Chapter 6
Analysis of Epithelial Injury and Repair

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Abbreviations

α-SMA	Alpha smooth muscle actin
ALI	Acute lung injury
ABCA3	ATP-binding cassette sub-family A member 3
AQP5	Aquaporin 5
ATI	Alveolar epithelial type I cell
ATII	Alveolar epithelial type II cell
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
COPD	Chronic obstructive pulmonary disease
ECAD	E-cadherin, epithelial cadherin
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
EMT	Epithelial-to-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
GFP	Green fluorescent protein
FACS	Fluorescence Activated Cell Sorting
IPF	Idiopathic pulmonary fibrosis
KGF	Keratinocyte growth factor
LAMP3	Lysosome-associated membrane glycoprotein 3
PARP	Poly (ADP-ribose) polymerase
RAGE	Receptor for advanced glycosylation end products
SPA	Surfactant protein A
SPB	Surfactant protein B
SPC	Surfactant protein C
SPD	Surfactant protein D
TGF-β	Transforming growth factor beta

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T1α	Podoplanin
TJ	Tight junctions
TJP1	Tight junction protein 1
TUNEL	TdT-mediated dUTP-biotin nick end labeling
WNT	Wingless-type MMTV integration site family member

Introduction

Acute and chronic lung diseases constitute a significant health burden worldwide and a better and deeper understanding of the mechanisms that initiate and drive disease progression [1–3]. Alveolar epithelial injury represents a hallmark of acute lung injury (ALI) as well as chronic lung diseases such as idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) [1, 4, 5]. In the healthy adult lung, alveolar epithelial type I (ATI) and alveolar epithelial type II (ATII) cells are the main cell types that form the alveolar epithelium and establish the alveolar epithelial barrier [6]. ATI cells represent large, thin squamous epithelial cells that cover an enormous surface area (95 % of the alveolus) and are in close vicinity to the underlying capillary endothelium to facilitate gas exchange [7–9]. ATII cells, however, display a cuboidal shape and one of their main functions is the production, storage and release of surfactant. Surfactant consists of an intricate combination of proteins and lipids which lines the alveolar epithelium, lowers the surface tension in the lung and plays an important role in host defense mechanisms [10, 11]. Both ATI and ATII cells participate in ion transport in the lung and contribute to the fluid balance within the alveolus [7, 12, 13]. In ALI, the alveolar epithelial barrier, formed by ATI and ATII cells as well as endothelial cells of the alveolar capillary, represents the first point of injury. Disruption of the barrier structure with subsequent accumulation of protein-rich edema fluid in the alveolar air spaces is a main feature of ALI [14–16]. Tight junctions (TJ) localizing to the cell–cell junctions connecting alveolar epithelial cells are essential for normal epithelial barrier function [17]. ATII cells are a critical cell population driving repair in the alveolar epithelium [18]. ATII cells are able to proliferate, self-renew and serve as a progenitor cell population for ATI cells in injury and repair processes induced by a variety of different triggers. Thus, ATII cells are considered one of the important epithelial stem cell populations in the adult distal lung [19–22]. Restoration of the normal epithelial barrier requires the spreading and migration of cells in close proximity to the injury to cover the denuded basement membrane. This is followed by migration and proliferation of progenitor cells to compensate for the cellular loss. Finally differentiation processes have to be initiated to restore a functional epithelium [14, 23, 24]. However, the loss of reparative function of ATII cells and a shift towards pro-fibrotic functions has been described for ALI as well as for IPF [25–29]. The elucidation of mechanisms driving alveolar epithelial cell responses in a beneficial versus a potentially detrimental direction during lung

injury and repair is therefore of prime interest and the development of novel models and methods to study those mechanisms is of utmost importance.

