

Impact of the epithelial hypoxia-inducible factor 2 α /
fetal liver kinase-1 system on murine lung development

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1. Introduction

1.1. Lung development

Lung development is a delicate and tightly regulated process which aims to create an organ in which air flow and blood flow are unified to allow the central function of gas exchange and oxygenation of the blood. Accordingly, two main systems are combined in the lung: the airway and the blood vessel systems. These two systems develop in parallel during the different stages of lung development. A variety of different cell types and structural compartments of the lung interface with one another to support this central function of gas exchange. For example, the surface tension of each single alveolus has to be reduced to prevent collapse of the gas exchange region. This is facilitated by surfactant production by alveolar type two cells, and secretion of surfactant into the surface mucoid liquid layer (Rugonyi et al., 2008). Furthermore, the lung has to be cleared of invaded particles which accumulate in the lung surface mucus. This is achieved by the coordinated movement of the cilia of the ciliated bronchial epithelial cells. Thus, during lung development, each single cell has to differentiate into a certain cell-type with a given phenotype to facilitate its individual impact on lung function. To characterise the different developmental processes involved, lung development has been divided into six different stages.

1.1.1. Stages of lung development

The six different stages of lung development begin with the first appearance of the tracheal bud in the developing embryo at gestational day 26 (Burri in: McDonald, 1997 p. 3-4), and last until early childhood. The early and prenatal stages comprise the embryonic, the pseudoglandular, the canalicular and the sacular stages (Figure 1). During these stages, the primitive airways and blood vessels are laid down in the surrounding interstitial mesenchyme, which gives rise to the so-called primary septa. Maturation of the airway system and the capillary network, respectively, is accompanied by a thinning of the interseptal mesenchyme. The fifth stage, the alveolar stage, begins shortly before birth and overlaps with the final stage of microvascular maturation which starts postnatally (Zeltner and Burri, 1987). In particular, the completion of alveolar formation takes place during the early postnatal phase, and it is still a matter of debate whether this process continues late into postnatal life (Alescio and Cassini, 1962; Burri, 1997).

1.1.1.1. The embryonic stage

The embryonic stage comprises the early phase of the first 1-7 weeks after fertilization. Most organs are laid down during this period. At gestational day 26, a ventral outpouching of the foregut initiates lung development. The appearance and deepening of the laryngotracheal grooves which start to separate the lung bud from the prospective esophagus, gives rise to the lobar bronchi after elongation and dichotomous division of the lung bud. The outpouching of the foregut is maintained and develops into the hypopharynx, which is the entrance to the larynx. At the age of 4-5 weeks, two sacculi on the left-hand side and three sacculi on the right-hand side invade the surrounding mesenchyme. Successive dichotomous division of the growing lung sacculi gives rise to the future airway tree. By the end of the 7th week, the airway tree is completely preformed, down to the subsegmental branches. The interaction of the mesodermally-derived mesenchyme and the endodermally-derived epithelial system is a key function that determines the developmental processes of the embryonic phase (Alescio and Cassini, 1962; Spooner and Wessells, 1970; Taderera, 1967). The development of the vascular system is also initiated during this stage. Vasculogenesis (the generation of blood vessels by endothelial progenitor cells) and angiogenesis (the formation of blood vessels out of existing blood vessels) have been described as the two major processes of vascular development (Risau, 1997). In particular, the process of angiogenesis has been investigated during lung development (Patan et al., 1996). The pulmonary arteries are derived from the six pairs of aortic arches. The pulmonary veins grow out of the left atrial portion of the heart. By the end of the 7th week, due to the invasion of the epithelial tubes into the mesenchyme, the morphological shape of the lung resembles a gland. This marks the beginning of the pseudoglandular stage.

1.1.1.2. The pseudoglandular stage

This stage was initially described as the “bronchial phase” of lung development. But further studies revealed that by the end of this stage at week 17, not all conducting airways have been built (Kitaoka et al., 1996). Hence, the terminal bronchi are built in this period. The transition of the pseudoglandular to the canalicular stage has been described as the period of the birth of the acinus (Burri in: McDonald, 1997 p. 4-6). Epithelial cells at the distal part of the airway tree have been identified as the precursor cells of the alveolar epithelium in this stage (Ten Have-Opbroek, 1979; Ten Have-Opbroek, 1981). These epithelial cells are loaded with glycogen which is a key substance required for further cell differentiation (Burri in: McDonald, 1997 p. 4-6). Furthermore, ciliated cells, goblet cells and basal cells appear in the

central epithelial tube at this stage, and are spread latterly to the more peripheral regions. Finally, cartilage is found in the pseudoglandular stage (Bucher and Reid, 1961). Regarding vascular development, the growth and division of the arteries occurs like that of the airway branching (Hislop and Reid, 1972). In contrast, the veins run in connective tissue septa between the airway branches (Verbeken et al., 1996). When the airway branches have formed the early acinus, the transition to the canalicular phase occurs.

1.1.1.3. The canalicular stage

This stage begins around week 16 and lasts until the end of week 26. Multiplication of the capillaries is one major developmental consequence of this stage. Therefore, it was designated the “canalicular” stage, because the lung parenchyma becomes canalized by the growing three dimensional capillary network (Burri in: McDonald, 1997 p. 6-8). Later, the airways were named canaliculi, which was misleading concerning the initial origin of the name of this stage. Indeed, the airways undergo an important change during this stage: the distal primitive epithelial system becomes flat, and single glycogen-rich cells differentiate into type I and type II pneumocytes (Burri in: McDonald, 1997 p. 6-8). This flattening of the peripheral regions and the multiplication of the capillary network leads to the first appearance of the gas exchanging tissue. Closer alignment of the capillaries and the flattened epithelial cell layer proceeds the maturation of the inter airway space towards the primary septa which are built in the saccular stage. At the same time, type II epithelial cells accumulate lamellar bodies, which represent the intracellular storage of surfactant. Thus the flattening of the peripheral regions is accompanied by the production of surface-active material. Mercurio and Rhodin have described that the cells of the undifferentiated epithelium already contain lamellar bodies before they differentiate into type I and type II pneumocytes (Mercurio and Rhodin, 1976; Mercurio and Rhodin, 1978). This has led to the suggestion that adult type II pneumocytes might be the precursors of type I pneumocytes. Taken together, the canalicular stage is a key developmental step: due to the appearance of the gas exchange region and the surface-active substances, by the end of the canalicular stage, a prematurely born baby in the canalicular stage may survive to adulthood (Figure 1).

1.1.1.4. The saccular stage

The saccular stage is primarily the stage of widening and growing of the so-called transitory airspaces and capillary network (Burri in: McDonald, 1997 p. 8-9). This process is accompanied by a reduction of the inter-airway septum thickness which gives rise to the primary septum. Within these primary septa a double-capillary network between the adjacent

distal air way walls still exists. The thickness of the intra-septal mesenchyme will be further reduced in the later alveolar stage: The primary septa will give rise to the first primary alveoli by generation of thinner secondary septa, which still contain two inter-septal capillaries. The earlier saccular stage begins at week 24 and finally overlaps with the alveolar stage shortly before birth, by which time it comes to the end. In summary, the saccular stage comprises a period of further maturation of the air spaces and capillary network, which gives rise to the primary septa. The transition into the alveolar stage begins shortly before birth. At birth, the saccular stage ends. During the subsequent alveolar stage, the process of further differentiation of the very distal air spaces continues.

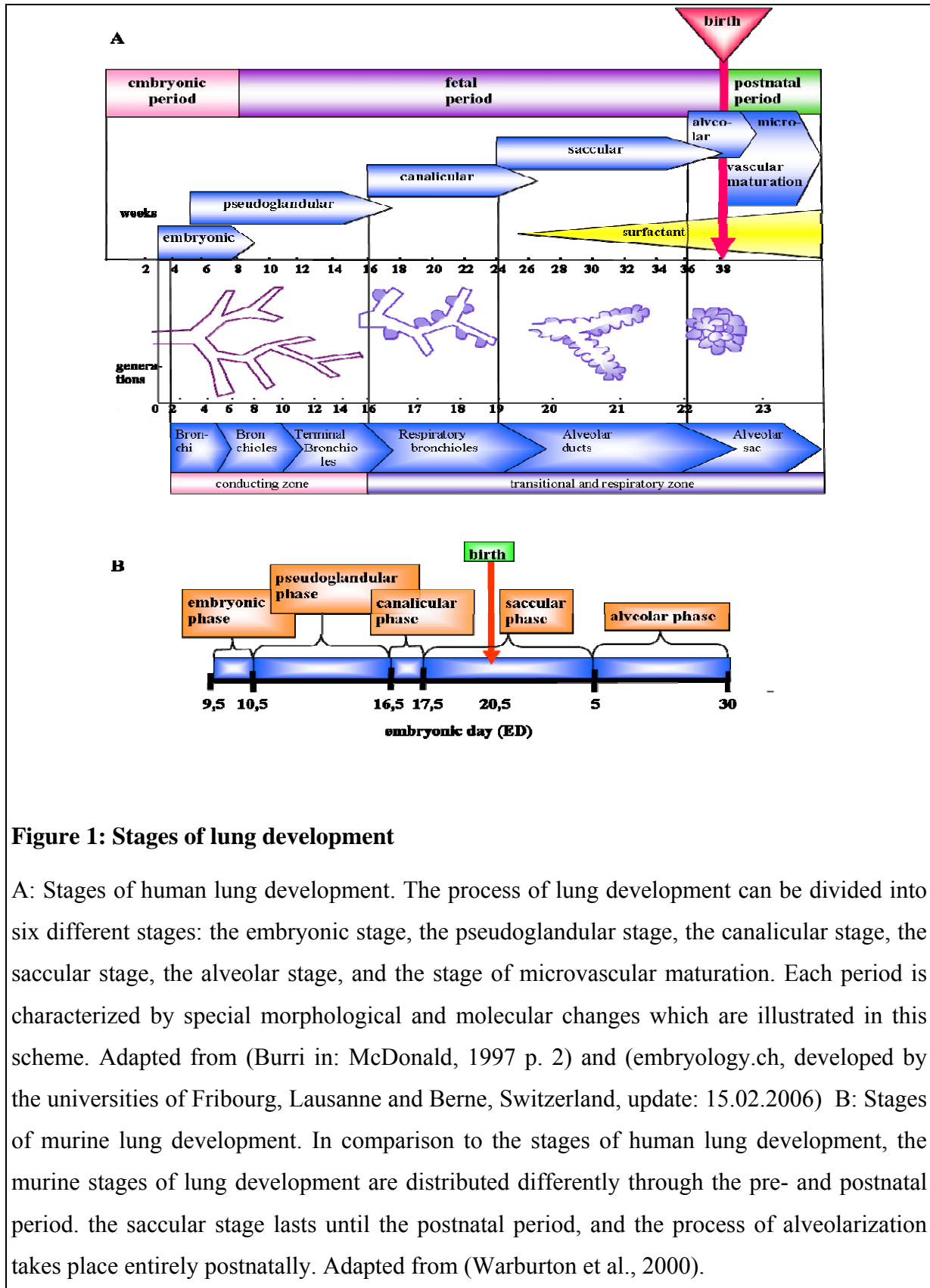
1.1.1.5. The alveolar stage

The alveolar stage begins shortly before birth at week 36 and ends in the postnatal period (Figure 1). In this stage alveoli appear for the first time as outgrowths from the distal transitory air spaces which were already laid down in the former stages. The peripheral gas exchange regions widen, and the transitory channels and saccules are subdivide into smaller units. Elastin deposition in the saccular parts of the lung leads to an outpouching of the transitory distal air spaces and gives rise to the first appearance of primitive alveoli (Burri in: McDonald, 1997 p. 12-15). The outpouching of the primary septa leads to the formation of the secondary septa, which still contain two intra-septal capillaries, but the inter-alveolar space is already reduced. Thus, the alignment of the capillaries with the primitive alveolar walls becomes closer. Advanced maturation causes the secondary septa to thin out further. This thinning out is preceded by the stage of microvascular maturation, where the inter-alveolar capillary network continues to develop.

