THE ROLE OF SOX2 IN LUNG AIRWAY EPITHELIAL DIFFERENTIATION

DE ROL VAN SOX2 IN DE DIFFERENTIATIE VAN LONG EPITHEEL

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Born in Homa-Bay, Kenya.

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"Sheilah and Adrian, thank you"

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Chapter 1

General Introduction and scope

The foregut is crucial for development of respiratory organs including the lungs. Foregut morphogenesis starts around embryonic day 8.0 in mouse when the endoderm epithelial sheet folds ventrally during gastrulation [1,2]. At embryonic day 9.0, the ventral folding is accompanied by a process in which the rod-like notochord delaminates from the endoderm and becomes closely associated with the neural tube [3]. Signals from the notochord are essential for the dorsal-ventral patterning of the neural tube and its subsequent tissue morphogenesis [4]. Once the foregut tube is formed, it exhibits dorsal-ventral patterning of signaling molecules and transcription factors in both the epithelium and the surrounding mesenchyme which are essential for normal anterior foregut morphogenesis. The dorsal region of the tube results in the oesophagus while the ventral section forms the trachea and lung buds.

The lung: Architecture and Cellular organization

The lung is an essential respiratory organ playing a crucial role in maintaining life by exchanging oxygen from the atmosphere with carbon dioxide in the blood. The structure of the lung represents an evolutionary adaptation which optimizes the area of the functional gas exchange unit, the alveolus, to accommodate the energetic needs of the individual. The alveolus is closely associated with very small blood vessels, capillaries, to minimize the distance between the external atmosphere and internal blood. In medical terms matters relating to the lung often begin with *pulmo-*, from the Latin word pulmonarius ("of the lungs") or with pneumo- (Greek for "Lung"). The branching pattern of the airways of the lung causes a single main airway, the trachea, to eventually divide into millions of alveoli.

The lung is divided into distinct compartments known as lobes. In humans, the left lung is trilobed, whereas the right lung is bilobed. In many quadrupeds including rodents, the lobes of the lung are organized in a slightly different configuration. In most species of rodents, the lung is comprised of a single lobe on the left and 4 lobes on the right [5]. The four lobes encompassing the right lung are commonly referred to as the cranial, middle, caudal, and accessory lobe.

Functionally, the lung is divided into two regions, the conducting and the respiratory region. The conducting region transports air to the respiratory part and is essential for moistening and warming up the air. Moreover, it is a physical barriers and the first line of defence against environmental toxins and pathogens. The respiratory region begins at the level where alveoli

first appear in the final branches of the bronchioles and are important for gas exchange between the alveolar lumen and capillaries appositional to the alveolar walls.

Components of airway structure vary considerably from the trachea to the alveoli. In human, the larger airways are surrounded by cartilage for support, which gradually decrease and are absent in the bronchioles. The trachea is surrounded by C shaped rings of cartilage but as the bronchi enter the lung, the cartilage rings are replaced by irregularly shaped cartilage plates which become less prevalent as the airways get smaller. A layer of smooth muscle cells surrounding the airways becomes more prevalent around the terminal bronchioles, and this layer becomes thinner at the transition from the terminal bronchioles to the alveoli.

The respiratory epithelium is composed of diverse and distinct cell types that differ in abundance along the proximal-distal axis [6,7,8]. The conducting airways are basically composed of basal, Clara, ciliated and goblet cells, but their distribution greatly varies from trachea to bronchiole. The distal alveolar epithelium is mainly lined with two structurally distinct cell types of : the alveolar type I cells, which provide the thin-walled gas exchange surface area, and cuboidal alveolar type II cells, producing numerous secretory vesicles filled with surfactant composites, including surfactant-associated protein C [9,10].

Also present in the lungs are small numbers of neuroendocrine cells appearing in clusters, the neuroendocrine bodies (NEB), which are surrounded by Clara-like cells [11]. Aside from acting as focal sites of secretory cell differentiation in the conducting airway, NEB are also important for regulating proliferation of adjacent cells [12,13]. Moreover, the highly specialized NEBs are closely associated with sensory nerve endings involved in sensing and transducing changes in the airway environment, such as hypoxia [14,15,16].

The mesenchymal compartment of the lung is less well studied and characterized, but interactions of the mesenchyme with the epithelium are crucial for lung branching morphogenesis. Early endoderm separated from the surrounding mesenchyme loses expression of genes that mark early cell fate decisions. However, these markers are completely restored by coculturing the isolated endoderm with mesenchyme or by the addition of FGF4 [10,17]. The mesenchymal compartment mainly consists of blood vessels, lymph vessels, nerves and structural cells, such as fibroblast and lipofibroblasts. At the site of the upper airways and trachea, cartilage rings and plates surround the airways for support.

Lung development

The lung primordium arises from the ventral foregut, just anterior to the developing stomach around embryonic day 9.5 in mouse or 4 weeks in human when a primary bud appears [18] [19,20]. The lung bud splits in two buds, the future left and right bronchus, elongates and the proximal part separates into oesophagus and trachea[19]. Early branching morphogenesis is highly stereotyped and uses four basic modes of branching: domain branching, planar branching, orthogonal bifurcation [21] and a more recently described, trifurcation, which was initially associated with the ureteric bud morphogenesis [22]. These modes of branching act as (i) a master branch generator, driving three routines (periodicity, rotator, bifurcator), (ii) rotational generator, orienting branches around the axis of the airway, and (iii) branch tip division. Precisely, domain branching involves the orderly sprouting of new buds at specific distances from the tip of the stalk and at positions that are either dorsal/ventral or medial/lateral to the parent stalk. Planar and orthogonal branching modes relate to the bifurcation of the tip depending on the axis along which the two new buds are formed. Trifurcations, recently described involves dominant bi/trifurcation at the expense of lateral branching [22]. These modes of branching and morphogenesis are used in a consecutive and reiterative manner in order to build the highly stereotyped bronchial tree [23,24]. Early lung branching pattern is intricately regulated by a network of hard-wired genetic program and signaling factors like Nkx2-1, Fgf10, Wnt signaling and Sox2 among others.

A vascular network is already present at the onset of lung formation, which is in close contact with the developing airways and expands through angiogenesis [25]. The presence of vascular network is evident at E11.5, a time point at which blood flows within the capillary plexus in mouse lung [19,25,26]. The vascular network most likely plays an important, guiding role during branching morphogenesis [26,27,28].Other studies suggested that endothelial cells of the splanchnic mesoderm may be involved in prepatterning of the presumptive lung region as has been shown with endothelial cells of the liver [25,29].

Phases of Lung development

The developing lung progresses through five overlapping phases based on histology: embryonic, pseudoglandular, canalicular, saccular and alveolar [30]. The earliest phase, embryonic, occurs between embryonic days 9.5 to 11.5 in mouse. During this phase, the morphologic changes observed are mediated by diffusible signals from the surrounding mesenchyme (Reviewed in [10,17]). Early endoderm isolated from surrounding mesenchyme

shows loss of expression of genes marking early cell fate decisions. This loss of expression is partially rescued by FGF4 in culture, suggesting its importance during this period [17]. Other signaling molecules affecting early embryonic cell fate decisions from the mesenchyme include TGF-β, BMP, Sonic Hedgehog, WNT, and FGF families [10,17]. As development of the lung advances, the embryonic endoderm undergoes progressive fate decisions that generate epithelial progenitor cells with increasingly restricted developmental potential over time. During this period, Nkx2.1 expression can be used as a marker to distinguish the future lung from other foregut derivatives in endoderm. Most factors involved in branching morphogenesis have been subject of discussion and are addressed in recent reviews [10,18,19,31]. The pseudoglandular phase is characterized by the commencement of differentiation of epithelial cells, which proceeds from embryonic day 12 till 15. Early stages of pseudoglandular phase are marked by the appearance of neuroendocrine bodies which serve as precursor cells of the conducting airways [32,33], whereas late stages of this phase are marked with the expression of ciliated cell marker FoxJ1 [34]. At embryonic day 16.5 lung development switches from branching morphogenesis to the canalicular and saccular stages [19]. During the canalicular phase, the terminal tubes narrow giving rise to small saccules and the endoderm begins to differentiate into specialized alveolar type I and type II cells [19]. The alveolar phase is characterized by the establishment of secondary septa resulting into alveolar formation, which mainly takes places after birth. Several signaling molecules like Pdgfa and fibrillin1 are important during alveolar septation as recently reviewed in [19].

Regulation of foregut and lung development

The primitive gut is gradually regionalized by the interaction with the surrounding mesenchyme, and the expression of Sox2 and Cdx2 mark the anterior and posterior parts, respectively [17]. The morphogenesis of the foregut and lung is subsequently regulated by a myriad of transcription factors and signaling cascades. The molecular and cellular events contributing to lung development and the separation of the trachea and esophagus have been extensively described in recent reviews [10,19,35]. Regionalization of the different parts of the gut is controlled by the localized expression of *Hox* genes [36]. Specifically *Hoxa3* and *Hoxb4* are expressed in the foregut endoderm, whereas *Hoxc5* and *Hoxa13* are respectively expressed, in the midgut and hindgut endoderm [36,37]. Sonic Hedgehog (Shh), is expressed in the entire endodermal layer other than the pancreas [36]. During tracheal-eosophagus development, Shh is specifically and dynamically expressed [38]. Shh is expressed early

during the patterning of the ventral foregut whereas its expression is transiently inhibited in the tracheal endoderm [38].

Transcription factors that show specific expression profiles in the endoderm, include SRY-related High-Mobility Group (HMG) transcription factors *Sox2* (foregut endoderm) and *Sox9* (midgut/hindgut endoderm) [39,40,41]. The homeodomain transcription factor *Nkx2.1* (TTF1) is specifically expressed in the anterior part of the foregut and in the endoderm of the developing trachea but not of the esophagus [42]. The spatial expression of these genes in the foregut suggests that foregut derived malformations may be caused by aberrant regulation of these genes.

Sox2 is expressed throughout the early foregut epithelium, but becomes restricted to the dorsal epithelial cells at embryonic day 9.5, whereas Nkx2.1 is reciprocally expressed in the ventral epithelium [20,41,43]. The proper dorsal-ventral patterning of Sox2 and Nkx2.1 is critical for foregut morphogenesis. Down-regulation of Sox2 leads to the formation of Esophageal Atresia/Tracheo-Esophageal Fistula (EA/TEF) in Sox2 hypomorphic mutants [20], whereas deletion of Nkx2.1 leads to defects in foregut separation and the formation of EA/TEF associated with high Sox2 expression in the epithelium [20,44]. Similarly, the epithelial cells in the fistula of Sox2 hypomorphic mutants express high levels of Nkx2.1 suggesting that low level of Sox2 is required for Nkx2.1 expression to expand dorsally and reprogram the dorsal epithelium to a respiratory fate [20]. These findings suggested that the dorsal-ventral arrangement of Sox2 and Nkx2.1 is essential for foregut separation and the subsequent differentiation of epithelial progenitor cells into oesophageal and tracheal epithelium and lung buds.

It has been shown using human embryonic stem cells, that SOX2 binds the promoter region of the NKX2.1 gene inhibiting its transcription [45]. Another study using in vitro organ cultures demonstrated that signaling molecule Fgf10 inhibits Sox2 expression in the mouse foregut [20]. In early embryogenesis, FGF10 is secreted from the visceral mesenchyme in the vicinity of the heart [46]. Mesenchymal FGF10 functions as a chemoattractant for airway branching by binding to the epithelial expressed FGF receptor 2b (FGFR2b), which subsequently causes the outgrowth of lung buds [47,48]. The functional interaction between Fgf10 and Fgfr2b was shown by the high similarity between the Fgf10-null and Fgfr2b-null mouse mutants [49,50]. Fgf10 knockout mice developed normal trachea, but completely lacked lung structures [49,51], whereas targeted deletion of Fgfr2b prevented branching, causing the trachea to

terminate as a blind-ended sac [52]. Conditional gene inactivation studies further demonstrate that both Fgf10 and Fgfr2b are required for a normal branching program and proper proximal–distal patterning of the lung [53]. Interestingly,Fgf10 and Dkk1 overexpression rescues defects in branching and proximal-distal differentiation suggesting that Fgf10 functions as a permissive factor but not important for branching morphogenesis [54].

It was recently shown that ubiquitous overexpression of Fgf10 throughout the lung could rescue lung agenesis in Fgf10 knockout mice, suggesting that precise localization of Fgf10 expression is not required for lung branching morphogenesis in vivo. Moreover, mesenchymal expression of Fgf10 is regulated by Wnt signaling [54]. This study further demonstrated that Fgf10 overexpression is not sufficient to rescue the lack of specification of primary lung field in embryos overexpressing secreted modulator of Wnt signaling, Dkk1. However, after this developmental time point when lung initiation has occurred, the overexpression of Fgf10 rescues the Dkk1 overexpression induced defects in branching and proximal-distal differentiation [54]. Additionally, Fgf10 activates β-catenin signaling and prevents the distal epithelial progenitors from differentiating into airway epithelial cells by inhibiting Sox2 expression, suggesting that Fgf10 regulates proximal-distal differentiation by maintaining cells in a branch-responsive state [20,54,55,56,57,58]. In addition, FGF signaling via the Erk/MAPK pathway can phosphorylate the Wnt co-receptor Lrp6 on Ser1490 and Thr1572 and phosphorylates β-catenin directly on Tyr142, thereby releasing it from cadherin complexes [59]. In turn, epithelial β-catenin activation participates in the induction of Fgfr2b expression to further increase the effect of Fgf10 signaling [60]. This regulation of distal epithelial progenitors by β-catenin suggests a progressive signaling cascade where Fgf10 regulates branching morphogenesis via the Wnt pathway.

Several studies analysed the role of Wnt signaling in foregut morphogenesis. Wnt proteins constitute a large family of highly conserved, secreted glycoproteins, which are crucial in mediating cell-cell signaling during embryogenesis [61,62,63]. Wntless (Wls), a cargo receptor protein important for directing Wnt ligands has recently been shown to be important for lung differentiation and vasculature development [64]. Nineteen different WNT proteins are identified in humans, most of which are expressed in a tightly regulated spatiotemporal manner depending on the ligand engagement [65]. Wnt signaling is transduced through either the canonical Wnt/β-catenin pathway or the non-canonical, β-catenin-independent pathway. Transduction of the signal through the canonical pathway is activated after binding of the Wnt3a or Wnt7b ligands to their receptors, Frizzled (Fzd) and LDL-receptor-related proteins

(Lrp) -5/6, preventing the phosphorylation of β-catenin by glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). Stabilization of β-catenin promotes its accumulation and consequent translocation into the nucleus where it interacts with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family to activate the transcription of target genes [61,66].

Canonical Wnt/β-catenin signaling displays a dynamic pattern, both before and after the separation of the trachea and oesophagus. At embryonic day 9.5, Wnt signaling is active in the ventral side of the unseparated foregut tube, where Wnt ligands Wnt2 and Wnt2b proteins are highly expressed [43,67]. Wnt2 and Wnt2b are expressed in the mesenchyme of the ventral foregut, while the Wnt signal receiving cells are located in the epithelium. Deletion of β-catenin in the foregut epithelium using Shh-Cre leads to abnormal separation of the foregut tube and complete lung agenesis [43,67]. Additionally it has been shown at cellular level that Wnt/β-catenin abrogation reduces cell proliferation by diminishing Cyclin D1 protein levels [67]. Furthermore, Shh-Cre;β-catenin$^{loxp/loxp}$ mutants, in which β-catenin has been deleted in the foregut epithelium, show a disruption in dorsal-ventral patterning of Sox2/Nkx2.1 characterized by an expansion of the Sox2 expression domain in the ventral region at the expense of the Nkx2.1-domain [43]. Consistent with the importance of Wnt expression in foregut separation, it has been demonstrated that deletion of Wnt2 and Wnt2b results in a similar phenotype [67]. Wnt7b is expressed in the endoderm of the early foregut and its deletion does not disrupt foregut separation, but results in irregular lung branching morphogenesis and vasculature development [68]. Wnt2 and Wnt7b cooperate to drive high levels of Wnt signaling activity specifically in mesenchymal lineages mediated by the Frizzled receptor [69]. Mesenchymal inactivation of either Wnt2 or Wnt7b leads to hypoplastic lungs with defects in smooth-muscle development [67]. Deletion of Wnt5a or Wnt11 has no separation defects in the foregut, possibly due to functional redundancy of non-canonical signaling [70,71]. Gain-of-function studies on activity of canonical Wnt signaling using a constitutive active form of β-catenin have recently demonstrated that Wnt signaling has no effect on alveolar development, although these mice exhibited air space enlargement [56]. Canonical Wnt signaling is a key regulator in foregut - and lung development, but it may be possible that additional signaling cascades are also involved in the process.

Bmp signaling plays prominent roles in foregut separation and lung development [72,73], but the molecular mechanisms controlling temporal-spatial Bmp signaling dynamics in foregut organogenesis are poorly understood. In the unseparated foregut tube, Bmp4 is expressed in

the ventral mesenchyme while Bmp7 and the Bmp antagonist Noggin are enriched in the dorsal endoderm [74]. Disruption of dorsal-ventral patterning by Noggin deletion leads to the formation of EA/TEF. Noggin deletion also induces abnormal delamination of the notochord from the early definite endoderm epithelial sheet, resulting in epithelial cells of endodermal origin being present in the notochord [75]. In addition, Noggin deletion leads to increased Bmp signaling in the foregut. Deletion of either Bmp4 or Bmp7 in the Noggin null mice rescues the separation defects [74,75]. Furthermore, tissue specific ablation of Bmp4 using Foxg1-Cre expressed in early foregut endoderm results in tracheal agenesis accompanied by reduced cellular proliferation in the epithelial and mesenchymal compartments. However, the trachea does not separate from the foregut and Nkx2.1 expression is conserved to the ventral endodermal epithelium, suggesting that Bmp4-mediated signaling is essential for separation but not for the initial specification of the tracheal epithelium [76]. Similarly, conditional inactivation of Bmp receptors 1a and 1b in the foregut leads to tracheal agenesis, a decrease of Nkx2.1 expression and a ventral expansion of Sox2 expression. However, these mutants have normal Wnt signaling activity in their foregut, demonstrating that Wnt signaling does not function downstream of Bmp during foregut separation. Complementing the role of Wnt signaling in foregut morphogenesis, Wnt ligand subfamilies have been shown to cooperate in promoting high levels of signaling in a cell lineage specific manner through Pdgf signaling [69]. Deletion of Sox2 in a $Shhcre^{/+}$; $Bmpr1a^{fl/-}$; $Bmpr1b^{-/-}$ mouse rescued the foregut separation defect, showing that Sox2 is downstream of Bmp signaling [77]. BMPs can also signal via Smad-dependent intracellular pathways whereby Smad1/5/8 and Smad4, are the key mediators of the pathway. Upon Bmp ligand binding, Smad1/5/8 are phosphorylated and associate with Smad4, followed by nuclear translocation and activation of downstream target gene transcription [78]. Smad4 is most active at embryonic day 9.5 in the mesenchyme and epithelium of the ventral foregut. Deletion of Smad4 using Nkx2.5-Cre at embryonic day E9.5 does not affect foregut separation, suggesting that simultaneous loss of Bmp and Tgfβ signals rescues foregut separation defects [79]. Sox17, a member of Sox family of transcription factors inhibits Tgfβ/smad3 signaling to initiate progenitor cell behaviour in the lung respiratory epithelium [80]

Sox Genes

Sox genes are a family of highly conserved transcription factors identified and named based on their homology to Sry [81]. All Sox transcription factors are composed of an N-terminal region, a 79-amino acid high-mobility group (HMG)-box domain, and a C-terminus

containing either a transactivation or repression function [82] [83]. The Sox gene family was discovered through the cloning of Sry, the mammalian sex-related Y-linked testis-determining gene [81,84,85]. In mice and humans, there are 20 Sox genes, classified into 8 different groups from A to H on the basis of the HMG box domain [81,86,87]. Sox proteins in the same group share a high level of homology both within and outside of the HMG box domain, whereas proteins from different groups share much less homology [88] [89]. All Sox proteins recognize and bind a DNA sequence motif related to CTTTG [86,90,91,92,93]. Sox transcription factors bind the minor groove of the DNA and induce a bend in the DNA helix [94,95,96]. In addition to its role in DNA binding and bending, the HMG domain also mediates protein–protein interaction [96,97,98] and nuclear localization [99]. Because Sox transcription factors have similar DNA binding specificities, their ability to trigger specific biological processes is thought to be mediated by selective interactions with cofactors. Sox proteins generally exhibit their gene regulatory functions by forming complexes with partner transcription factors [97,100,101], for instance Sox2 cooperates with Oct4 to induce pluripotency [81,86,102,103,104]. Therefore, a functional Sox DNA binding site usually is flanked by a binding site for another transcription factor that cooperates with the Sox protein to regulate transcription. Known factors for proper function of Sox proteins are POU domain factors, zinc finger proteins, basic helix–loop–helix and leucine zipper proteins [105,106,107,108].

To date, four members from Sox gene family are known to be involved in lung organogenesis, Sox2, Sox9, Sox11 and Sox17 [41,80,109,110,111,112,113]. *Sox9* was found throughout lung morphogenesis as a downstream gene of Sonic Hedgehog (Shh) and modulated by bone morphogenesis protein 4 (Bmp4) and Noggin. Using gain/loss function models in the epithelium, Sox9 has been shown to play a crucial role in branching morphogenesis through controlling a balance between proliferation and differentiation [114]. In another study, knock out of Sox9 in the mesenchyme demonstrated that it plays a crucial role in differentiation of the lung tracheal epithelium [115]. Sox9 is required for formation and patterning of tracheal cartilage by a mechanism mediated by fibroblast growth factor 18 (FGF18) [116,117]. Sox9 promotes proper branching morphogenesis by controlling the balance between proliferation and differentiation and regulating the extracellular matrix [114] However, specific inactivation of Sox9 later in lung development in respiratory epithelial cells does not alter lung structure, postnatal survival, or repair oxygen injury, indicating that Sox9 might not play an essential role in the respiratory epithelial cells [118]. *Sox11* has been suggested to be involved in development and plays a key function in tissue remodeling including the lung

[111]. Sox11 deficient mice die immediately after birth because of significant lung hypoplasia and other tissue defects [111]. *Sox17* was shown to be crucial for the formation of definitive endoderm, which gives rise to the lung, liver, pancreas, stomach, and gastrointestinal tract [119]. Sox17 is expressed in respiratory epithelial cells of the fetal lung at embryonic day 18 and is restricted primarily to ciliated cell in the postnatal and adult lung [80,113,120,121]. Sox17 is important for activation of the cell cycle, re-initiation of multipotent progenitor cell behavior in mature lung cells, as well as other development processes such as cardiovascular development, fetal hematopoietic stem cell maintenance, and angiogenesis [80,120,122,123,124]. Ectopic expression of Sox17 in the epithelial cells of the mouse embryonic lung inhibits peripheral epithelial cell differentiation and disrupts branching morphogenesis [80]. Additionally, its conditional expression in mature respiratory epithelial cells of mice displays formation of hyperplastic clusters of cells and respecification of alveolar progenitor cells toward proximal airway lineages [80,113]. As mentioned previously, Sox17 has been shown to induce reduction in the expression of TGFβ1 responsive inhibitors, p15, p21 and p57, while inhibiting TGF-β1 and Smad3 transcriptional activity indicating its importance in regulation of crucial processes in the lung [80].

Sox2

Sox2 plays a critical role in the formation of many different tissues and organs during embryonic development. In mice, expression of Sox2 starts from the 4-8 cell stage of embryo development and is initially expressed in the inner cell mass (ICM) and extra-embryonic ectoderm of blastocyst [125]. Sox2 is expressed exclusively in the conducting airways from foregut to mature lung, where it plays a crucial role in proliferation and differentiation of respiratory epithelial, trachea, airway branching, and Clara cells [41,109,112,126]. Previous studies in transgenic mice that express a doxycycline inducible Sox2 in the epithelium, demonstrated that ectopic expression of Sox2 during embryonic development leads to distal lung abnormalities and induction of basal cell differentiation [41]. Selective deletion of Sox2 in Clara cells lead to the progressive loss of ciliated cells, the ability of goblet cell differentiation and mucus production in response to allergen-induced lung damage [126]. Conditional expression of Sox2 in a subset of respiratory cells induces proliferation in non-ciliated airway epithelial cells [112]. In the same study, the subset of Sox2 positive alveolar epithelial cells expressed proximal epithelial markers and showed hyperplastic lesions, however, expression of Sox2 did not result in tumors [112].

Sox2 in human pathology

Heterozygous mutations in SOX2 have been associated with AEG syndrome, in diverse clinically recognizable features known as Anophthalmia (lack of eyes), Esophageal atresia (Discontinuous oesophagus) and Genital abnormalities (AEG syndromes) [127]. A related congenital syndrome is CHARGE, and because of the overlapping features, AEG and CHARGE patients are sometimes misdiagnosed. The chromohomeodomain protein CHD7 has been linked to CHARGE and recently a physical and functional association of SOX2 and CHD7 was shown [128]. Loss of function experiments in mice has demonstrated that Sox2 is necessary for development of multiple tissues such as the eye, brain and oesophagus/trachea. Genomic screenings of human lung squamous cell carcinoma indicated that Sox2 is the most amplified locus in human squamous carcinoma and is differentially expressed between squamous cell carcinoma and adenocarcinoma [129,130,131,132]. Ectopic expression of Sox2 in fetal mice resulted in localized macrocystic or microcystic lung malformations, depending on the duration of overexpression [41]. Although these cysts resembled the human Congenital Cystic Adenomatoid Malformation (CCAM), the genetic background of the origin of CCAMs is unknown.

Congenital lung Malformations

Congenital malformations of the lung constitute a spectrum of lesions that originate during the embryonic period. Incidences of congenital lung malformations are in the range of 1 in 11.000-35.000 pregnancies (WHO). Patients with congenital lung malformations are presented with a broad range of respiratory symptoms that either cause significant illnesses at birth presenting patients with respiratory distress and repeated chest infections or remain asymptomatic for long periods. Congenital lung malformations occur either in isolated cases or as part of a complex syndrome. The causes of most of these malformations are still unknown.

There are different types of congenital lung malformations:

1. **Congenital lobar emphysema** (**CLE),** also known as infantile lobar hyperinflation, is a rare lung malformation, with incidence ranging from 1:20,000 births to 1:30,000 births [133,134,135]. CLE is characterised by distended, hyperlucent lobe distal to the terminal bronchiole with destruction of the lobe walls. CLE usually affects the left upper or right middle lobe [136]. This localized area of emphysema presents as respiratory distress in early infants. Histologically, the parenchyma of CLE shows non-specific distension.

2. **Pulmonary sequestrations (PS),** commonly referred to as bronchopulmonary sequestrations (BPS), are characterized by normal, non-functioning lung tissue that has no connection with the bronchial tree and receives blood supply from the systemic circulation. Pulmonary sequestrations can be classified as extra-lobar or intra-lobar depending on their location in relation to the adjacent normal lung and their visceral pleural covering [137,138,139].

3. **Congenital Cystic Adenomatoid Malformation (CCAMs),** are lung lesions that result from disordered development of the lower respiratory tract. CCAMs are characterized by a multicystic mass of lung tissue with proliferation of bronchial structures and lung tissue showing aberrant, differentiated architecture, with various degrees of cyst formation [136] [140]. They have a wide spectrum of severity and vary substantially in size and composition. There are five types of CCAM mainly classified according to cyst size, of which type I and type II are the most frequent forms [140,141]. Type I CCAM is composed of single or multiple large cysts, while type II CCAM is characterized by multiple small cysts. Three additional CCAM types exist; type 0 CCAM involving all lung lobes, is a state not compatible with life, type 3 are predominantly solid lesions with cysts, and type 4 CCAM lesions are large peripheral thin-walled cysts [140].

The possible mechanisms and the exact period during gestation by which CCAM develop are yet to be determined, however there are previously reported theories suggesting the cause of the disease. One theory hypothesize that CCAM is caused by airway obstruction. The different presentation and types of lesions are accounted for by the timing and location of obstruction [142]. Another theory claims that CCAM originates as a result of imbalance between cell proliferation and apoptosis during airway branching [143,144]. Alterations of genetic expression of factors controlling normal lung development have been shown to result in CCAM-like phenotypes. SOX2 (Chapter 4), FGF10 [145], HOXB5 [146], Fatty acid binding protein [147] and CC10 [148] in rat and mouse models suggesting that these genes may play a role in CCAM pathogenesis reviewed in [149]. Moreover, published microarray data revealed a 6 fold up-regulation of SOX2 in the CCAM material when compared with control [147].

Sox2 in Lung Injury and Repair

The respiratory tract has an extensive cell surface area that is directly exposed to toxicants, pathogens and particles. These cells mediate gas exchange, surfactant homeostasis, mucociliary clearance as host defence to pathogens in order to maintain lung stability and

sterility A number of pharmacological approaches have been used for studying damage and repair processes in the adult lung. In all systems, the response of the epithelium varies with the region being studied, upper or lower airway damage, and mouse strain and sex. Details on the roles of Sox2 in lung damage are still scarce. Sox2 is exclusively expressed in the upper airways of mouse lungs [41,126], however Sox2 upregulation had been reported in the intermediate cuboidal cells two days after lung damage [120]. Physical damage of the epithelium allows tissue exposure to both exogenous and endogenous infectious and damaging agents which leads to airway wall structural changes, such as goblet cell hyperplasia, squamous metaplasia, disruption of tight junctions, loss of structural integrity, desquamation of epithelial cells and denudation of basement membrane, which in turn lead to inflammatory reaction and tissue damage. One widely used pharmacological injury model is naphthalene exposure, which destroys most of the secretory Clara cells. Naphthalene is administered by intraperitoneal injection and is metabolized by bronchiolar Clara cells into a toxic by-product that causes apoptosis in the majority of the Clara cells. After naphthalene induced injury, neighbouring epithelial cells start to spread and migrate along the basement membrane to cover the denuded area in a process called restitution. Different cell populations have been identified that proliferate in response to naphthalene injury. These include the basal cells in the trachea and primary bronchi, the naphthalene-resistant Clara cells in the more distal bronchi, bronchioles and the bronchoalveolar duct junction (BADJ) [150,151]. It has been shown that naphthalene-resistant Clara cells indirectly proliferate, giving rise to other cell lineages. The repair process does not occur if all Scgb1a1 cells are ablated, including both the naphthalene-sensitive Clara cells and the naphthalene resistant Clara (putative stem) cells [152]. Additionally, cells isolated from the BADJ can give rise to several differentiated cell types when cultured in vitro [153]. Evidence that basal cells in the upper airways can both proliferate and function as stem cells after naphthalene injury is based on in vivo lineage labelling [150,152].

Damage of the lungs may also be induced by bleomycin. It has been shown by in vivo and in vitro studies that bleomycin, a cancer chemotherapeutic agent, generates toxic O_2-derived species within the lung [154]. Particular susceptibility to bleomycin toxicity in the lung may depend on the fact that bleomycin is preferentially distributed in lung tissue and that the lung is relatively deficient in the hydrolase enzyme that detoxifies bleomycin [155].

SCOPE OF THE THESIS

Previously, our laboratory showed that Sox2 is crucial for branching morphogenesis and differentiation of cells in the lung [41]. Ectopic Sox2 expression induced the emergence of basal cells, which initiated the work described in chapter 2 to elucidate the molecular basis of this observation. Aside from the fact that we found that Sox2 directly regulates the transcription of the basal cell master gene Trp63, we also found that Sox2 regulates Gata6, another transcription factor possibly involved in the formation of bronchio-alveolar stem cells. Next, we investigated the plasticity of terminally differentiated cells by ectopically expressing Sox2 in alveolar type II cells, as described in chapter 3. Furthermore, we examined whether the observed phenotype in the Sox2 expressing mouse model resembled the pathogenesis of human Congenital Cystic Adenomatoid Malformation (CCAM), which is described in chapter 4. Surprisingly, we found that the affected areas of lungs from human Congenital Cystic Adenomatoid Malformation type II patients express SOX2 and TRP63, but type I patients do not. This could hint at a possible difference in the origin of these two types of CCAM. As part of the interest in epithelial differentiation, we investigated the role of the hypoxia inducible factor 3α (Hif3α) and the development of alveolar type II cells (chapter 5). Lastly, we discuss our findings in chapter 6 and indicate the relevance of our study.

