All-*trans* retinoic acid recruits Smad3 to drive TGF-β signaling in lung fibroblasts: new insights for the management of bronchopulmonary dysplasia with retinoids

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1 List of abbrevations

ACA	angiotensin-converting enzyme
ALK	activin-like kinase
AMH	anti-Müllerian hormone
ATRA	all-*trans* retinoic acid
BAL	bronchoalveolar lavage
BMP	bone morphogenetic proteins
BPD	bronchopulmonary dysplasia
CLD	chronic lung disease
cm	centimeter(s)
CMV	cytomegalovirus
CNIPPV	continuous nasal intermittent positive pressure ventilation
COPD	chronic obstructive pulmonary disease
co-Smad	common Smad
CPAP	continuous positive airway pressure
cpm	counts per minute
CT	computer tomography
ctgf	connective tissue growth factor
DLR	dual luciferase assay
DNA	deoxyribose nucleic acid
E	embryonic day
ECM	extracellular matrix modeling
ELBWI	extremely low birth weight infant
FEV_1	forced expiratory volume in one second

FiO_2	fraction of inspired oxygen
g	gram(s)
GA	gestational age
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
GDFs	growth and differentiation factors
GM-CSF	granulocyte macrophage-colony stimulating factor
GST	glutathione-S-transferase
h	hour(s)
HEK	human embryonic kidney
HL	human leukemia
HMD	hyaline membrane disease
IgG	immunoglobulin G
IL	interleukin
iNO	inhaled nitric oxide
IPTG	isopropyl–β thioglactopyranoside
IQ	intelligence quotient
I-Smad	inhibitory Smad
kDa	kilodalton
KGF	keratocyte growth factor
LAP	latency-associated peptide
Luc	luciferase
L-TGF–β	latency associated TGF–β molecule
MAPK	mitogen-activated protein kinase

mg	milligram(s)
MH	mad homology
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
MMP	matrix metallproteinases
MvILu	mink lung cells
NICHD	National Institute of Child Health and Human Development
NIPPV	nasal intermittent positive pressure ventilation
ng	nanogram(s)
nm	nanometer(s)
nM	nanomolar
NO	nitric oxide
nCPAP	nasal continuous positive airway pressure
P	postnatal day
PAI-1	plasminogen activator inhibitor 1
PCR	semi-quantitative polymerase chain reaction
PDA	patent *ductus arteriosus*
PDGF-BB	platelet derived growth factor BB
PMA	post menstrual age
ppm	part per million
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferating response elements

PPV	positive pressure ventilation
RA	retinoic acid
RAR	retinoic acid receptor
RDS	respiratory distress syndrome
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
R-Smad	receptor-regulated Smad
RXR	retinoic X receptor
s	second
S.D.	standard deviation
SARA	Smad anchor for receptor activation
SBE	Smad binding element
scr	scrambled
SDS	sodium dodecyl sulphate
serpine1	serine peptidase inhibitor 1
si	small interfering
SIDS	sudden infant death syndrome
SNIPPV	synchronized nasal intermittet positive pressure ventilation
SP	surfactant protein
TGF–α	transforming growth factor α
TGF–βR1	TGF–β receptor 1
TGF–βR2	TGF–β receptor 2
TGF–βR3	TGF–β receptor 3

TIMP	tissue inhibitor of metallproteinase
TNF	tumor necrosis factor
µg	microgram(s)
µl	microliter(s)
µM	micromolar
VEGF	vascular endothelial growth factor
VLBW	very low birth weight
WPC	weeks post conception

2 Introduction

Studies reported here will address the interaction of the transforming growth factor (TGF)–β and retinoid signaling systems in the context of lung development. The development of the mature, adult lung from the primitive foregut is a complex and lengthy process that is known to be regulated, at least in part, by both the TGF–β and retinoid signaling systems. No study to date has addressed how these two systems interact in the context of lung development. This is all the more important, since retinoid supplementation is being considered as a clinical management strategy in premature infants, where lung development is impacted. However, controversy remains as to whether this clinical approach has positive or deleterious effects, and understanding the interaction of the TGF–β and retinoid systems may yield insights into the effects of retinoids on the development of the immature lung.

2.1 Normal lung development

2.1.1 Stages of normal lung development

Lung development serves to maximize the gas exchange surface area while minimizing at the same time the thickness of the blood-air barrier. This aim is achieved by the process of branching, where the formation of conducting airways takes place and septation, where subdivision of airspaces increases the surface area of the lung [1]. Lung growth is physically influenced by the increasing size of the intrathoracic space, by lung liquid volume and pressure as well as the amniotic fluid volume [2]. During lung development there are several signaling pathways involved such as the canonical TGF–β pathway and the vitamin A pathway, which drive lung development by growth factor-mediated communication [3]. During the process of branching and septation, retinoic acid (RA) was shown to be an important mediator of development [4]. In later studies it was shown that dysregulation of TGF–β, vitamin A and other growth factors can effect normal development and, therefore, disturb normal organ function for the entire period of life [5]. The phases of normal development of the lung can be divided into five chronological stages [2]. The lung developmental stages correspond roughly to different weeks of gestation as follows: the embryonic period (1st - 7th week post conception, (WPC)), the pseudoglandular (7th - 16th WPC), the canalicular (16th until 26th - 28th WPC), saccular (26th - 28th until 32nd - 36th WPC) as well as the alveolar stage (32nd - 36th WPC until the 2nd year of life)[6]. The beginning and ending of the developmental stages described vary individually and, therefore, differ minimally from person to person [7].

During the embryonic stage (1st - 7th WPC), initial ventral outpouching initiates

from the primitive foregut, later generating the primary bronchi. During further development, two main bronchi, constructed of respiratory epithelial cells, grow into the mesenchyme and later divide in a dichotomic manner. Pulmonary arteries are formed next to the bronchial tree and pulmonary veins develop in the mesenchymal septa. The stage ends with the development of broncho-pulmonary segments [8]. The pseudo-glandular stage, with further branching, is placed in-between the embryonic and fetal period of child development (7th - 16th WPC). The mesenchyme tissue differentiates around the epithelial tubes into smooth muscle, connective tissue and cartilage. During the canalicular stage of lung development (16th - 27th WPC) the surface area increases by an increasing number of bronchioles and capillaries forming the prospective gas-exchange area [9]. In addition, the cuboidal epithelium differentiates into type I cells which form the structure of the alveolar wall and type II cells capable of producing surfactant [10]. During the saccular stage (24th WPC until birth) the air spaces increase in size while the lung parenchyma becomes thinner. The previously described type I cells build the endings of the airway tree by forming transitory airspaces or sacculi. The type II cells now start to produce surfactant, which increases pulmonary compliance and reduces surface tension, necessary for later lung unfolding. Following the saccular stage, the process of alveolarization takes place as early as in the 32nd week of gestation. It is characterized by an increasing number of alveolar spaces, primarily developing after birth. The division of secondary septa is called septation. During this process, the sacculi are further subdivided by secondary septa, forming smaller units, called the alveoli [1]. The amount of connective tissue decreases between the two layers of capillary meshwork, reducing the distance of the blood-air barrier. The complex process of capillary remodeling takes place until around one and a half years of age when the lung begins to resemble the adult structure [11]. Microvascular maturation concomitantly takes place with alveolarization, starting from the first month after birth until the age of two to three years of age [12].

2.1.2 Reference time points of lung development in mice and humans

Lung development follows a similar course in mice and humans, yet in mice over a much shorter period of time. In mice, stages are measured in days post partum, in humans time points are distinguished by weeks post conception. The developmental steps correspond to each other, as listed in table 1 modified from [1].

Table 1: Lung development stages in humans and mice. Event stages are differentiated by weeks post conception (WPC) for humans and embryonic (E) and days post partum (P) for mice.

event phase	humans	mice
embryonic stage	WPC 4–7	*E9.5–E12*
pseudoglandular	WPC 5–17	*E12–E16.5*
canalicular	WPC 16–26	*E16.5–E17.5*
saccular	WPC 24–38	*E17.5–P4*
alveolar	WPC 36–infancy	*P4–P14*

2.2 Historical background of bronchopulmonary dysplasia

The disease bronchopulmonary dysplasia (BPD) was first described by Northway *et al.* as a chronic pulmonary syndrome in preterm infants [13]. BPD resulted from prolonged mechanical ventilation and oxygen therapy applied to infants suffering from respiratory distress. In the past, another term for BPD was hyaline membrane disease (HMD) due to the histopathological picture of hyaline membrane formation and necrosis of lung parenchyma [13]. The diagnostic criteria described by Bancalari *et al.* included signs of chronic respiratory disease requiring oxygen supplementation with intermittent positive airway pressure ventilation for more than 28 days during the first weeks of life as well as an abnormal chest X-ray [14]. Today, this definition still has relevance but new diagnostic and therapeutic possibilities in neonatal intensive care medicine require a more complex definition of the disease [15]. When steroid application to mothers with suspected preterm infants was established after first the trials in the 1980s [16], severity and occurrence rates of this neonatal lung disease were reduced significantly [17]. Today, early surfactant administration [18] is routinely performed in preterm infants and has a proven positive outcome [19, 20, 21]. Clinical outcome and survival rates have improved over the past years also following new treatment options such as inhaled nitric oxide (iNO) [22] as well as inhaled prostacyclin [23]. Due to the available treatment options mentioned, the clinical and histopathological presentation of the disease have changed over time, resulting in two descriptions: namely old and new BPD, further described in sections 2.4, 2.5 and 2.6.

2.3 Defining bronchopulmonary dysplasia

There are two classifications used today for the diagnosis of BPD. Jobe and Bancalari proposed in their 2001 Workshop Summary a definition based on three severity grades [24]. This classification of BPD used by the National Institute of Child

Health and Human Development (NICHD), is defined by the three different levels, mild, moderate and severe, depending on the amount and duration of oxygen supplementation, described in table 2.

Table 2: Diagnostic criteria for bronchopulmonary dysplasia according to Jobe and Bancalari. This table is modified from Jobe and Bancalari [24]. Abbreviations: BPD, bronchopulmonary dysplasia; nCPAP, nasal continuous positive pressure; PMA, post menstrual age; PPV, positive pressure ventilation.

Gestational Age	<32 weeks	≥32 weeks
Time point assessment	36 weeks PMA or discharge to home	>28 but <56 days postnatal age or discharge to home
	Treatment with oxygen >21% for at least 28 days, plus	
Mild BPD	Breathing room air at 36 weeks PMA or discharge*	Breathing room air by 56 days postnatal age or discharge*
Moderate BPD	Need for <30% oxygen at 36 weeks PMA or discharge*	Need for <30% oxygen at 56 days postnatal age or discharge*
Severe BPD	Need for ≥30% oxygen and/or positive pressure (PPV or nCPAP) at 36 weeks PMA or discharge*	Need for ≥30% oxygen and/or positive pressure (PPV or nCPAP) at 56 days postnatal age or discharge*

*Whichever comes first

This definition is well accepted and employed in several representative studies [25]. A second definition described by Walsh *et al.* in 2003 is based on the physiological parameters oxygen saturation, the need for oxygen supplementation, and ventilatory support. This definition was described as a reliable, feasible and precise diagnosis of BPD. According to Walsh *et al.* the diagnosis of BPD was certain when an infant needed continuous positive airway pressure, mechanical ventilation or oxygen with a concentration above FiO_2 0.30. Infants failing to tolerate a stepwise reduction in oxygen supplementation <0.30 were also diagnosed with BPD. Patients were excluded from the diagnosis when saturation exceeded 88% while treated with room air or when a patient tolerated the previously described reduction test [26]. The existence of multiple diagnostic criteria and definitions confounds the assessment of incidence of BPD. Walsh *et al.* redefined these previously described criteria by changing the cutoff point for oxygen supplementation from 88% to 90%, observing a significant change in incidence rates [27].

Table 3: Diagnostic criteria for bronchopulmonary dysplasia according to Walsh *et al.*. This table is modified from Walsh *et al.* [26]. Abbreviations: BPD, bronchopulmonary dysplasia; CPAP, continuous positive pressure; PMA, post menstrual age.

Time of assessment	36 weeks PMA
BPD	>30% and/or ventilatory support or CPAP
	or
	>0_2 saturation <88% after at room air reduction test, or 0_2 saturation <88% after 1 hour observation

2.4 The "old" bronchopulmonary dysplasia

Old or classic BPD was first described by Northway *et al.* in 1967 where oxygen toxicity and lung overexpansion due to high pressure ventilation was postulated as a major reason for the development of BPD. Electron microscope analysis revealed the histopathological changes, where BPD was described as a bronchiolitic presentation with fibrosis [28]. Further investigations by O'Brodovich, Mellins and colleagues in 1985 revealed deeper insights into the pathogenesis of the disease. Their studies supported the hypothesis that mechanical ventilation contributed to the development of BPD [29]. Ventilatory support was needed in preterm infants due to respiratory distress even though it was known already that positive pressure ventilation may lead to permanent impairment of alveolar development [30]. The clinical features and pathological remodeling consisted of extrapulmonary air leaks,

emphysematous changes, epithelial metaplasia and peribronchial fibrosis [31, 32]. Further histopathological changes were metaplasia of the squamuus epithelial layer, destruction of the ciliated epithelium as well as an inhomogeneous lung structure [6]. Most infants suffering from BPD were born between 28 and 32 weeks of gestation with birth weights varying from 1000 g up to 1500 g. Surviving infants sometimes developed a massive fibrosis of lung vessels causing pulmonary hypertension including cor pulmonale resulting in death via right heart failure [28, 33]. Later studies regarding the pathological sequelae of BPD did reveal the existence of a different histopathological presentation of BPD. Preterm infants suffering from BPD had almost no interstitial fibrosis and presented with uniform enlargement of air spaces [34]. Further investigation of these findings contributed to the introduction of a definition of "new" BPD.

2.5 The "new" bronchopulmonary dysplasia

Changes in the past years from the old to new BPD occurred due to widespread use of prenatal corticosteroids, postnatal surfactant replacement therapy, less invasive ventilatory support and better nutritional options [35]. The new BPD has a different histopathological picture compared to the old BPD, characterized primarily by excessive elastin and collagen deposition, smooth muscle overgrowth, interstitial fluid accumulation and alveolar and capillary hypoplasia disrupting distal lung growth rather than parenchymal fibrosis and fibroproliferative airway damage [36, 37]. The new treatment options (mentioned in section 2.8) resulted in a less severe presentation of the disease with subsequent higher surviving rates in preterm infants causing BPD to be the most frequent presentation of chronic lung disease (CLD) of early infancy [21]. According to recent studies, approximately two thirds of the infants born with a birth weight below 1000 g later develop BPD even though never suffering from serious RDS [35]. The development of new BPD is thought to be driven by a combination of exposure to oxygen enriched gas and inflammatory processes [38].

2.6 Incidence of bronchopulmonary dysplasia

In the United States of America, 20.0000 out of the 500.000 infants born prematurely present with a birth weight below 1000 g, defined also as extremely low birth weight infants (ELBWI). Ehrenkranz *et al.* identified the relevance of the birth weight as a major risk factor BPD, since infants who later developed BDP had significantly lower birth weights [15]. Another study by Stevenson *et al.* in 1998 found the incidence rates of BPD to be around 29% of the preterm infants with a birth weight

from 501 g to 1500 g [39]. Another survey concluded in 2001 incidence rates of BPD in ELBWI as low as 23% [40]. Due to different BPD definitions and a different cutoff for weight, these data can only be in partly compared. However, incidence rates of BPD as well as the time of hospitalization did change over the last years. A large study using the US nationwide database revealed a decrease in incidence over the years 1993-2006, correlating with an increase in the use of non-invasive respiratory support [41]. Recent publications in Germany report BPD to be present in 29% of infants born less than 32 weeks of gestational age [42], and as high as 51% for infants born in-between 26-27 weeks of gestation. Mortality rates from affected infants differed depending on the gestational age (GA). Premature infants born at GA below 26 weeks had mortality rates as high as 28.6%, whereas infants affected by BPD at a GA between 31-32 weeks had a mortality rate of 8.8% [43]. A European cohort study with 4.185 preterm infants born in 2003 revealed strong regional incidence differences of BPD, possibly explained by different local practices [44]. However, a low GA appeared to be the strongest predictor for the development of BPD, rather than the geographic region [45, 44].

2.7 Bronchopulmonary dysplasia in the context of other preterm infant diseases

BPD seems to have a relevant impact on motor skills such as speech development and gross motor function. A correlation was seen in infants with the disease where speech development was impaired as well as retarded psychomotor development [46, 47]. Not only motor skills seem to be impaired in infants suffering from BPD but also academic and cognitive achievements seem to be lower. This was shown in an eight-year follow-up study including n=98 infants suffering from BPD compared to n=75 infants having only very low birth weight (VLBW). Cognitive function and intelligence quotient (IQ) scores were significantly lower, and enrollment in special education speech and language programs was more frequent in infants diagnosed with BPD [48]. Re-hospitalization was seen in up to 50% of diseased children in the first two years of life due to recurrent respiratory insufficiency requiring hospital stays. A lot of readmissions are reported from children receiving home oxygen therapy subsequently infected with the respiratory syncytial virus (RSV) [49]. Although the peak of re-hospitalization is during the first three years of life, school aged children as well as adolescents earlier diagnosed with BPD still suffer more frequently from respiratory symptoms than their colleagues, requiring higher rates of utilization of health service resources emphasizing the prolonged significance of the disease in later life [50]. Home oxygen requirement in the first years of life as well as infections

with RSV seem to be associated with increased chronic respiratory morbidity causing high rates of readmission and increased costs of care [49, 51]. The question was addressed whether sudden infant death syndrome (SIDS) and BPD are somehow associated. Werthammer and colleagues published incidence rates of SIDS to be seven times higher in infants with BPD compared to the control group [52]. Later studies, however, could not detect an increased incidence of SIDS in preterm infants diagnosed with BPD [53]. Long-term studies in adult life indicate an association of BPD in early childhood with a decline in forced expiratory volume in one second (FEV_1) [54]. As FEV_1 is an important measure for chronic obstructive pulmonary disease (COPD) as well, reduced values might indicate susceptibility of infants suffering from BPD to develop COPD in later life. Since physiological changes such as alveolar loss and increased airway size are partly characteristics of both diseases, the hypothesized association has to be looked at with caution since physiological features of BPD and COPD overlap to some degree [55, 56]. Emphysema, a key characteristic of COPD also has been identified in young adults with a history of BPD. Computer tomography (CT) scans were employed as well as measurements of FEV_1 which is inversely linked to the extent of radiological emphysema [57, 58]. Even though the pathological changes described for childhood have the potential to reduce in severity in later adult life due to remodeling of the lung architecture, impairment in lung function objectively measured by reduced expiratory flow rates can still be appreciated [59, 50].

2.8 Current treatment options for bronchopulmonary dysplasia

2.8.1 Nasal continuous positive airway pressure

Nasel continuous positive airway pressure (nCPAP) is a non-invasive ventilatory support, which is a continuous airflow performed without intubation or mechanical ventilation, thus the risks of infection, barotrauma or volutrauma are extremely low. Rapid initiation of nCPAP especially for infants born at 25 to 28 weeks of gestation reduces the likelihood of later intubation, and the incidence rates of BPD and death [60]. Controversially, in a study comparing the outcome of early intubation or nCPAP in preterm infants, higher incidence rates of pneumothorax were seen in the group receiving nCPAP. This did not adversely affect infants even thought half of the infants that develop a pneumothorax subsequently underwent mechanical ventilation. Thus, application of nCPAP was still recommended [61]. Even though positive long-term effects can thus be appreciated, delivery room intubation rates

[62] for preterm infants with a GA below 28 could not be reduced by early application of nCPAP [63]. In summary, less interventional ventilation is linked to reduced incidence rates of BDP and lower death rates, suggesting the use of late intubation and early non-invasive ventilatory support such as nCPAP are favored in infants suffering from respiratory distress [64, 65, 66].

