Alveolar epithelial cell-specific gene expression *in vivo*: Effect of TGF-β1 stimulation

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Acvr1	Activin type I receptor
AEC	Alveolar epithelial cell
ALI	Acute lung injury
ALK-1/8	Activin receptor like kinase-1/8
APS	Ammonium persulfate
AQP-5	Aquaporin-5
ARDS	Acute respiratory distresss syndrome
ATI / II	Alveolar epithelial cell type I / II
Atp1α/β1	Adenosine triphosphate Na^+/K^+ transporting alpha/beta 1 polypeptides
BAL	Bronchoalveolar lavage
BAMBI	BMP and activin membrane-bound inhibitor
BEC	Bronchial epithelial cells
BMP	Bone morphogenic protein
BPD	Bronchopulmonary dysplasia
CC-10 / CCSP	Clara cell-10 / Clara cell secretory protein
Cdk	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
Co-Smad	Common Smad
CREB	Cyclic AMP-regulated enhancer-binding protein
CTGF	Connective tissue growth factor
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetracetic acid
EGF	Epidermal growth factor
EL	Epithelial lavage
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal regulated kinase
FACS	Fluorescence-activated cell sorting

FCS	Fetal calf serum
FKB12	FK506-binding protein of 12 kDa
Foxp2	Forkhead box P2
GABRP	Type A γ–aminobutyric acid ($GABA_A$) receptor π subunit
GADD45β	Growth arrest and DNA-damage-inducible 45 beta subunit
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF	Growth and differentiation factors
GI	Guanidinium isothiocyanate
GF	Growth factor
HAT	Histone aceyltransferase
HDAC	Histone deacetylases
HPRT	Hypoxanthine guanine phosphoribosyl transferase
Id	Inhibitor of differentiation
i.p.	Intraperitoneal
IPF	Idiopathic pulmonary fibrosis
IL	Interleukin
IN	Intranasal instillation
I-Smad	Inhibitory Smad
IT	Intratracheal instillation
JNK	Jun N-terminal kinase
JunB	Junb oncogene
LAP	Latency-associated peptide
LCM	Laser capture microdissection
LPCAT	Lysoposphatidylcholine acyltransferase
LPS	Lipopolysaccharide
LTBP	Latent TGF-beta-binding protein
MAPK	Mitogen-activated protein kinase
MH1 / 2	Mad homology domain 1 / 2
MMP	Matrix metalloproteinase
MS	Microspray instillation
NB	Nebulisation administration method
NF-κB	Nuclear factor of κ-light polypeptide gene enhancer in B cells
OT	Orotracheal instillation method

PAI-1	Plasminogen activator inhibitor-1 / Serpine1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PECAM-1	Platelet-endothelial-cell adhesion molecule-1
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PF	Pulmonary fibrosis
RLE-6TN	Rat lung epithelial-T-antigen negative
RNA	Ribonucleic acid
R-Smad	Receptor Smad
RT	Reverse transcriptase
ROS	Reactive oxygen species
SBE	Smad binding element
SMC	Smooth muscle cell
Smurf	Smad mediated ubiquitin regulatory factor
SP	Surfactant protein
T1α	Podoplanin
TAE	Tris acetic acid EDTA
TAK1	TGF-β-activated kinase 1
TEMED	*N, N, N', N'* - tetramethyl ethylene diamine
TF	Transcription factor
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor-alpha
TTF-1	Thyroid transcription factor 1
TβRI / II	Transforming growth factor-beta type I / II receptor
ZO-1	Epithelial sealing junction zonula occludens-1
α-SMA	alpha-smooth muscle actin
β-ENaC	Epithelial sodium potassium channel beta subunit

SUMMARY

Lung function is critically dependent on the integrity of the pulmonary epithelial cell layer, which is largely comprised of bronchial, Clara, and type I and II alveolar epithelial cells (AEC). While primary AEC culture has increased our knowledge of AEC gene expression *in vitro*, a comprehensive analysis of epithelial cell gene transcription *in vivo* has not yet been attempted. Therefore, we sought to profile the epithelial cell gene expression in the murine lung *in vivo*. We established a method to obtain epithelial cell RNA-enriched fractions using a diluted guanidinium isothiocyanate solution, administered intratracheally. After lavaging the lungs twice with saline, the optimal dilution and retention time were optimised. This resulted in an enriched epithelial cell RNA fraction with low contamination by RNA from fibroblasts, smooth muscle, or endothelial cells, as assessed by marker gene expression. This novel methodology, named as epithelial lavage (EL), thus allows for the selective profiling of lung epithelial-specific gene expression patterns *in vivo*.

Furthermore, in order to mimic the high levels of transforming growth factor beta 1 (TGF-β1), a cytokine expression of which is dramatically upregulated in the lungs of patients with idiopathic pulmonary fibrosis (IPF), the recombinant TGF-β1 was instilled into the murine lung. It was demonstrated that the orotracheal (OT) administration of TGF-β1 stimulated Smad-dependent signalling in pulmonary epithelial cells, and induced transcription of TGF-β1-responsive genes. The activation of the downstream signalling pathway, assessed by Western blotting, microarray, and reverse transcriptase-polymerase chain reaction (RT-PCR) from lung homogenates, was achieved within short stimulation time-points and with either low or high concentrations of TGF-β1.

Moreover, TGF-β1-induced gene transcription was studied specifically to the lung epithelium *in vivo* after OT administration of TGF-β1 in combination with the EL methodology. A single dose of TGF-β1 stimulated the regulation of some markers after 8 h, as assessed by quantitative RT-PCR, demonstrating induction or inhibition in expression of various epithelial gene expressing markers *in vivo*. Therefore, the EL technique represents a novel methodology to isolate RNA from the lung epithelium and study the gene expression profile of these cells under different conditions, like the TGF-β1 effect assessed in this study.

ZUSAMMENFASSUNG

Die Funktion der Lunge hängt wesentlich von der Intaktheit der Epithelschicht ab, welche hauptsächlich aus Bronchialzellen als auch aus Clarazellen und TypI und TypII Pneumocyten, den Alveolarepithelzellen, besteht. Während mit Hilfe der Zellkultur von primären Zellen bereits viel über die Genexpression in Pneumocyten *in vitro* erforscht werden konnte, wurde bisher noch keine weitergehende Studie über die Genexpression im Lungenepithel *in vivo* durchgeführt. Deshalb haben wir in unserer Studie die Genexpression von Epithelzellen der Mauslunge *in vivo* untersucht. Wir etablierten eine Methode, bei der RNA-Fraktionen aus dem Lungenepithel gewonnen werden, indem verdünntes Guanidinisothiocyanat intratracheal in die Mauslunge appliziert wird. Nachdem die Lunge zwei mal mit Salzlösung gespült worden war, wurden die Verdünnung und die Verweilzeit der Lösung in der Lunge optimiert. Dadurch erhielten wir eine Fraktion, die mit epithelialer RNA angereichert war, mit geringer Kontamination durch RNA von Fibroblasten, glatten Muskelzellen oder Endothelzellen. Dies zeigten wir durch die Untersuchung der Expression von Markergenen. Diese neue Methode, Epitheliale Lavage (EL) genannt, ermöglicht also eine selektive Darstellung der Expressionen von lungenepithelspezifischen Genen *in vivo*.

Um die hohen Konzentrationen des transforming growth factor beta 1 (TGF-β1) zu imitieren, einem Zytokin, dessen Expression in der Lunge von Patienten mit idiopathischer Lungenfibrose (IPF, Idiopathic Pulmonary Fibrosis) stark hochreguliert ist, wurde TGF-β1 als rekombinantes Protein in die Mauslunge instilliert. Dadurch konnte gezeigt werden, dass eine oro-tracheale Administration von TGF-β1 den intrazellulären Smad-abhängigen Signaltransduktionsweg in den Lungenepithelzellen stimuliert, sowie die Expression der Gene, die durch TGF-β1 aktiviert werden. Die Aktivierung der nachgeschalteten Signalwege wurde bereits nach kurzer Stimulationszeit sowie mit niedrigen und hohen Konzentrationen des Liganden TGF-β1 durch Westernblot, Microarray und semi-quantitativen RT-PCR den Lungenhomogenaten nachgewiesen.

Wir untersuchten weiter die TGF-β1-induzierte Gentranskription in dem Lungenepithelium *in vivo* nach orotrachealer Applikation von TGF-β1 in Kombination

mit der epithelialen Lavage-Technik. Acht Stunden nach einer einmaligen Gabe von TGF-β1 konnten wir mit Hilfe der quantitativen RT-PCR einen Effekt auf die Regulation der Expression einiger Marker feststellen. Dadurch konnten wir die TGF-β1-regulierte Induktion der Genexpression im Lungenepithel *in vivo* zeigen. Aufgrund unserer Ergebnisse konnten wir zeigen, dass EL eine neue Methode darstellt, RNA aus dem Lungenepithel zu isolieren und das Genexpressionsprofil dieser Zellen unter verschiedenen Bedingungen zu untersuchen, wie der Effekt von TGF-β1 in der vorliegenden Studie.

1 INTRODUCTION

General

The lung is the organ that makes our respiration possible, being continuously exposed to air that contains a variety of infectious, inflammatory, and toxic agents. The host and the environment meet at the respiratory acini, a site of possible bacterial colonization. Therefore, the diseases of the respiratory system have become a major challenge in medical care, causing multiple socio-economic problems. The incidence of genetic and malignant lung diseases is steadily increasing. According to the World Health Organisation (WHO), of the top ten leading causes of mortality worldwide, four are diseases of the lung: tuberculosis, chronic obstructive pulmonary disease (COPD), pneumonia, and lung cancer. In Europe, respiratory diseases rank second after cardiovascular diseases in terms of mortality, incidence, prevalence, and costs (Murray and Lopez, 1997).

Modern medicine is faced with great difficulties treating pulmonary illnesses. Pneumonia is the most frequent lethal infectious pathology of all infectious diseases described, with 17,000 fatal cases per year in Germany alone. Acute lung injury (ALI) and pneumogenic sepsis represent the most common hospital-acquired diseases leading to death. The flu, caused by *influenza* viruses, reaches 12,000 deaths per year in Germany. Furthermore, fibrotic lung diseases, totalling almost 80,000 cases in Germany is without any therapeutic option, a fatal disease (Loddenkemper et al., 2003).

The onset and natural history of such diseases are dependent on individual genetic predispositions, cigarette smoke, and environment, among others. The molecular mechanisms of several genetic diseases are now starting to be unravelled. This will facilitate the development of new drugs that can prevent, treat, and cure respiratory illnesses.

1.1 ACTIVE SIGNALLING IN THE LUNG

1.1.1 Transfrorming growth factor-beta (TGF-β) superfamily

The transforming growth factor-beta (TGF-β) superfamily of ligands controls a large number of processes including cell proliferation, lineage determination, cell differentiation, adhesion, motility, and death. This family of cytokines plays a major role in the development, homeostasis, angiogenesis, and repair of most tissues in metazoan organisms (Massague, 1998). It comprises a large number of structurally-related proteins, such as the TGF-β ligands themselves, bone morphogenetic proteins (BMPs), the activins and inhibins, and other growth and differentiation factors (GDFs) (Mehra and Wrana, 2002; Miyazawa et al., 2002).

The term transforming growth factor (TGF) was coined in the early eighties, and it was applied to peptides that had the ability to confer a transformed phenotype on untransformed fibroblastic cells *in vitro*. Two different classes of TGF were defined: the alpha (TGF-α) and the beta (TGF-β) (Roberts and Sporn, 1985). While TGF-α is related to epidermal growth factor (EGF) and binds to the EGF receptor, TGF-β is not structurally or functionally related either to TGF-α or EGF, and binds to different receptors (Coffey et al., 1992).

Briefly, TGF-β and related factors transmit signals in the cell as follows: upon ligand binding to the type I and type II serine/threonine transmembrane kinase receptors, activation of the receptor-associated cytoplasmic effector molecules, Smads occurs. The receptor Smad (R-Smad) phosphorylation leads to association with the common Smad (Co-Smad), also known as Smad4. This transcription factor (TF) complex then translocates into the nucleus, where it activates or represses gene transcription, in association with DNA-binding partners (Figure 1.1).

Although this pathway appears to be simple, the combinatorial interactions, for example between the receptors themselves and with the R-Smads, allows enormous diversity in the TGF-β cell responses (Derynck and Zhang, 2003). Moreover, the differential effects on the cell depends on ligand concentration, the activated downstream molecules, and on the responsiveness of the target cell (Massague, 2000). For instance, in epithelial cells, the TGF-β ligands inhibit cell growth and induce differentiation including epithelial-to-mesenchymal transition (EMT); whereas BMP ligands weakly induce epithelial cell growth and do not induce cell differentiation

(Derynck and Zhang, 2003; Kowanetz et al., 2004). Moreover, the TGF-β1 ligand can bind to various type I receptors, either the ALK-1 (solely expressed in endothelial cells) or ALK-5, inducing Smad1/5/8 or Smad2/3 phosphorylation, respectively and subsequently, diverse responses inside the cell are driven (Lebrin et al., 2005).

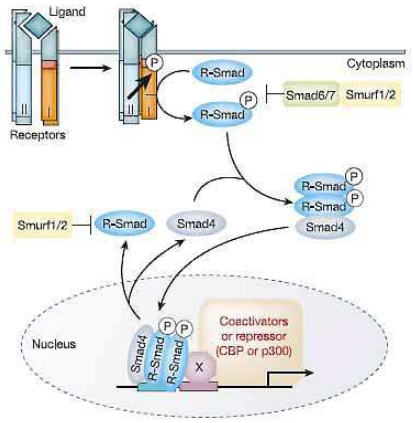


Figure 1.1: Schematic diagram of the TGF-β signalling pathway from the cell membrane to the nucleus. The arrows indicate signal flow. At the cell surface, the ligand binds to the type I / type II receptor complex and induces phosphorylation of the GS segment (red) in the type I receptor. Consequently, R-Smad are phosphorylated at the C-terminal serines, and then form a complex with Smad4. This complex translocates into the nucleus to regulate transcription of target genes, assisted by either co-activators or co-repressors. R-Smads and Smad4 shuttle between the nucleus and the cytoplasm. Smurf1, Smurf2, Smad6, and Smad7 function as inhibitors of TGF-β signalling (after Derynck and Zhang, 2003).

1.1.1.1 TGF-β ligands

A large number of ligands are present in the TGF-β superfamily with the same structural profile: a dimer (homo- or heterodimer) held together by hydrophobic interactions (Sun and Davies, 1995). The TGF-β ligands are synthesised as propeptide precursors, and are then processed and secreted as inactive homodimers, noncovalently bound to a latency-associated peptide (LAP) (Massague, 1998). The latent form becomes active extracellularly through proteolysis by thrombin and plasmin or retinoids, tissue transglutaminase, reactive oxygen species (ROS), low pH and thrombospondin (Camoretti-Mercado and Solway, 2005). The $\alpha_v\beta_6$ integrin-expressing cells also induce spatially restricted activation of TGF-β1 (Munger et al., 1999). The $\alpha_v\beta_6$ integrin is principally expressed by epithelial cells, at low levels in healthy adult lung tissues and highly and rapidly upregulated in injury and inflammatory conditions (Breuss et al., 1995).

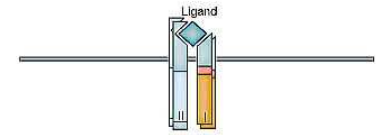
Three distinct TGF-β isoforms are secreted in mammals (TGF-β1, 2 and 3), which are encoded by different genes, and exhibit diverse expression patterns *in vivo*, due at least in part to differences in their promoter regions (Taipale et al., 1998). The

TGF-β1 isoform is induced by oncogenes and immediate early genes, whereas the other two isoforms are more developmentally and hormonally regulated (Letterio and Roberts, 1996). The diversity in biological activity correlates with the binding affinity of the ligands to the different TGF-β receptors (Wrana et al., 1992) and with the assembled accessory receptors, such as endoglin and betaglycan (Massague, 1998).

Specifically, the TGF-β1 isoform is produced by a large variety of cell types, such as, platelets and immune cells including lymphocytes, macrophages, and mast cells. Specifically in the lung, the endothelial, and smooth muscle cells (SMC), fibroblasts and epithelial cells synthesise this cytokine. Infiltrated inflammatory cells, including eosinophils and lymphocytes, also secrete TGF-β1 under stress conditions (Duvernelle et al., 2003). Interestingly, the TGF-β1 isoform has been implicated in diverse human pathologies, such as parasitic, autoimmune and fibroproliferative diseases, affecting distinct organs like the kidney, lung and liver (Border and Ruoslahti, 1992; Wahl, 1994). Therefore, the TGF-β1 isoform has been deeply studied during the last two decades, mainly for its role in inflammatory responses and healing disorders.

1.1.1.2 TGF-β receptors: classification and structure

The TGF-β receptor family comprises highly conserved transmembrane protein serine/threonine kinases (Krishnaveni and Eickelberg, 2006). Based on their structural and functional properties, the signalling receptors are divided into two subfamilies: type I (or activin receptor-like kinase, ALK-1 to -7) and the constitutively-active type II receptors, like the TβRII and BMPRII (de Caestecker, 2004). In mammals, five type II receptors and seven type I receptors have been identified. All of them contain a short extracellular domain, a cytoplasmic kinase domain, and in the case of type I receptors, additionally possess a GS domain, which is phosphorylated by the type II receptors, activating the signalling complex (Massague, 1998) (Figure 1.1 and 1.2). Upon ligand binding, the heterotetrameric receptor complex is formed, composed of two molecules each of type I and type II receptors (Kirsch et al., 2000). The possible combinations bridging the several type I and type II receptors together are large (Figure 1.2). Additionally, two accessory receptors called betaglycan and endoglin, classified as type III receptors, facilitate ligand binding (Krishnaveni and Eickelberg, 2006; Lutz and Knaus, 2002).



Type II	Type I	R-Smad
BMPRII	ALK-2 (ActRI) ALK-3 (BMP-RIA) ALK-6 (BMP-RIB)	Smad1, Smad5, Smad8
ActRII, ActRIIB	ALK-4 (ActRIB)	Smad2
ActRIIB	ALK-7	Smad2
TβRII	ALK-5 (TβRI) ALK-1 ALK-2	Smad2, Smad3 Smad1, Smad5
AMHR	ALK-3 ALK-2 ALK-6	Smad1, Smad5

Figure 1.2: Overview of the combinatorial interactions between type I and type II TGF-β signalling receptors and their corresponding R-Smads. Only the best-characterised interactions are listed (after Derynck and Zhang, 2003).

1.1.1.3 Intracellular signalling molecules: Smads

Activated type I receptors subsequently phosphorylate their cytoplasmic substrates, the Smad proteins. The Smad family of transcription factors, whose name is derived from the founding members of this family, the *Drosohila melanogaster* protein MAD (Mothers Against Decapentaplegic) and the *Caenorhabditis elegans* protein SMA (Small Body Size), is composed of eight proteins, Smad1-8, divided into three subclasses based on their structure and function (Miyazawa et al., 2002; Moustakas et al., 2001):

i) Receptor-regulated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5, and Smad8.

The R-Smads are the receptor-associated cytoplasmic effector molecules, which are directly phosphorylated by the type I receptor kinases. They are subdivided in two groups: 1) The activin/TGF-β activated R-Smads, Smad2 and Smad3, are also phosphorylated by activin, nodal, ALK-4, -5 and -7 receptors (Macias-Silva et al., 1996; Zhang et al., 1996). 2) The BMP activated R-Smads, Smad1, Smad5 and Smad8, are also phosphorylated by ALK-1, -2, -3, -6 and BMP type I receptors (Kretzschmar et al., 1997).

ii) Common-partner Smad (Co-Smad): Smad4.

In vertebrates, only one member is contained in this group, the so-called Smad4. Smad4 is necessary for the interaction between the R-Smads and various DNA-binding proteins, and in the activation of gene transcription (Lagna et al., 1996; Liu et al., 1997).

iii) Inhibitory Smads (I-Smads): Smad6 and Smad7.

The I-Smads are also called antagonistic Smads. They associate with the activated type I receptors in order to prevent R-Smad binding and activation, and therefore, inhibiting the signalling cascade. Both the TGF-β/activin and BMP signalling pathways are repressed by Smad7 (Hanyu et al., 2001; Nakao et al., 1997), whereas Smad6 specifically inhibits BMP signalling and competes with the Co-Smad in binding to active R-Smads (Hata et al., 1998; Imamura et al., 1997). The I-Smads are activated by several signals, including TGF-β- and BMP-induced negative feedbacks (Ishida et al., 2000). The duration and magnitude of the TGF-β / BMP signalling effects are thus determined by the Smad6 and Smad7 expression levels (Miyazawa et al., 2002; ten Dijke and Hill, 2004).

Structurally the Smad molecules contain two well-characterised, highly conserved, Mad homology (MH) domains, MH1 and MH2, present at the N- and C-terminal ends, respectively. A divergent middle linker segment is flanked by these two domains. The MH2 region is present in all three Smad subclasses, whereas the MH1 domain is absent in the I-Smads. The phosphorylation target site on the R-Smads contains the SSXS (S: serine; X: any amino acid except proline) motif at the C-terminal end (Massague, 1998; Moustakas et al., 2001). In the basal state, MH2 domain activity is inhibited by the MH1 region by physical association (Hata et al., 1997). Upon ligand binding and thus receptor activation, the interaction between both domains is disrupted, allowing the R-Smads to form a hetero-oligomer with the Co-Smad through their MH2 domains (Mehra and Wrana, 2002).

Once activated and translocated into the nucleus, the MH1 and MH2 domains perform different functions. The MH1 region is involved in DNA binding (Kusanagi et al., 2001) and the MH2 domain is able to complex with several DNA-binding factors and activate or repress gene transcription (Dennler et al., 1998).

1.1.1.4 Regulation of the TGF-β pathway

The presence of DNA-binding factors is required in order to achieve high-affinity, selective interactions with specific DNA-binding sequences. The number of DNA-binding partners described thus far is large (Miyazawa et al., 2002). They are divided into two groups, co-activators (CBP/p300, SMIF) (Feng et al., 1998; Itoh et al.,

2000) and co-repressors (c-Ski/SnoN, c-Myc) (Alexandrow et al., 1995; Liu et al., 2001; Luo et al., 1999) The co-activators bind to or possess intrinsic histone acetyltransferase (HAT) activity, which facilitates gene activation. On the contrary, the co-repressors recruit histone deacetylases (HDAC) to the complex, whose effect generally leads to chromatin condensation and thus, represses Smad transcriptional activity (Figure 1.4) (Berger, 2002; Massague, 2000).

The negative feedback represents another source of regulation at the intracellular level of the TGF-β signalling pathway (Figure 1.1 and 1.4). At the receptor level, FK506-binding protein of 12 kDa (FKBP12) binds to type I TGF-β receptor (ALK-5) impairing its phosphorylation by the type II TGF-β receptor (TβRII) at the basal state (Chen et al., 1997). Furthermore, Smad6 and Smad7 impair the R-Smad binding to the corresponding receptors (Stopa et al., 2000). Finally, the ubiquitin ligases, Smad-ubiquitination-regulatory factor 1 and 2 (Smurf1 and Smurf2), antagonise TGF-β signalling by interacting with the R-Smads and targeting them for degradation (Figure 1.1 and 1.3) (Arora and Warrior, 2001; Zhu et al., 1999).

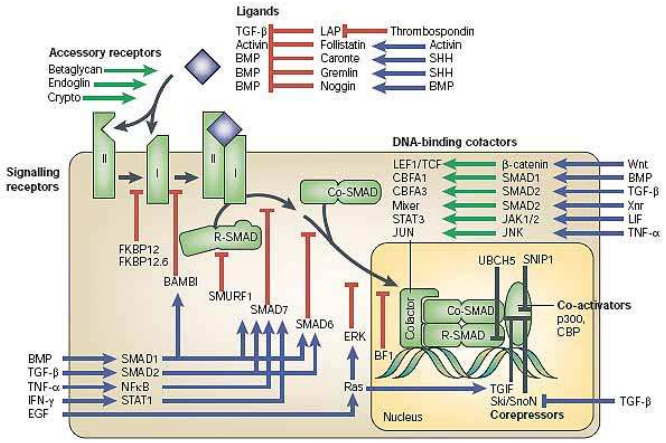


Figure 1.3: A network controlling the TGF-β signalling pathway. Regulation of the TGF-β pathway can take place at receptor level by accessory inhibitor proteins (*e.g*. FKBP12 and BAMBI); downstream cytoplasmic molecules by Smad6, Smad7, Smurf1 and Smurf2; and inside the nucleus by co-activators and co-repressors (CBP/p300 and c-Ski/SnoN). In addition, crosstalk pathways control TGF-β signaling (*i.e*. Wnt pathway, NF-κB). The blue arrows indicate the agonists, and the green and red arrows indicate activation and inhibition of the pathway, respectively (after Massague, 2000).

1.1.1.5 TGF-β target genes

TGF-β-induced gene transcription or repression is dependent on the cell type, promoter sequence and DNA-binding proteins (Moustakas et al., 2001). The inhibition of the cell-cycle progression by TGF-β stimulation in many cell types is well-characterised. TGF-β1 induces the activation of $p15^{Ink4b}$ (Hannon and Beach, 1994) and $p21^{Cip1}$ (Datto et al., 1995), which are cyclin-dependent kinase (cdk)

inhibitors that control the G1 phase. Conversely, TGF-β represses the oncogene *c-myc* (Alexandrow et al., 1995), the Cdk-activating phosphatase *cdc25A* (Iavarone and Massague, 1997) and the inhibitor of differentiation (Id) family. The Id proteins act as negative regulators of cell differentiation and positive regulators of cell proliferation (Korchynskyi and ten Dijke, 2002; Norton et al., 1998).

Several other genes are reported to be upregulated upon TGF-β1 ligand binding through Smad2/3 activation. Among them, genes encoding plasminogen activator inhibitor-1 (PAI-1) (Dennler et al., 1998; Hua et al., 1999), type I collagen (Zhang et al., 2000), Smad7 (Stopa et al., 2000), JunB (Pertovaara et al., 1989) and connective tissue growth factor (CTGF) (Grotendorst et al., 1996). Additionally, TGF-β1 promotes the accumulation of many extracellular matrix (ECM) proteins by increasing their synthesis while inhibiting the production of matrix-degrading enzymes (Branton and Kopp, 1999; Selman et al., 2001).