1.1.1.6. The microvascular maturation stage

This stage is marked by a strong reduction in the interstitial volume, while the total lung volume is constantly increasing. At the same time maturation of the capillary network gives rise to the “adult” septa which appear at the end of this stage. The characteristic feature of these septa is a single capillary within the inter-alveolar walls. There is strong discussion about the morphogenesis of this change from two capillaries to one single capillary in the adult septum: Burri et al. demonstrated that the most likely explanation was a process of capillary fusion (Burri in: McDonald, 1997 p.17-21), which would result in the formation of the pores of Kohn (Weiss and Burri, 1996). Taking together, the maturation of the capillary network is a crucial developmental step affecting alveolar maturation. Further preferential

growth of the merged areas contributes to further maturation and leads into the adult phase of growth of the lung.



1.2. Studying lung development as basis for lung tissue regeneration

Lung development is a highly complex process which is tightly regulated and involves a variety of different factors. Growth and maturation of the lung combine different remodeling processes: tissue proliferation and differentiation (for example forming the airways and capillary networks) occur at the same time as tissue reduction by apoptosis (for example in the interstitial mesenchyme and promoting capillary fusion). Even though lung development has been studied extensively, the numerous processes involved and the underlying mechanisms remain obscure. Since pulmonary diseases are often also accompanied by remodeling processes (Capelozzi, 2008; Chin and Rubin, 2008; Henson et al., 2006; Mauad et al., 2007; Pardo et al., 2008) a better understanding of the tissue and cell interactions relevant to lung development might be helpful, to understand the pathological remodeling processes which are activated during pulmonary diseases. Furthermore, a detailed knowledge of the factors involved in tissue generation during lung development might reveal targets that could be induced therapeutically in lung tissue regeneration in the future. One of those possible targets has been addressed in the present study: the HIF 2 α /flk-1 system.

1.3. The HIF 2 α /flk-1 system

The HIF 2 α /flk-1 system is a key system mediating physiological and pathological blood vessel formation (Carmeliet, 2005; Elvert et al., 2003; Elvert et al., 1999; Flamme et al., 1997). In particular, flk-1 (fetal liver kinase-1; kinase domain region, KDR; VEGF-receptor 2) is expressed during embryogenesis in the early primitive vascular network (Flamme et al., 1995; Risau, 1997; Yamaguchi et al., 1993) and in growing tumor vessels (Carmeliet, 2005). It has been demonstrated that flk-1 is regulated by HIF 2 α (Elvert et al., 2003). In mice *in vivo* knock-out approaches of HIF 2 α and flk-1 lead to severe vascular defects generating a lethal phenotype (Duan et al., 2005; Shalaby et al., 1995). Furthermore, HIF 2 α is highly expressed in the lung (Ema et al., 1997) and plays a critical role in lung development, since loss of HIF 2 α led to infant respiratory distress syndrome which could be overcome by intratracheal postnatal or intrauterine administration of VEGF (vascular endothelial growth factor) (Compernelle et al., 2002). This suggests that the role of the HIF 2 α /flk-1 system is not restricted to the development of vascular system. Furthermore, it has been described that interactions between the mesenchyme, early endothelial progenitor cells and primitive epithelial cells are important to regulate stages of lung development (Burri in: McDonald,

1997 p. 3-4). In particular for the VEGF/VEGF receptor system, the importance of the epithelial and endothelial cross-talk has been suggested (Del Moral et al., 2006). Thus, it is of relevance to the further understanding of developmental processes of the lung to characterize the localization and function of the HIF 2 α /flk-1 system during lung development.

1.3.1. Flk-1

Flk-1 belongs to the family of angiogenic vascular endothelial growth factor receptors (Mustonen and Alitalo, 1995). This tyrosine kinase receptor family comprises flt-1 (fms like tyrosin kinase/VEGF-receptor 1), flk-1 (fetal liver kinase-1/KDR, kinase domain region/VEGF-receptor 2) and Flt-4 (VEGF-receptor 3) (Figure 2). Vasculogenesis and angiogenesis have been shown to be critically dependent on the VEGF/VEGF receptor system (Flamme et al., 1995; Risau, 1997; Risau and Flamme, 1995). Two further members of the receptor family are neuropillin 1 and 2 (NRP-1, NRP-2) (Neufeld et al., 2002; Soker et al., 1998). They have been shown to influence vascular development and to interact with flk-1 (Figure 2)(Kawasaki et al., 1999; Soker et al., 1998). The VEGF-receptors are activated upon ligand binding of the vascular endothelial growth factor (VEGF) family members. Five predominant family members have been described: VEGF-A, VEGF-B, VEGF-C, VEGF-D and PLGF (placental growth factor)(Figure 2) (Ferrara et al., 2003). Some homologs of VEGF have also been identified in the parapoxvirus Orf virus and have been shown to have VEGF-A-like functions, and these homologs are gathered together and are collectively termed VEGF-E (Lyttle et al., 1994). VEGF-A is the key mediator of blood vessel formation. VEGF-C and VEGF-D are involved primarily in lymphatic angiogenesis (Karkkainen et al., 2002). VEGF-B and PLGF contribute to flt-1 function (Olofsson et al., 1998). Alternative splicing of VEGF-A leads to four different isoforms consisting of 121, 165, 189 and 206 amino acids in humans (Ferrara et al., 2003). The VEGF-A isoforms differ primarily in their capacity to bind to the extracellular matrix and cell surface via heparin-binding domains, and thereby generate gradients for angiogenic sprouting (Carmeliet, 2005; Ruhrberg et al., 2002). Furthermore, VEGF-A belongs to the hypoxia-regulated genes and is regulated by the hypoxia inducible factor 1 α (HIF-1 α) (Liu et al., 1995). Accordingly, it is secreted by hypoxic tissues (for example in developing organs or tumor tissue) to attract the growing vessels. During lung development, VEGF-A is secreted by peripheral respiratory epithelial cells at the tip of developing respiratory tubes (Ng et al., 2001), creating a VEGF gradient that guides the vascular network to follow and surround the growing bronchi (deMello et al., 1997). VEGF receptors are highly expressed in the mesenchymal endothelial and haematopoietic progenitor

cells and in the early primitive vascular network (Carmeliet et al., 1996; Fong et al., 1995; Kappel et al., 1999; Shalaby et al., 1995). Accordingly, *in vivo* gene deletion and mutation studies of VEGF, flk-1 andflt-1 demonstrated severe vascular defects with lethal phenotype. Loss of a single VEGF-A allele led to a lethal phenotype due to defective blood vessel formation (Carmeliet et al., 1996; Ferrara et al., 1996). Analogous effects were observed in homozygous flk-1 mutants (Shalaby et al., 1995). Furthermore, mutation of theflt-1 locus led to the formation of abnormal vascular channels (Fong et al., 1995). However, flk-1 has been shown to be the key mediator of the mitogenic, angiogenic and permeability enhancing effects (Clauss et al., 1996; Gille et al., 2001). Thus, flk-1 seems to be the most pivotal mediator of endothelial cell differentiation (Flamme et al., 1995; Kappel et al., 1999; Risau, 1997). It is also the first receptor known to be expressed in the primitive mesoderm (Yamaguchi et al., 1993). The flk-1 receptor is a 200 kDa protein with a high affinity for VEGF-A, VEGF-E, VEGF-C and VEGF-D (Takahashi and Shibuya, 2005). Flk-1 undergoes dimerization upon ligand binding which leads to phosphorylation of different tyrosine residues (Ferrara et al., 2003; Matsumoto and Claesson-Welsh, 2001). Flk-1 signaling gives rise to a variety of vascular cellular functions, such as vascular permeability, cell migration, cell survival, and cell proliferation, depending on the phosphorylation site (Figure 3) (Olsson et al., 2006). In contrast to the hypoxia-dependent regulation of the ligand VEGF by HIF 1 α , the receptor flk-1 is regulated by HIF 2 α (Elvert et al., 2003). Within the flk-1 promoter, a HIF 2 α -binding site has been demonstrated which is distinct from the hypoxia responsive element [(HRE) the common binding site of the HIF transcription factors] (Elvert et al., 2003). HIF 2 α is highly expressed in the lung (Ema et al., 1997). In particular, during lung development the generation of the vascular system is a key process regulating stages of lung development (Burri in: McDonald, 1997 p. 17-22). Angiogenesis and vasculogenesis are initiated at early stages of lung development (Gebb and Shannon, 2000; Schachtner et al., 2000). In sum, the role of the HIF 2 α /flk-1 system is of key importance in lung development.

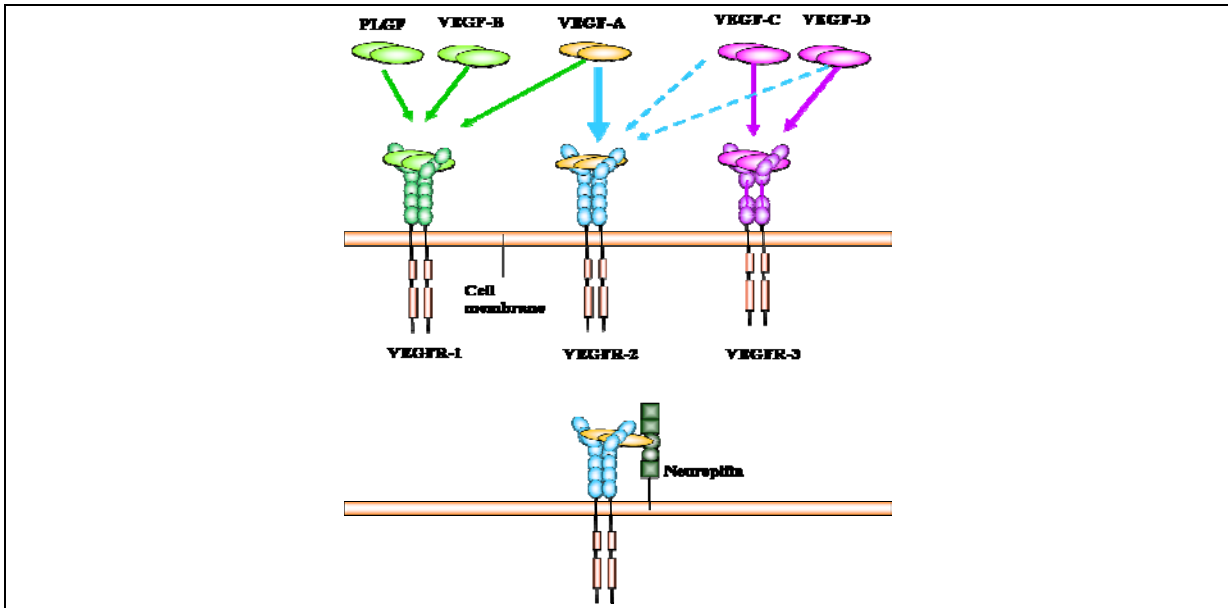


Figure 2: The vascular endothelial growth factor receptor family

The vascular endothelial growth factor receptor (VEGFR) family comprises four members: VEGFR-1/flt-1 (fms like tyrosine kinase-1), VEGFR-2/flk-1 (fetal liver kinase-1), VEGFR-3 and neuropilin, which bind the VEGF A, B, C and D ligands with different affinity. Adapted from (Olsson et al., 2006).