2.8.2 Nasal intermittent positive pressure ventilation

Nasal intermittent positive pressure ventilation (NIPPV) is another non-invasive ventilatory technique and has similar advantages than nCPAP over invasive options such has endotracheal tube ventilation [62]. No matter whether NIPPV or synchronized nasal intermittet positive pressure ventilation (SNIPPV) was used, both treatment options resulted in lower BPD rates compared to nCPAP given to preterm infants [67]. Even though several meta-analysis data favors NIPPV over nCPAP, further clinical trials have to be completed since investigated study groups still remain small and these data can only be partly compared since the time of administration of surfactant differed [66].

2.8.3 Surfactant

Exogenous surfactant, introduced twenty years ago into clinical practice, is nowadays routinely used in preterm infants at risk for BPD [68]. Surfactant was investigated in several clinical trials examining the prophylactic application of natural as well as synthetic exogenous surfactant therapy. Surfactant has proven to reduce mortality from BPD, but incidence rates of BPD could not be decreased with prophylactic treatment [20, 69, 19, 70]. The new generation protein-containing surfactant, also referred as lucinactant, could not reduce occurrence rates of RDS or BPD when applied prophylactically [71, 72, 73, 74].

2.8.4 Vitamin A

The importance of vitamin A for the development of several organs such as the genitals, kidneys, eyes and lung was described decades ago [75]. Further investigation on the specific role of vitamin A in lung development hinted that vitamin A deficiency might lead to impaired branching of the lobar units, leading to a decrease in normal lung function [76, 77]. A meta-analysis revealed vitamin A to be beneficial in the reduction of infant death, or the requirement of supplemental oxygen at one month of age. Further positive side effects of vitamin A did reveal reduced incidence of retinopathy of prematurity, another important disease of preterm born infants [78].

Effective incidence reduction rates of BPD were reported by combining antenatal vitamin A supplementation of mothers at risk for preterm infants, with continuing supplementation of vitamin A to newborns [79].

2.8.5 Caffeine

Even though caffeine (methylxanthine) is a well-known substance with a long history as a medical drug, routine application in neonatology is fairly new. A convenient advantage of the drug caffeine is the very wide therapeutic index where constant drug monitoring is not necessary [80]. Application of caffeine is dated back to 1977 [81], where respiratory outcome was positively influenced by methylxanthine application. Recent studies support these data where incidence rates of BPD could be reduced while survival rates improved under therapy. Application of the drug also demonstrated safety with respect to side effects such as neurological development, supporting the overall benefits of methylxanthine therapy for preterm infants at risk for BPD [82].

2.8.6 Steroids

Prenatal application of corticosteroids to mothers for suspected preterm birth are described as promising treatment options to reduce severity and occurrence of neonatal lung disease. Antenatal corticosteroid administration resulted in a reduction of RDS and neonatal death by 50% but incidence rates of RDS were not reported to be lower [83, 68]. Data from clinical trials are still rare and further investigations need to be performed [17, 84]. Late (>7 days) application of corticosteroids was assessed in a study described in the Cochrane Database. Side effects like adverse neurological outcome from corticosteroid therapy were identified, limiting the recommendation for steroids only to infants who cannot be weaned of from mechanical ventilation [85]. Early (<8 days) postnatal application of steroid did not reduce incidence rates of BPD either and adverse reactions such as abnormal neurological examination and cerebral palsy was more often seen, reducing the recommendation of for application in preterm born infants [86]. New options with inhaled corticosteroids were discussed to be advantageous over systemic application due to fewer side effects [87]. However, the efficiency of inhaled corticosteroids in children at risk for BPD could still not be proven until now since the seperate or combined outcomes of death or BPD could not be reduced [88]. Further studies are needed to evaluate the long terms risks and benefits of inhaled corticosteroids.

2.8.7 Inhaled nitric oxide

The physiological effects of nitric oxide (NO) administrationto to the lung were studied already twenty years ago. NO given to preterm infants had potentially positive effects on respiratory distress, and further investigation suggested especially the early use of minimal amounts of inhaled nitric oxide (iNO) to improve oxygen saturation levels and decrease the likelihood of developing severe hypoxemic respiratory failure [42, 57]. Some studies did report consistent saturation improvements and positive short-term effects under application of NO [89, 90, 91] while others still question the positive clinical outcome from iNO [92]. In summary, due to an absence of long term results and enormous treatment costs, the routine use of iNO is currently not recommended for preventive application of BPD [93].

2.9 Pathogenesis of bronchopulmonary dysplasia

The pathogenesis of BPD is multifactorial. Oxidative stress, fetal and postnatal infections, inflammation, antenatal steroids and nutritional support during early infancy can modulate severity and progression of the disease. These factors will now be considered in detail. Preterm infants especially in earlier days were ventilated with very high oxygen concentrations when suffering from respiratory distress [94]. Early approaches suspected the toxicity of oxygen free radicals to overwhelm the anti-oxidant defense of the host organism and thus be important for the development of the disease [95, 13]. After the toxicity of oxygen was identified, lower concentrations of supplemental oxygen were suggested and employed for ventilation [96]. But not only oxygen was identified to be damaging to the lung, recent studies showed that prolonged mechanical ventilation with physiological oxygen concentrations directly inhibited septation and angiogenesis with increasing apoptosis of the lung parenchyma. Thus, not only free oxygen radicals, but prolonged mechanical ventilation itself contributes to the development of BPD [97]. Brown and colleagues were the first to describe an association of BPD with the occurrence of a patent *ductus arteriosus* (PDA) [98]. This phenomenon was of interest in several studies, confirming that a PDA increases the risk for developing CLD [99, 100]. Gonzales *et al.* found an increased risk for the development of BPD in infants with a PDA in combination with a birth weight below 1000 g [101]. Increasing incidence rates of BPD were also seen in infants with a PDA and simultaneous comorbidities such as sepsis [98, 102].

Infections with several species such as cytomegalovirus (CMV), mycoplasma and ureaplasma spp. are suspected to predispose for the development of BPD. Several

studies showed incidence rates of BPD to be twice as high compared to non-infected infants [38, 103, 104]. Prenatal infections such as chorioamnionitis can contribute to disrupted alveolar development, partly mediated by bacterial endotoxin able to effect the fetus, leading to a decreased number of alveoli [105]. Several cytokines involved in this inflammatory process, such as interleukin (IL)-6, IL-8 and IL-1β, precede the neutrophil influx, and contribute to the inflammatory cascade. Hence, prenatal infections are linked to increasing incidence rates of BPD, supporting the inflammatory process to be crucial for development of BPD [106, 107, 108, 109].

Vitamin A is important for the development of the lung by stimulating alveolarization and reepithelialization, therefore, several studies aimed to test the therapeutic benefit of vitamin A supplementation. Tyson *et al.* and Darlow *et al.* reported reduced vitamin A deficiency with subsequent slightly decreased incidence rates of BPD in ELBWI receiving vitamin A supplementation [110]. Several studies aimed to find out the optimal amount of applied vitamin A to reduce incidence rates of BPD. A study from the NICHD recommended 5000 IU vitamin A three times per week for a maximum of four weeks via intramuscular injections. When vitamin A levels were studied in those infants receiving vitamin A, 25% remained with low vitamin A stores. Thus, later trials used higher doses while examining incidence rates of BPD. However, these new regimens tested were not able to diminish rates of BPD. Likewise, metabolites of vitamin A including RA await further investigation [111, 112, 110]. Interestingly, vitamin A is both important for proper immune system function as well as for the development of the lung. Patients suffering from inflammatory conditions exhibit low levels of vitamin A and increased levels of IL-1β [113]. Increased IL-1β levels caused downregulation of proteins involved in mediation of the cellular response to vitamin A in mice that are necessary for lung development. In sum, IL-1β was shown to disturb normal lung development by inhibiting the proper action of vitamin A [114]. Special nutritional regimens including vitamin A and inositol are important for the prevention and recovery of BPD. Significant reductions in the incidence and death from BPD were reported with oral administration of vitamin A, suggesting vitamin A supplementation even though evidence-based guidelines do not exist at this point of time [115, 116, 110].

Twin studies have revealed that the status of the first twin is a highly significant predictor for BPD for the second twin. Genetic factors could account for as much as 53% of liability for BPD [117]. Recent studies with interest in genetic susceptibility to BPD identified several genes contributing to the development of BPD. Several polymorphisms in genes such as angiotensin-converting enzyme [118] glutathione-S-transferase (GST) surfactant protein (SP)-A, SP-B and

tumor necrosis factor (TNF)-α-238G→A are able to modify incidence rates of BPD [119, 120, 121, 122, 123]. Surfactant is necessary for lung unfolding, thus surfactant deficiency contributes to the development of BPD. Surfactant genes such as SP-A, SP-B, SP-C and SP-D modulate surfactant levels. Genetic polymorphisms such as dominant mutations, deletions and allelic variations in these suspected genes have been shown to modulate incidence rates of RDS and BPD [124, 122, 123].

Many signaling and transcription factors are known to regulate lung morphology, among them thyroid transcription factor-1, β-catenin, GATA, SOX, and the ETS family members. Also proteins including FOX1, POD1, GLI and HOX, TGF–β, vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF) and granulocyte macrophage colony stimulating factor (GM-CSF) were shown to effect lung development and have been the aim of several studies [125, 126, 127, 128, 129, 130, 131, 132, 133]. VEGF is an invaluable stimulant necessary for vascular and alveolar development, involved in lung repair mechanisms, and suspected to be involved in the pathogenesis of BPD. Bronchoalveolar lavage (BAL) fluid analysis from infants suffering from BPD showed low levels of VEGF. Thus, low VEGF concentrations found in BAL from infants suffering from BPD could well reflect a mechanistic process of adverse lung development, even leading to the proposition to identify preterm infants at risk for BPD by measurement of VEGF levels [134, 135].

In recent years, the significance of growth factor cascades such as TGF–β have been investigated with increasing interest in disrupted lung development. It is already known that TGF–β is necessary for lung development including morphogenesis and epithelial differentiation and repair processes, since concentrations of TGF–β are physiologically elevated during development [136]. TGF–β is also involved in fibrotic changes in lung tissue, correlating with severity of lung function abnormalities [137]. In addition, it was shown that infants suffering from CLD had signs of inflammatory processes in their BAL with increased levels of cytokines such as IL-6 and IL-8, neutrophils and neutrophil elastase [138, 139, 140]. To study the role of TGF–β in inflammatory processes, BAL was performed in infants at risk for CLD. BAL analysis revealed increased concentrations of TGF–β. Thus it was hypothesized that dysregulation of the TGF–β machinery may contribute to disrupted lung development leading to BPD with characteristics such as abnormal alveolar structure and vascular development [106, 141, 142, 143]. Connective tissue growth factor (*ctgf*) is an important regulatory molecule for tissue development as well as a downstream mediator of the TGF–β signaling. The expression of CTGF is not only upregulated by TGF–β, but also by several factors contributing to the clinical picture of BPD, such as mechanical ventilation and oxygen [144]. Recent studies performed

in vivo and *in vitro* provided evidence that *ctgf* induces epithelial and endothelial cell dysfunction resulting in pathological changes to the lung structure [145, 146]. Another important target gene of the TGF–β machinery is the plasminogen activator inhibitor (PAI)-1, encoded by the *serpine1* gene. PAI-1 was analyzed in a study where samples of BAL were obtained from preterm infants at risk for BPD. Increased gene expression levels correlated with a more severe presentation of RDS. Thus, PAI-1 and *ctgf* seem to be important genes involved in the pathogenesis of BPD [147, 148].

2.10 Pathophysiology

Tachypnea, respiratory wheezing, shallow and paradoxical breathing characterizes the clinical sequelae of BPD. On thorax auscultation, rales, wheezes and coarse rhonchi can be appreciated [68]. The areas of the lung not participating in gas exchange are described as dead ventilatory space, located in the upper respiratory tract. Due to shallow breathing, the ventilatory dead space is more ventilated. In addition, damage to the airways (described in 2.9) results in ventilation of unperfused lung segments, worsening ventilation-perfusion matching. Edema, intestinal fibrosis, small airspace narrowing and atelectasis further damage the lung, leading to a decrease in lung compliance. The pathological features described all contribute to deteriorating oxygen saturations, an important hallmark for the diagnosis of BPD. Looking at follow-up pulmonary function tests in children who suffered from BDP, a typical course can be appreciated. First, pulmonary function is reduced in early stages, due to atelectasis. This is followed by gas trapping with subsequent hyperinflation. Even though this pattern is characteristic, it has to be interpreted with care since excessive chest wall distortion in patients with BPD my act as a confounder, leading to huge individual differences in lung volume [149, 150]. Pulmonary function tests are also used for long term follow-up, depicting substantial airway damage even in later years of childhood [59]. Structural changes to the lung vessels result in reduced diameter and subsequent increase of the pulmonary arterial pressure. In addition, the pulmonary arterial vessels present an abnormal vasoreactivity, contributing as well to pulmonary hypertension with consecutive development of *cor pulmonale* [149, 151]. Pulmonary hypertension, even though the pathogenic mechanisms are still not fully understood, somehow correlate with a more severe progression of BPD [152, 153, 154].

2.11 Vitamin A and its receptors

Vitamin A is involved in regulating numerous organ development steps of the lung, heart, urogenital tract as well cell apoptosis [155, 156]. Vitamin A (retinol) and vitamin A active derivatives (retinoids) can be classified according to biochemical structure. Retinoid derivatives include all-*trans* RA (ATRA), the 9-*cis* RA and the 13-*cis* RA (isotretinoin). It is believed that several actions from the 13-*cis* RA are mediated by ATRA, or possibly 9-*cis* RA after isomerization to these isoforms. However, 13-*cis* RA also directly mediates some biological response [157]. Two nuclear RA receptors, the retinoid acid receptor (RAR) [125] and the retinoid X receptor (RXR), each consist of three separate subtypes α, β and γ. RAR is able to bind all isoforms of RA whereas RXR only binds 9-cis RA stereoisomers. RAR's form heterodimers with the three RXR subtypes and RXR's form heterodimers with members of nuclear receptors, peroxisome proliferator activated receptor (PPAR) [158]. The PPAR consists the three isoforms α, β/δ and γ each having distinct ligand specificities. Gene transcription is then modulated by ligand-activated PPAR-RXR heterodimers binding to peroxisome proliferating response elements (PPREs). In sum, PPAR can act as activators and repressors in gene transcription also affecting other transcriptional factors with trans-repressions. The RA signaling pathway is crucial in numerous steps in organ developing with a highly complex interplay of the RXR, RAR and PPAR receptors [159].

2.12 TGF–β signaling

TGF–β signaling is involved in physiological and pathological processes such cell division, differentiation, migration, organization, adhesion, immune functions, extracellular matrix modeling (ECM), tumor invasion, angiogenesis, cell-death and apoptosis [160]. The TGF–β signaling encompasses ligand binding to the receptor, activation of the receptor and downstream signaling to the nucleus via Smad proteins [160, 161]. The pathway will now be described in detail.

2.12.1 The TGF–β signaling pathway

The current understanding of TGF–β signal transduction suggests the following course of events. First, an active TGF–β ligand binds to the serine/threonine kinase domain of the type II receptor. The TGF–β receptors type I is than phosphorylated in the GS domain by the type II receptor, leading to activation of the kinase with subsequent intracellular signaling to the nucleus. The cytoplasmic transport to the nucleus occurs via phosphorylation of mediators belonging to the Smad family.

Receptor-regulated Smad (R-Smad) proteins (further described in section 2.12.4) are directly activated by phosphorylation of the type I receptor kinases. The Co-Smads such as Smad4 form heteromeric complexes with the R-Smads. These complexes are translocated into the nucleus where they act as transcriptional factors, modulating gene response via transcription. This is done via direct interaction with DNA or in association with DNA binding elements. In humans, inhibitory (I)-Smads (Smad6 and Smad7) are also expressed which act as inhibitors of the TGF–β/bone morphogenetic proteins (BMPs) signaling pathways. Inhibition occurs through competition of the inhibitory Smads and the R-Smads for binding at the activated receptor [162, 163].

2.12.2 The TGF–β ligands

The TGF–β family consists of more than 30 members encompassing the TGF–β family as well as the BMPs, growth and differentiation factors, activins, inhibins, anti-Müllerian hormone (AMH), growth and differentiation factors (GDFs), nodal and others [161, 164]. TGF–β signaling is initiated by binding of one of the isoforms termed TGF–β_1, –β_2 and –β_3, all present in mammals, where TGF–β_1 is mostly present in the lung. However, prior to proper function of the three isoforms, biological processing is performed intracellularly and extracellularly. The precursor proteins, called latent associated (L)-TGF–β molecule contain a carboxy-terminal region also called latency-associated peptide (LAP) [114], and the amino-terminal region, serving as the potentially bioactive region. Activation is accomplished by either cleavage of the LAP region from L-TGF–β or by a conformational change in which LAP is not released but exposed to the TGF–β receptor binding site enabling activation of the TGF–β cascade [165].

2.12.3 The TGF–β receptors

TGF–β ligands bind to three different receptors, which are classified as type I (53 kDa) type II (73-95 kDa) and type III (110 kDa). The type I and the type II receptors contain a serine/threonine protein kinase, whereas the type III receptor lacks this domain. The type I receptor is also called TGF–βR1, or activin-like kinase (ALK)-5 or Acvrl1 (also called ALK-1) depending on the cell type. The type II receptor is sometimes also described as TGF–βR2 [166]. A unique feature of the type I receptor is the GS domain, named after the characteristic SGSGSG region. The GS domain serves as a key regulatory part, involved in controlling of the catalytic activity of the type I receptor. Signal transduction is initiated by binding of a ligand to the type II receptor. This complex causes phosphorylation

of the GS region with subsequent activation of the type I receptor kinase. The activated kinase then phosphorylates Smad proteins, mediating gene response from TGF–β ligand binding [167, 168]. TGF–β signaling is modulated by the type III receptor, consisting of Tgfbr3 (also called betayglycan) and endoglin. The type III receptor lacks any cytoplasmic serine/threonine kinase domain, thus leading to the assumption that the receptor is not directly involved in signal transduction but might control ligand binding to the receptor, since cells expressing betaglycan showed elevated binding of TGF–β [169].

2.12.4 The Smad proteins

Smad proteins function in downstream signal transduction to the nucleus when ligands bind to the TGF–β receptor. The name is a fusion of the two genes Mad *Drosophilia* mothers against decapentaplegic and *sma* genes in *Caenorhabditis* [170]. According to their function, the eight members of the Smad family are subdivided as followed:

- R-Smads, acting as regulatory substrates from the TGF–β family (Smad1, Smad2, Smad3, Smad5 and Smad8)
- common-Smads (co-Smads) Smad4 and Smad4–β serve as partners for the R-Smads
- I-Smads interfering with Smad-Smad and receptor interactions (Smad6 and Smad7)

The R-Smads can be further divided into two distinct groups:

- Smad2 and Smad3 as substrates of the TGF–β, nodal and activin receptors
- Smad1, Smad5 and Smad8 serve as substrates of the BMP and anti-Müllerian receptors

The Smad proteins consist of two Mad-Homology (MH) domains. MH1 is located at the N-terminal part, MH2 is located at the C-terminal end. When cytosolic R-Smads interact with their MH2 domain on the type I TGF–β receptor, the C-terminal serine residue becomes phosphorylated. The MH2 domains interact with the Co-Smads while forming heteromeric complexes. This complex is transported to the nucleus where interaction with DNA target genes controls gene transcription. The role of the I-Smads is by direct interaction with the type I receptor, regulating response to the R-Smads. The Co-Smads forming complexes with the R-Smads also have

the MH1 domains previously mentioned. Due to a different configuration at the C-terminus though, the Co-Smads cannot be activated by the type I receptor directly. Gene transcription is then initiated by binding of the MH domain from the R-and Co-Smads to specific Smad-binding elements (SBE) on the DNA. A single Smad protein interacting with one SBE has only very little affinity. Sufficient binding affinity is achieved by the oligomeric presentation of the Smad proteins, causing concomitant binding to several SBEs at the same time [171, 161]. Smad proteins, as mentioned in section 2.12.1 build complexes with the TGF–β receptors prior to realization of its nuclear effects. This Smad protein/TGF–β receptor interaction can be modified by several factors, such as the Smad anchor for receptor activation (SARA), Dok-1, Disabled-2 and several others. These factors are interesting due to their ability to modify TGF–β signaling by a varying affinity of the Smad proteins to the TGF–β receptor, showing that several molecules can interact with the TGF–β cascade [172, 161, 173].