1.1.1.6 TGF-β transgenic mice

Studies on mice harboring null mutations for the different TGF-β isoforms revealed that each isoform exerts discrete, nonredundant functions during murine development (Goumans and Mummery, 2000). Initially TGF-β1 null-mice suggested that the ligand was only required postnatally (Shull et al., 1992); however, further reports have proven that transplacental and lactational transfer of maternal TGF-β1 were sufficient to rescue the development and growth of the pups (Letterio et al., 1994). Different approaches have later shown that TGF-β1 is required for at least two distinct phases during embryogenesis; for preimplantation and for the vasculogenesis and haematopoiesis of the yolk sack (Kallapur et al., 1999). Moreover, TGF-β1 null-mice suffer from an excessive inflammatory response, with massive infiltration of leukocytes into many organs resulting in a multifocal inflammatory disease, organ failure, and early death (Kulkarni et al., 1993; Shull et al., 1992). The heart and the lungs were the mainly affected organs, leading to myocarditis, perivascular cuffing and interstitial pneumonia, due to an uncontrolled inflammatory response (Kulkarni et al., 1993; Shull et al., 1992).

TGF-β2 null-mice suffer cardiac, lung, spinal column, carniofacial, limb, inner ear, eye, and urogenital defects; and the mutant is perinatally lethal (Sanford et al., 1997). TGF-β3 null-mice die shortly after birth and present with a cleft palate. They suffer delayed lung maturation with alveolar hypoplasia, lack of alveolar septal

formation and diminished expression of surfactant protein C (SP-C) (Proetzel et al., 1995).

1.1.2 Additional signalling cascades active in the lung

It is well known that components of the TGF-β signalling cascade are targets of other signalling pathways and vice versa. Indeed, this complex network of crosstalk modifies and amplifies the cell responses to TGF-β signalling, depending on the cell conditions and cell type (Derynck and Zhang, 2003; Massague, 2000). Moreover, TGF-β ligands can signal through several Smad-independent pathways that can as well be regulated by other signalling pathways (Miyazawa et al., 2002) (Figure 1.3).

1.1.2.1 Wnt pathway

The Wnt signal transduction pathway has been identified as a key player in various developmental processes (Akiyama, 2000). The Wnt family comprises 19 secreted glycoproteins, which bind to cell surface receptors called Frizzleds (Fz). The best characterised intracellular signalling pathway is the canonical β-catenin pathway (Pongracz and Stockley, 2006).

Smad-dependent gene transcription can be modulated by the transcription factor β-catenin and vice versa (Lei et al., 2004; Nishita et al., 2000). On the other hand, Smad2 also regulates the β-catenin pathway (Nishita et al., 2000). Furthermore, both the Wnt and TGF-β pathways play a common role in the process of EMT in lung (Pongracz and Stockley, 2006), during embriogenesis and tumor metastasis (Nawshad et al., 2005) and heart development (Liebner et al., 2004).

1.1.2.2 MAPK pathway: JNK, p38 and ERK MAP kinases

The mitogen-activated protein kinases (MAPK) are a large group of proteins acting as signal transducing enzymes, since they facilitate extracellular signals to reach the cell surface to rapidly activate nuclear TF (Javelaud and Mauviel, 2005). Several extracellular stimuli can activate these serine-threonine kinases, which will subsequently phosphorylate nuclear kinases or TF (Mulder, 2000).

The mammalian MAPKs have been classified into four groups according to the activating molecule: 1) the extracellular signal-regulated kinases (ERK1 and ERK2 or also designated as p44 and p42, respectively), 2) p38/MAPKs, 3) the stress-activated

protein kinases known as c-Jun N-terminal kinases (JNK1, JNK2 and JNK3), and 4) ERK5 (Chang and Karin, 2001).

The MAPK activity is controlled by a sequential activation of several MAPK kinase kinase (MAPKKK or MEKK) and MAPK kinase (MAPKK, MEK or MKK) enzymes (Chang and Karin, 2001). The phosphorylated upstream kinase directs the activation of a certain downstream pathway. The ERK-mediated pathway, initiated by mitogens and several growth factors (GF), is involved in cell proliferation and differentiation and considered anti-apoptotic. The p38/MAPK and JNK-signalling pathways are activated by stress stimuli, such as, UV light, osmotic shock or inflammation, leading either to cell proliferation and differentiation or apoptosis, depending on the cell system (Javelaud and Mauviel, 2005).

TGF-β can interact with all the pathways described above, except the ERK5 pathway (Javelaud and Mauviel, 2005). Furthermore, the TGF-β-induced MAPK activation can follow either slow kinetics in a Smad-dependent pathway or rapid kinetics (5-15 min) in a Smad-independent pathway (Massague, 2000).

The biochemical mechanism and the biological consequences of the crosstalk are currently poorly understood. Thus far, one well-characterised interaction is through the TGF-β-activated kinase 1 (TAK1), a MAPKKK family member, which is involved in the activation of both the JNK and p38 pathways (Shibuya et al., 1996; Zhou et al., 1999). The MAPK pathways have been also implicated in the regulation of TGF-β responses. For instance, ERK phosphorylates the MH1 domain of Smad2 and the linker segments of Smad1, Smad2 and Smad3 (Derynck and Zhang, 2003; Kretzschmar et al., 1999). Moreover, activation of MEKK1 can also result in Smad phosphorylation through the ERK and JNK pathways (Brown et al., 1999). Furthermore, JNK can directly phosphorylate Smad3 at its linker region (Billings et al., 2000; Mori et al., 2004).

1.1.2.3 NF-κB pathway

Nuclear factor of κ-light polypeptide gene enhancer in B cells (NF-κB) is a generic name for a group of TF, which can induce or repress gene transcription, and are thus implicated in the regulation of cell proliferation, development, and apoptosis. NF-κB plays a major role in the innate- and adaptive-immune responses (Perkins, 2007; Schmitz et al., 2004).

In the majority of cell types, NF-κB complexes are retained in the cytoplasmic compartment by the inhibitors of NF-κB (IκBs). The activation of the pathway requires IκB phosphorylation by IκB kinases (IKK) (Perkins, 2007). A large number of stimuli can lead to the NF-κB pathway initiation and most of them represent stressful or precarious conditions (Schmitz et al., 2004).

TGF-β can stimulate the NF-κB pathway and vice versa. These two pathways act in an antagonistic manner in the regulation of immune-cell responses (Massague, 2000). For instance, through TAK1 activation, which then phosphorylates and activates the IKK, leading to the activation of the NF-κB pathway (Yamaguchi et al., 1999). Conversely, Smad7 levels can increase in response to TNF-α through the NF-κB pathway, inhibiting the Smad signalling pathway (Bitzer et al., 2000).

1.1.2.4 PI3K/AKT pathway

Phosphoinositide 3-kinases (PI3K) generate specific inositol lipids, which control cell growth, survival, differentiation, proliferation, and cytoskeletal changes. A well-characterised target of PI3K lipid products is the protein kinase AKT or also called protein kinase B (PKB) (Vanhaesebroeck and Alessi, 2000). AKT is a multifunctional cytoplasmic kinase, activated by various stimuli and has been implicated in cancer development (Barnett et al., 2005).

Crosstalk between the PI3K/AKT and TGF-β pathways has also been reported. For instance, the upregulation of the type I collagen expression by TGF-β was proposed to be mediated by TGF-β-induced AKT phosphorylation in Swiss 3T3 cells (Runyan et al., 2004). It has been also suggested an essential role of the PI3 kinase in TGF-β-mediated EMT and cell migration in NMuMG mammary epithelial cells (Bakin et al., 2000).

In conclusion, the interaction between downstream molecules in distinct signalling pathways may underlie diverse forms of integration and reciprocal regulation inside the cell (Massague and Chen, 2000). However, the implications of the Smad and other signalling molecule activation are still not well understood *in vivo*.

1.2 LUNG ANATOMY, PHYSIOLOGY AND FUNCTION

The principal function of the lung is to ensure efficient gas exchange, providing oxygen to the pulmonary and systemic blood, and removing the carbon dioxide

produced by cell respiration. The organ anatomy represents a well-optimised structure with a large exchange surface and a thin basement membrane, which enables rapid gas exchange by passive diffusion. The basement membrane is composed of both the endothelial and epithelial cell membranes fused together, minimizing the gas exchange surface to 0.2 μm, forming the alveolocapillary units. However, not only a thin basement membrane is needed for an optimal gas exchange, but also an optimal matching ratio between ventilation (V, air flow) and perfusion (Q, blood flow) is indispensable (Seeger et al., 1993; Von Euler, 1946).

The pulmonary parenchyma represents about 85% of the total lung volume (respiratory bronchioles, alveolar ducts, alveolar sacs and alveolar capillary network), the conducting airways cover only about 6 to 10% (trachea, bronchi, bronchioles, terminal bronchioles) and the remaining part of the lung consists of nervous and vascular tissue (Gehr, 1984). The lung is divided into three functional regions: the ventilation (conducting and respiratory airways), the perfusion (bronchial and pulmonary blood supply), and the lymphatic areas.

Several tissues are contained in the lung. The epithelial layer consists of at least 13 cell types, 11 epithelial and 2 mesenchymal cell types. The interstitium contains 36% of the total number of cells, among them, the fibroblasts and myofibroblasts, cells that synthesise collagen and elastic fibers and the pericytes and SMC, which compose the capillaries and vessels. The endothelium contains about 30% of the total cell number, representing the largest capillary endothelial surface of the body and so, facilitating the gas exchange (Simionescu and Antohe, 2006). Moreover, cartilage synthesised by chondrocytes, is also present in the lung, though only surrounding the trachea and bronchi. At the alveoli, specialized cells are responsible for host defense, such as, the alveolar macrophages that accomplish phagocytosis (9% of total cell number); the plasma cells in charge of antibody production; granulocytes and also the mast cells, which store histamine and heparin (Corrigan and Kay, 1991).

1.3 THE LUNG EPITHELIUM

1.3.1 Cell types and function

Epithelia are sheets of cells that are situated at the interface of two biologically distinct compartments. The epithelial layers form a semipermeable boundary and regulate the transport of ions and molecules between both compartments. Specially, the

pulmonary epithelium is necessary for an optimal gas exchange at the alveoli, and represents the first defense barrier between the host and the environment. The lung epithelium can be divided into proximal and distal, representing the respiratory epithelium and the gas exchange region, respectively. An intermediate area is found between them (Figure 1.4) (Breeze and Turk, 1984).

The distal alveolar epithelium, included in the sacci and ducti alveolari, is responsible for the gas exchange. In the adult lung, it consists of two highly specialized alveolar epithelial cells (AEC): squamous type I (ATI) and cuboidal type II (ATII) cells (Wise, 2002). The ATI, or also called type I pneumocytes, are large and very thin (0.2 μm in depth) cells. They cover over 95% of the alveolar surface (5,000 μm^2) and represent the 8% of the total cell number in the lung. The passive gas diffusion takes place at the ATI and endothelial cell junctions (McElroy and Kasper, 2004). Additionally, they form a barrier against fluid leakage into the alveolar spaces, but allow a selective exchange of physiological solutes and water between blood and alveoli (Schneeberger and Lynch, 2004; Williams, 2003). Recent reports assume that the ATI cells retain the ability to trans-differentiate not only *in vitro*, but also *in vivo* (Flecknoe et al., 2002; Uhal, 1997).

On the other hand, the ATII cells comprise only the remaining 5% of the respiratory lung surface area (180 μm^2) and the 16% of the total pulmonary cells. These cells can be named granular AEC (as they contain lamellar bodies), large AEC (due to their big size), corner cells (they are normally found at the edges of the alveolar spaces) or type II pneumocytes. These cells synthesise, secrete and re-uptake surfactant proteins (SP), which are lipoprotein complexes of the lung innate immune system that reduce surface tension at the air-liquid interface and serve as pulmonary host defenses (Wright, 2005). Four different surfactant proteins have been defined: SP-A, SP-B, SP-C and SP-D, with SP-A and SP-D being specifically involved in the host defense (Mason et al., 1998). The type II pneumocytes store the SP in so-called lamellar bodies, which are specialised secretory lysosomes (Gunther et al., 2001; Weaver et al., 2002). The ATII cells are multifunctional in that they regulate the alveolar lining fluid and its resolution and alter inflammation by GF and cytokine secretion (Manzer et al., 2006; Matthay et al., 2002). While the ATI cells are highly vulnerable to injury, the ATII cells are more resistant and act as progenitor cells for the re-epithelialisation process after epithelial

injury, by proliferation and trans-differentiation into ATI cells (Evans et al., 1975; Uhal, 1997) (Figure 1.5.C).

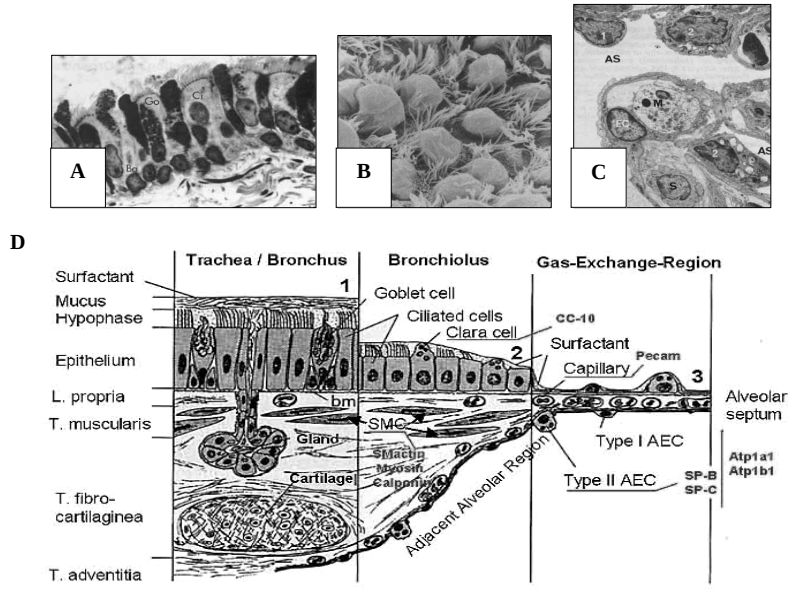


Figure 1.4: Anatomy and histology of the lung. The lung contains several cell-types present at different regions. These areas are divided into a proximal region containing the trachea and bronchi (A), a medial region that possesses the bronchioli, internally covered with Clara cells and ciliated cells (B) and a gas-exchange-region at a distal position (C). The epithelium varies from stratified (respiratory epithelium) to flattened cells and underlying it, other cell types are encounted (D). The cell type-specific markers for type I and II AEC, Clara, endothelial and smooth muscle cell (SMC) are indicated. Go: goblet cells, Ci: ciliated cells, Ba: basal cells, M: monocyte, AS: alveolar space, EC: endothelial cell, S: smooth muscle cell, 1: type I AEC and 2: type II AEC (modified after Breeze and Turk, 1984).

At the intermediate region, where the bronchioli are situated, the respiratory epithelium is cuboidal with ciliated columnar and non-ciliated cells, the latter named Clara cells. The Clara cells are implicated in the production and secretion of the surfactant components, SP-A, SP-B and SP-D, as well as the Clara cell secretory protein (CCSP), also called CC-10, which is a key regulator of inflammation in the lung (Chang et al., 2000; Stripp et al., 2002). The Clara cells are considered to be the progenitor cells at the proximal airways and essential in detoxifying inhaled foreign material (Bishop, 2004; Brody et al., 1987) (Figure 1.5.B).

The epithelial layer lining in the proximal region consists of seven cell types: basal, goblet, ciliated, brush, serous, Clara, and neuroendocrine cells. These cells cover the tracheobronchial airways forming the so-called respiratory epithelium. Of note, the ciliated and goblet cells play a key role in duct clearing by mucus secretion and entrapment and transportation of inhaled airway insults (Rogers, 1994) (Figure 1.5.A).

1.3.2 Cell type-specific markers

A cell type-specific marker is a molecule present in a particular cell type, facilitating cell characterisation, identification, and perhaps cell isolation (Atkinson et al., 2001). Furthermore, biological markers or biomarkers are cell type-specific markers that help to diagnose a disease, as they are highly expressed or absent in pathological conditions compared to physiological levels (Hirsch et al., 2002; Tzortzaki et al., 2007).

Some epithelial cell markers currently used in cell characterisation have been previously mentioned, for example the four surfactant proteins synthesised by ATII cells, and the CC-10 marker, unique to Clara cells. Some additional markers characterise type II pneumocytes. For instance, the type A γ-aminobutyric acid ($GABA_A$) receptor π subunit (GABRP) (Chen et al., 2004) recently discovered with an as-yet undefined function in the lung or lysophosphatidylcholine acyltransferase (LPCAT), which is required for surfactant phospholipid biosynthesis as it remodels the phosphatidylcholine, an essential lipid for SP production (Chen et al., 2006). Moreover, SP synthesis is dependent on the combined actions of multiple TF. Among them, the thyroid transcription factor 1 (TTF-1/Nkx2.1), which is a common positive regulator of their promoter activity (Bohinski et al., 1994) and the forkhead box P2 (Foxp2) that acts as a transcriptional repressor for murine CC-10 promoter, regulating proximal *versus* distal epithelial cell differentiation (Shu et al., 2001).

Podoplanin (T1α) was the first molecular marker identified for ATI cells (Williams et al., 1996). T1α is essential for optimal alveolar sacculation (Millien et al., 2006). Moreover, the water channel aquaporin (AQP) 5, key for fluid permeability between the airspace and the capillaries, also specifically identifies this cell type in the lung (Borok et al., 1998; Nielsen et al., 1997; Verkman et al., 2000).

The type I and II pneumocytes also share the expression of some markers (McElroy and Kasper, 2004), including the Na^+/K^+ transporting ATPase α and β 1 polypeptides (Atp1α1 and Atp1β1, respectively) present at the basolateral surface of the AEC (Machado-Aranda et al., 2005; Matthay et al., 2002). Additionally, the β subunit of the epithelial sodium channel (β-ENaC) is present in both cell types (Jernigan and Drummond, 2005; Matthay et al., 2002).

Other pulmonary cell types express different specific markers. Among them, the collagen $I\alpha_1$, synthesised and secreted by fibroblasts and myofibroblasts, forms part of the ECM (Oda et al., 1988). The contractile proteins α-smooth muscle actin (α-SMA),

calponin, and smooth muscle-myosin heavy chain are mainly expressed by vascular SMC (Shanahan and Weissberg, 1998). The platelet-endothelial-cell adhesion molecule-1 (PECAM-1) is a marker restricted to platelets and endothelial cells (Ilan and Madri, 2003).

Following injury, the quantity of the newly synthesised cell marker proteins differ from physiological levels. Thus, these changes in expression levels may facilitate early diagnosis of pulmonary disease and thus reduce the associated mortality, by allowing characterisation of novel molecular pathways that could be targeted in therapeutic interventions (McElroy and Kasper, 2004).

1.3.3 Lung epithelium-related diseases

Epithelial barrier disruption requires a rapid and efficient re-epithelialisation of the denuded basement membrane. However, the epithelial repair process may be disturbed, leading to aberrant alveolar epithelial cell populations (*e.g.*, cells co-expressing epithelial and mesenchymal markers) (Willis et al., 2005) and thus to a deregulation of cytokine secretion (increase levels of TGF-β, the central regulator of -PF-) (Selman et al., 2001), abnormal pulmonary surfactant production, and alveolar collapse (Taskar et al., 1997). Epithelial injury is evident in many lung diseases, including bronchopulmonary dysplasia (BPD) (Jobe and Bancalari, 2001), acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) (Geiser, 2003b; Martin et al., 2005), cystic fibrosis (Matthay et al., 2002) and idiopathic pulmonary fibrosis (IPF) (Geiser, 2003a; Selman et al., 2001). Abnormal airway remodelling and partial lost of epithelium is also observed in chronic obstractive pulmonary disease (COPD) (Puchelle et al., 2006).

Different types of insults provoke repeated microscopic injuries at the pulmonary epithelial layer and it is thought that a combination of events trigger the disease. Among the known epithelial damage insults are tobacco smoke (Van Winkle et al., 2001; Wistuba et al., 2002), pollutants, bacteria, viruses (Puchelle et al., 2006), and gastroesophageal reflux (Selman and Pardo, 2006).

1.4 TGF-β AND EPITHELIAL LUNG DISEASES

1.4.1 Expression and role of TGF-β in the lung

The TGF-β cytokines are part of a regulatory molecule network that control lung homeostasis and homeodynamics, required during lung branching and lung tissue repair. The three TGF-β ligand-coding genes are highly expressed in the mouse during lung morphogenesis. TGF-β1 is found as early as day 11 in the cytoplasm of stromal and epithelial cells of the primordial ducts and play a major role in lung branching (Schmid et al., 1991). The constitutive activation of TGF-β1 in a transgenic mouse model led to lung morphogenesis arrest and inhibition of epithelial cell differentiation, where neither pro-SP-C nor CC-10 were detected. On the contrary, type I collagen was present in the terminal airways and the distribution of α-SMA was markedly altered (Serra et al., 1994; Zhou et al., 1996). TGF-β1 also inhibited surfactant synthesis and epithelial cell maturation in human lung explants (Beers et al., 1998; Zhou et al., 1996). In the human developing lung, all three TGF-β isoforms are highly expressed. They play a decisive role in branching morphogenesis and epithelial cell differentiation with the concomitant surfactant maturation (Bartram and Speer, 2004; Chapman, 2004).

All three TGF-β isoforms are also found in the adult murine lung, primarily expressed at the bronchial epithelium. There is considerable controversy regarding the TGF-β ligand localization in other cell types, as there are studies reporting expression either in the AEC, in the vascular endothelium, or mesenchymal cells (Coker et al., 1996; Pelton et al., 1991). In line with the mouse TGF-β ligand expression pattern, the TGF-β ligands in the adult human lung are primarily expressed in the bronchial epithelial cells (BEC) (Magnan et al., 1994), alveolar macrophages, other inflammatory cells, vascular and airway SMC, and mesenchymal cells (Coker et al., 1996; Magnan et al., 1994).

1.4.2 TGF-β-related lung disorders

A common characteristic of many diseases is an inflammatory process related to tissue injury, followed by a phase of repair. Elevated concentrations of active TGF-β1 are found in patients, which suffer inflammatory lung diseases (Table 1) (Bartram and Speer, 2004). Moreover, in different organs, including the liver and kidney, high levels of this isoform have been also detected (Branton and Kopp, 1999; Sime and O'Reilly,

2001). This results in an enhanced matrix deposition and a downregulation of the inflammatory response after injury. These imbalanced levels of the cytokine, due to an exaggerated repair process, may generate fibrotic lesions in the organs (Bartram and Speer, 2004; Letterio and Roberts, 1996). TGF-β1 is the predominant TGF-β isoform in disease conditions, although the upregulation of the other two isoforms has also been described (Bartram and Speer, 2004).

Table 1: TGF-β1-related lung diseases

Disease	**Source**
Chronic lung disease of prematurity	(Kotecha et al., 1996)
Idiopathic pulmonary fibrosis (IPF)	(Khalil et al., 1991)
Giant-cell interstitial pneumonia	(Corrin et al., 1994)
Occupational diseases (silicosis, asbestosis, pneumoconiosis)	(Jagirdar et al., 1997) (Vanhee et al., 1994)
Sarcoidosis	(Limper et al., 1994)
Lymphangioleiomyomatosis (LAM)	(Evans et al., 2004)
Cystic fibrosis	(Wojnarowski et al., 1999)
Chronic obstructive lung disease (COPD)	(de Boer et al., 1998)
Asthma	(Aubert et al., 1994)
Transplantation-related diseases (bronchiolitis obliterans)	(Magnan et al., 1996)

Table 1: TGF-β1-related lung diseases. The main respiratory diseases associated with an imbalanced TGF-β1 expression are listed (modified after Bartram and Speer, 2004).

The consequences of abnormally high concentrations of TGF-β1 in the lung have been studied in a variety of models. The mouse model of bleomycin-induced lung fibrosis perfectly mimicks the upregulation of the three different isoforms (Santana et al., 1995). Furthermore, TGF-β1 overexpression in transgenic mice using tissue-specific promoters has provided various model systems to study the effects of an increased TGF-β1 activity in the intact organism (Bottinger and Kopp, 1998; Zhou et al., 1996). Additionally, TGF-β1 overexpression, assessed by intratracheal instillation of replication-deficient adenovirus vectors into the rat lung, demonstrated that only the active form of the cytokine resulted in pathological conditions. A severe interstitial and pleural fibrosis resulted, characterised by extensive deposition of the ECM proteins,

such as collagen, fibronectin, and elastin, and by the emergence of cells with a myofibroblast phenotype (Sime et al., 1997).

1.4.3 Idiopathic Pulmonary Fibrosis (IPF)

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating disease with unknown aetiology. The disease normally develops from the middle age onwards, with men twice as likely as women to be affected from IPF. Currently there is no available treatment, and after diagnosis the median survival is between three and five years (Selman and Pardo, 2002). The long-prevailing hypothesis of inflammation being the main trigger of the disease is been currently debated, since the response to steroids is usually poor in IPF, and the use of other immunosupressors has largely failed to increase the survival rate (Geiser, 2003a). Furthermore, studies on transgenic animal models indicate that the inflammatory response and the fibrotic response can be dissociated (Munger et al., 1999).

It has been recently proposed that IPF may result from a sequential epithelial injury, leading to the loss of the epithelial cell integrity, which is a consistent finding in IPF (Coalson, 1982; Kasper and Haroske, 1996). The injurious agent will affect both the increase in AEC apoptosis (Maeyama et al., 2001) and the inhibition of AEC proliferation, trans-differentiation and migration (Kasper and Haroske, 1996). All together, this would impair the normal re-epithelialisation of the denuded area, leading to an abnormal wound healing (Selman and Pardo, 2002) (Figure 1.5.A).

The remaining AEC after epithelial injury have been shown to produce a number of soluble factors, like TGF-β1 (Khalil et al., 1991), TNF-α (Kapanci et al., 1995) or platelet-derived growth factor (PDGF) (Antoniades et al., 1990), that are known to promote fibroblast activation. The active fibroblasts, the so-called myofibroblasts, migrate to the denuded area, where they undergo abnormal proliferation, leading to the formation of the so-called fibroblast foci (Figure 1.5.B). These myofibroblasts synthesise large amounts of ECM molecules and a variety of cytokines, including the profibrotic TGF-β1 (Serini and Gabbiani, 1999). Increased amounts of TGF-β1 mRNA and protein have been encountered. The abnormal deposition of ECM molecules results in the destruction of the alveolocapillary units, leading to impairment of normal lung function. An inefficient re-epithelialisation occurs over the fibroblastic foci; however,

the distance between the AEC and endothelial cells is too large, impeding gas-exchange (Figure 1.5.B).