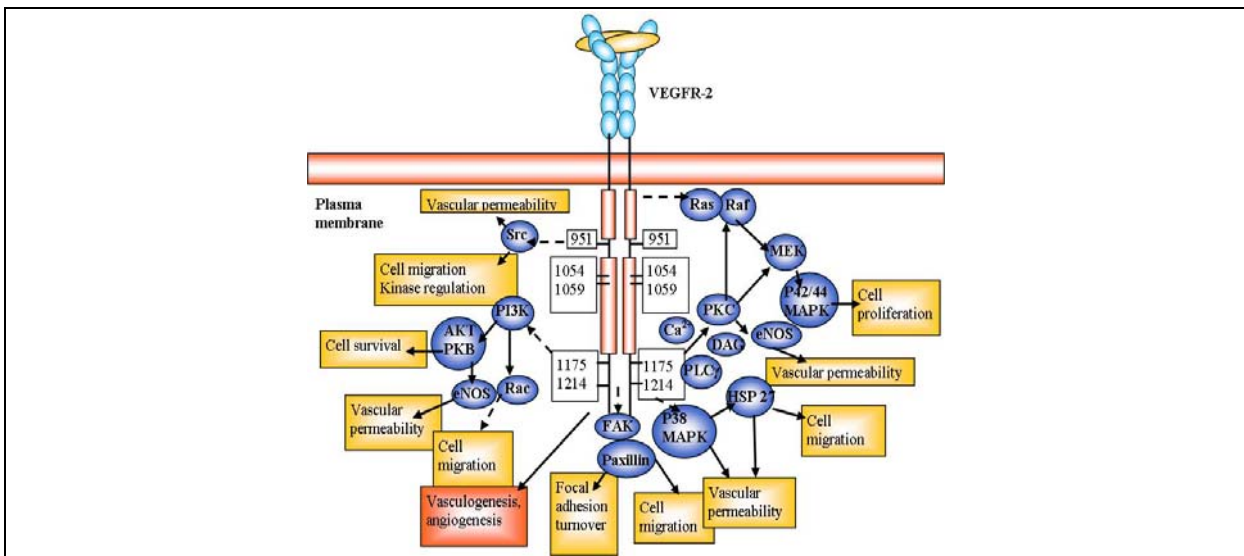


Figure 3: VEGFR-2/flk-1 signaling

Flk-1 is a homodimeric 200 kDa protein. Ligand binding leads to dimerization and downstream autophosphorylation of specific tyrosine residues which activates further downstream signaling. Cell survival, vascular permeability, cell migration and cell proliferation may all be activated via flk-1 signaling. Adapted from (Olsson et al., 2006)

1.3.2. The role of flk-1 in lung development

The importance of flk-1 function in lung development arises from its pivotal role in angiogenesis and vasculogenesis. These processes are initiated at early stages of lung development (Gebb and Shannon, 2000; Schachtner et al., 2000). Furthermore, it has been shown that the development of the endothelial system is crucial for effective lung development (Burri in: McDonald, 1997 p. 17-22). However, Gebb and Shannon have suggested that tissue interactions between the mesenchyme and the pulmonary epithelium play an important role in the developing vasculature, demonstrating that flk-1 expression in the early mesenchyme is located close to the pulmonary epithelium throughout early lung development. Hence, they suggest that the spatial and temporal expression of flk-1 may direct pulmonary vascularization through signals delivered from the epithelium (Gebb and Shannon, 2000). Accordingly, it has been reported that VEGF-A is secreted by peripheral respiratory epithelial cells at the tips of developing respiratory tubes (Ng et al., 2001), creating a VEGF gradient that guides the vascular network to follow and surround the growing bronchi (deMello et al., 1997). Furthermore, Yamamoto et al. suggested that primary septum formation is dependent on reciprocal interactions between the respiratory epithelium and the underlying vasculature, involving epithelium-derived VEGF and possibly endothelium-derived Hepatocyte growth factor (HGF) (Yamamoto et al., 2007). In the same line, Del Moral et al., reported that flk-1 knock-down by antisense oligodesoxynucleotides (ODN) in embryonic lung cultures resulted in reduced epithelial branching morphogenesis (Del Moral et al., 2006). Similar effects were demonstrated by Jakkula et al., where treatment of newborn rats with SU 5416 (a specific flk-1 inhibitor) led to decreased alveolarization and decreased arterial density (Jakkula et al., 2000). Furthermore, VEGF receptor inhibition has been reported to induce lung cell apoptosis and emphysema (Kasahara et al., 2000) and pulmonary hypertension and abnormal lung structure (Le Cras et al., 2002). In contrast, Akeson et al. demonstrated the disruption of pulmonary vascular assembly and reciprocal defects in branching morphogenesis in response to overexpression of VEGF-164 in the pulmonary epithelium (Akeson et al., 2003). Further effects of VEGF on lung development were reported by Le Cras showing pulmonary hemorrhage and enlargement of the airspaces (Le Cras et al., 2004). Taken together, these reports demonstrate the crucial role of the VEGF-flk-1 ligand receptor system in lung development. Nevertheless, the majority of reports suggests that flk-1 function primarily impacts the endothelial system, with secondary effect on the epithelial system and branching morphogenesis (Figure 4). In contrast, recent reports demonstrate flk-1 expression in isolated rat fetal alveolar type II cells (Raoul et al., 2004) and in distal airway

epithelial cells in human fetal lungs *in vitro* (Brown et al., 2001). Accordingly, a direct role of flk-1 on the epithelial system might be suggested (Figure 4). Taking into account that HIF 2 α is the primary transcription factor of the flk-1 system, the phenotype of HIF 2 α knock-out mice supports this suggestion, where Compernelle et al. demonstrated infant respiratory distress syndrome due to HIF 2 α deletion *in vivo* which could be overcome by administration of VEGF (Compernelle et al., 2002). Thus, we hypothesized that the HIF 2 α flk-1 system has a direct function in the pulmonary epithelium (Figure 4). Accordingly, with regard to flk-1, we characterized flk-1 expression during lung development at daily intervals from ED (embryonic day) 12.5 until postnatal stages, and in isolated AEC.

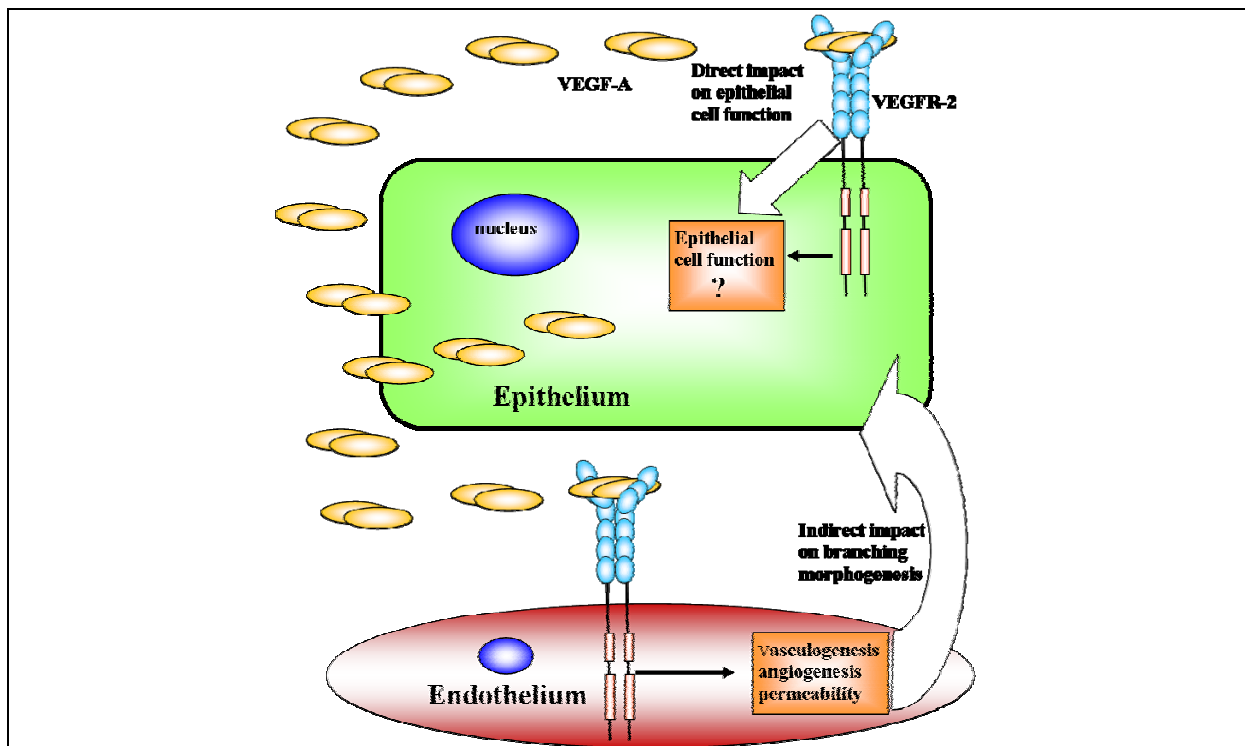


Figure 4: Possible role of flk-1 in the endothelial-to-epithelial crosstalk during lung development

Flk-1 has been shown to be highly expressed in the developing vascular system (Flamme et al., 1995). Accordingly, distinct reports suggest that flk-1 is involved in intercellular crosstalk between the endothelial, mesenchymal, and epithelial systems respectively. Signals from the developing vascular system may be directing epithelial function and branching morphogenesis (Del Moral et al., 2006; Gebb and Shannon, 2000; Yamamoto et al., 2007). In contrast, further reports suggest a direct function of flk-1 in the pulmonary epithelial system (Brown et al., 2001; Raoul et al., 2004).

1.3.3. HIF 2 α

HIF 2 α belongs to the family of hypoxia-inducible factors. This family comprises three different members, HIF 1 α , HIF 2 α and HIF 3 α , each of which form a heterodimer with HIF 1 β (also termed ARNT; aryl hydrocarbon nuclear translocator) to translocate under hypoxic conditions into the nucleus to initiate the transcription of hypoxia-responsive genes (for example erythropoietin, VEGF) (Wang and Semenza, 1993b) via the transactivation domains (TAD) (Figure 5A and 5B). HIF 1 α was the first member of this family to be discovered (Semenza et al., 1991; Semenza and Wang, 1992; Wang and Semenza, 1995). Identification of a minimal hypoxia-responsive element (HRE) in the 3' enhancer of the erythropoietin (EPO) gene led to the identification of a hypoxia- and phosphorylation-dependent DNA-binding protein (Wang and Semenza, 1993a). Further analysis revealed a heterodimeric complex consisting of this protein and HIF 1 β . This complex belongs to the basic helix loop helix (bHLH)/Per, ARNT and Sim (PAS) transcription factors. Biochemical analysis revealed two highly conserved regions within these HIF family members: the bHLH and the PAS domains (Figure 5A). The basic region consists of approximately 15 primarily basic amino acids which are responsible for direct DNA binding (Moore et al., 2000). The PAS domain comprises about 200-300 amino acids which give rise to two weakly-conserved regions: the PAS A and PAS B domain (Taylor and Zhulin, 1999). These domains form a secondary dimerization interface between the HIF family members, and have additional functions in targeting gene specificity, as shown in *Drosophila* for Tracheless (Trh) and single minded (Sim), but the underlying mechanism remains unclear (Zelzer et al., 1997). A further characteristic of HIF is oxygen-dependent regulation at the protein level. Under normoxic conditions, prolyl hydroxylases 1-3 hydroxylate prolyl residues of HIF α subunits (Pro402 and Pro564) within the oxygen dependent degradation domain (ODDD) which leads to proteasomal degradation of HIF (Figure 5A and Figure 6). Under hypoxic conditions, hydroxylation is inhibited and HIF α subunit levels increase in the cytoplasm (Pugh and Ratcliffe, 2003). A further hydroxylation step by the HIF asparaginyl hydroxylase (factor inhibiting HIF; FIH) is described, where hydroxylation of an asparaginyl residue prevents the interaction of HIF α subunits with the p300 coactivator which is required for transcriptional activation (Pugh and Ratcliffe, 2003) (Figure 6). Shortly after the discovery of HIF 1 α , a novel cDNA clone was isolated from a murine hypothalamus cDNA library. Using the yeast two hybrid system with the bHLH-PAS domain of Arnt as a prey, a protein of 97 kDa and exhibiting high homology with HIF 1 α was found (Ema et al., 1997). Sequence analysis