To summerize, TGF–β signal transduction is initiated by binding of a TGF–β ligand to the TGF–β RII. The TGF–β RI recruits with the TGF–β RII, resulting in phosphorylation of the TGF–β RI. After phosphorylation of the TGF–β RI, phosphorylated Smad2 and phosphorylated Smad3 molecules dissociate from the receptor and complex with Smad4. The Smad2/3/4 complex translocates to the nucleus where gene transcription together with other cofactors is then modulated [163].

2.13 The role of TGF–β and vitamin A in bronchopulmonary dysplasia

The new BPD displays marked histopathological changes including capillary and vascular dysplasia, excessive elastin and collagen deposition, smooth muscle overgrowth and disrupting distal lung growth [37, 36]. After TGF–β and RA were identified to be both important for normal lung development [75, 174], several subsequent studies investigated a possible involvement of these molecules for the development of the disease BPD. The first insight that TGF–β signaling might be dysregulated during BPD was obtained when BAL was performed on infants suffering from BPD, and revealed increased concentrations of TGF–β [106]. Further investigation of the TGF–β signaling cascade was done by Vicencio *et al.* in 2002, showing in a transgenic mouse model that overexpression of TGF–β_1 resulted in disrupted alveolar development, resembling the histological picture of BPD. Another study done by Gauldi and coworkers, using a rat model, could produce supporting data that excess TGF–β during lung development impairs branching and morphogenesis of the

alveolar structure [175, 143]. A study done by Chen and colleagues compared lung morphology in Smad3 knockout mice with wild type mice. Smad3 is an important downstream protein, transducing TGF–β signals from the receptor to the nucleus [162]. Comparison of both groups showed retarded alveogenesis in neonatal mouse lungs followed by centrilobular emphysema in the Smad3 knockout mouse. Thus, impaired TGF–β signaling, here done via Smad3 knockout seems to impair proper lung development underlining the significance of TGF–β for proper alveolarization [176].

Other studies employed a hyperoxic mouse model to mimic a major contributing factor for the pathogenesis of BPD (please refer to section 2.9 for more details). The hyperoxic mice exhibited arrest in alveolar formation and septation while TGF–β signaling was up-regulated [177]. The inhibition of TGF–β signaling was successful performed by using TGF–β neutralizing antibodies by Nakanishi and colleagues. Dampening of TGF–β signaling was achieved with subsequent restoring of normal alveolar architecture [178]. The studies mentioned and numerous others investigating the pathogenesis of BPD in early and late lung development have led to TGF–β signaling being accredited with a key role in the process of alveolarization as well as in the maintenance of alveolar structure. In sum, both excess and a lack of TGF–β appears to affect proper lung development negatively, and dysregulation of the signaling cascade seems to modulate the development steps [179].

Vitamin A is known to alter numerous processes in lung development including maturation as well as lung repair after injury and to support the maintenance and integrity [180]. The crucial role of vitamin A for the respiratory system led to close investigation for a possible treatment option for distorted lung architecture. Massaro *et al.* investigated RA effects in rats, where elastase-induced emphysema was abrogated by treatment with RA [181]. RA was not only studied in the pathogenesis of emphysema, but as well in a hyperoxic mouse model, done by Nabeyrat and colleagues. In their model, TGF–β signaling was upregulated in the presence of RA, whereas toxic oxidants which induced growth arrest could be reduced by administration of RA [182]. ATRA was also studied in other cells lines such as human leukemia (HL)-60 cells where ATRA is known to be an inhibitor of cell differentiation and apoptosis. Combined treatment with TGF–β_1 and ATRA was able to inhibit the ATRA-induced apoptosis and increases cell viability [118]. Other studies in HL-60 cells reported ATRA to inhibit TGF–β_1 induced phospho-Smad2 and phospho-Smad3 levels and nuclear accumulation. These data showed evidence of a cross-talk between ATRA and TGF–β pathways [183]. In other studies using different cells, it was reported that RA was able to prevent TGF–β induced collagen

production by lung fibroblasts [184] and downregulation of TGF–β receptors type I and type II was reported in a rat model of glomerulonephritis [185]. In sum, RA has different effects on the TGF–β machinery depending on the cell type and the experimental setup. Until now, RA was accepted as central role in triggering alveolar development and maintaining alveolar structure. However, clinical trials using vitamin A could only demonstrate minimal beneficial effects of RA administration. A better understanding of the biological mechanisms including interactions with other signaling pathways, especially the TGF–β machinery which is a key player in the disease BPD, are wanted [186, 187].

3 Aims of the study

The TGF–β family is one of the most extensively studied growth factor families. Involvement of the TGF–β/BMP family members have been described in numerous processes, regulating a wide range of responses in differentiation, proliferation, adhesion, migration and cell apoptosis. Vitamin A and its biochemical derivatives are essential in several organ development steps, repair processes and have an increasing importance as therapeutic agents. TGF–β and vitamin A have been implicated not only in the development of the human lung but also in the onset and progression of the disease BPD. This study aims to describe crosstalk of ATRA with the TGF–β machinery in NIH/3T3 cells and MLE-12 cells, analyzed in genes known to be important in connective tissue remodeling contributing to the clinical picture BPD. In this context, the research focus was:

- Expression analysis of TGF–β responsive genes in MLE-12 and NIH/3T3 cells
- Effects of RA on the TGF–β canonical pathway

4 Materials and reagents

4.1 Materials

ABI PRISM 7500 Sequence Detection System	Applied Biosystems, USA
Cell Culture Incubator; Cytoperm2	Heraeus, Germany
Developing machine; X Omat 2000	Kodak, USA
Electrophoresis chambers	Bio-Rad, USA
Film cassette	Sigma-Aldrich, Germany
Filter Tip FT: 10, 20, 100, 200, 1000	Greiner Bio-One, Germany
Filter units 0.22 µm syringe-driven	Millipore, USA
Freezer -20 °C	Bosch, Germany
Freezer -40 °C	Kryotec, Germany
Freezer -80 °C	Heraeus, Germany
Fridge +4 °C	Bosch, Germany
Fusion A153601 Reader	Packard Bioscience, Germany
Gel blotting paper 70 × 100 mm	Bioscience, Germany
Glass bottles: 250, 500, 1000 ml	Fischer, Germany
GS-800™ Calibrated Densitometer	Bio-Rad, USA
Incubator for cell culture	Heraeus, Germany
Luminometer	Berthold Technologies GmbH, Germany
Mini spin centrifuge	Eppendorf, Germany
Miniox II monitor	Catalyst Research, USA
Multifuge centrifuge, 3 s-R	Heraeus, Germany
Multipette® plus	Eppendorf, Germany
Nanodrop®	Peqlab, Germany
PCR-thermocycler	MJ Research, USA
Petri dish	Greiner Bio-One, Germany
Pipetboy	Eppendorf, Germany
Pipetman: P10, P20, P100, P200, P1000	Gilson, France
Pipette tip: 200, 1000 µl	Sarstedt, Germany
Pipette tip: 10, 20, 100 µl	Gilson, USA
Power Supply; Power PAC 300	Bio-Rad, USA
Radiographic film X-Omat LS	Sigma-Aldrich, Germany
Radiographic film	Kodak, Germany
Serological pipette: 5, 10, 25, 50 ml	Falcon, USA
Test tubes: 15, 50 ml	Greiner Bio-One, Germany
Thermo-Fast® 96 PCR Plate	Thermo Scientific, USA

Tissue culture dish 100 mm	Greiner Bio-One, Germany
Tissue culture flask 250 ml	Greiner Bio-One, Germany
Tissue culture plates: 6, 96 well	Greiner Bio-One, Germany
Western Blot Chambers: Mini Trans-Blot	Bio-Rad, USA
Vortex machine	Eppendorf, Germany
Vacuum centrifuge	Eppendorf, Germany

4.2 Reagents

13-*cis* retinoic acid	Sigma-Aldrich, Germany
all-*trans*-retinoic acid	Sigma-Aldrich, Germany
Acetone pure	Merck, Germany
Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
Agarose	Invitrogen, UK
Albumine, bovine serum	Sigma-Aldrich, Germany
Ammonium persulfate	Promega, Germany
Bromophenol blue	Sigma-Aldrich, Germany
Cell lysis reagent	Promega, USA
Citrate buffer pH 6.0	Invitrogen, UK
Complete™ protease inhibitor cocktail	Roche, Germany
D-(+)-Glucose	Sigma-Aldrich, Germany
D-MEM + GlutaMAX™ -I (1×) medium	Gibco BRL, Germany
D-MEM/F12 + GlutaMAX™ -I (1×) medium	Gibco BRL, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Germany
DNA Ladder (100 bp, 1 kb)	Promega, USA
Dulbecco's phosphate buffered saline 10×	PAA Laboratories, Austria
Dulbecco's phosphate buffered saline 1×	PAA Laboratories, Austria
ECL Plus Western Blotting Detection System	Amersham Biosciences, UK
β-estradiol	Sigma-Aldrich, Germany
Ethanol absolute	Riedel-de-Haën, Germany
Ethidium bromide	Roth, Germany
Ethylendinitrilo-N,N,N′,N′,	Promega, USA
-tetra-acetic-acid (EDTA)	
Ethylene glycol-bis	Sigma-Aldrich, Germany
(2-amino-ethylether)-N,N,N′,N′	
-tetraacetic-acid (EGTA)	
Foetal calf serum (FCS)	PAA Laboratories, Austria
Gel extraction kit Promega, Germany	Roth, Germany

L-Glutamine 200 mM (100×)	PAA Laboratories, Austria
Glutathione Sepharose beads	GE Healthcare, Germany
Glycine	Merk, Germany
GoTaq® Flexi DNA polymerase	Promega, USA
Hydrocortisone	Sigma-Aldrich, Germany
Hydrochloric acid	Sigma-Aldrich, Germany
2-(4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate (HEPES)	Sigma-Aldrich, Germany
Igepal CA-630	Sigma-Aldrich, Germany
Insulin	Invitrogen, UK
Lipofectamine™ 2000	Invitrogen, UK
Luria-Bertani Medium	Invitrogen, UK
Magnesium chloride	Sigma-Aldrich, Germany
Methanol	Fluka, Germany
MuLV Reverse Transcriptase	Applied Biosystems, USA
N,N,N′,N′-tetramethyl-ethane-1,2-diamine (TEMED)	Bio-Rad, USA
Opti-MEM medium Gibco	BRL, Germany
PCR Nucleotide Mix	Promega, USA
Penicillin-streptomycin	PAA Laboratories, Austria
PeqGold total RNA kit	Peqlab, Germany
Potassium acetate	Sigma-Aldrich, Germany
Potassium chloride	Merck, Germany
Potassium phosphate	Sigma-Aldrich, Germany
Precision Plus Protein™ Standards	Bio-Rad, USA
2-Propanol	Merck, Germany
Pure Yield Plasmid Midiprep System	Promega, Germany
Quick Start™ Bradford Dye Reagent	Bio-Rad, USA
Random Hexamers (50 µM)	Applied Biosystems, USA
RNase inhibitor	Applied Biosystems, USA
RNaseZAP®	Sigma-Aldrich, Germany
Roti®-Quick-Kit	Roth, Germany
Select Agar	Invitrogen, UK
siRNA control (10 µM)	Ambion, Germany
Sodium acetate	Sigma-Aldrich, Germany
Sodium chloride	Merck, Germany
Sodium dodecyl sulfate (SDS)	Promega, USA

Sodium *ortho* vanadate	Sigma-Aldrich, Germany
Sodium phosphate	Sigma-Aldrich, Germany
Sulfo-NHS-LC-Biotin	Pierce Biotechnology, USA
SuperSignal® West Pico Chemiluminescent Substrate	Thermo Scientific, USA
Streptavidin Agarose Resin	Thermo Scientific, USA
SYBER® Green PCR Kit	Invitrogen, UK
T4 DNA ligase	Promega, Germany
Transferin	Invitrogen, UK
Transforming growth factor (TGF)–β_1	R&D Systems, USA
Tween 20	Sigma-Aldrich, Germany
Tris	Roth, Germany
Triton X-100	Promega, USA
Trypsin/EDTA	Gibco BRL, Germany

4.3 Software

Adobe Photoshop CS6®	Adobe Systems GmbH, Germany
Microsoft Excel®	Microsoft, USA
Microsoft Power Point®	Microsoft, USA
Microsoft Word®	Microsoft, USA
Graph Pad Prism 5®	GraphPad Software Inc., USA
Lyx 1.6.9©	Lyx Document Processor, USA
BibDesk 1.5.4©	BibDesk Bibliographer, USA

5 Methods

5.1 Cell lines

The NIH/3T3 mouse fibroblast-like cell line (CRL-1658™) and the mouse distal alveolar epithelial cell-derived MLE-12 cell line (CRL-2110™) were obtained from the American Type Culture Collection (ATCC, http://www.atcc.org).

5.2 Cultivation of cell lines

In this study the NIH/3T3 cell line was employed. These cells are mouse fibroblasts, which were first derived at the National institute of Health (NIH) Bethesda, USA. The NIH/3T3 cells were divided in three equal portions every third day with trypsin for further growth [188]. The medium used for cell culture in this study is described below. The second cell line used was the MLE-12 cell line. Kathryn A. Wikenheiser first established this cell line from pulmonary mouse tumors [189]. The medium employed is listed below and was mixed after recommendation from the American Type Culture Collection, (Catalog number CRL-2110). The cell lines were processed twice weekly by harvesting. The cell layer was washed with 1× PBS and directly removed via suction, followed by detaching the cells with trypsin. The cells were diluted with medium and seeded in a 1:3 ration in 75 cm^2 flasks. All cultures were maintained in an incubator (Heraeus) at 37 °C in a 95% humidified atmosphere with 5% CO_2 and 21% O_2. When the cells reached 70-90% confluence after 3 or 4 days, they were seeded for further experiments.

5.3 Cell line stimulation

The following table (table 7) depicts the experimental set-up employed in this study. For all experiments performed in this study, stimulation of NIH/3T3 cells and MLE-12 cells were initiated after cells reached a confluency of 70-90%. When not differently described, the reagents used for cell line stimulation were: TGF–β 0.2 ng/ml and ATRA 10 μM. The ATRA employed was diluted in DMSO (0.5%), thus, 0.5% DMSO served as a vehicle control.

Table 6: Medium for MLE-12 cells and NIH/3T3 cells

Medium for MLE-12 cells
D-MEM Dulbecco's medium
Insulin 0.005 mg/ml
Transferrin 0.01 mg/ml
Sodium selenite 30 nM
Hydrocortisone 10 nM
β-estradiol 10 nM
HEPES 10 mM
L-glutamine 2 mM (in addition to that in the base medium)
fetal calf serum 2%

Medium for NIH/3T3 cells
D-MEM Dulbecco's medium
fetal calf serum 10%

Table 7: NIH/3T3 cell and MLE-12 cell line stimulation

cells were grown until 70% confluency medium change was done, followed by stimulation for 12 h with			
DMSO	DMSO	ATRA	ATRA
↓	↓	↓	↓
medium change was done, followed by stimulation for 12 h with			
DMSO	DMSO + TGF–β	ATRA	ATRA + TGF–β
↓	↓	↓	↓
Harvesting and lysis was followed by processing cells for protein isolation, mRNA isolation and luciferase assay readout			

5.4 Plasmids employed in this study

The plasmids employed in this study fall into two separate groups: (i) expression plasmids, for the over-expression of molecules of interest in the cells under study, and (ii) reporter plasmids, where a luciferase-based system was used to quantify changes in the level of active TGF–β signaling in the cells under study.

(i) For the expression plasmid, the gene encoding Smad3 (NM_016769, 5090 bp mRNA) was kindly by provided by Dr. Wang, Duke University Medical Center Durham. The 1277-bp *Mus musculus smad3* open-reading frame was amplified using forward (5'-**GAA TTC** CAT GTC GTC CAT CCT GCC CTT C-3') and reverse (5'-**GAA TTC** CTA AGA CAC ACT GGA ACA GCG-3') primers containing built-in EcoRI sites (in bold), T/A cloned into pGEM-T Easy (Promega, appendix figure 12) and subcloned using EcoRI into pIRES-hrGFPII (Agilent, appendix figure 13). The resulting *pIRES::smad3* construct was over-expressed in cells of interest

by transient transfection under control of the SV40 promoter (appendix figure 14). The *pIRES::smad3* construct was validated by sequencing.

(ii) For reporter plasmids, the Smad-binding element (p(CAGA)$_9$) was employed along with the pGL3–basic (appendix figure 15) or pRL–TK (appendix figure 16) vectors, which served as normalization standards for the luciferase readout. In case of the pGL3–basic, this normalization standard represents the "empty" vector, carrying a promoterless firefly luciferase gene while the pRL–TK carries the *Renilla* luciferase control reporter vector and serves as an internal control value to which expression of the firefly luciferase reporter gene is normalized. In this study we used the (CAGA)$_9$-firely luciferase [p(CAGA)$_9$-luc] [190] construct, obtained from Dr. Daizo Koinuma, University of Tokyo.

These plasmids were transformed into competent *E. coli* cells. Thereby, the growth of the transfected bacteria increased the amount of transfected plasmids, respectively. The following steps, including plasmid maxi preparation was done to clean and increase the concentration of the DNA. To verify the plasmids, enzyme digestion with restriction endonucleases, followed by agarose gel-electrophoreses was performed. The plasmids were stored for later use at -20 °C.

5.5 Elution of DNA

The acquired plasmids were eluted in 100 µl Tris solution by incubation for 30 min at room temperature. For later experimental use, 2 µl of DNA solution were stored on ice, the leftover was stored at -20 °C.

5.6 Preparation of competent *E. coli* DH5α bacterial cells

The competent *E. coli* DH5α bacterial cells used for transformation of the plasmids and later maxipreparation were produced after the protocol from Hanahan [191]. Competent cells were stored in the -80 °C freezer for later use.

5.7 Transformation of plasmid DNA into competent *E. coli* cells

5 µl of plasmid DNA were mixed with 80 µl of competent *E. coli* cells slowly thawed on ice. After incubation on ice for 30 min, a heat shock for 45 s at 42 °C was performed, allowing the plasmid to enter the bacteria. Cells were cooled down on ice for 5 min before 900 µl of LB medium was added and the culture was further incubated for 1.5 h at 37 °C with gentle shaking.

5.8 Plating and cultivation of *E. coli* cells

An aliquot of transfected *E. coli* cells were spread on LB-agar plates, containing the appropriate antibiotic for the construct used, and incubated at 37 °C for 8–15 h. On the following day, only antibiotic-resistant colonies were grown on the LB plates. One colony was selected from the plate and transferred to liquid culture. The selected *E. coli* colony was added to 500 ml LB media containing the appropriate antibiotic to suppress non antibiotic resistant bacteria. For bacterial growth, the culture was shaken at 37 °C for 8-16 h. The medium was then ready to use for maxi preparation.