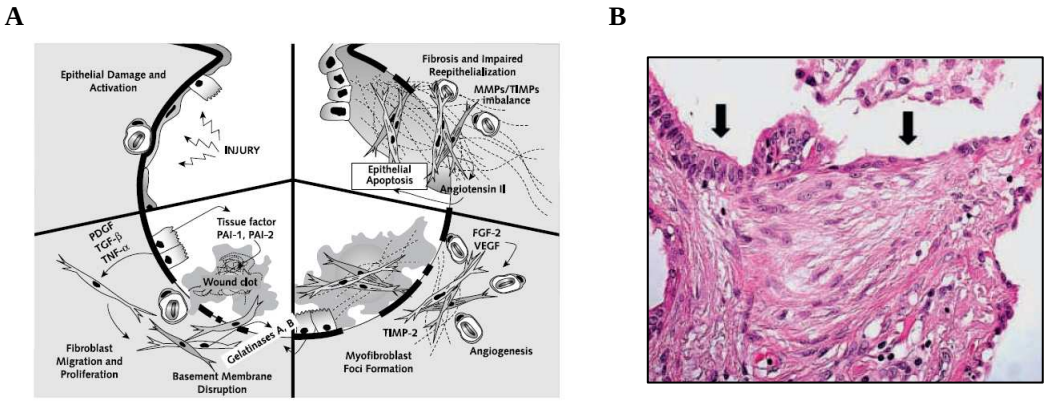


Figure 1.5: Hypothetical model of impaired wound healing for IPF. A) Constant and multiple microinjuries damage the AEC (*top left*) and the remaining AEC synthesise several GF, including TGF-β1 (*bottom left*). The denuded area is covered by migrating fibroblasts, where they proliferate forming fibroblast foci (*bottom right*). The myofibroblasts synthesise angiogenic factors to induce neovascularisation. The production of angiotensin II promotes AEC re-epithealization; however, the gas-exchange process is impaired by the fibroblast foci (*top right*) (after Selman M. *et al*., 2001). B) Haematoxylin and eosin staining of a fibroblast foci in IPF lung (after Geiser T., 2003b).

The fact that the myofibroblasts are the principal cell type participating in the pathogenesis of IPF is well described, as the progression of the IPF is associated with fibroblastic foci in the lung (King et al., 2001). The source for these activated spindle-shape cells, which express various mesenchymal makers, is currently under discussion. Three hypotheses have been proposed thus far to explain their origin:

- The first postulates the activation and proliferation of local fibroblasts, upon stimulation (Phan, 2002). TGF-β1 promotes differentiation of cultured isolated primary rat fibroblasts into myofibroblasts through the Smad3 signalling pathway (Hu et al., 2003).
- The second hypothesis claims that an engraftment of bone marrow-derived progenitor cells occurs. These cells then differentiate into myofibroblasts in the respiratory tract (Epperly et al., 2003; Hashimoto et al., 2004).
- The recently postulated hypothesis is related to EMT, where epithelial cells are considered to be the source of the mesenchymal cells by TGF-β1 induction (Zavadil and Bottinger, 2005).

The EMT process is a complex and extreme manifestation of epithelial cell plasticity, in which the polarized epithelial cells convert to fibroblastoid cells capable of locomotion (Thiery, 2002). The required cellular changes are the loss of the epithelial cell-cell and cell-matrix adhesion contacts and activation of *de novo* synthesis of ECM

molecules. At the transcriptional level, a simultaneous repression of epithelial markers (occludin and E-cadherin) and an upregulation of mesenchymal markers (α-SMA and vimentin) are observed (Ikenouchi et al., 2003).

The transmembrane proteins, occludin and ZO-1, are responsible for the tight junction maintenance and the E-cadherin extracellular domains to sustain adherens junctions, which maintain the epithelial cell polarity by interacting intracellularly with the actin cytoskeleton via α- and β-catenins (Zavadil and Bottinger, 2005). When the disassembly of cell-cell junctions including tight, gap and adherens junctions, and desmosomes occurs, integrity of the epithelial layer is lost. Simultaneously, the trans-differentiated epithelial cells synthesise vimentin intermediate filaments, which form the motile elements of the cellular architecture, along with the actin-containing microfilaments (Helfand et al., 2004). The fibroblast specific protein 1 (FSP1), also known as S100A4, has been described as a marker for differentiated fibroblasts (Strutz et al., 1995).

Many GF like EGF, hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and TGF-β; endothelin-1 (ET-1) and members of the Wnt signalling pathway promote EMT (Jain et al., 2007; Radisky, 2005). Interestingly, both the EMT process and fibrogenesis are common in epithelial-enriched organs, like the lung and kidney (Willis et al., 2006). Specifically, the TGF-β1 ligand promotes, through the Smad-dependent pathway (principally by the Smad3) (Roberts et al., 2006), other TF including Slug, Snail, and β-catenin, which are related to the epithelial cell trans-differentiation into mesenchymal cells (Kalluri and Neilson, 2003; Masszi et al., 2004). TGF-β1-induced EMT has been demonstrated in the human epithelial A549 cell-line (Kasai et al., 2005), on rat lung epithelial-T-antigen negative (RLE-6TN) and isolated primary ATII cells (Willis et al., 2005). Additionally, colocalisation of mesenchymal and epithelial markers in the same cell was demonstrated in IPF lung sections (Kim et al., 2006; Willis et al., 2005).

1.5 STUDIES ON LUNG EPITHELIUM

Research on the pulmonary epithelial layer has been accomplished by either isolation of various primary cell types, following microdissection, or fluorescence-activated cell sorting (FACS), thus avoiding tissue homogenates, which have the disadvantage of masking genetic deviations or expression changes of an

individual cell type by the surrounding cell bulk (Curran et al., 2000). However, the enzymatic digestion methods to obtain single cell suspensions for both FACS and short-term cell cultures may induce cellular changes as they are subjected to different environmental factors.

Several enhanced methods for isolating and culturing primary adult rat and mouse cells and fetal human type II pneumocytes are currently available (Wang et al., 2007). Their gene and protein expression under different culture conditions and their cell response upon stimulation is also well characterised. However, it has been demonstrated that isolated primary ATII cells change their phenotype upon culture (Gonzalez et al., 2005). They gradually trans-differentiate into ATI-like cells, as they loose their lamellar inclusion bodies, become flat and change their gene expression profile (Chen et al., 2004; Paine and Simon, 1996). This trans-differentiation occurs between day 3 and 5 after isolation, and cell type-specific markers from both the ATII and the ATI cell types are localized simultaneously, indicating the existence of a possible intermediate cell type (Bhaskaran et al., 2007; Chen et al., 2004; Danto et al., 1995). Interestingly, a trans-differentiation of ATII cells into mesenchymal cells has also been demonstrated *in vitro* and *in vivo* (Willis et al., 2005). Moreover, the trans-differentiation process depends, to a great extent, on the presence or absence of matrix on which the ATII cells are plated (Gonzales et al., 2002). When they are cultured on uncoated dishes (on a plastic surface), their phenotype is rapidly altered (Chen et al., 2004; Paine and Simon, 1996). The matrices can be composed of different molecules (fibronectin or laminin-5 alone), where the ATII cells change their phenotype (Guo et al., 2001; Olsen et al., 2005), on a mixture of fibronectin/collagen/laminin-5 or on Matrigel along with some GF, where the type II phenotype is preserved longer (Olsen et al., 2005; Wang et al., 2007). Additionally, the EMT process can differ depending on the ECM composition onto which ATII cells are attached (Zeisberg et al., 2001).

Recently, laser capture microdissection (LCM) has improved the current knowledge on cell gene and protein expression, as LCM enables fine microdissection of a specific pool of cells (Betsuyaku et al., 2001; Fink et al., 2006). However, the microdissection machinery is extremely expensive and the technique demands manual dexterity, and is prone to some operator bias. Furthermore, the mode of tissue section preparation may also modify the results (Curran et al., 2000; Kim et al., 2003) and a

high probability of RNA degradation by endogenous RNases has been reported (Kohda et al., 2000).

Differentiated ATII cell-lines are not currently available. Nevertheless, several cell-lines with an epithelial origin have been established. For instance, the NCI-H441 cell-line derived from a human pulmonary papillary adenocarcinoma highly expresses SP-A (O'Reilly et al., 1988). The A549 cell-line, which is frequently used in studies on the lung epithelium, was explanted from a human lung carcinomatous tissue, although any SP expression is absent (Mason and Williams, 1980). In addition, cell-lines with a rodent origin, such as, murine lung epithelial (MLE) 12 (Alejandre-Alcazar et al., 2007) and RLE-6TN (Willis et al., 2005) have been usually utilised. However, the cell-lines are limited in that they are immortalised and in many cases transfected and transformed, processes that completely change their gene expression patterns (Malkinson et al., 1997).

Many different screening approaches have been performed thus far in order to ascertain novel biomarkers for the various pulmonary diseases. For instance, some using cell-lines and arrays (Koike et al., 2004), studying the trans-differentiation of ATII cells into ATI *in vitro* by microarrays (Chen et al., 2004) or also comparing freshly isolated ATI, ATII and cultured ATII using microarray analysis (Gonzalez et al., 2005). Thus, the currently used cell type specific markers have been developed *in vitro* and thus far, a comprehensive analysis of the epithelial gene transcription *in vivo* has not been yet attempted.

2 AIM OF THE STUDY

While primary AEC culture has increased our knowledge of AEC gene expression *in vitro*, a comprehensive analysis of epithelial cell gene transcription *in vivo* has not yet been attempted. The aim of the study was to profile the epithelial cell gene expression in the murine lung *in vivo* by developing a new technique, in which RNA from the lung epithelium would be isolated directly from the mouse lung, avoiding cell plating.

Furthermore, the influence on the epithelial gene expression profile of TGF-β1, a cytokine related to many different lung diseases, was studied by direct administration of the ligand into the murine lung.

By combination of these two optimised approaches, the study of the TGF-β1 influence on the epithelial gene expression *in vivo* was assessed.

In summary, the specific aims of this work are as follows:

1. The establishment of a novel method to isolate RNA directly from epithelial cells in the murine lung, avoiding cell plating. This has been termed “epithelial lavage”.
2. Characterisation of epithelial gene expression *in vivo*.
3. Comparison of RNA expression between the epithelial lavage samples, primary isolated ATII cells, and epithelial cell-lines.
4. Orotracheal administration of the TGF-β1 cytokine.
5. Analysis of the TGF-β1-induced signal transduction of both Smad-dependent and -indendent pathways.
6. Analysis of TGF-β1 early-responsive genes in the epithelial layer.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 General chemicals

Table 2: General laboratory chemicals

Compounds	Company
β-mercaptoethanol	Sigma-Aldrich
3MM Whatman paper	Schleicher and Schnell
100 bp DNA ladder	Promega
Agarose	Invitrogen
Ammonium Persulfate (APS)	Promega
Biotin-16-2´-deoxyuridine 5´-triphosphate (dUTP)	Roche
Bradford reagent	Bio-Rad
Complete™ (Protease inhibitor cocktail tablets)	Roche
Dispase	BD Biosciences
Dithiothreitol (DTT)	Promega
Dual colour precision protein standards	Bio-Rad
ECL Western blotting detection reagents	Pierce
Eythelene diamino tetra acetic acid (EDTA)	Promega
Ethidium bromide	Roth
GEArray Q Kit	SuperArray
Glycerol	Promega
Glycogen	Sigma-Aldrich
GoTaq® Flexi DNA Polymerase	Promega
Isoflurane	Baxter
Herring Sperm DNA	Promega
Isotonic sodium chloride solution	DeltaSelect
Ketavet® (Ketamine)	Pharmacia
Methanol	Fluka
M-MLV Reverse Transcriptase	Promega

Mouse Genomic DNA	Promega
Mouse Universal Reference Total RNA	Clontech Laboratories
Nitro-cellulose membrane	Bio-Rad
Non-fat dry milk powder	Roth
Nonidet P-40	Sigma-Aldrich
Oligo$(dT)_{15}$ Primer	Promega
PCR Nucleotide Mix	Promega
Platinum® SYBR® Green qPCR SuperMix UDG	Invitrogen
Phosphate-buffered saline (PBS)	PAA
RNase-Free Dnase set	Qiagen
RNase inhibitor	Promega
RNaseZAP	Sigma-Aldrich
Rompun® (Xylazine)	Bayer Vital
Rotiphorese® gel 30	Roth
Roti-Quick-Kit (Solution 1, 2 and 3)	Roth
Sodium deoxycholate	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Promega
Sodium ortho vanadate	Sigma-Aldrich
TEMED	Bio-Rad
TGF-β1	R&D Systems
Toluidine Blue O	Sigma-Aldrich
Tris	Roth
Tween-20	Sigma-Aldrich

3.1.2 Cell culture reagents

Table 3: Cell culture reagents

Compounds	Company
β-estradiol	Sigma
Dulbecco's modified Eagle medium (DMEM)	Gibco
Fetal calf serum (FCS)	PAA

HEPES	PAA
Hydrocortisone	Sigma
Insulin/Transferrin/NaSelenit	Gibco
Trypsin-EDTA	PAA

3.1.3 Primers

The primers were designed using the Primer3 Input Internet programme (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), assuring specificity by the nucleotide-nucleotide BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST). Primers were synthesised by Qiagen, Germany, at the synthesis scale of 0.05 μmol. The primer sequences are listed in the Appendix (Table A.17).

3.1.4 Antibodies

Table 4: Primary antibodies

Catalogue No.	Description	Supplier
A 5228	α-smooth muscle actin	Sigma
9272	AKT	Cell Signaling
SC-601	CDK4 (H-22)	Santa Cruz Biothechnology
9212	p38	Cell Signaling
9102	p44/42 MAPK	Cell Signaling
4058	Phospho-AKT (Ser473) (193H12) Rabbit mAb	Cell Signaling
9211	Phospho-p38 (Thr180/Tyr182)	Cell Signaling
9106	Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) mouse mAb	Cell Signaling
SC-8979	PAI-1 (H-135)	Santa Cruz Biotechnology
3101	Phospho-Smad2 (Ser465/467)	Cell Signaling
9514	Phospho-Smad3 (Ser423/425) / phospho-Smad1 (463/465)	Cell Signaling
610843	Total Smad 2/3	BD Biosciences

61-7300	ZO-1	Zymed

Table 5: Secondary Antibodies

Name	Description	Supplier
Mouse horse-radish peroxidase	Anti-mouse IgG (H + L)	Cell Signaling
Rabbit horse-radish peroxidase	Anti-rabbit IgG (H + L)	Cell Signaling

3.2 METHODS

3.2.1 Mammalian cell culture

All cells were maintained at 37 °C in a humidified incubator in 5% (v/v) CO_2 and 95% (v/v) air. All cells were grown in cell culture flasks. Storage of cells was done by harvesting cells and adding 1 ml of freezing medium (10% (v/v) DMSO in fetal calf serum (FCS)). The cells were transferred to a cryovial, placed on ice for 1 h and transferred to –80 °C for 1 day prior to storage in liquid nitrogen. To thaw cells, the cryovial was removed from liquid nitrogen and placed at 37 °C for 10 min The cells were transferred to 10 ml of pre-warmed fresh medium followed by a light centrifugation before being re-plated onto cell culture dishes in fresh medium.

3.2.1.1 Cell isolation

Primary alveolar type II (ATII) cells were isolated from adult female C57BL/6N mice (18-20 g) as previously described (Corti et al., 1996). Briefly, mice were sacrificed by intraperitoneal injection of a mixture of ketamine and xylazine (1:1:2 - Ketavet®:Rompun®:saline in a final volume of 200 µl). The thoracic cavity was opened carefully to avoid puncturing the lung. Trachea was exposed by a midline neck incision and a catheter was inserted and ligated. The abdomen was opened, the renal artery was separated and pneumothorax was drawn. Afterwards, lungs were perfused with saline and lavaged. Next, the proteolytic enzyme dispase (1.2 ml) was introduced to dissociate the pulmonary epithelial cells from their basement membrane, followed by agarose instillation (0.4 ml), maintained in liquid form at 55 °C. Agarose solution was allowed to solidify for 2 min inside the lungs and the respiratory organ was then separated from the thorax and incubated in 1 ml dispase solution for 45 min at room temperature.

After dispase disaggregation, the purification method, based on the differential adherence to IgG-coated Petri dishes of immune, contaminating cells, was performed. The ATII cell preparations used were > 95% viable, as determined by trypan blue dye exclusion (cells were incubated for 2 min with the dye, blue-stained cells were considered non-viable) and 92–96% pure, as determined by lamellar body staining with Nile Red. Briefly, the Nile Red staining was performed as follows: 20 µl of cell suspension in 500 µl DMEM were combined with 1:20 Nile Red solution, vortexed and incubated for 5 min at room temperature. Stained cells were counted under UV-light. The ATII pneumocytes were then plated either on uncoated or fibronectin-coated dishes (100 µg/ml) in conditioned medium.

3.2.1.2 Cell culture

For sub-culturing, cells were washed twice with ice-cold 1× PBS and 2-3 ml of trypsin-EDTA was added and incubated at 37 °C until the cells were well dispersed. To these cells, 7 ml of fresh complete growth medium was added and the cells were aspirated gently by pipetting and finally aliquoted into new culture vessels. The murine lung epithelial cell-line MLE 12 was cultured in HITES medium supplemented with 2% FCS, according to the recommendations of the American Type Culture Collection (www.atcc.org). The ATII cells were cultured in DMEM with 10% FCS.

3.2.2 Molecular biology techniques

3.2.2.1 Epithelial lavage extraction

Adult male C57BL/6N mice were sacrificed by an intraperitoneal injection of ketamine/xylazine (1:1:2 - Ketavet®:Rompun®:saline in a final volume of 200 µl) and tracheostomised. The lungs were then lavaged twice with saline. This fraction was centrifuged for 5 min, at 13,000 r.p.m. and snap frozen. Subsequently, 600 µl of a 1:20 guanidinium isothiocyanate Roti-Quick1 dilution (Roti-Quick-Kit, Roth) was applied and retained for 30 seconds inside the lung. Immediately after, 600 µl of saline were introduced into the lung, withdrawn, and added to the previous extraction. The final volume of ~ 1100 µl was then added onto 200 µl of undiluted Roti-Quick1 solution and snaped frozen.

3.2.2.2 RNA isolation from cultured cells

Total RNA from MLE12 cells and freshly isolated and subsequently cultured ATII cells was isolated using the QIAshredder columns (Qiagen) to disrupt the DNA and then followed the Qiagen RNeasy MiniKit protocol.

3.2.2.3 RNA isolation from lung homogenates

Total RNA was isolated from unfixed lung tissue after epithelial lavage extraction. The right lobe was ground to powder under liquid nitrogen with a mortar and pestle. Guanidinium isothiocyanate (Roti-Quick1) was added to the tissue to isolate RNA, which was separated from the sheared DNA and proteins by adding phenol/chloroform (Roti-Quick2), and precipitated overnight with isopropanol (Roti-Quick3). Further steps were performed according to the manufacturer's protocol.

3.2.2.4 RNA isolation from epithelial lavage samples

Total RNA isolation from the epithelial lavage samples was performed with the Roti-Quick-Kit as described above. In order to improve the RNA precipitation, and therefore, the final RNA concentration, glycogen (1 µl/700 µl per extraction) was added as a co-precipitant for the overnight precipitation with isopropanol.

3.2.2.5 Reverse transcription (RT) reaction

A reverse transcription (RT) reaction was performed with the Sensiscript RT Kit (Qiagen) suitable for low quantities of RNA. According to the manufacture's protocol, 50 ng of high quality total RNA and diethylpyrocarbonate (DEPC)-treated water to a final volume of 13 µl were combined and heated to 65 °C for 5 min The reaction was snap-chilled on ice to allow annealing of Oligo$(dT)_{15}$ to the poly A tail of the mRNA. Meanwhile, a master-mix of the other reagents was prepared, in a total volume of 7 µl, as follows:

Table 6: Master-mix preparation for RT reactions

RT reaction component	Volume
10× buffer	2 µl
dNTP Mix (5 mM each dNTP)	2 µl
Oligo$(dT)_{15}$	1 µl (10 units)

Rnase inhibitor	1 µl
Sensiscript Reverse Transcriptase	1 µl

Later, master-mix was added to the reaction tube and transferred to a PCR machine programmed for maintaining 37 °C for 60 min

3.2.2.6 Polymerase chain reaction (PCR)

3.2.2.6.1 Semiquantitative PCR

All semiquantitative PCRs were performed using the PCR kit from Promega (Madison, WI, USA), in a total volume of 25 µl as follows:

Table 7: Master-mix preparation for semiquantitative PCR reactions

Components	Volume/Amount
Template cDNA	10 ng (1µl)
Forward primer	10 pmole (0.5µl)
Reverse primer	10 pmole (0.5µl)
40 mM dNTP mix	0.5 µl
5× PCR buffer	5 µl
25 mM $MgCl_2$	2 µl
Go Taq Polymerase	0.25 µl
Double-distilled water to 25 µL	15.25 µl

The tubes were then transferred to a PCR machine (Peltier Thermal Cycler-200, MJ Research, Ramsey, MN, USA) set for 20 cycles in the case of housekeeping genes and 35 cycles for the rest of genes, following the next steps:

Table 8: Programme for semiquantitative PCR reactions

PCR step	Temperature	Duration
First denaturation	95 °C	2 min
Second denaturation	95 °C	45 s
Annealing	60 °C	45 s
Extension	72 °C	1 min
Final extension	72 °C	10 min

Primer efficiency and optimal annealing temperature test were also performed by following a gradient PCR programme in DYAD™ machine (MJ Research, Ramsey, MN, USA):

Table 9: Programme for gradient PCR reactions

PCR step	Temperature	Duration
First denaturation	95 °C	5 min
Second denaturation	95 °C	45 s
Annealing	58-62 °C	30 s
Extension	72 °C	1 min
Final extension	72 °C	10 min

3.2.2.6.2 Quatitative PCR (qPCR)

All quantitative PCRs were performed using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), in a total volume of 25 µl as follows:

Table 10: Master-mix preparation for quantitative PCR reactions

Components	Volume/Amount
Template cDNA	5 ng (1 µl)
Forward primer	10 pmole (0.5 µl)
Reverse primer	10 pmole (0.5 µl)
50 mM $MgCl_2$	4.0 mM (0.5 µl)
2× PCR buffer	13 µl

The plate was then transferred to the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) set for 45 cycles. The gene expression was analysed by the 7500 Fast System Software. The programme set was as follows:

Table 11: Programme for quantitative PCR reactions

PCR step	Temperature	Duration
Activation of polymerase enzyme	50 °C	2 min
First denaturation	95 °C	5 min
Second denaturation	95 °C	5 s

Annealing	59 °C	5 s
Extension	72 °C	30 s
Dissociation step 1	95 °C	15 s
Dissociation step 2	60 °C	1 min
Dissociation step 3	95 °C	15 s
Dissociation step 4	60 °C	15 s

The specific amplification of the PCR products was confirmed by melting curve analysis and gel electrophoresis. Additionally, ΔCt values were calculated as follows: Δ Ct = Ct reference gene (*hprt*) – Ct target gene; denoting Ct the cycle number where the fluorescence value is above the determined threshold and each unit of ΔCt represents a 2-fold change in the target gene mRNA expression (Kwapiszewska et al., 2005). ΔCt values were plotted in Box and Whisker graphs, in which the box denotes the interquartile range (25-75%) and the horizontal line indicates the median. The whiskers or error bars indicate the 95% confidence interval and possible "outliers" of ΔCt values. ΔCt mean values were also blotted as mean ±SD in column graphs.

3.2.2.7 DNA agarose gel electrophoresis

The DNA sample along with the loading buffer (0.01% (m/v) Bromophenol blue; 40% (v/v) glycerol; 1× TAE buffer) was loaded onto either a 1% (m/v) agarose gel (semiquantitative RT-PCR) or 2% (m/v) agarose gel (quantitative RT-PCR). Electrophoresis was performed for 30-45 min with 5 V/cm (Bio-Rad, USA), where the negatively charged DNA migrates from the cathode to the anode. To visualise DNA, the gel was treated with ethidium bromide (0.5 µg/ml). The size of the DNA fragments was determined by a DNA molecular weight standard.

3.2.2.8 Oligo microarray

The RNA amount used to perform the gene expression analysis of the TGF-β treated lungs on the Oligo GEArray® Mouse TGF-β/BMP Signalling Pathway Microarray (OMM-035, SuperArray®, Frederick, MD, USA) was 3 µg. The RNA served as template to synthesise a biotin-labeled cDNA probe. Two different master-mixes were made as follows:

Table 12: Labelling master-mix for cDNA probe synthesis

Components	Volume
5× GEA labelling Buffer (BN)	8 µl
Biotin-16-dUTP (1µM)	4 µl
RNase inhibitor (40 U/µl)	2 µl
Reverse transcriptase (200 U/µl)	0.5 µl
RNase-free water	5.5 µl

Table 13: Annelling master-mix for cDNA probe synthesis

Components	Volume
Total RNA (3 µg)	x µl
GEAprimer Mix (Buffer A)	3 µl
RNase-free water	10-x µl

The annelling master-mix was placed in a PCR machine (Peltier Thermal Cycler-200, MJ Research, Ramsey, MN, USA) and warmed to 70 °C for 3 min to denature secondary structures in the RNA strands, and then cooled to 42 °C for 2 min. Both master-mixes were combined and the labeling reaction was performed at 42 °C for 90 min

In the meanwhile, the GEArray® membrane was pre-hybridisated by adding 2 ml of de-ionized H_2O and 2 ml of GEAhyb® hybridisation solution containing heat-denatured herring sperm DNA (100 µg DNA/ml). The GEArray® membrane was placed for 1 h into an oven (60 °C) with a continuous agitation (5 r.p.m.).

In order to stop the labelling reaction, 2 µl of 10× stop solution (Buffer C) was added. Immediately after, the sample was denatured, when 2 µl of the pre-warmed 10× denaturing solution (Buffer D) were added and incubated for 20 min at 68 °C. Afterwards, 25 µl of 2× pre-warmed neutralisation solution (Buffer E) were combined and incubated at 68 °C for 10 min

During this time, the pre-hybridisation solution was discarded from the membrane and the cDNA probe (50 µl) was mixed with another 1 ml of GEAhyb® hybridisation solution and was added onto the membrane. The hybridisation to the membrane was performed overnight in an oven with continuous agitation at 60 °C.

On the following day, the membrane was washed (2× 10 min) with solution 1 (2× SSC and 1% SDS) and again (2× 10 min) with solution 2 (0.1× SSC and 0.5% SDS) at 60 °C. Pre-warmed GEA blocking solution Q was added to the membrane and incubated for 40 min in a lab rotator. Afterwards, incubation with alkaline phosphate-conjugated streptavidin (diluted at 1:8000 in 1× washing buffer F) for 10 min with gentle shaking was performed, followed by three washing steps with 1× washing buffer F. Afterwards, the membrane was rinsed twice with 1× AP-assay buffer G, incubated with 1 ml CDP-Star chemiluminescent substrate, and signal was finally detected by chemiluminescence. Data analysis was performed on web-based GEArray® Analysis Suite software. Intensity levels on the blank samples were substracted from gene spots and data was normalized to the *gapdh* gene expression.