revealed a striking similarity to the amino acid sequence of HIF 1 α : the bHLH within the amino terminal half comprised 83.9%, the PAS domain 66.5% the following PAS domain distal region consisting of aminoacids 345-559 exhibited less similarity, with 36.4% and the C-terminal half consisted of variable parts and a small region, composed of amino acids 824-874, with 63% homology. Chromosomal localization revealed a region distinct from the HIF 1 α gene locus, which is located on the murine chromosome 12 (Semenza et al., 1996), whereas the new protein was localized to murine chromosome 17 (Ema et al., 1997). Analysis of dimerization, DNA-binding and transcription-activating properties revealed similar properties to HIF 1 α . Coimmunoprecipitation assays and the yeast two hybrid system demonstrated that the new protein was coimmunoprecipitated by an anti-Arnt antibody when incubated with Arnt, to the same extent as HIF 1 α . Furthermore, both proteins interacted selectively with Arnt in the yeast two hybrid system. Hence Ema et al. named the newly discovered protein “HIF-like factor” (HLF/ later HIF 2 α). Furthermore, the HIF 2 α /Arnt complex recognized and bound the HRE sequence of the EPO promoter with similar intensity to the HIF 1 α /Arnt heterodimer (Ema et al., 1997; Semenza and Wang, 1992). Moreover, the transcriptional activation of the HRE sequence of the VEGF promoter by the HIF 2 α /Arnt complex resembled the transcriptional activation by the HIF 1 α /Arnt complex. Hence, Ema et al. demonstrated that the biochemical and transcription activating properties of HIF 2 α were similar to HIF 1 α . The tissue distribution of HIF 2 α and HIF 1 α mRNA in the brain, heart, lung, liver, kidney testis and skeletal muscle reveal important differences between these two proteins. HIF 2 α was shown to be predominantly expressed in the lung compared to other organs. Even though both proteins were expressed ubiquitously in these organs, the expression levels of HIF 2 α were generally much higher than those of HIF 1 α at the mRNA level. Interestingly, the expression pattern of VEGF which is known to be regulated by HIF 1 α (Forsythe et al., 1996) was similar to that of HIF 2 α . In particular, during parturition, the expression levels of both factors increased. Beginning on ED 17.5, persisting to day P0 and becoming highly abundant in the adult lung, VEGF and HIF 2 α were strongly expressed. In contrast, HIF 1 α mRNA expression levels were constantly low during lung development (Ema et al., 1997). A detailed expression analysis revealed the expression of HIF 2 α in vascular endothelial cells in the primitive vascular network at embryonic day 9.5 and 10.5. Later stages exhibited the expression of VEGF and HIF 2 α in alveolar epithelial cells of murine lungs at day P0 (Ema et al., 1997). Some gene deletion studies *in vivo* exhibited different phenotypes. Scortegagna demonstrated an impact of HIF 2 α on hematopoiesis

(Scortegagna et al., 2003b) and ROS production (Scortegagna et al., 2003a). Further studies on HIF 2 α deletion revealed a crucial role for HIF 2 α on vascular development and remodeling (Duan et al., 2005; Peng et al., 2000). In particular, Duan et al. were able to show that overexpression of HIF 2 α in the endothelium of HIF 2 α knock-out mice could rescue the vascular phenotype (Duan et al., 2005). In contrast, Tian et al. suggested that the main function of HIF 2 α related to the catecholamine metabolism, where HIF 2 α knock-out mice suffered from cardiac dysfunction and bradycardia due to a decrease in systemic catecholamine levels (Tian et al., 1998). They postulated that HIF 2 α expression in the organ of Zuckerkandl leads to an increase in catecholamine production upon hypoxia. Investigations by Busselmann et al. supported these suggestions, demonstrating that heterozygous HIF 2 α mice are protected against vascular remodeling and pulmonary hypertension due to diminished levels of endothelin-1 and catecholamines (Brusselmans et al., 2003). In contrast, the same authors described that homozygous HIF 2 α deletion had an impact on the response to hypoglycemia, and not on reactions induced by hypoxia (Brusselmans et al., 2001). A further function of HIF 2 α was suggested by Guber et al., who demonstrated that loss of HIF 2 α led to anemia, affecting the development of the bone marrow-derived cells (Gruber et al., 2007). Taken together, the phenotype of HIF 2 α knock-out mice shows a heterogeneous pattern. One possible explanation could be the difference in mouse strain background. Furthermore, different approaches to gene deletion and differences in the experimental setup might have an impact on the phenotype, since different domains of HIF 2 α might have been deleted. However, Compernelle et al. reported a severe phenotype concerning lung function, where HIF 2 α deletion led to severe respiratory distress syndrome which could be overcome by the administration of VEGF (Compernelle et al., 2002). Furthermore, it has been reported that flk-1, the key regulator of vascular development, is a distinct target gene of HIF 2 α (Elvert et al., 2003). Since respiratory distress syndrome is generally caused by epithelial cell dysfunction, it might be suggested that the HIF 2 α /flk-1 system has a direct impact on the pulmonary epithelial system. This idea is supported by investigations by Raoul et al. and Brown et al., who demonstrated pulmonary epithelial expression of flk-1 (Brown et al., 2001; Raoul et al., 2004). Thus, direct epithelial functions of the HIF 2 α /flk-1 system might have impact on lung development. Accordingly, in the present study epithelial function of the HIF 2 α /flk-1 system during lung development was investigated using a transgenic approach.

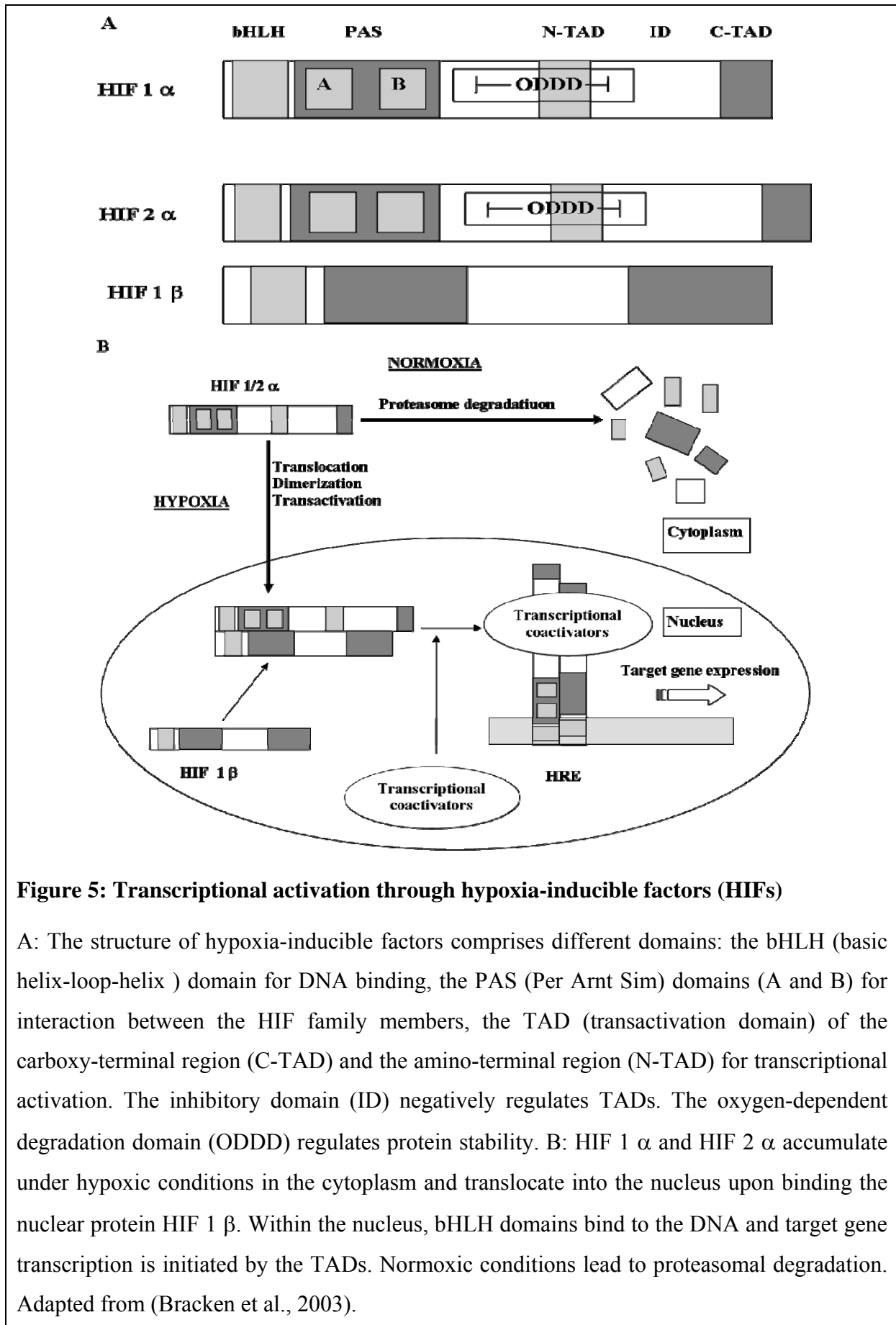


Figure 5: Transcriptional activation through hypoxia-inducible factors (HIFs)

A: The structure of hypoxia-inducible factors comprises different domains: the bHLH (basic helix-loop-helix) domain for DNA binding, the PAS (Per Arnt Sim) domains (A and B) for interaction between the HIF family members, the TAD (transactivation domain) of the carboxy-terminal region (C-TAD) and the amino-terminal region (N-TAD) for transcriptional activation. The inhibitory domain (ID) negatively regulates TADs. The oxygen-dependent degradation domain (ODDD) regulates protein stability. B: HIF 1 α and HIF 2 α accumulate under hypoxic conditions in the cytoplasm and translocate into the nucleus upon binding the nuclear protein HIF 1 β . Within the nucleus, bHLH domains bind to the DNA and target gene transcription is initiated by the TADs. Normoxic conditions lead to proteasomal degradation. Adapted from (Bracken et al., 2003).

