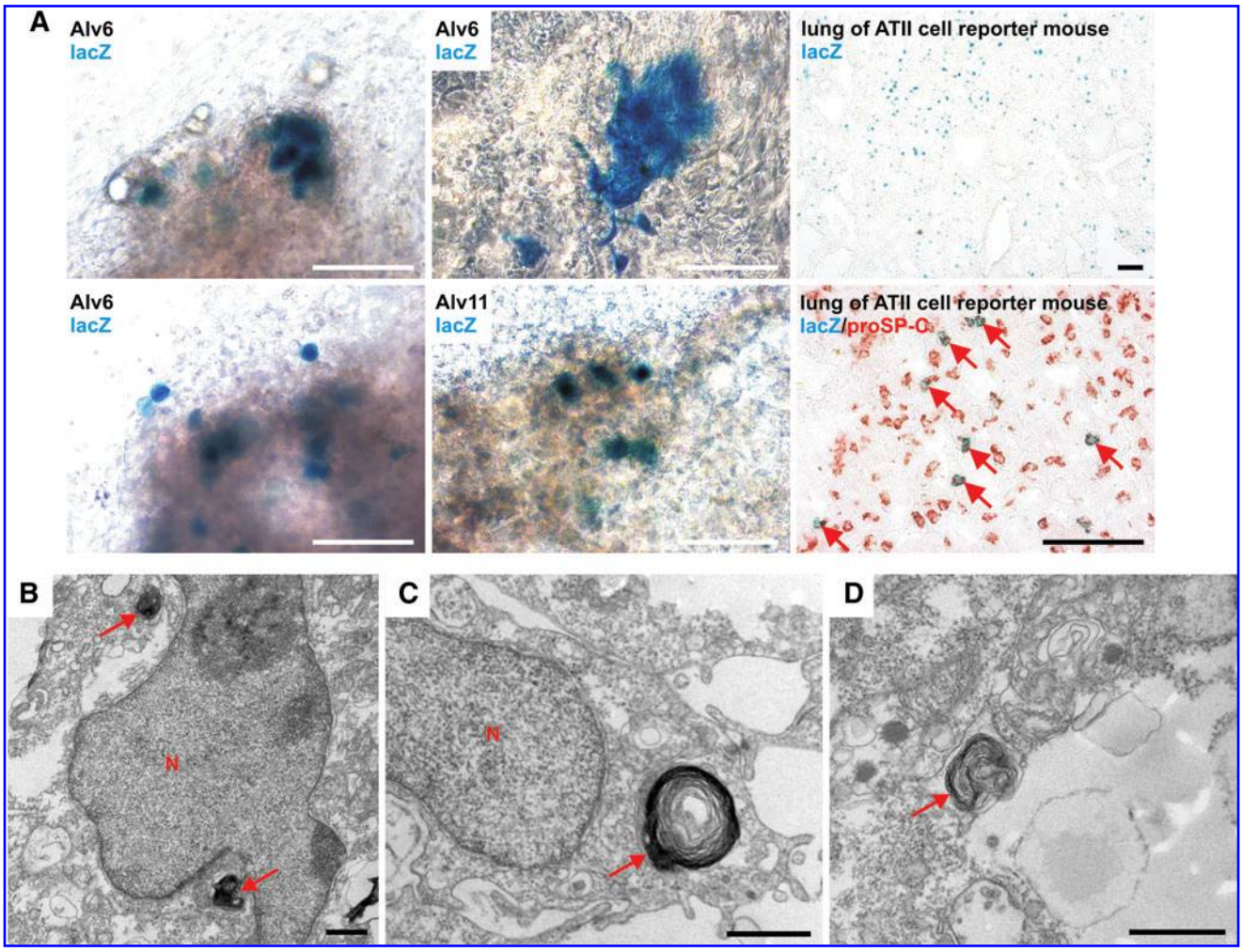

FIG. 5. ATII cell reporter iPSCs differentiate into lacZ expressing ATII-like cells. **(A)** X-gal stained lacZ-positive cells (blue) in d26 differentiation cultures of iPSC clones Alv6 and Alv11. Both clones are derived from an ATII cell reporter mouse (CCSP-rtTA2^s/GFP-tetO$_7$-lacZ), which shows lacZ (X-gal, blue) expression in ATII cells (proSP-C, red) following Dox treatment (see lung of ATII cell reporter mouse on the right). LacZ and proSP-C double-positive ATII cells are marked by red arrows. Scale bars: 100 μm. **(B-D)** TEM pictures of ATII-like cells in X-gal-positive areas of d26 iPSC (Alv6) differentiation cultures. ATII-like cells containing lamellar body-like structures (red arrows). **(B)** The lower lamellar body-like structure seems to fuse with a multivesicular body. **(D)** Cell with a lamellar body-like structure at the beginning of exocytosis. N, nucleus. Scale bars: 0.5 μm. Color images available online at www.liebertpub.com/tea