Methods of Alveolar Epithelial Type II Cell Isolation

A prerequisite for analyzing alveolar epithelial type II cell characteristics and functional properties in vitro is the isolation of a pure population of the respective primary cell type. This requires the identification of specific ATII cell markers within the tissue together with morphology and localization. Several markers expressed in adult ATII cells have been described over the past decades. These include the surfactant proteins A, B, C, and D (SPA, SPB, SPC, and SPD), of which SPC has been reported to be specific for ATII cells [30]. ATII specific expression of ATP-binding cassette sub-family A member 3 (ABCA3), a membrane component of lamellar bodies [31], in which surfactant proteins are stored [32], has been reported. In addition, lysosome-associated membrane glycoprotein 3 (LAMP3) [33] and pepsinogen C [34] have been proposed as ATII markers. Immunization strategies by Boylan [35] and Gonzalez [36] used ATII cells as immunogens to generate monoclonal antibodies for ATII cell surface proteins for rat and human. The monoclonal antibody MMC4 recognized a novel antigen on the apical surface of rat ATII cells, but also bound to rat club cells [35]. An antibody generated against human ATII cells recognized a protein of 280- to 300-kDa on the apical plasma membrane, which was termed HTII-280 and, by analysis of its biochemical characteristics, represents an integral membrane protein [36].

Two main isolation strategies have been widely used to isolate ATII cells from rodent and human tissue. Both strategies share a common procedure of enzymatic dissociation of lung tissue to obtain a single cell suspension of the lung. Enzymes most frequently used include porcine elastase, dispase, or collagenase as well as different combinations thereof [37–40]. In case of the murine lung, enzymes are directly instilled into the parenchyma via the cannulated trachea [38]. In case of human tissue, direct instillation into the alveolar region via a bronchus can only be applied for closed lung segments [28, 39]. Alternatively, minced human distal lung tissue can be subjected to enzymatic digestion [40]. Following digestion, alveolar tissue is dissected from the lager airways and minced mechanically. After sequential filtration of cells through nylon meshes of different pore sizes ranging from 100 to 10 μm to obtain a single cell suspension [38, 39], ATII cells can be isolated via positive or negative selection or a combinatorial approach making use of positive as well as negative selection markers.

Several different separation methods can be applied subsequently using different marker combinations. The most commonly used methods for depletion of specific subset of cells include the use of antibody coated cell culture plates, where cells expressing the respective markers adhere to the plate and non-adherent cells are collected [41–43], or using antibodies coupled to magnetic beads [38, 44, 45], and similarly the non-bound cells are collected.

Antibodies directed against CD45, CD14, and CD16/32 for hematopoietic lineages such macrophages, neutrophils and lymphocytes are commonly used [21, 27, 38, 43, 45–47] for negative selection. Species-specific IgG antibodies binding with their Fc domain to the Fcγ-receptors on the cell surface of phagocytes, B-lymphocytes, natural killer cells and dendritic cells are also widely used to eliminate these cell populations from the preparation [41–43, 45, 48–50]. Depletion of CD31 positive endothelial cells is often included in different protocols [44]. Furthermore, a fluorescence activated cell sorting (FACS) based approach can be utilized by using fluorescently labeled antibodies and subsequent sorting of cells, displaying no positive signal for any of the utilized markers [51]. Isolation protocols applied for human ATII cells frequently contain previous enrichment of ATII cells for the downstream depletion strategy by subjecting the crude singe cell suspension after enzymatic digestion to a discontinuous Percoll density gradient (1.04–1.09 g/ml). After centrifugation ATII cells and macrophages will be found in the same layer of the Percoll gradient. The cells in this layer can be isolated and subsequently a depletion of alveolar macrophages can be performed [41, 42, 45].

Positive selection strategies are mainly applied for the isolation of lineage labeled ATII cells in murine mouse models by FACS. For this strategy SPC-driver lines are used, which express GFP or EGFP under the control of the SPC promoter [7, 52–54]. In this context, it has been shown that transgenic mice exhibiting GFP expression under the control of the human SPC promoter in the murine system display GFP expression in only a subset of ATII cells or additionally in bronchiolar epithelial cells [55, 56]. However, the use of murine SPC promoters was reported to generate a higher specificity of labeling ATII cells [52, 53] although the efficiency of labeling was also described to be dependent on the age of mice [52] and isolated ATII cell displayed some heterogeneity as cells with bronchiolar and alveolar epithelial gene signatures were detected under three-dimensional culture conditions [52]. The positive selection of human ATII cells by FACS using an antibody against the previously described HTII-280 membrane protein has been initially described by Gonzalez and colleagues [36].