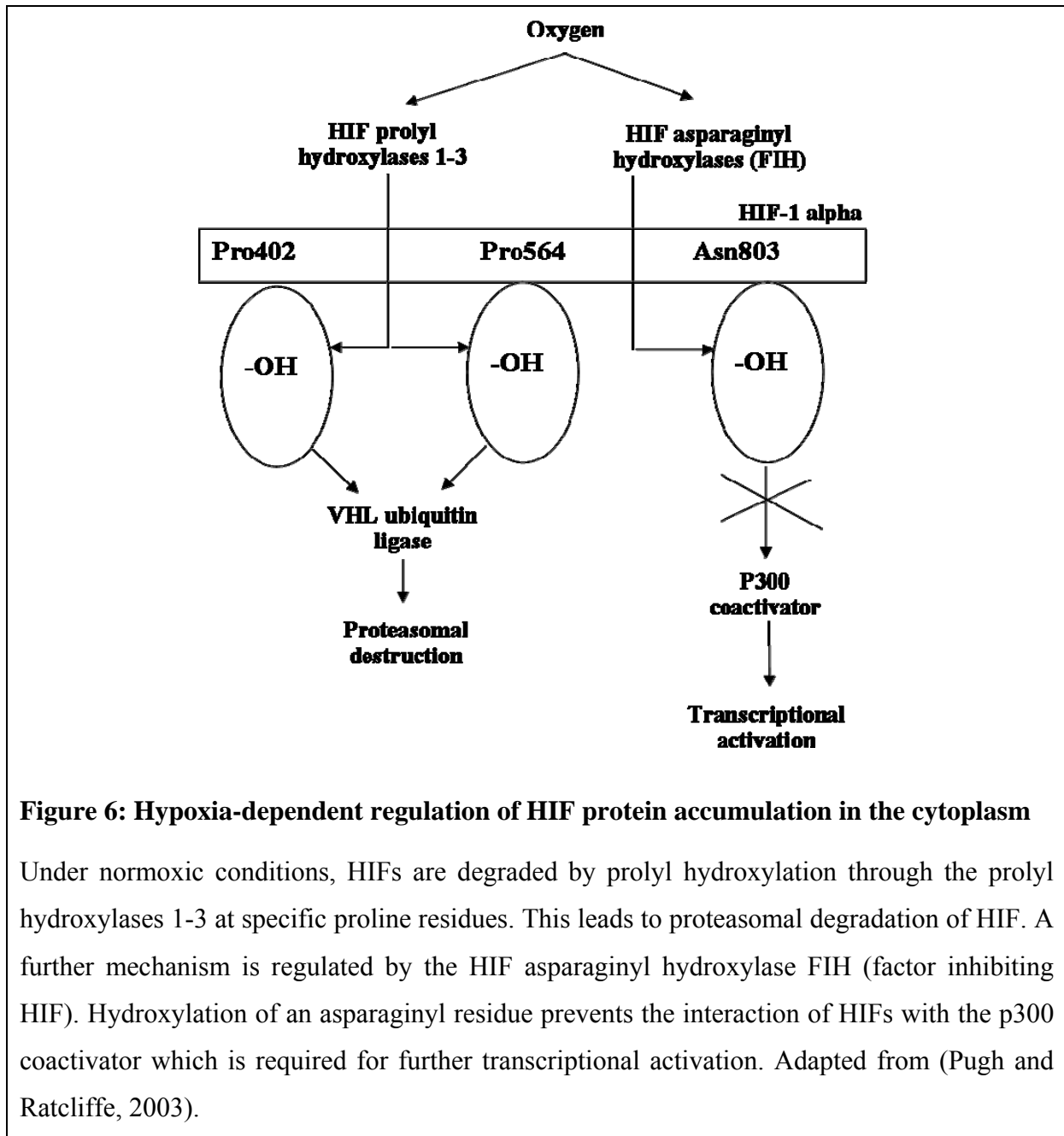


Figure 6: Hypoxia-dependent regulation of HIF protein accumulation in the cytoplasm

Under normoxic conditions, HIFs are degraded by prolyl hydroxylation through the prolyl hydroxylases 1-3 at specific proline residues. This leads to proteasomal degradation of HIF. A further mechanism is regulated by the HIF asparaginyl hydroxylase FIH (factor inhibiting HIF). Hydroxylation of an asparaginyl residue prevents the interaction of HIFs with the p300 coactivator which is required for further transcriptional activation. Adapted from (Pugh and Ratcliffe, 2003).

1.3.4. The role of HIF 2 α in lung development

HIF 2 α is crucial for vascular development (Duan et al., 2005; Elvert et al., 1999). Furthermore, it has been shown to be a crucial transcription factor of flk-1 (Elvert et al., 2003), which is a key regulator of angiogenesis and vasculogenesis (Risau, 1997; Risau and Flamme, 1995). Since the development of the vascular system is a key process regulating stages of lung development, the HIF 2 α /flk-1 system impacts a pivotal developmental step. Furthermore, Compennolle et al. demonstrated a severe pulmonary phenotype due to homozygous HIF 2 α deletion *in vivo*: HIF 2 α knock-out mice exhibited a severe respiratory distress syndrome which could be overcome by the administration of VEGF (Compennolle et

al., 2002). Since the respiratory distress syndrome was accompanied by epithelial dysfunction including reduced SPC production, a direct role of the HIF 2 α /flk-1 system on the pulmonary epithelial system might be suggested. Accordingly, data by Ema et al. and Wagner et al. support this hypothesis, demonstrating that HIF 2 α is expressed in the bronchial and alveolar regions, respectively of the developing lung (Ema et al., 1997; Wagner et al., 2004). Taken together, the HIF 2 α /flk-1 system is crucial for the development of the lung because it is a key regulator of the vascular development. However, due to distinct reports on pulmonary epithelial cell function (section 1.4), it might be suggested that the HIF 2 α /flk-1 system has direct functional impact on the pulmonary epithelial system.

1.4. The role of pulmonary epithelial HIF 2 α and flk-1 during lung development

Differentiation of the pulmonary epithelial system is tightly connected to the differentiation of the vascular system (Burri in: McDonald, 1997 p. 3-21), Since flk-1 is a key mediator of blood vessel formation (Flamme et al., 1995; Risau and Flamme, 1995; Shalaby et al., 1995), and is regulated by HIF 2 α (Elvert et al., 2003). Several studies illustrated the role of the VEGF/flk-1 system in intercellular crosstalk during pulmonary development (Del Moral et al., 2006; Gebb and Shannon, 2000) Accordingly, these studies point out the relevance of endothelial flk-1 mediating effects on the epithelial system, and conversely, epithelial VEGF function also impacts the vascular system. It has also been demonstrated that deletion of HIF 2 α led to a severe disruption of vascular development (Duan et al., 2005). Interestingly, a further HIF 2 α deletion study pointed out that global HIF 2 α deletion resulted in a respiratory distress syndrome which could be overcome by the application of VEGF (Comperolle et al., 2002). Thus, it might be suggested that the HIF 2 α /flk-1 system has a direct impact on the pulmonary epithelium. This is supported by studies by Raoul et al. and Brown et al., who demonstrated epithelial flk-1 expression in fetal isolated rat alveolar type two cells and human fetal lung explants, respectively (Brown et al., 2001; Raoul et al., 2004).

2. Hypothesis and aims of the study

We hypothesize that the HIF 2 α /flk-1 system has a direct impact on the pulmonary epithelial system. Epithelial flk-1 expression regulated by HIF 2 α might have direct roles in the epithelial system during lung development (Figure 7). To test this hypothesis, the aims of the present study were:

1. Characterization of flk-1 expression from ED 12.5 at daily stages throughout lung development until postnatal stages, and in freshly-isolated alveolar epithelial cells from adult mice (Ahnbrecht et al., 2008).
2. Generation of triple-transgenic mice to create an inducible pulmonary epithelial deletion of HIF 2 α , to evaluate the impact of epithelial HIF 2 α deletion on lung development *in vivo*.
3. Characterization of the phenotype of pulmonary epithelial HIF 2 α knock-out mice, in comparison with control mice.

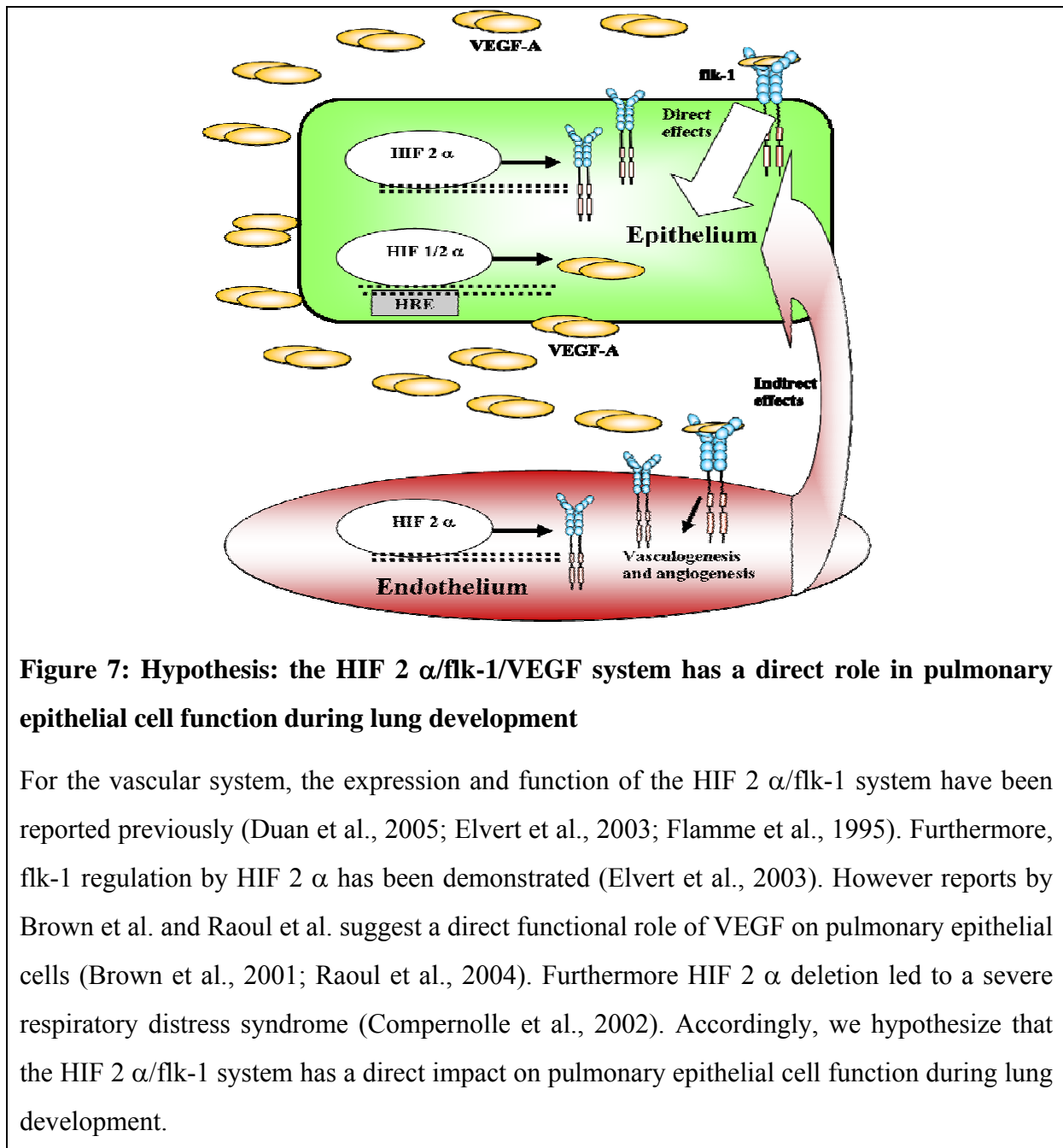


Figure 7: Hypothesis: the HIF 2 α /flk-1/VEGF system has a direct role in pulmonary epithelial cell function during lung development

For the vascular system, the expression and function of the HIF 2 α /flk-1 system have been reported previously (Duan et al., 2005; Elvert et al., 2003; Flamme et al., 1995). Furthermore, flk-1 regulation by HIF 2 α has been demonstrated (Elvert et al., 2003). However reports by Brown et al. and Raoul et al. suggest a direct functional role of VEGF on pulmonary epithelial cells (Brown et al., 2001; Raoul et al., 2004). Furthermore HIF 2 α deletion led to a severe respiratory distress syndrome (Compennolle et al., 2002). Accordingly, we hypothesize that the HIF 2 α /flk-1 system has a direct impact on pulmonary epithelial cell function during lung development.

3. Methodical basis for *in vivo* transgenic engineering

3.1. *Transgenic animal models*

The ability to investigate single gene function *in vivo* is accompanied by several advantages compared to *in vitro* cell culture: gene function can be investigated in the context of the whole organism, where the impact of the function of a single gene on development can be elucidated. Accordingly, the use of transgenic animal models is now widespread, and a variety of different approaches have been developed. The key discovery which contributed to the transgenics was made by mammalian embryologists. In 1972, Brinster et al. developed a technique for the removal of embryos, embryo culture *in vitro* and reimplantation of embryos into foster mothers. Normal embryogenesis was observed in these reimplanted embryos (Brinster in: Rothblat, 1972 p. 251-286). This was the essential step in the production of chimeric animals, by combining embryonic cells from one embryo with those of another. This particular step gained importance because, methods for introducing selectable genes into tissue culture were developed by geneticists and virologists at the same time. Further techniques to manipulate the embryo were carried out by infecting early embryos with intact viruses or viral DNA (Jaenisch, 1976). Combining these two key findings, transgenic engineering was born. A variety of different ways to introduce foreign DNA into embryonic cell cultures and tissues has been further developed (Palmiter and Brinster, 1986). In particular, the pronucleus method (section 3.2) and an alternative method using embryonic stem (ES) cells (section 3.3) are currently used to produce transgenic animals. The availability of gene transfer technology gave rise to the different transgenic approaches to address different questions. In general, gain of function or loss of function can be investigated, either by overexpression of a selected gene (section 3.1.1.1) (gain of function) or overexpression of a dominant-negative protein or decoy receptor (loss of function). Furthermore, techniques exist for the genetic deletion of an endogenous gene to create “knock out” models for an alternative approach to study of loss of function (section 3.1.1.2). The third group of transgenic approaches comprises the introduction of reporter genes into the genetic background (section 3.1.1.3). Using this tool the *in vivo* localization, expression or function of a certain gene and controlling targeted deletion of genes became possible. Further sophisticated approaches allow for tissue-specific and inducibility of selected genes (section 3.1.2.).