A short overview of the GEArray® procedure is provided in the Appendix (Figure A.1). The array layout table containing the short and long name, and location of the murine genes on the microarray is attached in the Appendix (Table A.18).

3.2.3 Immunological methods

3.2.3.1 Immunohistochemistry

Three-µm paraffin sections mounted on poly-L-lysine-coated slides were dewaxed by immersion in xylene (3× 10 min) and rehydrated by immersion in ethanol (5 min, 2× 100%, 2× 95%, and 2× 70% v/v) followed by a 1× phosphate-buffered saline wash (PBS). After antigen retrieval in 1× citrate buffer (in a pressure cooker for 20 min followed by a 10 min-warming step), endogenous peroxidase activity was blocked with 3% (v/v) H_2O_2 for 20 min. The blocking reagent was applied for 10 min to prevent non-specific binding. Sections were incubated with the relevant primary antibody overnight at 4 °C. On the following day, tissue slides were incubated with the relevant biotinylated secondary antibodies for 10 min, followed by streptavidin-conjugated enzyme for another 10 min, and chromogen substrate incubation for 10 min (Zymed; Histostain *Plus* Kit). All the steps described above were preceded by washing 2× 5 min with 1× PBS. Finally, sections were counterstained with haematoxylin for 8 min and washed under running tap water for 10 min. Sections were mounted by mounting medium and examined under a bright field microscope (Leica Mikroscopie und Systeme GmbH).

3.2.3.2 Western blotting

i) Protein isolation from lung tissue

The lung tissue was ground to powder under liquid nitrogen and lysed in RIPA lysis buffer (1× PBS without calcium and magnesium, 0.5% (m/v) sodium deoxycholate, 1% (m/v) SDS, 1% (v/v) Nonidet P-40) containing a mixture of protease and phosphatase inhibitors. The lysates were vortexed in a period of 30 min every 5 min and centrifuged at 13,000 r.p.m. for 15 min. The supernatant protein concentration was measured and stored at -80 °C.

ii) Protein separation by poly acrylamide gel electrophoresis (PAGE)

The separating gel mixture (Table 15) was poured between two glass plates and allowed to polymerise. The stacking gel mixture (Table 16) was poured on the top of the separating gel and the comb was inserted in the gel to form the wells. The gel was run in 1× Laemmli-loading buffer (25 mM Tris-Cl pH 8.3, 0.2 M glycine and 0.1% (v/v) SDS) until the desired distance had been reached.

iii) Immunoblot

After gel electrophoresis, proteins were transferred to a nitrocellulose membrane using a Bio-Rad transfer chamber containing transfer buffer (24 mM Tris base, 193 mM glycine, 10% (v/v) methanol), at room temperature for 1 h at 100 V. Blots were incubated for 1 h in blocking buffer (5% (m/v) non-fat dry milk powder in PBS containing 0.1% (v/v) Tween-20) at room temperature. Incubation with the respective primary antibodies (1 µg/ml) was in blocking buffer for 1 h at room temperature or overnight at 4 °C in the case of anti-phospho antibodies. After 3× 5 min wash with washing buffer (1× PBS, 0.1% (v/v) Tween-20), blots were incubated with the respective HRP-labeled secondary antibodies (0.3 µg/ml) at room temperature for 40 min After 4× 10 min washing with washing buffer, the blots were developed by enhanced chemoluminescence and visualised with Hyperfilm ECL (Kodak). To re-probe with another antibody, the blots were stripped with stripping buffer (0.063 M Tris-Cl pH 6.8, 2% (m/v) SDS, 0.7% β-mercaptoethanol) at 52 °C for 30 min, and then probed as described above.

Table 14: Composition of 10% resolving gels (40 ml)

Component	Volume
Distilled water	15.9 ml
30% acrylamide mix	13.3 ml
1.5 M Tris-Cl (pH 8.8)	10 ml
10% (m/v) SDS	400 µl
10% (m/v) APS	400 µl
TEMED	16 µl

Table 15: Composition of 5% stacking gels (20 ml)

Component	Volume
Distilled water	13.6 ml
30% acrylamide mix	3.32 ml
1 M Tris-Cl (pH 6.8)	2.52 ml
10% (m/v) SDS	200 µl
10% (m/v) APS	200 µl
TEMED	20 µl

3.2.4 Animal handling

Six to eight week-old male C57BL/6N mice with body weight of 20 to 22 g were purchased from Charles River Laboratories (Sulzfeld, Germany). Animals were housed in humidity- and temperature-controlled rooms on 12:12 hs light-dark cycle with free access to rodent chow and tap water. All experiments were conducted according to the institutional guidelines that comply with national and international regulations.

3.2.4.1 Instillation of substances into the murine lung

Liquid instillation and nebulised aerosols are the predominant delivery methods for pulmonary drug administration in experimental setups. The different solutions used were: for optimisation, 1% (m/v) Toluidine Blue O in saline solution, which eases the comparison of the deposition yield between five different delivery procedures; and for TGF-β application, the recombinant protein diluted in saline.

3.2.4.1.1 Orotracheal administration (OT)

The mice were lightly anesthetised with halothane breathing. Afterwards, the animals were placed under the binocular microscope suspended by the superior cranial incisors. The mouth was wide opened, the tongue was held with blunt forcers (to block the swallow reflex), and the nasal orifices pinched close with a pair of curved forceps, forcing breath through the mouth. In this way, the visualization of the oropharyngeal cavity and pharynx was possible. Tongue restraint was maintained until at least two breaths were completed and then the mice were allowed to recover by themselves. Of note, all animals completely recovered after this procedure.

3.2.4.1.2 Intranasal administration (IN)

Mice were lightly anesthetized with ketamine/xylazine (1:1:2-Ketavet®:Rompun®:saline in a final volume of 200 µl) intraperitoneally (i.p.) and settled in a recumbent position or with an angle of 45° to ease normal breathing. Small drops of 1% (m/v) Toluidine Blue O solution were given to the nose every 15 s, to a final volume of 40 µl.

3.2.4.1.3 Intratracheal administration (IT)

The animals were deeply anesthetized i.p. with ketamine/xylazine solution (1:1:2-Ketavet®:Rompun®:saline in a final volume of 200 µl) and placed on a heating pad to maintain body temperature in the physiological range. The trachea was exposed by a midline neck incision and a canula, connected to a needle, was inserted. The 1% (m/v) Toluidine Blue O solution was slowly administrated. When necessary, animals were artificially ventilated after this procedure and the survival rate was 100%.

3.2.4.1.4 Microspray (MS)

Mice were lightly anesthetised i.p. with ketamine/xylazine solution (1:1:2 -Ketavet®:Rompun®:saline in a final volume of 200 µl). The mouth was opened, a canula was intubated into the trachea under microscopic guidance. The final volume of Toluidine Blue O solution was instilled with the help of the Microsprayer™ (Penn-Century Inc., PA, USA).

3.2.4.1.5 Nebulisation (NB)

Animals were anesthetized intraperitoneally with ketamine/xylazine (1:1:2 -Ketavet®:Rompun®:saline in a final volume of 200 µl) and placed on a heating

pad to maintain body temperature at physiological range. Mice were tracheostomized and artificially ventilated with room air with the use of an Aeroneb® Professional Nebulizer System (Aerogen) respirator. This ultrasonic nebulizer was positioned in the respiratory limb of the ventilator tubing. The Aeroneb® nebulizer produced an aerosol with a mass median aerodynamic diameter (MMAD) of < 3.0 μm and a geometric standard deviation (GSD) ≤ 2.2.

3.2.5 Statistical analysis

The ΔCt mean values from the indicated groups were compared using the two-tailed Student's t-test. A level of $p<0.05$ was considered statistically significant. Standard deviations were calculated as $SD= \sqrt{(SD_1)^2 + (SD_2)^2}$.

4 RESULTS

4.1 OPTIMISATION OF LOCAL DELIVERY TO THE LUNG

Five different techniques for administering liquid substances into the lung have been described (Sakagami, 2006; Sweeney and Brain, 1991). These are the intratracheal (IT), intranasal (IN), microspray (MS), orotracheal (OT) and the nebulisation (NB) methods. Direct inhalation approaches are not commonly performed, as they require specialised equipment and consume large amounts of the particulate insult, and the exact dose inhaled cannot be possible estimated (Hanson et al., 1985). For this reason, many investigators choose to mix the molecule of interest with a carrier liquid such as saline, to prevent the particles from agglomeration (Driscoll et al., 2000; Schermuly et al., 1997). For repetitive dosing, the liquid must be carefully instilled in order to prevent airway destruction, disturbance of normal breathing and induction of coughing, which would lead to a rapid lung liquid clearance. The first four procedures mentioned above are commonly utilised in pharmacodynamic investigations, although they are reported to be nonphysiological, as a large volume is administered into the lung (Driscoll et al., 2000).

The deposition efficacy was compared for the five techniques using a 1% (m/v) Toluidine Blue O solution for a quick and easy visual assessment (Lakatos et al., 2006; Visweswaraiah et al., 2002) (Figure 4.1). A detailed description of each method is given in the Materials and Methods section. After ink administration, mice were immediately sacrificed and lungs were explanted.

1) The classical intratracheal (IT) method is regularly utilised in animal laboratories, because the dose delivery can easily be measured and it minimises the risks for laboratory workers, compared to inhalation methods (Driscoll et al., 2000). Two different instillation volumes were used in this study, 100 and 200 µl. When the lower volume was utilised, only a small area was covered by the ink. However, the 200 µl volume was sufficient to cover around 85% of the lung external surface.
2) The intranasal (IN) method is a non-invasive method and has often been used for induction of viral infections and allergic sensitization (Cates et al., 2004;

Leong et al., 1998; Lundstrom, 2001). The total ink volume utilised was 40 µl. Firstly, the animal was positioned horizontally. The deposition rate yielded very low (approximately 2%), as it was only visualised in the trachea and principal bronchi. Then the animal was re-positioned at 45°. This facilitated the uptake of the entire volume, as the nose ceased to produce bubbles. Around 15% of the lung surface was covered by the ink. However, a large deposition of ink to the stomach was observed.

3) The microspray (MS) technique was reported to dispense uniformly and dispersed to the discrete pulmonary airway via bronchoscopy (Hoover et al., 1993; Sakagami, 2006). For the MS method, two different volumes were employed, 100 and 200 µl. Both demonstrated effective deposition covering at least ~70% of the lung surface.

4) The orotracheal (OT), also called transtracheal or oropharyngeal aspiration, has been recently described (Lakatos et al., 2006; Rao et al., 2003). Through this method, 80% of the lung surface was covered, when 80 µl of the 1% Toluidine Blue O solution was utilised. An additional experiment was performed using several ink volumes: 80, 100, 120, and 140 µl (Figure 4.2). The higher the volume instilled, the better the deposition. However, 80 µl was considered sufficient to reach the majority of the lung surface.

5) The nebulisation method (NB) employed a nebuliser system, which converts liquid into an aerosol (Leong et al., 1998; Schermuly et al., 1997). The NB method was not efficient, as the lung did not contained much 1% Toluidine Blue O solution, except for a small area on the lung surface and in the trachea (5%), although high volumes were utilised (100 and 200 µl). Interestingly, the ink was visualized in the nebuliser system, leading to the conclusion that the losts of nebulised substances is very high by this method.

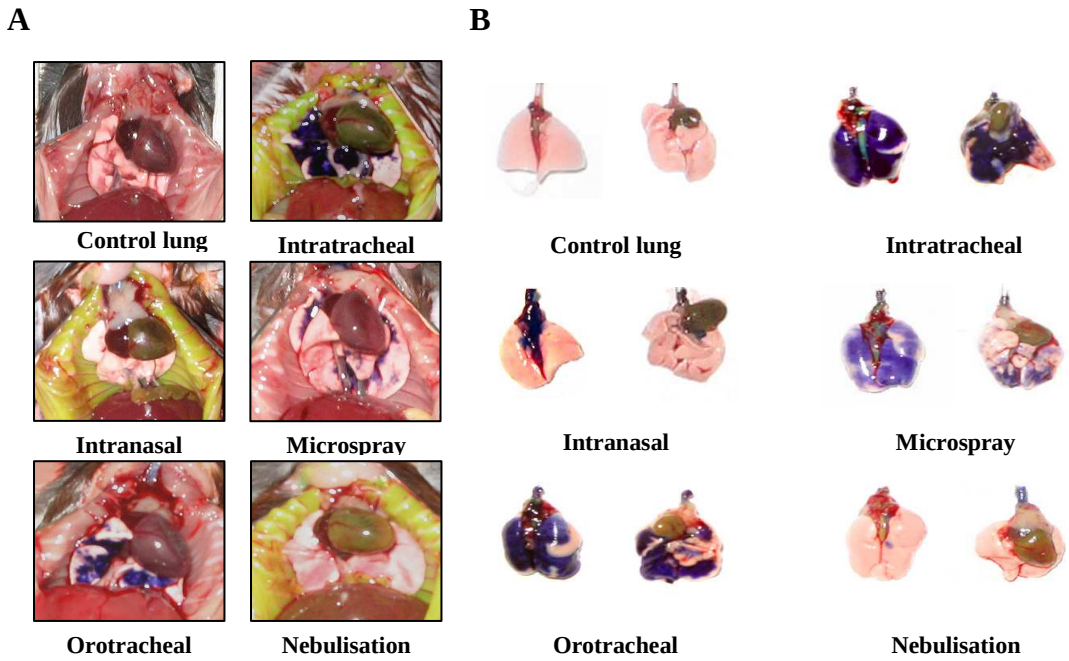


Figure 4.1: Optimisation of local delivery to the murine lung. Five different instillation approaches (intratracheal -200 µl-, intranasal -40 µl-, microspray -100 µl-, orotracheal -80 µl-, and nebulisation -100 µl-) to administer substances into the lung were compared (n = 2 per condition). Mice were instilled with different volumes of 1% Toluidine Blue O solution, depending on the technique used. The deposition rate in the lung surface of 1% Toluidine Blue O solution was observed A) *in situ* and B) in the lung explants (ventral and dorsal views). The top left panel depicts a mouse lung that was not treated but inflated with air (control lung).

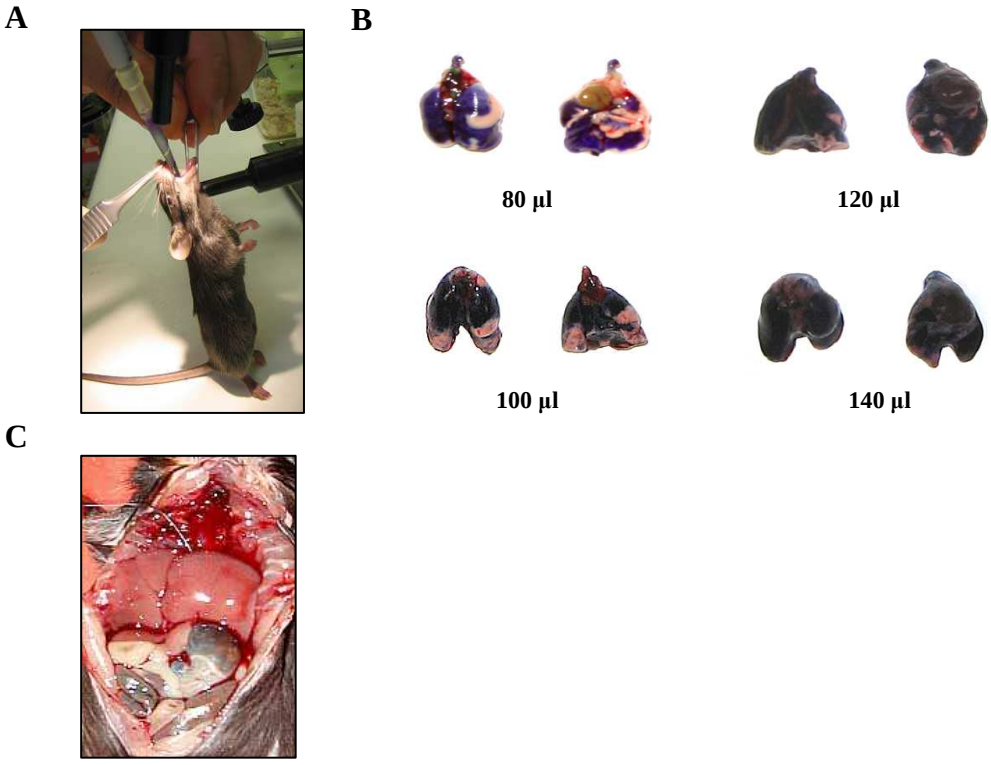


Figure 4.2: Overview of the OT method. A) The OT method was performed as follows: mice were lightly anesthetised, then suspended by the cranial incisors under the binocular microscope, the nose was pinched closed by forceps, mouth was opened, and the desired volume was pipetted into of the mouth. B) Several volumes of 1% Toluidine Blue O solution (80, 100, 120 and 140 µl) were orotrachally administered into the lung (n = 4 per condition). C) Deposition of ink could be observed in the digestive system.

Only three methods were efficient enough to cover more than 70% of the lung surface. These were the IT, MS and OT techniques (Figure 4.1). For further experiments, the OT aspiration was chosen, as it yielded the best deposition (applied volume/lung covered area), relatively small amounts of ink leaked into the stomach, due to indirect flux, and for its rapidness and easiness (Figure 4.2). In addition, it was the only approach in which light anesthesia was used, and there was no perianesthetic death associated with this procedure.

4.2 TGF-β1 ADMINISTRATION

After choosing the OT method as the most efficient approach for administration of substances to the distal lung, due to its rapidity, simplicity, and use of light anaesthesia, the TGF-β1 recombinant ligand was applied. Furthermore, the OT technique was previously reported to be useful for the delivery of antigens, infectious agents, vectors and silica particles, thereby inducing PF (Lakatos et al., 2006; Singh et al., 2004).

Our first aim was to analyse whether the ligand reached the lung, and once there, was still active; thus phosphorylation of the TGF-β1 downstream molecules was assessed by Western blotting. Several ligand concentrations (0.25, 0.5, 2.25, and 2.5 ng/µl) diluted in saline (total volume 80 µl) were instilled into the murine lung and secondly, different retention times of the recombinant cytokine (30 min, 2h, and 6 h) within the lung were analysed. At the same time, in order to distinguish between the TGF-β1 effect itself and the stress produced in the lung during the instillation procedure, non-treated (naïve) and saline-treated (80 µl of NaCl) mice were included in the study.

Furthermore, the following aim was to ascertain which genes were activated after OT ligand delivery in the lung homogenates, as the TGF-β1 early-responsive genes have not been yet described *in vivo*. Using the same ligand concentrations and retention times as mentioned above and later isolating RNA from the lung homogenates, the induction or repression in target gene transcription, was assessed by semi-quantitative, quantitative RT-PCR, and oligo microarrays. Moreover, the identification of the cell-type responsive to TGF-β1 was determined by immunohistochemistry.

4.2.1 Orotracheally-instilled TGF-β1 activates the Smad-dependent pathway

Mice were instilled with several TGF-β1 concentrations (0.25, 0.5, 2.25, and 2.5 ng/µl) and sacrificed at different times (30 min, 2h, and 6 h). Then proteins were extracted and blotted against phospho-Smad2, phospho-Smad3, phospho-ERK1/2, phospho-p38, and phospho-AKT, in order to investigate which pathway (Smad-dependent or -independent) was activated by TGF-β1.

The results depicted in Figure 4.3 are a representation of untreated, saline-treated and mice treated with several TGF-β1 concentrations (n = 2-3 per condition). As evident from Figure 4.3.A, the recombinant ligand reached the respiratory organ and was still able to activate intracellular signalling pathways, as assessed by phosphorylation of the downstream molecules. Interestingly, only the R-Smad proteins were phosphorylated after TGF-β1 administration. Phospho-Smad2 and phospho-Smad3 activation was independent of the TGF-β1 concentration, as the phosphorylation signal was observed after administration of low (0.25 ng/µl) or high (2.5 ng/µl) concentrations of ligand. Phosphorylation of AKT was observed in every mouse submitted to the OT instillation, indicating that the AKT activation was not a TGF-β1-specific response. Nevertheless, the phosphorylation state is stronger upon TGF-β1 presence in the longest studied period (6 h) and high ligand concentrations. This phosphorylation may be induced by both the stress produced in the animal during the OT instillation and the TGF-β1 cytokine. Phosphorylation on other MAPK downstream molecules was not observed (p38 or ERK1/2) (Figure 4.3.A).

Moreover, in order to compare the same ligand concentration and its effect after different time points, the lung protein content was extracted 30 min or 6 h after TGF-β1 ligand was instilled (2.5 ng/µl) (Figure 4.3.B). As earlier assessed by Western blotting, only the Smad pathway was activated, but AKT phosphorylation was as well observed. Neither p38 nor ERK1/2 molecules were phosphorylated (Figure 4.3.B).

Taken together, Figures 4.3.A. and 4.3.B. indicate that phosphorylation of Smad2 occurred solely after TGF-β1 instillation. However, the phospho-Smad3 signal was observed on saline-treated and naïve murine lungs, as well suggesting that Smad3 activation also occurs at basal state in the lung, and this activation was enhanced when TGF-β1 was instilled (Figure 4.3.B). In addition, TGF-β1-mediated Smad3

phosphorylation kinetics demonstrated an early response (after 30 min) and a late response (after 6 h) (Figure 4.3.B).

In conclusion, only the Smad-dependent pathway was specifically activated by TGF-β1, as no specific phosphorylation on the related Smad-independent downstream signalling molecules was observed (MAPK pathways). However, the phosphorylation of AKT, due to stress conditions produced during the treatment, may lead to additional intracellular responses. The concomitant activation of both R-Smad and AKT occurred under low and high concentrations of the recombinant cytokine, and at early and late time points after ligand delivery.

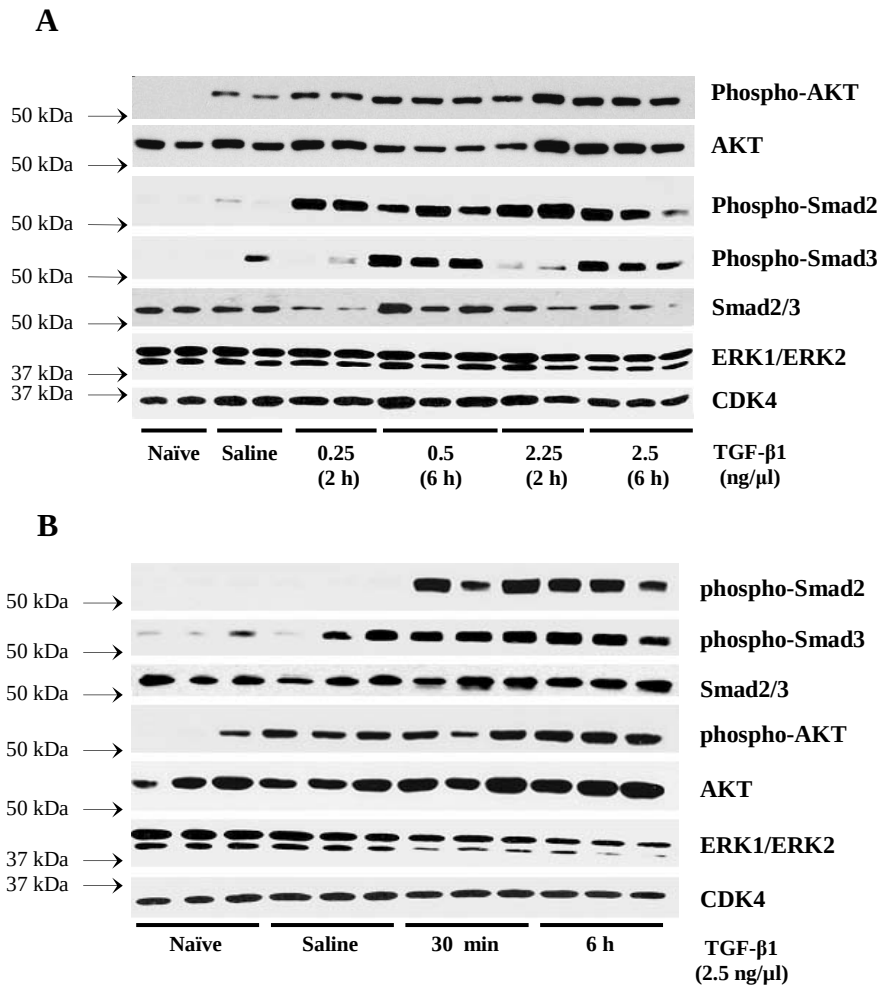


Figure 4.3: TGF-β1-induced phosphorylation of the downstream signalling molecules. A) Protein expression levels after administration of several TGF-β1 concentrations (0.25, 0.5, 2.25, and 2.5 ng/µl) and B) cytokine retention time-points within the lung (30 min and 6 h., 2.5 ng/µl TGF-β1) were compared to control mice (naïve) and saline-treated mice by Western blotting. Membranes were incubated with anti-phospho-Smad2, -Smad3, -AKT, -p38, and -ERK1/ERK2 and pan antibodies anti-AKT, -Smad2/3, -p38 and -ERK1/ERK2. CDK4 served as internal loading control. Arrows indicate standard protein molecular weight (n = 6).

4.2.2 TGF-β1-induced gene transcription

In order to study the transcriptional induction of TGF-β1 on the respiratory organ, total RNA from lung homogenates orotrachally instilled with various concentrations (0.25, 0.5, 2.25, and 2.5 ng/µl) and retention time-points (2h, and 6 h) of ligand, was isolated and reversed transcribed (n = 2-5 treated mice per condition). Next, semi-quantitative RT-PCR for well-characterised TGF-β1 target genes was performed. These genes were the connective tissue growth factor (*ctgf*), growth arrest and DNA-damage-inducible 45 β gene (*gadd45b*) (Takekawa et al., 2002), the junB oncogene (*junb*), plasminogen activator inhibitor-1 (*serpine1*) and Smad7 (*smad7*). As is evident in Figure 4.4, the *ctgf*, *gadd45b*, *junb*, *serpine1*, and *smad7* genes were upregulated in the presence of the TGF-β1 ligand. The *ctgf* and *serpine1* genes exhibited the major response to TGF-β1, compared to their basal expression in the naïve and saline-treated mice. The gene expression changes were neither dependant on the duration or on the concentration of the stimulus. However, the gene expression levels were not elevated in some mice, meaning that some animals did not react to TGF-β1. This may be explained by the fact that some animal movement during the OT application could have occurred, leading to improper deposition of the applied liquid, or to the intervariability found between individuals of the same species.