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Chapter 2

SOX2 REGULATES THE EMERGENCE OF LUNG BASAL CELLS BY DIRECTLY ACTIVATING THE TRANSCRIPTION OF *TRP63*

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SOX2 REGULATES THE EMERGENCE OF LUNG BASAL CELLS BY DIRECTLY ACTIVATING THE TRANSCRIPTION OF *TRP63*

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Running title: Sox2 controls basal cells by regulating Trp63

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Abstract

Lung development is determined by the coordinated expression of several key genes, and previously, we and others have shown the importance the SRY (sex determining region Y)-box 2 (Sox2) gene in lung development. Transgenic expression of Sox2 during lung development resulted in cystic airways and here we show that modulating the timing of ectopic Sox2 expression in the branching regions of the developing lung results in variable cystic lesions resembling the spectrum of the human congenital disorder Congenital Cystic Adenomatoid Malformation (CCAM). Sox2 dominantly differentiated naïve epithelial cells into the proximal lineage, irrespective of the presence of Fgf10. Sox2 directly induced the expression of Trp63, the master switch towards the basal cell lineage. In addition, Sox2 induced the expression of Gata6, a factor involved in the emergence of bronchioalveolar stem cells. In addition, we showed that SOX2 and TRP63 are co-expressed in the lungs of human CCAM type II patients. The combination of premature differentiation towards the proximal cell lineage and the induction of proliferation finally resulted in the cyst-like structures. Thus, we show that Sox2 is directly responsible for the emergence of two lung progenitor cells, basal cells by regulating the master gene Trp63, and bronchioalveolar stem cells by regulating Gata6.

Keywords

Mouse, lung, Trp63; Sox2, basal cells, Gata6, BASC cells

Introduction

Development of the lung starts early after gastrulation when cells at the ventral site of the foregut form a primitive bud and invaginate the surrounding mesenchyme. Subsequently, this lung bud branches in a coordinated, repetitive process, called branching morphogenesis, to generate the primary bronchial tree (1-2). Failure to complete the lung morphogenesis program correctly may lead to congenital abnormalities, such as congenital diaphragmatic hernia and congenital cystic adenomatoid malformation of the lung (CCAM) (3). CCAMs are rare developmental lung defects characterized by cystic areas and adenomatous overgrowth of the terminal bronchioles (4). The pathology of the CCAMs displays a large variation and therefore patients are classified according to the different types of lesions (types 0-4). The incidence of CCAM is 1 in 35,000 live births and the pathogenesis of CCAM development is still unknown.

A putative role for the high mobility group (HMG) box transcription factor Sox2 in lung development was based on its expression pattern in the foregut, and recently, we and others showed the importance of Sox2 in lung development (5-7). Sox2 is cell-autonomously required in both embryonic and extra-embryonic cell types, and the lack of Sox2 results in peri-implantation lethality (8). Later in development, Sox2 is involved in several processes during neurogenesis and sensory organ formation, such as structures in the ear and eye (reviewed in (9)). Moreover, Sox2 is important to maintain pluripotency and self-renewal in embryonic stem (ES) cells as well as epithelial stem cells in multiple tissues (9). It is also one of the original factors required for reprogramming somatic cells into pluripotent stem cells (iPS) (10). In lung, Sox2 is expressed in epithelial cells of the foregut, which later develops into the pharynx, oesophagus, trachea, bronchi, and bronchioles (5). However, Sox2 expression is excluded from the peripheral and alveolar regions of the lung. Hypomorphic mouse mutants with reduced Sox2 expression demonstrated that Sox2 is involved in the morphogenesis of the trachea and oesophagus, as well as the differentiation of the oesophageal epithelium (11). We showed that Sox2 is involved in branching morphogenesis and epithelial cell differentiation, since ectopic expression of Sox2 led to an increase in committed precursor-like cells, such as neuroendocrine and

basal cells (5). Ectopic expression of Sox2 in Clara cells of adult mice resulted in the development of p63-positive carcinomas (12).

Basal cells are relatively undifferentiated cells present in the epithelium of the trachea and upper airways, and are the residing stem cell population of the upper airways that regenerates damaged epithelium (13-14). In mouse lungs, basal cells make up 30% of the pseudostratified mucociliary epithelium and they are characterized by the expression of the transcription factor Trp63 (p63) (14-15). Trp63 is a homologue of the tumor-suppressor p53 and functions as a master regulator of epidermal development. Trp63 is expressed in the basal or progenitor cell layer of stratified epithelia (e.g., squamous, urothelial, bronchial), in the basal cells of some glandular epithelia (e.g., prostate), as well as in myoepithelial cells of breast and salivary glands, trophoblasts and thymic epithelial cells (16). Mutations in Trp63 leads to dominantly inherited clinical conditions, like ectrodactyly (split hand/foot malformation), orofacial clefting and ectodermal dysplasia with defects in skin, hair, teeth, nails and exocrine glands. Mice deficient of Trp63 lack normal epidermis and die shortly after birth (15, 17-19). The Trp63 -/- mice completely lacked basal cells in the trachea and esophagus, and instead developed a highly ordered, columnar ciliated epithelium. These observations indicated that Trp63 is for the commitment of early stem cells into basal cell progeny and the maintenance of basal cells (15).

Regulation of Trp63 is complex by the usage of different promoters, generating six isoforms , which are grouped into trans-activating (TAp63) and non-transactivating (ΔNp63) isoforms. Both the TAp63 and ΔNp63 transcripts are differentially spliced at the 3′ end generating proteins with unique C-termini, designated as α, β, and γ isoforms. The β and γ are shorter isoforms, while the longer Trp63α isoforms harbour a Sterile Alpha Motif (SAM) domain, which is thought to mediate protein-protein interactions. ΔNp63 is the predominant isoform in basal cells and can transcriptionally activate or repress target gene expression (20-23). Recently, we showed that ectopic Sox2 induces the expression of the ΔNp63 isoform in the lungs (5).

In order to study the role of Sox2 in the regulation of basal cells, we ectopically expressed Sox2 in mouse distal epithelial cells starting early in gestation, when the mouse lung starts to branch until the later phases of gestation. We observed a correlation between the time of transgene induction and the appearance and size of cystic lesions, which resembled the different forms of human CCAMs. Furthermore, we showed that Sox2 directly binds and transactivates the Trp63 promoter of the ΔN isoform, which indicates a direct involvement of Sox2 in the emergence of $Trp63^{+}$ basal cells. In addition, we showed that Sox2 directly influences the emergence of bronchioalveolar stem cells (BASCs) in the lung by regulating Gata6 expression. Lastly, we analysed the expression of SOX2 in lung samples of human type I and type II CCAM patients.

Materials and Methods

Detailed materials and methods sections is available as supplemental data published on-line

Tissue preparation and Immunohistochemistry

Human lung samples were retrieved from the archives of the Department of Pathology, Erasmus MC, Rotterdam, following approval by the Erasmus MC Medical Ethical Committee. Mouse lungs were processed according to routine protocols (5, 24).

Mesenchyme free cultures s

Embryonic lung mesenchyme was isolated and cultures in Matrigel^{TM} (BD Bioscience) with 250 ng/ml Fgf10 (R&D systems) and 0.6μM doxycycline.

*Cell Transfection***.**

HEK-293T cells were transiently transfected using Lipofectamine LTX (Invitrogen) with appropriate luciferase constructs and expression plasmids. Luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega, Germany).

Chromatin Immunoprecipitation assays

ChIP assays were performed essentially as previously described, using $6x10^7$ A549 cells cultured in 150 μM $CoCl_2$ to induce hypoxia (24). PCR primers are listed in Table 1

Results

Timed Sox2 expression in the lung leads to cyst-like malformations in distal lung

We first studied the morphological abnormalities caused by ectopic expression of Sox2 in the developing lung using the SPC-rtTA transgenic driver mouse line (iSox2$^{SPC\text{-}rtTA}$; (25)). The induction of Sox2 in the epithelial cells of the developing airway from the onset of lung development resulted in the appearance of large, cystic lesions, as we previously reported (Fig. 1C) (5). Next, we analysed whether modulation of the transgenic Sox2 expression had an effect on the size of the cystic abnormalities. Therefore, we varied the administration of doxycycline to express the transgenic Sox2 at later time points during gestation. Administration of doxycycline was started at the onset of branching morphogenesis (E10.5), at the pseudoglandular stage (E12.5) and at the canalicular stage (E15.5) to monitor the development of the lungs (Fig. 1A). Lungs were isolated at gestational age 18.5, just prior to birth, and morphological analysis of the lungs showed that iSox2$^{SPC\text{-}rtTA}$ lungs had mild to severe cyst-like defects in the distal regions, which correlated with the duration of transgene induction (Fig. 1D-1F). Lungs from wild type control mice, single transgenic animals, or non-induced iSox2$^{SPC\text{-}rtTA}$ animals appeared healthy and did not show apparent abnormalities (Fig. 1B).

Histological analysis of the lungs showed that prolonged expression of Sox2 resulted in dilation of the developing airways with limited branched airways, suggesting that ectopic expression of Sox2 in the airway epithelial cells prevented branching morphogenesis (Fig. 1G-1K). Microscopic examination of the iSox2$^{SPC\text{-}rtTA}$ lungs revealed a disorganized epithelium lining the cystic lesions (Fig. 1 M, N; arrows), which was absent in control lungs (Fig. 1L). Analysis with specific epithelial markers was done and showed a distribution as previously reported, with all differentiated cells being present, albeit their distribution was slightly changed depending on the penetration of the SPC-rtTA expression (data not shown; (5)). In order to analyse whether switching off iSox2 expression would restore normal lung branching and growth, the transgene was activated early in development at E6.5, after which the doxycycline was removed at different time points (E12.5, E14.5 or E16.5; Suppl. Fig. 1A). Analysis of the lungs at E18.5 showed that withdrawal of doxycycline led to the initiation, or reactivation, of the lung developmental program from the time the switching off of the transgene occurred (Suppl. Fig. 1B-E). The lungs showed cystic structures more internally in the lung and less to the periphery as

compared to continuous iSox2 expression (compare Suppl. Fig. 1F-I with Fig. 1H). Together, these data show that variation in cyst size is dependent on the timing and duration of transgenic Sox2 expression. Moreover, late induction of Sox2 resulted in normal development of the primary branches up to the point when expression of Sox2 was induced by doxycycline, suggesting that it has a dominant effect on the cells involved in branching.

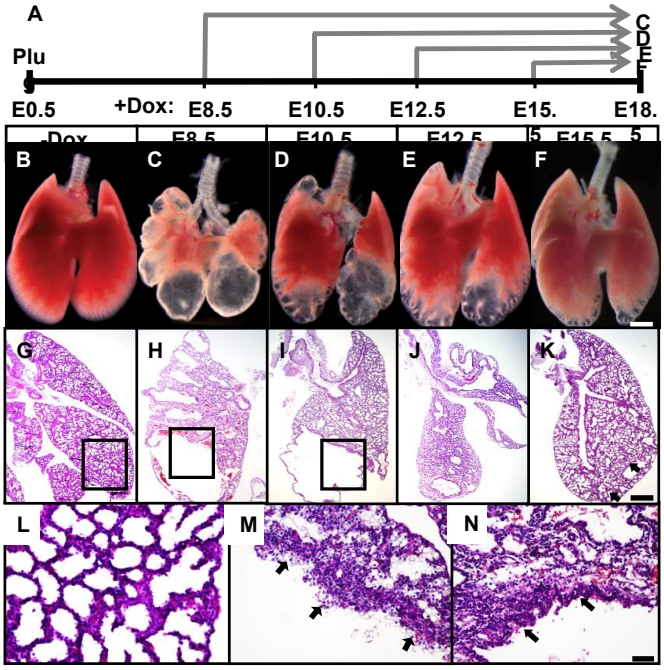


Figure 1: Ectopic Sox2 expression causes variable cyst sizes in the lung. Control and iSox2$^{Spc\text{-}rtTA}$ mice were treated with doxycycline for a specified period as indicated in the schematic overview. Lungs were isolated at E18.5 and analysis of the external appearances shows varied cystic abnormalities in lung structure, consisting of loss of peripheral airspaces and disruption of branching morphogenesis (B–F). Low (G-K) and high power images (L-N, enlarged boxes of G-I) of haematoxylin/eosin stained sections show the cystic malformations in the Sox2 transgenic lungs. Arrows indicate examples of disorganized epithelium. Scale bars: 2 mm (F, K) and 50 µm (N).

Sox2 inhibits the response of cells to Fgf10

Since ectopic expression of Sox2 severely affected branching morphogenesis, we analysed whether expression of the iSox2 transgene affected the normal response of the epithelial tip cells to the branch-

inducing growth factor Fgf10. Therefore, fetal lung explants were cultured in the presence of PBS or Fgf10 coated heparin beads. The airways of the control lungs that were in close proximity to the Fgf10 beads clearly grew towards these beads and started to engulf them within four days (Fig 2A-C, arrows). In contrast, the airways of the doxycycline induced iSox2$^{SPC\text{-}rtTA}$ lung explants showed a severely reduced attraction towards the beads, indicating that expression of the Sox2 transgene inhibited tip cells from responding to Fgf10 (Fig 2E-G). Subsequent immunostaining of the cultured explants revealed that the unresponsive regions expressed the transgenic Sox2 (Fig. 2H). Moreover, the staining also showed the incomplete penetrance of the transgene, allowing epithelial cells that do not express the transgene to respond to Fgf10. The latter explained why the iSox2$^{SPC\text{-}rtTA}$ lungs would still show signs of branching. Next, we examined whether the lack of responsiveness of the epithelial tip cells was an autonomous effect of the airway epithelium expressing exogenous Sox2. Therefore, mesenchyme-free lung endoderm of E11.5 embryonic iSox2$^{SPC\text{-}rtTA}$ and control lungs were cultured in matrigel supplemented with Fgf10 in the presence or absence of doxycycline. Branching was readily detected in the control and non-induced iSox2$^{SPC\text{-}rtTA}$lung endoderm cultures (Fig. 2I/2L and 2J/2M). When doxycycline was added to the endoderm cultures from iSox2$^{SPC\text{-}rtTA}$ lungs, the branching process was severely hampered (Fig. 2K and 2N; quantification in Fig. 2O), indicating that ectopic expression of Sox2 either prevented the response towards Fgf10, or induced the differentiation of cells which lead to the loss of FgfR2b expression. Additionally, we analysed whether cells of the iSox2$^{SPC\text{-}rtTA}$ lungs were viable by performing a specific immunostaining on lung explants using the mitotic marker Phh3 (Suppl. Fig. 2). This showed that non-induced lung explants had only a few proliferative cells, whereas the iSox2$^{SPC\text{-}rtTA}$ lungs had significantly more cycling cells. Taken together, these data showed that Sox2 expression in lung buds inhibits the response of the cells to Fgf10 without the ability to proliferate.

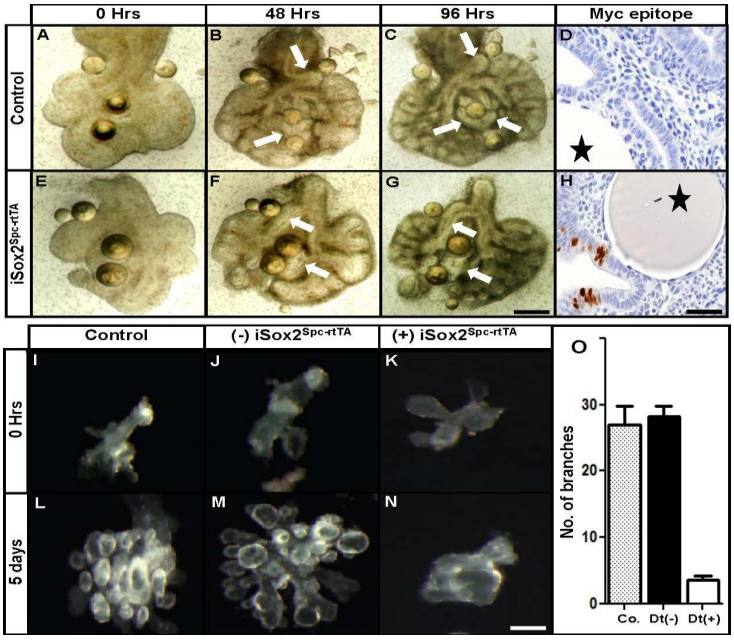


Figure 2: Transgenic Sox2 prevents Fgf10 induced branching. Lung explants of control (A-D) and iSox2$^{Spc\text{-}rtTA}$ (E-H) mice were grown for 96 h in medium containing doxycycline. Heparin beads coated with Fgf10 were placed with the explants. The airways of control lungs that are in close proximity of the beads grow towards the Fgf10 source (arrows in B, C), whereas the iSox2$^{SPC\text{-}rtTA}$ derived lungs show severe reduction of this effect (arrows in F, G). Sections were stained with the myc epitope to show expression of the transgene (D, H; stars indicate position of the beads). Scale bars: 500 µm (A-C; E-G) and 50 µm (D, H). Mesenchyme free lung endoderm from control and iSox2$^{SPC\text{-}rtTA}$ lungs was cultured in growth factor reduced matrigel supplemented with Fgf10, in the presence (I, K, L, N) or absence (J,M) of doxycycline. The control endoderm (I, L) and the iSox2$^{SPC\text{-}rtTA}$ derived endoderm grown in the absence (-) of doxycycline (J, M) clearly showed branching after five days of culture. iSox2$^{SPC\text{-}rtTA}$ derived endoderm grown in the presence of doxycycline showed no branching at all (K, N). Quantification of the number of branches revealed a six fold decrease in branching in Sox2-induced endoderm cultures (O). Scale bars: 100 µm.

Prolonged Sox2 expression affects basal cell differentiation in the respiratory epithelium

The emergence of basal cells during development showed a correlation with the duration of iSox2 expression (Suppl. Fig. 3). However, it is unclear if the emergence of basal cells depended on continuous expression of iSox2, or that precursor cells are primed to become basal cells, after which

Sox2 is no longer required. We hypothesized that varying the timing and duration of the transgenic Sox2 expression would result in fluctuations in the number of basal cells. Therefore, the differentiation towards basal cells was examined after induction of Sox2 starting at embryonic, early and late pseudoglandular phases of gestation. The expression and distribution of iSox2 in correlation with the cystic lesions was first examined using an antibody against the myc-epitope present at the N-terminus of the iSox2 transgene. Strong nuclear staining was detected in the iSox2$^{SPC\text{-}rtTA}$ lungs when the transgene was induced from embryonic phase (Fig. 3D, E) or pseudoglandular phase (Fig. 3G, H), whereas no myc-epitope positive cells were observed in lungs of wild type or non-induced iSox2$^{SPC\text{-}rtTA}$ animals (Fig. 3A, B). Varying the time of induction of Sox2 resulted in changes in the total number of differentiated basal cells. In control lungs, Trp63 and myc immunoreactive cells were not observed in the distal epithelium (Fig. 3A-C), although Trp63 positive cells were observed in the trachea (Fig. 3K). However, in iSox2$^{SPC\text{-}rtTA}$ lungs, we detected cells expressing both the transgenic Sox2 and Trp63 using sequential sections of the distal epithelium of the lung, correlating with the time of doxycycline exposure (Fig. 3D-I). Quantification of the number of Trp63 positive basal cells revealed a gradual decrease of basal cells correlating with the onset of induced expression of Sox2 in the iSox2$^{SPC\text{-}rtTA}$ lungs (Fig. 3J). Only a few differentiated basal cells were identified when the transgene was induced late in gestation, whereas high numbers of basal cells emerged with an early induction of the transgene in the intra-lobular airways. Moreover, dual immunofluorescence staining revealed that Trp63 and Sox2 were expressed in the same cell, both in the trachea of control and iSox2$^{SPC\text{-}rtTA}$ mice , as well as in the aberrant Trp63 positive cells in the distal lungs of iSox2$^{SPC\text{-}rtTA}$ mice (Fig. 3K, L). Together, these results demonstrate that a linear correlation between the expression of iSox2 and of the appearance of p63^{+} basal cells.

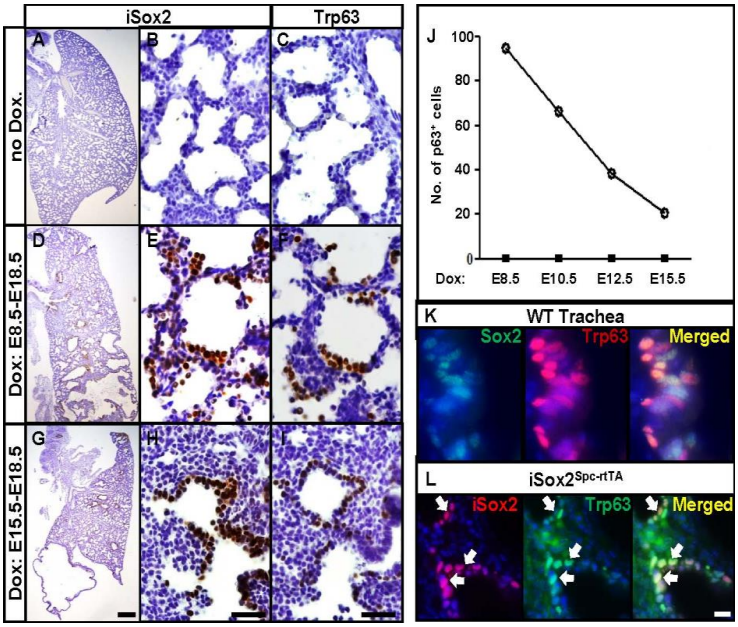
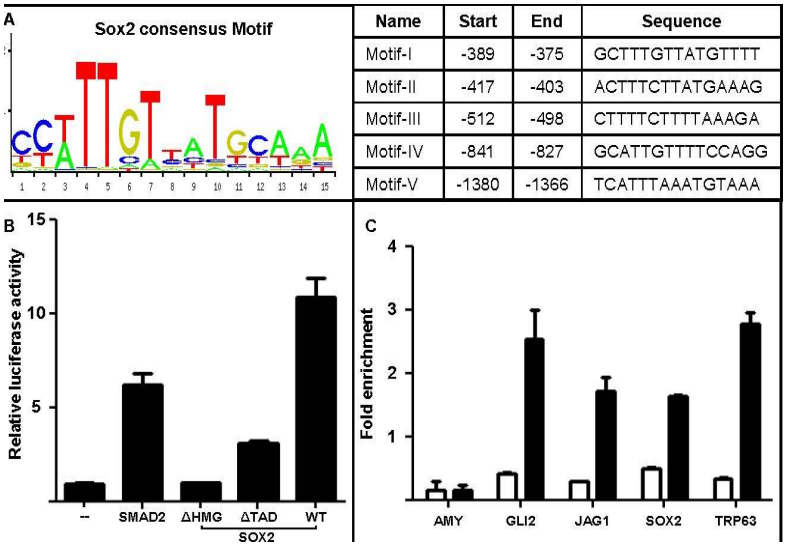


Figure 3: **Progressive increase of basal cells in the alveolar epithelium with prolonged Sox2 induction.** Control (A-C) and iSox2$^{SPC\text{-}rtTA}$ lungs exposed for 10 days (E8.5-E18.5; D-F) or 3 days (E15.5-E18.5; G-I) of doxycycline were isolated and analysed for the appearance of basal cells. The expression of the iSox2 transgene was evaluated using the myc-epitope specific antibody (A, D, G with corresponding details in B, E, H). Basal cells were detected in sequential sections with the Trp63 specific antibody in the distal, iSox2 positive regions of the iSox2$^{SPC\text{-}rtTA}$ lungs (C, F), but were completely absent in the controls (I). iSox2/Trp63 double positive cells were confirmed with immunofluorescence (L; iSox2 in red, Trp63 in green, double positive cells in yellow), and endogenous Trp63/Sox2 double positive cells were detected in the trachea of positive controls (K, Sox2 green, Trp63 red, Sox2 and Trp63 double positive cells in yellow). In iSox2$^{SPC\text{-}rtTA\ lungs,}$ the Trp63 expression decreased with the shorter time of doxycycline exposure. Quantification (J) of the number of differentiated basal cells (Y-axis) showed a linear correlation with iSox2 expression (start of expression indicated on X-axis). Open circles represent basal cell counts from the iSox2SPC-rtTA lungs, black boxes represent the control lungs. Three microscopic fields with a 40-times objective were analysed for intra-lobar Trp63 positive cells. The results are expressed as the average of the number of positive cells per field. Scale bars: 2mm (G), 50µm (H, I,) 25 µm (L).

The basal cell specific Trp63 gene is a direct target of Sox2

Based on the correlation between the number of basal cells and the induced Sox2 expression, as well as the colocalization of Sox2 and Trp63 (Fig. 3L), we analyzed whether Sox2 could directly regulate

the expression of the Trp63 gene. Previously, we showed that the ΔNTrp63 isoform was predominantly induced in iSox2$^{SPC\text{-}rtTA}$ lungs (5), so we analyzed a 1500 bp region immediately upstream of the Trp63 ΔN isoform transcriptional start site (TSS) and identified five putative Sox2 binding motifs (Fig. 4A). Based on the highest similarity scores for motifs I and II, a fragment covering 520 bp immediately upstream the TSS was subcloned upstream of a luciferase reporter construct. SMAD2 was used as positive control since it was previously shown to transactivate the Trp63 promoter (26). The full length Sox2 induced the Trp63 minimal promoter constructs containing the putative Sox2 consensus binding sites (Fig. 4B). However, two mutant Sox2 constructs, lacking either the HMG DNA binding motif (ΔHMG) or the transactivating domain at the C-terminal part (ΔTAD), could not activate the Trp63 promoter constructs, suggesting that Sox2 transactivates the Trp63 promoter. Next, we performed a chromatin immunoprecipitation (ChIP) to analyse whether Sox2 would directly bind to the minimal promoter region of the Trp63 gene in vivo. Therefore, we used chromatin isolated from A549 human bronchiolar lung cells and showed that SOX2 bound to the region covering the putative conserved SOX2 binding sites in the 500 bp Trp63 promoter region (Fig. 4C). Collectively, our data indicate a direct regulatory role for Sox2 in the transcriptional activation of the Trp63 gene, leading to the differentiation of naive epithelial cells into committed basal cells.



Name	Start	End	Sequence
Motif-I	-389	-375	GCTTTGTTATGTTTT
Motif-II	-417	-403	ACTTTCTTATGAAAG
Motif-III	-512	-498	CTTTTCTTTTAAAGA
Motif-IV	-841	-827	GCATTGTTTTCCAGG
Motif-V	-1380	-1366	TCATTTAAATGTAAA

Figure 4: Sox2 transactivates the Trp63 gene. (A) Schematic presentation of Sox2 consensus binding motif (left), and a table with the in silico analysis of Sox2 binding regions in a 1500 bp

sequence immediately upstream the Trp63-ΔN promoter region. (B) Relative luciferase activity as a measurement of the transcriptional activity of the 500 bp Trp63-ΔN minimal promoter. The activity was significantly induced by the full length Sox2 (WT), but mutation of the HMG domain (ΔHMG) or the transactivation domain (ΔTAD) abolished the Sox2- mediated luciferase activity. SMAD2 served as positive control for the activation of the minimal promoter. (C) PCR analysis of A549 chromatin precipitated with IgG control immunoglobulins (white bars) or the Sox2 specific antibody (black bars). Negative control (AMY; Amylase), positive controls (GLI2 JAG1, SOX2), and the TRP63 specific primers flanking the TRP63-ΔN minimal promoter region were used in a standard PCR assay. Graphs depict representative results of 3 separate experiments performed in duplicate and expressed as mean standard deviation.

Aberrant Sox2 expression induces transcription factor Gata6

Recently, it was shown that numerous human squamous cell carcinomas showed increase expression of Trp63, which would promote proliferation and an increase in Gata6 expression (27). Based on the importance of Gata6 in the proximal-distal patterning of the lung (28) (29) and the capacity of Sox2 to differentiate naïve progenitors into Trp63 expressing basal cells (5), we investigated whether the induction of Trp63 by the transgenic Sox2 would also lead to an increased expression of Gata6. Dual immunofluorescence staining showed co-localization of Gata6 and iSox2 positive cells in induced iSox2$^{SPC\text{-}rtTA}$ lungs (Fig. 5A). In contrast, very few Gata6 positive cells were detected in control lungs. In-silico matrix analysis of a minimal promoter region of the Gata6 gene surprisingly identified two putative Sox2 binding sites within 500 bp of a minimal Gata6 promoter. Subsequently, we showed that this fragment could be transcriptionally activated by the full length Sox2 in vitro, but not by the ΔHMG and ΔTAD mutant Sox2 (Fig.5B). Moreover, SOX2 specifically bound this promoter region in vivo using a SOX2-specific ChIP on chromatin isolated from A549 cells (Fig. 5C). Taken together, these data demonstrate that Sox2 directly regulated Gata6 expression in the lung.

Next, we showed that the Sox2 transgene induced proliferation by staining for phospho-Histone H3 (Phh3; Fig. 5D), supporting the observed proliferation in the lung explants (Fig. 2P-T), and that these Sox2/Phh3 double positive cells also expressed Gata6 (Fig. 5E). Interestingly, the Gata6 positive cells also co-localized with the Trp63^{+} basal cells in the distal epithelium (Fig 5F). Quantification of the different cell types, revealed that 17% of the Sox2^{+} cells were also Gata6^{+}, 21% was Sox2^{+}/Trp63^{+} and

15% was positive for all three factors (Fig.5G). Thus, our data indicated that Sox2 induced the activation of Trp63 and Gata6 leading to cellular changes, which eventually contributed to the morphological abnormalities in the $Sox2^{SPC\text{-}rtTA}$ lungs.

SOX2 expression in lungs of human CCAM patients

Since we observed a phenotype that resembled the human CCAM pathology, we analyzed the expression of SOX2 in lungs of human type I and type II CCAM patients. Surprisingly, no SOX2 positive cells were observed in the affected regions of the type I patients, but a prominent staining was present in the type II CCAM lungs (Fig. 6A-F). In age-matched control lung samples no SOX2 positive cells were observed in the lung, although we did find positive cells in the upper bronchi. The SOX2 positive cells in the type II CCAM lungs co-expressed the TRP63 protein, which paralleled the findings in the $Sox2^{SPC\text{-}rtTA}$ mouse lungs.

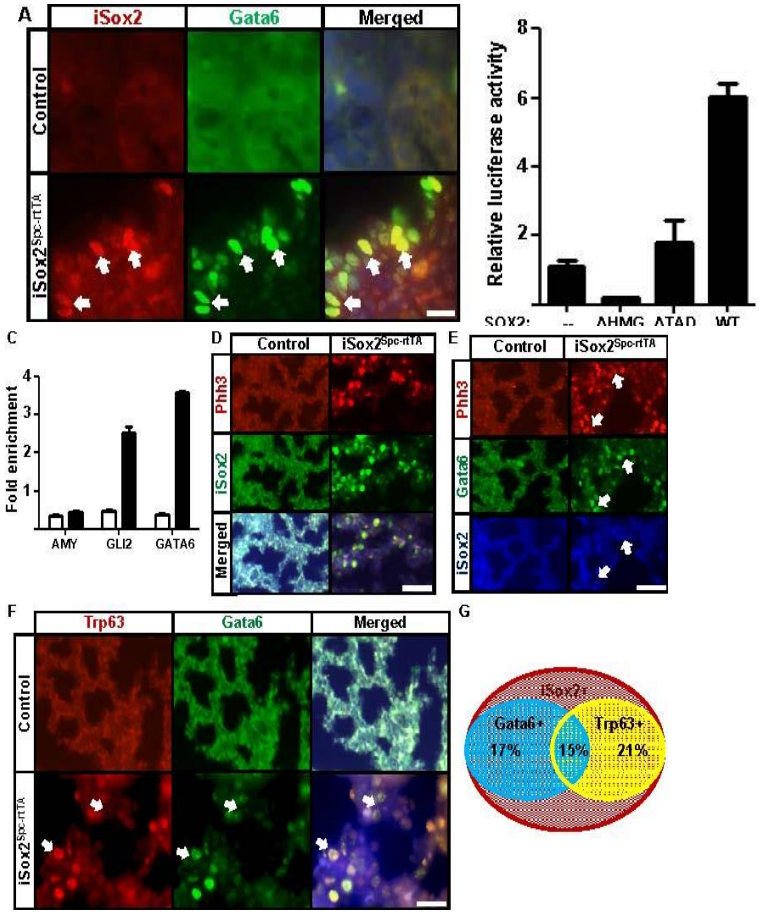


Figure 5: Sox2 induces proliferation and Gata6 expression. (A) Immunofluorescence detection of Myc-epitope (iSox2; red) and Gata6 (green) in E18.5 lung tissue from control (top) and iSox2$^{SPC\text{-}rtTA}$ (bottom) illustrating the co-expression of Gata6 and iSox2 (yellow, arrows). The full length Sox2 (WT) transactivates a 500 bp Gata6 promoter fragment (B), whereas the HMG domain (ΔHMG) or the transactivation domain (ΔTAD) mutated Sox2 did not. SOX2-specific ChIP analysis showed specific binding to this fragment in vivo (C; white bars represent the IgG immunoglobulin controls, black bars represent the SOX2 specific precipitation). Negative control (AMY, Amylase), positive control (GLI2) and GATA6 specific enrichment are indicated. Co-localization studies revealed that (D) Sox2 positive cells proliferate (Phh3 in red and myc-epitope in green), that (E) Sox2 (iSox2; blue) and Gata6 (green) double positive cells also proliferate (Phh3 in red), and that (F) Trp63 (red) and Gata6 (green) co-localize (yellow). Arrows in A, E and F indicate examples of positive cells. (G) Percentage of the total number of iSox2^{+} cells that are iSox2^{+}/Gata6^{+} (17%), iSox2^{+}/Trp63^{+} (21%) or iSox2^{+}/Gata6^{+}/Trp63^{+} (15%). Scale bars: 100 μm (D, E) and,25 μm (A, F).