3.1.1. Constitutive transgenic models

Stable transgenic animal models can be generated by three different strategies: 1) genes can be overexpressed to investigate a gain of function; 2) deletion of genes can be used to elucidate the impact of loss of function of a gene on developmental processes or under challenge conditions; and 3) reporter genes can be introduced, to localize gene expression *in vivo*.

3.1.1.1. The overexpression model

Overexpression models are frequently used to investigate gene function *in vivo*. The impact of overexpression on development (Akeson et al., 2005) or on different pathological stimuli (Hanstein et al., 2008) can be investigated. Furthermore, studies on oncogene expression gain increasing importance in the elucidation of mechanisms of malignant tumor growth (Acker et al., 2005). A variety of different methods for the stable introduction of foreign DNA into the genome have been described by Palmiter and Brinster (Palmiter and Brinster, 1986). Due to stable and reliable gene expression and targeting, the pronucleus method and the ES cell method are currently the most frequently used methods. Furthermore, the constant development of transgenic approaches introduced the possibility of cell type-specific and inducible overexpression. In addition to the gain of function approaches, investigations using loss-of-function models provide a further insight into gene function *in vivo* and are of particular importance for developmental investigations. Overexpressed proteins can also be fused to certain amino acid sequences (tags) which can be easily detected by commercially available antibodies raised against these tags, facilitating the localization of the expressed protein.

3.1.1.2. The knock-out model

In vivo gene deletion is a key tool for developmental research. Investigations on the phenotype of certain knock-out animals gave rise to the key findings which constituting the milestones of developmental research. In particular, the key process of vascular development was elucidated by these approaches (Shalaby et al., 1997; Shalaby et al., 1995). However, this is only one example out of the variety of knock-out investigations. The discovery of homologous recombination (section 3.3.1) in combination with embryonic stem (ES) cell culture led to the development of a technique for direct gene integration into a specific region of the genome. Accordingly, it became possible to disrupt endogenous gene expression by insertion of foreign DNA. Furthermore, site-directed mutagenesis could be integrated into *in*

vivo models by integration of mutated target vectors. A further DNA recombination process was used to generate gene deletion: The discovery of the Cre loxP system (section 3.1.2.2) gave rise to site-directed gene deletion *in vivo*. The combination of the Cre loxP system with tissue-specific and inducible systems comprises a further key methodology for developmental research. The deletion of genes can be combined with the integration of reporter genes to investigate localization and the extent of ablation of gene expression (section 3.1.1.3).

3.1.1.3. Reporter gene transgenic animals/knock-in models

The possibility of site-directed integration of DNA into the genome gave rise to the so called “knock in” method. Reporter genes were integrated into the genome at specific locations. This led to the deletion of a specific gene, and also the localization of the replaced gene could be investigated by visualization of a reporter gene (Shalaby et al., 1995). Reporter genes are genes which code for fluorescent proteins [for example green fluorescence protein (GFP) and red fluorescence protein (RFP)] or enzymes [which can be visualized by addition of a substrate [for example alkaline phosphatase (AP), β -galactosidase, (β -gal); lacZ gene]. The expression of reporter genes can be restricted to a specific cell-type by a specific promoter [for example the surfactant protein C (SPC) promoter or Clara cell specific promoter (CCSP)] (Perl et al., 2002a) or can be expressed globally if introduced under the control of a housekeeping gene promoter (Soriano, 1999). Currently, reporter genes are often combined with inducible systems to control the transgene expression (Perl et al., 2002a; Perl et al., 2002b).

3.1.2. Inducible tissue-specific overexpression and knock-out models

The inducible tissue-specific transgene expression or gene deletion comprises a variety of methods to investigate gene function during development, and the impact of gene expression on different pathological stimuli. Furthermore, the particular cell-type specific role of gene expression can be investigated. Phenotypes of global knock-outs can be compared to phenotypes of tissue-specific knock-out animals. Furthermore, gene deletion which led to a very early lethal phenotype can be investigated by later induction of the gene deletion. Taken together, tissue-specific and inducible gene deletion is a key tool to investigate gene function *in vivo*. A variety of different systems has been developed [reviewed by Lewandowski 2001 (Lewandoski, 2001)], but three systems are currently used for inducible tissue-specific expression: the tetracycline-induced system (section 3.1.2.1), and the RU 486 and the tamoxifen systems. The tetracycline-induced system is used in this study, hence, it will be describe in more detail in section 3.1.2.1. The RU486/tamoxifen system requires another gene

targeting system, the Cre-loxP system (section 3.1.2.2) and can be used for inducible gene deletion. Mutation of the progesterone-/estrogen receptor leads to a higher affinity for RU486/tamoxifen than to the endogenous binding peptides. Furthermore, the Cre recombinase is fused to one or two synthetic hormone binding sites which can bind to RU486/tamoxifen. Without RU486/tamoxifen the Cre recombinase fusion protein is expressed in the cytoplasm and inactive, because the nucleus is its site of action. Administration of RU486/tamoxifen causes the nuclear translocation of the Cre recombinase fusion protein by binding to RU486/tamoxifen, which also binds to the progesterone/estrogen receptor. Accordingly, the entire complex is able to translocate into the nucleus. In the nucleus, the Cre recombinase is active and can lead to gene deletion between two inserted loxP sites (Lantinga-van Leeuwen et al., 2006; Nagy et al., 2003 p. 291-293). In the present study, generation of inducible lung epithelial HIF 2 α deletion was generated using the SPCrtTA tetO system (section 3.1.2.1) driving Cre recombinase expression (section 3.1.2.2) in SPC-positive alveolar epithelial cells in the presence of doxycycline, which led to HIF 2 α deletion due to the inserted loxP sites (section 3.4).

3.1.2.1. The rtTA tetO system

The ability to create a system for regulation of gene expression in an “on and off” manner was initially discovered by Gossen and Bujard in 1992 (Gossen and Bujard, 1992). They fused the control element of a tetracycline resistance operon of *Echerichia coli* to the activation domain of virion protein 16 (VP 16) of the herpes simplex virus, to generate a tetracycline-controlled transactivator (tTA). To activate transcription, this tTA protein has to be combined with a minimal promoter sequence of the human cytomegalie virus (CMV) which is fused to a tet operon sequence. Accordingly, the activation of the expression is initiated by binding of the tTA to the tet operon (Figure 8 A). Tetracycline prevents tTA from binding to the tet operon, thus its presence inhibits gene expression (Figure 8 A). Fusing a luciferase gene to a tTA-controlled promoter, Gossen and Bujard were able to show *in vitro* that luciferase expression could be controlled by administration of tetracycline in HeLa cell clones. They showed a dose-dependent reduction in luciferase activity with increasing concentrations of tetracycline. Thus, this system was also called the tetOff system (Figure 8 A). Removal of tetracycline-containing media led to an increase in luciferase activity (Gossen and Bujard, 1992). This was a remarkable finding because gene expression could be temporally controlled. Nevertheless, this model has two major disadvantages: 1) constant tetracycline administration was required, and 2) the time-point of induction of the transgene was dependent on the efficacy of

tetracycline clearance rate of the HeLa cells. Thus Gossen and Bujard further developed their model and published in 1995 the mode of action of the reverse tetracycline transactivator (rtTA) system (Gossen et al., 1995). In this model, the VP 16 activation domain was fused to a mutated Tet repressor operator of *Escherichia coli* which gave rise to the rtTA, which requires tetracycline derivatives like doxycycline (dox) for DNA binding. Thus, administration of dox to HeLa cells constitutively-expressing the rtTA and containing the tet operon with the minimal CMV promoter controlling expression of a reporter gene (for example lacZ) leads to reporter gene expression (Figure 8 B). Thus, the “tetOn system” was born. Furthermore, the ability of inducible transgene expression was combined with tissue-specific expression of the rtTA *in vivo*. Constructs were integrated into the mouse genome using the pronucleus method (section 3.2). Thus, a complex model was available to address developmental and biological questions. Perl et al combined the rtTA system with the SPC promoter and the CCSP promoter respectively, to investigate pulmonary epithelial transgene expression in mice (Perl et al., 2002a) (Figure 8 C). This model was particularly beneficial and important for developmental studies, because transgene expression could be switched on at different stages of lung development. Several studies have relied on this model to investigate the impact of pulmonary epithelial transgene expression on lung development (Akeson et al., 2005; Akeson et al., 2003).

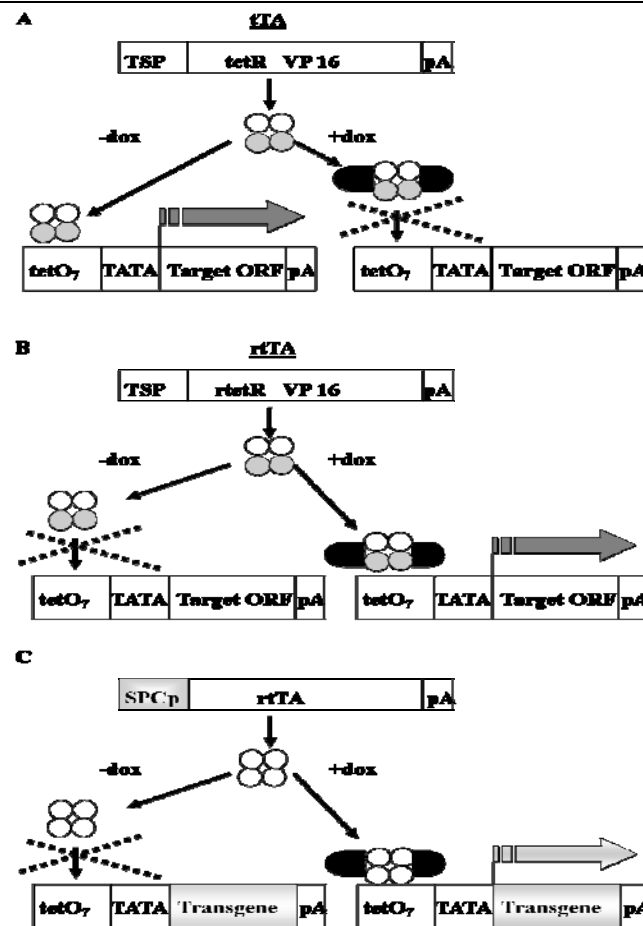


Figure 8: Inducible tissue-specific transgene expression

A: The tTA system: The tetracycline transactivator (tTA) is expressed under the control of a tissue-specific promoter (TSP). The tTA binds the tetO₇ sequence (seven tandemly-repeated 19-bp tetO sequences) in the absence of doxycycline (dox) to induce expression of the transgene (target ORF [open reading frame]). Upon dox application, binding is prevented and the transgene is not expressed (tetOff system). B: The rtTA system. The reverse tetracycline transactivator (rtTA) binds the tetO sequence only in the presence of dox to induce expression of the transgene (tetOn system). In the absence of dox, the rtTA is not able to bind the tetO sequence and expression of the transgene is not induced. Adapted from (Gossen et al., 1995; Lewandoski, 2001). C: Tissue-specific overexpression using the SPCrtTA system: the expression of the rtTA is driven by the SPC promoter (alveolar epithelial cell-specific promoter of the surfactant protein C [SPCp]). Thus, upon dox application, the transgene is exclusive expressed in the alveolar epithelial cells. (Abbreviations: tetR: tetracycline repressor; VP 16: transactivation domain of the herpes simplex virus protein VP 16; TATA: minimal promoter containing TATA box, pA: poly-adenylation site). Adapted from (Gossen et al., 1995; Lewandoski, 2001; Perl et al., 2002b).