Table 8: *E. coli* cultivation components

LB Medium (Luria-Bertani Medium)
1% (m/v) bacto tryptone
0.5% (m/v) bacto yeast extract
1% (m/v) NaCl
1.5% (m/v) agar*

Additives
50 µg/ml ampicillin
30 µg/ml kanamycin
20 µg/ml X-gal
0.1 mM isopropyl-β-thiogalactopyranoside (IPTG)

*only for plate preparation

5.9 Plasmid maxi preparation

According to the manufacture's instructions from Pure Yield™ Plasmid Midiprep System from Promega, plasmid purification was performed. All steps were done at room temperature, as advised in the protocol. The liquid LB medium containing the propagated transfected *E. coli* bacterial was distributed into Falcon tubes. The tubes were centrifuged for 10 min at 4500 g. The lysate was poured into clearing columns containing a filter and incubated for 2 min. To fully clean the lysate, centrifugation of the columns was done twice for 5 min with 1500 g. The filtered lysate was poured through a binding column and centrifuged for 3 min at 1500 g. To clear the lysate from RNA, endotoxin, endonucleases and protein, was washed with 5.0 ml of endotoxin removal wash solution, provided from the kit and centrifuged at 1500 g for 3 min. The lysate was poured into DNA binding columns, washed with 20 ml wash solution containing alcohol and centrifuged at 1500 g for 5 min and again for 10 min with 1500 g to ensure complete removal of ethanol. The eluate was proceeded to DNA concentration measurement, described in section 4.10.2. To verify the plasmids, vector digestion was done. Plasmids were verified by sequencing.

5.10 Vector digestion using DNA restriction endonucleases for plasmid verification

For the verification of the plasmids, restriction endonucleases were employed. In this study, the following restriction endonucleases with the recognition and cleavage sites shown in brackets were employed: SalI (G↓TCGAC), XhoI (C↓TCGAG), NotI (GC↓GG
CCGC). These nucleases recognize short DNA sequences and cut DNA by digestion of phosphodiester bonds in-between nucleotides. The enzymes are able to recognize specific palindromic sequences on the DNA strand and therefore cut DNA bindings very specific. The cut DNA strands are short base pair DNA sequences that are compatible and able to be ligated. This property is also described as 'sticky ends'. DNA digestion was performed at 37 °C for 2 h using the following reaction mix.

Table 9: Vector digestion components. Abbreviations: EDTA, Ethylendinitrilo-N,N,N′,N′-tetra-acetic-acid.

Components	Amount
10× restriction endonuclease buffers	2 µl
Restriction endonuclease	1 to 5 U/µg DNA
DNA sample in H_2O or Tris-EDTA-buffer	0.1 to 4 µg
Autoclaved, deionized water	up to 20 µl

5.11 Luciferase assay

The luciferase assay system is an extremely sensitive technique for quantitative measurement of gene activity in cells, represented by different levels of luminescence activity of the promoter-tagged luciferase gene. A further advantage of this readout assay is its linear result over at least eight orders of magnitude of enzyme concentration. The light emitted from the luciferase assay is stable for at least one minute and detectable with a luminometer, in our case the Fusion A Reader (Packard Bioscience, Germany) was employed.

5.12 Transient transfection using Lipofectamine™ 2000

Either NIH/3T3 cells or MLE-12 cells were grown for 24 h on a 96-well dish until 60% confluence. On the following day, the transfection process was performed. OptiMEM (GIBCO, 31985) was first added to a suspension containing the firefly luciferase

plasmid construct p(CAGA)$_9$-luc and the *Renilla* luciferase control reporter pRL-SV40, to form a total volume of 50 µl. This suspension was mixed carefully and left to incubate for 15 min at room temperature. Then, 49.25 µl of OptiMEM were added to 0.75 µl of lipofectamine (Lipofectamine 2000™, 1 mg/ml, Invitrogen P/N 52887) and also left to incubate for 15 min at room temperature. These two mixtures were then added together to form a total volume of 100 µl (amount/well), mixed carefully and left to incubate for another 20 min at room temperature. Media was then removed from each well and cells were washed with ice cold 1× PBS solution. After removing the 1× PBS solution from each well, 100 µl of the transfection mixture was pipetted into each well and left to incubate for 5 h at 37 °C, 5% CO_2, and 95-100% humidity. Depending on the experiment, cells were also exposed to Opti-MEM or Lipofectamine™ 2000 only, transfected with the constitutively active *Renilla* luciferase plasmid pRL-SV40 for luminescence-based dual luciferase assay (DLR) or the promoterless firefly luciferase gene pGL3-basic to control the transfection process.

5.13 NIH/3T3 cell line transfection with small interfering RNA

The NIH/3T3 cells were trypsinized and plated in growth medium for approximately 16 h to obtain 60-70% confluency prior to transfection. Specific small interfering RNA (siRNA) oligonucleotides against mouse *smad3* (obtained from Santa Cruz Biotechnology, USA) as well as silencer® negative control siRNA (Ambion, Germany) were used in this study. NIH/3T3 cells were transiently transfected with 200 nM of specific *smad3* siRNA as well as non specific siRNA to serve as a negative control for siRNA-mediated ablation of the according gene mRNA expression. Lipofectamine™ 2000 was added to OptiMEM and incubated for 5 min while siRNA was combined with OptiMEM before mixing both solutions. The mix was incubated for another 15 min at room temperature. The siRNA-transfection reagent complexes were added to the cells. After this last incubation period, a certain amount of this mixture was then added directly to cell medium (see section 5.12). Following transfection, cells were used for further experiments (section 5.15 and table 7). Luciferase assay readout was done and efficiency of knockdown was monitored by Western blot analysis.

5.14 Transfection of cells with overexpression plasmid constructs

The gene encoding *smad3* (provided by Dr. Wang, Duke University Medical Center Durham) was over-expressed in cells of interest by transient transfection of the plasmid carrying this gene. Cells were transfected with overexpression plasmids (section 5.4) using lipofectamine™ 2000 and OptiMEM. In order to control these experiments, cells were also treated with empty pIRES hrGFPII pcDNA plasmids to serve as a negative control. First, OptiMEM was added to the plasmid construct to form a total volume of 50 µl. This suspension was mixed carefully and left to incubate for 15 min. Then, 49.25 µl of OptiMEM were also added to 0.75 µl of lipofectamine™ 2000 and also left to incubate for 15 minutes at room temperature. These two mixtures were then added together to form a total volume of 100 µl (amount/well), mixed carefully and left to incubate for another 20 min at room temperature. Media was then removed from each well and cells were washed with ice cold 1× PBS solution. After removing 1× PBS solution from each well, 100 µl of transfection mixture was pipetted into each well and left to incubate for 5 h at 37°C, 5% CO_2, and 95-100% humidity. After 5 h of incubation, transfection mixture was removed from each well and cells were stimulated according to protocol (see sections 5.15 and 5.26).

5.15 Luciferase assay

The experimental setting in this study was applied to both MLE-12 cells and NIH/3T3 cells. The cells were grown on 96-well plates in the incubator. After 24 h, upon reaching 60-70% confluence, the transfection was performed as described in section 5.11. Following transfection, the cells were stimulated for 2× 12 h with different media: TGF–β (R&D Systems, USA) and ATRA (Sigma-Aldrich, Germany R2625). The first stimulation was done for 12 h as follows:

Medium alone, or
Medium plus ATRA (10 µM)

After 12 h, a medium change was performed by gently removing the medium via aspiration. New medium was then added to the wells in the setting as described below for again 12 h.

Medium alone, or
Medium plus TGF–β (0.2 ng/ml), or
Medium plus TGF–β (0.2 ng/ml) plus ATRA (10 µM), or
Medium plus ATRA (10 µM)

After stimulation, the plates were taken out of the incubator and washed three times with Dulbecco's phosphate-buffered saline (PBS). The PBS was removed via aspiration and the plates were then either directly subjected to cells lysis followed by analysis, or stored in the -80 °C freezer for later use.

5.16 Protocol for cell lysis using cell culture lysis 5× reagent

Prior to use, the cell lysis buffer was equilibrated to room temperature. Four volumes of purified water were added to one volume of cell culture lysis 5× reagent (Promega, E 194 A). For the preparation of the 96-well dishes for readout the growth medium was removed by suction and each well was washed with 1× PBS solution. 100 µl of the 1× cell lysis reagent was added to the wells. The plates were placed on a rocking motion shaker, ensuring complete lysis of cells.

5.17 Protocol for luciferase measurement

When the cells were completely lysed, 40 µl of the cell lysate was transferred from each well of the 96-well plates into a different 96-well plate designed for luciferase analysis. Finally, 50 µl of a fluorescent luciferase reagent (Promega, USA) was added to each well in a strictly defined order. The flourescence which is emitted during the enzymatic reaction was measured by a microplate luminometer (Berthold Technologies GmbH & Co. KG, Germany). The result was expressed as an absolute value and transferred to an excel file. All samples were measured as a quadruplicate of 4 and their mean value was calculated.

5.18 RNA isolation, cDNA synthesis and PCR

RNA isolation

Isolation of RNA from cultured cells material was performed according to the manufacturer's instructions provided with Roti® Quick-Kit.

Determining RNA concentration

The concentration of isolated RNA was determined according to a protocol from Peqlab by applying 1.5 µl of the sample to a Nanodrop® spectrophotometer.

Reverse transcriptase polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) is an enzymatic process carried out by reverse transcriptase, which synthesizes complementary (c) DNA using RNA as a template. In order to perform RT-PCR, 500 ng of mouse total RNA was added to autoclaved water up to 10 µl of total volume. The reaction mixture was heated to 70 °C for 15 min and chilled on ice. Finally, the following RT reagents were added:

Table 10: Reverse transcriptase reaction components Abbreviations: RT, reverse transcriptase; dNTP, deoxynucleotide triphosphate; RNA, ribonucleic acid.

RT reaction component	Volume	Final concentration
10× RT Buffer II ($MgCl_2$ free)	2 µl	1×
25 mM $MgCl_2$	4 µl	5 mM
10 mM dNTP mix	1 µl	0.5 µM
Random hexamers (50 µM)	1.5 µl	3.75 µM
RNAse inhibitor (20 U/µl)	0.5 µl	10 U
Reverse transcriptase (50 U/µl)	1 µl	50 U
RNAse free water	variable	

For the amplification of cDNA, the reaction mixture was incubated at 20 °C for 10 min, then at 43 °C for 75 min and at 99 °C for 5 min. Synthesized cDNA was stored either at -20 °C or used for other experiments immediately.

5.19 Polymerase chain reaction

The polymerase chain reaction (PCR) is a method that allows million-fold amplification of DNA segments. The enzyme DNA polymerase amplificates the segments using cDNA, previously reverse-transcribed from RNA. Each PCR cycle consists of three steps:

Denaturation:	separation of double-stranded DNA into single strands
Annealing:	primer binding to the target sequence of single DNA strands
Elongation:	amplification of a sequence of interest by DNA polymerase

5.20 Semi-quantitative reverse transcription polymerase chain reaction

For a semi-quantitative RT-PCR, amplification of DNA was performed according to the manufacturer's instructions provided with the GoTaq® Flexi DNA Polymerase. The following components were combined in a 0.5 ml microcentrifuge tube, on ice.

Table 11: Semi-quantitative PCR reaction components Abbreviations: PCR, polymerase chain reaction; dNTP, deoxynucleotide triphosphate; DNA, deoxyribose nucleic acid.

PCR reaction component	Volume	Final concentration
5× PCR Buffer (free $MgCl_2$)	10 µl	1×
25 mM $MgCl_2$	5 µl	2.5 mM
10 mM dNTP mix	1 µl	0.2 µM
10 µM forward primer*	1 µl	0.2 µM
10 µM reverse primer*	1 µl	0.2 µM
Taq DNA Polymerase (5u/µl)	0.25 µl	1.25 U
cDNA template	1 µl	not applicable
H_2O (autoclaved)	up to 50 µl	not applicable

* All primer sequences are listed in table 20.

To perform effective amplification of DNA, the following program was run:

Table 12: Semi-quantitative reverse transcription polymerse chain reaction cylces

Step	Time	Temperature
First denaturation	5 min	95 °C
Second denaturation	1 min	95 °C
Annealing	0.5-1 min	57-60 °C
Elongation	1 min	72 °C
Final extension	10 min	72 °C

The steps were repeated for 22-33 cycles depending on the amplified sequence please refer to table 20, for the exact cycle number used. Newly generated DNA was immediately separated by agarose gel electrophoresis and visualized by staining with ethidium bromide.

5.21 Real-time reverse transcription polymerase chain reaction

Simultaneous quantification and amplification of specific DNA sequences can be realized by using quantitative real-time RT-PCR. The procedure follows the PCR strategy but the DNA is quantified after each amplification cycle. Quantification is performed by means of fluorescent dye–SYBR® Green I – which directly binds to double-stranded DNA. The attached dye generates a signal that is proportional to the DNA concentration. Reactions were performed according to the manufacturer's instructions provided with a SYBR® Green PCR Kit. PCR reaction mix was prepared as follows:

Table 13: Real-time reverse transcription polymerase chain reaction components Abbreviations: PCR, polymerase chain reaction; DNA, deoxyribose nucleic acid.

Polymerase chain reaction component	Volume	Final concentration
Platinum®Sybr®Green qPCR SuperMix-UDG	13 µl	1×
50 mM $MgCl_2$	1 µl	2 mM
10 µM forward primer*	0.5 µl	0.2 µM
10 µM reverse primer*	0.5 µl	0.2 µM
cDNA template	2 µl	not applicable
H_2O (autoclaved)	up to 25 µl	not applicable

The amplification and quantification of cDNA was carried out by means of following program:

Table 14: Real-time reverse transcription polymerase chain reaction cycles

Step	Time	Temperature
Activation of polymerase enzyme	2 min	50 °C
First denaturation	5 min	95 °C
Second denaturation	5 s	95 °C
Annealing	5 s	59-60 °C
Elongation	30 s	72 °C
Dissociation step 1	15 s	95 °C
Dissociation step 2	1 min	60 °C
Dissociation step 3	15 s	95 °C
Dissociation step 4	15 s	60 °C

The steps were repeated for 45 cycles. A ubiquitously and equally-expressed gene that is free of pseudo genes was used as the reference gene in all quantitative real-

time RT-PCR reactions. The relative transcript abundance of a gene was presented as ΔCT values (ΔCT = CT reference – CT target). Relative changes in transcript levels compared to controls were displayed as ΔΔCT values (ΔΔCT = ΔCT treated – ΔCT control).

5.22 DNA agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate and analyze nucleotide acid fragments obtained by PCR. Depending on the size of the DNA amplicon 1-2% agarose gels were used. Agarose gels contained 0.5 μg/ml ethidium bromide, prepared in 1× tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA, pH 8.0). The components were heated to allow agarose to melt and after adding ethidium bromide (a fluorescent intercalating dye that enables visualization of the DNA fragments under ultraviolet light), the gel solution was poured in a casting frame provided with a comb for the wells. The DNA samples along with the loading dye (0.01% bromphenol blue, 40% glycerol, 1× TAE buffer) were loaded into the gel wells and electrophoresis was performed at 5 V/cm in 1× TAE buffer for 45-60 min. Finally, the electrophoresis result was illuminated with short wavelength ultraviolet light (λ=254 nm), and photographed with Kodak camera connected to analyzing software. The size of the DNA fragments was determined by a DNA molecular weight standard marker.

5.23 Protein isolation

The protein isolation from NIH/3T3 cells and MLE-12 cells was equally performed by the following protocol:

After washing twice with PBS, confluent monolayers of cells were detached by scraping of the layers, which were previously covered with cell lysis buffer. Cell lysis buffer consisted of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Igepal CA-630 and 1 mM Ethylene glycol-bis (2-amino-ethylether)-N,N,N′,N′-tetraacetic-acid (EGTA). Collected cells were passed 5–8 times through a 0.9 mm gauge needle to obtain a homogenous lysate. Lysates were then incubated for 30 min on ice and centrifuged 15000× g for 15 min at 4 °C. Resulting supernatants were used as cell extracts and stored at -20 °C for further experiments. In addition, 1 mM Na_3VO_4, phosphatase inhibitor was added immediately prior to homogenization Complete™, protease inhibitor mix – added immediately prior to homogenization.

Table 15: Loading-buffer ingredients. Abbreviations: SDS, sodium dodecyl sulphate.

625 mM Tris-HCl, pH = 6.8
50% (v/v) glycerol
20% (w/v) SDS
9% (v/v) β-mercaptoethanol
0.3% (w/v) bromophenol blue

5.24 Protein quantification measurement

Protein concentrations in cell extracts were spectrophotometrically determined using Quick Start™ Bradford Dye Reagent and a Fusion A153601 Reader according to the manufacturer's instructions. The protein assay is based on the color change of Coomassie Brilliant Blue G-250 dye after binding proteins. The dye binds primarily to basic and aromatic amino acids residues. Ten µl of sample was mixed with 200 µl of Bradford Dye Reagent and transferred to a 96-well plate. Six dilutions of protein standard, bovine serum albumin, 0.05–0.5 µg/µl, were prepared and mixed with Bradford Dye Reagent in the same ratio as the sample of unknown concentration. Reaction mixtures were incubated for 15 min at room temperature. The absorbance of the samples was measured at 570 nm. The unknown amount of protein in the sample was determined by interpolation, reading the concentration of protein on the standard curve that corresponded to its absorbance.

5.25 Separation of proteins by SDS poly-acrylamide gel electrophoresis

For the analytical separation of proteins, extracts were separated with the SDS poly-acrylamide gel electrophoresis (SDS-PAGE). Before loading, 20 µg of protein were mixed with 10× SDS-loading buffer and denaturated by heating for 5 min at 95 °C. The separation of proteins was performed in gels consisting of 10% stacking gel and 10% resolving gel. The separation gel mix was poured between two glasses with space holders and the gel was allowed to polymerase. After polymerization, the stacking gel was poured of top of the separating gel and a comb was inserted to form the wells. Electrophoresis was carried out in SDS-running buffer at 120 V.

Table 16: Stacking gel (for 20 ml). Abbreviations: APS, ammonium persulphate; SDS, sodium dodecyl sulphate; TEMED, N,N,N′,N′-tetramethyl-ethane-1,2-diamine.

Component	Volume
dH_2O	13.6 ml
30% Acrylamide	3.32 ml
1.5 M Tris-HCl, pH 6.8	2.25 ml
10% SDS	200 µl
10% APS	200 µl
TEMED	20 µl

Table 17: Resolving gel (for 40 ml). Abbreviations: APS, ammonium persulphate; SDS, sodium dodecyl sulphate; TEMED, N,N,N′,N′-tetramethyl-ethane-1,2-diamine.

Component	Volume
dH_2O	15.9 ml
30% Acrylamide	13.3 ml
1.5 M Tris-HCl, pH 8.8	10 ml
10% SDS	400 µl
10% APS	400 µl
TEMD	16 µl

5.26 Western blotting

Proteins separated by SDS-PAGE were transferred to 0.25 µm nitrocellulose membrane using Bio-Rad transfer chambers. Transfer was performed in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 120 V for 1 h.

For protein detection, membranes were blocked in blocking solution (5% non-fat dry milk in PBS and 0.1% (vol/vol) Tween-20) for 1 h at room temperature and incubated overnight at 4 °C with the desired primary antibody. Primary antibody concentration varied depending on the antibodies used in the experiment and are represented in table 18. After transfer of the antibody, membranes were washed three times for 10 min with 1× Phosphate-buffered saline + 0.1% Tween 20 (PBST) buffer, incubated with horseradish peroxidase-labeled secondary antibody (Pierce Biotechnology, Rockford IL) for 1 h at room temperature, and then washed five times for 10 min with 1× PBST each.