in more detail. Pluripotency of both iPSC clones was demonstrated by the expression of the pluripotency-associated markers Oct4, Sox2, Nanog, stage-specific embryonic antigen 1 and alkaline phosphatase, and the ability to form teratomas (Supplementary Fig. S2).

Next, iPSCs (Alv6) were differentiated in the presence of KGF (d0–24), DCI (d14–24), or the combination of KGF (d0–24) and DCI (d14–24) versus the untreated (BM) control. In parallel, ESCs were differentiated as well (experimental setup, see Fig. 4A). In addition to 20 ng/mL, a fivefold higher concentration of KGF was applied to test whether this has an even potentiating effect on lung epithelial marker expression.

In line with the former data for ESCs, the application of 20 ng/mL KGF and DCI to differentiating iPSCs synergistically induced SP-C mRNA (32.5 ± 21.7-fold increase vs. BM; $n = 4$) and SP-B mRNA (5.4 ± 1.8-fold increase vs. BM; $n = 4$) expression measured on d24 (Fig. 4B,C). By increasing the KGF concentration to 100 ng/mL, the synergistic effect on ATII cell markers was strongly accelerated resulting in a 179.7 ± 126.5-fold higher SP-C ($n = 4$) and a 22.0 ± 11.0-fold higher SP-B ($n = 4$) expression versus the BM control. In contrast, ESCs did not respond to the elevated KGF concentration as the iPSCs did (Fig. 4B,C).

Nkx2.1 mRNA expression was highest following the combined treatment with KGF and DCI mediating a slightly synergistic stimulating effect. In combination with DCI, 20 ng/mL KGF induced a 4.0 ± 0.8-fold higher (vs. BM; $n = 4$) and 100 ng/mL KGF induced a 5.4 ± 1.0-fold higher (vs. BM; $n = 4$) Nkx2.1 expression in d24 iPSC differentiation cultures (Fig. 4D). Similar expression levels were detected in d24 ESC cultures following combined treatment with DCI and 20 ng/mL KGF (4.3 ± 1.0-fold increase vs. BM; $n = 4$) or 100 ng/mL KGF (6.0 ± 1.8-fold increase vs. BM; $n = 4$). Notably, in this experiment, the overall expression level of Nkx2.1 was

considerably lower compared to the former experiment (Fig. 2D) resulting in a much higher expression level in MLE12 cells relative to the respective BM control.

Concerning the ATI cell marker aquaporin 5 (Aqp5),[38] the single treatment with DCI (d14–24) induced a 7.5±2.8-fold ($n=4$) higher expression in iPSC cultures and a 5.0±1.3-fold ($n=4$) higher expression in ESC cultures compared to the respective BM without factors (Fig. 4E). The additional presence of KGF (d0–24) had no further stimulating effect on the Aqp5 expression, not even in the presence of 100 ng/mL of KGF.

Finally, both iPSC clones were differentiated in the presence of KGF (d0–26) plus DCI (d14–26) to induce ATII cell reporter gene expression and indeed, lacZ expressing cells could be detected in d26 differentiation cultures of both iPSC clones (Fig. 5A). Similar to differentiated ESCs, the TEM analysis of the lacZ-positive areas revealed cells with lamellar body-like structures (Fig. 5B–D).

Discussion

Current protocols for alveolar differentiation of PSCs are typically inefficient, the lack of specific and reliable detection and enrichment tools complicates the definite proof of PSC-derived ATII cells,[6] and valid data demonstrating a significant effect of specific inductive factors during alveolar differentiation of PSCs are scarce.

With respect to these methodological restrictions, we now aimed at (1) identifying key factors for the differentiation of PSCs into ATII-like cells and (2) generating transgenic iPSC lines expressing reporters specific for ATII cells, both prerequisites for the development of a more efficient and robust differentiation of PSCs into functional ATII cells.