3.1.2.2. The Cre-loxP system

In 1998 a key tool for genetically engineering the mouse genome was discovered: Rossant and McMahon elucidated the mode of action of the Cre-loxP recombinase system (Rossant and McMahon, 1999). Due to the discovery of this system, site-directed DNA recombination within two integrated loxP sites became feasible. The DNA recombination could either lead to loss-of-function or gain-of-function. In the late '80s and early '90s Sauer and Henderson demonstrated that the Cre recombinase could work *in vitro* in eukaryotic cells (Sauer and Henderson, 1988; Sauer and Henderson, 1989; Sauer and Henderson, 1990). Accordingly, Lakso et al. and Orban et al. demonstrated, that Cre recombinase worked in the mouse model as well, when expressed as a transgene (Lakso et al., 1992; Orban et al., 1992). The possibility of combining this system with the existing inducible tissue-specific systems gave rise to highly sophisticated models of genetically engineering. A remarkable statement by Andreas Nagy in 2000 characterized the importance of this finding: “...When we acquired the Cre/loxP recombinase system as a tool, we reached the level of sophistication of “no more practical limitations” in tailoring the mouse genome...” (Nagy, 2000).

The Cre recombinase belongs to a family of site-specific recombinases which are collected together in the integrase family. The Cre recombinase is a 38 kDa protein of the P1 bacteriophage and catalyzes recombination between two so-called loxP recognition sites (Hamilton and Abremski, 1984). These loxP sites comprise a 34 bp consensus sequence which contains a core spacer sequence of 8 bp and two 13 bp flanking palindromic sequences. The asymmetric core sequence defines an orientation to the loxP site. The process of recombination occurs by the formation of tetramers: each palindromic sequence of a loxP site is bound by a single recombinase molecule. These molecules then form a tetramer and bring the loxP sites close together (Voziyanov et al., 1999). After recombination, the new loxP sites consist of the two complementary halves of the prerecombined sites (Figure 9). The outcome of the recombination depends on the location of the loxP sites and the actual insertion site. This can lead to excision of a sequence, or to inversion of the targeted sequence. Accordingly a variety of different consequences is possible, as described by Nagy (2000) (Nagy, 2000): Deletion of a functional part of a gene can lead to a knock-out condition. Inversion of a sequence could give rise to a new open reading frame (ORF), thus the expression of a new protein or mutations can be introduced by recombination. A further tool is to activate reporter gene expression by recombination, where the introduction of a stop codon, flanked by the loxP sites in front of a reporter gene, can lead to reporter gene expression after recombination and stop-codon removal. Taken together, the most common use of Cre recombinase is tissue-

specific inducible gene deletion, but for particular developmental and biological questions, the use of Cre recombinase system is a reliable and well-established tool. In the present study, Cre recombinase was inducibly expressed in pulmonary alveolar epithelial cells (Figure 12), using the rtTA system under the control of the SPC promoter, as established by Anne Karina Perl (Perl et al., 2002b). This Cre expression led to HIF 2 α deletion due to the inserted loxP sites, as established by Celeste Simon (Gruber et al., 2007).

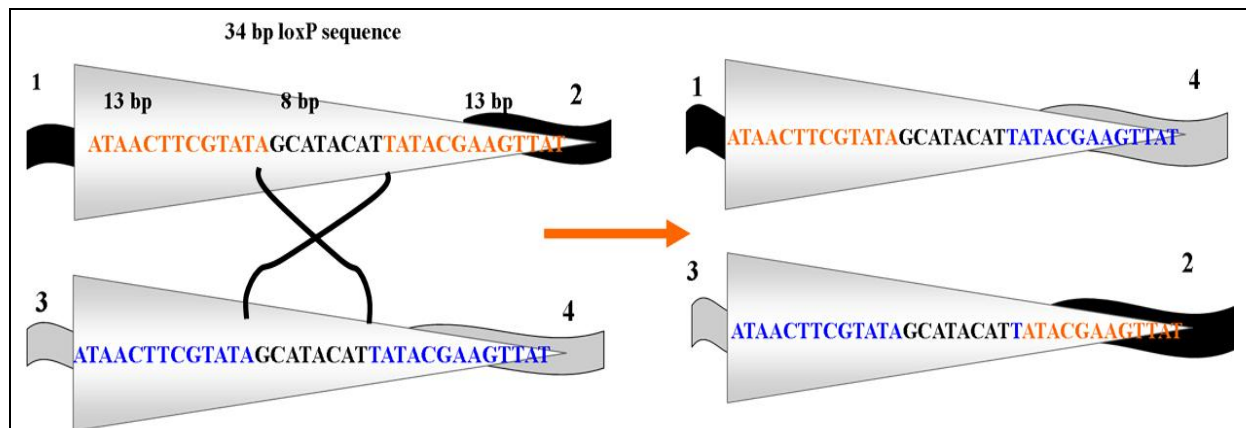


Figure 9: Cre recombinase-based excision of a gene

Excision of a gene can occur upon Cre recombinase-based recombination between two loxP recognition sites. They contain an 8 bp core sequence which is flanked by two 13 bp palindromic sequences. Each palindromic sequence is recognized by a single Cre recombinase molecule. Formation of a tetramer brings the loxP sites together and initiates recombination. After recombination the loxP sites consist of two complementary halves of a prerecombined loxP site and the initially flanked sequence is deleted. Adapted from (Nagy, 2000).

3.2. Generation of transgenic mice by the pronucleus method

The integration of DNA into the mouse genome by the pronucleus method is the most frequently and successfully used method to generate transgenic mice. The foundations of this method were laid down by Lin in 1966 (Lin, 1966), who demonstrated that mouse zygotes could survive the insertion of a fine glass needle into their pronuclei. Furthermore, the injection of macromolecules such as bovine serum albumin (BSA) into zygotes, and their transfer into pseudopregnant female gave rise to viable mice. This finding was ahead of time because recombinant DNA technologies had not yet been developed. But in 1980 Gordon et al. were able to generate transgenic mice (mice stably carrying foreign DNA in their genome) by the pronucleus method (Gordon et al., 1980), where foreign DNA was injected into a fertilized zygote which was reimplanted into a pseudopregnant female mouse. The offspring

comprised the so called founder animals which carried the injected foreign DNA, the “transgene”, in their genome (Figure 10). Further reports were published over the following years (Brinster et al., 1981; Costantini and Lacy, 1981; Gordon and Ruddle, 1981). Today the pronucleus method has become standard procedure to generate transgenic mice (Nagy et al., 2003; Palmiter and Brinster, 1986), and the maximum permissible size of the integratable DNA fragments has increased over time. Strouboulis et al. reported the integration of a 70-kb fragment (Strouboulis et al., 1992). The integration of DNA into the genome by the pronucleus method results in stable integration of foreign DNA in 10-40% of mice generated in the first step, the founders (Nagy et al., 2003 p. 293-294). Integration into the DNA takes place randomly (Nagy et al., 2003 p. 293-294). Thus, the integration site and number of the copies is not predictable. Accordingly, each generated founder animal has a unique integration pattern, site of integration and number of copies. Shortly after injection of the DNA into the fertilized zygote, DNA integrates into the genome before the first cleavage. Thus, all somatic and germ cells will contain the transgene. If integration is delayed, mosaics will be created (Wilkie et al., 1986). Accordingly, the foreign DNA will not be integrated into all somatic and germ cells. Thus these founders will give rise to less than 50% of transgenic mice, when bred with non transgenic mice. The mechanism of DNA integration is still not known in detail (Palmiter and Brinster, 1986) but methods to optimize the process of integration have been developed (Brinster et al., 1981; Brinster et al., 1982). Generated founder animals have to be characterized, to generate a stable transgenic mouse line (section 3.2.1).

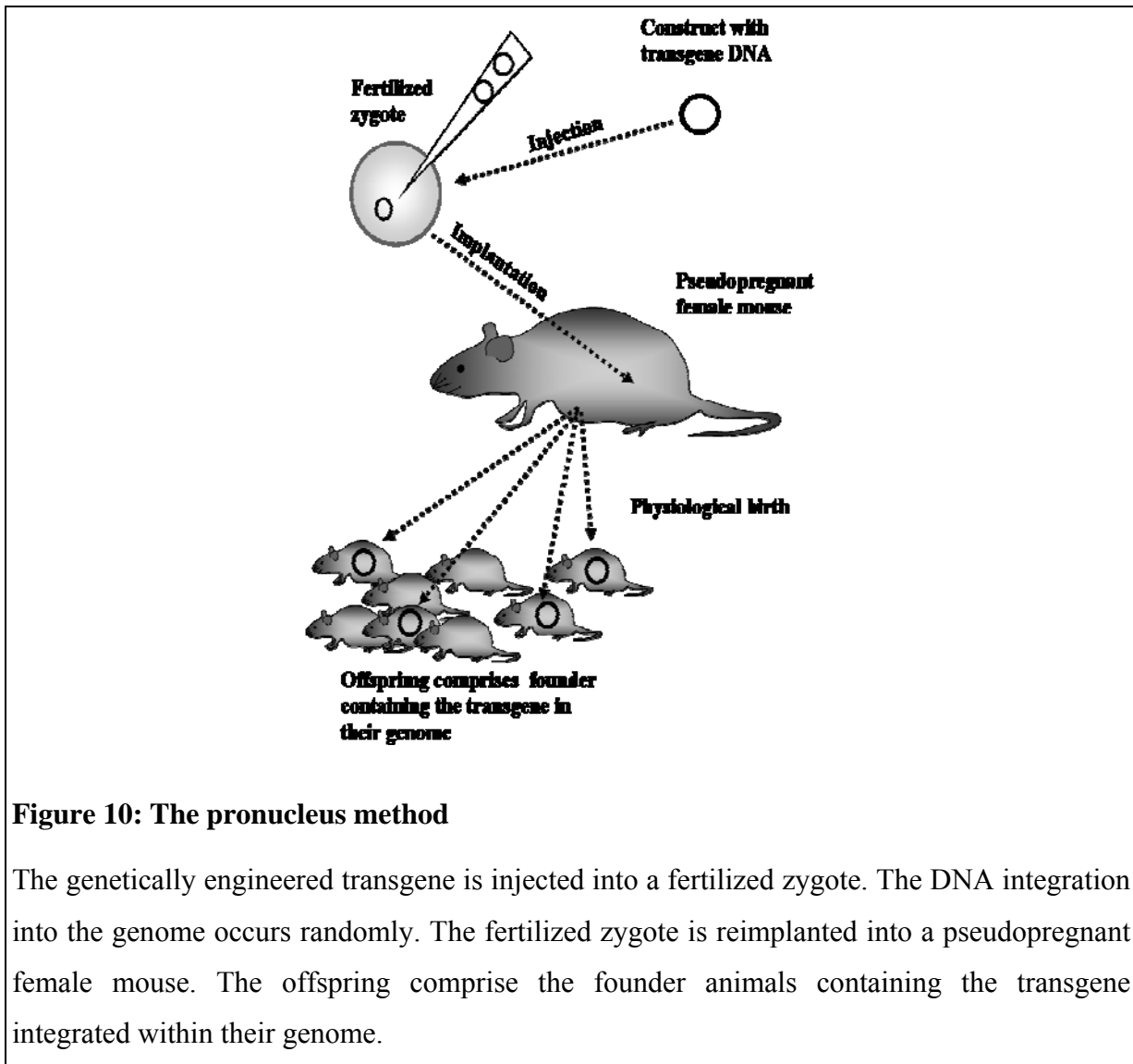


Figure 10: The pronucleus method

The genetically engineered transgene is injected into a fertilized zygote. The DNA integration into the genome occurs randomly. The fertilized zygote is reimplanted into a pseudopregnant female mouse. The offspring comprise the founder animals containing the transgene integrated within their genome.