Combined negative and positive selection strategies are applied using different combinations of negative depletion for of a variety of cell types, and are listed in Table 6.1. These markers include markers for cells from hematopoietic lineages [22, 28, 40, 57–60], and ATI cells (T1α positive cells) [40] and a positive selection for general epithelial markers such as EpCAM [22, 40, 57, 59–61] and E-cadherin [28, 58] or a lysosomal marker such as the fluorescent dye LysoTracker [59], labeling acidic organelles within live cells.

Overall, the different ATII cell isolation strategies result in some variability of cell purity (between 80 and 99 %). The choice of the appropriate isolation method is widely discussed in particular with respect to the utilization for the isolation of ATII cells from different models of lung disease or the subsequent usage of cells for different downstream applications (direct analysis versus culture for functional assays). When choosing which methods to apply, several points need to be taken under consideration. In general, the use of positive selection markers might result in higher cell purity—and better characterized cell (sub)populations, however, changes

Table 6.1 Markers for the depletion or enrichment of specific cell populations

	Marker	Targeted cell population	Reference
Negative selection *Depletion of unwanted cell populations*	CD45	Differentiated hematopoietic cells, except erythrocytes and platelets	[22, 27, 28, 38]
	CD16/32	Macrophages, monocytes, B-cells, NK cells, neutrophils, mast cells, dendritic cells	[38, 44, 46]
	CD11c	Macrophages, monocytes, NK cells, dendritic cells, granulocytes, subsets of B- and T-cells	[51]
	CD11b	Macrophages, monocytes, NK cells, dendritic cells, granulocytes, subsets of B- and T-cell	[51]
	F4/80	Macrophages	[51]
	CD14	Macrophages, monocytes, dendritic cells, granulocytes	[45, 60]
	CD19	B-cells, follicular dendritic cells	[51]
	CD31	Endothelial cells	[44]
	T1α	Alveolar epithelial type I cells	[40]
Positive selection *Enrichment for wanted cell population*	HTII-280	Alveolar epithelial type II cells	[22, 36]
	EpCAM	Epithelial cells	[22, 40, 59–61]
	ECAD	Epithelial cells	[28]
	SPC-GFP	SPC expressing alveolar epithelial type II cells and bronchiolar epithelial cells (lineage–labeled)	[28, 52, 53, 56]
	LysoTracker	Lysosomal rich cells	[59]

in the expression pattern of single specific markers used for selection might be altered in different disease models, which in turn changes the population analyzed.

Alveolar Epithelial Type II Cell Analysis

Obtaining insight into molecular mechanisms of alveolar epithelial injury and repair is of prime interest to identified potential targets for therapeutic intervention needed for the treatment of various lung diseases, including ARDS.

Alveolar Epithelial Type II Cell Culture

Analyzing freshly isolated ATII cells from rodent injury models or human diseased tissue using microarray technology is a powerful tool to determine disease related altered phenotypes in lung injury and repair [27, 40]. However, for functional analysis, the culture of primary ATII cells is of utmost importance. Challenged by

the fact that ATII cells possess the intrinsic properties to differentiate into ATI cells when placed into normal cell culture, a wide range of culture conditions and media compositions for ATII cells are described. A careful selection of culture conditions is crucial to obtain meaningful results. Depending on the applied assay, culture vessels as well as the presence of specific media supplements might influence experimental outcomes. Plating fresh ATII cells on plastic dishes induces the gradual loss of ATII cell characteristic [62, 63]. Coating cell culture dishes with extracellular matrix (ECM) components such as fibronectin, collagen or laminin or a combination thereof, will lead to differences in the dynamics of trans-differentiation processes. Additionally, culturing of ATII cells on trans-well filter inserts has been described to result in stabilized monolayers of ATII cells and allow cultures at the air liquid interphase [61]. Furthermore, the supplementation of commonly used cell culture media (e.g., DMEM or DMEM/F12) with KGF [64–67] and glucocorticoids in the combination with cAMP [68, 69] has been described to promote ATII cell phenotype in culture.