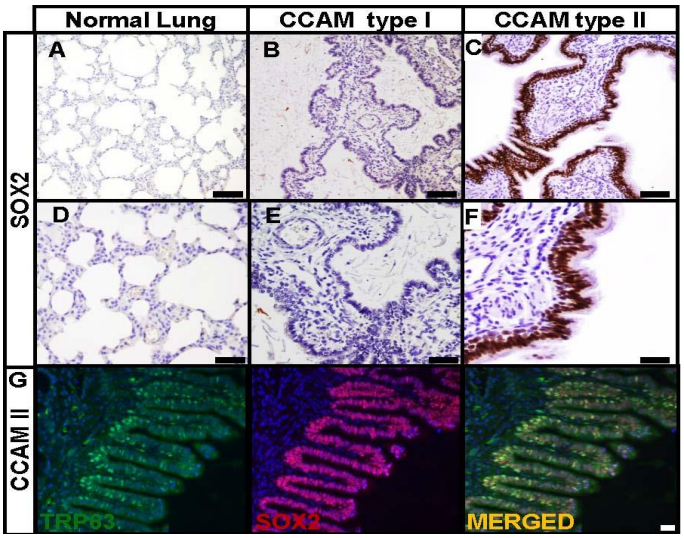


Figure 6: SOX2 expression in human lungs of type II CCAM patients. SOX2 expression is detected in lungs of human type II CCAM patients (C, F), but is absent in control (A, D) and type I CCAM samples (B, E). Moreover, the SOX2 positive cells (red) co-expressed the TRP63 (green) protein as detected by immunofluorescence (G). Scale bars: 200 µm (A-C) and 100 µm (D-G)

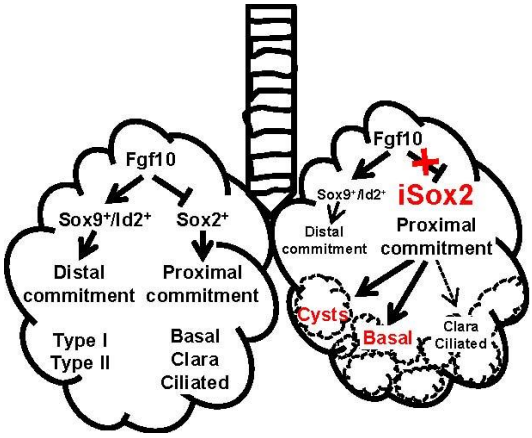


Figure 7: Schematic representation of our findings. Left part shows the normal developmental context of Sox2, whereas the right part shows the effect of the iSox2 expression. See text in the discussion for details.

Discussion

The mammalian lung is a complex, highly branched organ consisting of airways and closely associated blood vessels required for life to exchange catabolic carbon dioxide and oxygen. The development from a primary bud protruding from the ventral foregut to the bronchial tree with a myriad of alveolar spaces can be described in five histologically defined stages: embryonal, pseudoglandular, canalicular, saccular and alveolar. However, at the cellular and molecular level it is more difficult to describe the development in separate stages, and the formation of the lung requires the intimate interaction between the endodermal epithelial cells and the surrounding mesenchyme. Several signaling cascades have been described that interact at different levels with each other to drive the expansion of the primitive branches. Over the years, many factors have been shown to be involved in the different processes, many of them are expressed in waves of cyclic expression patterns (1-2, 30). Sox2 is a key transcription factor involved in cell fate decision of neural and epithelial tissues (review by (9) and several studies by us and others have revealed specific roles of Sox2 in lung development by varying the level of expression (5-7). We have shown that ectopic expression of Sox2 induces the emergence of basal cells and neuroepithelial cells (5). Here we show that Sox2 directly regulates the master gene Trp63, resulting in the differentiation of epithelial cells into basal cells. Moreover, we show that Sox2 directly regulates the expression of Gata6, which is possible involved in the emergence of the BASC cells.

Ectopic expression of iSox2 in the developing lung resulted in abnormal distal cysts resembling the human congenital disorder CCAM, which are congenital abnormalities of the respiratory tract characterized by multicystic lesions in pulmonary tissue with proliferation of bronchial structures (4). CCAMs can be detected by prenatal ultrasonography and the clinical manifestation ranges from total absence of abnormalities at birth till fetal polyhydramnios and hydrops fetalis. CCAMs are classified in five groups, depending on the size and histological appearance of the cyst. Type I CCAMs contain relatively large cysts, which can be solitary or multiple cysts; type II has multiple, small cysts, whereas type III is characterized by solid microcysts. Aside from these original three types of CCAMs, two additional types were added, type 0 with a bronchial-like histology, and type IV, with distal acinar

lesions (4). The pathogenesis of CCAM is still elusive and most likely linked to developmental defects of the lung. So far, only a few studies have identified genes that may be associated with this abnormality, such as Fgf10, Hoxb5, CC10 and FABP-7 (reviewed in (31)). We analysed the expression of SOX2 and TRP63 in the lungs of human CCAM type I and type II patients. Surprisingly, SOX2 was only detected in the affected areas of the type II CCAM samples and was completely absent in the type I CCAM samples. This indicates that the affected epithelium in the type I CCAMs is composed of totally different cell types than the type II CCAM epithelium. Moreover, it suggests that the developmental origin of the two types of CCAM is different, maybe related to the timing of the onset of the disease.

In order to further evaluate the putative involvement of Sox2 in CCAM pathogenesis, we modulated the timing of Sox2 transgene expression. This resulted in variable cysts in the iSox2$^{SPC\text{-}rtTA}$ mouse lungs, ranging from small to large, inversely correlating with the time of iSox2 expression. We speculated that the cysts were induced as a result of the premature differentiation of epithelial tip cells, leading to an arrest in branching of the epithelium. Therefore, we first wanted to know whether cells expressing the transgenic iSox2 were able to respond to the branch-inducing Fgf10 growth factor. Using lung explants and mesenchyme free endoderm cultures we showed that the cells expressing iSox2 do not respond to the branch inducing signaling molecule Fgf10. In situ hybridization and quantitative PCR analysis revealed no change in the expression of the FgfR2 isoforms IIIb and IIIc, indicating that Sox2 acts downstream of this signaling cascade ((5); data not shown). In addition, we previously showed that tip cells in the lungs of the iSox2SPC-rtTA mouse did not express nuclear phosphorylated ERK1/2 (phosphor-p42/44 MAP kinase), the activated downstream mediator of the Fgf10/FgfR signaling pathway. Instead, the activated mediator ot Bmp4 signaling, nuclear phosphorylated SMAD1/5/8, was increased, indicating that the cells were differentiating (5). This is in line with previous data from us and others showing that Fgf10/FgfR2 signaling acts upstream of Sox2 and is not influenced by manipulating the level of Sox2 expression (5, 7). Fgf10 signaling in tip cells activates β-catenin signaling, which in turn inhibits Sox2 expression and prevents Id2^{+} progenitor cells from differentiating into epithelial cells (11, 32-34). Indeed, the inability of the iSox2$^{SPC\text{-}rtTA}$ cells to respond to Fgf10 is caused by the dominant effect of Sox2 on cellular differentiation by directing cells

towards a proximal cell fate, such as basal cells. In addition, we also found that Sox2 induces proliferation in embryonic lungs and in fetal lung explants, extending previous results obtained in adult mice (6). Collectively, our data suggest that the cystic structures are caused by a combination of the fact that tip cells are induced by Sox2 to differentiate into cells with a proximal fate, thereby losing their ability to respond to Fgf10, and the fact that Sox2 induces proliferation. The proliferation caused the epithelial buds to grow without forming new branches, resulting in a ballooning of the existing bud.

Similar cyst-like abnormalities to those we observed in iSox2$^{SPC\text{-}rtTA}$ mice have been reported in several other studies. Ectopic expression of the Wnt downstream mediator β-catenin in the airway epithelium resulted in the loss of Sox2 and p63 expression in proximal derivatives, indicating that Wnt signaling inhibits the differentiation of Sox2 positive proximal cells (34). The mesenchymal-specific TgfβRII knockout showed increased levels of Ptc and Gli1, two downstream mediators of Shh signaling (35). Inhibition of the Wnt signaling pathway by either expressing the Dkk1 antagonist, or by tissue-specific deletion of β-catenin resulted in an expansion of proximal cell types at the expense of distal cells. Moreover, β-catenin and Lef/Tcf activated the promoter of Bmp4 (36). It was also shown that conditional deletion of the two Bmp receptors Bmpr1a and Bmpr1b early in lung development resulted in trachea agenesis, ectopic formation of lung buds and an expansion of Sox2 and p63 positive cells, indicating that Bmp signaling inhibits the differentiation of Sox2 positive cells, at least early in development (37). Later in development, Bmp signaling is required for the proliferation and survival of distal tip cells (38). It is clear that complex interactions of different signaling pathways, such as Wnt, Fgf and Bmp, are required for the correct development of the lung, and interfering with one of the players may affect the formation of the lung at different levels. Recently, it was shown that the molecular programs involved in airway branching to create the bronchial tree, and alveolar formation to increase the gas exchange area are mutual exclusive (39). Activating the branching program by expressing a hyperactive Kras, which is downstream of Fgf10/Fgfr signaling, in lung progenitors resulted in the abrogation of the alveolar program. In contrast, the conditional deletion of Sox9, a marker of distal, alveolar cells, resulted in reduced

branching(39). These data perfectly fit with our data that premature differentiation of cells into the proximal airway fate prevents further branching.

We show that the induced cellular changes observed in the iSox2$^{SPC\text{-}rtTA}$ transgenic lungs are the result of the direct regulation of the basal cell specific Trp63 gene by Sox2. Previously, we showed by microarray analysis and RT-PCR that lungs from iSox2$^{SPC\text{-}rtTA}$ mice had increased levels of p63 and specifically the ΔNp63 isoform. Here, we show that Sox2 transactivates a minimal ΔNTrp63 promoter construct and also binds to the ΔNTrp63 promoter in vivo. These data now provide molecular evidence for previous findings by us and others that Sox2 would be upstream of Trp63 (5, 12, 34). Interestingly, Fgf10 prevented lung progenitor tip cells from differentiating into the proximal, Sox2^{+} lineage, but once epithelial cells were committed to the Sox2^{+} lineage, Fgf10 would positive regulate basal cell differentiation. However, the latter may also be regulated by other Fgfs, such as Fgf7 (40). Interestingly, it was reported that p63 directly transactivated the Fgfr2 gene, the major receptor for Fgf7 and Fgf10 (41). Since we did not find changes in the expression of Fgfr2, it may suggest that even the ectopically expressing Sox2 cells still express the Fgfr2, which would be required for correct differentiation into the basal cell lineage. Based on the putative relation between Trp63 and Gata6 (27), and the important role of Gata6 in the regulation of yet another cell with progenitor properties, the bronchioalveolar stem cells (42), we analysed the expression of Gata6 in our mouse model. Surprisingly, when we analysed the transgenic lungs for the expression of Gata6, we found that Sox2 directly regulates transcription of Gata6, as shown by the co-localization of Sox2 and Gata6, luciferase activity assays and by ChIP technology. Gata transcription factors are zinc finger proteins which are an essential cis-acting element in the promoters and enhancers of a variety of genes. Gata6, a member of this family, is expressed during lung development and in proximal bronchiolar epithelium (28). Since the full knockout of Gata6 is lethal early during gestation (43), the effect of Gata6 on lung development is based on the manipulation of the expression levels (28, 42). These studies showed that Gata6 is required for lung branching and late epithelial cell differentiation (28). Moreover, conditional depletion of Gata6 showed its role in the emergence of lung CC10^{+}/SPC^{+} progenitor cells (42). Therefore, the induced expression of Gata6 by Sox2 may contribute to the emergence of the observed phenotype. Moreover, the association of Gata6 with Wnt signaling (44), and the complex formation of

Sox2 with the Wnt downstream mediator β-catenin (34) suggests a complex network of transcription factors and signaling molecules involving Sox2, Gata6 and Wnt. The present findings that Sox2 transactivates Gata6 support a potential regulatory role for Sox2 in Gata6 expression in late phases of lung development.

In conclusion, our results show for the first time that Sox2 directly binds and transactivates the ΔN Trp63 promoter, thereby initiating the emergence of the basal cell lineage in lung epithelium. Moreover, we show that Sox2 also activates the Gata6 promoter, which contributes to the development of bronchioalveolar stem cells. In normal lung development, Fgf10 is required to maintain the $Id2^{+}$ population and the naïve epithelium in order to branch (40, 45). As cells become more distant from the Fgf10 source, they start to adopt the proximal cell fate and express Sox2, leading to the emergence of proximal cell types, such as basal cell, ciliated cells and Clara cells. Ectopic expression of Sox2 from an exogenous promoter (SPC) results cells that are not responsive anymore to Fgf10. Instead, the cells immediately become proximal cells, leading to a reduced branching. Moreover, the cells proliferate, thereby forming a cyst-like structure (Fig. 7). Importantly, we correlated our findings of the $Sox2^{Spc\text{-}rtTA}$ mice with human CCAM patient material, which surprisingly showed that SOX2 and TRP63 are expressed in lungs from type II CCAM patients, but not in lungs from type I CCAM patients.

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Table 1: Primers used for ChIP and PCR-cloning

	Primers used for quantitative-PCR
hSOX2	F 5' CATGCACCGCTACGACG – 3'
	R 5' CGGACTTGACCACCGAAC – 3'
h JAG1	F 5' GCA GAG CGG TAA GCA CTT AAT -3'
	R 5' GTT TGG ATG GCG GTT TAT TT 3'
hGLI2	F 5' TAG AAT TGC TCC TGC ACT TC
	R 5' ATG TCG GAT GAC CCT TTC TC
H AMYLASE	F 5'GGG AAAA GGC AGC ATA TTG
	R 5'CAC GCT AAA TTG CCT GTG AA
TRP63	F 5' ATG GGA AAG GCT TTG CCA CC 3'
	R 5' CAG TCT CTT CTT GCT AGG TA 3'
GATA6	F 5'ATCTTGGTTAAAGCGGCGATGG 3'
	R 5'CAGCGGTGAATCAAGCGGTA 3'
	PCR and Cloning Oligos
0,5 kb Trp63 Promoter	F 5' CAGCTCGAGATGGCAAGACAAGTTACTTC
	R 5' CAGAGATCTCTAGACAACAGAATGGTCAA
0,5 kb Gata6 Promoter	F 5'CAGAGATCTCAATGACCTTTCCGGCAACC 3'
	R 5'CAGCTCGAGCCAACGATACGGGTCACCTT 3'

Chapter 3

Differentiated type II Pneumocytes can be reprogrammed by ectopic Sox2 expression

SUBMITTED: PLOS ONE

DIFFERENTIATED TYPE II PNEUMOCYTES CAN BE REPROGRAMMED BY ECTOPIC SOX2 EXPRESSION

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Running title: Sox2 induces reprogramming in vitro in the lung

Key words: Sox2, lung, reprogramming, type II cells, basal cells

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Kim Schilders: Acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content

Heleen Kool: Acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content

Anne Boerema-de Munck: Aquisition of data and technical support; analysis and interpretation of data

Marjon Buscop-van Kempen: Aquisition of data and technical support; analysis and interpretation of data

Frank Grosveld: Critical revision of the manuscript for important intellectual content

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Robbert J. Rottier: Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; obtained funding; study supervision

Abstract

The adult lung contains several distinct stem cells, although their properties and full potential are still being sorted out. We previously showed that ectopic Sox2 expression in the developing lung manipulated the fate of differentiating cells. Here, we addressed the question whether fully differentiated cells could be redirected towards another cell type. Therefore, we expressed an inducible Sox2 construct in type II pneumocytes, which are situated in the distal, respiratory areas of the lung. Within three days the type II cells start to proliferate and form clusters of cuboidal cells. Prolonged Sox2 expression resulted in the reversal of the type II phenotype towards a more embryonic, precursor-like cell, being positive for the stem cell markers Sca1 and Ssea1. Moreover, the cells started to co-express Spc and Cc10, characteristics of bronchioalveolar stem cells. We demonstrated that Sox2 directly regulates the expression of Sca1. Subsequently, these cells expressed Trp63, a marker for basal cells of the trachea. So, we show that the expression of one transcription factor in fully differentiated, distal lung cells changed their fate towards proximal cells through intermediate cell types. This may have implications for regenerative medicine, and repair of diseased and damaged lungs.

Introduction

The mammalian lung is a complex organ with a large and highly vascularized epithelial surface area. The airway epithelium is lined with a diversity of cell types that vary in abundance along the proximal-distal axis. The conducting airways have a pseudostratified epithelium to facilitate mucociliary transport, which gradually transforms into a simple columnar and cuboidal epithelium. Finally, the respiratory part of the lungs consists of squamous epithelium for efficient gas exchange. Cellular homeostasis is important for the maintenance of the lung, and in mature lungs, cell turnover and proliferation is low (Kauffman, 1980). However, after bronchiolar injury, either infections or mechanical insults such as artificial ventilation to the lung, the respiratory epithelium extensively proliferate to regenerate and repair the injured lung, indicating the presence of lung progenitor cells (Rock and Hogan, 2011) (Wansleeben et al., 2013).

In general, lung stem/progenitor cells should have the capacity to self-renew and differentiate into specialized cell lineages. In mouse, endogenous adult progenitor/stem cells function to repopulate the damaged lung epithelium (Driscoll et al., 2012; Rackley and Stripp, 2012; Reynolds et al., 2012). Several distinct populations of stem/progenitor cells have been described to be present in the conducting and respiratory epithelium (H. Chen et al., 2012; McQualter and Bertoncello, 2012; Rackley and Stripp, 2012; Rock and Hogan, 2011; Weiss, 2013; Weiss et al., 2011). Lineage tracing studies in mice have shown that the proximal airway basal cells act as stem cells, giving rise to Clara and ciliated cells during lung injury (Ghosh et al., 2011; Rock et al., 2010). On the other hand, recent data suggest that Clara cells may differentiate into Trp63 positive basal cells in damaged lung parenchyma and into alveolar type II cells upon bleomycin treatment or influenza infection (Rock and Hogan, 2011; Zheng, Limmon, et al., 2013). Other putative proximal stem cells include a subpopulation of toxin-resistant Clara cells that function as bronchiolar stem cells located

within two discrete cell niches: the neuroepithelial body (NEB) and the bronchoalveolar duct junction (BADJ) (Ghosh, et al., 2011; Giangreco et al., 2009; Hong et al., 2001). Moreover, several studies have shown the differentiation of type II cells into type I cells (Chapman et al., 2011; Rock and Hogan, 2011). Thus, intrinsic cell populations exist in the lung that may be triggered to differentiate into distinct cell types.

Sox2 is among other transcription factors essential for lung development and maturation (Gontan et al., 2008; Que et al., 2009; Tompkins et al., 2011). Sox2 is a member of the highly conserved HMG box family of transcription factors and required early in embryonic development to maintain pluripotency and self-renewal in embryonic stem (ES) cells. In mice, Sox2 is required for normal morphogenesis and homeostasis of diverse tissues, including neural stem cells; retinal stem cells taste buds; hair sensory follicles in the ear; and epithelia of trachea, lung, and esophagus (Graham et al., 2003; Kiernan et al., 2005; Okubo et al., 2006; Que, et al., 2009). Sox2 is one of the original factors together with Oct4, Klf4, and c-Myc required for the reprogramming of somatic cells (Takahashi and Yamanaka, 2006). In the embryonic lung, Sox2 is expressed in the developing respiratory epithelium (Gontan, et al., 2008), whereas in adult lungs, expression of Sox2 is restricted in epithelial cells, in the adult trachea, airway/bronchiolar epithelium and the conducting airways but is not detected in the alveolus. (Que, et al., 2009; Que et al., 2007; Tompkins et al., 2009).

Many reports have described variations of the original cocktail of factors to generate multipotent iPS cells in vitro (reviewed in (Ladewig et al., 2013); (Graf and Enver, 2009)). Lineage conversion or trans differentiation have recently been reported in vitro and in vivo (review (Sancho-Martinez et al., 2012);(Abad et al., 2013; Niu et al., 2013; Zhou et al., 2008). Mouse and human fibroblasts and other types of cells have been trans-differentiated directly into post-mitotic neurons with combinations of transcription factors (Ambasudhan et al., 2011); (Caiazzo et al., 2011); (J. Kim et al., 2011); (Marro et al., 2011); (Pang et al., 2011);

(Vierbuchen et al., 2010). It was recently reported that the combination of three or more factors can reprogram mouse fibroblasts into induced neural stem cells (iNSCs) with self-renewing ability (Xue et al., 2013); (Lujan et al., 2012); (Sheng et al., 2012); (Ji et al., 2013). Recently, we showed that ectopic expression of Sox2 during lung development induced the differentiation of embryonic epithelial cells into basal and neuroendocrine cells (Gontan, et al., 2008). Since subsets of epithelial cells in the developing lung may still be multipotent, we wondered whether ectopic expression of Sox2 could change the faith of fully differentiated alveolar type II cells in vivo. Therefore, we ectopically expressed Sox2 in alveolar type II cells using a tet-inducible, bi-transgenic approach. We show that conditional expression of Sox2 in the alveolar epithelium results in emphysematous lungs concomitant with the emergence of aberrant structures containing cuboidal cells in the periphery of the lungs. Moreover, Sox2 was found to induce progenitor-like cells which become proliferative and differentiate into cuboidal and basal-like cells, implying that fully differentiated type II cells can be reprogrammed with a single transcription factor to develop into cells expressing proximal markers.

Results

Expression of Sox2 in alveolar type II cells results in morphological changes

Ectopic expression of iSox2 in relative uncommitted, naïve epithelial cells during development resulted in their differentiation into proximal epithelial cells, primarily basal cells (Gontan, et al., 2008) (Ochieng et al unpublished data). We wondered whether induced expression of iSox2 in fully differentiated cells would affect the cells in an autonomous manner. Therefore, we expressed the iSox2 transgene in adult lungs using the SPC-rtTA transgene, which was reported to be expressed in a subset of type II cells in adult lungs (Perl et al., 2002). The immediate effect of the expression of the transgene was evaluated after one, three, six, and nine days of doxycycline induction, as well as prolonged expression for four weeks. The mice did not show typical lung-related phenotypic abnormalities, such as breathing problems, and were indistinguishable from control mice. However, histological analysis of HE stained sections revealed significant structural abnormalities after treatment with doxycycline for four weeks in the lungs of the $Sox2^{SPC\text{-}rtTA}$ mice, which resembled an emphysematous-like appearance (Figure 1B, stars). The air spaces in the lungs of $iSox2^{SPC\text{-}rtTA}$ mice were significantly enlarged and accompanied by the destruction of the normal alveolar architecture compared to control mice (Figure 1A-C). The disrupted areas were interspersed within relative normal alveolar regions, indicating that the phenotype was not uniform throughout the lung of $iSox2^{SPC\text{-}rtTA}$ mice. The normal appearance of lung architecture in some regions was an indication of incomplete penetrance of the transgene (Figure 1B arrow). There was no evidence of inflammatory cells in the alveolar parenchyma and airway spaces, nor were any fibrotic changes observed. Careful analysis of the lungs exposed to doxycycline showed the emergence of clusters of cuboidal cells already after three days of doxycycline treatment (Figure 1C, arrows). Next, we analyzed the extend of the transgene expression after the specified time points by immunohistochemistry with an

antibody against the myc epitope (Myc), which is present at the N-terminus of the iSox2 transgenic protein. In contrast to lungs of wild type control or non-induced transgenic mice (Figure 1D), clear positive cells were already detected after one day of doxycycline exposure in subsets of type II cells in the lungs of the iSox2$^{SPC\text{-}rtTA}$ mice (Figure 1E), which progressively increased with prolonged exposure to doxycycline for 3, 6, 9 and 28 days (Figure 1F-I). Although the appearance of positive cells after one day did not change the overall structure of the lung, the gradual increase of Sox2 positive cells caused cellular changes, from typical type II cells to cuboidal shaped, proximal cells. Thus, expression of iSox2 in peripheral respiratory epithelial cells expanded with the duration of doxycycline administration and induced the appearance of clusters of cuboidal shaped cells, leading to disorganized alveolar septa and loss of the normal lung architecture.

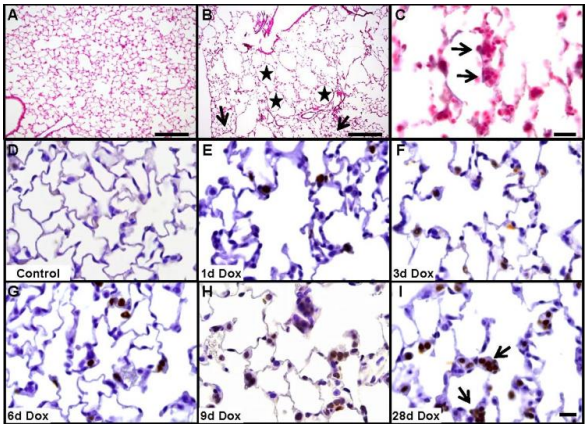

Figure 1: Ectopic Sox2 expression induces emphysematous-like lungs with abnormal cell clusters. Representative HE staining of a control lung (A) demonstrating the normal lung architecture and of iSox2$^{SPC\text{-}rtTA}$ lungs after 4 weeks doxycycline treatment (B) showing numerous, enlarged emphysematous structures (asterisks) with cell clusters (C). Representative IHC staining for the myc-epitope in wild type control (D) and iSox2$^{SPC\text{-}rtTA}$ lungs (E-I) after 1, 3, 6, 9 and 28 days of doxycycline treatment. Transgenic iSox2 positive myc staining is already evident after 1day of dox administration (E), which gradually increased in time (F-I). The positive cells clearly form clusters (arrows in I). Scale bars 200µm (A, B), 100µm (C, I).

Sox2 induces proliferation in alveolar type II cells in vivo

Although cell turnover and proliferation is low during homeostasis in mature lungs, we and others previously showed that Sox2 induced proliferation in lung epithelium (Gontan, et al., 2008; Tompkins, et al., 2011) (Ochieng et al unpublished data). Since Sox2 induced the appearance of an increasing number of cell clusters within the alveolar epithelium, we analyzed whether Sox2 induced proliferation in fully differentiated alveolar type II cells using an antibody against the mitotic cell marker phospho-histone H3 (Phh3). Contrasting the non-proliferative, homeostatic lungs derived from control lungs, Phh3 positive cells were observed in the cuboidal clusters of the lungs of iSox2$^{SPC\text{-}rtTA}$ exposed to doxycycline (Figure 2A versus 2B-D). The number of proliferative cells increased with the duration of transgene expression in iSox2$^{SPC\text{-}rtTA}$ (Figure 2E). Next we determined if iSox2 expressing cells also expressed one of the known Sox2 target genes, cyclin D1 (Ccnd1), which is expressed at the G1/S phase of the cell cycle. Dual immunofluorescence staining revealed co-localization of Ccnd1 and iSox2 in the induced clusters of alveolar cells, confirming that proliferation occurred within the transgene-expressing cells (Figure 2F). So, iSox2 induced type II cells to proliferate and induced cellular changes, ultimately leading to abnormal lung architecture.

iSox2 induces expression of Clara-like and basal-like cells in AVTII cells

Since iSox2 induced the emergence of proximal cell types when expressed during lung development (Gontan, et al., 2008) (Ochieng et al unpublished data), we analyzed if the expression of iSox2 in terminal differentiated type II cells also induced genes specific for proximal airway cells differentiation. Therefore, the expression of Cc10 and Trp63, two markers of the proximal Clara cells and basal cells, respectively, was analyzed. Although Cc10 positive cells were present in the proximal epithelium of the control lungs, the alveolar regions were completely devoid of them (Figure 3A, C, E). In contrast, Cc10 positive cells were readily detected in the alveolar regions in the lungs of the iSox2$^{SPC\text{-}rtTA}$ mice, even after

one day of doxycycline induction, and increased with prolonged exposure (Figure 3B, D, F). Interestingly, these CC10 positive cells also expressed the type II cell marker Spc, indicating that the iSox2 transgene induced the emergence of a transient, bronchioalveolar stem cell (BASC)-like population ($CC10^{+}/Spc^{+}/iSox2^{+}$; Figure 3G). The BASC population has been described to serve as a progenitor like population which is induced upon damage to repopulate the airway epithelium (C. F. Kim et al., 2005; Rawlins et al., 2009). Furthermore, Trp63 positive cells were detected in the clusters of the lungs of transgenic mice exposed to doxycycline for four weeks, indicating the ectopic appearance of basal-like cells in the distal epithelium of the $iSox2^{SPC\text{-}rtTA}$ lungs (Figure 4).

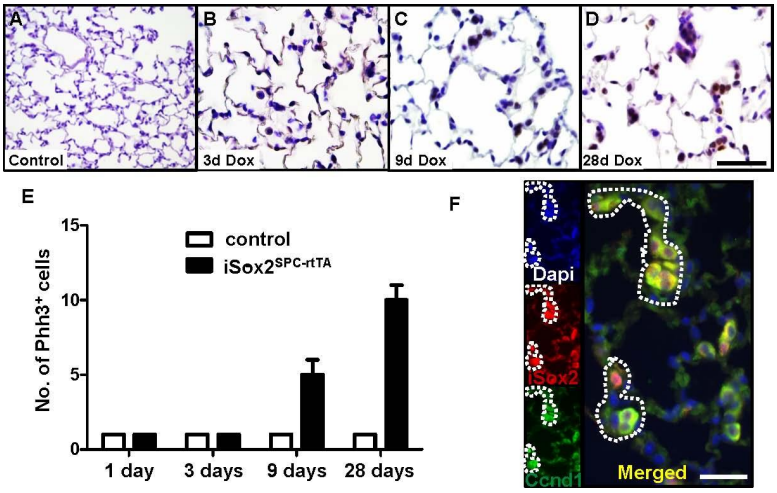

Figure 2: Sox2 induces proliferation in terminally differentiated alveolar type II pneumocytes. (A-D) Lungs of control (A) and iSox2SPC-rtTA mice treated with doxycycline for 3 (B), 9 (C) or 28 (D) days were analyzed with the mitotic cell marker antibody Phh3. Representative images show proliferation in individual type II cells after 3 days of iSox2 induction (B), which gradually develop into proliferative clusters of cells (C, D). (E) Quantification of Phh3 staining indicates the correlation between the increase of Phh3 positive cells and time of doxycycline expression. (F) Colocalization of iSox2 and cell cycle marker CyclinD1 (Ccnd1) is shown by dual immunofluorescence labeling after 28 days of doxycycline exposure (examples in dotted areas). Scale bars: 100µm (A), 50µm (F)

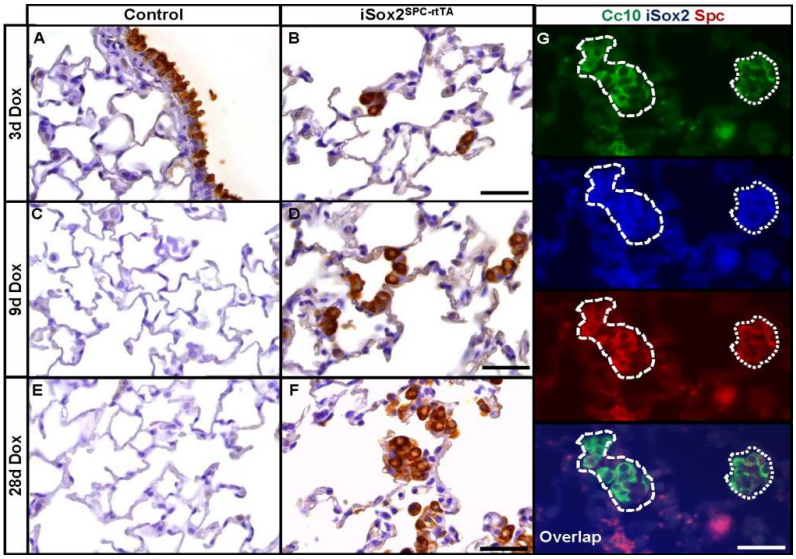

Figure 3: Sox2 induces Clara-like cells and BASC cells. Lungs of control (A, C, E) and iSox2$^{SPC\text{-}rtTA}$ (B, D, F) lungs treated with doxycycline for 3 (A, B), 9 (C, D) and 28 (E, F) days were stained with the Clara cell marker Cc10. Endogenous expression of Cc10 is demonstrated in the conducting airways (A), which also shows the absence of Cc10 positive cells in the distal airways (A, C, E) of control lungs. The progressive increase in number of Clara-like cells (Cc10^{+}) in the iSox2$^{SPC\text{-}rtTA}$ lungs with prolonged induction of iSox2 is noticeable (B, D, F). Colocalization of the iSox2 transgene (blue), Cc10 (green) and Spc (red) is demonstrated with triple immunofluorescence staining (G), indicating the emergence of BASC cells (dotted areas indicate examples). Scale bars: 100 µm (B, D, F), 50µm (G).