The use of KGF in our differentiation protocol was based on the hypothesis that this factor may specifically induce proliferation/maturation of PSC-derived ATII cells similar to primary ATII cells.[27–38] Indeed, KGF application led to considerably increased SP-C expression after 24 days of differentiation. However, as opposed to late KGF application starting on d17 of differentiation, this increase was significant, only, in case of an earlier onset of KGF application.

Similar to the treatment of primary ATII cells,[46] the supportive effect of KGF on the ATII cell differentiation of PSCs, as suggested by elevated SP-C and SP-B expression on d24 of differentiation, was potentiated in both, ESC and iPSC differentiation cultures by the additional supplementation with the three-factor combination DCI (Figs. 2A,B and 4B,C). Also, in combination with DCI, KGF was most effective in case of earlier application.

This result suggests that KGF, in addition to the hypothesized very late effect on maturation and proliferation of PSC-derived ATII cells, exerts another effect at least in the intermediate phase of differentiation (second stage, Fig. 6). Arguing against an additional very early KGF effect on the formation of (mes)endoderm (first stage, Fig. 6), and confirming recent data of Peterslund and Serup,[55] no increased formation of Bry-GFP^{pos} or Foxa2-huCD4^{pos} ESC derivatives was observed after early KGF application (Supplementary Fig. S3C,D). Moreover, despite a disproportional high number of differentially expressed lung-associated genes, KGF does obviously not affect the formation of Nkx2.1^{pos} progenitors (Supplementary Table S2 & Supplementary Fig. S3B).

Further studies will have to show whether KGF, supported by DCI, in the intermediate phase of alveolar differentiation directly promotes further differentiation and maturation of respiratory progeny, or whether it exerts indirect effects via unknown third party cell types that provide other inductive factors. Since FGFR2-IIIb, the only known receptor of KGF, is typically not expressed on embryonic mesenchyme,[56] at least KGF-activation of mesenchymal cells as the underlying mechanism appears unlikely.

In general, ESCs and iPSCs behaved quite similar; however, a direct comparison between iPSCs and ESCs revealed some differences. Whereas in ESC differentiation cultures, a fivefold higher concentration of KGF only slightly enhanced ATII cell marker expression compared to the standard concentration of 20 ng/mL KGF, application of 100 ng/mL KGF led to a four to sixfold increased expression of SP-C and SP-B in iPSC derivatives (Fig. 4B,C). However, we assume that this observation reflects cell line-specific variations, which are typically observed among ESC as well as iPSC lines,[57,58] rather than a specific feature of iPSCs.

During the revision of this manuscript, a study of Longmire *et al.* confirmed the general maturational effect of KGF

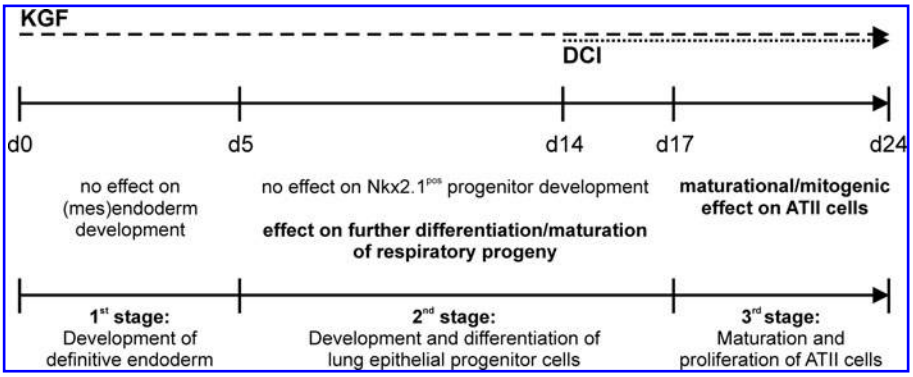

FIG. 6. Scheme of the potential effect of KGF plus DCI on the alveolar differentiation of PSCs. KGF applied in the second stage of PSC differentiation plus DCI most likely induce further differentiation/maturation of respiratory progeny rather than the development of Nkx2.1^{pos} progenitor cells. In the third and last stage of PSC differentiation, KGF together with DCI seems to support maturation and/or proliferation of the generated ATII cells. KGF supplementation also during the first stage of PSC differentiation might be useful for the alveolar differentiation, but is at least not mediated by accelerated (mes)endoderm formation.

and DCI on the progeny of purified murine ESC-derived Nkx2.1^{pos} progenitor cells, however, this was not analyzed in detail as in the present study, and KGF plus DCI was applied, only, as a late maturational stimulus during the last 3 days of differentiation.[5] Although, Longmire *et al.* applied KGF and DCI to cultures derived from pure populations of those Nkx2.1^{pos} progenitors, the induction of SP-C mRNA expression over undifferentiated ESCs was about 10 times less than in our nonpurified d24 ESC differentiation cultures following treatment with KGF and DCI (Fig. 2E). This result may confirm our observations and further supports the need for an earlier application.