In conclusion, a change in the gene expression profile induced by TGF-β1 in the murine lung was confirmed. The TGF-β1 target genes *ctgf*, *gadd45b*, *junb*, *serpine1*, and *smad7* were rapidly (after 2 h) upregulated and low ligand concentrations (0.25 ng/µl) were efficient enough to enhance their transcription.

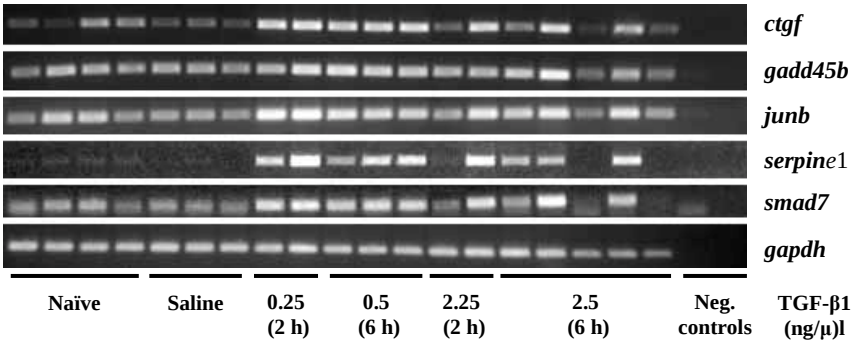


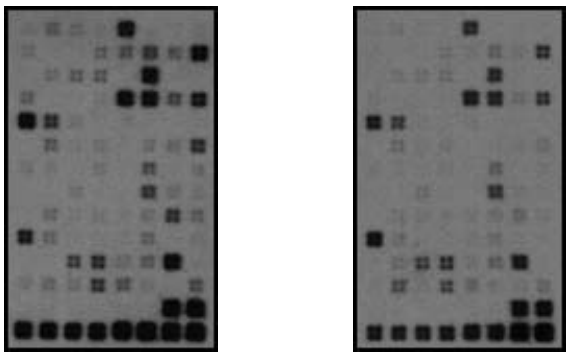
Figure 4.4: TGF-β1-induced upregulation of early-responsive target genes. Four different TGF-β1 concentrations (0.25, 0.5, 2.25, and 2.5 ng/µl) were instilled into the murine lung. Mice were sacrificed 2 h (0.25 and 2.25 ng/µl) or 6 h (0.5 and 2.5 ng/µl) after instillation. Total RNA was isolated from lung homogenates, along with control (naïve) and saline-treated murine lungs. Upregulation of TGF-β1 responsive genes was assessed by semi-quantitative RT-PCR. GAPDH served as internal loading control. Negative controls contained H_2O as template for the RT and PCR steps (n = 6).

Additionally, murine oligo GEArray® membranes were utilised in order to further investigate the TGF-β1-regulated transcriptional genes within the TGF-β superfamily members. For these reason, total RNA was isolated from untreated mice and mice OT instilled with a concentration of 2.5 ng/µl of TGF-β1. Afterwards, the amplified, biotin-labelled antisense RNA was hybridised onto a TGF-β/BMP signalling pathway microarray and signal was visualised by chemiluminiscence (Figure 4.5.A and B). The TGF-β1-induced gene transcription or repression is represented in Figure 4.5.C. The spotted genes have been listed in the Appendix section, along with the short and long names (Table A.18).

Interestingly, many genes were repressed when TGF-β1 was instilled (Figure 4.5.B and C). Among them, many members of the BMP family, several BMP ligands (e.g. Bmp4), the *acvrl1* receptor, and the inhibitory *smad6*. In addition, downregulated were members of the collagen family, the TβRIII (*tgfbr3*) or also called betaglycan, and the distal-less homebox 2 (*dlx2*). From the Id family, solely the *idb2* member was significantly expressed to a lesser extent when the ligand was added. On the contrary, some genes were upregulated after TGF-β1 administration, like *serpine1*, which upregulation was already observed in the semi-quantitative RT-PCR analysis; the type III TGF-β receptor known as BAMBI, several members of the growth differentiation factor (GDF) family, IL-6, the TGF-β induced (*tgfbi*) gene, and the BMP inhibitor Noggin, which binds to the BMP-4 ligand (Figure 4.5). However, transcriptional regulation of the R-Smads in the lungs was not observable upon TGF-β1 instillation, but on protein level by activation via phosphorylation (Figure 4.3).

In order to confirm some of the results obtained by the semi-quantitative RT-PCR and the microarray technique, quantitative RT-PCR was performed on the same homogenate samples acquired from untreated or TGF-β1-treated mice (2.5 ng/µl for 6 h) on 5 animals per condition. For instance, the expression level of a member of the collagen family, collagen Iα_1, exhibited a faint downregulation after TGF-β1 application, following the same pattern as in the microarray analysis. The TGF-β1-induced downregulation of the *idb2* gene observed by the microarray (highest repression by TGF-β1) approach was corroborated by quantitative RT-PCR.

A **B**



C

Acvr1	Acvr1b	Acvr2	Acvr2b	Acvrl1	Amh	Bambi	Bglap1
0.03±0.13	-0.11±0.21	-0.11±0.22	0.08±0.09	-0.10±0.01*	0.08±0.05	0.16±0.03*	0.03±0.08
Bmp1	Bmp10	Bmp15	Bmp2	Bmp3	Bmp4	Bmp5	Bmp6
0.07±0.26	0.12±0.07	0.14±0.07	-0.19±0.16	-0.24±0.36	-0.40±0.09*	-0.28±0.14	-0.16±0.06
Bmp7	Bmp8a	Bmp8b	Bmpr1a	Bmpr1b	Bmpr2	Cdc25a	Cdkn1a
0.14±0.07	-0.06±0.04	-0.23±0.08	-0.07±0.14	0.02±0.18	-0.08±0.06	0.16±0.10	0.12±0.06
Cdkn2b	Cer1	Chrd	Grem1	Col1a1	Col1a2	Col3a1	Dlx2
-0.11±0.18	0.21±0.03*	0.22±0.07*	0.07±0.02	-0.04±0.02	-0.08±0.02	-0.39±0.07*	-0.20±0.16
Lefty1	Eng	Evi1	Fkbp1b	Fos	Fst	Gdf1	Gdf11
-0.05±0.01	-0.04±0.09	0.08±0.04	0.25±0.10	0.08±0.12	0.17±0.15	0.15±0.05*	0.15±0.04*
Gdf2	Gdf3	Gdf5	Gdf6	Gdf8	Gdf9	Idb1	Idb2
0.19±0.05*	-0.22±0.14	0.13±0.05	0.11±0.09	0.13±0.02*	-0.03±0.02	0.14±0.31	-0.50±0.13*
Idb3	Idb4	Cd79a	Igf1	Il6	Inha	Inhba	Inhbb
0.06±0.01	0.16±0.04*	0.09±0.22	0.25±0.18	0.19±0.05*	-0.41±0.21	0.19±0.05*	-0.02±0.12
Inhbc	Inhbe	Itgb5	Itgb7	Ivl	Jun	Junb	Lap3
0.18±0.07	0.18±0.04*	0.24±0.13	0.22±0.08	0.17±0.09	0.04±0.09	-0.09±0.10	0.09±0.04
Lefty2	Igfbp3	Smad1	Smad2	Smad3	Smad5	Smad6	Smad7
0.17±0.05*	-0.07±0.10	0.12±0.09	0.06±0.03	0.20±0.13	0.08±0.04	-0.44±0.08*	-0.14±0.11
Smad9	Nbl1	Nodal	Nog	Pdgfb	Plat	Plau	Runx1
-0.01±0.08	0.05±0.15	0.27±0.24	0.20±0.07*	0.15±0.14	-0.13±0.06	0.28±0.21	0.17±0.07
Runx2	Serpine1	Sox4	Stat1	Tgfb1	Tgfb1i1	Tgfb1i4	Tgfb2
0.33±0.17	0.38±0.15*	-0.15±0.26	0.00±0.08	0.03±0.07	-0.07±0.12	-0.02±0.02	0.17±0.04*
Tgfb3	Tgfbi	Tgfbr1	Tgfbr2	Tgfbr3	Tgif	Timp1	Zfhx1a
0.06±0.05	0.26±0.14	0.39±0.32	-0.07±0.04	-0.18±0.05*	0.18±0.07	0.19±0.07	-0.11±0.12
PUC18	PUC18	PUC18	Blank	Blank	Blank	Gapd	Gapd
Ppia	Ppia	Ppia	Ppia	Rpl13a	Rpl13a	Actb	Actb

Figure 4.5: Gene transcription regulation by TGF-β1 in the murine lung (microarrays): The gene expression pattern on A) naïve and B) TGF-β1-treated lungs (2.5 ng/µl, for 6 h) was compared by the GEArray® TGF-β superfamily gene array. Total RNA was isolated and hybridised onto the specific membrane. C) The short name of the genes and their corresponding position on the membrane, the regulation factor and error standard deviations are depicted. Comparison between the arrays (TGF-β1 vs. naïve) was performed by the on-line GEArray® Analysis Suite Software. Intensity of spots was normalized to *gapdh* gene expression and background from blank samples was substracted. The penultimate row contains the negative controls and the bottom row is spotted with housekeeping genes (n = 3).

The *serpine1* gene expression, already demonstrated to be upregulated by semi-quantitative RT-PCR and microarray techniques (highest induction by TGF-β1), was again significantly upregulated in the murine lungs after cytokine administration. Furthermore, the upregulation of the *junb* gene transcription by semi-quantitative RT-PCR could not be confirmed by microarray analysis, where the TGF-β1 induction was not observed. Neither the upregulation of the *ctgf* gene as it was not spotted in the utilised microarray.

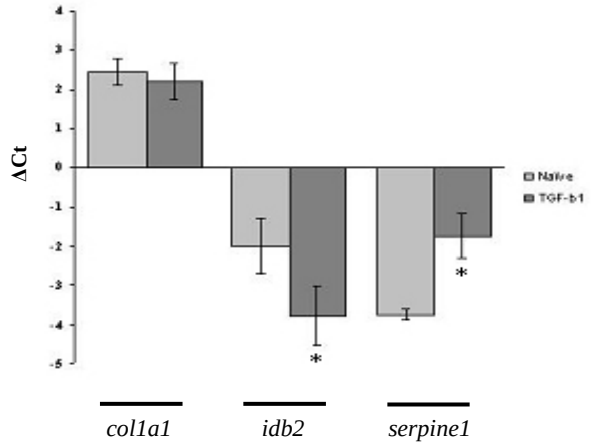


Figure 4.6: Regulation of gene transcription by TGF-β1 in the murine lung (quantitative RT-PCR). Expression of TGF-β1-regulated genes was analysed by real-time RT-PCR, using RNA from lung homogenates from untreated and TGF-β1-treated mice (2.5 ng/µl, for 6 h). *: $p \leq 0.05$ for the comparison between the naïve and TGF-β1-treated fractions (n = 2).

In conclusion, low concentrations of instilled TGF-β1 rapidly induced gene repression of many members of the BMP family; inhibition that was expected as the TGF-β and BMP signalling pathways have antagonistic effects on the cell. Furthermore, inhibition of *idb2* transcription was observed under TGF-β1 stimulation, in contrast to the previously reported fast induction upon BMP ligand binding (Miyazono and Miyazawa, 2002). Regarding the consistent and increased *serpine1* expression levels, three different techniques demonstrated that this gene is highly and rapidly upregulated upon TGF-β1 stimulation in the lung *in vivo*.

4.2.3 Immunohistochemistry on saline and TGF-β1-treated murine lungs

The TGF-β1 effect on the lung was also analysed by immunohistochemistry, so that, the cells responding to the ligand could also be visualised. Two molecules were chosen as representing downstream molecules: PAI-1, which upregulation was

determined previously on RNA level (Figure 4.6) and phospho-Smad2, as immediate responsive molecule after TGF-β1 administration (Figure 4.3).

Saline- and TGF-β1-treated lungs (0.5 ng/µl) were fixed 6 h after ligand administration and stained for PAI-1 (Figure 4.7.A) and phospho-Smad2 (Figure 4.7.B). The bronchoalveolar cells (BEC) and the alveolar epithelial cells (AEC) were the only immunoreactive cells for both target proteins. PAI-1 staining was evident in the cytoplasmic compartment of these cells, whereas the phospho-Smad2 nuclear staining was indicative of its translocation into the nucleus upon TGF-β1 stimulation.

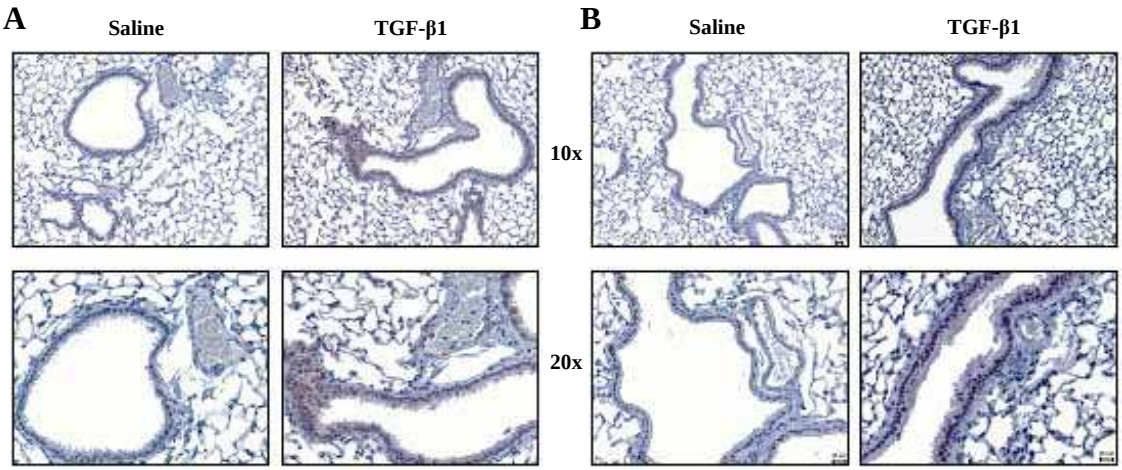


Figure 4.7: Bronchial and alveolar epithelial cells respond to TGF-β1 instillation. Immunological representative fields of A) PAI-1 and B) phospho-Smad2 are illustrated. 0.5 ng/µl of recombinant protein diluted in saline were instilled into the murine lungs; control lungs received only saline (80 µl final volume). The organs were fixed and explanted after 6 h (n = 3).

In conclusion, TGF-β1 application through the OT method was efficient, in that the ligand could reach the lung and activate gene transcription and phosphorylation of the downstream signalling molecules. The signal transduction was active at low and high ligand concentrations and at early and late time points. The TGF-β1-responsive cells were situated at the epithelial layer; specifically two cell types were reactive: the BEC and AEC localised the activated TGF-β1 downstream signalling molecules.

4.3 EPITHELIAL LAVAGE

The cells found to be responsive to TGF-β1 were present in the epithelial layer (both the BEC and the AEC). Due to the necessity to find novel pulmonary epithelial cell-specific markers expressed *in vivo*, a novel approach was developed to isolate the RNA contained in the epithelial cells of the murine lung. The advantage of this method

would be to avoid cell plating, process that potentiates the expression of different markers differently than *in situ* (Chen et al., 2004; Paine and Simon, 1996), and thus studying the gene expression profile in the *in vivo* situation. In this way, modification of gene expression profiles reported to occur during cell culture conditions would be obviated (Bhaskaran et al., 2007; Chen et al., 2004). The technique developed was performed similarly to a bronchoalveolar lavage (BAL) (Connett, 2000); and therefore, was named “epithelial lavage” (EL).

Briefly, the EL proceeded as follows: mice were sacrificed and tracheostomised. Lungs were lavaged twice with saline and an appropriate dilution of guanidinium isothiocyanate (GI) was then instilled into the organ. The solution was withdrawn from the lung and finally RNA was isolated using the phenol/chloroform precipitation method (Huang et al., 1995). An epithelial RNA fraction with minimal contamination of adjacent cells was successfully recovered, as assessed by cell-specific marker gene expression (semi- and quantitative RT-PCR).

4.3.1 Epithelial lavage: optimisation of the guanidinium isothiocyanate concentration

In order to isolate RNA from epithelial cells lining the respiratory surface of the lung, a solution containing GI was used, as this chemical disrupts the cells, denatures proteins, and suppresses RNase activity. This solution is normally employed for RNA isolation from tissues and cells.

Before GI application, two consecutive saline lavages (500 µl each) were performed to eliminate the maximal amount of lining fluid and non-epithelial cells covering the epithelium, like immune and dead cells (Connett, 2000). An appropriate dilution of the GI solution became necessary, as an undiluted solution led to the immediate rupture of the lung. Therefore, a series of dilutions, including 1:20 dilution of GI and 1:40 dilution were instilled (600 µl) (n = 4 mice per group). The volume was then recovered and maintained on ice, together with 200 µl of undiluted GI, to accelerate cell disruption and prevent RNase activation. Afterwards, a supplementary saline fraction was instilled to facilitate the recovery of additional disrupted cells. This fraction was extracted from the lung and added to the previously withdrawn solution. Total RNA was isolated from the extracted volume by the EL method, the BAL fraction

and the remaining lung homogenates, using the phenol/chlorofom method, as described in the Materials and Methods section (Huang et al., 1995).

Cell type-specific marker expression in the EL fractions was analysed by semi-quantitative RT-PCR, including epithelial and non-epithelial markers. As evident from Figure 4.8, the Clara cell marker CC-10 (*scgb1a1*), and the AEC marker ATPase, Na^+/K^+ transporting beta 1 polypeptide (*atp1b1*) and the ATII cell marker pro-surfactant protein B (*sftpb*) were amplified in the 1:20 dilution fractions. This amplification indicated that RNA was isolated from both the proximal and the distal epithelium, respectively. The non-epithelial markers analysed were characteristic for SMC and endothelial cells, so as to exclude any contamination from adjacent cells and thus ensure the extraction of RNA solely from epithelial cells. The platelet-endothelial-cell adhesion molecule 1 (*pecam1*), restricted to platelets and endothelial cells, was not amplified. Additionally, the commonly used marker for SMC, α-smooth muscle actin (*acta2*) was also not detected. The 1:40 dilution only disrupted the proximal epithelium, evident by the CC-10 marker amplification.

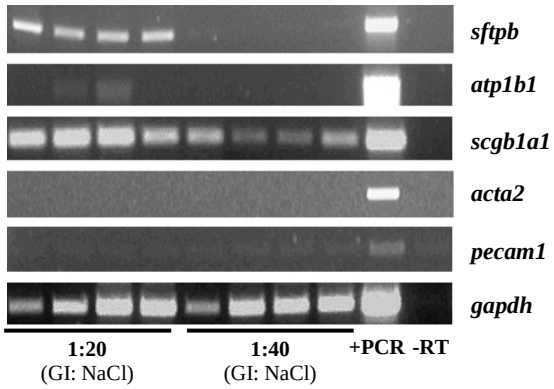


Figure 4.8: Optimisation of the required dilution of GI solution for the EL technique. Two different dilutions of GI were applied to four mice each, 1:20 and 1:40. Amplification of epithelial and non-epithelial cell type-specific markers was assessed by semi-quantitative RT-PCR. GAPDH served as internal loading control. +PCR: positive control for the PCR step containing mouse genomic DNA. -RT: negative control for the RT step lacking RNA template.

Taken together, it could be concluded that a diluted solution of GI was necessary in order to recover RNA from the pulmonary epithelial layer. The 1:20 dilution of GI was the optimal concentration to isolate a pure epithelial fraction from both the distal and the proximal areas of the lung, without any RNA contamination from adjacent cells. The 1:40 dilution was not sufficient to disrupt the epithelial cells situated in the gas-exchange region.

4.3.2 Epithelial lavage: retention time optimisation

After optimising the required dilution of GI, different retention times of the 1:20 diluted GI within the lung were investigated, as the final RNA concentration and possible contamination of adjacent cells might vary. The times selected were the following: introducing the solution and subtracting it immediately (in and out, ~ 1 s) and after 10, 30, and 60 s. As observed in Figure 4.8, the 30 s time point fraction yielded the highest RNA concentration and exhibited epithelial marker specificity, as assessed by semi-quantitative RT-PCR. Solely in the representative fraction of 30 s, the AEC gene marker, *atp1b1*, was amplified, as well as, the Clara cell marker, *scgb1a1*. The specific markers of other cell-types, PECAM-1 and α-SMA, were not amplified. A longer retention time (1 min) did not yield a higher total RNA concentration. Therefore, in order to prevent possible contaminations and to achieve the highest RNA concentration, the 30 s time point was utilised for further analysis.

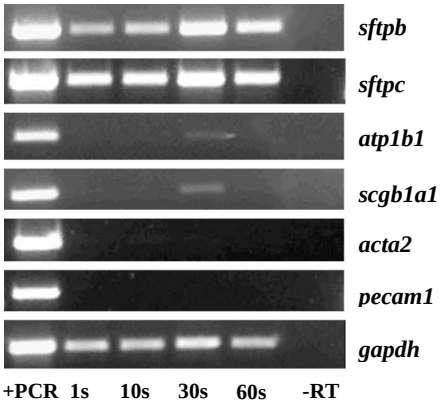


Figure 4.9: Optimisation of the required retention time of GI within the lung for the EL technique. The EL fractions obtained by the 1:20 dilution of GI at different retention time points (1 s, 10 s, 30 s and 60 s) were analysed for cell type-specific markers by semi-quantitative RT-PCR. GAPDH served as internal loading control. -RT: negative control for the RT step lacking RNA template (n = 2).

4.3.3 Reproducibility of the epithelial lavage technique

The next question to be answered was if the novel experimental approach could be repetitively performed without variations on the marker expression profile. In this respect, the EL was conducted using the 1:20 dilution of GI maintained for 30 s within the lung (n = 4 animals). The gene expression analyses from the collected EL fractions (E1-E4), along with the corresponding remaining lung homogenates (L1-L4) and the cellular debris from the BAL samples (BAL) were assessed by semi-quantitative RT-PCR.

As shown in Figure 4.10.A, high expression of the epithelial markers was observed in the EL fractions (E1-E4). Especially the *scgb1a1* (CC-10), *sftpc* (SP-C), and *sftpb* (SP-B) genes were highly expressed. In the epithelial samples the pump markers present in the AEC, Atp1α1 and Atp1β1, were also expressed. Moreover, the binding protein E-cadherin, encoded by the *cdh1* gene, responsible for the maintenance of the epithelial cell polarity, was also amplified in the EL samples. On the contrary, the water channel aquaporin (AQP) 5, used as a marker for ATI cells, was not detectable. It was then suspected that the EL procedure might not be suitable for isolating RNA from type I pneumocytes.

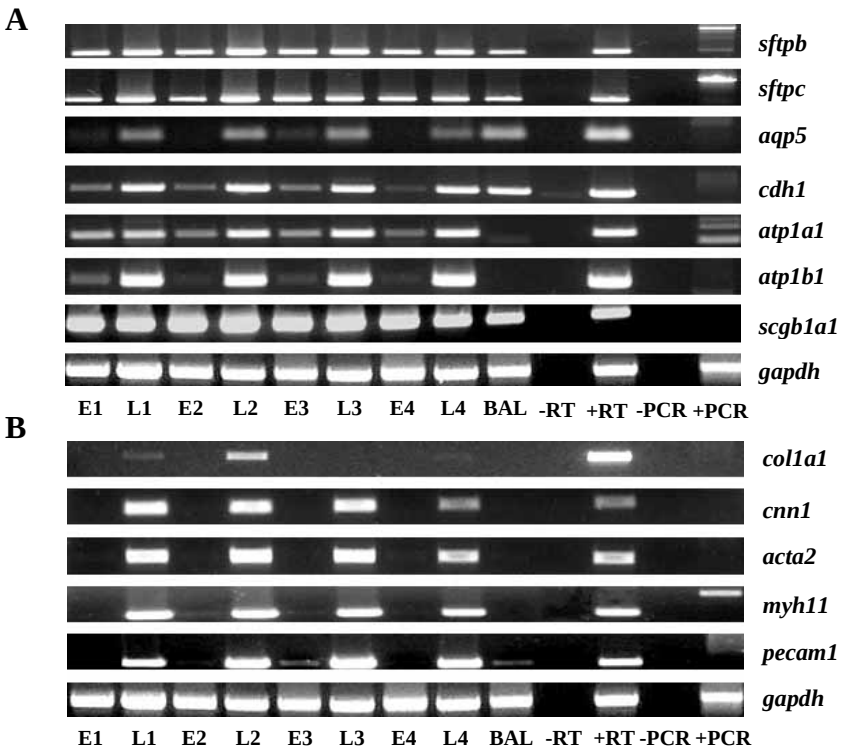


Figure 4.10: Reproducibility of the EL technique. Cell type-specific marker expression analysis from four EL fractions (E1-E4) and their corresponding lung homogenates (L1-L4) were analysed by semi-quantitative RT-PCR. Cell debris from a bronchoalveolar lavage (BAL) sample was also included. A) Epithelial cell type-specific marker amplification. B) Amplification of fibroblast, smooth muscle and endothelial cell type-specific markers. GAPDH served as internal loading control. -RT: negative control for the RT step lacking RNA template. +RT: positive control for the RT step containing murine total RNA. -PCR: negative control for the PCR step with H_2O as template +PCR: positive control for the PCR step containing mouse genomic DNA (n = 4).

As depicted in Figure 4.10.B, many non-epithelial markers were analysed, such as the fibroblast marker collagen Iα_1 (*col1a1*) and the SMC markers: calponin (*cnn1*), α-SMA and myosin (*myh1l*). None of the previously mentioned markers were amplified in the EL samples, indicating a reproducible pure RNA epithelial isolation by this

method. Expression of the *pecam1* gene was observed in the sample E3. As this contamination was only elevated in one sample, it was not considered a major drawback of the method. The cellular debris, contained in BAL fluid, exhibited almost the same gene expression pattern as the EL fractions. This could be explained by the fact that the majority of the cells present at the respiratory acini are macrophages, whose main function, at basal state, is to clear the alveolar ducts and alveoli of inhaled antigens and dead cells. These macrophages possibly express epithelial markers as they also phagocytace dead epithelial cells. Possible could be also that the BAL procedure itself disrupted partially the epithelium, dragging along some epithelial cells.

Taken together, a constant reproducibility of the method was demonstrated by the similar gene expression profiles from the four different EL. Both RNA from proximal and distal situated cells was collected. This RNA highly expressed epithelial markers and non-epithelial marker expression was absent. In addition, the use of intron-spanning primers could ensure genomic DNA free samples.