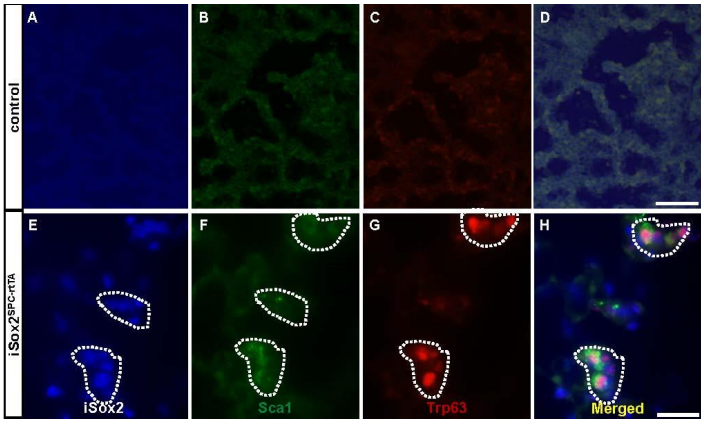

Figure 4: iSox2 induces expression of Sca1 and Trp63 in type II cells. Triple immunofluorescence staining of lungs of control (A-D) and of iSox2$^{SPC\text{-}rtTA}$ mice exposed to doxycycline for 28 days (E-H) with antibodies against the myc epitope (iSox2; A, E, Blue),

Sca-1 (B, F, Green) and Trp63 (C, G, Red). The merged image (D, H) clearly shows the co-localization of the three proteins in the iSox2$^{SPC\text{-}rtTA}$ derived lungs (dotted areas). Scale bar 100μm.

Ectopic Sox2 expression induces progenitors-like cells

Since we observed the emergence of proximal markers in the distal type II cells and the appearance of Spc^{+}/CC10^{+} double positive BASC-like cells, we wondered whether the differentiation of proximal Clara and basal cells occurred through intermediate, progenitor-like cells. Therefore, we analyzed the lungs of control and iSOX2$^{SPC\text{-}rtTA}$ mice, isolated at different time points after doxycycline treatment, for the expression of Sca1 and Ssea1, two markers normally expressed in progenitor cells (Holmes and Stanford, 2007) (Solter and Knowles, 1978). Sca1and Ssea1 were readily detected in the alveolar epithelium in iSox2$^{SPC\text{-}rtTA}$ after 3 days of doxycycline exposure to these mice (Figure 5B, F). This pattern of expression progressively increased after 9 days of transgene induction (Figure 5C, G), and after four weeks of doxycycline induction virtually all clusters of cells expressed Sca1 and Ssea1 (Figure 5D, H). Sca1 and myc-epitope double immunofluorescence staining on lungs of control and iSox2$^{SPC\text{-}rtTA}$ animals exposed for four weeks to doxycycline clearly showed co-localization of Sca1 with the transgenic protein (Figure 5L). Moreover, the previously identified Spc^{+}/CC10^{+} cells also appeared to express Sca1, as shown by triple immunofluorescence staining (Figure 6A, arrowheads). In addition, Sca1 also co-expressed with the basal cell marker Trp63 after four weeks of iSox2 induction, suggesting that the Sca-1^{+} cells gradually differentiate into more committed cells (Figure 4). Thus, Sca1 was specifically induced in the subset of AVTII cells that expressed transgenic iSox2, which indicates that iSox2 is able to induce progenitor-like cells in terminally differentiated type II cells.

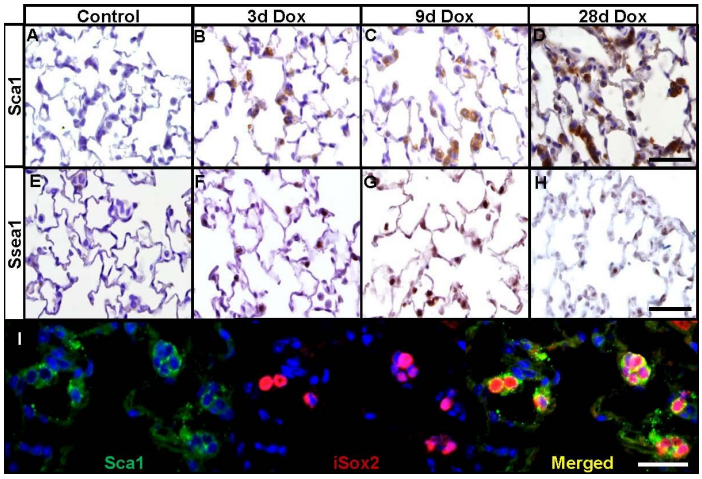

Figure 5 iSox2 induces the appearance of stem cell markers. Immunohistochemistry for Sca1 (A-D) and Ssea1 (E-H) was performed on lungs of controls (A, E), and of iSox2$^{SPC\text{-}rtTA}$ mice after 3 (B, F), 9 (C, G) and 28 (D, H) days of doxycycline exposure. Sca-1 and Ssea1 expressing cells are completely absent in control lungs (A, E), but readily detectable after 3 days of exposure and progressively increased with duration of transgene activation. Sca1 is clearly associated with iSox2^{+} cells, as shown by dual immunofluorescence staining (I). Scale bar 100µm (D, H, K), 50µm (J).

Sox2 activates promoter-Luciferase construct for Sca-1

Next, we wondered whether Sca1 expression is directly regulated by Sox2, or not. Therefore, the Sca1 gene was analyzed for putative Sox2 binding sites and within a region of 500 bp directly upstream of the transcriptional start site, a well-conserved Sox2 binding motif was found. The functionality of this potential Sox2 site was tested in vitro using a luciferase reporter assay. The full length Sox2 protein (WT) induced the transcriptional activity of the Sca1-luciferase construct sevenfold as compared to baseline expression, whereas mutant Sox2 proteins lacking either the transactivation domain (ΔTAD) or the HMG domain (ΔHMG) did not transactivate the minimal Sca1 promoter (Figure 6B). Next, the in vivo binding of SOX2 to this putative Sox2 binding site was analyzed by chromatin immunoprecipitation (ChIP), which revealed that SOX2 directly bound to the Sox2 recognition site in the human

bronchiolar cell line A549. (Figure 6C). These results demonstrated the direct activation of the Sca1 promoter by Sox2, and thus highlights a novel transcriptional target gene of Sox2.

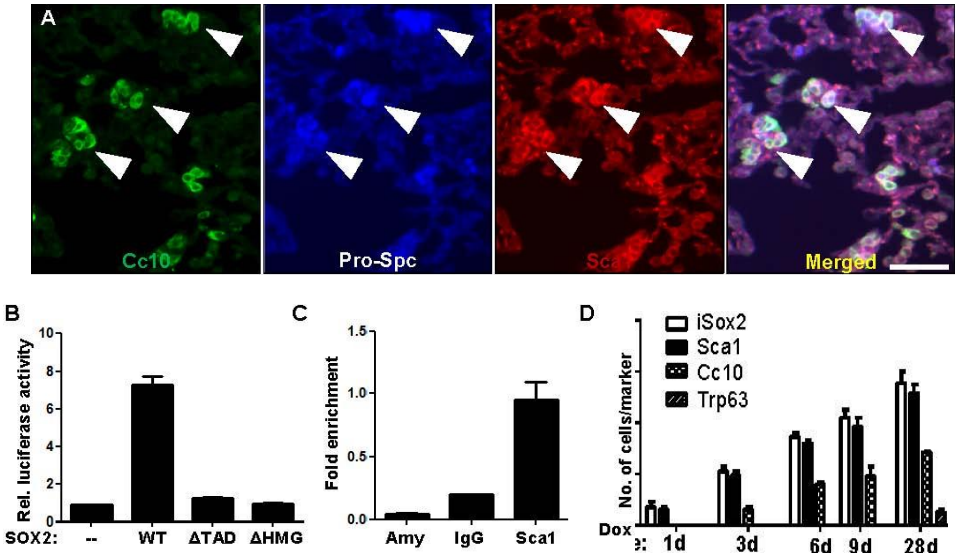

Figure 6: Sox2 directly transactivates the Sca-1 gene. (A) Triple immunofluorescence staining with Cc10 (green), Spc (Pro-Spc, blue) and Sca1 (red) on lungs of iSox2$^{SPC\text{-}rtTA}$ animals treated for 28 days with doxycycline (arrowheads indicate triple positive cells) demonstrate the emergence of BASC-like progenitor cells. (B) Luciferase assay shows in vitro transactivation of the Sca1 minimal promoter by the full length Sox2 (WT), but not by deletion constructs lacking the transactivation domain (ΔTAD) or the HMG domain (ΔHMG). (C) SOX2 specific ChIP analysis showing specific enrichment of the Sca1 promoter region used in the luciferase assay. (D) Quantification of the expression of the different lung markers in vivo after the indicated time of doxycycline exposure. Arrows indicate the immunopositive cells. Scale bar: 50µm (D). Values are represented as SEM.

Transgenic Sox2 induces proximal markers in primary AVTII cells

Quantification of the appearance of the different markers and cell types in relation to the timing of doxycycline exposure, suggested that Sox2 first initiates the appearance of markers associated with progenitor-like cells (Figure 6D; Sca1^{+}). In time, differentiation markers emerge, as evidenced by the number of Cc10^{+} and Trp63^{+} cells. To determine the observed plasticity of ATII cells in vitro, we isolated type II cells from 4 weeks old non-doxycycline

exposed iSox2$^{SPC\text{-}rtTA}$ mice and cultured these primary cells with or without doxycycline. After three, six and nine days the cultures were analyzed for the expression of the myc epitope (iSox2), Cc10, Sca1 and Trp63. The non-doxycycline treated cells expressed Spc, indicative for AVTII cells, but obviously lacked expression of Cc10, Sca1 and Trp63 (Figure 7A). However, in cells treated with doxycycline for 3 days (Figure 7B), 6 days (Figure 7C) and 9 days (Figure 7D), AVTII cells expressed Sca1, and Cc10, while Trp63 positive cells were observed only in the cultures exposed to doxycycline for nine days, which correlated with the in vivo observations (Figure 6D). Taken together, these data demonstrate that AVTII cells also exhibit phenotypic plasticity in vitro.

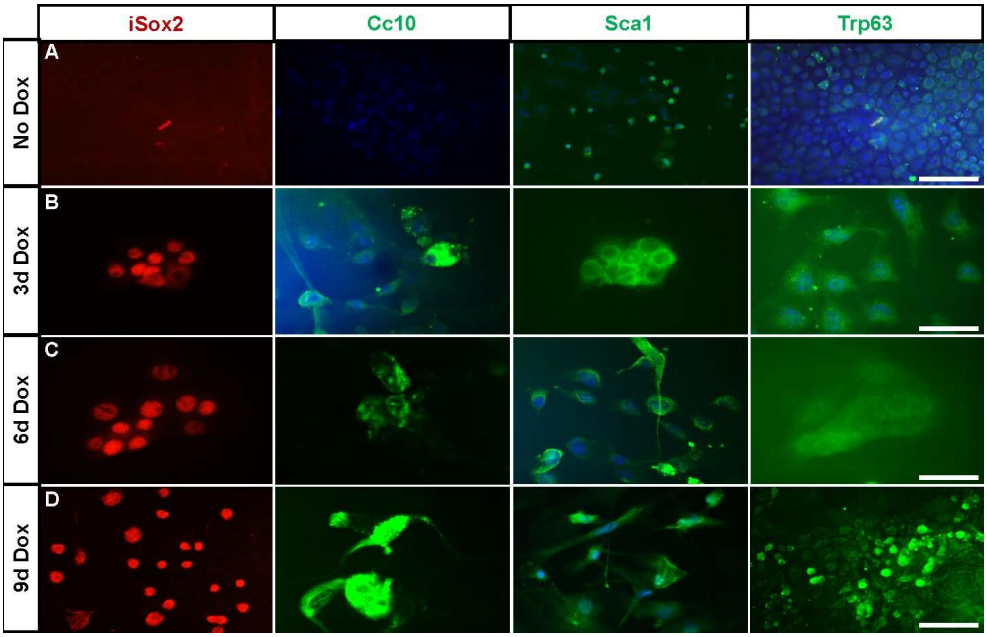

Figure 7: Primary alveolar type II cells can be reprogrammed in vitro Representative immunofluorescence images showing expression of iSox2 (Red), Cc10 (Green), Sca1(Green) and Trp63 (Green) after culturing the primary type II cell cultures without doxycycline (A), or with doxycycline for 3 days (B), 6 days (C) and 9 days (D). The results demonstrate that AVTII cultured in vitro have comparable phenotypic plasticity as in vivo AVTII cells.

Discussion

The idea that the future potential of differentiated cells is limited has been gradually adapted, starting with the seminal work of nuclear replacement in frogs and culminated in the complete reversal of differentiated fibroblast into multipotent cells by only four factors (Gurdon, 1962; Takahashi and Yamanaka, 2006). These experiments have led to an expansion of experimental approaches to manipulate and modify cells to give them multipotent potential (Lensch and Mummery, 2013). Several adaptations to this original combination of four factors have been shown to result in pluripotent cells, depending on the starting source of cells used as target (Liu et al., 2013; Najm et al., 2013; Nemajerova et al., 2012; Wu et al., 2011). Recently, in vivo reprogramming has been show, for instance of resident brain astrocytes in both young adults and mice with Sox2 only (Niu, et al., 2013)

Our previous study established that ectopic expression of Sox2 during lung development caused cystic lesions and aberrant differentiation of the epithelial cells (Gontan, et al., 2008). Recently, we showed that Sox2 directly induced Trp63 and Gata6 expression, which caused naïve epithelial cells to be become unresponsive to the branch inducing signal Fgf10 (Ochieng et al unpublished data). This led to a skewing of the developmental potential of the uncommitted cells towards a proximal cell fate, primarily basal cells. In the current study we investigated whether Sox2 would be sufficient to redirect the fate of terminally differentiated alveolar type II cells.

After bleomycin induced injury, type II cells start to proliferate and repopulate the alveolar epithelium by self-renewal and functioning as progenitor cells for type I cells (Rock et al., 2011). We showed that induction of Sox2 expression in alveolar type II cells resulted in the immediate emergence of proliferative cells. Sox2, together with the Wnt downstream mediator β-catenin, was shown to directly regulate the promoter of Ccnd1 in breast cancer cells (Y. Chen et al., 2008), and we show that the iSox2 positive cells were expressing Ccnd1.

Sox17 was also suggested to regulate the Ccnd1 promoter directly, although no ChIP analysis was done (Lange et al., 2009). Tompkins et al. added that upon Sox2 expression several other genes involved in cell cycle initiation and progression were upregulated (Tompkins, et al., 2011). Within the first week of doxycycline induction, the iSox2 expressing cells started to express the Sca1 and Ssea1 stem cell markers (Holmes and Stanford, 2007; Solter and Knowles, 1978). Moreover, we showed a direct binding and transactivation of the Sca1 promoter by Sox2, thereby initiating a progenitor-like program. This suggests that iSox2^{+}/Sca1^{+}/Ssea1^{+} cells represent an initial sign of dedifferentiating type II cells, indicating that Sox2 may initiate alveolar epithelial cell plasticity by first regulating the emergence of proliferative intermediate cells, perhaps progenitor-like cells. Thus, our data suggest a mechanism where Sox2 first induces proliferative Ssea1^{+}/Sca1^{+} progenitor cells, which increase over time and subsequently promote differentiation of these cells into proximal epithelial cells. Detailed analysis of the progenitor-like, iSox2/Sca1^{+}/Ssea1^{+} cells showed a gradual differentiation towards proximal epithelial cell fate, since these cells started to express Cc10 and Trp63 after longer exposure to doxycycline, both in vivo and in vitro. A number of these cells co-expressed Spc and Cc10, a population also referred to as the bronchioalveolar stem cells (BASC), which are normally located ate the bronchio-alveolar junctions (C. F. Kim, et al., 2005). These authors also showed that the BASC cells expressed Sca1, which became proliferative after naphthalene injury. The cells with characteristics of BASC cells in the iSox2 expressing lungs also expressed Sca1 (Spc^{+}/Cc10^{+}/Sca1^{+}), contrasting earlier findings obtained with ectopic Sox17 expression that did not find these triple positive cells (Lange, et al., 2009). The Sca1 marker has been used to purify BASC through fluorescence activated cell sorting (FACS) using CD45^{-}CD31^{-}Sca1^{+} (C. F. Kim, et al., 2005; D. Kim et al., 2009), but other studies described this population to be more heterogeneous (McQualter et al., 2010). BASC cells self-renew and have the potential to differentiate into proximal and distal

epithelial cells (J. Kim, et al., 2011). Lineage tracing experiments using a CCSP-CreEr followed by hyperoxia injury suggested that BASC cells did not give rise to alveolar cells (Rawlins, et al., 2009), but after bleomycin injury they did (Rock, et al., 2011). This would suggest that BASC cells respond differently to various triggers. Recently, lineage tracing studies using bleomycin induced lung damage in Scgb1a1-CreER mice showed that basal cells ($Trp63^{+}$) in the damaged parenchyma were directly derived from Clara cells ($Scgb1a1^{+}$) (Zheng, Yin, et al., 2013). Moreover, upon SO_2-induced damage or viral infection, Clara cells also dedifferentiated into basal cells (Tata et al., 2013). Exposure to naphtalene or hyperoxia revealed that Clara cells may contribute to maintenance and repair of the conducting airways without dedifferentiating into basal cells (Rawlins, et al., 2009). Our current findings demonstrate that Clara and basal cells may originate from $iSox2^{+}/Sca1^{+}$ progenitor cells. However, it remains to be determined whether these cells are derived from $Sca1^{+}$ progenitor cells by genetic lineage tracing experiments.

The continuous proliferation of iSox2 positive cells led to the emergence of clusters of cells in the alveolar walls with a cuboidal to columnar appearance, which became apparent after 9 days. Concomitant with the development of these clusters was the disruption of the alveolar structure, as evidenced by the emphysema-like phenotype observed after four weeks of iSox2 expression. The combination of proliferation and the induction of progenitor-like characteristics resulted in the loss of structural integrity. The conversion of cell fate combined with the increased proliferation induced by iSox2 may have changed the secretion and composition of the extra cellular matrix, which may have weakened the alveolar structure. This in turn may enhance the activity of local proteases to digest the tissue matrix and induce septal rupture, leading to emphysematous-like lungs.

Long term and high ectopic expression of Sox2 in Cc10 positive cells was shown to result in adenocarcinomas in fifty percent of the mice (Lu et al., 2010), but we and others did not find

evidence that Sox2 induced lung cancer in our mouse models (Tompkins, et al., 2011). The difference in the various transgenic approaches may contribute to this discrepancy. However, SOX2 has been associated with human squamous cell lung tumors (Bass et al, 2009; Hussenet et al, 2010; Yuan et al, 2010).

The only solution for patients with end-stage, severe chronic lung disease, like COPD and idiopathic pulmonary fibrosis, is lung transplantation. However, the shortage of suitable donors may result in a significant mortality of patients. Therefore, a potential future treatment for these severe lung diseases is a (temporary) transplantation with engineered lungs or stem/progenitor cells (Green et al., 2011; Mou et al., 2012; Van Haute et al., 2009). However, the approaches for generating these cells have been limited to the use of combination of factors in vitro (Han et al., 2012; J. Kim, et al., 2011; Ring et al., 2012; Takahashi and Yamanaka, 2006). In addition, a variety of in vitro protocols exist for differentiating a range of pulmonary epithelial cell types, including alveolar type II cells (Green, et al., 2011; Longmire et al., 2012; Mou, et al., 2012; Rippon et al., 2006; Van Haute, et al., 2009; Wang et al., 2007). Recently, the direct conversion of cellular fate has been reported in vivo in a study demonstrating that neurons can be generated from endogenous mouse astrocytes that are reprogrammed by viral delivery in situ (Torper et al., 2013) In addition, it has been shown that SOX2 is also capable of converting resident astrocytes into proliferative neuroblasts (Niu, et al., 2013). We showed that Sox2 alone is sufficient to induce alveolar plasticity in resident lung alveolar type II cells into progenitors in adult mice. Our study demonstrates a feasible strategy for using Sox2 to reprogram alveolar type II cells in vivo and in vitro. In the future, studies to identify the signaling pathways that regulate the differentiation of progenitors and the induction of proliferation in alveolar type II cells will be critical to facilitate the understanding of alveolar plasticity for future regenerative medicine. Lineage reprogramming would be applicable in translational medicine if this event can be triggered by a factor,

whether transcription factor or small molecule, which acts transiently and exerts a complete effect.

In conclusion, we ectopically expressed one of the Yamanaka reprogramming factors, Sox2, in type II cells cells using our previously described system ((Gontan, et al., 2008) Ochieng etal 2013). We show that these dedifferentiate into progenitor-like cells and subsequently commit to the proximal pulmonary epithelial cell lineages, like basal cells, extending previous findings with Sox2 and Sox17 (Lange, et al., 2009; Tompkins, et al., 2011). Moreover, we show that aside from directly activating the promoter of the key gene in basal cell development, Trp63 (Ochieng et al unpublished data), Sox2 also binds and activates the progenitor cell marker Sca1, providing molecular evidence for a direct role of Sox2 in the dedifferentiation process.

Materials and methods

Mouse breeding and genotyping

Mice were kept under pathogen-free conditions and all experiments were performed according to the guidelines of the local ethics committee. Generation of the Sox2 transgenic mouse line has been described before (Gontan, et al., 2008). Lung-specific expression of the myc-Sox2 transgene was obtained by breeding the myc-Sox2 line with the SPC-rtTA transgenic mice (generous gift of Jeffrey Whitsett, Cincinnati), subsequently referred to as iSox2$^{SPC\text{-}rtTA}$. Doxycycline was administered in the drinking water (2 mg/ml doxycycline, 5% sucrose), and lungs were harvested after 1, 3, 9, or 28 days. Transgenic mice were genotyped by PCR of tail-tip DNA using transgene specific primers as previously described. Each experiment was executed on at least three independent lungs of iSox2$^{SPC\text{-}rtTA}$, single transgenic and wild type pups.

Immunohistochemistry and Immunofluorescence

Lungs of adult mice were inflated with 4% paraformaldehyde/phosphate-buffered saline (PBS), subsequently fixed by immersion overnight at 4°C, and processed according to standard protocols for paraffin embedding. Immunohistochemistry (IHC) was performed as previously described using primary antibodies for goat-anti Sox2 (1:500 immune system), mouse anti myc (1:1000, Roche) rabbit anti-phospho-histone H3 (1:1000; Millipore), rabbit anti-CCSP (1:1000, 7-Hills), Mouse anti Ssea (1:200, Millipore), rabbit anti-cyclin D1 (1:500; Abcam), rat anti-Sca-1 (1:500; Abcam), rabbit anti-proSP-C (1:1000; Seven Hills), Mouse anti-Trp63 (1:200; Santa cruz), and Goat anti-CCSP (1:1,000; Seven Hills) (Huang et al., 2012). Briefly, sections (5 μm) were deparaffinised, rehydrated and microwave treated for antigen retrieval in 10 mM citric acid buffer, pH 6.0. Slides were incubated with primary antibodies diluted in PBS/0,5% Triton/0.5% BSA overnight at 4°C, followed by incubation

with the appropriate secondary antibody (1:100; Vector Labs) and amplification with ABC reagent (Vectastain Elite ABC kit; Vector Labs). Antigen localization was detected with nickel-diaminobenzidine. Sections were counterstained with haematoxylin and coverslipped using Permount (Fisher Scientific). Quantification of IHC staining (Figure 2E, 6D) was performed by counting positive cells in 5 separate images of three independent experiments,

Immunofluorescence was performed as described above, but substituting the secondary antibodies with fluorophore labelled antibodies (Alexa Fluor-350, Alexa Fluor-488, and Alexa Fluor-594; Molecular Probes). Sections were mounted with Vectashield anti-fade reagent containing DAPI (Vector Labs). Brightfield and fluorescent images were obtained using a Zeiss Axioplan2 microscope equipped with AxioVision Software.

Isolation and culturing of alveolar type II cells (AVTII)

Alveolar epithelial cells were isolated from the lungs of $Sox2^{SPC\text{-}rtTA}$ mice by Dispase (BD, Pharmingen) digestion as described previously with few modifications (Corti et al., 1996). Lungs were exsanguinated by perfusing through the right ventricle with 4 ml PBS after opening the peritoneum, clipping the vena cava inferior and removing the ribcage. 1 ml Dispase (BD, Pharmingen) was instilled over a tracheal cannula into the lung, immediately a sterilized suture (Braun) was used to tighten a node around the cannulised trachea. Lungs were isolated, incubated for 45 minutes in 1 ml Dispase at room temperature and transferred to a culture dish containing 5 ml medium (DMEM/F12 (1:1) (Gibco) with 0.04 mg/ml DNase I (AppliChem), 3.6 mg/ml D-(+)-Glucose (AppliChem) and 1% Penicillin/Streptomycin (P/S)). The small airways were gently removed and the obtained cell suspension was serially filtered through 100, 70 and 40 µm nylon meshes and centrifuged at 200 g for 10 minutes at 15°C. The supernatant was discarded and the cell pellet was resuspended in 500 µl medium containing FCS (DMEM/F12 (1:1) (Gibco) with 3.6 mg/ml D-(+)-Glucose (AppliChem), 1%

Penicillin/Streptomycin (P/S) and 2% FCS). AVTII cells were cultured in DMEM/F10 containing 10% FCS and 1% P/S in tissue culture cover slip immersed in 12 well plates (Corning, NY) previously coated with collagen (Inamed); cultures were maintained in a 5% CO_2/air incubator.

Plasmids, Cell culture and Luciferase Reporter Assays

A 500 bp promoter fragment immediately upstream of the Sca-1 transcriptional start site was PCR amplified from genomic DNA using the primers Forward 5'-TAAACGCGCACACGTTTCTC and Reverse 5'-GGCCAGCATCTGACCTCTTT-, cloned into the pGL4 luciferase. Human embryonic kidney HEK 293T cells maintained under standard culture conditions were plated on 6-well plates (3.5×10^5 cells per well). 24 hours after plating, the HEK-293Tcells were transiently transfected using Lipofectamine LTX (Invitrogen) with 2.5 µg of the following Firefly luciferase reporter plasmids (pGL4-Sca-1 1µg of Renilla luciferase plasmid (transfection control), and 2.5µg of empty vector (pcDNA3) or plasmids expressing full length Sox2 (Wt-Sox2), or one of the mutant Sox2 . After 24 hr, cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega). The lysate was assayed for luciferase and Renilla activity using the GloMax 96 Microplate Luminometer with Dual Injectors (Promega, Madison, WI), according to the Dual-Luciferase Reporter Assay system kit protocol (Promega). The luciferase activity was calculated relative to the TK Renila. All reporter assays were performed in triplicate, and the bars in the figures denote the standard error of mean (SEM).

Chromatin Immunoprecipitation

A549 cells were cultured in hypoxic conditions. ChIP assay was performed essentially as described previously using $6x10^7$ cells (Raghoebir et al., 2012) (Ochieng et al unpublished

data). The immunoprecipitated DNA was analysed with region specific primers in a Q-PCR assay for the enrichment of the promoter sequences of, Amylase, Sca-1 or Gli2 genes. Amount of DNA immunoprecipitated with Sox2 and IgG beads was calculated based on threshold cycle [C(t)] using the ΔC(t) method and normalized to input samples. Results are expressed as fold enrichment of Sox2 immunoprecipitated samples relative to IgG controls. PCR primers are listed in Table 1

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Chapter 4

IN PROGRESS:

Expression of SOX2 and TRP63 in congenital Cystic Adenomatoid Malformation

EXPRESSION OF SOX2 AND TRP63 IN LUNGS OF PATIENTS WITH CONGENITAL CYSTIC ADENOMATOID MALFORMATION (CCAM) TYPE II, BUT NOT IN TYPE I

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Abstract
Congenital cystic adenomatoid malformation (CCAM) of the lung in fetuses is a very rare lung malformation with incidence rate of 1 in 35000 live births. However, pathogenesis CCAM is unknown and its natural history is currently unpredictable. SOX2 has been previously described in lung development and cancer tumors. In addition, our Sox2 overexpression studies in mouse model resulted in phenotypes resembling CCAM pathology. But, SOX2 has never been described in CCAM before. We used immunohistochemistry to investigate SOX2 expression in CCAM. Our results demonstrated strong expression of SOX2 in CCAM type II compared with CCAM type I and normal fetal lung suggesting SOX2 may have a role in the development of CCAM type II.

Introduction

Congenital Cystic Adenomatoid Malformations (CCAMs) are rare lung lesions resulting from disordered development of the respiratory tract [1- 3]. CCAMs vary in size and composition, and the clinical manifestations vary from severe respiratory insufficiency at birth towards recurrent infections or being completely asymptomatic throughout life. CCAMs are classified in different types of lesions (types 0-4), some associated with cystic areas and adenomatous overgrowth of the terminal bronchioles [4-5]. The incidence of CCAM is 1 in 35,000 live births [6]. Although the pathogenesis of CCAM development remains unknown, it is postulated that an imbalance between cell proliferation and apoptosis during organogenesis may be involved [7-8]. In addition, misregulation of HOXB5 has also been linked to the etiology of the disease [9].

SOX2 belongs to the highly conserved SRY-related HMG-box family of embryonic developmental transcription factors [10-12]. It plays a critical role in the development of the respiratory tract and the central nervous system [13-15]. SOX2 is expressed in the epithelial cells of the non-branching parts of the developing airways, and later in the adult trachea and extra-pulmonary airways [13, 16-17]. SOX2 plays a critical role in maintaining the stem cell-like phenotype [13]. SOX2 has been found to be amplified and overexpressed in several malignancies including head and neck, esophageal, breast, gastric, and colon carcinomas [18-20]. Recently, we showed that ectopic expression of Sox2 in epithelial cells during lung development resulted in aberrant epithelial differentiation combined with the emergence of abnormal large cysts, which showed phenotypic resemblance to the human CCAM pathology ([13]; Ochieng et al., unpublished). Moreover, we found that Sox2 directly activated the transcription of the basal cell master gene, Trp63, leading to a significant expansion of the number of basal cells, which are involved in maintenance and repair of the upper airways ([13]; (Ochieng et al, 2014). Furthermore, in vitro experiments with embryonic lung explants demonstrated that Sox2 induced proliferation and prevented cells from responding to the branch inducing signaling molecule Fgf10. Instead of bifurcating, the airways would balloon out, forming cystic structures (Ochieng et al., unpublished). Although, ectopic expression of Sox2 in the distal airway epithelium in mice showed phenotypic similarities with human CCAM pathology, it is not known whether human CCAMs express SOX2 and the downstream target TRP63 within the cystic lesion.

Previously, immunohistological analysis of CCAM material had demonstrated the expression of several lung markers, such as type I cell-associated antigen (T1 cell-Ag), surfactant

proteins and surfactant protein precursors (SP-A, SP-B, proSP-B, and proSP-C), neuroendocrine cells (GRP), Clara cells (UP-1), and the adhesion molecule CD44v6 [21]. Based on our previous mouse studies, we investigated the expression of SOX2 in human CCAM disease. We demonstrate that lungs derived from human type II CCAM patients aberrantly expressed SOX2 and TRP63, which suggests the presence of basal cells within the cystic lesion. Unexpectedly, lungs samples from type I CCAM patients did not show expression of SOX2 or TRP63, suggesting that these lesions may have a distinct etiology.