It should be noted that any discrepancies of significance levels between different qRT-PCR analyses of the same marker are mostly due to from qRT-PCR to qRT-PCR varying numbers of repeated experiments per differentiation approach and varying numbers of differentiation approaches statistically analyzed.

In contrast to the ATII cell markers, the generation of ATI cells in iPSC and ESC differentiation cultures as indicated by Aqp5 mRNA expression was supported by DCI, but appeared not to be affected by KGF (Fig. 4E). A potential explanation for this result may be that KGF supplementation leads to a higher number of ATII cells that in turn could theoretically enhance ATI cell generation due to their putative ATI progenitor function, but as KGF simultaneously inhibits further differentiation into ATI cells as described for primary cells,[38] the total Aqp5 expression remains unaffected.

Furthermore, the single application of DCI seems also to be the most effective treatment for the stimulation of airway epithelial CCSP expression (Fig. 2C). Notably, the additional presence of KGF in the second and third (d5–24) or only in the third (d17–24) stage of ESC differentiation is apparently inhibitory to CCSP expression, or directly counteracts the DCI effect, exceeding recent findings on lung morphogenesis.[32] Interestingly, the additional presence of KGF also in the first phase of differentiation (d0–24) led to less reduced CCSP expression levels compared to KGF treatment starting as early as d5 or 17. One might speculate from the qRT-PCR data that in the latter case, earlier KGF stimulation of respiratory differentiation, either directly, or through third party cell types, matrix-bound or soluble factors, may antagonize the late inhibitory KGF effect on CCSP expression.

Moreover, we aimed at further confirming the appearance of SP-C expressing ESC-derived ATII-like cells using one of the commercially available anti-proSP-C rabbit sera. Clearly, the common anti-proSP-C sera represent valuable tools for the detection of ATII cells in histological lung sections, since tissue structure, cell localization, and morphology enable quite reliable discrimination from other pulmonary cell types. In contrast to other cell types with very characteristic staining patterns, such as cross-striation of cardiomyocytes,[2] clear-cut immunofluorescence-based identification of ATII cells is frequently impossible in differentiation cultures with their multitude of extrapulmonary lineages and the typical high degree of autofluorescence. Thus, the lack of appropriate controls, such as isotype controls or nonimmunized sera, may easily lead to false positive results and an overestimation of differentiation efficiencies. In our hands, staining with different lots of nonimmunized control sera of several manufacturers led to putative-positive cells in ESC differentiation cultures (Supplementary Fig. S8), a finding that emphasizes the need for appropriate controls to avoid misinterpretation of an immunostaining with anti-proSP-C antibodies.

Addressing the observed unspecific staining with rabbit immune sera, we did not directly stain our differentiation cultures. Instead, the adherent cells were scraped off from the culture dish, pelleted, and cryosectioned to enable alternate immunohistochemical staining of the same structure on adjacent cryosections with the anti-proSP-C serum and the control serum. Although this approach largely destroyed the grown morphology and prohibited quantification of proSP-C^{pos} cells, it enabled reliable detection of true proSP-C^{pos} epithelial-like cellular structures (Fig. 2F,G).

The presence of ESC-derived ATII-like cells was finally confirmed by ultrastructural analyses. After treatment with KGF (d0–24) and DCI (d14–24), the differentiation cultures contained epithelial structures showing features of distal (bronchiolar and alveolar) lung epithelium (Fig. 3A). ATII-like cells were observed, which contained composite body-like and more mature lamellar body-like structures (Fig. 3B–F), typical organelles of ATII cells.[21,22] The lamellar body-like structures had striking similarities with lamellar bodies of ATII cells in lungs of rodents.[59] Interestingly, some ATII-like cells exhibited lipid droplets and intermediate forms of lipid droplets and lamellar body-like structures (Fig. 3E), which have been reported as features of ATII cells under certain conditions.[59,60]