Alveolar Epithelial Type II Cell Proliferation

Due to their role as progenitor cells, the proliferative capacity of ATII cells is a critical feature in lung injury and repair processes within the lung. Thus, the assessment of proliferative behavior of this cell population is one of the most assessed cell characteristics. For the determination of in vitro proliferation capacity several different methods can be applied. Determination of gene and protein expression of genes related to cell cycle progression such as Ki67, Ccng1, and Ccng2 [27], are widely used to compare proliferative capacities of injured versus non-injured ATII cells and furthermore their response to different stimuli. The analysis of protein expression of proliferation markers Ki67 [27, 70], PCNA [71, 72] and phosphorylated histone H3 [27, 73] in cells in vitro as well as in in vivo models by immunofluorescence/immunohistochemistry represents a complementary approach. Direct functional assays for the detection of proliferating cells include the use of metabolic activity assays such as the WST-1 assay [74, 75] where a tetrazolium salt is converted in a colored formazan by endogenous dehydrogenates displaying a proportional relationship to cell number. Furthermore, the incorporation of bromodeoxyuridine (BrdU) [21] or [3H]thymidine [27] into the DNA of proliferating cells represents the gold standard for determining proliferation. Usage of several of these techniques provided insight in the reprogrammed phenotype and aberrant proliferative capacity of fibrotic ATII cells and the observation that targeting this phenotype attenuates pulmonary fibrosis in different models [27, 70].

Alveolar Epithelial Type II Cell Apoptosis

Besides changes in the proliferative behavior of injured ATII cells, the presence of apoptosis is another important parameter when analyzing injury and repair processes in the lung epithelium. The most commonly applied strategies are the analysis of caspase activity [76, 77] and the caspase mediated cleavage of endogenous substrates such as PARP [77], a crucial step in the apoptotic process. Furthermore, early apoptotic changes such as the flip of phosphatidylserine from the inside to the outside of the cell plasma membrane is used as a surrogate marker for apoptosis and detected by Annexin V binding and further analysis by flow cytometry [77]. TdT-mediated dUTP-biotin nick end labeling (TUNEL), a method to detect DNA fragmentation occurring in apoptotic cells, can be used for in vitro studies as well as for the detection of apoptotic cells in tissues of in vivo models of lung injury [70, 78]. The use of TUNEL staining provided data on the presence of increased numbers of apoptotic alveolar epithelial cells in fibrotic mouse models, as well as IPF tissue [70, 78, 79].

Epithelial to Mesenchymal Transition (EMT) of ATII Cells

Besides the described imbalance of proliferation and apoptosis in models of epithelial injury, the occurrence of EMT is widely discussed in the context of attempted alveolar repair processes. In vitro studies of EMT of cultured epithelial cells regularly use the cytokine TGF-β1 for EMT induction, which has been demonstrated in several organs including the lung [80–82]. Monitoring of decreased expression of epithelial marker genes such as E-Cadherin, cytokeratin and TJ-proteins is performed for the characterization of epithelial integrity on gene expression level as well as on protein level. Moreover, analysis should include the expression of EMT transcription factors, such as Snail, Slug, Zeb, or Twist as well as mesenchymal markers. Several mesenchymal markers, including αSMA, Calponin, and ECM related proteins such as collagen1, fibronectin and vimentin are used to describe the gain of mesenchymal cell characteristics [27, 80, 83, 84]. Importantly, co-expression of epithelial and mesenchymal markers by immunofluorescence/immunohistochemistry staining should be analyzed and has been demonstrated in human tissue of different lung diseases [28, 85, 86]. These descriptive investigations should be further complemented with functional cell assays, such as cell migration, which is a prominent feature of EMT.

For studying the in vivo relevance of findings generated by in vitro cultures, lineage tracing animals can be used to determine the cell fate in the context of lung injury. These studies utilize different transgene mouse strains, which express traceable markers under the control of the surfactant protein C promotor [19, 86, 87].