Materials and Method

Paraffin embedded tissue from the archives of the department of pathology of the Erasmus Medical Center of 20 patients with CCAM were obtained, 10 with a type I and 10 with a type II anomaly, as well as 10 age-matched healthy control lungs. Details of the patients are described in table 1. Sections of 5 μm were used for immunostaining. Sections were deparaffinized and rehydrated, followed by antigen retrieval with microwave treatment in 10mM citric acid pH 6.0 [13]. Sections were blocked with 1% BSA in PBS for 10 min and incubated with primary antibody diluted in 1% BSA overnight at 4 ^{0}C. Primary antibodies used are Sox2 1:500 (Immune system), Trp63 1:200 (Santa cruz) and Keratin5/14 1:40 (Abcam) and Sma 1:1000 (Thermo Scientific). Secondary antibodies against the correct IgG species were conjugated with peroxidase followed by DAB colorimetric detection. Nuclear were counterstained with Hematoxyline

Results

Immunohistochemical localization of SOX2 in CCAM

To determine whether SOX2 is expressed in human CCAM tissue, we used immunohistochemistry to evaluate the expression pattern of the protein in normal human lungs, and lungs of patients with CCAM type I and type II. Endogenous SOX2 protein expression was readily detected in the trachea and larger bronchi of control and CCAM patients, resembling previously reports for the pattern of mouse Sox2 [13, 15, 17]. However, the CCAM type II samples displayed a robust expression of SOX2 in the epithelium lining the cystic lesion as compared with similar distal regions of normal lungs, where no SOX2 expression was observed (Fig. 1C, F). In contrast, this pattern of expression of SOX2 was not detected in CCAM type I samples (Fig. 1B, E). Together, these analyses demonstrate the expression of SOX2 in human CCAM type II-disease tissues at aberrant sites.

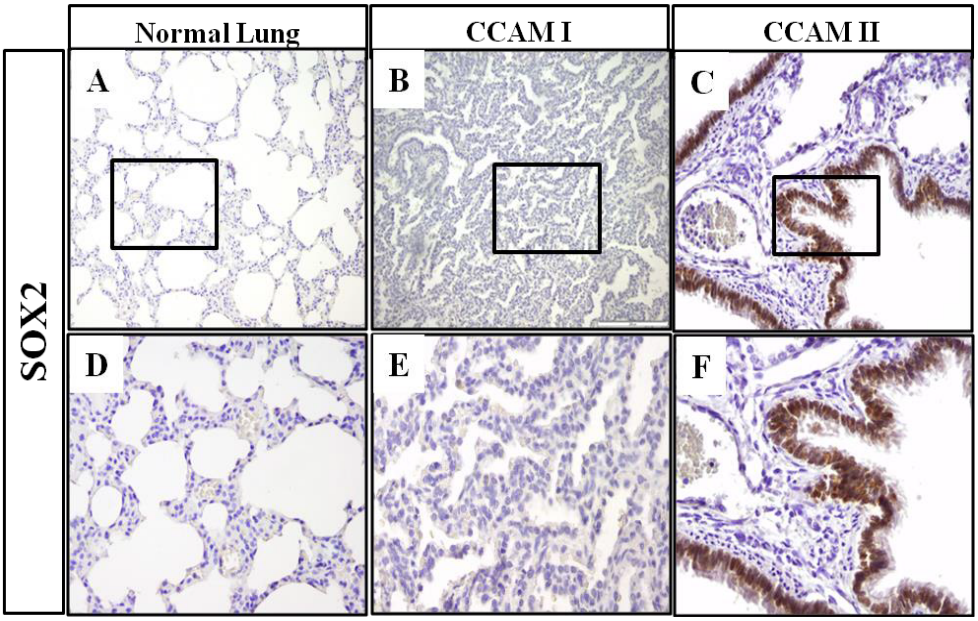

Figure 1: Lungs from human type II CCAM patients, but not type I, aberrantly express SOX2
Representative pictures of SOX2 immunostaining on human lung samples from control (A, D), type I (B, E) and type II (C, F) CCAM patients showing positive staining in the epithelium lining the cysts of the type II CCAM lungs. Scale bars: 200 μm (A-C), 40 μm (D-F)

Detection of TRP63expression in CCAM lesion

We recently showed that ectopic expression of Sox2 induced the emergence of basal cells. [13], and that Sox2 directly regulates the transcription of the basal cell master gene Trp63

(Ochieng et al., 2014). To determine whether aberrant expression SOX2 in human CCAM lungs is associated with TRP63 expression, we performed immunohistochemistry on lung sections of human CCAM type I and type II patients, as wells as control lungs. We found no TRP63 positive cells in CCAM type I lung samples except for the trachea (Fig. 2B, E). In contrast, all epithelium lining the cystic lesion in lungs of CCAM type II patients displayed intense TRP63 nuclear immunoreactivity (Fig. 2C, F). Strikingly, we noticed a gradual reduction of staining in less affected regions of CCAM lungs. Moreover the affected cystic lesions consisted of multilayered cells. Analysis of other cell markers like cGPR and GATA6 by immunohistochemistry did not detect neuroendocrine bodies (data not shown), contrasting a previous report describing the presence of cGRP in CCAM lungs [21].

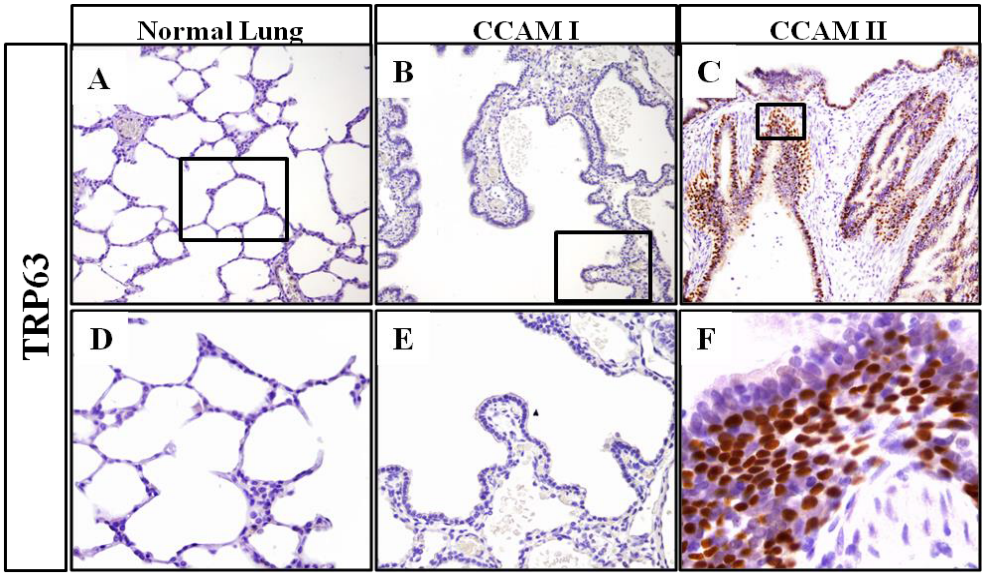

Figure 2: Lungs from human type II CCAM patients, but not type I, aberrantly express TRP63. Representative pictures of TRP63 immunostaining on human lung samples from control (A, D), type I (B, E) and type II (C, F) CCAM patients showing positive staining in the epithelium lining the cysts of the type II CCAM lungs. Scale bars: 200 µm (A-C), 40 µm (D-F)

Colocalization of SOX2 and TRP63 in CCAM

Having shown that SOX2 and TRP63 were detectable around the CCAM type II lesions in lungs of patients, we analyzed whether these proteins are co-expressed in the same cell. We previously established in the iSox2$^{Spc\text{-}rtTA}$ mouse model that Sox2 positive cells also expressed Trp63 [13](Ochieng et al., unpublished). We therefore performed dual immunofluorescence staining to investigate the co-localization of SOX2 and TRP63 in lungs of patients with

CCAM disease. As expected, no staining was observed in the lungs of patients with type I CCAM or in control lungs (Fig. 3A, B). However, an overlap of SOX2 expressing cells with TRP63 in CCAM type II samples was observed, whereas no staining was detected in the non-affected regions of these lungs (Fig.3C). In addition, the SOX2 and TRP63 double positive cells also co-expressed cytokeratin 5 (CYT5), which showed that these cells are basal cells (Fig. 3D).

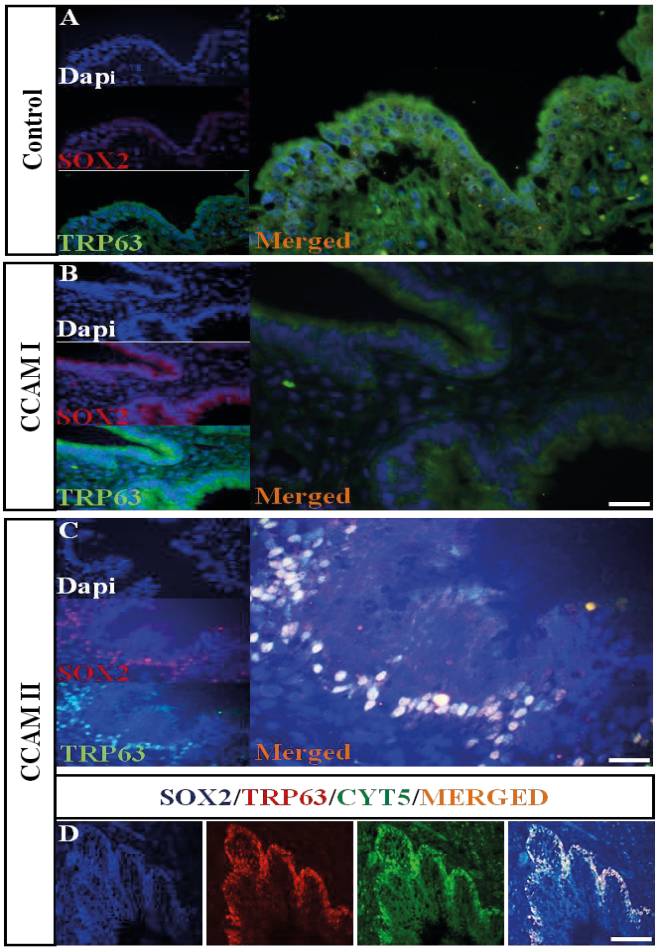

Figure 3: co-localization of SOX2 and TRP63 in cystic epithelium of type II patients

Dual immunofluorecence staining of SOX2 and TRP63 in human lung samples of control (A), type I (B) and type II (C) CCAM patients. No SOX2 and TRP63 expression was observed in the control and type I CCAM human lungs, but strong colocalization of SOX2 and TRP63 was detected in type II CCAM. A triple immunofluorescence staining using SOX2, TRP63 and CYT5 was performed on the type II CCAM samples (D). This showed that the SOX2/TRP63 double positive cells also expressed CYT5, indicating that these cells were indeed basal cells. Scale bar: 50µm

Evaluation of α-SMA in CCAM

Gross histologic examination of the cystic regions in CCAM suggested an extensive muscularization of the lesion compared to unaffected areas of the lungs. Therefore, we tested whether human CCAM lungs expressed α-SMA. Clear expression of the smooth muscle marker was observed in all specimens of CCAM I and II patients, which was absent in healthy control samples (Fig. 4A-C). The tissue surrounding lesions were α-SMA positive but not in healthy lungs from the control group.

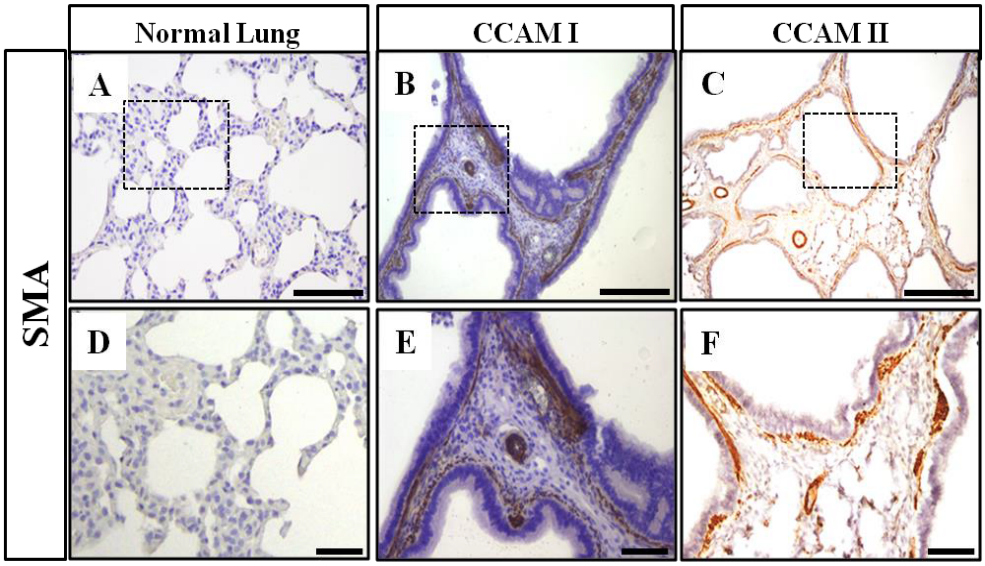

Figure 4: Aberrant expression of α-SMA in human CCAM samples Representative images of α-SMA immunostaining on lungs of human control (A, D), type I (B, E) and type II (C, F) CCAM patients. Although no α-SMA positive cells were observed in the human distal lung area, significant staining was detectable in the CCAM patients. Scale bar: 200 μm (A-C), 40μm (D-F).

Discussion

The pathogenesis of CCAM is still unclear, but several hypothesis are raised to explain the pathogenesis, such as misregulation of HOXB5 gene [9], disordered cell proliferation and apoptosis [7-8], and anomalous vascular development [22]. We suggested that aberrant expression of Sox2 may be a possible candidate for the etiology of CCAM-like lesions based on our mouse model [13] (Ochieng et al, unpublished). SOX2 is a transcription factor involved in branching morphogenesis, development of the respiratory tract and differentiation of epithelial cells [13, 16-17]. In addition, we showed that Sox2 directly regulates the expression of Trp63, the key gene for differentiation of epithelial cells towards basal cells. Here, we analyzed the expression of SOX2 and TRP63 in lungs of human CCAM type I and type II patients. Our current results show that both proteins are strongly expressed in lungs of patients with CCAM type II, but not in type I. Interestingly, this expression pattern of SOX2 was consistent with the observation we previously reported in a mouse model expressing Sox2 in the distal regions of the developing lung [13].

Previously, analysis of cellular differentiation has been performed in lungs of human CCAM patients [21]. On the basis of several differentiation markers, this study highlighted a distinction in expression pattern, suggesting that CCAM type I, II and III belong to a bronchiolar subtype, and type IV to a acinar-alveolar subtype. We present a novel finding by demonstrating the expression of SOX2 in the epithelial lining of the cystic lesions of the lungs of CCAM type II patients. Importantly, these $SOX2^{+}$ cells also co-expressed TRP63, suggesting that these cells are indeed basal cells. Additionally, our study presents a striking resemblance between animal and human data, possibly indicating that SOX2 may play a role in the pathogenesis of CCAM. Interestingly, CCAM type II tissues showed intense SOX2 and TRP6 expression within the affected lesion compared to the normal fetal lung, whereas neither of the proteins were detected in the diseased regions of the lungs of type I CCAM patients. Our results indicate a possible difference in the pathogenesis of CCAM type I and type II, and suggest a possible difference in timing of the onset of the pathogenesis. This could fit with the different cystic lesions we have observed in the ectopic Sox2 expressing mouse model [13](Ochieng et al., 2014). The exact role of SOX2 in CCAM remains unknown, however, we have shown that SOX2 directly regulate genes responsible for basal cell differentiation (Unpublished data). SOX2 and TRP63 are normally expressed in the trachea and conducting regions of fetal and adult lungs [13, 17, 23]. Taken together, we hypothesize that aberrant expression of SOX2 within the cystic lesion led to differentiation of

basal cells (TRP63+). Additionally, our finding suggests that ectopic or aberrant expression of SOX2 in CCAM type II may be responsible for the autonomous growth and proliferation previously reported in CCAM. Importantly, our results are the first findings to report the expression of SOX2 and TRP63 in lungs of human CCAM patients. Thus, our data suggest that SOX2 form part of the genes that could be of potential interest to be investigated in understanding the pathogenesis of CCAM

We also demonstrated the expression of the smooth muscle marker, α-SMA, which appeared significantly higher expressed within the cystic lesions of both CCAM I and CCAM II lungs compared to control lungs and non-affected regions. The observation of SMA cells in CCAM lesions affirmed the hypothesis that the epithelial lining of cystic lesions in CCAM becomes more proximal and thus muscularized. We hypothesize that cells lining the cystic lesions aberrantly expressed SOX2, thereby inducing proliferation and differentiation towards the proximal lineage, as we have observed in our mouse model [13](Ochieng et al, unpublished). As a result, these cells did not respond to the branch-inducing Fgf10 growth factor, but instead the regions containing these cells started to expand, forming small cysts. Depending on the developmental stage when this event started, the cysts grow and the epithelial cells start to signal to the underlying mesenchyme to differentiate into α-SMA-positive smooth muscle cells [24-25].

In summary, aberrant expression of SOX2 and TRP63 were observed in lungs of human type II CCAM patients compared to the adjacent normal lung tissue, but not in type I patient material. This suggests that there may be a fundamental difference in the origin or onset between CCAM type I and type II. However, our study focused on the two major types of CCAM, therefore the examination of surgical specimens from patients with other types of CCAM may provide additional data on the possible mechanisms of the pathogenesis.

Acknowledgements

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Sex	Diagnosis	Age/site of resection
	CCAM I	
	CCAM I	
	CCAM I	
Female	CCAM I	4 years, 2 months
Male	CCAM I	6 weeks; wedge resection l upper lobe
Female	CCAM I	4 days, lobectomi of lower lobe
Male	CCAM I	
Male	CCAM I	7 days, lobectomy right lower lobe
Male	CCAM I	
	CCAM I	
	CCAM II	
	CCAM II	
	CCAM II	
	CCAM II	
Male	CCAM II	3 years, 2 months right lower lobectomy
Male	CCAM II	1 day, bilobectomie right lung
Male	CCAM II	
Male	CCAM II	7 years, 11 months, right lower lobectomy
Female	CCAM II	13 days, left lower lobectomy
Female	CCAM II	2 years, left upper lobe
	Controls	1 day, at term neonatal death
	Controls	2 days, fetal hydrops, cardiac trombus
	Controls	perinatal asphyxia, Tr 21 term
	Controls	IUFD 39w, chorioamnionitis
	Controls	Intracerebral hemmorhage due to delivery term 2d
	Controls	Turner S, TOP 16+2w
	Controls	TOP 19w toxoplasmosis, no abnormalities found.
	Controls	TOP 20 w hydrocephalus
	Controls	TOP 21w Anencephaly
	Controls	TOP 18+1 Anencephaly

Chapter 5

Hypoxia Inducible Factor 3a Plays a Critical Role in Alveolarization and Distal Epithelial Cell Differentiation during Mouse Lung Development

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Abstract

Lung development occurs under relative hypoxia and the most important oxygen-sensitive response pathway is driven by Hypoxia Inducible Factors (HIF). HIFs are heterodimeric transcription factors of an oxygen-sensitive subunit, HIFα, and a constitutively expressed subunit, HIF1β. HIF1α and HIF2α, encoded by two separate genes, contribute to the activation of hypoxia inducible genes. A third HIFα gene, *HIF3α*, is subject to alternative promoter usage and splicing, leading to three major isoforms, HIF3α, NEPAS and IPAS. HIF3α gene products add to the complexity of the hypoxia response as they function as dominant negative inhibitors (IPAS) or weak transcriptional activators (HIF3α/NEPAS). Previously, we and others have shown the importance of the Hif1α and Hif2α factors in lung development, and here we investigated the role of Hif3α during pulmonary development. Therefore, HIF3α was conditionally expressed in airway epithelial cells during gestation and although HIF3α transgenic mice were born alive and appeared normal, their lungs showed clear abnormalities, including a post-pseudoglandular branching defect and a decreased number of alveoli. The HIF3α expressing lungs displayed reduced numbers of Clara cells, alveolar epithelial type I and type II cells. As a result of HIF3α expression, the level of Hif2α was reduced, but that of Hif1α was not affected. Two regulatory genes, Rarβ, involved in alveologenesis, and Foxp2, a transcriptional repressor of the Clara cell specific Ccsp gene, were significantly upregulated in the HIF3α expressing lungs. In addition, aberrant basal cells were observed distally as determined by the expression of Sox2 and p63. We show that Hif3α binds a conserved HRE site in the Sox2 promoter and weakly transactivated a reporter construct containing the Sox2 promoter region. Moreover, Hif3α affected the expression of genes not typically involved in the hypoxia response, providing evidence for a novel function of Hif3α beyond the hypoxia response.

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Introduction

The lung originates from the primitive foregut early in the development of land dwelling organisms, and through a complex interplay of signaling molecules the future airway epithelium and surrounding mesenchyme develop into the highly structured arbor-like bronchial-vascular tree (reviewed in [1,2,3]). Normal development in mammals occurs in a relative hypoxic environment, which is beneficial for lung organogenesis [4,5]. Cellular responses to different levels of oxygen are important for development and homeostasis [6], and the most important oxygen-sensing mechanism to protect cells from oxygen toxicity is the transcriptional response mediated by Hypoxia Inducible Factors (HIF), which are also expressed in the lungs [7].

HIFs are critical mediators of the hypoxic cellular response and regulate cellular adaptation by transactivating genes involved in angiogenesis, metabolism and cellular homeostasis (for recent reviews see [6,8,9]). HIFs are heterodimeric transcription factors which have two structurally related subunits, an oxygen sensitive HIFα subunit and a constitutively expressed HIFß or ARNT subunit (Aryl hydrocarbon Receptor Nuclear Translocator). Both subunits belong to the transcription factor family containing a basic Helix-Loop-Helix (bHLH) and a Per/ARNT/Sim (PAS) domain at the N-terminus, which mediate heterodimerization and DNA binding [10,11]. HIFß is expressed ubiquitously and as such, the level and expression patterns of the HIFα proteins are mostly determining the activity of the heterodimers [12]. Currently, three genes have been identified in human and mouse that encode HIFα isoforms, *HIF1α* [10,11], *HIF2α* or *EPAS1* [13,14,15], and *HIF3α* [16,17,18,19,20]. Aside from the N-terminal bHLH/PAS domain, the HIFα subunits contain an Oxygen-Dependent Degradation Domain (ODDD) in the center of the protein, an N-terminal transactivation domain (NTAD) and a C-terminal transactivation domain (CTAD) [21,22,23,24,25]. The CTAD is absent in the HIF3α subunit, which significantly reduces the transcriptional activity of the protein [16,26]. The three α subunits are post-transcriptionally regulated by prolyl hydroxylase domain-contain-

ing enzymes (PHD1-3), which hydroxylate with different specificity the HIFα subunits at two critical prolyl residues in the ODDD under normoxic conditions [22,27]. The PHD proteins are dioxygenases which require oxygen for their function and as such are sensitive to oxygen concentrations, losing their activity under low oxygen concentration [22]. The hydroxylated HIFα proteins are poly-ubiquitinylated and targeted for 26S proteosomal degradation through the von Hippel-Lindau (pVHL)/Elongin BC/Cul2 ubiquitin-ligase complex [28,29,30,31,32,33,34]. Under low oxygen conditions, the PHD proteins are inactive, so the HIFα proteins are not hydroxylated and stable. They will translocate to the nucleus and dimerize with HIF1β, leading to the transcription of target genes, such as EPO and VEGF, through the binding to specific DNA seqences (Hypoxia Responsive Elements, HRE) [8,35,36]. Aside from the regulation of the stability of the HIFα isoforms by PHDs, additional regulatory activities are identified. The oxygen-dependent asparaginyl hydroxylase Factor Inhibiting HIF (FIH), a member of the Fe(II) and 2-oxoglutarate-dependent dioxygenase, hydroxylates a conserved asparaginyl residue in the CTAD, preventing the association of HIFα with the p300 coactivator [37,38,39]. In addition to these hydroxylation dependent regulation of HIFα isoforms, several other posttranslational modifications have been identified (for review, see [8,40,41]).

The regulation and functions of the HIF3α gene and isoforms is very complex, contrasting HIF1α and HIF2α. The *HIF3α* locus gives rise to different splice variants, resulting in three protein isoforms, HIF3α, NEPAS (neonatal and embryonic PAS) and IPAS (inhibitory PAS) [19,20,42]. HIF3α and NEPAS only differ in the first eight N-terminal amino acids due to alternative exon usage. IPAS and NEPAS are hypoxia inducible, whereas HIF3α is not because of alternative usage of promoters [43,44]. HIF3α expression is induced under hypoxia in several organs, including cortex, hippocampus, lung, heart, kidney, cerebral cortex [17,45,46]. NEPAS is almost exclusively expressed during late embryonic and neonatal stages of development, especially in the lung and heart, while HIF3α mRNA is rarely detectable during embryonic and neonatal stages [42]. HIF3α has a high homology to HIF1α and HIF2α at the N-terminus, but only a low degree of sequence similarity across the C-terminus [26]. The HIF3α/HIF1ß (HIF3) and NEPAS/HIF1ß dimers suppress basal and hypoxia induced reporter gene activation, as well as HIF1 (HIF1α/HIF1ß) or HIF2 (HIF2α/HIF1ß) driven expression [16,42]. HIF3 binds to HRE sites in promoter regions, but the transcriptional activity is much weaker than that of HIF1 and HIF2, because it lacks the CTAD [16,26,42]. Therefore, both HIF3α and NEPAS serve as competitors of HIF1 and HIF2 dependent transcription, not only by occupying identical promoter regions, but also by associating with the same HIF1ß partner [16,42]. The splice variant IPAS lacks both the NTAD and CTAD domains producing a dominant negative regulator of the HIF1α and HIF2α dependent pathway [16,18,43]. It was shown that IPAS directly associates with HIFα isoforms, thereby displacing Hif1β, and the resulting IPAS/Hifα dimer is unable to bind to DNA [18]. Both short HIF3α isoforms related to IPAS in human and the IPAS in mouse have antagonistic effects on the expression of HIF1 and HIF2 dependent hypoxia regulated target genes [47]. Thus, the *HIF3α* locus encodes isoforms generally thought to act as negative regulators of the hypoxic response.

The importance of the hypoxia response was shown by the identification of mutations in the VHL-HIF pathway in different human diseases (reviewed in [9]). Specific gene ablation studies in mice also added to the knowledge on the pleiotropic effects of the members of the hypoxia response pathway. Complete ablation of this pathway through inactivation of Hif1ß resulted in a severe lethal phenotype with defective angiogenesis of the yolk sac and branchial arches, stunted development and embryo wasting [48,49]. Hif1α knockout mice also died early during development with cardiac malformations and vascular defects [50]. Hif2α null mice displayed a pleiotropic phenotype ranging from premature death until postnatal abnormalities, depending on the background of the mouse strain [51,52,53,54]. The neonates that survived suffered from breathing problems and did not produce sufficient surfactant phospholipids and surfactant associated proteins [51]. It is interesting to note that the inactivation and ectopic activation of Hif2α showed comparable phenotypes, suggesting that type II cells require different levels of Hif2α at distinct phases of type II cell maturation [51,55]. Homozygous mutant NEPAS/Hif3α$^{-/-}$ mice were alive at birth, but displayed enlarged right ventricle and impaired lung remodelling, suggesting that NEPAS/Hif3α is important in lung and heart development during embryonic and neonatal stages [42]. Interestingly, the *Hif3α* gene contains hypoxia response elements in its promoter region and has been shown to be a transcriptional target of Hif1α [56].

In order to understand the precise role of Hif3α during pulmonary epithelium development, we generated transgenic mice with an inducible *HIF3α* gene. Mice expressing the *HIF3α* transgene in the developing airways showed a post-pseudoglandular branching defect with a reduced number of airspaces and a clear reduction in the number of alveolar type I and type II cells. Importantly, expression of the HIF3α transgene did not lead to changes in the levels of Hif1α, but affected Hif2α. The lungs of the HIF3α expressing mice showed an upregulation of genes normally expressed in the proximal parts of the lung, while genes only expressed in distal parts of the lung were downregulated. Specifically, Foxp2, a repressor of distal cell markers, and Rarß were induced in the lungs of Hif3α expressing mice, which may explain the reduction in the number of distal cell types. Furthermore, we showed that Hif3α binds a conserved HRE in the Sox2 promoter and induces the expression of a Sox2 promoter driven reporter gene, explaining the appearance of aberrant Sox2- and p63 positive cells. Collectively, our results show that Hif3α is involved in modulating correct development of the lung epithelium.

Materials and Methods

Generation of transgenic animal

The myc epitope encoding sequence (EQKLISEEDL) was cloned directly after the endogenous ATG start codon of the full length human HIF3α cDNA (GenBank: BC080551) and subcloned into a modified pTRE-Tight vector [55]. Transgenic lines were produced by pronuclear injection of FVB/N fertilized eggs, and tail tip DNA of transgenic lines was initially genotyped by Southern blot analysis, after which positive lines were routinely checked by PCR, using transgene-specific primers (sense: 5′-GTCAAGCTTATGGCGCTGGGGCTGCAGCG; antisense 5′-GCATCTAGATCAGTCAGCCTGGGCTGAGC). Three independent lines were initially analyzed, which all produced the same phenotype as described in this manuscript. Lung-specific expression of the HIF3α transgene, i-Tg-mycHIF3α, was obtained by crossing the mycHIF3α lines with the SPC-rtTA transgenic mice (A generous gift of Jeffrey Whitsett). Administration of doxycycline to pregnant mothers from gestational age 6.5 onward in the drinking water (2 mg/ml, 5% sucrose) resulted in lung epithelium-specific expression. Each experiment was performed with at least three independent litters containing double transgenic, single transgenic and wild type pups. All double transgenic animals receiving doxycycline expressed mycHIF3α in the pulmonary

epithelium and showed the described phenotype. Mice were housed under standard conditions at 40–50% relative humidity and 21±1°C (12/12 hour dark/light cycle) with food and water ad libitum. All animal experiments were performed according to the Dutch and European guidelines and approved by the local ethics committee (DEC Nr 1657, 1833 and 2206).

Immunohistochemistry

Immunohistochemistry was essentially performed as previously described [57]. Briefly, lungs were dissected and fixed in formal saline (BDH) overnight at 4°C before processing for paraffin embedding according to routine protocols Antigen retrieval was performed with microwave treatment in 10 mM citric acid buffer pH 6.0 or Tris-EDTA. Sections were blocked with 5% BSA or 5% ELK in PBS for 10min and incubated with primary antibody diluted in 5% BSA or 5% ELK in PBS overnight at 4. The following antibodies were used: Myc (9E10, Roche; 4A6, Millipore), Hif3α (Ab2165, Abcam; NBP1-03155, Novus Biologicals), ß-tubulin IV (bioGenex), proSP-C (Chemicon), T1α (University of Iowa Hybridoma bank), Ttf1 (Thermo), Ccsp (seven hills), Sox2 (seven hills), Foxp2 (Abcam), Lpcat1 (Seven hills Bioreagents), α-Sma (Thermo), Ki67, cGRP Secondary antibodies against the correct IgG species were conjugated with peroxidase (Dako).

Lungs were imaged using an Olympus BX41 microscope and DP71 camera (Olympus, Zoeterwoude, The Netherlands). Subsequent airspaces counting were performed with SIS Softward Cell D (Olympus). Three independent samples of control and double-transgenic lungs of gestational age E18.5 were used to count the number of airspaces on a selected surface area (140000 μm^2) on those selected lung samples.

Microarray analysis

Lungs of three control and three double transgenic embryos were dissected at E18.5 and the middle and caudal lobes were used for total RNA isolation with Trizol reagent according to the manufacturer's instructions (Invitrogen life technologies, Carlsbad, CA, USA). RNA was purified using the RNeasy MinElute Cleanup kit. (Qiagen, Valencia, CA, USA) and cDNA was synthesized from 3 µg RNA using the GeneChip Expression 3′-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA, USA). Biotin-labelled cRNA synthesis, purification and fragmentation were performed according to standard conditions. Fragmented biotinylated cRNA was subsequently hybridized onto Affymetrix Mouse Genome 430 2.0 microarray chips. After normalization, the data were analysed with OmniViz software, version 3.6.0 (Omniviz, Inc., Maynard, MA, USA).

Functional annotation of the statistical analysis of microarrays results was done using Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA) and DAVID (http://david.abcc.ncifcrf.gov). The results are shown for biological processes, which are significantly ($P < 0.05$) enriched after multiple testing.

RT-PCR

RNA isolation and subsequent quantitative PCR analysis was essentially performed as previously described [7]. Gene-specific primer sets were Abca3: 5′-TTACGGTCCAAGTTCCTGAG-3′ and 5′-TAACATCAGCACCTTAGAGCC-3′; Aqp5: 5′-GTGGTCATGAATCGGTTCAG-3′ and 5′-CAAGTAGAAGTAGAGGATTGCAG-3′; Epas1: 5′-CTGTGACGACAGAATCTTGG-3′ and 5′-GGCATGGTAGAACTCATAGG-3′; Foxp2: 5′-TGTCATCAGAGATTGCCC-3′ and 5′-ATAGCCTGCCTTATGAGTG-3′; Rarß: 5′-AACTGCGTCATTAACAAGGTC-3′ and 5′-TCATTCCTAACAGACTCTTTGG-3′; Scd1: 5′-GAGCCACAGAACTTACAAGG-3′ and 5′-GTACACGTCATTCTGGAACG-3′; Sftpd: 5′-GGAAGCAATCTGACATGCTG-3′ and 5′-GAGGCTCTTCATTTCTGCTC-3′. Standard deviations of the duplicates are calculated with the SPSS program (Independent-samples T test), which also generated the P values.