Finally, we have addressed the obvious lack of suitable ATII cell-specific reporter lines by using iPSCs generated from well defined transgenic reporter mice.[47] Although, controlled by a CCSP promoter element, reporter gene expression induced by the activator mouse line 38 is restricted to ATII cells instead of bronchial epithelium,[47] most likely due to positioning effects. Interestingly, however, lungs of the CCSP-rtTA2^s/GFP-tetO$_7$-lacZ double-transgenic mice express lacZ in ~20% of the ATII cells, only (Fig. 5A). While such nonuniform marker expression in ATII cells of transgenic mice is a known phenomenon,[61] it can be expected to impede quantification and will probably lead to underestimation of the percentage of ATII-like cells in differentiation cultures of iPSCs derived from these mice. Nevertheless, the lacZ expressing iPSC derivatives that were detected in d26 differentiation cultures of both tested iPSC clones clearly indicate the formation of ATII-like cells (Fig. 5A). The TEM-based detection of cells containing lamellar body-like structures in those X-gal-positive areas further confirmed the ATII cell phenotype (Fig. 5B–D). Thus, the generated iPSC lines represent a valuable tool for further optimization of alveolar differentiation protocols and identification of additional key differentiation factors. Moreover, the application of commercially available fluorescence substrates for *E. coli* β-galactosidase detection in living cells may enable transgene-based cell sorting and enrichment of iPSC-derived ATII-like cells.

Conclusion

In conclusion, this is the first report demonstrating the respiratory differentiation of iPSCs taking advantage of ATII cell-specific reporter expression. We have shown KGF and DCI to synergistically support the generation of ATII-like

cells from both, murine ESCs and iPSCs. Surprisingly, our data reveal the need for a supplementation with these key factors during PSC differentiation starting earlier as expected. The combined application of these factors will facilitate, as one important component, a more robust and efficient generation of PSC-derived ATII cells for future basic research and potential therapeutic application. Further studies will be required to investigate whether KGF and DCI evoke similar effects in human PSCs and whether our protocols can be directly applied to human ESCs and iPSCs.

Acknowledgments

We thank P. Gadue (Children's Hospital of Philadelphia) and G. Keller (McEwen Center for Regenerative Medicine, Ontario Cancer Institute, Toronto, Canada) for providing us with the transgenic murine Bry and Foxa2 ESC lines, and Amgen Inc., CA for providing us with the human recombinant KGF. We are grateful to J. Whitsett (Cincinnati Children's Hospital Medical Center) for the supply of the MLE12 cell line, D. N. Kotton (Boston University) for the transgenic murine Nkx2.1-GFP knockin reporter ESC line, and R. Sprengel (Max-Planck-Institute for Medical Research, Heidelberg, Germany) for the GFP-tetO$_7$-lacZ reporter mice. Finally, we would like to acknowledge the technical assistance of S. Menke, W. Schulze, J. Paijo, S. Hirtz, and J. Schatterny. This work was funded by the German Research Foundation (KFO123; MA 2331/5-1,-2,-3; MA 2081/4–1), by the German Federal Ministry of Education and Research (CARPuD, 01GM0854, 01GM0857; German Center for Lung Research [DZL], 82DZL00201, 82DZL00401), the Fritz Thyssen Stiftung and by the Cluster of Excellence "REBIRTH" (EXC 62/1).

Disclosure Statement

No competing financial interests exist.