To re-probe the membranes with another antibody, the blots were stripped with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM β-mercaptoethanol) for 15 min at 52 °C at and then probed again with a new antibody as described previously. The detection of specific bands was performed by using the Enhanced Chemiluminescence Immunoblotting System Plus reagent (Amersham Biosciences,

Table 18: Primary antibodies employed in Western blot analysis

Primary Antibodies	Host	Dilution	Company
Phospho-Smad2	mouse	1:1000	Cell Signaling
Smad2/3	mouse	1:1000	Cell Signaling
Phospho-Smad3	rabbit	1:1000	Cell Signaling
Lamin	rabbit	1:10000	Cell Signaling
β-Actin	rabbit	1:1000	Cell Signaling

Table 19: Secondary antibodies employed in Western blot analysis

Secondary Antibodies	Host	Dilution	Company
HRP-conjugated anti-mouse IgG	goat	1:3000	ZyMax
HRP-conjugated anti-rabbit IgG	goat	1:3000	ZyMax

Uppsala, Sweden) on a radiographic film (Kodak). In order to re-probe membranes with another antibody, membranes were stripped with stripping buffer for 15 min and subsequent protein detection was performed as described above. Primary antibody concentrations varied depending on the antibodies used in the experiment, and are presented in table 18. Densitometric analysis of immunoblot bands was performed using the Multi Gauge MFC Application version 3.0.0.0.

5.27 Statistical analysis of data

Values are indicated as mean ± S.D. Statistical comparisons were made between two samples with an unpaired Student´s *t*-test, or by one way ANOVA followed by a Bonferroni *post-hoc* test (for more than two samples) or Dunnett´s test for multi-one-comparisons, to evaluate changes between mean values. All experiments were performed at least three times. *P*-values of $p<0.05$ shown as *, $p<0.001$ shown as ** or $p<0.0001$ shown as *** were considered statistically significant, depending on the experiment.

5.28 Primer table

Table 20: Primer employed for semi-quantitative RT-PCR

GenBank™ Accession Number	Forward Primer 5´-3´	Reverse Primer 5´-3´	Amplicon size bp	Cycle Number	Annealing temp. (°C)
acvrl1 NM_009612.2	ATGACCTTGGGGAG CTTCAG	GAGGACCGGATCT GCAGCCAG	1380	23	60
gapdh NM_008084.3	ACCCAGAAGACTGT GGATGG	TGTGAGGGAGATGC TCAGTG	548	21	60
endoglin NM_001146348.1	GAGTCGGCTGTGAT CTACAGCCTGTGG	CTGATGATCACCTC ATTGCTGACC	898	22	60
tgfbr1 NM_009370.2	CTGTGAGACAGATG GTCTTTGC	CATCACTCTCAAGG CCTCAC	1273	27, 29	60
tgfbr2 NM_009371.2	CCAAGTCGGATGTG GAAATGGAA	CTATTTGGTAGTGT TCAGCGAGCCATC	1694	25	60
tgfbr3 NM_011578.3	CCCTGTGTTTGTCCT GATGAGCGCCTGCC	CCTATGTCTAGTAC CACAGCCATTC	1501	27	60
serpine1 NM_008871	ATGCAGATGTCTTC AGCCCTTG	CTCTGAGGTCCACT TCAGTC	931	25	60
ctgf NM_010217.2	ATGCTCGCCTCCGT CGCAGGTCCC	TTACGCCATGTCTC CGTACATCTT	1047	27	62
smad2 NM_010754.3	CTCCGGCTGAACTG TCTCCTACT	TTACAGCCTGGTGG GATCTTACA	405	26	60
smad3 NM_016769.4	GAATTACGGGCCAT GGAGCTCTGT	TCGGGAATGGAATG GCTGTAGTCA	182	27	69
smad4 NM_008540.2	ACAGTGTCTGTGTG AATCCA	TCAGTCTAAAGGCT GTGGGTCC	1286	25	60

Table 21: Primer employed for real time RT-PCR

GenBank™ Accession Number	Forward Primer 5´-3´	Reverse Primer 5´-3´	Amplicon size bp	Amplicon Region	Annealing temp. (°C)
ctgf NM_010217.2	AATGCTGCAAGGAG TGGTGTGTG	AGGCAGTTGGCTCG CATCATAG	124	757-880	60
tgfbr3 NM_011578.2	ATGGCAGTGACATC CCACCACAT	AGAACGGTGAAGCT CTCCATCA	152	1-152	60
gapdh NM_008084.3	ATGGTGAAGGTCGG TGTGAAC	TCATACTGGAACAT GTAGACC	143	50-210	60
endoglin NM_001146348.1	CGTGCTACTCATGT CCCTGAT	CAGGACAAGATGGT CGTCAGT	162	1052-1214	60
smad3 NM_016769.4	CTGGATGACTACAG CCATTCCATT	CTGTGGTTCATCTG GTGGTCACTG	140	456-596	60
smad4 NM_008540.2	TCATCCTAGCAAGT GTGTCACC	CTCCACAGACGGGC ATAGAT	101	603-704	60
tgfbr1 NM_09370.2	CAGAGGGCACCACC TTAAA	AATGGTCCTGGAAG TTC	110	502-622	60

6 Results

6.1 ATRA enhances TGF–β signaling assessed by p(CAGA)$_9$ induction

Activation of the TGF–β responsive Smad3 binding element p(CAGA)$_9$ was measured in this study by using a DLR. The firefly luciferase p(CAGA)$_9$–luc construct was employed to assess activation of the TGF–β machinery by ATRA (or 13-*cis* RA) using DLR. The NIH/3T3 cells were transfected with the p(CAGA)$_9$–luc reporter construct and expression levels were assessed using DLR as described in section 5.11. First, NIH/3T3 cells were stimulated with ATRA (0, 0.01, 0.1, 1, 10 µM) or 13-*cis* RA (10 µM) for 12 h. This was followed by co-stimulation with TGF–β (0.2 ng/ml) and ATRA or 13-*cis* RA for a further 12 h. TGF–β activated the p(CAGA)$_9$ element of the p(CAGA)$_9$–luc construct, and this activation was significantly enhanced by ATRA in a dose-dependent manner (figure 1 A). All-*trans*-retinoic acid and 13-*cis* RA alone were not able to increase activation of the p(CAGA)$_9$–luc construct in NIH/3T3 cells (figure 1 B).

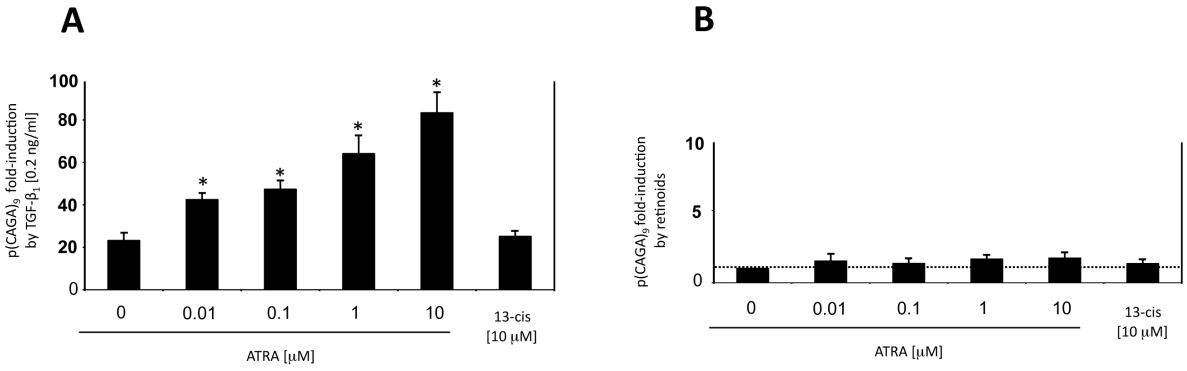

Figure 1: **ATRA enhances TGF–β signaling in NIH/3T3 cells.** Expression analysis of the p(CAGA)$_9$-luc construct in NIH/3T3 cells after stimulation with ATRA (0, 0.01, 0.1, 1, 10 µM), 13-*cis* RA (10 µM), and TGF–β (0.2 ng/ml), assessed by dual-luciferase promoter assay. NIH/3T3 cells were stimulated with ATRA or 13-*cis* RA for 12 h and subsequently exposed to TGF–β (A). In panel (B) NIH/3T3 cells were exposed to ATRA or 13-*cis* RA only. All values were normalized for firefly luciferase transcriptional acitivity using the pGL3-basic control construct. The values used are representative for at least three independent experiments. Data indicate mean from quadruplicates ± S.D. Statistically significant differences ($P<0.05$; by ANOVA followed by Dunnett´s test for multi-one-comparisons) are marked by *.

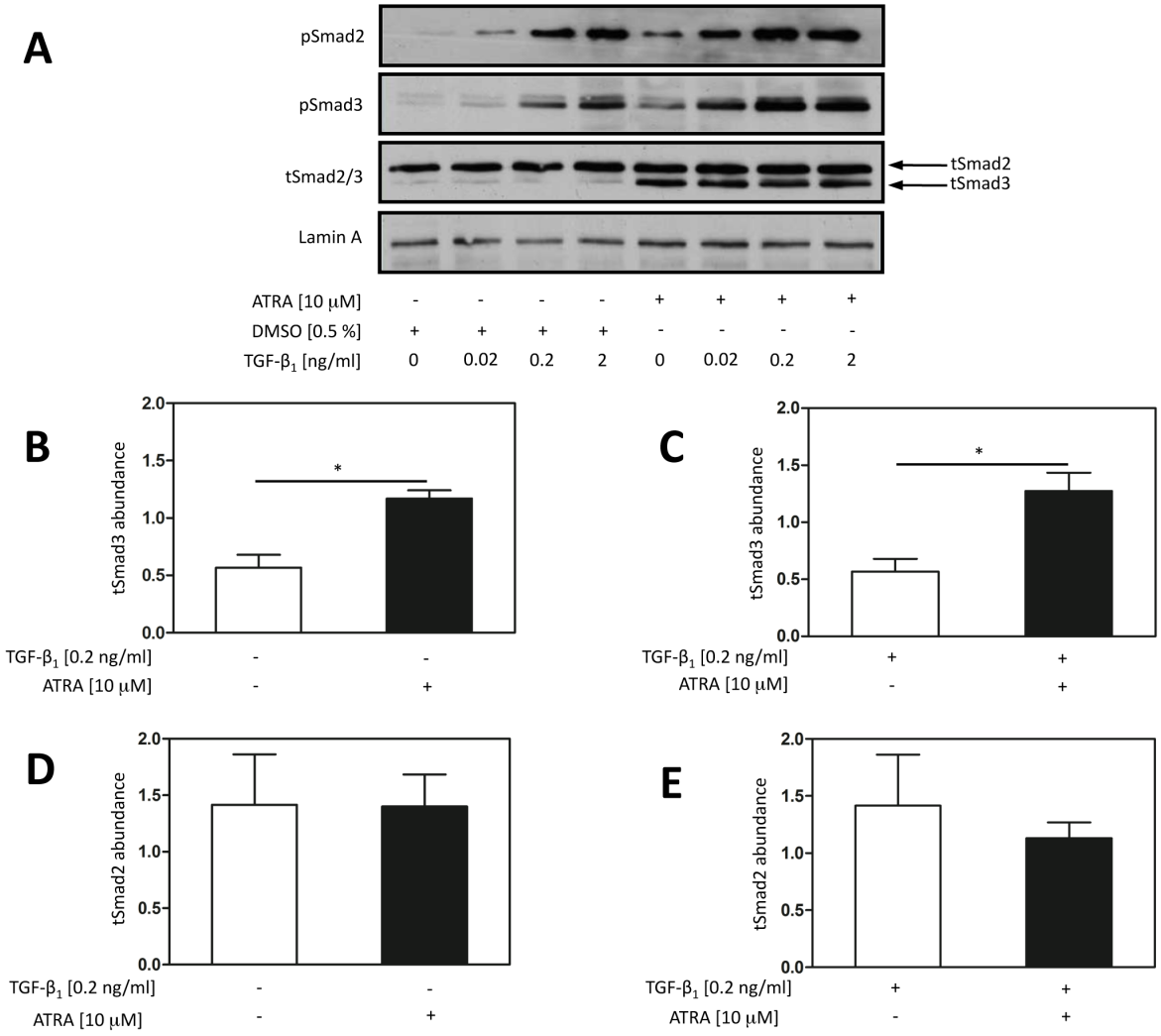

Figure 2: **ATRA effects on phospho-Smad2 and phospho-Smad3 levels in NIH/3T3 cells.** Phospho-Smad2, phospho-Smad3 and total Smad2/3 levels were assessed in NIH/3T3 cells. Stimulation was done with ATRA (10 μM) or DMSO (0.5%) for 12 h, followed by stimulation with TGF–β (0, 0.02, 0.2 and 2 ng/ml). (A) Phospho-Smad3 as well as phospho-Smad2 levels are affected by TGF–β and ATRA. The toal Smad3 levels only respond to stimulation with ATRA, whereas toal Smad2 levels remain unchainged. (B-E) Data were quantified by densitometry for toal Smad2 and total Smad3, where data indicate mean ± S.D. from three plots. *P*-values were assessed by unpaired Students's *t*-test. Gels are representative of three independent experiments.

6.2 ATRA effects on TGF–β signaling assessed by Smad phosphorylation using Western blot

In this set of experiments, Smad proteins were studied in NIH/3T3 cells after exposure to TGF–β and ATRA. Smad proteins are known as intracellular mediators transducing downstream signaling of the TGF–β machinery, after phosphorylation, to the nucleus, able affect transcription activity of target genes [167, 161].

The NIH/3T3 cells were grown in the presence of ATRA (10 μM in 0.5% DMSO) or with DMSO (0.5%) as a control for 12 h. This was followed by stimulation with TGF–β for 30 min at different dilutions. Cellular proteins were analyzed by SDS–PAGE where total Smad2 and Smad3 and phospho-Smad2 and phospho-Smad3 as well as lamin A antibodies were used in Western blotting (further described in table 18). NIH/3T3 cells had increased levels of phospho-Smad2 after stimulation with ATRA, when co-stimulated with TGF–β phospho-Smad2 levels were as well increased. To further study this phenomenon, phospho-Smad3 and total Smad3 levels, which are also part of the regulatory Smad family, were assessed in NIH/3T3 cells (regulatory Smads are described in detail in section 2.12.4). Stimulation with higher concentrations of TGF–β resulted in higher levels of phospho-Smad3. Co-stimulation with ATRA resulted in even higher levels of phospho-Smad3 (figure 2 A).

Data was quantified by densitometry and examplary, results are shown for stimulation of the cells with 0.2 ng/ml TGF–β for total Smad3 and total Samd2. The levels of total Smad3 were significantly increased without co-stimulation with TGF–β (figure 2 B) and with co-stimulation of the cells with TGF–β (figure 2 C). Total Smad2 levels did not change throughout the experiments (figure 2 D, E). These results further support the hypothesis of a possible influence of ATRA on the TGF–β machinery in NIH/3T3 cells, specifically on total Smad3.

6.3 Identification of TGF–β-responsive genes in NIH/3T3 cells and MLE-12 cells

To determine mRNA levels of TGF–β responsive genes, visualization and quantification was conducted by means of semi-quantitative RT-PCR analysis. Genes potentially responsive to TGF–β stimulation were studied both in NIH/3T3 cells and MLE-12 cells. Cells were stimulated with 0.2 ng/ml TGF–β or 10 ng/ml TGF–β or with DMSO (0.5%) for 12 h. The *serpine1* gene was responsive in both cell lines to TGF–β stimulation. The *ctgf* gene exhibited similar trends. The mRNA level of the loading control gene *gapdh* used in this study did not change expression levels throughout TGF–β stimulation (figure 3).

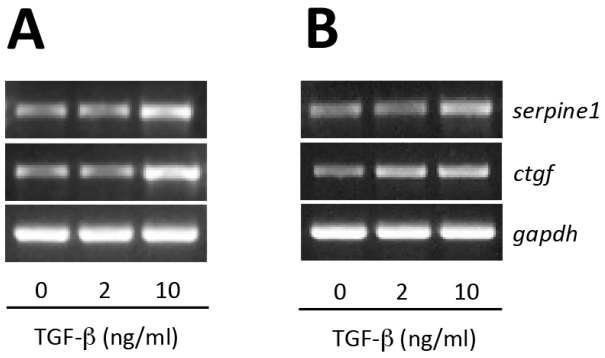

Figure 3: **TGF–β responsive genes in NIH/3T3 cells and MLE-12 cells.** Gene expression patterns of TGF–β responsive genes performed in NIH/3T3 cells (A) and MLE-12 cells (B). Stimulation was done with TGF–β (0.2, 2 and 10 ng/ml). The mRNA expression analyses assessed by semi-quantitative RT-PCR. The TGF–β responsive genes *ctgf* and *serpine1* were responsive to TGF–βstimulation in both cell lines. The *gapdh* mRNA was used as a loading control. Gel pictures are representative of three independent experiments.

6.4 ATRA impacts expression of TGF–β-responsive genes in NIH/3T3 cells

After the identification of genes responsive to TGF–β stimulation in NIH/3T3 cells, the question was addressed whether these genes are also responsive to ATRA. Therefore, the following four different experimental set-ups were employed in 100 mm petri dishes as described in table 7 and analyzed by means of semi-quantitative RT-PCR.

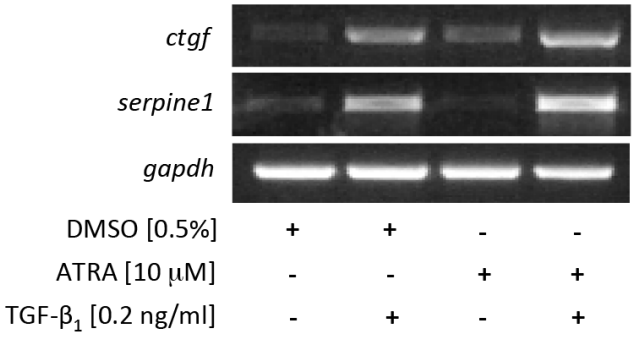

Figure 4: **TGF–β target gene expression patterns in NIH/3T3 cells.** Gene expression patterns in NIH/3T3 cells after stimulation with ATRA (10 μM) or DMSO (0.5%) with or without TGF–β (0.2 ng/ml). The mRNA level expression analyses were done by semi-quantitative RT-PCR. The *gapdh* mRNA was used as a loading control. Gel pictures are representative of three independent experiments.

NIH/3T3 cells stimulated with 0.2 ng/ml TGF–β alone demonstrated upregulation of mRNA levels both for the *ctgf* gene as well as for the *serpine1* gene. Exposure

to ATRA (10 μM) alone resulted in the *ctgf* gene in a minimal upregulation of gene expression, the *serpine1* gene was minimally downregulated (figure 4). Exposure to 12 h of ATRA (10 μM), followed by 12 h of ATRA (10 μM) together with TGF–β 0.2 ng/ml resulted in marked upregulation of the *serpine1* gene. The *ctgf* gene expression levels were as well increased in the double stimulation condition (TGF–β plus ATRA). The *gapdh* mRNA was used as a control.

To verify the results from the semi-quantitative RT-PCR, the same experimental setting mentioned above was employed and analyzed with real-time RT-PCR. To get a deeper understanding of the different effects of retinoids, this study was extended by exposure of the cells to 13-*cis* RA. Time of exposure and concentration of the 13-*cis* RA was equal to ATRA.