Luciferase reporter activity assays

HEK293T cells were transfected in duplo with Lipofectamine 2000 (Invitrogen) with a total concentration of 500 ng DNA/well, using 9*HREluc (Gift from Manuel Landazuri), pGL3-mpSox2 and pGL3-mpSox2delta (Named Sox2-Luc and ΔSox2-Luc; Gift from Victoria Moreno), Hif2α-pcDNA3, (gift from Carole Peyssonnaux), Hif3α-pcDNA3 or pcDNA3. Cells were lysed with passive lysis buffer (Promega) 24-hours after transfection and processed for lucifease analysis by the addition of the LARII reagent (Promega), which was subsequently quantified with the VICTOR luminometer. A construct containing the renilla gene (10 ng/well) was co-transfected in each well to serve as an internal control for transfection efficiency. The renila luciferase activity was quantified by addition of Stop&Glio reagent and also detected with the VICTOR luminometer. The experiment was repeated three times, and all samples were measured at least in duplo. The average luciferase activity was calculated and divided by the average of renilla activity. Standard deviations were measured with the SPSS program (Independent-samples T test), which also generated the P values.

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed essentially as previously described [58], with some modifications. Chromatin-protein complexes of confluent A549 cells were fixed by adding 1% formaldehyde to the cultures. Nuclear extracts were made and chromosomal DNA was fragmented by sonication. Equal amounts of DNA was diluted 1:10 with ChIP dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1 and 167 mM NaCl) and the samples were pre-cleared with 80 µl prot A/G agarose beads for 1 hour, after which the sample was split in equal volumes and incubated O/N with 6 µg antibody specific for HIF3α (NBP1-03155) or control IgG (rabbit). Immune complexes were subsequently purified by adding 80 µl of prot A/G beads, which were washed several times before the immune-precipitated DNA was eluted with elution buffer (1% SDS and 0.1 m $NaHCO_3$). After de-crosslinking the DNA-protein complexes by incubation at 65°C O/N with 200 mM NaCl, the eluted DNA was phenol-extracted, precipitated and qPCRs were performed to analyze the enrichment of HIF3α specific binding to the HRE in the SOX2 gene using the following primer set 5′-CAAGTGCATTTTAGCCACAAAG-3′ and 5′-CCCAAGAGGGTAATTTTAGCCG-3′, while the primers for the ARRDC3 and EGLN3-D were described previously [36,59]. The data are the average of two independent ChIP assays, which were each analyzed by duplicate qPCRs, and are represented as the fold enrichment of the specific immune precipitation compared to the control IgG precipitation.

Results

Ectopic expression of mycHIF3α causes late branching defects

Previously, it was shown that homozygous NEPAS/Hif3α knockout mice were viable, but displayed an enlarged right ventricle and impaired lung remodelling, suggesting that Hif3α plays an important role during pulmonary development. However,

the precise role of Hif3α during the formation of the lung is not fully understood. We first analyzed the endogenous expression of Hif3α in normal fetal lungs isolated at the end of gestation (E18.5) and in lungs of adult mice (8 weeks). Hif3α positive cells were present in the epithelium of the developing lung, as well as in the type II pneumocytes of the adult lung (arrows in Figure 1A, B). In order to determine the precise role of Hif3α in the epithelium during lung development, and more specifically in type II pneumocytes, we generated transgenic mice carrying a myc-epitope tagged HIF3α under the control of a doxycycline-inducible tet-on promoter (i-Tg-mycHif3α; Figure 1C). Expression of mycHIF3α in embryonic lung epithelium was established by crossing the i-Tg-mycHIF3α transgenic line with the established SPC-rtTA line, which drives the expression of the rtTA gene in epithelial cells of the embryonic lung [60]. Pregnant females from timed matings between SPC-rtTA and i-Tg-mycHIF3α mice received doxycycline to induce the expression of the HIF3α transgene in double-transgenic fetuses. Lungs isolated from doxycycline-induced or non-induced single i-Tg-mycHIF3α or SPC-rtTA transgenic mice, or lungs from non-induced double transgenic i-Tg-mycHIF3α/SPC-rtTA animals appeared indistinguishable from wild type lungs. Doxycycline-induced, double-transgenic pups were born at Mendelian ratio and did not show obvious external abnormalities compared to their control litter mates.

In order to determine whether expression of mycHIF3α leads to pulmonary development defects, we analyzed lungs of double-transgenic animals and control lungs at different gestational ages. Macroscopic analysis of isolated lungs did not show clear abnormalities in double-transgenic animals at gestational ages E16.5, E17.5, E18.5 days and postnatal day 1 (PN1) (Figures 1E and F, I and J; Figure S1). Histological examinations at E16.5 did not show clear differences between control and mycHIF3α transgenic lungs (Figures S1C and D). However analysis of a series of developmental ages clearly showed aberrant alveolar airspaces in mycHIF3α expressing lungs starting at E17.5 compared to controls (Figure S1G, H). mycHIF3α expressing lungs contained significant fewer alveolar spaces compared to control ones at E18.5 and PN1 (Figures 1D). Staining with a specific antibody against the myc-epitope confirmed the expression of transgenic mycHIF3α protein in the epithelium of double-transgenic lungs (Figures 1H and L, Figure S1). The abnormal alveolar spaces remain present in the PN1 stages, but apparently, the mice do not suffer from respiratory distress, indicating that the initial requirements for life are present. So, we conclude that mycHIFα expression in epithelial cells leads to aberrant alveolar formation and affects late branching morphogenesis during pulmonary development.

This post-pseudoglandular branching defect prompted us to analyze the expression of the mycHIF3a at early embryonic stages of development. This showed that the transgene is expressed in a non-uniform manner in the epithelium of early E11.5 lungs (Figure 2A), but gradually all epithelial cells express the transgene (Figure 2B-D). Next, we analyzed whether the primary airway branches appropriately expressed some of the major branch-inducing genes [2]. Therefore, embryonic lungs of controls and double transgenic animals were isolated at gestational age 12.5. At this stage of development, the primary bronchi are already present, and these branches start to form secondary and tertiary branches. The expression of *Fgf10*, the growth factor with a very potent branch-inducing activity, was found in the mesenchymal compartment, alongside the epithelium that is in the process of branching (Figure 2E and I, arrows). Moreover, its receptor, *FgfR2*, was detected at the tips of the epithelium, in close proximity of the Fgf10 signal (Figure 2F and J). Next, we also analyzed the expression of two genes known to be induced as a result of the Fgf10-FgfR2 signalling, *Shh* and *Bmp4*. Both genes were also expressed in the epithelium at the same location as the FgfR2, indicating that the Fgf10-FgfR2 signalling cascade is intact (Figure 2G and K; H and L). In addition, quantitative PCR analysis of embryonic lungs isolated at E12.5, E15.5 and E17.5 of controls and double transgenic mice using primers specific for FgfR2, FgfR2-IIIb, FgfR2-IIIc, Bmp4 and Spry did confirm the absence of differential expression of these important branch-inducing genes (data not shown). In conclusion, no differences in expression pattern were observed for the early branch-inducing genes between controls and double transgenics, suggesting that the initiation of the branching process occurred normally.

mycHIF3α expression inhibits Clara cells differentiation

Since we observed significant alveolar changes and aberrant branching morphogenesis, we analyzed the integrity and differentiation potential of fetal transgenic lungs by immunohistochemistry with cell-specific markers. The smooth muscle cell component of the mesenchyme (α-Sma) did not reveal striking differences between control and transgenic lungs (Figures 3A, B). Thyroid transcription factor (Ttf1) was expressed in nearly all epithelial cells in both control and transgenic lungs (Figures 3C, D). Ciliated cells (β-tubulin) and neuroendocrine cells (cGRP) were present in proximal conducting airways of control and transgenic lungs at gestational age E18.5 (Figures 3E, F and 3G, H, arrows). Moreover, both type I (T1α; Figure 4A, B) and type II pneumocytes (Lpcat1; Figures 4C, D) were present in the alveolar regions. These results indicate that differentiation into the various epithelial cell types is not hampered by Hif3α, although the total number of each cell type may be different. In addition, no differences were observed in the proliferation of epithelial or mesenchymal cells between control and transgenic lungs as indicated by Ki67 staining (Figure 4E, F).

Next, three mycHIF3α-expressing lungs and three control lungs were processed at gestational age 18.5 days for microarray analysis, to elucidate the origin of the aberrant branching morphogenesis. Hierarchical clustering of differentially expressed genes revealed large differences between controls and double transgenic lungs (Figure 5A) and the major biological processes (Figure 5B) and molecular functions (Figure 5C) are indicated. Although mycHIF3α does not prevent the differentiation of epithelial cells into Clara cells, we noticed that the number of Clara cells was significantly reduced. Both in the microarray analysis as well as the qPCR validation showed downregulation of the Ccsp gene in mycHIF3α transgenic mice. These gene expression results were confirmed by immunohistochemistry, showing that Ccsp positive cells were less prominent in the proximal airways of the Hif3α expressing lungs compared to control lungs (Figures 6A-D). Quantification of the total number of Clara cells revealed a significant reduction in the double transgenic mice (Figures 6H). So, our data show that mycHIF3α expression inhibits Clara cells differentiation during pulmonary development.

mycHIF3α induces airway epithelial cells to differentiate into proximal cell types

Analysis of the microarray data revealed that genes associated with proximal cell types of the lung appeared to be upregulated, whereas genes specifically expressed in distal epithelial cells were downregulated (Table 1 and Table 2). The induction of proximal markers is reflected by the significant downregulation of genes specific for the distal lung epithelium. The type 1 pneumocyte cell

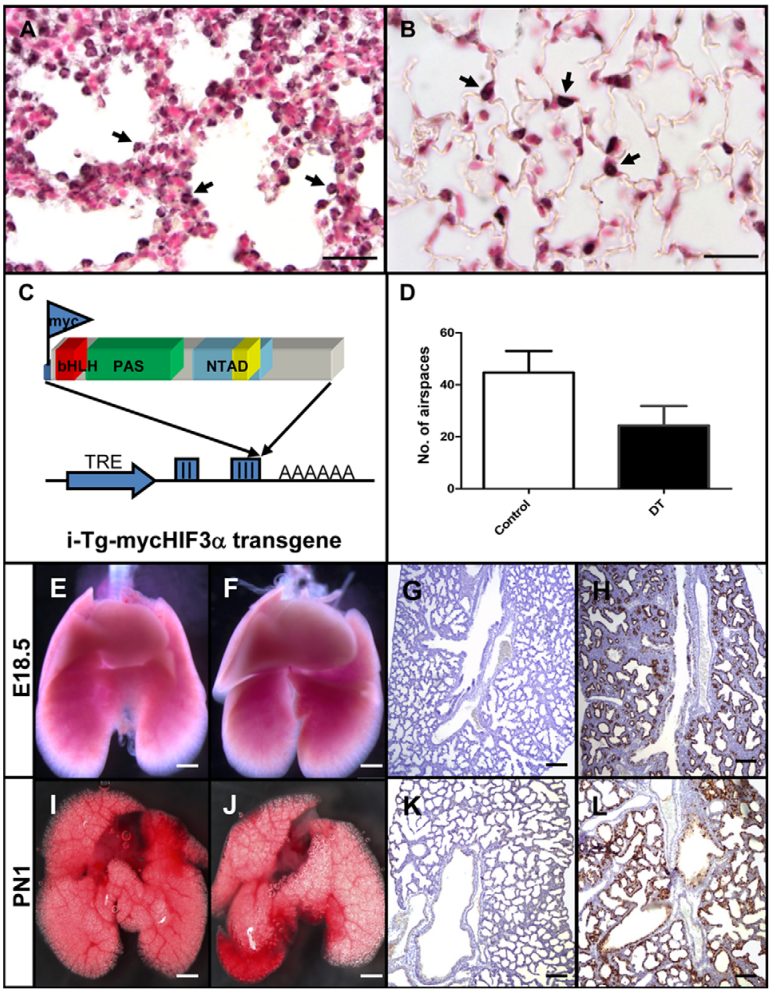

Figure 1. Enhanced expression of HIF3α results in late branching defect. Endogenous expression of Hif3α was detected in epithelial cells at gestational age E18.5 (A, arrows) and in type II pneumocytes in adult mice (B, arrows). (C) Graphic representation of the tet-inducible Hif3α/NEPAS cDNA construct used to generate transgenic mice. TRE is the Tet-responsive element containing minimal promoter, II and III refer to exon 2 and 3 of the ß-blobin gene and AAAAAA is the poly-adenylation signal. Indicated are the position of the myc-epitope, and the bHLH, PAS and NTAD domains (see text) (D) Quantification of the number of airspaces in the lung. Three independent samples of control and double-transgenic lungs at gestational age E18,5 were used to count the number of airspaces. External appearances of control (E, I) and double transgenic mycHIF3α (F, J) lungs at E18.5 days of gestation (E18.5) and post natal age 1 (PN1) do not show apparent differences. Histological analysis of control (G, K) and double transgenic lungs (H, L) showed decreased number of alveolar spaces and reduced branching in the double transgenic lungs (H and L). Anti-Myc epitope staining confirmed the expression of the mycHIF3α transgene in double transgenic lungs (H and L), which is absent in control lungs (G and K). Scale bars: Scale bars: 25 μm (A, B), 2 mm (E, F, I, J) or 200 μm (G, H, K, L).
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marker Aquaporin 5 (*Aqp5*) was dowregulated in the Hif3α expressing mice, as were three genes specifically expressed in type II pneumocytes, stearoyl-coenzyme A desaturase (*Scd1*), surfactant associated protein D (*Sftpd*) and ATP-binding cassette (ABC) subfamily A3 (*Abca3*) (Figure 6E) [61,62,63]. Quantification of the number of type II pneumocytes present in the Hif3α expressing lungs using *Sftpd* in reference to *Tff1* confirmed a significant reduction in these cells (Figure 6G). Since we are inducing the Hif3α family member of hypoxia inducible genes, we analyzed the expression of Hif1α and Hif2α in the transgenic lungs. Although no apparent difference could be detected for Hif1α (Figure 6F), but we did notice a significant downregulation of Hif2α (*Epas1*) (Figure 6E). Previously, we showed that Hif2α is involved in maturation of type II pneumocytes, so the reduction of *Epas1* expression could be directly related to the loss of type II cells.

Among the upregulated genes are two transcription factors known to play important functions during lung development, *Foxp2* and *Sox2* [57,64]. Foxp2 is important during lung

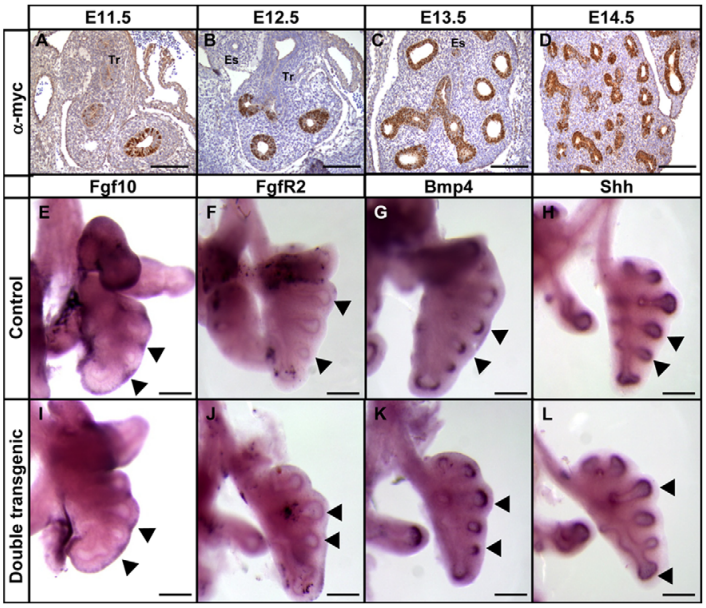

Figure 2. Expression of genes involved in branching morphogenesis. Analysis of the distribution of mycHIF3α early in lung development in double transgenic animals at E11.5 (A), E12.5 (B), E13.5 (C) and E14.5 (D). Whole mount in situ hybridization to detect the expression and localization of Fgf10 (E and I), FgfR2 (F and J), Bmp4 (G and K) and Shh (H and L) in lungs isolated at gestational age E12.5 from control (E–H) and mycHIF3α double transgenic animals (I–L). Tr: Trachea; Es: Esophagus. Scale bars: 200 μm.
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development and is expressed in the distal parts of the lung. It represses the transcription of several distal cell markers, such as T1α, Spc, and Ccsp [65]. In our microarray analysis, *Foxp2* was significantly upregulated, which we validated by quantitative PCR (Table 1 and Figure 7G). Staining with a Foxp2 antibody show that the distribution of Foxp2 positive cells in Hif3α double transgenic lungs was expanded compared to control lungs (Figures 7A, D), suggesting that Hif3α suppressed the transcription of genes specific for alveolar epithelial cells through the induction of Foxp2. In addition, *Rarβ*, which is expressed at proximal sites in the lung from embryonic day 11 to 12 and not in the distal epithelium of the lung [66,67], was significantly induced in Hif3α transgenic mice (Figure 7G), confirming the expansion of proximal cell makers in these lungs [64,65].

Sox2 is important for pulmonary branching morphogenesis, epithelial cell differentiation and is exclusively expressed in the proximal parts of the lung [57]. However, in mycHIF3α expressing lungs, Sox2 is present in epithelial cells of both proximal airways and certain alveoli at postnatal day 1, suggesting that Hif3α is able to induce proximal cell fate (Figures 7B, E, arrows). The basal cell marker p63 is expressed in the esophagal and tracheal epithelium, and previously we showed that ectopic Sox2 expression induced the appearance of p63 positive cells in the epithelium of the bronchioles and enlarged distal airspaces [57]. Therefore, we analysed the distribution of basal cells in the mycHIF3α expressing lungs and found that p63 is abnormally expressed in the alveolar epithelial cells of mycHIF3α expressing lungs, contrasting the unique expression in the trachea (Figures 7C insert, *arrows* F). Collectively, our data indicate that mycHIF3α expression leads to the induction of crucial genes, such as Sox2, Foxp2 and Rarß, which cause airway epithelial cells to differentiate into proximal cell types.

Hif3α binds the promoter region of Sox2 and induces transcription of Sox2

The promoter region of the *Sox2* gene contains two functional HREs, which are bound by Hif2α [68]. Since Sox2 is upregulated in Hif3α transgenic lungs, we analyzed whether Hif3α can directly induce the transcription of Sox2. Therefore, we first performed transcription reporter assays using a luciferase reporter construct under the influence of the Sox2 promoter containing two HREs, or two mutated HREs (Sox2-Luc and ΔSox2-Luc [68]). Hif3α induced the expression of the Sox2-Luc promoter about 2 fold, whereas the ΔSox2-Luc promoter was hardly induced compared to controls (Figure 7H). The positive control, HRE, was considerably induced by Hif2α, but only mildly by Hif3α, corresponding with the weak transcriptional activity of Hif3α [16,42]. Under hypoxia-mimicking conditions, induced by adding $CoCl_2$ to the medium, which inhibits prolyl hydroxylases by displacement of Fe(II) from their catalytic center [22], Hif3α could induce the 9*HRE-Luc considerably, and the difference with the Hif2α induced expression was much reduced (10 times versus 2 times). Moreover, the induction of the Sox2-Luc construct by Hif3α was 4 times higher than under normoxic conditions, and was comparable between Hif2α and Hif3α (Figure 7H). Subsequent analysis of the 1 kilobase region immediately upstream of the Sox2 transcriptional start site revealed that the most upstream of the two putative HRE sites was highly conserved between mice and human [68]. In order to investigate whether Hif3α could directly bind this conserved HRE site, we performed a chromatin immunoprecipitation of chromatin-protein complexes isolated

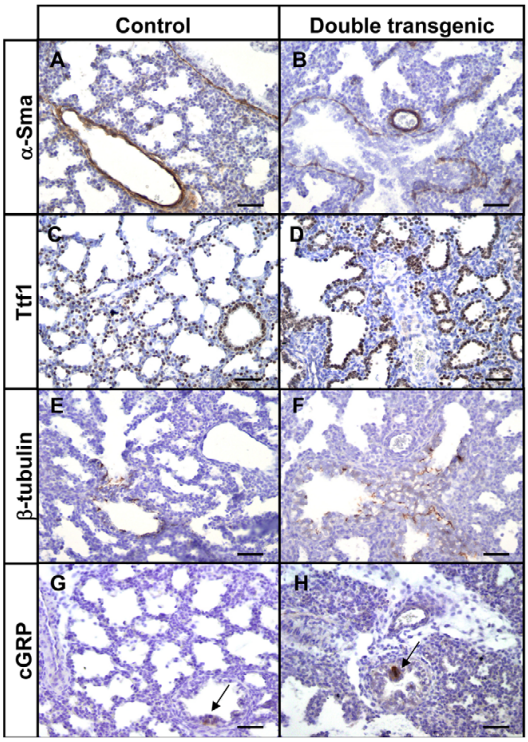

Figure 3. Normal differentiation of proximal epithelial cells in mycHIF3α transgenic lungs. The site and expression pattern of α-Sma (A and B), Ttf1 (C and D), β-tubulin (E and F) and cGRP (arrows in G and H) are comparable between control and mycHIF3α double transgenic lungs at gestational age E18.5. Scale bars: 100 μm.
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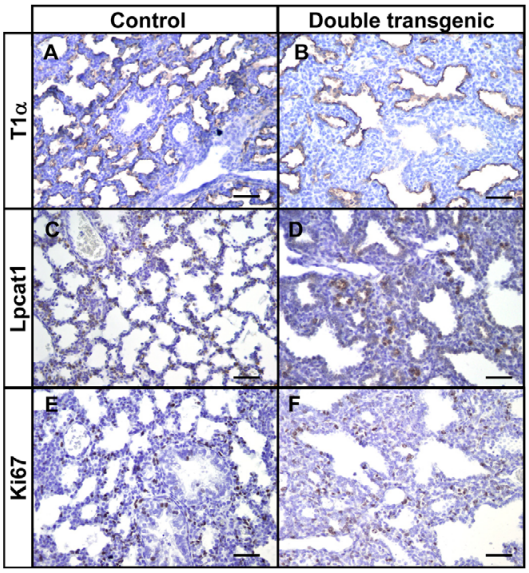

Figure 4. Normal differentiation of distal epithelial cells in mycHIF3α transgenic lungs. The site and expression pattern of T1α (A and B), Lpcat1 (C and D) and Ki67 (E and F) are comparable between control and mycHIF3α double transgenic lungs at gestational age E18.5. Scale bars: 100 μm
doi:10.1371/journal.pone.0057695.g004

from human A549 cells. Analysis of the HIF3α precipitated chromatin showed that the region containing the conserved HRE site in the SOX2 promoter region was indeed preferentially enriched compared to the IgG fraction (Figure 7I). ARRDC3 was used as a potential positive control, as it is bound by both HIF1α and HIF2α, and the enhancer region D of the EGLN3 gene served as negative control [36,59]. Indeed, HIF3α did not bind to the EGLN3-D region, but did bind to the ARDDC3-HRE. This indicated that HIF3α could bind the HRE site present in the Sox2 promoter, suggesting a potential direct regulatory role of Hif3α in the transcription of Sox2.

So, Hif3α binds to the conserved HRE in the Sox2 promoter and weakly induces Sox2 expression, resulting in an abnormal Sox2 expression in airway epithelial cells of HIF3α transgenic lungs.

Discussion

Hypoxia inducible factors are an important family of proteins involved in the regulation of the cellular response to hypoxia. Its functions are required from the earliest steps of mammalian life to the correct development of multiple organs and tissues, like the placenta, trophoblast formation, bone development, heart and vascular development (reviewed in [6,8]). The importance of the hypoxia response was shown by the identification of human mutations in the VHL-HIF pathway in different diseases [9]. Gene ablation studies in mice have revealed in more detail the specific and important roles of the different subunits of the Hifα/Hifß heterodimers. Inactivation of the stable subunit, Hif1ß, resulted in severe embryonic defects and premature death [48,49]. The disruption of the different Hifα genes identified specific roles for the individual Hifα isoforms. Hif1α knockout mice die early at gestation, have multiple developmental defects in neural tube-forrmation, vascularization, heart development, neural crest migration [69,70,71], whereas depending on the genetic background of the mouse strain, Hif2α knockout out mice ranging from early embryonic lethality to adulthood [51,52,53,54].

Hif genes and lung development

The lung is under continuous exposure of external oxygen and several (patho)-physiologic conditions trigger global or local hypoxia in the lung, resulting in pulmonary abnormalities to which HIFs contribute, such as lung cancer, acute lung injury and pulmonary hypertension (reviewed in [72]). Long term changes in oxygen levels, as experienced at high altitude gives rise to lung damage as a result of chronic mountain sickness. Recently, the *EPAS1* gene, encoding for HIF2α, was shown to be associated with adaptation of living at high altitude [73,74,75,76].

Inactivation of Hif2α in mice resulted in respiratory distress and surfactant deficiency in newborns on a mixed genetic background [51]. Remarkably, heterozygous Hif1$\alpha^{+/-}$ or Hif2$\alpha^{+/-}$ mice showed a reduced increase in pulmonary arterial pressure and right ventricular hypertrophy upon exposure to chronic hypoxia in comparison with wild type mice [77,78]. Ectopic expression of an oxygen-insensitive Hif1α transgene in lung epithelial cells during development resulted in defective branching, impaired epithelial maturation and respiratory distress. Moreover, increased expres-

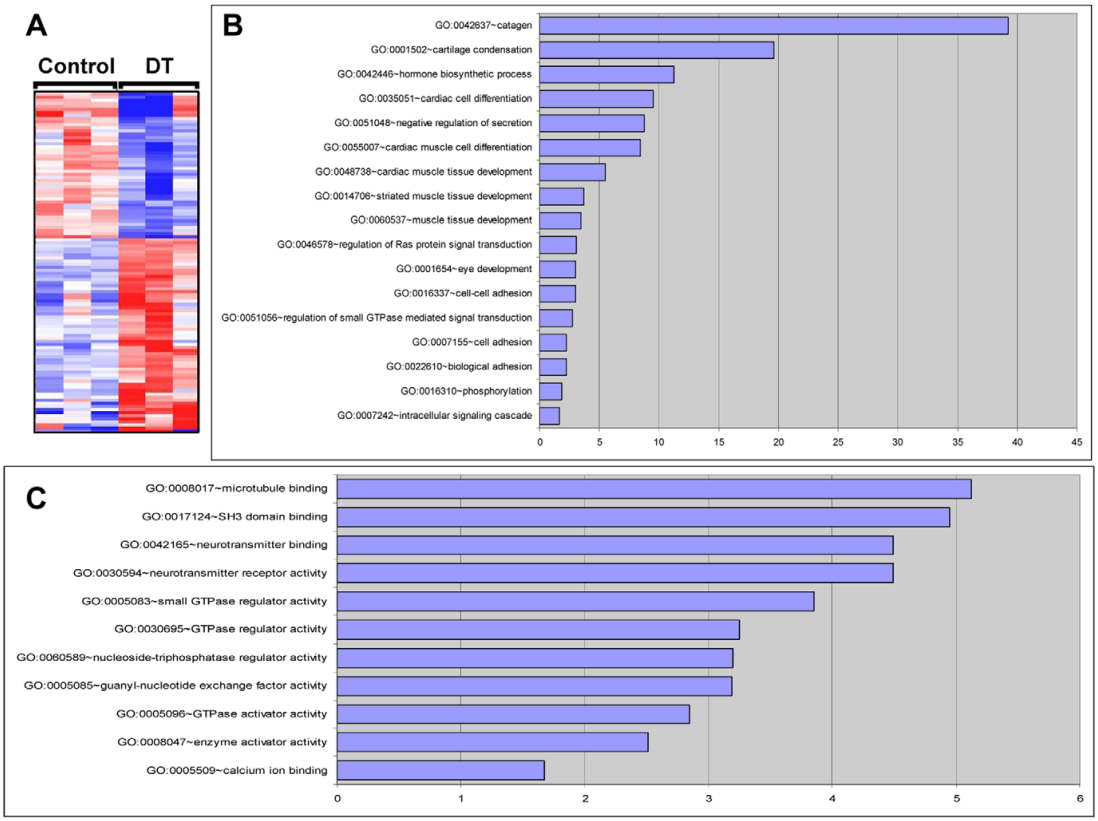

Figure 5. Transcriptome analysis of mycHIF3α expressing lungs. Treescape showing that the transcriptome of the lungs of the mycHIF3α expressing animals are significantly different from that of the control lungs (A). The red color indicates the upregulated genes and the blue color indicates downregulated genes. The expression of the genes presented in the treescape is at least 1.5 fold changed with a false discovery rate (FDR) of 10%. The top 10 biological processes (B) and molecular functions (C) of the differentially expressed genes are shown.
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sion of VegfA and VegfC was observed, leading to sub-pleural hemorrhaging [79]. We recently showed that the transgenic expression of an oxygen-insensitive mutant of Hif2α also lead to a late branching defects with enlarged alveoli and altered epithelial differentiation [55]. Contrasting the Hif1α transgenic study, we did not find increased levels of VegfA or endothelial abnormalities, even though the transgenes were expressed in the same manner. This indicates that Hif1α and Hif2α have different effects. In addition, the expression of Hif1α had not changed, whereas Hif3α expression was reduced in our Hif2α transgenic mice [55]. It seems that the effects of Hif1α are more widespread, whereas the number of affected genes by Hif2α is restricted, which is in line with previous reports describing target genes of Hif1α and Hif2α [35,36,80,81,82,83,84,85].

The occurrence of the Hif3α isoforms is well described transcriptionally, but the functional analysis is complicated by the appearance of different splice variants [19,26,42,43,86]. Hif3α isoforms act as negative regulators of the traditional Hif1 (Hif1α/Hif1ß) and/or Hif2 (Hif2α/Hif1ß) driven hypoxia response by functioning as dominant negative modulators, effectively resulting in the transcriptional competition with Hif1 and Hif2 [16,18,26,42,43]. Gene ablation of Hif3α/NEPAS/IPAS, resulted in mice that were born alive with enlarged right ventricles and impaired lung remodelling [42]. Furthermore, they showed that expression of endothelin-1 is negatively influenced by Hif3α/NEPAS, by regulating the binding of Hif1α and Hif2α to the HRE sites if the ET-1 promoter, which may contribute to the observed phenotype. Remarkably, the expression of Vegf, a direct target of Hif1 and Hif2, had not changed, even though the expression of Hif1α and Hif2α was not affected. This hinted at a selective regulation of target genes by NEPAS/Hif3α during pulmonary development. Therefore, we conditionally expressed mycHIF3α in airway epithelial cells during embryonic development in order to further elucidate the role of Hif3α in pulmonary development.

Cellular effects of mycHIF3α transgene expression

Since the NEPAS/Hif3α knockout mice suggested a selective regulation of genes by Hif3α, and our Hif2α transgenic mice showed a selective reduction in Hif3α expression, we conditionally expressed mycHIF3α in airway epithelial cells during embryonic development in order to further elucidate the role of Hif3α in pulmonary development. Analysis of mice expression a transgenic mycHIF3α in lung epithelium revealed a late branching morpho-

Table 1. Significant upregulated genes in the mycHIF3α expressing lungs.

Gene symbol	Gene name	Entrez ID	Fold Change
Dub2a	deubiquitinating enzyme 2a	384701	6,22
Naaladl2	N-acetylated alpha-linked acidic dipeptidase-like 2	635702	2,16
Cldn6	claudin 6	54419	2,14
Hspa1a	heat shock protein 1A	193740	2,14
Fbn2	fibrillin 2	14119	2
ATP6	ATP synthase F0 subunit 6	17705	1,87
Rimklb	ribosomal modification protein rimK-like family member B	108653	1,83
Sema3e	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3	20349	1,8
Tinag	tubulointerstitial nephritis antigen	26944	1,71
Mia1	melanoma inhibitory activity 1	12587	1,68
Plac1	placental specific protein 1	56096	1,68
Cdh16	cadherin 16	12556	1,64
Cnksr2	connector enhancer of kinase suppressor of Ras 2	245684	1,64
Mthfd2l	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	665563	1,63
Pcgf1	polycomb group ring finger 1	69837	1,61
Pfn2	profilin 2	18645	1,61
Hspe1	heat shock protein 1 (chaperonin 10)	15528	1,58
Fmod	fibromodulin	14264	1,54
Cdh3	cadherin 3	12560	1,54
Maob	monoamine oxidase B	109731	1,54
Rpl23a	ribosomal protein L23a	268449	1,53
Flrt2	fibronectin leucine rich transmembrane protein 2	399558	1,53
Lgals12	lectin, galactose binding, soluble 12	56072	1,53
Nnat	neuronatin	18111	1,53
Rasef	RAS and EF hand domain containing	242505	1,53
Egfl6	EGF-like-domain, multiple 6	54156	1,53
Ctnnd2	catenin (cadherin associated protein), delta 2	18163	1,52
LOC674930	similar to suppressor of initiator codon mutations, related sequence 1	674930	1,5
Sox2	SRY-box containing gene 2	20674	1,57
Foxp2	forkhead box P2	114142	1,51

doi:10.1371/journal.pone.0057695.t001

genesis defect with a reduced number of alveoli and changes in the differentiation of epithelial cell types.