References

1. Itsykson, P., Ilouz, N., Turetsky, T., Goldstein, R.S., Pera, M.F., Fishbein, I., *et al.* Derivation of neural precursors from human embryonic stem cells in the presence of noggin. Mol Cell Neurosci **30,** 24, 2005.
2. Mauritz, C., Schwanke, K., Reppel, M., Neef, S., Katsirntaki, K., Maier, L.S., *et al.* Generation of functional murine cardiac myocytes from induced pluripotent stem cells. Circulation **118,** 507, 2008.
3. Winkler, M.E., Mauritz, C., Groos, S., Kispert, A., Menke, S., Hoffmann, A., *et al.* Serum-free differentiation of murine embryonic stem cells into alveolar type II epithelial cells. Cloning Stem Cells **10,** 49, 2008.
4. Green, M.D., Chen, A., Nostro, M.C., d'Souza, S.L., Schaniel, C., Lemischka, I.R., *et al.* Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. Nat Biotechnol **29,** 267, 2011.
5. Longmire, T.A., Ikonomou, L., Hawkins, F., Christodoulou, C., Cao, Y., Jean, J.C., *et al.* Efficient derivation of purified lung and thyroid progenitors from embryonic stem cells. Cell Stem Cell **10,** 398, 2012.
6. Mauritz, C., and Martin, U. Embryonic stem cells: differentiation into respiratory cell derivatives. In: Singh, S.R., Mishra, P.K., and Hou, S.X., eds. Organogenesis and Cancer. Kerala, India: Transworld Research Network, 2010, pp. 17–38.
7. Ali, N.N., Edgar, A.J., Samadikuchaksaraei, A., Timson, C.M., Romanska, H.M., Polak, J.M., *et al.* Derivation of type II alveolar epithelial cells from murine embryonic stem cells. Tissue Eng **8,** 541, 2002.
8. Denham, M., Cole, T.J., and Mollard, R. Embryonic stem cells form glandular structures and express surfactant protein C following culture with dissociated fetal respiratory tissue. Am J Physiol Lung Cell Mol Physiol **290,** L1210, 2006.
9. Lin, Y.M., Zhang, A., Bismarck, A., and Bishop, A.E. Effects of fibroblast growth factors on the differentiation of the pulmonary progenitors from murine embryonic stem cells. Exp Lung Res **36,** 307, 2010.
10. Lin, Y.M., Zhang, A., Rippon, H.J., Bismarck, A., and Bishop, A.E. Tissue engineering of lung: the effect of extracellular matrix on the differentiation of embryonic stem cells to pneumocytes. Tissue Eng Part A **16,** 1515, 2010.
11. Qin, M., Tai, G., Collas, P., Polak, J.M., and Bishop, A.E. Cell extract-derived differentiation of embryonic stem cells. Stem Cells **23,** 712, 2005.
12. Rippon, H.J., Ali, N.N., Polak, J.M., and Bishop, A.E. Initial observations on the effect of medium composition on the differentiation of murine embryonic stem cells to alveolar type II cells. Cloning Stem Cells **6,** 49, 2004.
13. Rippon, H.J., Polak, J.M., Qin, M., and Bishop, A.E. Derivation of distal lung epithelial progenitors from murine embryonic stem cells using a novel three-step differentiation protocol. Stem Cells **24,** 1389, 2006.
14. Roszell, B., Mondrinos, M.J., Seaton, A., Simons, D.M., Koutzaki, S.H., Fong, G.H., *et al.* Efficient derivation of alveolar type II cells from embryonic stem cells for *in vivo* application. Tissue Eng Part A **15,** 3351, 2009.
15. Samadikuchaksaraei, A., and Bishop, A.E. Effects of growth factors on the differentiation of murine ESC into type II pneumocytes. Cloning Stem Cells **9,** 407, 2007.
16. Samadikuchaksaraei, A., Cohen, S., Isaac, K., Rippon, H.J., Polak, J.M., Bielby, R.C., *et al.* Derivation of distal airway epithelium from human embryonic stem cells. Tissue Eng **12,** 867, 2006.
17. Van Haute, L., De Block, G., Liebaers, I., Sermon, K., and De Rycke, M. Generation of lung epithelial-like tissue from human embryonic stem cells. Respir Res **10,** 105, 2009.
18. Van Vranken, B.E., Romanska, H.M., Polak, J.M., Rippon, H.J., Shannon, J.M., and Bishop, A.E. Coculture of embryonic stem cells with pulmonary mesenchyme: a microenvironment that promotes differentiation of pulmonary epithelium. Tissue Eng **11,** 1177, 2005.
19. Wang, D., Haviland, D.L., Burns, A.R., Zsigmond, E., and Wetsel, R.A. A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A **104,** 4449, 2007.
20. Wang, D., Morales, J.E., Calame, D.G., Alcorn, J.L., and Wetsel, R.A. Transplantation of human embryonic stem cell-derived alveolar epithelial type II cells abrogates acute lung injury in mice. Mol Ther **18,** 625, 2010.
21. Schmitz, G., and Muller, G. Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. J Lipid Res **32,** 1539, 1991.
22. Weaver, T.E., Na, C.L., and Stahlman, M. Biogenesis of lamellar bodies, lysosome-related organelles involved in storage and secretion of pulmonary surfactant. Semin Cell Dev Biol **13,** 263, 2002.