Alveolar Epithelial Trans-Differentiation

ATII cells expressing surfactant proteins are able to self-renew and trans-differentiate into ATI cells [20, 22, 88–90]. Depending on the type of injury applied, other epithelial cell populations, negative for SPC, can contribute to the attenuation of lung injury [58, 91]. Therefore, understanding how ATII cells differentiate into an ATI cell phenotype is under intense investigation including respective gene expression signatures as well as the morphological conversion from a cuboidal to a squamous cell shape. Early studies described the trans-differentiation of ATII cells into ATI-like cells in primary culture. These observations were based on the gradual loss of gene and protein expression of surfactant proteins as well as the loss of lamellar bodies, investigated by the use of electron microscopy [92]. Furthermore, the gain of features of ATI cells such as a flattened cell morphology and the expression of ATI cell-associated markers T1α (podoplanin) [93–96], aquaporin 5 (AQP5) [64, 97], receptor for advanced glycosylation end products (RAGE), and caveolin [98–100] were described. An overview of ATII and ATI cell specific markers is displayed in Table 6.2. Applying freshly isolated ATII cells to standard cell culture conditions is now widely used to mimic differentiation and repair mechanisms to investigate molecular cues in response to lung injury. The model has been utilized to study ATII cell trans-differentiation potential in various species including rat, mouse and human. Monitoring of epithelial cell identity and trans-differentiation is mainly achieved by gene and protein expression analysis of the respective markers in combination with microscopic evaluation of cell morphology.

Utilizing the spontaneous trans-differentiation of primary ATII cells in culture shed light into molecular programs that regulate this process and identified essential developmental pathways, such as the Wnt/β-catenin pathway [47, 60, 96, 103, 104] as well as TGF-β and BMP signaling [46, 105] to be involved.

However, it has to be taken into account that the model of ATII to ATI cell trans-differentiation in vitro does not fully resemble the processes occurring in vivo, as the specific trigger of injury has been shown to modulate a differential response of ATII and other progenitor cell populations. Furthermore, data indicate that the expression profile of freshly isolated ATI cells does not completely concur with the profile of ATI-like cells derived from trans-differentiation models in vitro [106].

Table 6.2 ATII versus ATI cell markers/characteristics for the determination of trans-differentiation

	Marker/characteristics	Reference
ATII cells	SPC	[30, 96]
	ABCA3	[31]
	LAMP3	[33]
ATI/AT1-like cells	T1α	[93–95]
	AQP5	[64, 97]
	HOPX	[22, 101]
	RAGE	[96, 102]
	Caveolin-1	[98–100]

To overcome the limitations of 2D cell culture models for studying ATII to ATI trans-differentiation and the regenerative potential of the ATII cell progenitor pool, the establishment of new 3D culture methods has been expedited extensively [22, 107], comparably to strategies for the generation of 3D organoids from colon, small intestine, and stomach [108]. For the purpose of mimicking the 3D microenvironment of the lung alveolus, primary ATII cells are seeded as a single cell suspension in an ECM mixture which is secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells (Matrigel), [22, 109]. Studies utilizing the co-culture of ATII cells with other cell populations such as fibroblasts or endothelial cells in matrigel describe the formation of lung organoids which display cuboidal ATII cells expressing SPC on the outer layer of the organoid. The organoid lumen, however, is lined by thin, squamous epithelial cells expressing markers of differentiated ATI cells such as AQP5 and T1α, indicating a self-renewal as well as a trans-differentiation capacity of ATII cells in this setting, and therefore representing an advanced model for studying mechanisms involved in this process in a more in vivo-related fashion [22, 101].

Short Summary

Alveolar epithelial cells play a crucial role in lung injury and repair processes in response to different stimuli and in the context of various lung diseases. A careful characterization of specific disease related alveolar epithelial phenotypes using comprehensive approaches and improved culturing methodologies will lead to important insights into novel therapeutic strategies targeting lung injury and repair.

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