Surprisingly, no apparent defects are observed early during lung development, even though the transgene is expressed. This may be due to the fact that at these stages of development, putative associating factors of Hif3α, like Hif2α and Hif2α, are not expressed yet. After the pseudoglandular stage of lung development, endogenous Hif2α becomes expressed in the cells positive for mycHIF3α and the effect of the mycHIF3α transgene starts to be noticeable. Histological analysis and gene expression profiling revealed changes in the differentiation of the developing pulmonary epithelium. We found reduced numbers of Clara cells, alveolar type I and type II cells, and in addition, basal cells were observed in atypical spatial positions. The expression pattern of diverse sets of genes was affected, and revealed that mycHIF3α expression mainly affects Hif2-directed transcription, although not all Hif2 target genes are equally affected. We show that expression of mycHIF3α in epithelial cells results in a down regulation of Hif2α, but not of Hif1α. This suggests that Hif3α is not a global regulator of the hypoxic response, but that Hif3α may selectively function to modulate Hif2α controlled target genes, supporting previous work [42]. The reduction in the expression level of Hif2α late in gestation may be due directly to the presence of mycHIF3α, or due to the impaired differentiation of the type II cells. However, it is clear that mycHIF3α does affect the differentiation of epithelial cells, and this could partly be explained by the aberrant activation of specific genes that are not part of the hypoxic response. Gene expression analysis does not show significant changes in typical hypoxia responsive genes, which indicates that Hif3α may have specific functions beyond the hypoxia response. Therefore, we provide first evidence for novel Hif3α functions beyond the hypoxia response.

The apparent increase in the mesenchymal compartment after the pseudoglandular stage does not seem to be induced by proliferation, as we did not observe an increase in mitotic cells in the mycHIF3α lungs. It may be due to either a delayed development of the double transgenic lungs, or, alternatively, to a specific response in epithelial cells triggered by mycHIF3α. Lysyl oxidase may be activated, which subsequently activates a cascade of proteins, such as Snail, involved in the repression of E-cadherin,

Table 2. Significant downregulated genes in the mycHIF3α expressing lungs.

Gene symbol	Gene name	Entrez ID	Fold Change
Olfr767	olfactory receptor 767	258315	0,45
Ass1	argininosuccinate synthetase 1	11898	0,53
Pgam2	phosphoglycerate mutase 2	56012	0,56
Gipr	gastric inhibitory polypeptide receptor	381853	0,58
Olfr6	olfactory receptor 6	233670	0,6
Igfbp6	insulin-like growth factor binding protein 6	16012	0,61
Nppa	natriuretic peptide precursor type A	230899	0,61
Dio3	deiodinase, iodothyronine type III	107585	0,63
Mphosph6	M phase phosphoprotein 6	68533	0,64
Plscr2	phospholipid scramblase 2	18828	0,64
Ccin	calicin	442829	0,65
Fabp5	fatty acid binding protein 5, epidermal	16592	0,65
Nudcd3	NudC domain containing 3	209586	0,65
Olfr171	olfactory receptor 171	258960	0,65
Rtl1	retrotransposon-like 1	353326	0,66
Rasgrf2	RAS protein-specific guanine nucleotide-releasing factor 2	19418	0,66
Fabp12	fatty acid binding protein 12	75497	0,66
Scnn1a	sodium channel, nonvoltage-gated 1 alpha	20276	0,66
Surfactant related genes			
Scd1	stearoyl-Coenzyme A desaturase 1	20249	0,31
Sftpd	surfactant associated protein D	20390	0,65
Clara cells marker			
Scgb1a1(ccsp)	secretoglobin, family 1A, member 1 (uteroglobin)	22287	0,65
Type I pneumocytes marker			
Aqp5	aquaporin 5	11830	0,65

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and ultimately leading to epithelial-mesenchymal transition, as described for metastatic tumors [87,88].

Genes affected by mycHIF3α

The appearance of proximal cells at the expense of distal cells in the mycHIF3α lungs is paralleled by transcriptional changes in several genes, such as Sox2, Rarß and Foxp2. At this point, it remains to be seen whether all effects observed are directly related to mycHIF3α, or that the expression of mycHIF3α affects Hif1α and Hif2α specific complexes, thereby interfering with transcription of specific genes. The increased expression of mycHIF3α could lead to the formation of complexes that normally are not present in the cell, which would shift the balance between Hif2α and Hif3α.

We observed Sox2 positive cells at unusual sites in the lung, which was supported by the aberrant presence of p63 positive basal cells. Previously, we showed that Sox2 directly induces the appearance of basal cells [57]. Since a link was found between Hif2α and Sox2 transcription [68], we analyzed the putative regulation of the Sox2 gene by Hif3α. We show that Hif3α is capable of inducing basal expression of a reporter construct under the control of the Sox2 promoter containing two HRE sites. In addition, we show that HIF3α binds to the conserved HRE sequence in the Sox2 promoter, which suggests that Hif3α may contribute directly to the regulation of Sox2 expression. However, the minimal transcriptional activity of Hif3α, as also shown previously, may explain the appearance of only scattered Sox2 positive cells in the lungs of mycHIF3α mice [16,26,42]. In addition, depletion of individual HIFα genes by siRNA in human ES cells suggested that HIF3α upregulates HIF2α, which subsequently induced the expression of stem cell marker genes, like SOX2 [89]. Although this hypothesis is intriguing, no direct relationship was established, yet. It was also shown that ectopic expression of HIFs in cancer cell lines can induce embryonic stem cell markers, like SOX2 and NANOG [90]. The combination of weak transcriptional activity and the ability to act as a dominant negative modulator of Hif2α may be responsible for the transcriptional regulation of Sox2. These results directly show that through the expression of HIF3α, $Sox2^+$ and $p63^+$ basal cells appear and suggest that the balance between Hif2α and Hif3α may function as a modulator of basal cell emergence [68].

Besides the aberrant induction of Sox2 and p63, the expression domain of *Rarβ* was expanded distally in the mycHIF3α transgenic lungs. Rarβ knockout mice exhibited premature septation, and formed alveoli twice as fast as wild-type mice [66,67,91]. So, upregulation of *Rarβ* in mycHIF3α transgenic mice may in part explain the observed inhibition of pulmonary alveoli formation. We also detected an increase of Foxp2, which is a transcriptional repressor able to inhibit the expression of Ccsp and markers specific for distal epithelial cells, such as Spc and T1α [64,65,92]. Therefore, the reduced numbers of Clara cells ($Ccsp^+$), alveolar type I ($Aqp5^+$) and alveolar type II ($Sftpd^+$) cells could be directly related to the upregulation of Foxp2. Recent findings showed that depletion of cells with CCSP promoter activity was associated with

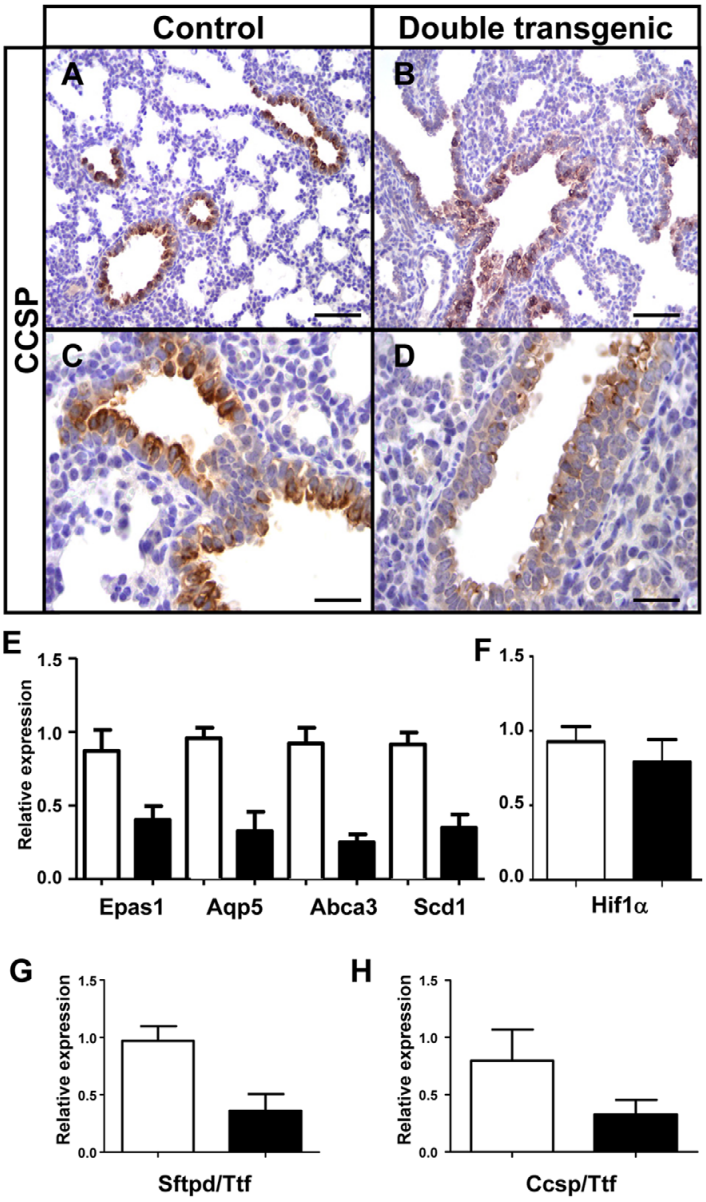

Figure 6. mycHIF3α reduces the number of Clara cells. The expression of the Clara cell marker, Ccsp, was strongly decreased in mycHIF3α transgenic lungs at gestational age E18.5 compared to controls (A and C versus B and D). (E) Alveolar epithelial cell markers are downregulated in Hif3α transgenic lungs at gestational age E18.5 as shown by quantitative PCR. *Epas1* (0,4 ± 0.1 versus control 0.87 ± 0.1, n = 3 each, P = 0.012), *Aqp5* (0.33± 0.1 versus control 0.96 ± 0.1, n = 3 each, P = 0.005), *Abca3* (0.25 ± 0.1 versus control 0.92 ± 0.1 n = 3 each, P = 0.002), *Scd1*(0.35 ± 0.1 versus control 0.92 ± 0.1, n = 3 each, P = 0.001). (F) There is no significant change in the mRNA expression of Hif1α gene (0,8 ± 0.1 versus control 0.7 ± 0.1, n = 3 each, P>0.05). Quantification of the number of (G) type II pneumocytes (*Sftpd* over *Ttf1*, 0.36 ± 0.1 versus control 0.9 ± 0.1; n = 3, P = 0.01) and (H) Clara cells (*Ccsp* over *Ttf1*, 0.3 ± 0.1 versus control 0.82 ± 0.1; n = 5, P = 0.01) showed a significant reduction of in the Hif3α double transgenic animals. White bars represent control lung samples, black bars represent mycHIF3α double transgenic lung samples. Scale bars: 100 μm (A, B) and 50 μm (C,D).
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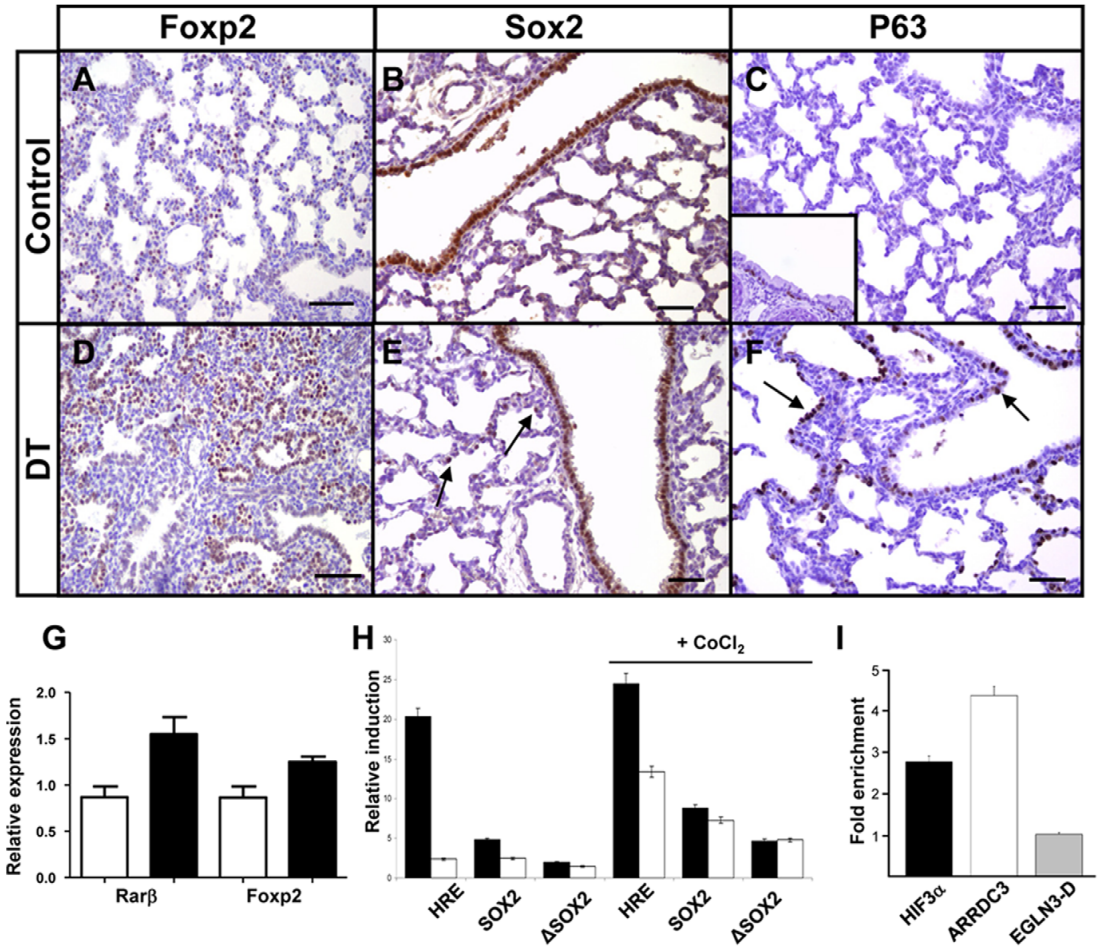

Figure 7. mycHIF3α induces the expression of proximal differentiation markers. mycHIF3α induces an expansion of the Foxp2 positive cells in the double transgenic lungs at gestational age E18.5 (A, D), as well as an expansion towards the distal parts of the lungs of Sox2 (B, E) and p63 (C, F). Sox2 was expressed in both proximal airways and alveolar epithelial cells in mycHIF3α transgenic lungs (*arrows*, E) at PN1. Basal cells are absent in control lungs (C), but are expressed in basal cells of trachea (C, insert). However, p63 is expressed in the proximal airways and alveolar epithelial cells in mycHIF3α transgenic lung (*arrows*, F). *Scale bar*: 200 μm (A and D) and 100 μm (B, C, E, F). (G) *Foxp2* and *Rarβ* are significantly upregulated in Hif3α transgenic lungs at gestational age E18.5 as shown by quantitative PCR. (Foxp2: 1.25 ± 0.1 versus control 0.87 ± 0.1, n = 3, P = 0.007; Rarβ: 1.55 ± 0.1 versus control 0.87 ± 0.1, n = 3, P = 0,009). White bars represent control lung samples, black bars represent mycHIF3α double transgenic lung samples. (H) Hif2α (black bars) and Hif3α (white bars) induce the 9*HRE-Luc (HRE) and Sox2-Luc (Sox2) as measured by the amount of luciferase activity. The fold induction of the HRE promoter is higher with Hif2α (20,3 fold and 24,5 fold under hypoxic conditions-$CoCl_2$) than with Hif3α (2,4 fold and 13,4 fold under hypoxic conditions-$CoCl_2$). The induction of the Sox2 promoter is higher with Hif2α than with Hif3α under normoxic conditions (4,8 versus 2,5), but equally strong under hypoxia mimicking conditions (8,8 versus 7,3). Data are presented as the induction (n-fold) relative to cells transfected with the corresponding reporter plasmid and control vector (pcDNA3). The values are the average of two duplicates, and standard deviations are: 0,04 (HRE-Hif2α), 0,02 (Sox2-Hif2α), 0,03 (ΔSox2-Hif2α), 0,08 (HRE-Hif3α), 0,24 (Sox2-Hif3α), 0,06 (ΔSox2-Hif3α), 0,53 (HRE-Hif2α+CoCl2), 0,007 (Sox2-Hif2α+CoCl2), 0,03 (ΔSox2-Hif2α+CoCl2), 0,88 (HRE-Hif3α+CoCl2), 0,02 (Sox2-Hif3α+CoCl2), 0,1 (ΔSox2-Hif3α+CoCl2). (I) Chromatin immunoprecipitation (ChIP) using anti-HIF3α antibody and chromatin isolated from A549 cells. Graph represents the fold enrichment of the HIF3α-specific binding to the conserved HRE of the SOX2 promoter compared to the IgG control ChIP. HIF3α also bound the ARRDC3 HRE region, and the enhancer region D of the EGLN3 gene served as negative control (EGLN3-D).
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alveolar hypoplasia and respiratory failure, adding to the idea that Ccsp downregulation as a result of Hif3α-mediated Foxp2 upregulation, directly leads to reduced numbers of Clara cells [93].

The increase d expression of key genes in lung development, which lead to major changes in epithelial differentiation, was confirmed by the loss of expression of other cell type specific markers,, such as *Sftpd*, *Scd1* and *Abca3* for type II cells. At this point it is not clear if the reduced expression of the type II cell markers is the cause, or the result of the loss of type II cells. Previously, we showed a significant downregulation of *Scd1* and

Abca3 in Hif2α expressing transgenic mice, which suffered from respiratory distress and surfactant deficiency [55]. However, the mycHIF3α transgenic mice appeared to produce sufficient levels of Scd1 and Abca3 to support respiration, even though the expression of Hif2α is decreased.

Thus, the increased expression of Sox2, Rarß and Foxp2 in the developing mycHIF3α lungs may directly contribute to the cellular changes observed and explain the phenotypic abnormalities observed in these lungs. The effects may also be cell type specific, as increased HIF3α expression in vascular cells resulted in an antagonistic effect on hypoxia induced HIF1/HIF2 target genes [47].

Concluding remarks

Although we cannot conclude that the dominant negative role of Hif3α as part of the hypoxic response is absent, our previous and current data do suggest that Hif2α and Hif3α have different target genes, during pulmonary development [55]. This is in line with previous findings describing common targets, as well as specific genes induced by Hif1α and Hif2α [80,81,82]. However, these studies used overexpression of Hif1α and Hif2α, which may cause aberrant complexes and loss of target gene specificity, as was reported for certain tumor cells [94]. Using siRNA and chromatin immuno-precipitation approaches, HIF1 and HIF2 target genes were identified [35,36,83,84,85]. Interestingly, it was shown that ETS transcription factors were involved in the regulation of HIF1 and HIF2 driven gene activation in MCF7 cells [83]. Knock down of ELK1 resulted in a reduction of hypoxia induced HIF2 dependent transcription. These data suggested a cooperation between ETS family members and HIF1 and HIF2 in the selection of target genes. An interesting idea is that target selection by HIFs may be cell specifically regulated by additional factors, adding to the complexity of the hypoxic response [8,95]. This is also observed in the analysis of the different transgenic mouse models expressing Hif1α [79], and our studies with HIf2α or Hif3α, showing similarities and differences [55].

Thus, in spite of the limited functional significance of Hif3α/NEPAS in development as a global regulator of the hypoxia response, we demonstrate that Hif3α does contribute by balancing the function of the Hif regulated genes. Furthermore, Hif3α contributes to late branching morphogenesis, alveolar formation and epithelial differentiation. Moreover, the level of *Hif3α*, as well as Hif1α and Hif2α, is tightly regulated to ensure balance between the total number of proximal cells and distal cells.

Supporting Information

Figure S1 Expression of mycHIF3α leads to late branching defect. External appearances of control (A and E) and mycHIF3α transgenic lungs (B and F) at E16.5 and E17.5 showed no apparent differences. Histological analysis of control (C and G) and mycHIF3α transgenic (D and H) lungs showed a gradual decrease in the number of air spaces and aberrant, late branching morphogenesis in mycHIF3α transgenic lungs. Anti-Myc epitope staining confirmed the expression of the mycHIF3α transgene in double transgenic lungs (D and H), which is absent in control lungs (C and G). Scale bars: 2 mm (A, B, E, F) or 200 μm (C, D, G, H). (TIF)

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Author Contributions

Conceived and designed the experiments: YH FG DT RJR. Performed the experiments: YH J-KO MB-vK AB-dM SS WvIJ RJR. Analyzed the data: YH J-KO MB-vK AB-dM SS WvIJ FG RJR. Contributed reagents/materials/analysis tools: YH MB-vK AB-dM SS WvIJ RJR. Wrote the paper: YH FG SS DT RJR.

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Chapter 6
General Discussion

Discussion

Sox2 is a member of a family of highly conserved transcription factors that play diverse roles in development and disease [1-3]. Sox2 is important for normal morphogenesis and homeostasis of diverse tissues, including neural stem cells, retinal stem cells, taste buds, hair sensory follicles in the ear, and epithelia of trachea, esophagus, and lung [4-9]. In normal mouse lung, Sox2 is exclusively expressed in conducting airway epithelial cells [10-12]. The importance of Sox2 in lung function has been demonstrated in previous findings by us and others, showing that Sox2 plays a critical role in branching morphogenesis and differentiation of basal cells [4,11,13], and it induces epithelial cell plasticity by partially reprogramming alveolar type II cells in-vivo (Chapter 3) [12]. Previous studies have shown that Sox2 is required for the expression of differentiation markers of different cell types in the lung including Sgb1a1 (Clara cells), goblet cells, and ciliated cells from a common precursor [2]. Extending previous findings from our laboratory [11], the current studies described in this thesis show a direct transcriptional control of the basal cell master gene Trp63 by Sox2. Sox2 induces cells to proliferate and to differentiate into proximal epithelium, leading to expansion of the lung epithelium which is unable to branch (figure 1). Subsequently, this results in the cystic lesion, as observed in the iSox2 mouse. This phenotype resembles a human pathology known as congenital cystic adenomatoid malformation (CCAM). In addition, we demonstrate in chapter 2 that prolonged induction of Sox2 expression during gestation induced Gata6 expression. Gata6 is predominantly associated with development and differentiation of endoderm derived organs, maturation of the lung and cancer development [14-16] It has also been shown that Gata6 regulates proliferation in the lung [17,18] and that Gata6 correlates with Wnt signaling activity in the developing lung [19].

Since full knockout of Gata6 in embryos leads to peri-implantation lethality [20], the lung epithelial specific conditional knockout of Gata6 showed that this transcription factor is required for proper lung epithelial regeneration [19,21,22]. Moreover, postnatal ablation of Gata6 was shown to result in an expansion of the BASC population and decreased differentiation [19]. Forced activation of Wnt signaling, which normally is activated during epithelial regeneration of the niche containing BASCs, leads to an increase in the number of BASC cells [19]. Blocking Wnt signaling was shown to increase the number of $Sox2^{+}$ cells [23]. We show that ectopic expression of Sox2 leads to the appearance of distal cystic lesion consisting of epithelium lined with $Gata6^{+}$ and $Trp63^{+}$ cells. However, our preliminary

analysis of BASC cells in the embryonic lung epithelium only detected very few positive cells in the bronchiolar epithelium (Chapter 2). Collectively, our study suggest a competitive action between Wnt and Sox2, where increased Sox2 expression in the lung epithelium blocked Wnt signaling, and thus BASC formation. In addition, we have also discovered an important role for Sox2 in the direct regulation of Gata6 expression, by promoting the differentiation of $Gata6^{+}$ cells late in gestation. Finally, these data suggests an association of Sox2 with the regulation of genes that could be of interest in understanding the pathogenesis of CCAM (Figure 1).

Our findings have potentially important consequences with regard to the role of Sox2 in human lung diseases. Sox2 is the most frequently amplified locus in human lung squamous cell carcinoma, but not in other lung cancer types, suggesting it may have oncogenic properties specific to tumor biology [24-30]. Therefore the induction of proliferation observed in iSox2 mouse suggests that long term expression of Sox2 may result in lung tumors. However, this hypothesis needs further exploration to determine exactly how Sox2 would contribute to tumors.

Sox2 and progenitor epithelial cells

The potential for repair of damaged airway epithelium with stem/progenitor cells is of great therapeutic interest. Regenerative medicine focuses on harnessing the power of stem/progenitor cells treatments in an attempt to reverse structural damage to the lungs, but the system is still very poorly investigated. The identification and characterization of lung epithelial stem/progenitor cells is the initial step to achieve this. Classification of progenitor cells has been challenging, and it is still controversial whether true adult lung stem cells exist. However, ongoing research into pulmonary stem cells has yielded valuable information concerning the origin and functions of stem cells at various anatomical niches, such as the trachea, bronchus and alveolus as extensively reviewed[31-36]. It is therefore crucial to further explore the lung stem/progenitor cells as they hold the key to treatments of lung abnormalities like emphysema, and the possibility to even regenerate the whole lung.

Studies in mice have shown that, under normal conditions, progenitor cells are sufficient to maintain the epithelium [37]. However, evidence for their capacity to regenerate the lung following acute injury is still lacking. Airway epithelial cells possessing the ability to enter the cell cycle after injury to the lungs have been identified and are called facultative

progenitor cells (Figure 2). These include: basal cells, Sgb1a1-like cells, Sgb1a1 cells, pulmonary neuroendocrine cells and alveolar type II cells [34,38]. These cells show high regional specialization of functions [39]. On the basis of these definitions and previous findings, we showed in chapter three that iSox2 rapidly induced the markers Sca1 and Ssea1, normally associated with progenitor cells [40,41]. These findings suggest that iSox2^{+}/Sca1^{+} cells represent an induced progenitor pool derived from de-differentiated type II cells. This indicates that Sox2 initiates alveolar epithelial cell plasticity by first regulating the emergence of progenitor-like cells in adult mouse lungs, although the subsequent steps into the proximal lineage remain speculative.

In chapter three, prolonged expression of iSox2 in the adult lungs showed the emergence of clusters of alveolar epithelial cells which expressed proximal cell markers, Scgb1a1 and Trp63. Furthermore, our results showed that the Scgb1a1^{+} and Trp63^{+} are derived from Sca1^{+} progenitor cells. In the upper airways, a discontinuous population of basal stem cells expressing Trp63 is found to be precursors of tracheal lineages [42-44]. Distal airway stem cells (DASC) possessing basal cell characteristics have also been identified after influenza virus H1N1 mediated lung injury in mouse lungs associated with regeneration of the alveolar epithelium [45]. After induction of iSox2 in adult mice, we also observed the induction of BASCs, which co-expressed Scgb1a1 and Spc. These cells had been described as stem cells since they possess the capacity to expand after naphthalene bronchiolar injury to repopulate damaged lung [46,47], further suggesting that besides the Sca1^{+} progenitor cell population, Sox2 induces other stem cell populations in the iSox2 mouse lung. We have demonstrated that Sox2 directly regulates Trp63, a master gene responsible for basal cell differentiation, as well as Sca1, a gene associated with progenitor cells, which may subsequently lead to the emergence of proximal lineages. Sgb1a1^{+} cells have been reported to self-renew and to generate ciliated cells [42]. We found that Sox2 induced populations of Sgb1a1^{+} cells in the alveolar epithelium, of which some co-expressed Sca1^{+}. In addition, lineage tracing studies using bleomycin-induced lung damage have shown that basal cells (Trp63^{+}) in the damaged parenchyma are directly derived from the labelled Scgb1a1^{+} [48]. Thus, we hypothesize that the dedifferentiation of the type II cells occurs through induction of intermediate cell type(s), Sca1^{+} or Scgb1a1^{+}/Spc^{+} (BASC), which subsequently differentiate into proximal cell types.

Lineage tracing experiments have shown that alveolar type II cells give rise to type I cells [49-51]. Recent studies demonstrated that Sgb1a1 cells gives rise to alveolar type I and

alveolar type II cells during the regeneration of alveolar epithelia in response to severe pulmonary damage [51]. In another study, basal cells differentiated into Sgb1a1 and ciliated cell after naphthalene mediated depletion of secretory and ciliated cells [52,53]. Collectively, these data show that upon damage epithelial cells can be activated and may differentiate into other cells. Our data show that the expression of Sox2 probably induces transcriptional programs that lead to a sequential dedifferentiation followed by a differentiation into selective proximal epithelial cells. Possibly, the emergence of progenitor-like cells coincides with expression of factors that may function as transcriptional partners of Sox2, which subsequently activates genes involved in proximal epithelial differentiation. The in vivo findings were supported by the in vitro culturing of primary type II cells that were induced to express iSox2, resulting in the emergence of the stem cell markers as well as differentiation markers (chapter 3).

Our results, together with previous findings, demonstrate that for growth and maintenance in adult lungs, there is a pool of inducible multipotent progenitor cells associated with a particular anatomical niche. Apparently, these cells may also exert their stem cell function at another location, whether these cells adapt to the new microenvironment, or not, requires more research. Based on our data, it seems as if the cells have either an intrinsic capacity to serve as progenitor cells irrespective of the microenvironment. Alternatively, it may be that these cells secrete specific factors that alter their microenvironment. We speculate that Sox2 can induce different lung progenitor cells by acting in multiple “hits”, like a model previously described in the Knudson cancer hypothesis, describing the inheritance of one mutant copy of a gene and mutation of the second normal allele during the life of the patient lead to retinoblastoma [54]. This would therefore suggest that multiple "hits" of Sox2 as a result of continuous activation are necessary to induce different lung progenitor stem cells that would result in plasticity. Taken together, the present study as described in chapter three provides the first evidence that Sox2 plays a crucial role in progenitor cell initiation in the lung through the regulation of Sca1. These results further suggest that a single transcription factor is sufficient to induce reprogramming of terminally differentiated cells. Further experiments are needed to clearly define the specific molecules that contribute in this process.

Control of differentiation in respiratory epithelium

Multiple transcription factor networks are involved in the development and maintenance of respiratory epithelium. Transcription factors related to differentiation include *Nkx2.1* (*Ttf1*),

necessary for lung morphogenesis, surfactant expression, and expression of the Sgb1a1 cell marker (*Ccsp*) [55-59]. Sox2 also plays crucial roles in the regulation of embryonic development (reviewed in [1]). In mice, expression of Sox2 starts from the 4-8 cell stage of embryo development and is initially expressed in the inner cell mass (ICM) and extra-embryonic ectoderm of blastocyst [60]. Sox2 is expressed exclusively in the conducting airways from foregut to mature lung, where it plays a crucial role in proliferation and differentiation of respiratory epithelial cells trachea epithelium and airway branching, Sgb1a1 cells and basal cells (Chapter two) [2,11,12,61]. Proper dorsal-ventral patterning of Sox2 and Nkx2.1 is critical for lung morphogenesis [13,62]. It has been shown that SOX2 binds the promoter region of the NKX2.1 gene inhibiting its transcription [63]. The distal tip epithelium of the developing lung contains a self-renewing population of progenitor cells positive for Id2, whose descendants exit the tip and give rise to all lung epithelial cell types [42]. Fgf10 plays a crucial role in maintaining the $Id2^{+}$ multipotent distal progenitor cells preventing them from exiting the distal tip [13,64]. Furthermore, it has been shown that as the ($Id2^{+}$) progenitor cells leave the distal tip zone, they lose expression of both Id2 and Sox9 and begin to express Sox2 [37]. Since Sox2 is downstream of Fgf10 signaling, forced expression of Sox2 surpasses the Fgf10 regulated maintenance of the Id2 cells. As a consequence, we hypothesize that Sox2 overexpression forces the exit of $Id2^{+}$ cells from their maintenance zone to differentiate away from the distal lung epithelium. This results in an abrogation of branching morphogenesis and the differentiation of cells into proximal cells. Phenotypically, these changes cause the airway epithelium to proliferate and differentiate as supported by the expression of Phh3, Trp63 and Gata6 without branching, leading to cystic abnormalities.