23. Zhou, L., Lim, L., Costa, R.H., and Whitsett, J.A. Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. J Histochem Cytochem **44,** 1183, 1996.
24. Fehrenbach, H. Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res **2,** 33, 2001.
25. Wert, S.E., Whitsett, J.A., and Nogee, L.M. Genetic disorders of surfactant dysfunction. Pediatr Dev Pathol **12,** 253, 2009.
26. Cardoso, W.V. Molecular regulation of lung development. Annu Rev Physiol **63,** 471, 2001.
27. Finch, P.W., Cunha, G.R., Rubin, J.S., Wong, J., and Ron, D. Pattern of keratinocyte growth factor and keratinocyte growth factor receptor expression during mouse fetal development suggests a role in mediating morphogenetic mesenchymal-epithelial interactions. Dev Dyn **203,** 223, 1995.
28. Mason, I.J., Fuller-Pace, F., Smith, R., and Dickson, C. FGF-7 (keratinocyte growth factor) expression during mouse development suggests roles in myogenesis, forebrain regionalisation and epithelial-mesenchymal interactions. Mech Dev **45,** 15, 1994.
29. Peters, K., Werner, S., Liao, X., Wert, S., Whitsett, J., and Williams, L. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. Embo J **13,** 3296, 1994.
30. Peters, K.G., Werner, S., Chen, G., and Williams, L.T. Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. Development **114,** 233, 1992.
31. Post, M., Souza, P., Liu, J., Tseu, I., Wang, J., Kuliszewski, M., *et al.* Keratinocyte growth factor and its receptor are involved in regulating early lung branching. Development **122,** 3107, 1996.
32. Shiratori, M., Oshika, E., Ung, L.P., Singh, G., Shinozuka, H., Warburton, D., *et al.* Keratinocyte growth factor and embryonic rat lung morphogenesis. Am J Respir Cell Mol Biol **15,** 328, 1996.
33. Chelly, N., Henrion, A., Pinteur, C., Chailley-Heu, B., and Bourbon, J.R. Role of keratinocyte growth factor in the control of surfactant synthesis by fetal lung mesenchyme. Endocrinology **142,** 1814, 2001.
34. Chelly, N., Mouhieddine-Gueddiche, O.B., Barlier-Mur, A.M., Chailley-Heu, B., and Bourbon, J.R. Keratinocyte growth factor enhances maturation of fetal rat lung type II cells. Am J Respir Cell Mol Biol **20,** 423, 1999.
35. Sugahara, K., Tokumine, J., Teruya, K., and Oshiro, T. Alveolar epithelial cells: differentiation and lung injury. Respirology **11 Suppl**, S28, 2006.
36. Ulich, T.R., Yi, E.S., Longmuir, K., Yin, S., Biltz, R., Morris, C.F., *et al.* Keratinocyte growth factor is a growth factor for type II pneumocytes *in vivo*. J Clin Invest **93,** 1298, 1994.
37. Yano, T., Mason, R.J., Pan, T., Deterding, R.R., Nielsen, L.D., and Shannon, J.M. KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in adult rat lung. Am J Physiol Lung Cell Mol Physiol **279,** L1146, 2000.
38. Borok, Z., Lubman, R.L., Danto, S.I., Zhang, X.L., Zabski, S.M., King, L.S., *et al.* Keratinocyte growth factor modulates alveolar epithelial cell phenotype *in vitro*: expression of aquaporin 5. Am J Respir Cell Mol Biol **18,** 554, 1998.
39. Liggins, G.C. Premature delivery of foetal lambs infused with glucocorticoids. J Endocrinol **45,** 515, 1969.
40. Roberts, D., and Dalziel, S. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane Database Syst Rev **3,** CD004454, 2006.
41. Odom, M.J., Snyder, J.M., and Mendelson, C.R. Adenosine 3′,5′-monophosphate analogs and beta-adrenergic agonists induce the synthesis of the major surfactant apoprotein in human fetal lung *in vitro*. Endocrinology **121,** 1155, 1987.
42. Whitsett, J.A., Pilot, T., Clark, J.C., and Weaver, T.E. Induction of surfactant protein in fetal lung. Effects of cAMP and dexamethasone on SAP-35 RNA and synthesis. J Biol Chem **262,** 5256, 1987.
43. Gonzales, L.W., Guttentag, S.H., Wade, K.C., Postle, A.D., and Ballard, P.L. Differentiation of human pulmonary type II cells *in vitro* by glucocorticoid plus cAMP. Am J Physiol Lung Cell Mol Physiol **283,** L940, 2002.
44. Ballard, P.L., Lee, J.W., Fang, X., Chapin, C., Allen, L., Segal, M.R., *et al.* Regulated gene expression in cultured type II cells of adult human lung. Am J Physiol Lung Cell Mol Physiol **299,** L36, 2010.
45. Gonzalez, R.F., Allen, L., and Dobbs, L.G. Rat alveolar type I cells proliferate, express OCT-4, and exhibit phenotypic plasticity *in vitro*. Am J Physiol Lung Cell Mol Physiol **297,** L1045, 2009.
46. Wang, J., Edeen, K., Manzer, R., Chang, Y., Wang, S., Chen, X., *et al.* Differentiated human alveolar epithelial cells and reversibility of their phenotype *in vitro*. Am J Respir Cell Mol Biol **36,** 661, 2007.
47. Duerr, J., Gruner, M., Schubert, S.C., Haberkorn, U., Bujard, H., and Mall, M.A. Use of a new-generation reverse tetracycline transactivator system for quantitative control of conditional gene expression in the murine lung. Am J Respir Cell Mol Biol **44,** 244, 2010.
48. Krestel, H.E., Mayford, M., Seeburg, P.H., and Sprengel, RA. GFP-equipped bidirectional expression module well suited for monitoring tetracycline-regulated gene expression in mouse. Nucleic Acids Res **29,** E39, 2001.
49. Fehling, H.J., Lacaud, G., Kubo, A., Kennedy, M., Robertson, S., Keller, G., *et al.* Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. Development **130,** 4217, 2003.
50. Mauritz, C., Martens, A., Rojas, S.V., Schnick, T., Rathert, C., Schecker, N., *et al.* Induced pluripotent stem cell (iPSC)-derived Flk-1 progenitor cells engraft, differentiate, and improve heart function in a mouse model of acute myocardial infarction. Eur Heart J **32,** 2634, 2011.
51. Wikenheiser, K.A., Vorbroker, D.K., Rice, W.R., Clark, J.C., Bachurski, C.J., Oie, H.K., *et al.* Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. Proc Natl Acad Sci U S A **90,** 11029, 1993.
52. Ikeda, K., Clark, J.C., Shaw-White, J.R., Stahlman, M.T., Boutell, C.J., and Whitsett, J.A. Gene structure and expression of human thyroid transcription factor-1 in respiratory epithelial cells. J Biol Chem **270,** 8108, 1995.
53. Kho, A.T., Bhattacharya, S., Mecham, B.H., Hong, J., Kohane, I.S., and Mariani, T.J. Expression profiles of the mouse lung identify a molecular signature of time-to-birth. Am J Respir Cell Mol Biol **40,** 47, 2009.
54. Perl, A.K., Tichelaar, J.W., and Whitsett, J.A. Conditional gene expression in the respiratory epithelium of the mouse. Transgenic Res **11,** 21, 2002.
55. Peterslund, J.M., and Serup, P. Activation of FGFR(IIIc) isoforms promotes activin-induced mesendoderm development in mouse embryonic stem cells and reduces Sox17 coexpression in EpCAM+ cells. Stem Cell Res **6,** 262, 2011.