When cells were stimulated with TGF–β compared to DMSO, a significant two-fold upregulation of the *ctgf* gene was assessed (figure 5). Exposure to ATRA compared to DMSO resulted in a significant 1.8-fold induction of *ctgf* gene expression levels. Compared to TGF–β treated cells, *ctgf* gene expression levels while cells were exposed to ATRA plus TGF–β resulted in a significant three-fold induction. When cells were stimulated with 13-*cis* RA, gene expression levels of *ctgf* did not change. The combined exposure of 13-*cis* RA plus TGF–β resulted in a significant two-fold upregulation compard to DMSO. Stimulation of cells with TGF–β caused a significant two-fold upregulation of the gene *serpine1* compared to DMSO. Cell stimulation with both ATRA or 13-*cis* RA caused significant downregulation of the *serpine1* gene compared to exposure to DMSO only. Combined exposure to ATRA and TGF–β resulted in a three-fold induction of *serpine1*. This degree of upregulation was significant when compared to stimulation with TGF–β only. Simultaneous stimulation of 13-*cis* RA and TGF–β caused a significant two-fold upregulation of gene expression levels, which were equivalent to that seen for TGF–β alone.

To summarize these results, different chemical derivatives of RA are able to have very complex effects on TGF–β target genes in NIH/3T3 cells. ATRA seems to enhance TGF–β mediated gene induction for the *ctgf* and *serpine1* gene in NIH/3T3 cells. ATRA alone is able to upregulate *ctgf* gene expression levels but does not have a significant effect on the *serpine1* gene. Gene expression levels are not altered by stimulation with 13-*cis* RA alone, but co-stimulated of cells with 13-*cis* RA enhances TGF–β mediated gene expression levels.

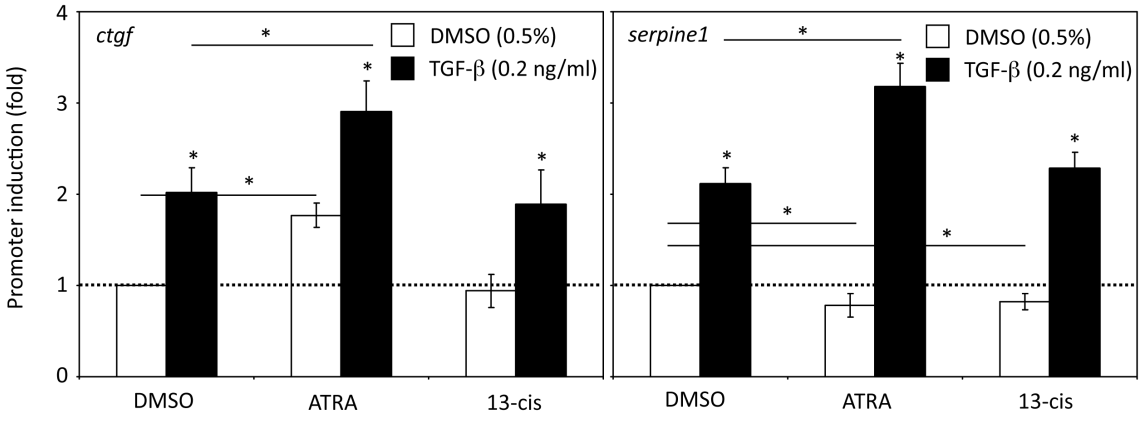

Figure 5: **TGF–β target gene expression patterns in NIH/3T3 cells after stimulation with ATRA.** Gene expression patterns in NIH/3T3 cells after stimulation with DMSO (0.5%)„ ATRA (10 μM) or 13-*cis* RA (10 μM) with or without TGF–β (0.2 ng/ml). The mRNA level expression analyses readout for *serpine1* and *ctgf* were done with real-time RT-PCR. The graphs shown are representative of three independent experiments. The *gapdh* gene was used a house keeping gene. Data indicate mean ± S.D. from two measurements. Statistically significant differences by ($P<0.05$; by ANOVA followed by a Bonferroni *post-hoc* test) are marked by *.

6.5 ATRA impacts expression of TGF–β-responsive genes in MLE-12 cells

TGF–β responsive genes studied previously in NIH/3T3 cells were now assessed in MLE-12 cells to identify possible changes in expression levels in epithelial cells. MLE-12 cells were stimulated with 0.2 ng/ml TGF–β and treated with ATRA (10 μM) or DMSO (0.5%) as a control (please refer to table 7 for further details where the experimental set-up is described). Upregulation of mRNA levels was present both for the *ctgf* as well as for the *serpine1* gene (figure 6). The *serpine1* and *ctgf* gene were upregulated after exposure with ATRA (10 μM) or ATRA (10 μM) followed by 12 h ATRA (10 μM) and TGF–β (0.2 ng/ml). The mRNA level of the loading control *gapdh* used in this study did not change expression levels throughout the experiments.

To verify the results from the semi-quantitative RT-PCR, the same experimental setting was analyzed with real time RT-PCR (figure 7). To get a deeper understanding of the different effects of retinoids, this study was extended by exposure of the cells to 13-*cis* RA. Time of exposure and dilution of the 13-*cis* RA was equal to ATRA. A significant 1.5-fold upregulation of the *ctgf* gene was assessed in the presence of TGF–β compared to DMSO. Exposure to ATRA only resulted in a significant 2.5-fold upregulation of the gene compared to DMSO. When cells were stimulated with ATRA plus TGF–β, the *ctgf* gene expression levels exhibited a three-fold in-

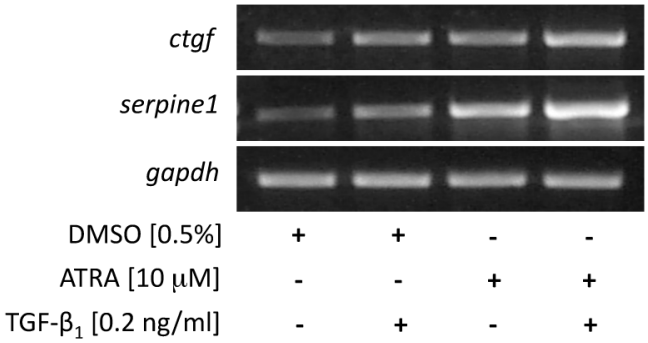

Figure 6: **Gene expression patterns in MLE-12 cells after stimulation with ATRA and TGF–β.** MLE-12 cells were stimulated with ATRA (10 µM) or DMSO (0.5%) with or without TGF–β (0.2 ng/ml). The mRNA level expression analyses readout of *ctgf* and *serpine1* was done with semi-quantitative RT-PCR. The *gapdh* gene was used as a loading control. Gel pictures are representative of three independent experiments.

duction of gene expression levels. Compared to stimulation with TGF–β only, this upregulation was significant. Neither exposure to 13-*cis* RA nor co-stimulation to 13-*cis* RA plus TGF–β did change gene expression levels of the *ctgf* gene significantly. When investigating the *serpine1* gene, stimulation of cells with TGF–β caused a significant two-fold upregulation of the gene compared to DMSO. Combined exposure of ATRA plus TGF–β resulted in a five-fold induction of *serpine1*. This altitude of upregulation was significant when compared to stimulation with TGF–β only. Cell stimulation with ATRA only resulted in a significant 3.8-fold induction of the *serpine1* gene compared to DMSO. Stimulation with 13-*cis* ATRA only or co-stimulation with 13-*cis* ATRA plus TGF–β did not result in markedly changes of *serpine1* gene levels.

To summarize these results, ATRA is able to enhance TGF–β mediated gene induction both of the *serpine1* as well as the *ctgf* gene in MLE-12 cells. Cell stimulation with ATRA alone does have notable effects on gene expression of both genes as well, but co-stimulation of TGF–β and ATRA seem to affect each other positively. When looking at 13-*cis* RA, treatment of the cells did not result in significant gene expression level changes, neither alone nor when co-stimulation with TGF–β was performed.

6.6 ATRA effects the expression of components of the TGF–β signaling machinery in NIH/3T3 cells

ATRA effects on the TGF–β receptor subunits were also studied in NIH/3T3 cells. We looked at *tgfbr1*, *tgfbr2* as well as on the accessory receptors *tgfbr3* and *endoglin*

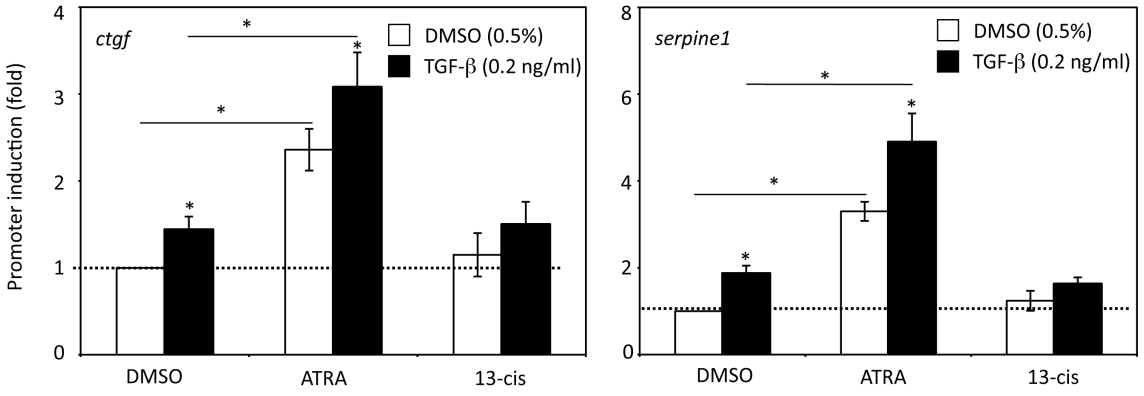

Figure 7: **Gene expression patterns of TGF–β target genes in MLE-12 cells after stimulation with ATRA.** Gene expression patterns in MLE-12 cells after stimulation with DMSO (0.5%), ATRA (10 μM) or 13-*cis* RA (10 μM) with or without TGF–β (0.2 ng/ml). The mRNA level expression analyses readout for *serpine1* and *ctgf* gene was done with real time RT-PCR. The graphs shown are representative of three independent experiments. The *gapdh* gene was used a house keeping gene. Data indicate mean ± S.D. from two measurements. Statistically significant differences ($P<0.05$; by ANOVA followed by a Bonferroni *post-hoc* test) are marked by *.

and *smad2*, *smad3* and *smad4* by using semi-quantitative RT-PCR. During the experiments, cells were exposed to ATRA (10 μM) dissolved in DMSO (0.5%) or only DMSO (0.5%) for 12 h, respectively. While stimulation did not significantly change *tgfbr1, tgfbr2* and *tgfbr3* gene expression levels, gene levels of *smad3* were strongly upregulated by ATRA. Gene expression levels of the other accessory receptor *endoglin* were higher compared to the DMSO control. The *Smad2*, *smad3* and *smad4* gene were studied to assess effects of ATRA on the downstream signaling proteins of the TGF–β machinery. While *smad2* and *smad4* did not presented with changes in gene expression levels, *smad3* was upregulated in the presence of ATRA (figure 8 A).

These data acquired from the semi-quantitative RT-PCR was quantitatively confirmed by means of real time RT-PCR. NIH/3T3 cells were grown and stimulated with the same conditions employed for the semi-quantitative RT-PCR using ATRA (10 μM) or DMSO (0.5%) treatment for 12 h. In NIH/3T3 cells treated with ATRA, the *smad3* gene expression levels changed by 2.844 ΔCT units. Gene expression levels of the *tgfbr1*, *tgfbr3, smad4* and *endoglin* gene did not change significantly in the presence of ATRA (figure 8 B). These results assessed by means of the real time RT-PCR quantitatively support findings from the semi-quantitative RT-PCR.

To summarize, ATRA has significant effects on the regulatory *smad3* gene (section 2.12.4) molecule, transducing downstream signaling from the TGF–β receptor to the nucleus is sensitive to stimulation with ATRA visible by the increased abun-

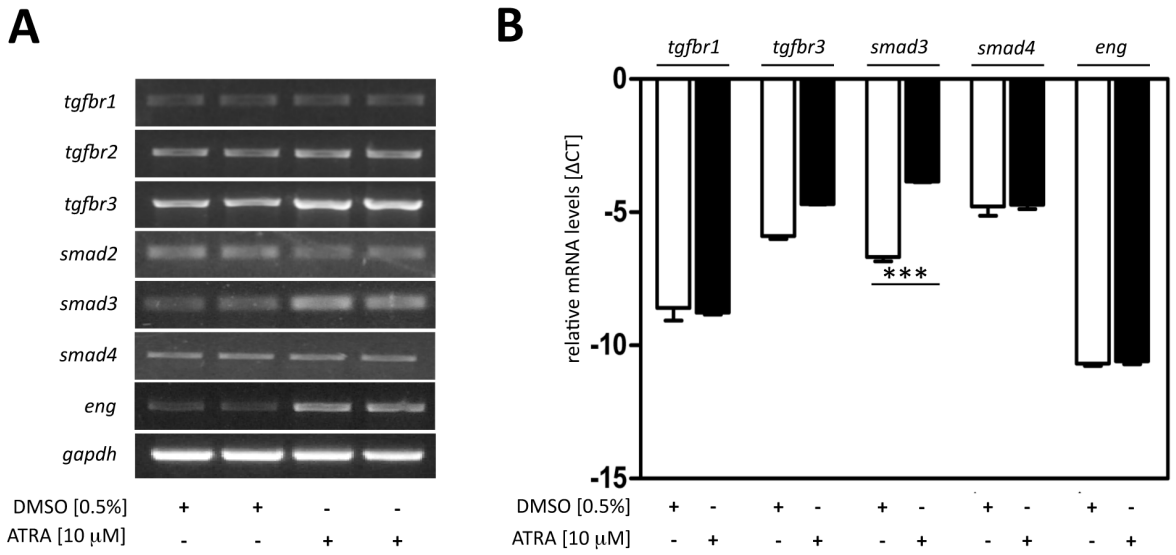

Figure 8: **Gene expression patterns of the TGF–β receptor and TGF–β signaling Smads in NIH/3T3 cells after stimulation with ATRA.** Expression analysis of the *tgfbr1* (using a cycle number of 27), *tgfbr2*, the accessory *tgfbr3*, and *endoglin* gene as well as downstream signaling genes *smad2, smad3, smad4* was assessed via semi-quantitative RT-PCR (A) and real-time RT-PCR (B). Cells were treated with ATRA (10 μM) or with vehicle (DMSO). The *gapdh* gene was used as a loading control. (B) Changes in mRNA expression were assessed by real time RT-PCR. Results are representative of three independent experiments. Data indicate mean ± S.D. from two measurements. Statistically significant differences ($P<0.001$; by ANOVA followed by a Bonferroni *post-hoc* test) are marked by ***.

dance of the gene (figure 8A,B). In sum, ATRA seem to have significant regulatory effects on the canonical TGF–β pathway most importantly on the downstream signaling molecule Smad3.

6.7 ATRA effects the expression of components of the TGF–β signaling machinery in MLE-12 cells

The effects of ATRA on the TGF–β receptor subunits were also studied in MLE-12 cells. We looked at *tgfbr1*, *tgfbr2* as well as on the accessory receptors *tgfbr3* and *endoglin* and *smad2*, *smad3* and *smad4* by using semi-quantitative RT-PCR. In the experimental setting, cells were exposed to ATRA (10 μM) for 12 h or to DMSO (0.5%) for 12 h. The *tgfbr2* gene was the only member of the receptor family able to be activated by stimulation with ATRA. Gene expression of the *tgfbr2* gene was slightly downregulated by ATRA. The mRNA levels of the accessory receptor endoglin were not affected by ATRA. The *tgfbr3* gene which is extremely weak expressed in MLE-12 cells was not detectable. Gene expression levels of *smad2, smad3* and *smad4* did not change during stimulation with ATRA (figure 9).

These trends assessed by semi-quantitative RT-PCR suggest that ATRA alone does not have significant effects on the TGF–β receptor in MLE-12 cells since the only subunit affected was the *tgfbr2* gene, which was downregulated. This hypothesis is further supported by the lacking response of the downstream signaling genes *smad2, smad3, smad4* following ATRA treatment.

6.8 Smad3 modulates TGF–β signaling in NIH/3T3 cells

In the previous studies (please refer to section 6.2 and 6.6 for more information), significant upregulation of mRNA and protein levels of Smad3 was observed in NIH/3T3 cells treated with ATRA. With the knowledge that ATRA drives up Smad3 levels, it was now asked in which way Smad3 affects TGF–β signaling. The following experiments were done to assess the impact of the smad3 molecule on the TGF–β machinery.

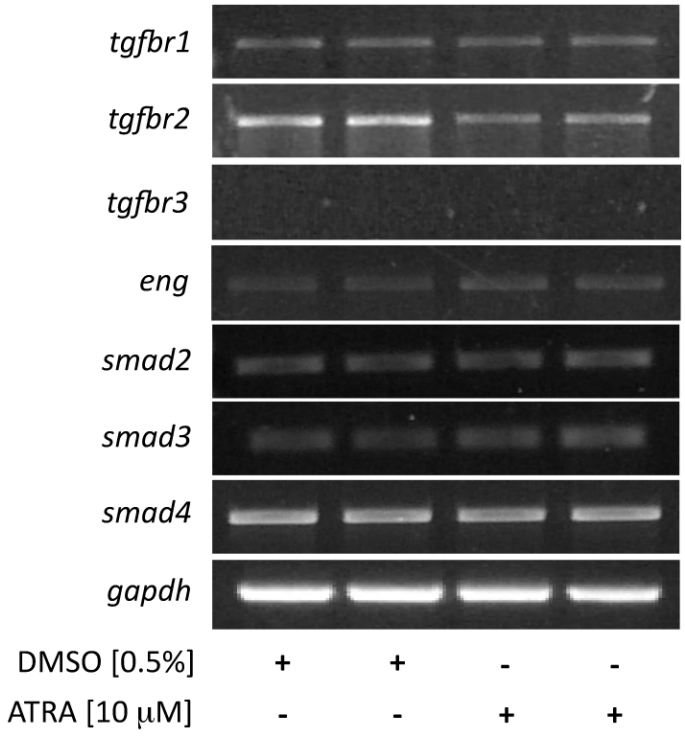

Figure 9: **Gene expression patterns of the TGF–β receptor and signaling Smads in MLE-12 cells after stimulation with ATRA.** Expression analysis of *tgfbr1* (using a cycle number of 29), *tgfbr2*, the accessory *tgfbr3*, *endoglin* and *smad2, smad3 and smad4* was performed using semi-quantitative RT-PCR in MLE-12 cells. Cells were exposed to the vehicle DMSO (0.5%), or to ATRA (10 μM). The *gapdh* gene was used as a loading control. Gels pictured are representative of three independent experiments.

First, NIH/3T3 cells were transfected with scrambled (scr) RNA or with specific

smad3 small interfering (si) RNA followed by treatment with TGF–β and ATRA, as described in section 5.13. Activity of the TGF–β machinery was measured by the p(CAGA)$_9$/*Renilla* ratio using DLR. The p(CAGA)$_9$/*Renilla* ratio was significantly downregulated in cells transfected with the *smad3* siRNA when stimulated with TGF–β or TGF–β and ATRA (figure 10).

In the second experimental setting, cells were transfected with the expression plasmid pIRES::*smad3* (section 5.12). Increased activity of the TGF–β machinery is represented by a higher value of the luciferase firefly p(CAGA)$_9$-luc/*Renilla* ratio using DLR. Overexpression of Smad3 increased significantly the p(CAGA)$_9$-luc/*Renilla* ratio in cells exposed to DMSO and TGF–β compared to cells transfected with scr siRNA (figure 10 A). The most profound increase in p(CAGA)$_9$-luc/*Renilla* ratio could be appreciated in Smad3 transfected cells exposed to ATRA and TGF–β. Ablation of Smad3 using siRNA resulted in a significant decrease in p(CAGA)$_9$-luc/*Renilla* ratio in cells exposed to TGF–β and ATRA plus TGF–β, compared to untransfected cells (figure 10 panel B). The siRNA mediated gene knockdown of Smad3 as well as overexpression was performed and assessed by Western blot to obtain further insights into changing Smad protein levels (figure 10 C and D). Overexpression of pIRES::*smad3* resulted in a profound increase of total Smad3 (figure 10 C). Ablation of Smad3 reduced Smad3 levels while Smad2 levels remained unchanged (figure 10 D).