4.3.4 Epithelial lavage effect on the lung morphology

Lung slices from both saline-lavaged and GI-lavaged organs, were probed by immunohistochemistry so to evaluate the effect of the EL technique on the lung architecture. For this reason, antibodies against two different layers of the lung were used: the epithelial sealing junction zonula occludens (ZO-1, or also called tight junction protein 1 -TJP1-), responsible for maintenance of the epithelial cell polarity (Abraham et al., 1999; Stevenson et al., 1986) and the α-SMA antibody, which was chosen to stain the smooth muscle layer of the lung.

As depicted in Figure 4.11.A, the pronounced ZO-1 staining on saline-treated lungs (control) was localised along with the intact epithelium. Furthermore, ZO-1 was localised on the GI-lavaged lung slices on the disrupted epithelial cells at the airways and in the remaining, non-disrupted epithelial layer. On the contrary, as illustrated in Figure 4.11.B, the staining for α-SMA was comparable after both saline and GI treatments.

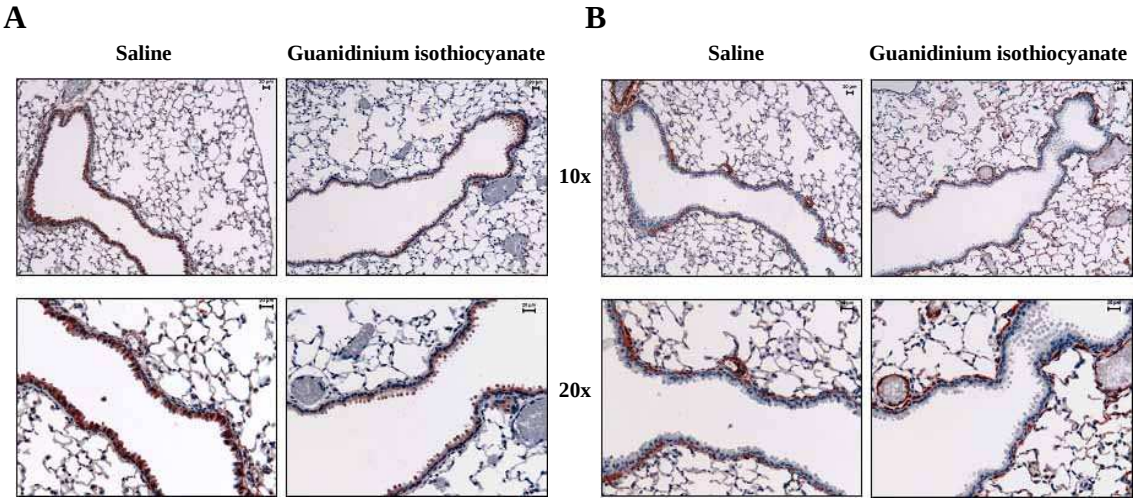


Figure 4.11: Epithelial cell disruption by GI. Immunohistochemistry of A) ZO-1 and B) α-SMA was performed on control lungs (saline-treated) and lungs treated with 1:20 GI for 30 seconds. Lower (10x, *upper* row) and higher magnification (20x, *lower* row) are depicted. Scale bar = 20 µm (n = 3).

The fainter staining of ZO-1 on the right columns indicated that the epithelial cells were disrupted by the GI solution. The same staining levels of α-SMA on the vasculature led to the conclusion that the EL technique only disrupted the epithelial cells and not deeper tissue layers of the lung. It could be then morphologically assured that within a period of 30 seconds, using a 1:20 dilution of GI solution, a sample, free of RNA from adjacent, non-epithelial cells, could be collected.

4.3.5 Steps to perform the epithelial lavage technique

After establishing the optimal dilution, retention time of GI inside the lung, ensuring the reproducibility of the experimental approach, and the disruption of solely epithelial cells, the steps of the EL technique can be summarised as follows:

1) Mice are sacrificed and placed in suprine position. The trachea is exposed via surgical dissection of the ventral neck and tracheostomised. A canula is inserted through and fixed.
2) The lungs are carefully lavaged twice with saline (~500 µl each time) through the canula with the help of a 1 ml syringe. Both samples are collected together and snap frozen.
3) Immediately after, 600 µl of 1:20 dilution of GI is carefully instilled and retained inside the lung for 30 s.
4) The GI solution is then extracted from the lung and maintained in an Eppendorf tube, which contains 200 µl of undiluted GI.

5) An extra saline solution is instilled into the lung in order to extract the maximum amount of epithelial cells, which are disrupted and still laying in the alveolar spaces. This sample is carefully, repeatedly introduced and withdrawn (maximum three times).
6) This fraction is subtracted and added on the sample obtained previously. Sample is either snap frozen or maintained in ice, if RNA is isolated immediately.
7) Finally, RNA is isolated using the phenol/chloroform precipitation method. Briefly, the guanidinium isothiocyanate fraction (Roti-Quick1) is combined with phenol/chloroform (Roti-Quick2) to separate the RNA from the sheared DNA and proteins. RNA is then precipitated overnight by isopropanol (Roti-Quick3). An overnight precipitation is highly recommended, as well as the addition of glycogen (1 µl per extraction). Afterwards, disrupting salts are washed with 70% (v/v) ethanol and final RNA concentration is measured.

4.3.6 Enrichment of the epithelial lavage fractions

To accurately quantify the expression of epithelial cell type-specific markers in the EL samples, real-time RT-PCR was performed. The specificity of the PCR product was confirmed by melting curve analysis and additionally, ΔCt values were also calculated and graphically represented as Box and Whisker plots, which indicate the median values ± 25% and 75% confidence intervals and possible "outliers".

The following genes were investigated by real-time RT-PCR: *aytl2* (LPCAT), *sftpc* (SP-C), *gabrp*, *titf1* (TTF-1), *foxp2*, *pdpn* (T1α), *tjp1* (ZO-1), *scgb1a1* (CC-10), *acta2* (α-SMA) and *pecam1*, with n = 4-7 EL extractions. The analysis demonstrated that the markers, which identify ATII cells, were highly expressed in the EL samples (*sftpc*, *titf1*, and *gabrp*) (Figure 4.12). Furthermore, the CC-10 marker exhibited the highest expression (ΔCt median = 7.25), indicating that the bronchial epithelium was effectively disrupted along with the alveolar epithelium during the EL approach. The *pdpn*, an ATI cell marker was also amplified (ΔCt median = 2.00), thus the novel technique was also able to disrupt type I pneumocytes. However, an amplification of *aqp5* (an ATI cell marker) by semi-quantitative RT-PCR was not observed (Figure 4.10). On the contrary, the control markers for contaminating cells like endothelial and

SMC were only present at very low levels (*pecam1*, ΔCt median = -3.56; *acta2*, ΔCt median = -1.9).

In summary, the quantification of marker expression by real-time RT-PCR demonstrated that the EL samples were enriched in epithelial markers and contained only RNA from cells present in both the bronchial and alveolar epithelium. These fractions expressed primarily Clara cell and ATII cell markers.

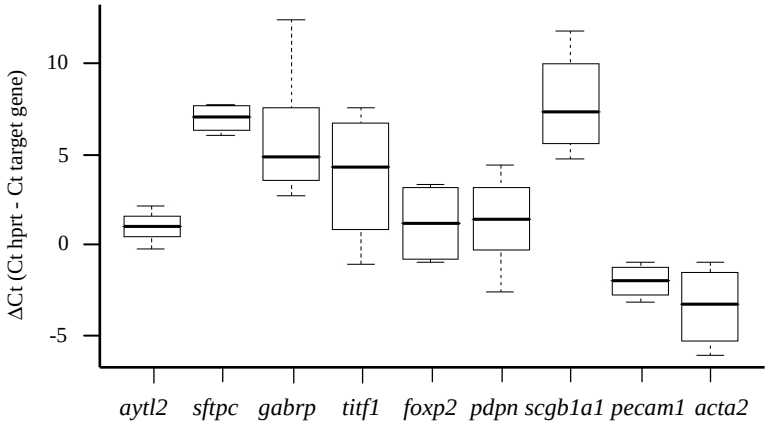


Figure 4.12: EL gene expression pattern at basal state. Quantification of the EL gene expression profile (ΔCt) was assessed by real-time RT-PCR and represented in a Box and Whiskers plot. Median values are indicated with the bold, horizontal line. Epithelial and non-epithelial cell type-specific markers were analysed (n = 3). HPRT was used as reference gene.

4.4 EPITHELIAL LAVAGE *VERSUS* PRIMARY ALVEOLVAR EPITHELIAL TYPE II (ATII) CELLS

It has been repeatedly demonstrated that isolated primary ATII cells change their phenotype upon culture. The ATII cells gradually trans-differentiate into AEC type I-like cells between days 3 and 5 after isolation (Chen et al., 2004; Paine and Simon, 1996). Moreover, the trans-differentiation degree depends on the matrix onto which these cells are plated (Gonzales et al., 2002) and on the presence of various stimuli (Thiery, 2002). Therefore, a comparison of gene expression levels between *in vivo* EL fractions and the type II pneumocytes plated for 1.5 days on uncoated dishes was performed. Moreover, the comparison included RNA of other samples obtained from the bronchoalveolar lavage cellular fraction (BAL), the corresponding remaining lung homogenates, and the mouse lung epithelial 12 (MLE 12) cell-line, and thus estimate how reliable, pure and enriched the EL fractions are. RNA was then isolated, and reverse transcribed. Large sample sizes (n = 4-7) from isolated primary ATII cells, lung

homogenates, BAL, and EL samples and a quantitative technique like real-time RT-PCR were utilised to analyse their correspondent gene expression profiles. The ΔCt values obtained were calculated and plotted in a Box and Whisker graph. Statistical analyses were evaluated between the epithelial lavage and the ATII cells fractions.

As depicted in Figure 4.13.A, the *gabrp* and *aytl2* markers were significantly higher expressed in the EL fractions than in the isolated ATII cells ($p \leq 0.01$). The higher expression of *gabrp* could be due to the fact that *gabrp* is also synthesised by BEC (Chen et al., 2004), cells that are also disrupted during the EL extraction. As expected, the relative expressions of *scgb1a1* and *pdpn* were also significantly higher in the EL fractions, although not absent in the ATII samples ($p = 0.02$ and $p = 0.04$, respectively) (Figure 4.13.B). The expression of *scgb1a1*, a cell type-specific marker for Clara cells positioned at the proximal area of the lung, in the isolated ATII cell fractions could mainly be due to bronchial cell contamination during cell extraction.

Interestingly, the analysed SMC marker (*acta2*) was not detected in the EL samples, in contrast to the high expression levels exhibited by the isolated primary ATII cells ($p = 0.09$), either due to a contamination of SMC during the extraction or it may be derived from a rapid trans-differentiation of ATII cells into mesenchymal-like cells (Figure 4.13.C). Moreover, the most abundantly expressed marker in the plated ATII cells was the *sftpc*. The *titf1* and *foxp2* markers were expressed at comparable levels in both the EL and isolated primary ATII cell samples, with *pecam1* expression completely absent in both fractions (Figure 4.13.A and 4.13.C). Thus, the EL samples represent a more pure fraction than do the isolated primary ATII cells, in that the cell type-specific markers for smooth muscle and endothelial cells were not amplified. Furthermore, cell-specific markers for BEC and type I pneumocytes were present in the ATII cell samples, as well as in the epithelial lavage fractions.

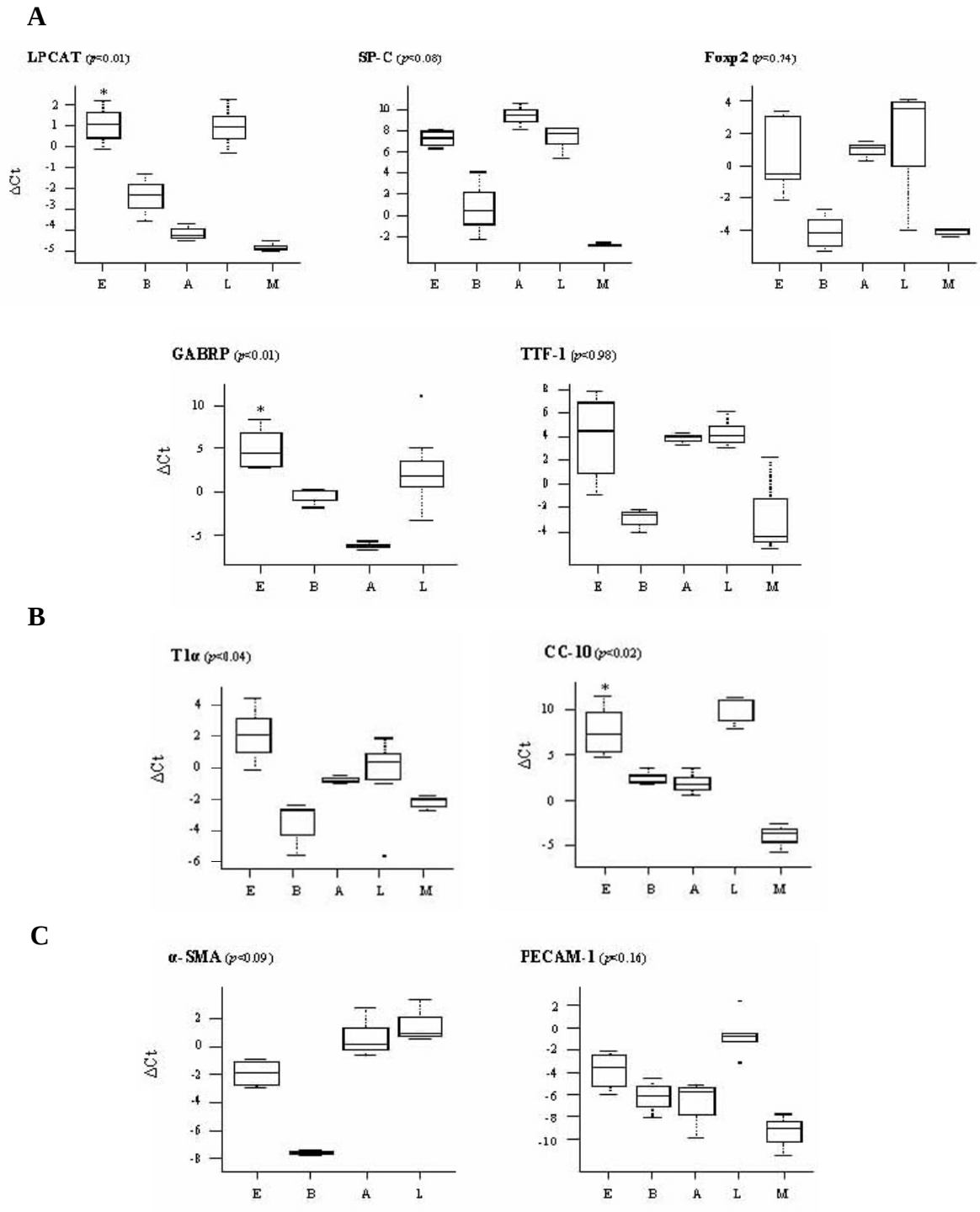


Figure 4.13: Comparison between the EL fractions with other RNA isolations by real-time RT-PCR. EL fractions (E) were compared to the bronchoalveolar lavage cells (B), isolated primary ATII cells plated for 1.5 days (A), lung homogenates (L) and the MLE 12 cell-line (M) by real-time RT-PCR. Box and Whisker plots represent median values (bold horizontal lane) ± 25% and 75% confidence intervals and possible "outliers" of ΔCt values. HPRT was used as reference gene. *: $p<0.05$ for the comparison between the EL and ATII cells fractions (n =2).

Previously it was shown by semi-quantitative RT-PCR that the EL gene expression pattern was similar to the BAL fraction (Figure 4.10). Therefore, the quantification by real-time RT-PCR was strictly required to analyse, if the EL contained

solely epithelial cells or also a proportion of immune cells. Once performed, it could be concluded that the EL samples were not contaminated with macrophages, as the expression pattern did not exhibit any similarities. It was then demonstrated that the EL samples only contained RNA from disrupted epithelial cells. Therefore, the two BAL performed prior to the EL extraction were efficient enough to clear the pulmonary airways of dead and immune cells. Additionally, lung homogenate samples highly expressed all of the studied markers, while the MLE 12 cell-line did not express any of the markers analysed.

In summary, the mRNA expression profiles proved that macrophages or dead cells did not contaminate the epithelial lavage samples, as the BAL expression profile did not compare with the epithelial lavage fractions. Surprising was the observation that the gene expression profile from MLE 12 did not demonstrate any expression of the markers analysed, although *sftpc* has been reported to be detectable on this immortalized cell-line (www.atcc.org). The comparison between the murine epithelial cell-line, freshly isolated ATII cells, lung homogenates and epithelial lavage extractions, led to the confirmation that the EL method is highly enriched in epithelial markers and it is not contaminated by mRNA from immune, smooth muscle or endothelial cells. Furthermore, as the gene and protein expression profile of the type II pneumocytes change upon culture (Chen et al., 2004; Paine and Simon, 1996), the physiological transcriptional and translational profiles reported thus far have not represented the real gene expression pattern of the ATII cells *in vivo*. The EL method would then be useful for exploring epithelial *in vivo* gene expression profile, technique by which cell isolation or tissue section preparation are avoided and thus modifications to the gene expression pattern are prevented.

4.5 MARKER GENE EXPRESSION IN EPITHELIAL LAVAGE SAMPLES AFTER TGF-β1-INSTILLATION

The efficient administration of the TGF-β1 ligand into the murine lung by the OT instillation and the isolation of pure RNA from the respiratory epithelial layer *in vivo* have been previously demonstrated (Figure 4.3 and 4.10, respectively). The combination of both techniques would represent a powerful tool to study marker gene expression in response to TGF-β1 in the epithelium *in vivo*. Thus far, it has been described that TGF-β1 can regulate gene expression in the respiratory epithelium,

playing an important role in the regulation of the epithelial pumps and channels (Frank et al., 2003) and in the induction of EMT on the ATII cells (Kim et al., 2006; Willis et al., 2005). This study was focused in the latter effect, the TGF-β1-induced EMT on the epithelium. For this reason, markers that determine the EMT process in the lung, previously demonstrated to be regulated by TGF-β1, were analysed. To do so 2.5 ng/µl of TGF-β1 ligand (80 µl total volume) were OT instilled to the mice and after 8 h, the EL technique was performed. Once the RNA was isolated and reverse-transcribed, the cDNA was subjected to real-time RT-PCR. The genes analysed included EMT markers, both epithelial (E-cadherin -*cdh1*-, and occluding -*ocln*-) and mesenchymal markers (fibroblast specific protein 1 -*s100a4*-, α-SMA -*acta2*-, and vimentin -*vim*-). The β-catenin1 -*ctnnb1*- marker can be considered both an epithelial and/or a mesenchymal marker, as it can drive or repress the EMT process, depending on the cell context. The transcriptional expression of TGF-β1 early target genes, *serpine1* and *ctgf* was also included, in order to control ligand activity inside the lung.

The gene expression patterns of both the untreated mice (naïve) and the saline-treated mice (NaCl, 80 µl total volume, sacrificed after 8 h) were also examined (n = 5 per condition). The different treatments were statistically compared by the Student's t-test, as follows: naïve *vs*. saline-treated group and saline- *vs*. TGF-β1-treated mice. The first comparison served as control for the unspecific gene regulation due to the stress induced to the animals during the instillation. The latter comparison served to analyse the specific TGF-β1-induced gene regulation.

As is evident in Figure 4.14, the *serpine1* and *ctgf* gene expression was upregulated when TGF-β1 was administered, indicating that the recombinant ligand reached the lung and was active *in situ*. However, an upregulation on both genes was also observed in the saline-treated mice. The comparison between the saline- and TGF-β1-treated animals led to the conclusion that only the *serpine1* gene expression was significant ($p<0.05$) and that the TGF-β1 itself could specifically induce *serpine1* upregulation.

Additionally, the epithelial markers *cdh1* and *ocln* were less expressed in the TGF-β1-treated mice, when compared to the saline-treated mice, being the downregulation significant. However, the *cdh1*gene expression remained at the same levels as the naïve expression. The dual functional molecule, ctnnb1, exhibited a significant upregulation in the TGF-β1-treated compared to the saline-treated mice

($p<0.01$). This leads to the conclusion that the *ctnnb1* gene was higher express, promoting the transcriptional activation of mesenchymal markers.

Furthermore, the mesenchymal markers included in the analysis, FSP1, α-SMA, and vimentin, did not demonstrate any significant specific regulation due to the TGF-β1 instillation, although a tendency to higher expression compared to untreated animals could be observed after 8 h.

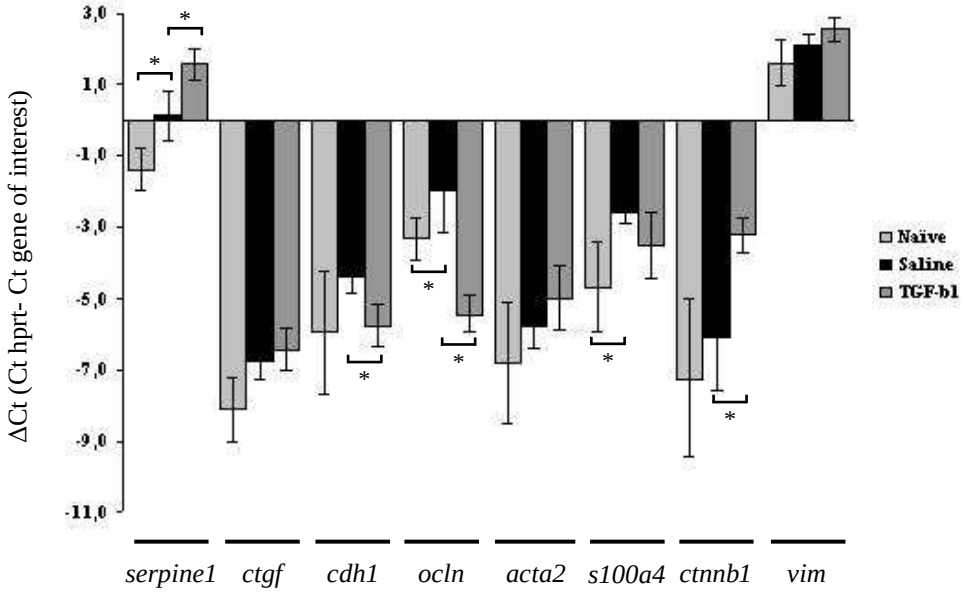


Figure 4.14: Marker gene expression in EL upon TGF-β1 stimulation. EL samples from mice orotracheally instilled with TGF-β1 or saline and control mice (n = 5 per condition) were subjected to real-time RT-PCR. Animals were sacrificed after 8 h and EL extractions were performed. Markers for controlling TGF-β1 stimulation (PAI-1 and CTGF) and the EMT process: epithelial markers (E-cadherin and occludin), mesenchymal markers (α-SMA, FSP-1, and vimentin), and epithelial-mesenchymal marker (β-catenin) were analysed. Data represent the mean ± SD. HPRT was used as reference gene. *: $p<0.05$ for comparison between naïve *vs.* saline-treated and saline- *vs.*TGF-β1-treated samples (n=3).

In summary, the expected upregulation of the mesenchymal markers after TGF-β1 instillation was not observed, when a single application of the recombinant ligand at a concentration of 2.5 ng/ml was utilised. However, a downregulation of the epithelial markers, *cdh1* and *ocln*, was evident. Furthermore, an upregulation of the β-catenin molecule, responsible for the maintenance of adherens junctions and co-activator of mesenchymal markers on liberated, non-attached, cells was significant. Therefore, this indicates that TGF-β1 can induce changes on the gene expression of epithelial cells, but 8 h of retention time within the lung may not be sufficient to study the EMT process by this method; or alternatively, the amount of epithelial cells undergoing EMT may be below the detection level of this thechnique.

5 DISCUSSION

5.1 OROTRACHEAL INSTILLATION

Direct drug delivery into the lung provides novel pathways, not only to treat the respiratory organ, but also to cure systemic diseases (Sakagami, 2006). The advantages of this therapeutic approach are the high rate of drug absorption that the lung exhibits, due to its highly vascularized respiratory mucosa, large absorptive surface area, and its relative low enzymatic activity (Gonda, 2006; Patton et al., 2004). Therefore, the above-mentioned properties facilitate the absorption of substances into the lung and further into the systemic vasculature.

The lung is an organ constantly exposed to airborne particles, potential pathogens, and toxic gases contained in the inspired air, what naturally leads to the development of efficient control barriers i.e. chemical (surfactant lipids), mechanical (sneezing), and immune defense mechanisms (Nicod, 1999; Twigg, 1998). These well-regulated, coordinated components need to be bypassed in order to ensure an efficient drug deposition and absorption. These pitfalls have challenged the efforts to deliver drugs into the lung, either for systemic or local healing.

In the murine model utilised in this study, the epithelial lining fluid, alveolar macrophages absorption, and the epithelium itself were the defense barriers needed to be overcome in order to facilitate an efficient drug delivery. Among them, it is believed that the epithelial layer is the main barrier restricting the movement of drugs, water, and solutes from the airway lumen into the blood circulation (DeFouw, 1983; Schneeberger, 1978).

Different methods were analysed in order to characterise the instillation approach that could yield the best deposition rates. A preliminary experiment was performed in which five different instillation techniques, using the 1% Toluidine Blue O solution, were compared. The technique that would result in a good peripheral distribution into all the five lobes of the lung, using a minimal volume, would be the approach to use in future experiments. Among them, the orotracheal aspiration (OT) of 80 µl of 1% Toluidine Blue O solution demonstrated a broad and homogenic distribution of ink throughout the lung, including the distal parenchyma, adjacent to the pleural surface, confirming previous observations, which utilised the Evans Blue dye

(Lakatos et al., 2006) (Figure 4.1). Moreover, it has been previously shown that this nonsurgical procedure results in a good bilateral and uniform distribution of suspended particles (Lakatos et al., 2006; Rao et al., 2003). Interestingly, the survival rate after the OT administration was almost 100% and multiple applications (reaching six within 28 days, data from a non-related project) were possible. Furthermore, the duration of the whole procedure was very fast (around two minutes), required minimal training, no sophisticated laboratory equipment, and the mice recovered within seconds without any sequelae.

Among the methods evaluated, the intratracheal (IT) delivery also yielded a good deposition rate; however, a larger volume (200 µl) was required to cover the same proportion of lung areas as in the OT approach. The IT method also resulted in a non-uniform distribution within the lung (the instilled particles were predominantly retained in the large conducting airways near the hilum) (Lakatos et al., 2006; Pritchard et al., 1985). Nevertheless, this method has been frequently utilised for the mouse model of bleomycin-induced PF (Lindenschmidt et al., 1986; Shahzeidi et al., 1993). Furthermore, direct intubation of the trachea, performed during the IT and microspray methods, is extremely challenging in mice and the mean survival rate is low (Guilbault et al., 2005). Intranasal aspiration has been reported to be adequate for inducing viral or bacterial invasion, but unsuitable for leading substances directly to the lung, as 50% of the administered material is trapped in the nasal passages (Eyles et al., 2001), a feature that was also recognised in the present study. The nebulisation approach exhibited some drawbacks, in that, only 5% of the lung was covered by the ink, specialised equipment was needed, and large amounts of the particulate insult were consumed, with the exact inhaled dose being impossible to estimate (Hanson et al., 1985).