56. Ornitz, D.M., and Itoh, N. Fibroblast growth factors. Genome Biol **2,** REVIEWS3005, 2001.
57. Bock, C., Kiskinis, E., Verstappen, G., Gu, H., Boulting, G., Smith, Z.D., *et al.* Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. Cell **144,** 439, 2011.
58. Boulting, G.L., Kiskinis, E., Croft, G.F., Amoroso, M.W., Oakley, D.H., Wainger, B.J., *et al.* A functionally characterized test set of human induced pluripotent stem cells. Nat Biotechnol **29,** 279, 2011.
59. Ochs, M. The closer we look the more we see? Quantitative microscopic analysis of the pulmonary ' system. Cell Physiol Biochem **25,** 27, 2010.
60. Bates, S.R., Tao, J.Q., Collins, H.L., Francone, O.L., and Rothblat, G.H. Pulmonary abnormalities due to ABCA1 deficiency in mice. Am J Physiol Lung Cell Mol Physiol **289,** L980, 2005.
61. Nesslein, L.L., Melton, K.R., Ikegami, M., Na, C.L., Wert, S.E., Rice, W.R., *et al.* Partial SP-B deficiency perturbs lung function and causes air space abnormalities. Am J Physiol Lung Cell Mol Physiol **288,** L1154, 2005.

Address correspondence to:
Ulrich Martin, PhD
Leibniz Research Laboratories for Biotechnology and Artificial Organs (LEBAO)
Hannover Medical School
Carl-Neuberg-Str.1
Hannover 30625
Germany

E-mail: martin.ulrich@mh-hannover.de

Received: February 2, 2012
Accepted: November 8, 2012
Online Publication Date: January 15, 2013