In summary, Smad3 levels impact the TGF–β signaling. When cells have lower amounts of Smad3, achieved by transfection with *smad3* siRNA, TGF–β signaling is damped via reduction of Smad3 and not Smad2. When cells contain more Smad3, the TGF–β signaling is enhanced, revealed by increased expression levels of the TGF-–β responsive element p(CAGA)$_9$. Hence, it can be concluded that the TGF–β signaling downstream molecule Smad3 is crucial for TGF–β signaling.

From the results acquired in this section, it can be concluded that Smad3 is crucial for TGF–β signaling. As described in section 2.12.4, TGF–β signaling is mediated downstream to the nucleus via the Smad proteins. Phospho-Smad2 and phospho-Smad3 are necessary for activation of TGF–β target genes in the nucleus. Interestingly, while phospho-Smad3 is able to directly bind DNA sequences, phospho-Smad2 does not has this property. Phospho-Smad2 can only affect TGF–β target genes when binding concomitantly with phospho-Smad3 to DNA [192, 193]. Several experiments in this section provide further evidence that Smad3 is a key signaling protein for TGF–β signaling. Overexpression of the Smad3 protein resulted in a strong activation of the TGF–β target gene sequence p(CAGA)$_9$, whereas Smad3 knockdown damped TGF–β signaling massively (figure 10). Interestingly, TGF–β

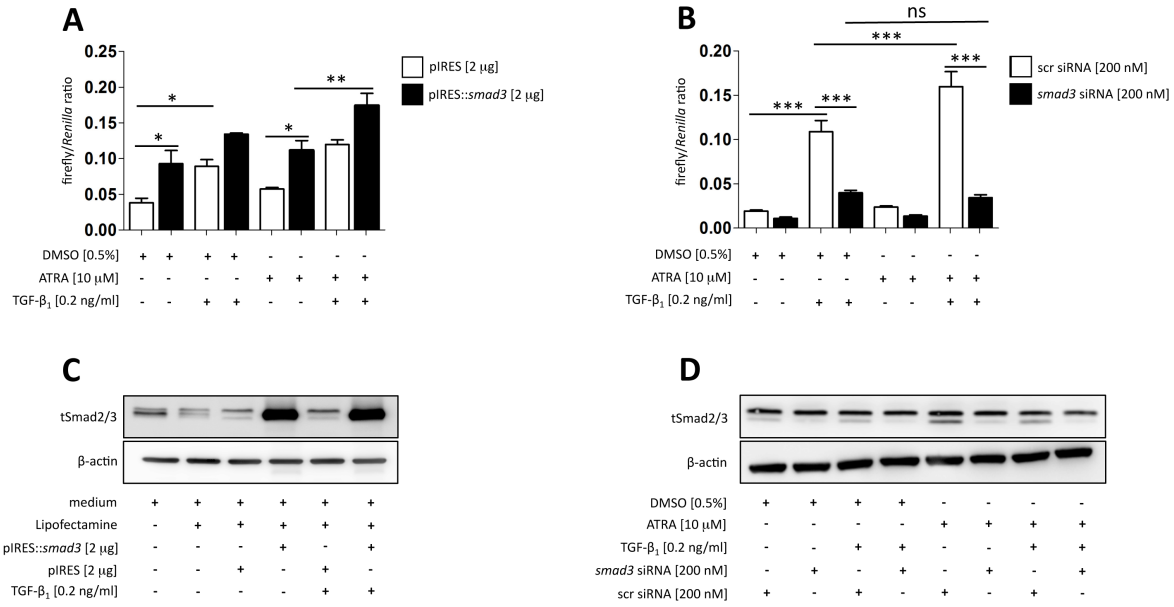

Figure 10: **Smad3 modulates responsiveness to TGF–β and ATRA in NIH/3T3 cells.** (A) To validate the expression of *smad3* (with ATRA alone or in combination with TGF–β) a luminescence-based dual luciferase assay was performed employing p(CAGA)$_9$-luc and pRL-SV40, in the presence of either pIRES::*smad3*, or pIRES as empty vector. (B) The *smad3* gene expression was knocked-down by siRNA transfection, and the effects of ATRA and TGF–β, alone, or in combination, were assessed in a luminescence-based dual luciferase assay employing p(CAGA)$_9$-luc and pRL-SV40. Data indicate mean ± S.D., statistically significant differences (P-values of $p<0.05$ shown as *, $p<0.01$ shown as ** or $p<0.001$ shown as ***) were studied by ANOVA followed by a Bonferroni *post-hoc* test. Samples were measured in quadruplicates (C). The impact of the overexpression of *smad3* in NIH/3T3 cells on Smad2/3 levels was assessed by immunoblot, employing pIRES::*smad3* for *smad3* overexpression, or pIRES as empty vector. (D), The impact of reduced *smad3* expression on total Smad2/3 levels induced by ATRA and TGF–β stimulation (alone, or in combination), was assessed by immunoblot. These data is representative of three independent experiments.

signaling was not totally abolished in cells lacking Smad3, evidencing that TGF–β downstream signaling is not only mediated by Smad3.

6.9 Smad3 is crucial for TGF–β signaling in NIH/3T3 cells

To examine the functional contribution of Smad3 to TGF–β signaling, the gene expression of *smad3* was ablated by transfection of NIH/3T3 cells with siRNA directed against *smad3* and with scrambled siRNA serving as a negative control. Performing real time RT-PCR *smad3* mRNA levels were reduced in a dose dependet pattern. Transfection with 25 nM siRNA resulted in 0.367 ± 0.1635-fold increase, 50 nM siRNA in 0.0558 ± 0.097-fold decrease, 100 nM siRNA in 1.323 ± 0.0686-fold and 200 nM siRNA in 1.722 ± 0.08372-fold decrease, respectively. Using a luciferase-based DLR assay, TGF–β signaling was damped with increased doses of *smad3* siRNA,

evidenced by a significant decrease of the TGF–β sensitive p(CAGA)$_9$-luc/*Renilla* ratio (figure 11 panel B), confirming that *smad3* is necessary for TGF–β signaling. Consistent with these data presented in panel B, immunoblot analysis from NIH/3T3 cells transfected with *smad3* siRNA resulted in decreased levels of *smad3* with increasing doses of *smad3* siRNA, (panel C). These DLR data as well as the real time RT-PCR and immunoblot data confirm that *smad3* impacts the responsiveness to TGF–β in a dose dependent manner. The means of quadruplicates ± S.D. are shown, representative of three independent experiments

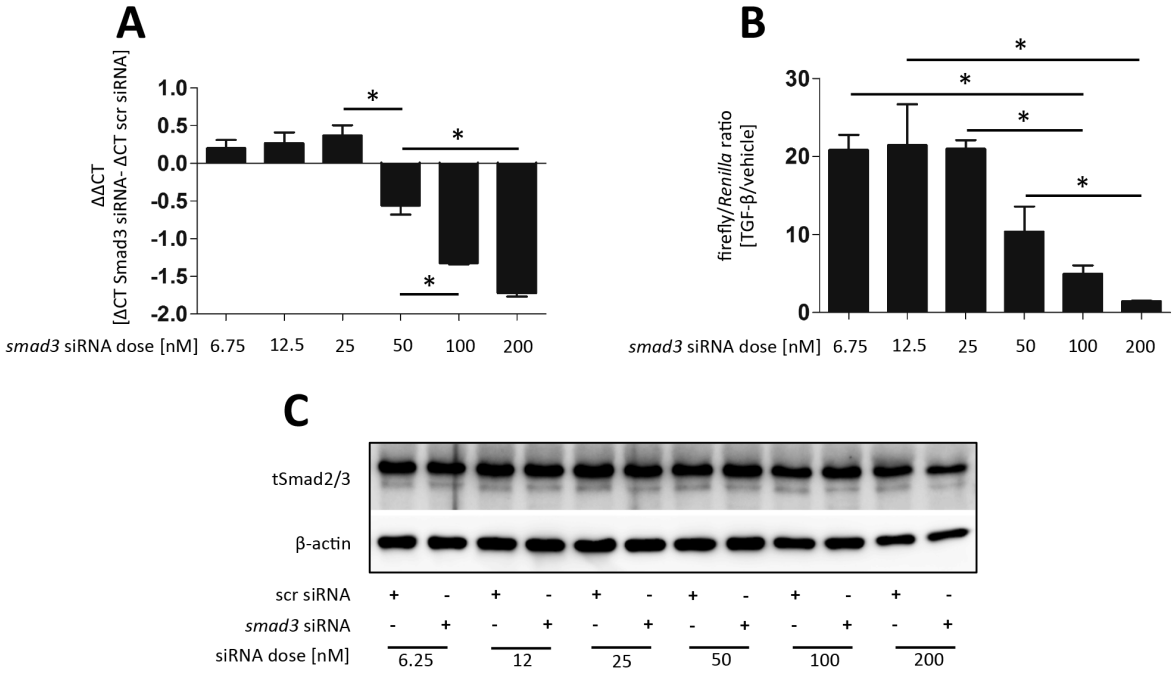

Figure 11: **Dose dependent effects of Smad 3 expression on TGF–β signaling.** Expression of the Smad3 molecule was assessed by real-time RT-PCR in NIH/3T3 cells. Expression of the *smad3* gene was significantly reduced with increasing doses of *smad3* siRNA. Changes in mRNA expression levels for the real time RT-PCR are reflected as fold change, ΔΔCT values. Data represent mean ± S.D., statistically significant differences (P-values of $p<0.05$; by ANOVA followed by a Bonferroni *post-hoc* test) are marked by *, n=3. Samples were measured in duplicates (A). The expression of *smad3* was ablated by siRNA-mediated knock-down, and the induction of p(CAGA)$_9$-luc was assessed in a luminescence-based dual luciferase assay employing p(CAGA)$_9$-luc and pRL-SV40. Data represent mean ± S.D., statistically significant differences (P-values of $p<0.05$; by ANOVA followed by a Bonferroni *post-hoc* test) are marked by *, n=3. Samples were measured in quadruplicates (B). The efficiency of Smad3 knock-down mediated by siRNA was confirmed by immunoblot, where the faint Smad3 band is almost lost from NIH/3T3 cell extracts. β-actin served as a loading control (C). Data are representative of three independent experiments.

7 Discussion

Bronchopulmonary dysplasia remains one of the main reasons for morbidity in preterm infants with a birth weight below 1000 g [40]. Depending on regional differences and treatment options, approximately 30% of the affected children later develop BPD and a lot of the diseased children are at risk for developing pulmonological and neurological complications [194]. Therefore, BPD bears an importance until adulthood since the associated complications are able to impact the entire period of life including emotional stress for affected families and high costs for health systems [195].

Considering the pathogenesis of BPD, a multifactorial etiology has been discussed and several factors are currently described which may contribute to the development of the disease. Hyperoxia is mentioned not as obligate, but as very important factor for disease progression [99, 37, 102]. Several studies validated this theory in animal models where chronic hyperoxia was able to negatively affect normal postnatal lung maturation [196, 95]. This study focused on the TGF–β signaling pathway which is known to be dysregulated in BPD [56] and retinoic acid which is an important regulator of lung development [75], and has been discussed as a treatment option for BPD [187, 186]. Particular attention was paid to the interaction of the TGF–β and retinoic acid pathway.

7.1 TGF–β signaling is critical for lung development

TGF–β signaling is required for both early and late lung development, although this requirement needs to be finely-tuned since too much and too little TGF–β signaling negatively impacts alveolarization [175, 143, 176]. Several studies have addressed the impact of TGF–β signaling in the developing lung [179, 177, 175]. The necessity of TGF–β signaling for lung development was proven in a study where complete blockade of TGF–β signaling by ablation of Smad3 in neonatal mice arrested alveolarization [176]. On the other hand, too much TGF–β signaling also negatively affects proper lung development as excessive TGF–β during lung development also inhibits alveolarization [143, 175].

In this study, the fibroblast derived NIH/3T3 cell line and the MLE-12 distal epithelial cell line were investigated. Both, fibroblast and epithelial cells are important for lung development and are present in the mouse lung [1, 197]. A critical hallmark of late lung development is the process of alveolarization, in which TGF–β plays a key role [9]. It was shown that TGF–β signaling molecules Smad2, Smad3 and Smad4 are present during this phase of late lung development [198]. Alejandre-

Alcázar *et al.* revealed changing patterns of expression of Smad2 and Smad3 during this phase of lung development, suggesting a critical role of these two molecules in the process of alveolariziation [179]. Generally speaking, as normal lung development proceeds, there is reduced TGF–β signaling and progressively decreasing Smad expression levels. In contrast, when lung development is disturbed by high oxygen concentrations, this pattern of Smad expression (and TGF–β signaling) is altered, where prolonged "high" Smad2 and Smad3 expression levels, and increased TGF–β signaling, are associated with disturbances to alveolarization [177].

The observations on Smad3 expression and TGF–β signaling reported here, where ATRA can increase Smad3 protein levels in NIH/3T3 cells, and can thus potentially increase TGF–β signaling in the developing lung, are consistent with findings from *in vivo* studies reporting that too much ATRA can negatively influence lung development [4]. Similarly, in a clinical setting, ATRA (or vitamin A) administration in clinical trails has failed to prove beneficial effects when used in pre-term infants at risk for BPD [199, 187].

7.2 Vitamin A is critical for lung development

Vitamin A is known to be involved in numerous processes in the lung including maturation, lung repair after injury, and the maintenance and integrity of lung structure [180]. The RA pathway can effect all stages of lung development, and RA signaling needs to be finely tuned to promote proper lung maturation [200, 201, 4]. Given the crucial role of vitamin A in the respiratory system, led to several studies which investigated the vitamin A pathway as a target to promote proper lung development and to restore normal alveolar spaces [181, 202]. As the lung architecture is damaged during BPD [68], several groups have assessed the impact or RA in models of BPD [203, 182, 184]. RA is discussed as a treatment option in preterm children suffering from BPD [204], even though clinical trials could reveal only minimal beneficial effects of RA administration [187, 199].

7.3 ATRA affects Smad3 protein levels in culture

In the present study, complex effects of ATRA on the TGF–β signaling cascade were identified. The expression of components of the TGF–β machinery, including downstream signaling molecules Smad2 and Smad3, as well as the levels of phospho-Smad2 and phospho-Smad3, were assessed in NIH/3T3 cells. ATRA was able to increase the phospho-Smad2 and phospho-Smad3 levels significantly in NIH/3T3 cells (analyzed via Western blot, section 6.2). This effect was enhanced when TGF–β was

administered to the ATRA treated cells concomitantly (section 6.2). Interestingly, while TGF–β alone (or vehicle alone) did not change total Smad3 levels, administration of ATRA alone was able to increase the abundance of total Smad3 significantly (figure 2). In addition, mRNA levels of Smad3 assessed by real time RT-PCR revealed a significant increase in *smad3* mRNA levels in NIH/3T3 cells exposed to ATRA (section 6.6). Interestingly, supporting data for an interplay of Smad3 and TGF–β signaling has been described by other authors in other cell types. One study has revealed that RA binds directly to and sequesters Smad proteins [205] in human lung fibroblasts; and another study identified RA to be able to increase both nuclear and cytoplasmic expression of Smad3 in adipocytes [206].

Smad3 and RA interaction was further investigated using a luciferase-based transcriptional reporter assay, employing the specific TGF–β responsive promoter element p(CAGA)$_9$. Increased TGF–β signaling in cells exposed to TGF–β and ATRA together were identified (figure 1). However, cells exposed to ATRA alone did not have an increased activity of the p(CAGA)$_9$ promoter element (figure 1). This important observation suggested that ATRA could alter the expression of the TGF–β signaling machinery in TGF–β treated cells, which would have consequences for TGF–β signaling in those cells. To this end, the expression of key TGF–β receptors and key TGF–β intracellular signaling molecules was determined in cells treated with ATRA (section 6.6). While ATRA did have the ability to influence expression levels of a variety of molecules, only Smad3 expression was significantly impacted by ATRA treatment (figure 8). This finding has, to our knowledge, not been described in literature so far.

7.4 Retinoids regulate TGF–β signaling at the level of Smad3 in NIH/3T3 cells

It was also aim of this study to investigate how changing Smad3 lelvels might affect TGF–β signaling in NIH/3T3 cells. Smad3 was overexpressed in NIH/3T3 cells, and the activity of the TGF–β machinery was assessed by DLR using the promoter element p(CAGA)$_9$ as well as by Western blot analysis for phospho-Smad2 and phospho-Smad3 (figure 10). In NIH/3T3 cells transfected with the *smad3* gene, increased baseline p(CAGA)$_9$ activity was noted, even in the absence of exogenous TGF–β. Thus, the overexpression of the *smad3* gene may have made the NIH/3T3 cells "more sensitive" to TGF–β already present in the fetal calf serum in the cell-culture medium. When cells overexpressing *smad3* were stimulated with exogenous TGF–β, the p(CAGA)$_9$ signal was dramatically elevated, consistent with this theory. Thus, it seems that when Smad3 levels are increased, as would be the case after

exposure to ATRA, the TGF–β signaling capacity of the cell is also increased. Interestingly, when NIH/3T3 cells that overexpress *smad3* were stimulated first with ATRA and then with exogenous TGF–β, a further enhancement of the p(CAGA)$_9$ signal was noted (figure 10). This may reflect the added Smad3 expression driven by ATRA, on top of the over-expression driven by the pIRES::*smad3* vector.

A Smad3 knockdown approach was also employed, where Smad3 was depleted from NIH/3T3 cells by a siRNA-mediated approach. When Smad3 was depleted from NIH/3T3 cells, a strong decrease in the p(CAGA)$_9$ signal was noted. Indeed, the same p(CAGA)$_9$ signal was obtained after TGF–β stimulation, irrespective of whether cells were concomitantly stimulated with ATRA or vehicle alone (figure 10). These data support the idea that the impact of ATRA on Smad3 signaling is attributable largely to the effects of ATRA on Smad3 levels in NIH/3T3 cells.

The question of whether the levels of Smad3 induced by ATRA in NIH/3T3 cells were sufficient to alter TGF–β signaling was also addressed. In this experimental setting a dose range of *smad3* siRNA was employed, to knock down *smad3* expression levels to varying degrees, and the impact of different *smad3* expression levels on TGF–β signaling (assessed by p(CAGA)$_9$ reporter) was investigated (figure 11). It is clear from these data that a change of one ΔCT in *smad3* expression levels is sufficient to generate a significant change in p(CAGA)$_9$ responsiveness to exogenous TGF–β stimulation. In NIH/3T3 cells treated with ATRA, the *smad3* expression levels changed by up to three ΔCT units. To summarize, the levels to which ATRA may influence *smad3* gene expression are certainly relevant in terms of TGF–β signaling.

Interestingly, other studies have reported an interplay of RA and TGF–β signaling in other cell types [207, 205]. It has been shown in mink lung epithelial (MvIlu) cells that TGF–β signaling and the RA pathway might cooperate with each other and thus result in an inhibition of cell growth via Smad proteins [207]. Further investigation revealed an interplay of Smad3 with TGF–β signaling in human embryonic kidney (HEK) 293 cells [207]. Smad3 phosphorylation by TGF–β was enhanced due to specific interaction of the RA receptor (RAR)-α and Smad3. It was shown that Smad3 and RAR-α cross-talk with each other in a TGF–β-dependent manner, extending the knowledge about an interaction of these two pathways [207]. Further evidence for an interplay of the two molecules was provided by Pendaries where the MH2 domain of Smad3 was identified to interact directly with the RARγ [205] and studies done in NIH/3T3 cells demonstrated RA to regulate nuclear uptake of Smad2 and the availability of Smad2 for phosphorylation [203]. Taken together, several studies support that RA is able to affect downstream signaling molecules

(Smads) of the TGF–β machinery.