Sox2 regulates differentiation of multiple cell types

The study described in chapter two demonstrated that *Sox2* is required for the differentiation of basal cells in the bronchiolar epithelium. Basal cells, so-named for their proximity to the underlying basal lamina, are relatively undifferentiated cells characterized by the expression of the transcription factor Trp63 (p63)[65-67] (reviewed in [68]. Lineage-tracing experiments using keratin 5-creER transgenic allele showed that basal cells in the mouse trachea can self-renew and generate both Sgb1a1 cells and ciliated cells during post-natal growth and after injury. These findings placed basal cells at the apex of the cellular hierarchy in generating and repair of the tracheal epithelium [69] reviewed in [37]. In light with the previous findings, our *in vitro* data demonstrated the activation of promoter-luciferase constructs for the differentiation marker genes *Trp63* (basal cell), supporting the concept that *Sox2* directly

regulate the differentiation of basal cells In addition, our results are supported by the findings that conditional knockdown of Sox2 leads to reduction in basal cells [2,4], whereas, knockout of p63 results in a total depletion of basal cells in the trachea and the development of an epithelium composed mainly of ciliated cells [65]. Thus, the data presented in chapter two of this thesis show a direct involvement of Sox2 in the regulation of the basal cell master gene, Trp63.

Gata6 is important in regulating lung endoderm progenitor differentiation and proliferation [19,21,22]. Moreover, Spc^{+}/$Scgb1a1^{+}$ cells, also called broncho-alveolar stem cells (BASCs) [46], have been shown to emerge in the lungs when Gata6 is conditionally depleted [19]. Therefore BASC have been proposed to represent lung epithelial progenitor cells able to contribute both to the alveolar (distal) and bronchiolar (proximal) lineages [46]. Similarly, our study found that iSox2 induced Spc^{+}/$Scgb1a1^{+}$ cell clusters suggesting a role of Sox2 in the regulation of BASCs. In addition, we provided evidence that Sox2 directly regulates the expression of *Gata6* (Chapter 2). Zhang *et al,* demonstrated that during early lung development β-catenin triggered the amplification of cells that neither express Spc nor Scgb1a1 [19]. Their finding suggested that these cells would give rise to double positive cells upon Wnt activation. Gata6 activates canonical Wnt signaling pathway by directly repressing transcription of the secreted Wnt antagonist Dickkopf-1 [23,70-72], whereas Sox2 forms a complex with the Wnt downstream mediator β-catenin [23] [71]. Within the context of these findings, the emergence of BASCs in adult lung upon ectopic Sox2 expression suggests that Sox2 controls the balance between progenitor initiation and differentiation necessary for the development and repair of damaged adult lung. Future investigation on the interplay between these transcription factors and the cross-talk with signaling pathways will further lead to understanding of the mechanisms involved in regulation of differentiation.

iSox2 mouse: a model for cystic congenital lung malformation?

Ectopic expression of Sox2 in the developing lung epithelium resulted in cystic lesions in the mouse lungs, which resembled the human condition CCAM. However, little is known about the pathogenesis of human CCAMs. CCAMs are classified based on pathologic features, type I and type II being the most frequent forms [73,74]. Type I CCAM is composed of single or multiple large cysts, while type II CCAM is characterized by multiple small cysts. Three additional CCAM types exist; type 0 CCAM involving all lung lobes, is a state not compatible with life, type 3 are predominantly solid lesions with cysts, and type 4 CCAM lesions are large peripheral thin-walled cysts [73]. Our results in chapter four showed that SOX2 was

expressed in the lesions of type II CCAM lung samples, but not in the type I lesions. This suggests a potential difference in the pathogenesis of type I and type II CCAMs, although the exact role of SOX2 remains unknown. SOX2 expression is predominantly expressed in the conducting airways [1,4,11,12] and we recently demonstrated that ectopic expression of Sox2 in mouse lungs induced basal cell differentiation and cystic abnormalities in the lung [11]. Interestingly, cells expressing the basal cell marker TRP63 were detected in CCAM type II, which we speculate to be associated with SOX2 expression. However, the CCAM study is limited, because of the rarity of samples of some CCAM types, the evaluated samples encompassed only two types of CCAMs (type I and type II).

Currently, few mouse models harboring distinct CCAM phenotypes exist. These mouse models demonstrating cystic lung phenotypes are almost exclusively the result of altered expression of factors controlling normal lung development [75-77]. A potential role of FGF10 in CCAM pathogenesis was recently highlighted in fetal rats where induction of localized lung FGF10 overexpression resulted in localized macrocystic or microcystic lung malformations, depending on the developmental stage and the site of overexpression [75]. FGF10 is a mesenchymal growth factor which acts on the epithelium through its receptor FGFR2b to control pulmonary morphogenesis [78]. FGF10 is known to play a crucial role in branching morphogenesis [79]. Complete gene expression profiling of CCAM patient material using microarray analysis found that the FABP- gene is differentially expressed in CCAM [80]. FABP-7, also called Brain-FABP, is involved in the spatial development of the cortex, and is strongly expressed in radial glial cells [81]. Overexpression of FABP-7 in transgenic mice resulted in increased ductal differentiation, although the lungs were not examined. Hoxb5 is necessary for normal mouse lung branching morphogenesis, and its overexpression causes specific alterations in airway branching resembling human CCAM [76]. TTF1, a transcription factor regulating early phases of lung development has also been shown to be associated with CCAM, given its potential role in the development of CCAM in fetal mice lungs at varying gestational ages [77] Clements and Warner suggested based on a mouse model that a sequence of events in lung development, is responsible for congenital anomalies. This is known as the ‘wheel’ theory assuming that any insult to the tip of a developing bronchus may lead to a different lesion depending on the timing and severity, rather than on the nature, of the insult [82]. Because the cystic phenotypes reported in previous studies are a result of abnormal expression of genes important during lung development, and the fact that

Sox2 is a crucial player in lung morphogenesis, it could be important to consider using iSox2 mouse as model to study CCAM which be beneficial to the field of congenital lung diseases.
In summary, ectopic Sox2 expression during embryonic lung development resulted in the differentiation of basal cells and Gata6^{+} cells, leading to the formation of distal cysts (chapter 2). Furthermore, ectopic expression of Sox2 in fully differentiated alveolar type II cells induced the gradual de-differentiation and subsequent induction of basal cells and BASCs (Chapter 3; summarized in Figure 2).

Conclusion

The lung is a complex organ with a diverse set of cell types performing multiple functions including barrier maintenance, clearance, fluid/electrolyte homeostasis, host defense, surfactant homeostasis, and gas exchange. Congenital lung abnormalities and induced lung injuries, such as emphysema, pulmonary fibrosis, and acute lung injury, cause serious health care problems. Therefore, mechanisms of repair and regeneration of the lung are emerging fields as newer approaches for the study of stem - and progenitor cells become available. We present data supporting the concept that Sox2 is capable of inducing cellular plasticity in vivo. This finding could potentially contribute to the field of regenerative medicine. For instance, methods and mechanism of isolating alveolar epithelial cells are in place that could be used to isolate alveolar type II cells and to modify them with a single transcription factor to establish progenitor cells. In vitro these cells may be used for (re-)populating de-cellularized lungs, or establishing artificial scaffolds, that can be transplanted in damaged lungs.
The work described in this thesis primarily focused on the role of Sox2 in epithelial differentiation, and the potential importance of this transcription factor in the reprogramming of fully differentiated cells. The results contribute to a better understanding of the function of Sox2 in the differentiation of airway epithelium of embryonic and adult mouse lungs and how the resulting phenotypes in mice may be related to human congenital pathologies.

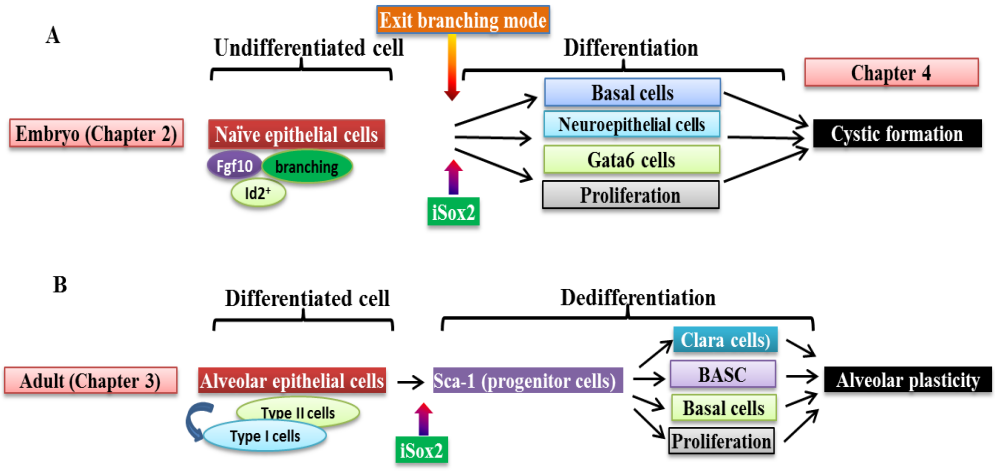

Figure 1: An illustrative scheme showing the role of Sox2 in differentiation in embryonic lungs and dedifferentiation of alveolar type II cells. Sox2 regulate the differentiation of basal cells, NEB and Gata6, and possess the capacity to initiate distal lung malformation (A). Ectopic expression of Sox2 in alveolar type II cells results in proximalization of the lung epithelium, consistent with regulation of progenitor cell initiation (B). Therefore, Sox2 is crucial for regulating the differentiation of basal and Gata6 cells during perinatal development and Sox2 induces cellular plasticity.

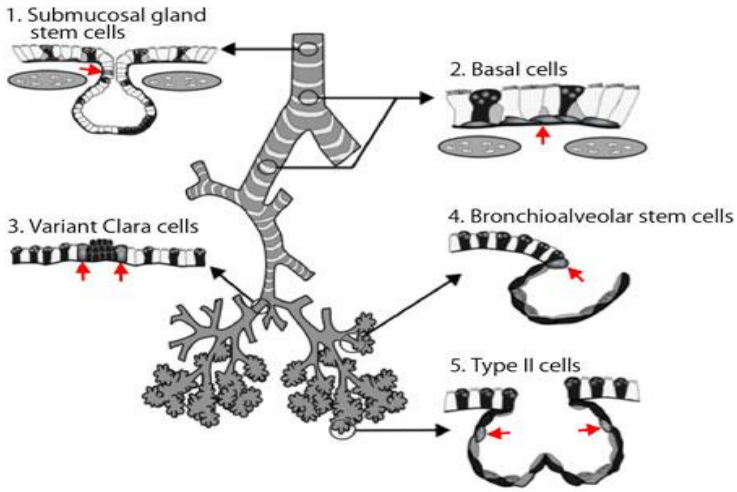

Figure 2: stem cells of the adult conducting airways (http://www.cdb.riken.jp)

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Hoofdstuk 7
Summary/Samenvatting

Summary

The lung is a complex organ whose primary function is exchanging oxygen from the atmosphere with carbon dioxide in the blood. Functionally, the lung can be divided into two regions, the conducting and the respiratory region. The conducting part transports the air to the respiratory part and is essential for moistening and warming up the air. Moreover, it is the first line of defense against environmental toxins and pathogens. The conducting part ends into millions of gas exchanging units, the alveoli. During development of the lung many factors are involved that coordinate several processes, such as branching of the airways and the formation of the vasculature. One of these factors is Sox2, a member of the highly conserved transcription factor containing the High Mobility Group (HMG). We and others have shown that this factor is important for branching morphogenesis and differentiation of the airway epithelial cells.

The lung originates at gestational age 9.5 days in mouse, or 4 weeks of gestation in human, as a primary epithelial bud from the ventral side of the foregut and invaginates the surrounding mesenchyme. We reviewed the processes implicated in lung development and highlighted some congenital lung abnormalities as examples where these processes are disturbed in **Chapter 1**. One part of this thesis focused on understanding the role of Sox2 in the emergence of Congenital Cystic Adenomatoid Malformations (CCAM), a group of congenital lung abnormalities. Previous work from our laboratory based on mouse embryonic lungs suggested a putative role for Sox2 in the pathogenesis of CCAM. The other part of the thesis focused on the plasticity of terminally differentiated cells in the lung.

To establish the role of Sox2 in the origin of distal lung malformations, we varied the duration of an inducible Sox2 transgene (iSox2) in mice during development. In **Chapter 2** we first examined the abnormalities in relation to the duration of iSox2 expression by histology. We showed a direct correlation between the size of the cysts and the timing of iSox2 expression. Lungs exposed to Sox2 for long period were characterized by severe reduction in the number of airways. Moreover, these aberrant structures contained high numbers of basal cells, as marked by the expression of the master gene Trp63. We showed that Sox2 directly bound to the minimal promoter of the Trp63 gene, and subsequently activated transcription. These findings demonstrate that Trp63 gene is a direct target of Sox2. In addition, we found that iSox2 also induced the appearance of $Gata6^{+}$ cells, which suggested a possible induction of Bronchio-Alveolar Stem Cells (BASC). We found that Sox2 directly activated the Gata6 transcription. In conclusion, we showed that aberrant Sox2 expression

induced proliferation and the differentiation towards a proximal cell fate, which prevented these lungs from responding to the branch inducing signal Fgf10. The unrestrained growth of the epithelial cells and the lack of branching resulted in the development of cyst-like structures.

In **Chapter 3,** we describe the in vivo reprograming by Sox2 of fully differentiated distal alveolar type II cells into proximal cells, which display characteristics of tracheal basal cells and bronchioalveolar stem cells. We investigated the *in vivo* and *in vitro* plasticity of differentiated alveolar epithelial cells by conditionally expressing Sox2 in alveolar type II cells. This reprogramming process occurs through intermediate cell types expressing progenitor – or stem cell-like markers, such as Ssea1 and Sca1. We demonstrate that Sox2 directly binds to the promoter of Sca1 and induces the transcription of this stem cell gene. In addition, we noticed the appearance of $Spc^{+}/Cc10^{+}$ double positive cells, suggesting that we Sox2 induced the emergence of BASC cells as well. Our data thus show the plasticity of terminally differentiated cells in vivo, which may have implications for regenerative medicine and for the development of putative therapeutic strategies to treat lung damage.

Given the link between Sox2 expression and the appearance of cyst-like structures in the Sox2 transgenic mice, we investigated the expression of SOX2 and its downstream basal cell marker gene TRP63 in lungs of human CCAM patients as described in **Chapter 4**. We show that SOX2 is expressed in lungs of CCAM type II patients, but not in type I patients, suggesting that the disease may have different pathogenesis depending on subtype. Moreover, we show the overlap of expression of basal cells with SOX2 expressing cells within the cystic lesion. These findings have potentially important consequences with regards to understanding the pathogenesis of CCAM. However, further experiments will be required that include all CCAM types to expand of the current knowledge of SOX2 expression in CCAM.

As part of our interest in airway epithelium development, we investigated the role of Hif3α in the airway epithelial cells during gestation, as described in **Chapter 5**. Conditional expression of Hif3α, another subunit of the family of hypoxia inducible transcription factors, results in aberrant branching morphogenesis, a disruption of alveolar structures and an increase in the number of proximal epithelial cells at the expense of distal epithelial cells. We also show that Hif3α directly activates Sox2, thereby triggering the differentiation of cells into proximal cells.

Thus, the data presented in this thesis show that Sox2 is a master regulator of key genes responsible for proximal epithelial differentiation. Moreover, Sox2 has a dominant effect on epithelial cells, even if these cells are already fully differentiated. The latter may

have future implication in finding ways to use regenerative medicine to address lung related diseases. Finally, we show that the expression of SOX2 is correlated with type II CCAMs, indicating that there may be a difference in the origin of this subtype compared to the other CCAMs.

Samenvatting

De longen zijn een complex orgaan met als belangrijkste functie de uitwisseling van zuurstof uit de lucht met kooldioxide in het bloed. Functioneel kunnen er twee gebieden worden onderscheiden, het transportgebied en het ademhalingsgebied. In het transportgebied wordt de lucht vervoerd naar het ademhalingsgebied, en dit is essentieel om de lucht te bevochtigen en te verwarmen. Bovendien vormt het de eerste verdedigingslinie tegen toxinen en pathogenen uit de omgeving. Het transportgebied eindigt in miljoenen orgaantjes waar de gasuitwisseling plaats vindt, de alveoli. Tijdens de longontwikkeling zijn vele factoren betrokken bij de coördinatie van verschillende processen, zoals het vertakken van de luchtwegen en de vorming van het vaatbed. Een van deze factoren is Sox2, een goed geconserveerde transcriptiefactor met genen uit de High Mobility Group (HMG). Wij en anderen hebben aangetoond dat deze factor belangrijk is voor de morfogenese van de luchtwegvertakking en de differentiatie van de epitheelcellen in de luchtwegen.

De longen worden aangelegd op dag 9.5 van de zwangerschap bij muizen, en 4 weken zwangerschap bij de mens, in de vorm van een primaire epitheelknop aan de ventrale kant van de voordarm die instulpt in het omringende mesenchym. We hebben literatuuronderzoek gedaan naar de processen die betrokken zijn bij de longontwikkeling, met speciale aandacht voor enkele aangeboren longafwijkingen waarbij deze processen zijn verstoord; dit is beschreven in **Hoofdstuk 1**. Een deel van dit proefschrift geeft een beter begrip van de rol van Sox2 bij het ontstaan van congenitale cysteuze adenomatoïde malformaties (CCAM's), een groep aangeboren longafwijkingen. Eerder onderzoek in ons laboratorium op muizenlongen wijst op een vermoedelijke rol van Sox2 bij de pathogenese van CCAM's. In het andere deel van dit proefschrift ligt de nadruk op de plasticiteit van terminaal gedifferentieerde cellen in de longen.

Om de rol van Sox2 bij het ontstaan van distale longmalformaties beter te begrijpen hebben we experimenten uitgevoerd waarin de duur van een induceerbare Sox2 trangene (iSox2) bij muizen tijdens de longontwikkeling werd gevarieerd. In **Hoofdstuk 2** hebben we eerst de afwijkingen histologisch onderzocht in relatie tot de duur van de expressie van Sox2. We konden een directe correlatie aantonen tussen de afmetingen van de cysten en de timing van de expressie van iSox2. In de longen die voor een lange periode waren blootgesteld aan Sox2 was het aantal luchtwegen sterk verminderd. Bovendien bevatten deze afwijkende structuren grote aantallen basaalcellen, gekarakteriseerd door de expressie van het mastergen Trp63. We konden aantonen dat Sox2 zich rechtstreeks bond aan de minimale promoter van

het Trp63-gen, en vervolgens de transcriptie activeerde. Deze bevindingen laten zien dat het Trp63-gen een directe target is van Sox2. Ook zagen we dat iSox2 het verschijnen van Gata6$^+$ cellen induceerde, wat wijst op een mogelijke inductie van bronchoalveolaire stamcellen (BASC). Sox2 activeerde de Gata6 transcriptie rechtstreeks. Samenvattend, afwijkende Sox2-expressie induceerde proliferatie en de differentiatie naar een proximale celbestemming, waardoor deze longen niet konden reageren op het vertakkingsinducerende signaal Fgf10. De ongecontroleerde groei van de epitheelcellen en het achterwege blijven van de vertakking resulteerde in de ontwikkeling van cysteuze structuren.

In **Hoofdstuk 3** beschrijven we de *in vivo* reprogrammering door Sox2 van volledig gedifferentieerde distale alveolaire type II cellen naar proximale cellen, die kenmerken vertonen van basaalcellen in de slokdarm en bronchoalveolaire stamcellen. De *in vivo* en *in vitro* plasticiteit van gedifferentieerde alveolaire epitheelcellen hebben we onderzocht door Sox2 voorwaardelijk tot expressie te brengen in alveolaire type II cellen. Dit reprogrammeringsproces vindt plaats via intermediaire celtypes die voorloper – of stamcelachtige markers, zoals Ssea1 en Sca1, tot expressie brengen. We laten zien dat Sox2 rechtstreeks bindt aan de voorloper van Sca1 en de transcriptie van dit stamcelgen induceert. Bovendien zagen we Spc$^+$/Cc10$^+$ dubbelpositieve cellen verschijnen, wat er op wijst dat Sox2 ook het verschijnen van bronchoalveolaire stamcellen induceerde. Onze bevindingen wijzen daarom op de plasticiteit van terminaal gedifferentieerde cellen *in vivo*, een eigenschap die implicaties kan hebben voor de regeneratieve geneeskunde en voor de ontwikkeling van mogelijke therapeutische strategieën om longschade te behandelen.

Gezien de link tussen Sox2-expressie en het vóórkomen van cysteuze structuren bij de Sox2 transgene muizen, hebben we de expressie van SOX2 en zijn downstream basaalcel marker gen TRP63 onderzocht in de longen van mensen met CCAM, beschreven in **Hoofdstuk 4**. We tonen aan dat SOX2 tot expressie wordt gebracht in de longen van patiënten met CCAM-type II, maar niet type I, wat doet vermoeden dat de ziekte een verschillende pathogenese kent afhankelijk van het subtype. Bovendien zagen we in de cysteuze beschadiging een overlap van expressie van basaalcellen met cellen die SOX2 tot expressie brengen. Deze bevindingen hebben potentiele belangrijke gevolgen betreffende ons begrip van de pathogenese van CCAM's. Verdere experimenten waarin alle CCAM-types zijn vertegenwoordigd kunnen meer kennis opleveren over SOX2-expressie in CCAM's.

Omdat we ook geïnteresseerd waren in de ontwikkeling van het luchtwegepitheel hebben we gekeken naar de rol van Hif3α in de epitheelcellen van de luchtwegen tijdens de zwangerschap, zoals beschreven in **Hoofdstuk 5**. Voorwaardelijke expressie van Hif3α, een

andere subunit van de familie van hypoxie-induceerbare transcriptiefactoren, resulteert in afwijkende morfogenese van de luchtwegvertakking, een verstoring van alveolaire structuren en een toename in het aantal proximale epitheelcellen ten koste van distale epitheelcellen. We tonen ook aan dat Hif3α rechtstreeks Sox2 activeert, en daarmee de differentiatie van cellen naar proximale cellen in gang zet.

Concluderend kunnen we zeggen dat Sox2 een master-regelaar is van de belangrijkste genen betrokken bij de proximale epitheeldifferentiatie. Bovendien heeft Sox2 een dominant effect op epitheelcellen, zelfs als deze cellen al volledig gedifferentieerd zijn. Het laatste kan van belang zijn bij het zoeken naar mogelijkheden om regeneratieve geneeskunde in te zetten bij de behandeling van long-gerelateerde ziekten. Ten slotte, we hebben aangetoond dat de expressie van SOX2 gecorreleerd is met type II CCAM's, wat er op wijst dat er een verschil kan zijn in het ontstaan van dit subtype vergeleken met andere CCAM's.

Curriculum Vitae

Bio Data

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Education and Research profile

1988-1995: Koduogo Primary School, Kenya Certificate of Primary Education (KCPE).

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2001-2005: Bachelor of Science (Bio/chemistry). Moi University Kenya, Department of Chemistry.

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2009-2013: PhD Biomedical Science. Department of Pediatric Surgery, Erasmus University medical Centre, Rotterdam, The Netherlands.

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PhD Portfolio

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1. PhD training	
	Year
General courses	
Laboratory Animal science (Art.9)	2009
Biochemistry and Biophysics	2010
Genetics	2010
Safety working in Laboratory	2011
Biomedical English Writing	2012
Specific courses (e.g. Research school, Medical Training)	
Literature courses	2011
From development to disease	2011
In vivo imaging: From molecules to organism	2012
Leica confocal introduction courses	2012
Seminars and workshops	
16th MCG PhD Student workshop, Cologne Germany	2010
17th MGC PhD student workshop, Maastricht, Netherlands **Poster presentation**	2011
19th MGC PhD student workshop, Luxembourg, Luxembourg **Oral presentation**	2013
NRS symposium Utrecht**,** Netherlands **Oral presentation**	2013
Presentations	
Monday Morning Meeting	2009-2013
Work discussions (Dr. Rottiers'group)	2009-2013
Literature discussion	2009-2013
(Inter)national conferences	
EuTRACC 2nd Young Scientist Meeting, Dubrovnik Croatia **Oral presentation**	2010
EuTRACC Scientific Meeting, Berlin Germany **Poster presentation**	2012
ERS Lung Science Conference, Estoril ,Portugal **Poster presentation**	2013
International CDH workshop, Rotterdam Netherlands, **Oral presentation**	2013
National Conferences	
MGC symposium, Leiden MC The Netherlands	2012
Supervising practical's and excursions, Tutoring	
Student supervision (Rowan van Sprundel)	2012

List of abbreviations

BMP	Bone morphogenetic factor
cGRP	Calcitonin Gene related protein
CC10	Clara cell protein 10
CCAM	Congenital cystic adenomatoid malformation
CLE	congenital lobar emphysema
ChIP	Chromatin immunoprecipitation
iSox2	induced Sry related HMG box
IHC	Immunohistochemistry
IF	Immunofluorescence
IP	Immunoprecipitation
Fgf	Fibroblast growth factor
H&E	Hematoxyline and Eosin
HMG	High mobility group
PS	pulmonary sequestration
qPCR	quantitative polymerase chain reaction
rtTA	reverse tetracycline transactivator gene
Sca-1	Stem cell antigen-1
Shh	sonic hedge hog
SPC	Surfactant protein C
SRY	Sex determining region of Y chromosome
SMA	Smooth muscle actin
Tgf	Thyroid growth factor

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Needless to say, any remaining errors, oversights and any other deficiencies are mine and mine alone.

Supplementary Figures [Chapter Two (AJRCMB)]

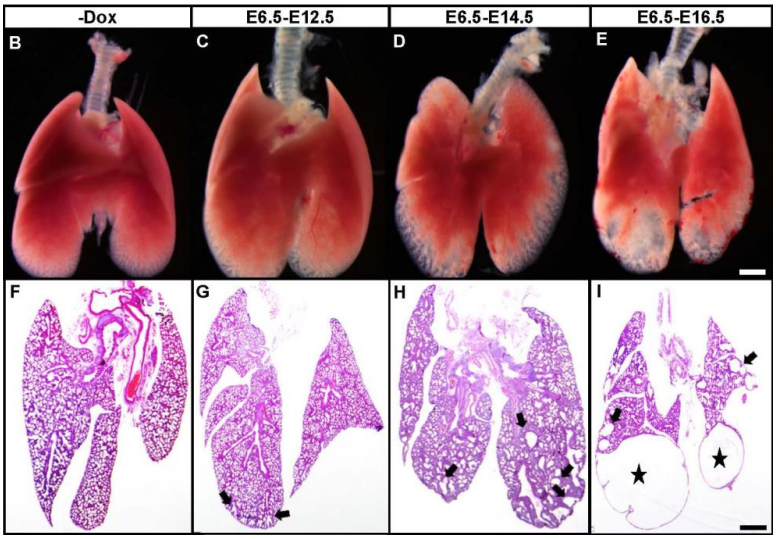


Figure1: Withdrawal of ectopic Sox2 expression causes variable cyst sizes in the lung. Control and iSox2$^{Spc\text{-}rtTA}$ mice were treated with doxycycline for a specified period then doxycycline administration withdrawn. Lungs were isolated at E18.5 and analysis of the external appearances shows varied cystic abnormalities in lung structure (C–E). Haematoxylin/eosin stained sections show the cystic malformations in the Sox2 transgenic lungs (F-I). Arrows indicate cysts in the epithelium. Scale bars: 2 mm

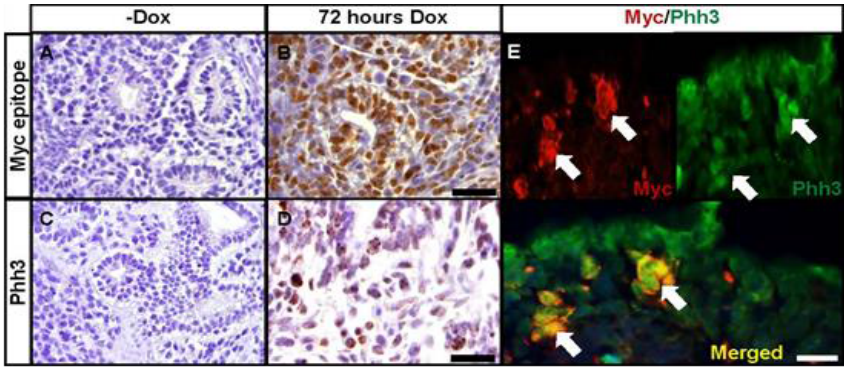


Figure2: Sox2 induces proliferation. (A-D) iSox2$^{SPC\text{-}rtTA}$ lung explant treated with(out) doxycycline for 72hours were analyzed with the mitotic cell marker antibody Phh3. Representative images show proliferation in individual type II cells after 72hours of iSox2 induction (D. (E) Colocalization of Myc epitope and proliferation Phh3 is shown by dual immunofluorescence labeling. Scale bars: 100µm (A-D), 50µm (E)

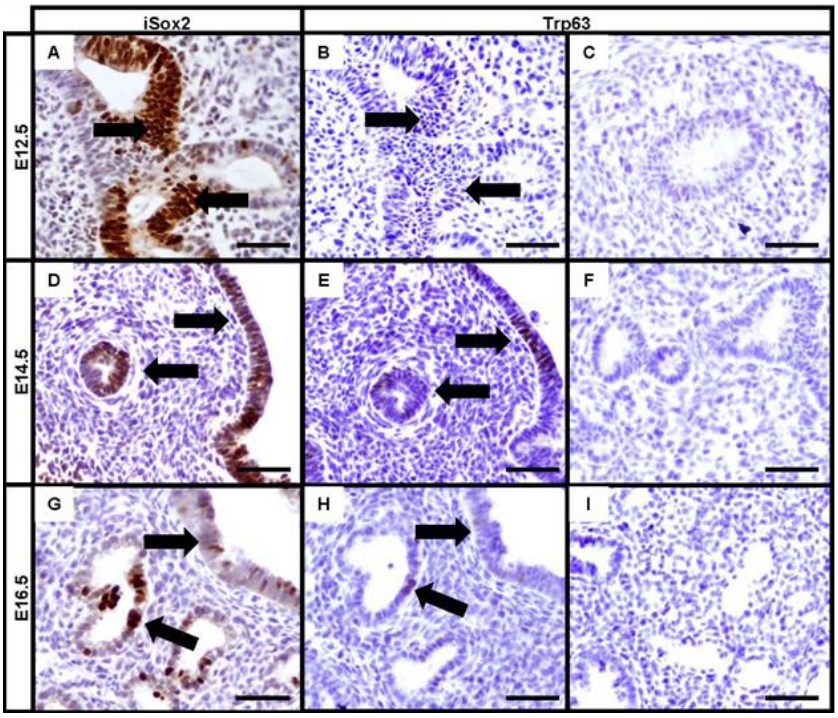
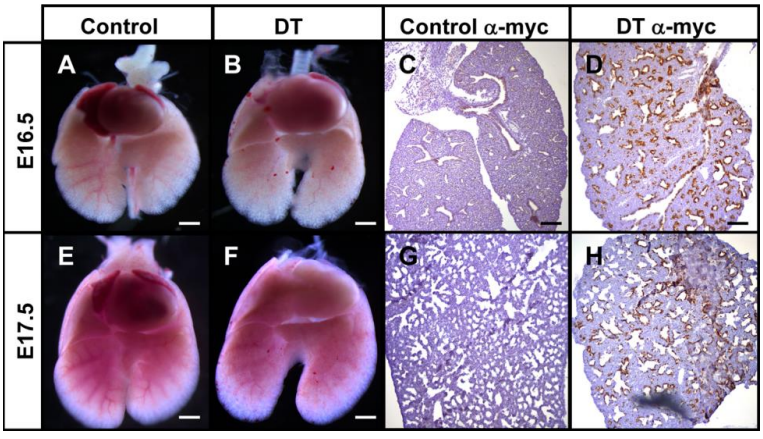


Figure3: Expression of basal cells in the alveolar epithelium with is directly associated with Sox2 induction. Immunohistochemistry of iSox2 and Trp63 in (A-C) E12.5, (D-F) E14.5 and (G-I) E.16.5 lungs Scale bars: 100μm

Supplementary figure PLOS ONE (Chapter 5)



Expression of mycHIF3α leads to late branching defect. External appearances of control (A and E) and mycHIF3α transgenic lungs (B and F) at E16.5 and E17.5 showed no apparent

differences. Histological analysis of control (C and G) and mycHIF3α transgenic (D and H) lungs showed a gradual decrease in the number of air spaces and aberrant, late branching morphogenesis in mycHIF3α transgenic lungs. Anti-Myc epitope staining confirmed the expression of the mycHIF3α transgene in double transgenic lungs (D and H), which is absent in control lungs (C and G). Scale bars: 2 mm (A, B, E, F) or 200 μm (C, D, G, H).

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(TIF)