In conclusion, the method chose to instill TGF-β1 into the lung was the OT approach, as it generated the best results when compared to other drug delivery methods, like the intratracheal, intranasal, nebulisation, and microspray techniques.

5.2 TGF-β1-INDUCED SIGNAL TRANSDUCTION AND GENE TRANSCRIPTION

The development of TGF-β1 transgenic animal models has overcome the necessity of multiple administrations of ligand and has provided useful insights about its role in the lung. However, some drawbacks are encountered along with these models.

For instance, the TGF-β1 transgene is expressed throughout the lifetime of the animal, resulting in abnormal lung development (Zhou et al., 1996). Furthermore, a transient transgene approach was also achieved. In this case, a recombinant, replication-deficient, and mutant adenovirus (AdTGF-$\beta1^{223/225}$), which renders the biological active form of TGF-β1, was intratracheally instilled into rats (Sime et al., 1997) or mice (Kolb et al., 2001). The animals showed mononuclear cell accumulation between day 3 and day 14 and a progressive fibroblast accumulation after day 14. This overexpression resulted in an extensive and persistent fibrosis, demonstrating the crucial role of TGF-β1 in the pathogenesis of PF. Moreover, the conditional overexpression of TGF-β1 has also been achieved using a triple-transgenic construct under a tetracycline operator coupled to the CC-10 promoter, as a model of BPD (Vicencio et al., 2004). However, through these methods, the induction or repression of the early-responsive gene transcription upon TGF-β1 *in vivo* could not be studied, as the exact initiation time of TGF-β1 signalling inside the cells cannot be determined.

The delivery of recombinant cytokines is limited by their short half-life, cost, and the difficulties encountered by a repeated ligand administration (lower survival rate). However, in this report the ligand was administered, not to initiate a fibrotic response, but to examine the epithelial cell response upon TGF-β1 in the early time points. A single instillation of ligand was sufficient to promote signal transduction and gene transcription in these cells.

The response to TGF-β1 was analysed in this report by using the Western blot, semi- and quantitative RT-PCR, specific TGF-β/BMP microarrays, and immunohistochemical approaches. It was demonstrated that the TGF-β1 reached the lung, was still active, and that the lung epithelium responded intracellularly to the ligand (Figure 4.7). It was also proved that the TGF-β1 promoted a Smad-dependent signal transduction inside the cells (Figure 4.3) and the repression and activation of early-induced gene transcription (Figure 4.4, 4.5, and 4.6).

During the analysis of downstream signalling in lung homogenates, only the R-Smads, Smad2 and Smad3, were studied, as these two molecules are specifically phosphorylated in response to TGF-β1. Additionally, it has been reported a TGF-β1-induced activation of the BMP Smads, Smad1 and Smad5. This signalling pathway requires the presence of the ALK-1 receptor on the cell membrane, accompanied by the endoglin accessory receptor. However, these receptors have only

been found to be expressed in endothelial cells (Goumans et al., 2002; Miyazawa et al., 2002), and not on epithelial cells, which are the target cells in the TGF-β1-instilled model used in this assay.

It has been well established that the TGF-β1 ligand may stimulate various intracellular signalling pathways. Therefore, the study of the TGF-β signalling pathways has become more complex, as not only the Smad-dependent pathway may be active, but also, among others, the MAPK, PI3K, and JNK pathways can be activated, either dependently or independently of the Smad pathway (Derynck and Zhang, 2003; Roberts, 2002). In the current model, the Smad pathway was activated 30 min after TGF-β1 stimulation. Phosphorylation of both Smad2 and Smad3 was specific to the TGF-β1 instillation and was independent of the TGF-β1 concentration, as the phosphorylation signal was observed after low (0.25 ng/µl) and high (2.5 ng/µl) concentrations of ligand. Nevertheless, the activation pattern was different between both R-Smads, while phospho-Smad2 was constantly activated, the Smad3 phosphorylation kinetics demonstrated an early response after 30 min and a late response after 6 h. However, the phospho-Smad3 signal was also found in saline-treated and naïve murine lungs, indicating that the Smad3 activation occurred at basal state in the lung, with the phosphorylation signal being stronger when TGF-β1 was present (Figures 4.3.A and 4.3.B).

Additionally, AKT phosphorylation was observed on every mouse submitted to OT instillation, and thus not only on mice treated with the TGF-β1-diluted fractions, but also in those treated with the carrier. These saline-treated samples served as controls for an indirect, unspecific pathway activation. It could be then concluded that the AKT phosphorylation was not a TGF-β1-specific response, but due to the stress conditions produced during the treatment (Figures 4.3.A and 4.3.B). However, this activation may be key, together with the activation of the Smad-dependent pathway, for EMT induction in the lung (Bakin et al., 2000). The other Smad-independent pathways, the MAPK and JNK pathways were activated by neither the OT method, nor the TGF-β1 instillation.

The TGF-β1-induced regulation of gene expression was studied at the RNA level by RT-PCR (Figure 4.4) and a TGF/BMP-specific microarrays (Figure 4.5). It was confirmed that the *serpine1*, *ctgf*, *gadd45b*, and *junb* were upregulated upon TGF-β1 application. However, the observed *smad7* and *junb* upregulation in the semi-quantitative studies could not be confirmed by microarray, but the *serpine1*

upregulation (*ctgf* and *gadd45b* genes were not present on the utilised membrane). The *gadd45b* upregulation was reported to be very fast upon TGF-β1 administration, mediating TGF-β-induced p38 activation (Takekawa et al., 2002). The importance of the *ctgf* overexpression was studied by adenoviral gene transfer in the rat lung; in which severe fibrosis was developed although only transiently in rat lungs (Bonniaud et al., 2003).

The specific TGF/BMP superfamily microarrays, spotted with genes known to be regulated by either the TGF-β and/or BMP family ligands, showed the downregulation of a high number of genes upon TGF-β1 administration (Figure 4.5). Having in mind that the BMP and TGF-β signalling pathways have opposing effects; it follows that TGF-β1 may downregulate many BMP-induced genes in the pulmonary organ. For instance, the gene transcription of the BMP-4 ligand was strongly repressed and simultaneously, its direct inhibitor, Noggin, was upregulated upon TGF-β1 stimulation. The inhibitor Noggin prevents BMP-4 interaction with the corresponding cell surface receptors (Zimmerman et al., 1996). The downregulation of the distal-less homeobox 2 (*dlx2*) by TGF-β1 stimulation in the lung has not been previously reported; however, BMP-2 ligand stimulates the expression of this gene in chondrocytes (Harris et al., 2003). Moreover, the RNA expression of the inhibitor of differentiation 2 (*idb2*) was also downregulated upon TGF-β1 stimulation, consistent with other studies (Kowanetz et al., 2004). It has also been previously demonstrated that the BMP-7 antagonises the TGF-β1-mediated collagen induction through the Id2 molecule (Izumi et al., 2006). The accessory TGF-β receptor type III betaglycan, was found to be downregulated in the murine lungs treated with TGF-β1 for 6 hours. Betaglycan may act as a TGF-β antagonist, as it does not have an intrinsic signalling function and thus impairs binding of the TGF-β ligand isoforms to their corresponding signalling receptors (Eickelberg et al., 2002; Lopez-Casillas et al., 1994). Thus, TGF-β1 may repress this antagonistic receptor to longer potentiate its effects on the target cells. Furthermore, the Smad6 downregulation observed upon TGF-β1 stimulation inhibits the negative-feedback regulation of the TGF-β pathway (Imamura et al., 1997).

On the contrary, various genes were upregulated upon TGF-β1 stimulation. The TGF-β1 induction has been previously demonstrated in other models for *serpine1* (Dennler et al., 1998; Jakowlew et al., 1997). Moreover, the role of *serpine1* in PF has already been reported by using PAI-1-deficient mice, which were protected from

bleomycin-induced PF, and by using PAI-1-overexpressing mice that showed higher hydroxyproline content (collagen accumulation) leading to impairment of the gas exchange (Eitzman et al., 1996). Furthermore, the *tgfbi* was also upregulated upon cytokine application, following previously published data (Skonier et al., 1992). The *tgfbi* gene is principally expressed in the bronchial smooth muscle cells (SMC) (Billings et al., 2000). Members of the GDF family were also upregulated upon TGF-β1 stimulation, more specifically the GDF1, GDF11, GDF2, and GDF8 members; however, the role of the GDF family in the lung under TGF-β1 influence has not been studied thus far. The BMP and activin membrane-bound inhibitor (BAMBI) was also upregulated by TGF-β1 stimulation. This transmembrane glycoprotein has been reported to be induced both in response to TGF-β and BMP signalling pathways, suggesting a probable negative feedback mechanism on the signalling of both subfamilies (Sekiya et al., 2004).

An additional method, like the immunohistochemical analysis, demonstrated that the TGF-β1-responsive cells were situated in the epithelial layer: the bronchial epithelial (BEC) and the alveolar epithelial cells (AEC), at the proximal and distal regions of the lung, respectively. Both the cell types localised for phospho-Smad2 and PAI-1, well characterised downstream molecules of the TGF-β pathway (Figure 4.7).

In Figures 4.3 and 4.4, it is evident that an activation of the downstream molecules (at the protein and RNA levels) does not always occur, but this was dependant on the samples utilised. This could be due to several reasons. Some silent pathological conditions affecting the murine lung (pulmonary edema, repeated mucosal injury, and inflammatory conditions), may lead to changes in the tissue composition and therefore, affect the permeability properties of the epithelium (Audi et al., 1999). In order to avoid these different responses, every treatment was performed on littermates and mice were always hosted under same conditions; however, it is sometimes not possible to distinguish if an animal is or was previously affected by any of these pathologies. Furthermore, the inter-animal variability found between individuals of the same species may play a crucial role in these variations. Another explanation is that the complete instilled dose of ligand may not reach the lungs, due to misdirected application of the liquid to the gut.

5.3 EPITHELIAL LAVAGE OPTIMISATION

Following the necessity of finding novel pulmonary epithelial cell-specific markers expressed *in vivo*, the development of a novel method for RNA isolation was indispensable. An "epithelial lavage" (EL) extraction was then optimised in the laboratory permitting the study of the lung epithelium gene expression profile *in vivo*. The novel method avoids contamination by other cell layers of the lung and provides a highly enriched and pure epithelial mRNA fraction.

Several optimisation steps were performed. The optimal dilution of guanidinium isothiocyanate (GI) (Figure 4.8) and the optimal retention time within the lung, conditions in which the maximum amount of epithelial cells could be disrupted, were assessed (Figure 4.9 and 4.11). Finally, the technique was demonstrated to be reproducible and the specificity and purity of the RNA epithelial samples were validated by quantitative RT-PCR (Figure 4.10 and 4.12).

Pulmonary epithelial tissue consists of many cell types, including Goblet, Clara, type I and II alveolar epithelial cells (AEC), cuboidal and cylindrical ciliated cells. These cells have a different morphology, secrete diverse products, but as a whole, they form the first barrier between the inspired air and the host and regulate the response to external stimuli. During EL extraction, RNA from different cell types present at the epithelium was isolated (Figure 4.10). Therefore, the results obtained through the EL samples are a representation of the gene expression profile from different epithelial cell types *in vivo*. In order to certainly ascribe a newly discovered marker to a specific cell type, immunohistochemisty or *in situ* immunofluorescence could be employed.

It is of importance to characterise the gene expression profile of the epithelial cells under normal conditions and compare this with the expression pattern of epithelial cells from injured lungs. The questions to be answered are, what is/are the trigger/s of these changes and which are the alterations that the cell suffers to become malignant, apoptotic, or hyperplastic. This knowledge will be essential for the development of new drugs, as the pulmonary epithelium plays a crucial role in various pulmonary diseases, for instance, in idiopathic pulmonary fibrosis (IPF) (Geiser, 2003a), or acute lung injury (ALI) (Geiser, 2003b).

Use of the novel method described in this report would help explore AEC gene expression profiles *in vivo*. However, a drawback of this method is that only between 400 and 1000 ng of total RNA can be collected, and thus a high efficiency reverse

transcriptase is required. With regard to the reverse transcribed cDNA from EL samples, it consistently and reproducibly amplified products up to 1000 base pairs, leading to the conclusion that the isolated RNA integrity was good. If a genomic microarray analysis was to be performed, larger quantities of RNA (micrograms) would be demanded (Jenson et al., 2003). To be able to achieve those quantities, a pool of samples or an additional pre-amplification step would be necessary. However, these solutions are sub-optimal, as the sample pool may mask some crucial gene regulation (Peng et al., 2003) and complex pre-amplification reactions may alter the relative abundance of specific transcripts and thus yield erroneous results in the subsequent microarray analyses (Wilhelm et al., 2006). For instance, the T7 pre-amplification technique leads to the underrepresentation of 5´-terminal transcripts (Wilhelm et al., 2006).

5.4 EPITHELIAL LAVAGE *VERSUS* ISOLATED PRIMARY ATII CELLS

In order to validate the specificity and purity of EL extractions, their gene expression profiles were compared to primary ATII cells, the murine lung epithelial 12 cell-line, the cellular fraction of the BAL fluid, or whole lung homogenates after EL by semi-quantitative and real-time RT-PCR. Using these techniques, it was demonstrated that not only the EL extraction, but also the primary isolated ATII cells, contained several types of epithelial cells like Clara and ATI cells. The isolated primary ATII cells were as well accompanied by non-epithelial cells (demonstrated by the amplification of the *acta2* transcript, specific marker for SMC) (Figure 4.13). Therefore, the EL fractions were more pure than the primary ATII cells, in that they did not contain markers from non-epithelial cell types. The gene expression of non- and epithelial markers found in the isolated primary type II pneumocytes fractions can be due to the trans-differentiation that these cells suffer upon culture. These cells trans-differentiate into intermediate cells, which express ATI and mesenchymal markers, independently on the matrix onto which they were plated (Chen et al., 2004; Gonzalez et al., 2005). This transition of epithelial to mesenchymal cells was not induced in the current study by any additional external stimulus, than from the cocktail of GF present in the serum added to the DMEM medium, in where the cells were grown. The exact concentration of TGF-β1 present in the commercial FCS is not clearly described, but some reports have estimated

the TGF-β1 secretion levels of ATII cells in culture to about 3.5 ± 0.5 ng/ml after 24 h (Kumar et al., 1996).

The EL fractions were not only compared to the isolated primary type II pneumocytes, but also to the MLE 12 cell-line, which did not express any ATII cell marker, although they have been reported to synthesise the SP-C (Wikenheiser et al., 1993). It was also ensured that the EL fractions did not contain immune cells, as the comparison between both fractions was completely different, as assessed by quantitative RT-PCR (Figure 4.13). The complete comparison at the mRNA level between epithelial and immune cells was not possible to pursue, as no markers at the transcriptional level are currently available to detect specifically the later cell-type. Immune cells are currently isolated and identified by protein markers at their cell surface, known as cluster of differentiation molecules (Woolfson et al., 2006; Zola, 2001). However, the unique possible comparison performed using epithelial, endothelial and SMC markers, between both fractions, was completely different and thus, it could be concluded that the two bronchoalveolar lavages, undertaken prior to the EL, were efficient enough to remove lining cells and fluid and so to avoid contamination of the EL samples by immune cells.

The gene expression profile from the remaining lung homogenates represented the cells, which were still present in the lung, after the EL extraction was completed. Expression of epithelial, endothelial, and SMC markers was evident. Interestingly, the *pdpn* and *gabrp* markers had a higher expression in the EL fractions than in the homogenates, leading to the conclusion that the ATI and ATII cells were highly disrupted by the EL (Figure 4.13).

Of note, many previously reported markers are not as specific as they were originally thought to be. For instance, the *atp1a2* was recently reported to be also expressed by type I pneumocytes and not only by ATII cells (Johnson et al., 2002). Moreover, different research groups have contradicting results on which cell-type expresses a specific biomarker, for example, the receptor for advanced glycation end products (RAGE) has been localised to both the ATI (Demling et al., 2006) and ATII cells (Morbini et al., 2006). Therefore, the definition of some cell-specific markers may vary with acquired data in the future.

After ascertaining that the EL was neither contaminated by immune cells nor endothelial or SMC, and that the fraction was highly enriched in epithelial markers; it

could be concluded that the EL technique represents a novel method for isolating RNA from the murine lung epithelium. Therefore, the study of the gene expression *in vivo* (where cell plating could be avoided) by the EL technique may constitute nowadays the unique approach to characterise the epithelial gene expression patterns at basal level.

5.5 TGF-β1 AND THE LUNG

Basal activity of the TGF-β pathway is required to regulate lung branching, maintain alveolar integrity and extracellular matrix (ECM) homeostasis, but an excessive, upregulated signalling would result in lung fibrosis, characterised by inhibited ECM degradation and enhanced ECM deposition. The TGF-β pathway has been implicated since many years in the pathogenesis of fibrosis based on its induction of matrix production and deposition on stromal cells *in vitro*, and its increased expression in fibrotic tissues from a variety of organs (Branton and Kopp, 1999).

Not only TGF-β1 overexpressing mouse models have been developed (Discussion 5.2), but also the TGF-β-related deficient mouse models. For instance, the Smad3 knock-out (KO) mouse model was developed as the most pro-fibrotic activities of TGF-β are mediated by the TF Smad3, and Smad3 deficient mice demonstrated no fibrotic responses after bleomycin instillation (Flanders, 2004; Roberts et al., 2001). Additionally, the TGF-β1 null mouse demonstrated fewer fibrotic lesions and decreased RNA and protein expression of collagen I and fibronectin than did the wild-type after bleomycin administration (Zhao et al., 2002). Furthermore, the Smad3 KO mouse has been subjected to high local concentrations of TGF-β1 using the AdTGF-$\beta1^{223/225}$ adenovirus. In this case, the loss of Smad3-dependent signalling prevented the TGF-β1-induced ECM gene expression and blocked fibrosis progression; however, an emphysema-like phenotype was observed (Bonniaud et al., 2004).

Regarding human disease, clinicians have found high TGF-β1 levels inside the lung under pathological conditions (El-Gamel et al., 1999). For instance, lung transplantation in the advanced medical therapies can be complicated by chronic rejection of the lung, which is associated with increased expression of TGF-β (El-Gamel et al., 1998). High levels of TGF-β1, both the active and latent form, have been found augmented following radiation therapy (Herskind et al., 1998), in accordance with the radiation mouse model (Rube et al., 2000).

Highly expressed TGF-β1 levels have been also related to other diseases, such as, ALI and bronchiolitis obliterans (BO), in which this cytokine plays a critical role. In the ALI disease, it have been demonstrated that TGF-β1 levels are critical to the development of pulmonary edema by increasing alveolar epithelial cell permeability (Dhainaut et al., 2003), whereas in BO the TGF-β1 is thought to induce BEC proliferation and myoblast trans-differentiation, leading to fibrous scarring of the respiratory and terminal bronchioli (Frost, 2002).

5.6 TGF-β1 AND THE LUNG EPITHELIUM

TGF-β1 and the pulmonary epithelial layer are highly integrated, as TGF-β1 is synthesised by many different cell types in the lung, but principally by the bronchial epithelium at basal state (Magnan et al., 1994). The activity of this ligand on the airways is related to cell growth, migration, and cell differentiation, which are directly relevant to airway physiology and pathophysiology (Duvernelle et al., 2003). Furthermore, TGF-β1 plays a key role in regulating airway production of ECM, as it induces the synthesis of matrix molecules, including fibronectin and tenascin, and represses the expression of proteases involved in matrix regulation, such as cathepsins. This cytokine also induces the production of integrins and adhesion molecules by epithelial cells (Branton and Kopp, 1999; Duvernelle et al., 2003). Furthermore, it promotes bronchial epithelial cell differentiation (Masui et al., 1986) and inhibits epithelial cell proliferation (Kasper and Haroske, 1996).

However, not only augmented ECM production but also ineffective epithelial layer repair has been related to high levels of TGF-β1. For instance, the cytokine has been reported to potentiate AEC apoptosis by activating the Fas-mediated apoptosis pathway in these cells (Wang et al., 1999) and thus augmenting the extrinsic oxidative stress (Arsalane et al., 1997; Thannickal and Fanburg, 1995).

The influence of the TGF-β1 cytokine on the epithelium has been studied on cell-lines and isolated primary ATII cells. Although differentiated ATII cell-lines are not currently available; nevertheless, several cell-lines with an epithelial origin have been submitted to TGF-β1 stimulation, such as, the A549 and RLE-6TN, and found to undergo EMT (Kasai et al., 2005; Willis et al., 2005). However, the cell-lines are limited in that they are immortalized and in many cases transfected and transformed, processes that change completely the gene expression of those cells (Malkinson et al.,

1997). Regarding the isolated primary cells, the reproducible isolation method of viable ATI cells for *in vitro* studies has been recently developed (Dobbs et al., 1998). On the other hand, the ATII cells have been extenually studied, as their isolation protocol was already optimised thirty years ago (Dobbs et al., 1986; Dobbs et al., 1997) and shown to undergo EMT under TGF-β1 stimulation (Willis et al., 2005). Moreover, not only the TGF-β1 effect has been studied on alveolar epithelial cells, but also on the airway epithelial cells, both the bronchial and the Clara cells, promoting their cell apoptosis and differentiation (Pelaia et al., 2003; Zeng et al., 2001).

5.7 ROLE OF TGF-β1 ON THE EPITHELIUM *IN VIVO*

Thus far, it has been described that TGF-β1 can regulate gene expression in the respiratory epithelium, playing an important role in the induction of EMT on the ATII cells (Kim et al., 2006; Willis et al., 2005) and in the regulation of the epithelial pumps and channels (Frank et al., 2003). The current study was focused more on the first regulationTGF-β1-induced EMT. The process of EMT occurs *in vivo*, playing a pivotal role in the cellular trans-differentiation during development and tumor invasiveness (Zavadil and Bottinger, 2005). The process of EMT is an example of epithelial cell plasticity, as during this trans-differentiation, the epithelial cell exhibits a loss of cell polarity, disassembly of cell adhesion systems (*cdh1* and *ctnnal* -α-catenin-gene expression is absent), and an increased synthesis of molecules responsible for cell-motility. These modifications lead to morphological changes and acquisition of fibroblast-specific markers like α-SMA, vimentin, or fibronectin, features that enable cell movement and morphogenesis (Thiery and Sleeman, 2006; Zavadil and Bottinger, 2005). The EMT process may be activated by various extracellular stimuli. TGF-β1-induced EMT was the first to be described in mammary epithelial cells (Miettinen et al., 1994). However, many members of the TGF-β superfamily initiate and maintain the EMT programme, by regulation of the Smad-dependent and -independent pathways (Thiery and Sleeman, 2006). On the contrary, BMP-7 has an opposite response as it notably inhibits EMT and fibrotic responses in several animal models (Zeisberg et al., 2003). Furthermore, other signalling pathways promote EMT, including the Wnt and ET-1 pathways, among others (Jain et al., 2007; Radisky, 2005).

The limitations of *in vitro* cell culture experiments in reflecting *in vivo* biology led to the initiation of the current study, as it has already been proven that

different specific cell culture conditions can affect the EMT response. For instance, the ECM on which the cells are attached dictates the EMT response (Willis et al., 2005; Zeisberg et al., 2001), maybe due to a combination of multiple factors including integrin signalling, cytoskeletal signalling through small G proteins, and signalling through GF bound to the matrix (Werb, 1997). Furthermore, the mixture of GF present in the serum can also modify the EMT response of these cells. Therefore, the EMT induction by TGF-β1 instillation, followed by direct RNA isolation of the epithelial cells, would shed light on the EMT process *in vivo*.

A single instillation of TGF-β1 (2.5 ng/µl, 8 h) was found to induce gene expression modifications in the epithelial cells (Figure 4.14). For instance, the *cdh1* and *ocln* genes were specifically transcriptionally repressed by TGF-β1 stimulation. Previous publications reported the Snail and Slug family to be responsible for the E-cadherin and occludin downregulation, this family of TF being as well positively regulated by TGF-β1 (Ikenouchi et al., 2003). The E-cadherin and occludin expression levels were found to be essential for the maintenance of the epithelial cell polarity (Zavadil and Bottinger, 2005). Therefore, since these two molecules related to adherens and tight junctions, were less expressed, it can be proposed that epithelial cell polarity was partially lost in the TGF-β1-treated mice.

The E-cadherin gene expression level, expressed by the epithelial lavage samples (Figure 4.10) was downregulated upon TGF-β1 application, as assessed by quantitative RT-PCR (Figure 4.14). It has been previously demonstrated that NMuMG cells (mouse mammary epithelial cells) overexpressing Id2, exhibited partial resistance to TGF-β1-induced EMT by inhibiting the helix-loop-helix factor E2A, which is responsible for the suppression of the E-cadherin promoter (Kondo et al., 2004). In the current model, the downregulation of the Id2 molecule was confirmed by microarray and quantitative RT-PCR, on mice treated with TGF-β1. The Id2 downregulation was observed after 6 h of stimulation, prior to the E-cadherin repression, which was observed after 8 h. Therefore, it can be hypothesised that the downregulation of E-cadherin could be due to the absence of the Id2 molecule.

Interestingly, no mesenchymal markers were significantly upregulated apart from the β-catenin gene. This molecule can be considered either an epithelial or a mesenchymal marker, as it can drive or repress the EMT process. β-catenin is both an intracellular binding partner of the E-cadherins, and thus responsible for the

maintenance of adherens junctions (Zavadil and Bottinger, 2005) and a transcriptional co-activator, when the cell is freed from cell contacts (Behrens et al., 1996). Functioning as a transcriptional co-activator can induce mesenchymal gene transcription like α-SMA expression (Masszi et al., 2004) and EMT progression of tumor cells (Gilles et al., 2003). Furthermore, it has been localised to the nuclei of the epithelial cells in patients suffering from IPF (Chilosi et al., 2003) and published that the TGF-β1 could induce β-catenin expression (Masszi et al., 2004; Tian et al., 2003), which correlates with the results of TGF-β1 OT instillation. Thus, the significant upregulation of β-catenin after 8 h could further promote the activation of transcription of mesenchymal markers.

Furthermore, the rest of the mesenchymal markers included in the analysis, FSP1, α-SMA, and vimentin, did not demonstrate any significant upregulation due to the TGF-β1 instillation, although a tendency to a higher expression compared to untreated animals could be observed after 8 h.