But what is the role of Smad3 for lung development, and how might lung maturation be affected by changing levels of Smad3? In this study, the impact of changing levels of Smad3 on TGF–β signaling in NIH/3T3 cells - a model for lung fibroblasts - was assessed. It was revealed that ATRA can increase Smad3 levels in NIH/3T3 cells, leading to increased TGF–β signaling (figure 2 and 1). Further evidence for a potentially negative impact of increased TGF–β signaling for lung development and specifically increased levels of Smad3 is given by other authors where it was shown that Smad3 ablation resulted in stimulation of embryonic lung branching and maturation [208]. As Smad3 levels were identified to be physiologically reduced during late lung development [179], and a potentially negative effect of too much Smad3 during lung development has been suggested [208], the increased abundance of Smad3 in response to ATRA administration seen in this study should be kept in mind when considering ATRA administration for the treatment of BPD.

As fibroblasts are believed to be key players in lung development, the modulation of fibroblast function by TGF–β is potentially important, particularly since increased levels of TGF–β signaling in the developing lung have been accredited with a negative impact on lung development [68, 209]. How might this occur? Fibroblasts are key producers of the lung ECM, including collagen, elastin, and other ECM components [177]. The production of ECM components by fibroblasts is largely TGF–β regulated [210]. Thus, the increased TGF–β signaling in fibroblasts, as a consequence of ATRA exposure, may well cause the production of too much ECM, or may cause an imbalance in the proper levels of the components of the ECM, leading to difficulties in forming the correct matrix structure, or the reshaping ("remodeling") of existing matrix structures. This may well cause problems in the formation of the lung architecture. Indeed, several studies have already reported that when ECM production and remodeling in the lung are disturbed, there is a strong impact on alveolarization [211, 212].

The use of retinoids such as ATRA and vitamin A, is currently debated as a means to improve lung development in patients where lung development is usually less effective or abnormal, such as in premature infants with respiratory distress syndrome, who are at risk for BPD [187, 199]. However, the clinical trials attempted to date have yielded conflicting and often unsatisfactory results [199, 79, 187]. It may well be that ATRA or vitamin A supplementation have different effects on different cell types in the lung, and the underlying pathology may influence whether ATRA or retinoids in general have an overall positive or negative effect. For example, ATRA is thought to impact epithelial cell differentiation [213], which is also relevant to lung

development. The balance of a "good" effect on epithelial cells may overcome the "bad" effect on fibroblasts, to yield an overall improvement, however the reverse may also be true. As such, the complex effects of retinoids on different cell types may account - in part - for the failure of retinoids as a therapeutic option in a clinical setting.

The morphogenesis of the developing lung is also affected by changing levels of RA binding receptors [159]. As previously said, ATRA exerts effects by binding to the RAR (section 2.11). During lung development, levels of RA are finely tuned by several enzymes causing degradation as well as synthesis of RA, which modifies the usage of RA [4, 214, 215]. Interestingly, prolonged high concentrations of RA exposure are able to activate RAR receptors in the lung [4], and there have been increasing evidence that abnormal activation of RARs in the lung seems to negatively influence lung maturation, characterized by failure to form typical distal buds [4]. Hence, it can be speculated that the constant application of vitamin A might have a negative affect on lung maturation through activation of RAR receptors in the lung.

Furthermore, the RARα was described to be critically involved in lung maturation and differentiation [216]. Downregulation of the receptor is necessary for development of alveolar type I and type II pneumocytes [217]. Interestingly, Smad3 has been described to directly interplay with the RARα [207], and in this study, increased levels of Smad3 have been identified in NIH/3T3 cells exposed to ATRA (section 6.4). Hence, it can be speculated that ATRA might negatively affected lung maturation by increasing Smad3 levels, which then might increase the activation of the RARα.

7.5 Perspectives and conclusion

To summarize these findings in this study, the TGF–β signaling pathway interplays with vitamin A. Increased activity of the TGF–β machinery could be appreciated in cells exposed to ATRA and TGF–β, and Smad3 is suggested to be involved in this process. In addition genes important for pulmonary remodeling, including *serpine1* and *ctgf* have also been modulated by vitamin A. In summary, novel findings in this study can be summarized as follows:

- ATRA is able to enhance TGF–β signaling in NIH/3T3 fibroblasts.
- ATRA exhibits an effect on TGF–β signaling through modulation of the expression of Smad3.

In this study, the NIH/3T3 cells and MLE-12 cell lines were employed, which are

both derived from the mouse. NIH/3T3 cells and MLE-12 cells have been used already in previous studies where TGF–β signaling has been investigated in the context of BPD. Numerous studies employed NIH/3T3 cells in order to understand lung fibrosis and TGF–β signaling in the developing lung [218, 219, 220, 221]. However, some studies employed at the same time primary cells lines or even performed experiments in animals [177, 212]. It is a weakness of this study that experiments were only performed in NIH/3T3 cells and MLE-12 cells rather than primary cell lines as well. Hence, results from this study have to be interpreted with caution and can only be in parts transferred to *in vivo* conditions. Also, it may well be that retinoids have very different effects on different cell types, which would be important to know, when considering the effects in the context of the entire lung, not only the fibroblast compartment.

Future studies should comply with current recommendations how to control gene expression levels. Currently, at least three house-keeping genes are recommended. Even though gene expression levels of the house-keeping gene *gapdh* did not change througout the represented experiments, it is a weakness of this study that only one house-keeping gene was employed and the here presented results should be intepreteted with caution.

There are established models for BPD in mice, and TGF–β signaling has been studied closely in several studies [177, 179, 202]. It would be advisable to study the effect of ATRA on the TGF–β machinery, with special focus on Smad3 in a mouse model of BDP. Investigating the interplay of ATRA and TGF–β *in vivo* from whole lung homogenates is advantageous since not only lung fibroblasts and epithelial cells which were the focus of this study are investigated, but also all the other cells which are parts of the lung including for example smooth muscle cells and endothelial cells. In addition, histomorphometric analyzation can then be performed from the mice lungs using the mean linear intercept which is inversely proportional to the alveolar surface area [1, 222, 223] and is commonly employed to study lung architecture. Furthermore, the mean septal thickness could be assessed, calculated by the width of alveolar septs divided by their number [5, 223] which might provide a further understanding of the lung architecture.

In summary, the present study reveals new and interesting evidence for a direct interplay of ATRA and TGF–β signaling in a lung fibroblast cell-line model. This interaction highlights the interaction of two key pathways that regulate lung development, extending the knowledge from previous studies about these systems. It is still not known what cross-talk exists of vitamin A and TGF–β signaling in primary cell lines and in *in vivo* models of BPD. Transferring experiments from this study

to primary cells as well as in a mouse model of BPD, which are closer to *in vivo* conditions might reveal further insights. It was mentioned, that TGF–β signaling cross-talks with other signaling pathways as well. It remains to be assessed whether the impact of ATRA on TGF–β signaling in lung fibroblasts is advantageous or deleterious. The crucial importance of vitamin A for lung development, as well as the desperate need for therapeutic treatment options for BPD justifies further experiments in this field.

8 Abstract

There is currently much interest in using retinoids such as all–*trans* retinoic acid (ATRA), or retinoid analogs such as vitamin A (retinol), in the management of premature neonates at risk for the development of bronchopulmonary dysplasia (BPD). Current clinical evidence is contradictory, with some reports suggesting benefits, while other reports suggest no impact, or a deleterious effect of retinoid administration. In contrast, retinoids generally perform very well in animal models of BPD, and serve to stimulate lung development, particularly alveolarization. In this study, we attempted to evaluate the impact of retinoids on the activity of the transforming growth factor (TGF)–β signaling pathway, a pathway that has been credited with high importance in driving lung development and alveolarization.

We employed NIH/3T3 mouse fibroblasts to study the impact of ATRA on TGF–β signaling. While the NIH/3T3 cell line differs in some important aspects to that of lung fibroblasts, it is reasonable to assume that NIH/3T3 have a similar biological behavior to that of airway fibroblasts in tissue. We observed that ATRA had a dramatic effect on TGF–β signaling in NIH/3T3 cells. Pre-treatment of NIH/3T3 cells with ATRA caused a strong increase in TGF–β signaling, as assessed by phosphorylation of Smad3, and by a luciferase-based promoter-reporter assay. Further studies revealed that this effect was caused by the ability of ATRA to strongly increase Smad3 expression levels in NIH/3T3 cells. Using plasmid-mediated overexpression of the *smad3* gene, we confirmed these observations, and showed that increased Smad3 levels in NIH/3T3 cells can indeed make the NIH/3T3 cells more responsive to TGF–β. To further support these data, we also down-regulated *smad3* gene expression using siRNA directed against Smad3, and demonstrated that by down-regulating *smad3* expression, the pro-TGF–β signaling effect of ATRA was lost. We also demonstrated that the degree of change in *smad3* gene expression caused by ATRA stimulation is sufficient to cause a significant change in the TGF–β signaling pathway.

Together, these data demonstrate that ATRA administration can upregulate TGF–β signaling in NIH/3T3 cells. This may have important consequences for lung development, since TGF–β drives multiple fibroblast functions such as extracellular matrix (ECM) production, as well as ECM reshaping in developing organs. Future studies should address the cross-talk of TGF–β and retinoid signaling on other lung cell types, primary cells, and in animal models of BPD, in order to complete the picture of how retinoids may influence lung organogenesis. With a better understanding about the impact of retinoids on lung fibroblasts function and lung development one might be able to optimize the administration of retinoids in patients with BPD and

thus improve clinical outcome.

9 Zusammenfassung

Aktuell besteht ein großes Forschungsinteresse in der Verwendung von Retinoiden wie Vitamin A Säure (englisch: all–*trans* retinoic acid (ATRA)) oder Retinoid-Analoga wie Vitamin A (Retinol) bei der Behandlung von Frühgeborenen, welche an Bronchopulmonaler Dysplasie (BPD) erkrankt sind. Der klinische Effekt von Vitamin A bei der Behandlung von BPD ist sehr widersprüchlich. Manche Studien berichten Erfolge, wobei andere Studien keinen Effekt oder sogar schädliche Auswirkungen bei der Verwendung von Vitamin A berichten. Hierzu steht im Widerspruch, dass Retinoide meist eine sehr gute Wirksamkeit in BPD Tiermodellen zeigen, wo sie die Lungen-entwicklung, insbesondere die Alveolarisierung fördern. In dieser Studie untersuchten wir die Auswirkung von Retinoiden auf die transformierende Wachstumsfaktor–β (eng-lisch: transforming growth factor (TGF)–β) Signalkaskade. Der TGF–β Signalkas-kade wird eine wichtige Bedeutung für die Lungenentwicklung und Alveolarisierung zugeschrieben.

Zur Untersuchung des Einflusses von ATRA auf die TGF–β Signalkaskade verwendeten wir NIH/3T3 Maus-Fibroblasten. Diese unterscheiden sich in einigen wichtigen Aspekten von Lungenfibroblasten, jedoch wird angenommen, dass NIH/3T3 Zellen ähnliche biologische Funktionen wie Lungenfibroblasten erfüllen. Wir konnten zeigen, dass ATRA einen dramatischen Effekt auf die TGF–β Signalkaskade in NIH/3T3 Zellen hat. Die Vorbehandlung von NIH/3T3 Zellen mit ATRA resultierte in einer starken Zunahme der TGF–β Signaltransduktion, was durch die Bestimmung der Phosphorilisierung von Smad3 und in einem Luciferase Promoter-Assay untersucht wurde. Weiterhin konn-te gezeigt werden, dass dieser Effekt durch ATRA hervorgerufen wurde, welches die Fähigkeit besitzt, die Smad3 Expression in NIH/3T3 Zellen zu erhöhen. Wir konnten mit Hilfe einer Plasmid-vermittelten Überexpression des *smad3* Gens diese Beobachtung bestätigen und konnten zeigen, dass durch eine Erhöhung von Smad3 in NIH/3T3 Zellen diese Zellen gegenüber TGF–β sensibilisiert werden. Um diese Beobachtung zu bestätigen, unterdrückten wir die Smad3 Gen Expression unter der Verwendung von siRNA gegen *smad3* und zeigten durch die Reduktion der *smad3* Expression einen Verlust der ATRA-abhängigen TGF–β Stimulation. Des Weiteren konnte beobachtet werden, dass das Ausmaß der *smad3* Gen Expression durch ATRA intensiv genug ist, um signifikante Veränderungen in der TGF–β Signalkaskade zur Folge zu haben.

Zusammenfassend konnte festgestellt werden, dass die Administration von ATRA

die Aktivität der TGF–β Signalkaskade in NIH/3T3 Zellen erhöhen kann. Diese Beobachtung könnte wichtige Auswirkungen auf die Lungenentwicklung haben, da TGF–β verschiedene Funktionen von Fibroblasten reguliert. Hierzu zählt die Produktion von extrazellurärer Matrix (ECM), aber auch der Umbau der ECM bei sich entwickelnden Organen. Ergebnisse dieser Studie bilden eine Basis für zukünftige Studien, welche die Interaktion von TGF–β und Retinoiden in anderen Zelltypen wie z.B. primären Zellen, aber auch in BPD Tiermodellen untersuchen sollten, um den Einfluss von Retinoiden bei der Organentwicklung weiter zu verstehen. Mit einem besseren Verständnis über die Bedeutung von Retinoiden auf die Funktion von Fibroblasten und auf die Lungenent-wicklung besteht das Potential, den therapeutischen Einsatz von Retinoiden bei BPD zu optimieren und den Verlauf der Erkrankung positiv zu beinflussen.

10 References

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11 Appendix

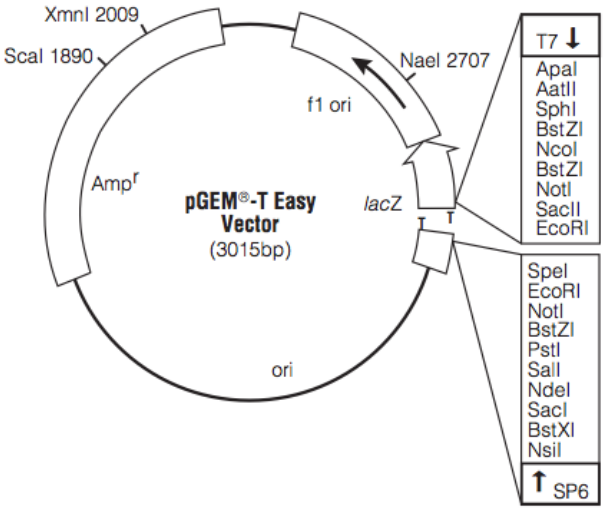

Figure 12: **The pGEM-T Easy vector map.** This figure is modified from Promega (Promega Germany, www.promega.com).

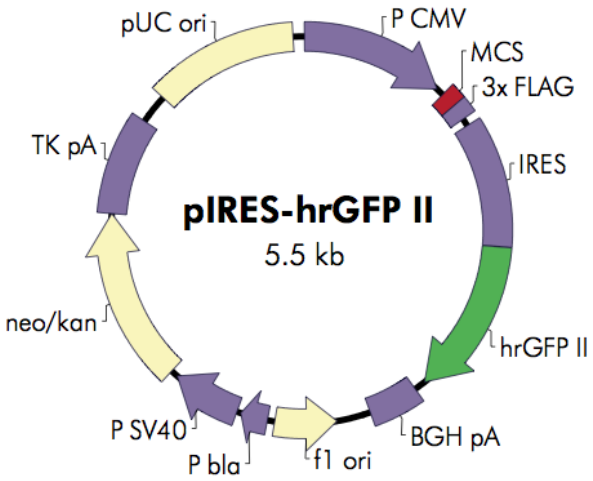

Figure 13: **The pIRES-hrGFPII vector map.** This figure is modified from Agilent (Agilent USA, www.agilent.com).

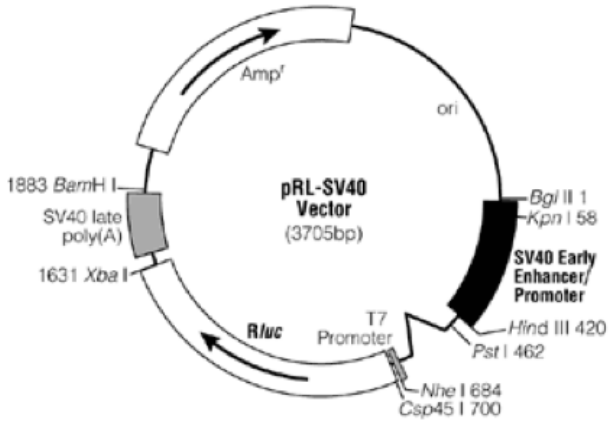

Figure 14: **The pRL-SV40 vector map.** This figure is modified from Promega (Promega Germany, www.promega.com).

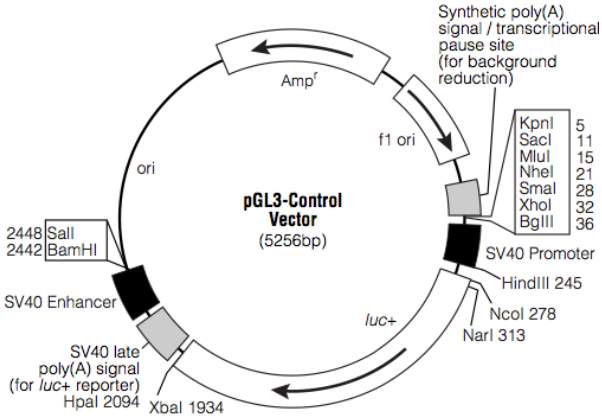

Figure 15: **The pGL3-basic vector map.** This figure is modified from Promega (Promega Germany, www.promega.com).

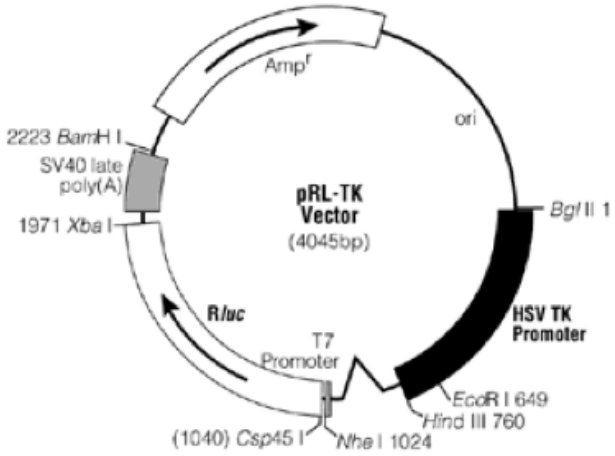

Figure 16: **The pRL–TK vector map.** This figure is modified from Promega (Promega Germany, www.promega.com).

12 Declaration

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13 List of publications

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Anagnostopoulou P, Egger B, Lurà M, **Usemann J**, Schmidt A, Gorlanova O, Korten I, Roos M, Frey U, Latzin P. Multiple breath washout analysis in infants: quality assessment and recommendations for improvement. Physiol Meas 2016 Mar

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European Respriratory Society Meeting 2009, Vienna. “Retinoids modulate TGF–β signalling in the lung through betaglycan and Smad3: implications for retinoid use in bronchopulmonary dysplasia“. Usemann J, Morty RE. Publication E 4753

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European Respriratory Society Meeting 2015, Amsterdam “YKL-40 levels in cord blood - assocations with respiratory symptoms and asthma in children?“ Usemann J, Frey U, Mack I, Schmidt A, Hartl D, Latzin P.

Journal article

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Der Lebenslauf wurde aus dieser Version der Arbeit entfernt.

The curriculum vitae was removed from this version of the paper.