To sum up, the expected significant upregulation of the mesenchymal markers after OT TGF-β1 instillation was not observed in the current model, when a single application of the recombinant ligand at a concentration of 2.5 ng/ml was utilised. However, a significant downregulation of the epithelial markers, *cdh* and *ocln* was evident. Furthermore, an upregulation of expression of the dual molecule β-catenin molecule was significant. Therefore, the experimental procedure may be useful in that TGF-β1 induced some changes on the gene expression of the epithelial cells. In order to improve assessment of changes, the retention time of TGF-β1 within the lung could be varied, as 8 h may not be sufficient to detect a complete initiation of the EMT process. Furthermore, the ligand concentration used could be augmented in order to induce a faster cell trans-differentiation. Additional OT instillations of the cytokine to the mice may also help in the induction of EMT.

The EMT process has been defined by simultaneous expression of epithelial and mesenchymal markers. However, it has not been established if the downregulation on epithelial markers occurs first, and later the upregulation of the mesenchymal markers is induced or vice versa. The simultaneous expression due to *de novo* synthesis of mesenchymal markers colocalising with previously expressed epithelial markers may occur sometime after the analysed 8 h.

Finally, it is important to stress that the potential source of the myofibroblasts may not be unique, but a combination of local, infiltrated bone marrow-derived cells, and trans-differentiated epithelial cells could contribute to the increase number of these active cells in the lung, which will form the fibroblastic foci, the hallmark of IPF.

5.8 CONCLUSIONS AND PERSPECTIVES

From the presented data, the following can be concluded:

1. The OT instillation yields the best deposition rate for local delivery to the lung, when compared with intratracheal, intranasal, microspray, or nebulisation methods (Figure 4.1-4.2).
2. The OT instillation of the TGF-β1 cytokine is sufficient to promote intracellular changes, both at the protein and RNA levels, at relatively low amounts of ligands and at early time points (Figure 4.3-4.6).
3. Cells activated by OT-instilled TGF-β1 include alveolar and bronchial epithelial cells (Figure 4.7).
4. The EL technique represents a novel approach to obtain an enriched RNA fraction from murine epithelial cells *in vivo*, without contamination of adjacent cell types (Figure 4.8-4.12).
5. Comparison of the gene expression pattern between the EL technique, primary ATII cells, cells from the bronchoalveolar lavage, and cell-lines, demonstrated that the EL fractions do not contain immune, smooth muscle, or endothelial cells and are enriched in ATI and ATII cell markers (Figure 4.13).
6. The TGF-β1 instillation combined with the EL demonstrated a loss of epithelial cell markers in response to TGF-β1 *in vivo* (Figure 4.14).

The EL technique, which makes use of a diluted GI solution administered intratracheally, represents a novel method to specifically isolate RNA from the pulmonary epithelial layer, comprising the distal and the proximal epithelium. The samples are free of genomic DNA and of endothelial and mesenchymal markers. The RNA obtained represents the gene expression pattern of the epithelial layer *in vivo* as it is extracted directly from the murine lung. In this way, cell isolation or tissue section preparations are avoided and thus modifications to the gene expression pattern are prevented. This represents a key milestone, as it has been published that the chosen

method for handling with the samples (Curran et al., 2000) or the high probability of RNA degradation by endogenous RNases can modify the *in vivo* gene expression profile (Gonzalez et al., 2005; Kohda et al., 2000).

Furthermore, the EL can also be used to analyse the uptake of different substances from the alveolar space into the epithelial cells, and their effect inside the cell (for example, the instillation of cytokines combined with the EL technique) can serve to study different processes in the murine lung *in vivo*.

The future perspectives would be to analyse the gene expression profile of many other markers in the EL fractions by semi-, quantitative RT-PCR, and microarray. The large number of thus far known cell-specific markers could not be covered by the current report. The microarray, using the SMART™ pre-amplification method (necessary due to the low RNA concentrations obtained), which avoids the underrepresentation of 5´-terminal transcripts (Wilhelm et al., 2006), would shed light on the knowledge from the murine epithelial gene expression *in vivo*. Additionally, using the microarray technique, different samples could be compared, for instance, the EL fractions *versus* the mouse isolated primary type II pneumocytes or *versus* lung homogenates, or other epithelial cell-lines. Moreover, the comparison between EL fractions obtained from saline- and bleomycin-instilled mice could reveal modifications to the gene expression patterns in the epithelial cells in this model of IPF.

A chromatin immunoprecipitation (ChIP) analysis could also be performed on these fractions (using the antibody against Smad3 as a precipitant) (Yu et al., 2008), in order to investigate TGF-β1 responsive genes *in vivo* in the epithelial layer.

The EL method could also be optimised in the rat, an animal that possesses a more similar lung morphology to the human organ than do the mice.

Furthermore, a more detailed study on the TGF-β1-induced EMT in the lung would be useful, to understand when EMT is initiated and which are the main early-modified markers *in vivo*. For this, different concentrations of TGF-β1, several stimulations and different retention time points could be studied.

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Table A.16 : Primer sequences

Gene	Forward primer sequence (5´-3´)	Reverse primer sequence (5´-3´)	GenBank™ Accesion Nr.
α-SMA	AGACAGCTATGTGGGGGATG	GAAGGAATAGCCACGCTCAG	NM_007392
α-SMA-RT	CTGACAGAGGCACCACTGAA	CATCTCCAGAGTCCAGCACA	NM_007392
β-catenin-RT	ACGCACCATGCAGAATACAA	GGTGACCCAAGCATTTTCAC	NM_007614.2
*β-EnaC	AAAGGCCTGCGCAGGAGGCG GCCACAG	GATGGCCTCCACCTCACTGTCCGACTC	NM_011325.1
Atp1α1	GGGAAGGGGGTTGGACGA	GGTGTGAGGGCGTTGGGG	NM_144900
Atp1β1	GGAGAAGAAGGAGTTTTTGGGCAG	CTTCCTCTCTCCCCGTTCGTG	NM_009721
AQP5-RT	CCTTATCCATTGGCTTGTCG	CTGAACCGATTCATGACCAC	NM_009701.4
Calponin	GGCCAAGACAAAAGGAAACA	GACCTGGCTCAAAGATCTGC	NM_009922
CC-10-RT	TGGATACCCTCCCACAAGAG	AGGGCAGTGACAAGGCTTTA	NM_011681.1
Col1agen1α1	ACGTCCTGGTGAAGTTGGTC	TCCAGCAATACCCTGAGGTC	NM_007742.3
CTGF	CCCGCCAACCGCAAGATT	AGGCGGCTCTGCTTCTCCA	NM_010217
CTGF-RT	GCAGACTGGAGAAGCAGAGC	GACAGGCTTGGCGATTTTAG	NM_010217
E-cadherin	AGTTTACCCAGCCGGTCTTT	AGGGTTCCTCGTTCTCCACT	NM_009864
E-cadherin-RT	CAAGGACAGCCTTCTTTTCG	TGGACTTCAGCGTCACTTTG	NM_009864
Fibronectin-RT	GAGTGGAAGTGTGAGCGACA	GCATCGTAGTTCTGGGTGGT	NM_010233
Foxp2-RT	CTTGGAAGAATGCAGTGCGTC	TTTTGTGACCTTCGCTTCTGG	NM_053242.3
FSP1-RT	TTGTGTCCACCTTCCACAAA	GCTGTCCAAGTTGCTCATCA	NM_011311.1
GABRP	GCGCCTTGCTCAGTACACAA	ACGTTCCTCCGAAGCTCAAAT	NM_146017.2
GADD45	AGGCGGCCAAACTGATGAATGT	AGGTCTCGGGCTTCGGTTGTG	NM_008655
GAPDH	ACCCAGAAGACTGTGGATGG	TGTGAGGGAGATGCTCAGTG	NM_001001303
HPRT-RT	TGCTGACCTGCTGGATTACA	TATGTCCCCCGTTGACTGAT	NM_013556.2
JunB	GCAGCTACTTTTCGGGTCAG	TTCATCTTGTGCAGGTCGTC	NM_008416.1
LPCAT	GGCTCCACATTCCTCCTACTTTG	ATCTCCTCCACTGTCTTCCTTCG	NM_145376.3
Myosin	GACAACTCCTCTCGCTTTGG	GCTCTCCAAAAGCAGGTCAC	NM_013607.1
Occludin-RT	GCTCTCTCAGCCAGCGTACT	ATAGCCTCTGTCCCAAGCAA	NM_008756.2
PAI-1	TCATCAATGACTGGGTGGAA	GCCAGGGTTGCACTAAACAT	NM_008871
PAI-1-RT	CTTTACCCCTCCGAGAATCC	GACACGCCATAGGGAGAGAA	NM_008871
PECAM-1	CCTTCACCATCAACAGCATCCA	ATGGGTTCTGACTCCTGCAATT	NM_008816
PECAM-1-RT	CACCTGCAAAGTGGAATCAA	AGCAGGACAGGTCCAACAAC	NM_008816
proSP-B	CCAAGAGTGTGAGGATATTGTCCA	AAGGGCAGGGGAATGGGG	NM_147779
proSP-C	CCAGGAGCCAGTTCCGCATC	AGTGGTAGCTCTCCACACAGG	NM_011359
proSP-C-RT	GCAAAGAGGTCCTGATGGAG	ATGAGAAGGCGTTTGAGGTG	NM_011359
Smad7	TCTCCCCCTCCTCCTTACTC	CAGGCTCCAGAAGAAGTTGG	NM_008543.1
Slug-RT	GAAGCCCAACTACAGCGAAC	AGGAGAGTGGAGTGGAGCTG	NM_011415.2
T1α-RT	ACAGGTGCTACTGGAGGGCTT	TCCTCTAAGGGAGGCTTCGTC	NM_010329.1
TTF-1-RT	AGCTTCCGAAGCCGAAGTATC	AGAACGGAGTCGTGTGCTTTG	NM_009442.1
Vimentin	TGAAGGAAGAGATGGCTCGT	TCCAGCAGCTTCCTGTAGGT	NM_011701.3
Vimentin-RT	TGAAGGAAGAGATGGCTCGT	TCCAGCAGCTTCCTGTAGGT	NM_011701.3

Table 17: Primer sequences used for semi-quantitative and real-time RT-PCR: The short gene name, forward and reverse primer sequences in 5´to 3´ orientation, and GeneBank™ accession number are listed. The specific primers used for quantitative RT-PCR are indicated by the addition of RT after the name of the gene. *: These primers were derived from Jernigan NL and Drummond HA, 2005.

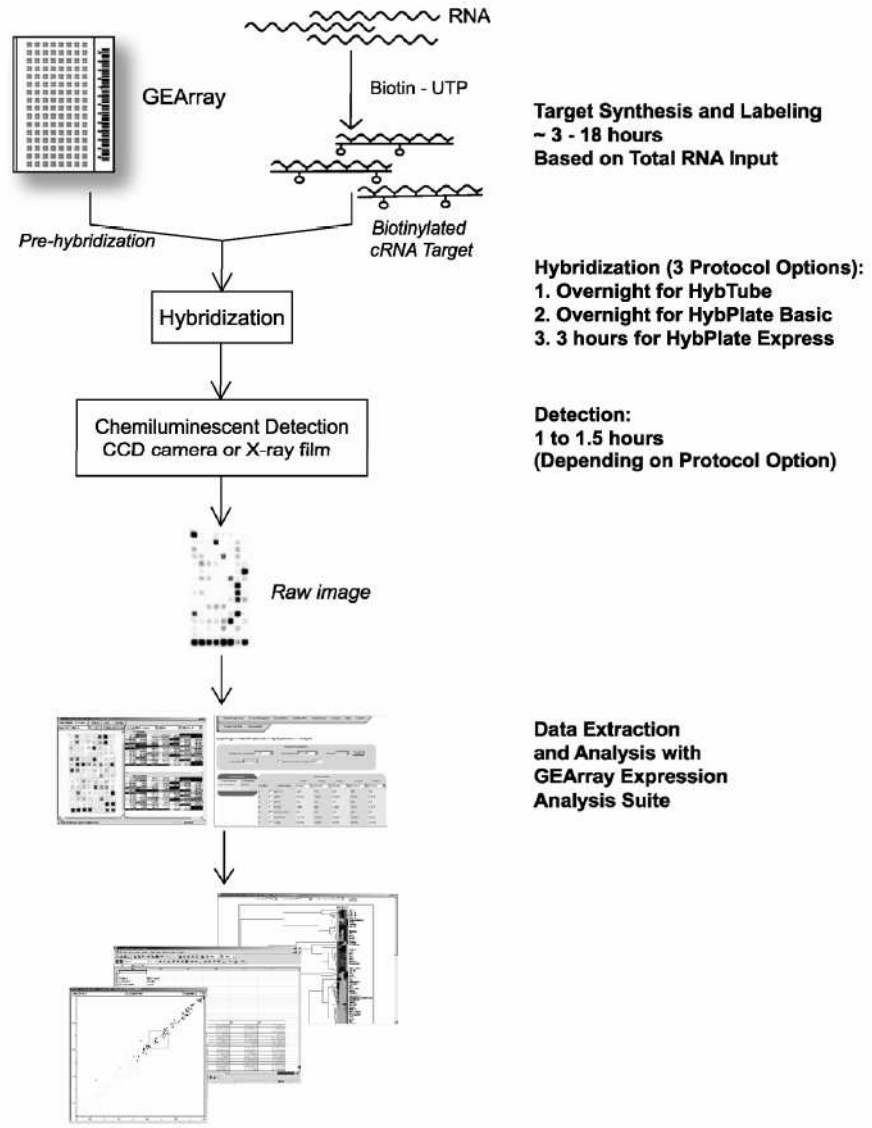

Figure A.1.: Overview of the GEArray® Procedure: Amplification and labelling by biotin-UTP of antisense RNA is followed by hybriditation on the GEArray

Table A.17: Oligo BMP/TGF-β superfamily microarray

Acvr1	Acvr1b	Acvr2	Acvr2b	Acvrl1	Amh	Bambi	Bglap1
Bmp1	Bmp10	Bmp15	Bmp2	Bmp3	Bmp4	Bmp5	Bmp6
Bmp7	Bmp8a	Bmp8b	Bmpr1a	Bmpr1b	Bmpr2	Cdc25a	Cdkn1a
Cdkn2b	Cer1	Chrd	Grem1	Col1a1	Col1a2	Col3a1	Dlx2
Lefty1	Eng	Evi1	Fkbp1b	Fos	Fst	Gdf1	Gdf11
Gdf2	Gdf3	Gdf5	Gdf6	Gdf8	Gdf9	Idb1	Idb2
Idb3	Idb4	Cd79a	Igf1	Il6	Inha	Inhba	Inhbb
Inhbc	Inhbe	Itgb5	Itgb7	Ivl	Jun	Junb	Lap3
Lefty2	Igfbp3	Smad1	Smad2	Smad3	Smad5	Smad6	Smad7
Smad9	Nbl1	Nodal	Nog	Pdgfb	Plat	Plau	Runx1
Runx2	Serpine1	Sox4	Stat1	Tgfb1	Tgfb1i1	Tgfb1i4	Tgfb2
Tgfb3	Tgfbi	Tgfbr1	Tgfbr2	Tgfbr3	Tgif	Timp1	Zfhx1a
PUC18	PUC18	PUC18	Blank	Blank	Blank	Gapd	Gapd
Ppia	Ppia	Ppia	Ppia	Rpl13a	Rpl13a	Actb	Actb

Gapdh: Glyceraldehyde-3-phosphate dehydrogenase
Acvr1 : Activin A receptor type 1
Acvr1b : Activin A receptor type 1B
Acvr2 : Activin A receptor type 2
Acvr2b : Activin A receptor type 2B
Acvrl1 : Activin A receptor type 2-like 1
Amh: Anti-Mullerian hormone
Bambi: BMP and activin membrane-bound inhibitor
Bglap1: Bone γ-carboxyglutamate protein 2
Bmp1: Bone morphogenetic protein 1
Bmp10: Bone morphogenetic protein 10
Bmp15: Bone morphogenetic protein 15
Bmp2: Bone morphogenetic protein 2
Bmp3: Bone morphogenetic protein 3
Bmp4: Bone morphogenetic protein 4
Bmp5: Bone morphogenetic protein 5
Bmp6: Bone morphogenetic protein 6
Cdkn2b: Cyclin-dependent kinase inhibitor 2B (p15)
Cer1: Cerberus 1 homolog
Chrd: Chordin
Grem1: Gremlin 1
Col1a1: Procollagen type Iα1
Col1a2: Procollagen type Iα2
Col3a1: Procollagen type IIIα1
Dlx2: Distal-less homeobox 2
Lefty1: Left right determination factor 1
Eng: Endoglin
Evi1: Ecotropic viral integration site 1
Fkbp1b: FK506 binding protein 1b
Fos: FBJ osteosarcoma oncogen
Fst: Follistatin
Gdf1: Growth differentiation factor 1
Gdf11: Growth differentiation factor 11

Gdf2: Growth differentiation factor 2
Gdf3: Growth differentiation factor 3
Gdf5: Growth differentiation factor 5
Gdf6: Growth differentiation factor 6
Gdf8: Growth differentiation factor 8
Gdf9: Growth differentiation factor 9
Idb1-4: Inhibitor of DNA binding 1-4
Cd79a: CD79 antigen (immunoglobulin-associated α)
Igf1: insulin-like growth factor 1
Il6: interleukin 6
Inha: inhibin α
Inhba: inhibin β-A
Inhbb: inhibin β-B
Inhbc: inhibin β-C
Inhbe: inhibin β-E
Itgb5: Integrin β 5
Itgb7: Integrin β 7
Ivl: Involucrin
Jun: Jun oncogene
Junb: Jun-B oncogene
Lap3: Leucine aminopeptidase 3
Lefty2: Left-right determination factor 2
Igfbp3: Insulin-like growth factor binding protein 3
Smad1: MAD homolog 1-7
Smad9: MAD homolog 9
Nbl1: Neuroblastoma, suppression of tumorigenicity 1
Nodal: Nodal
Nog: Noggin
Pdgfb: Platelet derived growth factor, B polypeptide
Plat: Plasminogen activator, tissue
Plau: Plasminogen activator, urokinase
Runx1: Runt related transcription factor 1
Runx2: Runt related transcription factor 2
Serpine1: Serpine (or cysteine) peptidase inhibitor, clade E, member 1
Sox4: SRY-box containing gene 4
Stat1: Signal transducer and activator of transcription 1
Tgfb1: Transforming growth factor β1
Tgfb1i1: Transforming growth factor β1 induced transcript 1
Tgfb1i4: Transforming growth factor β1 induced transcript 4
Tgfb2: Transforming growth factor β2
Tgfb3: Transforming growth factor β3
Tgfbi: Transforming growth factor β induced
Tgfbr1: Transforming growth factor β receptor 1
Tgfbr2: Transforming growth factor β receptor 2
Tgfbr3: Transforming growth factor β receptor 3
Tgif: TG interacting factor 1
Timp1: Tissue inhibitor of metalloproteinase 1
Zfhx1a: Zinc finger transcription factor 1a
PUC18: PUC18 plasmid DNA
Ppia: peptidylpropyl isomerase A
Rpl13a: ribosomal protein 13a
Actb: β-actin

Table A.17: BMP/TGF-β array: The layout of the array is listed, including gene symbol and corresponding position on the membrane. The specific array is spotted with BMP and TGF-β members and target genes.

9 DECLARATION

Ich erkläre: "Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

10 CURRICULUM VITAE

PERSONAL DATA

Name	:	Julia
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Date and place of birth	:	29/09/1980 Madrid
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Office address	:	Department of Medicine II, University of Giessen School of Medicine, Aulweg 123, Room 6-11, 35392, Giessen, Germany

STUDIES

10/2007 – to date	:	Postdoctoral Fellow in the laboratory of Proff. Dr. L. M. Schmitz (Professor of Biochemistry, Department of Biochemistry, Giessen, Germany). Project entitled "HIPK2 activation loop".
08/ 2004 – 09/2007	:	PhD Student in the laboratory of Dr. O. Eickelberg (Assistant Professor of Medicine, Department of Medicine II, Giessen, Germany). Project thesis entitled "Characterisation of AEC gene expression *in vivo* under TGF-betą stimulation".
09/ 2004 – 08/ 2006	:	Graduate student of the International Graduate Program "*Molecular Biology and Medicine of the Lung" (MBML)*
10/ 2003 – 07/ 2004	:	Research project in the laboratory of Dr. O. Eickelberg (Assistant Professor of Medicine, Department of Medicine II, Giessen, Germany. Project entitled "Discovery of TGF-beta Target Genes". Visiting student of the International Graduate Program "*Molecular Biology and Medicine of the Lung" (MBML).*
06/ 2003	:	Diploma degree in Biology (specialised in Genetics, Biochemistry and Microbiology) at the Faculty of Biology and Environmental Sciences, University of León, Spain.

10/ 2002 – 07/ 2003	:	Studies of Biology at the Justus-Liebig University of Giessen, Germany (ERASMUS/SOCRATES scholarship).
1998 – 2003	:	Studies of Biology at the Faculty of Biology and Environmental Sciences, University of León, Spain.
June 1998	:	Abitur
1994 – 1998	:	High School in the I.E.S. La Granja, León, Spain.
08 – 10/ 1996	:	High School at Banting High School in London, Canada with an exchange program.
1986 – 1994	:	Primary School, Colegio Camino, León, Spain.

PUBLICATIONS

M. A. Alejandre-Alcázar, P. D. Shalamanov, O. V. Amarie, J. Sevilla-Pérez, W. Seeger, O. Eickelberg, and R. E. Morty. "Temporal and spatial regulation of bone morphogenetic protein signalling in late lung development". *Developmental Dynamics* 2007 Sep 6 [Epub ahead of print].

M. A. Alejandre-Alcázar, G. Kwapiszewska, I. Reiss, O. V. Amarie, L. M. Marsh, J. Sevilla-Pérez, M. Wygrecka, B. Eul, S. Köbrich, M. Hesse, R. T. Schermuly, W. Seeger, O. Eickelberg, and R. E. Morty. "Hyperoxia modulates TGF-beta/BMP signalling in a mouse model of bronchopulmonary dysplasia". *Am J Physiol Lung Cell Mol Physiol* 292: L537–L549, Feb 2007.

J. Sevilla-Pérez, G. Kwapiszewska, O. V. Amarie, N. Weissmann, R. T. Schermuly, W. Seeger, O. Eickelberg. "Shroom expression is attenuated in experimental pulmonary arterial hypertension". (*In revision, Eur Respir J.*).

J. Sevilla-Pérez, R. Dumitrascu, R.T. Schermuly, W. Seeger, O. Eickelberg. "Epithelial lavage: novel method to isolate pulmonary epithelium". (*In preparation*).

CONFERENCES AND POSTERS

M.E.Yeager, V.L. Battula, J. Sevilla, O. Eickelberg. "Whole Genome Location Analysis of TGF-beta Responsive Genes in Fibroblasts – Potential Link to Pulmonary Fibrosis". Poster discussion. *Am. J. Resp. Crit. Care Med.*, Vol. 169 (7), April 2004.

J. Sevilla-Pérez, M. E. Yeager, L. V. Battula, W. Seeger, O. Eickelberg. "Immunoprezipitation neuer Zielgene von Smad3 in Fibroblasten: Bedeutung für Lungenfibrose". Poster discussion. *Pneumologie*, March 2005.

J. Sevilla-Pérez, H. Yu, R. Dumitrascu, M. A. Alejandre-Alcazar, O.V. Amarie, W. Seeger, R.T. Schermuly, O. Eickelberg. "*In vivo* Signalling of TGF-beta in Mouse Alveolar Epithelium". Poster discussion. FASEB SRC, TGF-beta Superfamily: Signalling and Development. June, 2005.

J. Sevilla-Pérez, M. A. Alejandre-Alcazar, O. V. Amarie, W. Seeger, O. Eickelberg. "Alveolar epithelial cell-specific gene expression in the mouse lung *in vivo*". Oral presentation. *Eur. Resp. J.* 2005, in press.

J. Sevilla-Pérez, R. Dumitrascu, M. A. Alejandre-Alcazar, R.T. Schermuly, W. Seeger, O. Eickelberg. "Alveolar Epithelial Gene Expression *in vivo*: Regulation by TGF-beta". Poster Discussion, DGIM, April, 2006.

J. Sevilla-Pérez, R. Dumitrascu, M. A. Alejandre-Alcazar, R.T. Schermuly, W. Seeger, O. Eickelberg. "Alveolar Epithelial Gene Expression *in vivo*: Regulation by TGF-beta". Poster Discussion, ATS, May, 2006.

J. Sevilla-Pérez, R. Dumitrascu, M. A. Alejandre-Alcazar, R.T. Schermuly, W. Seeger, O. Eickelberg. "Alveolar Epithelial Gene Expression *in vivo*: Regulation by TGF-beta". Poster Discussion, ICLF, September, 2006.

J. Sevilla-Pérez, G. Kwapiszewska, W. Seeger, O. Eickelberg. "Shroom expression is attenuated in hypoxia-induced pulmonary hypertension". Oral presentation, *Eur. Resp. J.* 2006, in press.

J. Milosevic, M. Konigshoff, J. Sevilla-Pérez, A. Jayachandran, A. Jahn, and O. Eickelberg. "Rapid loss of the alveolar epithelial cell phenotype during primary culture in a matrix-independent fashion". Poster presentation, ATS, May, 2007 / Poster presentation, 2nd summer course on Mass Spectrometry in Biotechnology and Medicine, July, 2007.

M. Konigshoff, J. Milosevic, A. Jayachandran, J. Sevilla-Pérez, and O. Eickelberg. "Matrix independent expression of novel markers during transdifferentiation of primary alveolar epithelial cells". Electronic poster discussion, *Eur. Resp. J.*, 2007.

PRACTICAL TRAINING AND SEMINARS

- "Biostatistics", 20th – 24th November, 2006
- "Scientific reasoning, project design, and paper writing", 6th – 10th February 2006.
- "High performance presentation workshop in English", 25th – 27th April 2005.
- "Lung physiology", 31st January – 4th February 2005.
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- MBML retreat presentations at Rauischholzhausen, Germany, August 2004-2005-2006.

TECHNICAL EXPERTISE

Molecular Biology	:	Cloning, isolation of RNA, DNA and proteins from both cells and tissues by several methods, reverse transcription PCR (RT-PCR), real-time PCR, Western blotting, reporter assays, micro-array.
Immunostaining	:	Histo- and cytological staining of mouse, rat and human lung sections.
Cell Culture	:	Culturing of cell lines and primary cells. DNA and siRNA transfections.
Animal handling	:	Breeding, treatment and tissue extraction of mice.
Software skills	:	MS Office, Adobe PhotoShop, Internet surfing, operating system Windows.

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Spanish	:	Native speaker.
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