3.2.1. Characterization of founder animals to generate stable transgenic mouse lines

Founder animals are generated by the pronucleus method to create transgenic mice. To produce a stable transgenic mouse line, the generated founder animals have to be characterized: thus three key features have to be investigated: 1) transgene integration, 2) transgene germ-line transmission and 3) transgene expression. Since integration of the transgene occurs randomly, and succeeds in only 10-40% of the founder animals (Nagy et al., 2003 p. 293-294), genomic integration has to be evaluated by genomic PCR to facilitate genotyping of the mice. Positive animals are bred with nontransgenic animals to check for germline transmission. Genotyping of the resulting offspring shows if the transgene is present in the offspring. If transmission is positive, the offspring still have to be tested for transgene expression. In case of a constitutive transgene expression the expression can be investigated in the first offspring at the RNA and protein level by RT PCR and immunohistology,

respectively. Usually tags are integrated into the transgene, to facilitate transgene detection. In case of reporter genes, the expression can be visualized. In case of inducible transgenic systems, the mice expressing the activator (for example rtTA) are bred with the mice carrying the responder transgene which should be induced (for example tetO-Cre). Single transgenic animals are bred (rtTA X tetO-Cre) to generate double transgenic mice, which are positive for both genes (tTA and tetO-Cre). After induction (for example application of dox), double transgenic mice should express the transgene. The double transgenic genotype has to be controlled by PCR to distinguish between control mice (single transgenic) and double transgenic mice. If single transgenic mice do express the transgene as well, the inducible system is leaky. Thus, it is effectively a constitutive system. In case the offspring of a founder do express the transgene, the founder can be used to generate a stable transgenic mouse line. It has to be kept in mind that mouse lines generated from distinct founder animals must not be intercrossed because the integration pattern of the DNA is different and can lead to phenotypic differences even though they are carrying the same transgene.

3.3. Generation of transgenic mice by the ES cell method

Generation of transgenic mice by the ES cell method is the most common method of site-specific deletion or gene targeting. In particular, the integration of loxP sites into the genome at distinct sites as required for the Cre loxP system is completed by this method. The mechanism of homologous recombination (section 3.3.1) allows the specific localization of gene targeting. The possibility to combine this method with the Cre loxP system gives rise to a variety of different approaches of genetic engineering. In contrast to the pronucleus method, the foreign DNA or the so-called targeting vector is transfected into cultured ES cells before injection into the blastocyst (Capecchi, 1989). This gives rise to the possibility of screening and enrichment of the successfully transfected cells prior to injection. After homologous recombination between the target vector and the endogenous DNA, ES cells are screened for recombination and enriched by positive and negative selection and clonal expansion (Figure 11 A and C). Enriched cells are injected into the recipient blastocyst which is implanted into a foster mother to give rise to usual development and birth (Figure 11 B). The first offspring are chimeric mice. For example, if the ES cells are derived from a mouse homozygous for black coat color allele, and the blastocyst is derived from an albino mouse, the offspring will have black patches on the white fur because the mice contain cells of both genotypes. Breeding of the chimeric mice to albino mice will give rise to some black mice. This indicates that the ES cells contributed to the formation of the germ-line. By genotyping of the offspring,

germ-line transmission should be controlled. Breeding of heterozygous transgenic mice will give rise to 25% of homozygous transgenic mice.

3.3.1. Alteration of the genome by homologous recombination

The mechanism of homologous recombination is a key process in generating transgenic animals by the ES cell method. It gives rise to site-specific gene alteration. Alterations can comprise a variety of different gene manipulations: Gene deletion, site directed mutagenesis, reporter gene integration and combination with the Cre loxP system. The targeting vector to delete a special gene by homologous recombination has to be constructed in a certain way: the vector contains two segments of homologous sequences from the gene of interest which are flanking a region essential for the gene function. Inbetween these two homologous sequences a positive selection marker (neo gene; resistance marker, resistant to the drug G 418) is placed (Figure 11 A and C). After homologous recombination, the positive selection marker will replace the gene of interest. Accordingly, ES cells which underwent homologous recombination successfully (and became null mutants for the gene of interest), will survive the G418 selection. Cells which do not contain the neo resistance gene due to failure of homologous recombination will die. Thus, only the null ES cells (-/- ES cells) will survive and can be expanded [Figure 11 A and C, (Capecchi, 1989; Nagy et al., 2003)]. Negative selection markers have been developed to improve the purity of -/-ES cell cultures: Integration of a negative selection marker (tk, thymidine kinase) outside the two sequences of homology leads to elimination of all cells with random integration, since random integration integrates the whole vector at non-specific regions into the genome, without homologous recombination. This leads to integration of the tk, which is usually excluded upon homologous recombination because it lies outside the two homologous regions. Addition of gancyclovir into the culture medium will select all cells without tk, and thus, all cells which underwent homologous recombination successfully. Cells containing tk will be deleted due to the increasing toxicity of gancyclovir mediated by tk expression (Figure 11 C). The effect of homologous recombination can be determined by choosing the sequence between the two sites of homology because this sequence will be integrated. Thus, a variety of different gene alterations can be produced by this method.

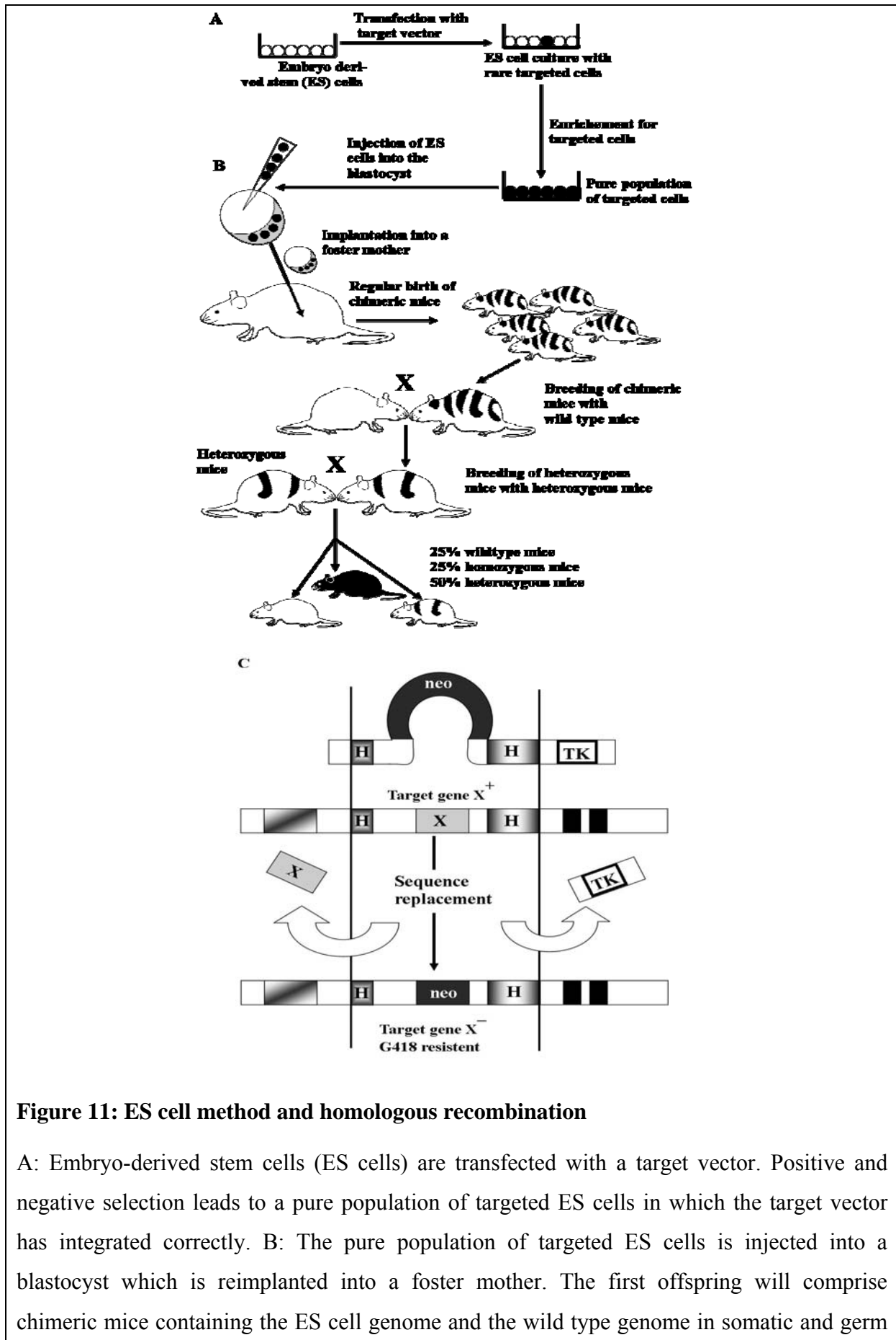


Figure 11: ES cell method and homologous recombination

A: Embryo-derived stem cells (ES cells) are transfected with a target vector. Positive and negative selection leads to a pure population of targeted ES cells in which the target vector has integrated correctly. B: The pure population of targeted ES cells is injected into a blastocyst which is reimplanted into a foster mother. The first offspring will comprise chimeric mice containing the ES cell genome and the wild type genome in somatic and germ

line cells. Further breeding of chimeric mice to wild-type mice will give rise to heterozygous mice containing the modified gene locus if germ-line transmission succeeded. Breeding of heterozygous mice will give rise to 25% of homozygous mice containing the modified gene locus. C: Homologous recombination, and positive and negative selection upon integration and homologous recombination. The target vector contains a resistance cassette [for example neo gene which is flanked by sequences (H) which are homologous to the sequences (H) upstream and downstream of the targeted sequence (X)]. Upon homologous recombination, the homologous sequences of the target vector anneal to the endogenous homologous sequences flanking the target gene (X). Recombination between these sites leads to the excision of the target gene (X) and integration of the resistance cassette containing the the neo gene. Accordingly, only the ES cells containing the neo gene will survive the G418 media cultivation. Cells lacking the gene will die. As a further strategy to increase purity of the cells containing the correctly-integrated vector, the negative selection based on tk and gancyclovir is used, where the tk (thymidine kinase) gene is integrated into the target vector outside of the homologous regions. Accordingly, the tk gene will be excluded upon correct integration. Thus, only cells undergoing random integration without homologous recombination will contain the tk gene and will be killed by gancyclovir treatment. Adapted from (Capecchi, 1989).

3.4. Generation of inducible pulmonary epithelial HIF 2 α knock-out mice

In this study, the impact of the pulmonary epithelial HIF 2 α flk-1 system on lung development was investigated. To address this question, an inducible *in vivo* knock-out of the pulmonary epithelial HIF 2 α gene was created, using a transgenic mouse model approaches. The Cre loxP system, in combination with the rtTA system, was used to generate pulmonary epithelial HIF 2 α gene deletion. Double transgenic mice carrying the rtTA activator under the control of the human SPC promoter and the Cre recombinase controlled by the tetO CMV promoter (Perl et al., 2002b) (Figure 12 A) were bred with homozygous HIF 2 α loxP mice (Gruber et al., 2007) (Figure 12 B) to generate triple transgenic SPCrtTA/Cre/HIF 2 α loxP mice. Further breeding strategies finally lead to triple transgenic, SPCrtTA positive, Cre positive and homozygous HIF 2 α loxP mice (Figure 14). Application of dox (by addition to the drinking water of the mice) led to expression of Cre in the pulmonary epithelial system. The Cre expression drove the recombination between the loxP sites, and hence, deletion of HIF 2 α , by creating multiple stop codons within the open reading frame (Figure 12).

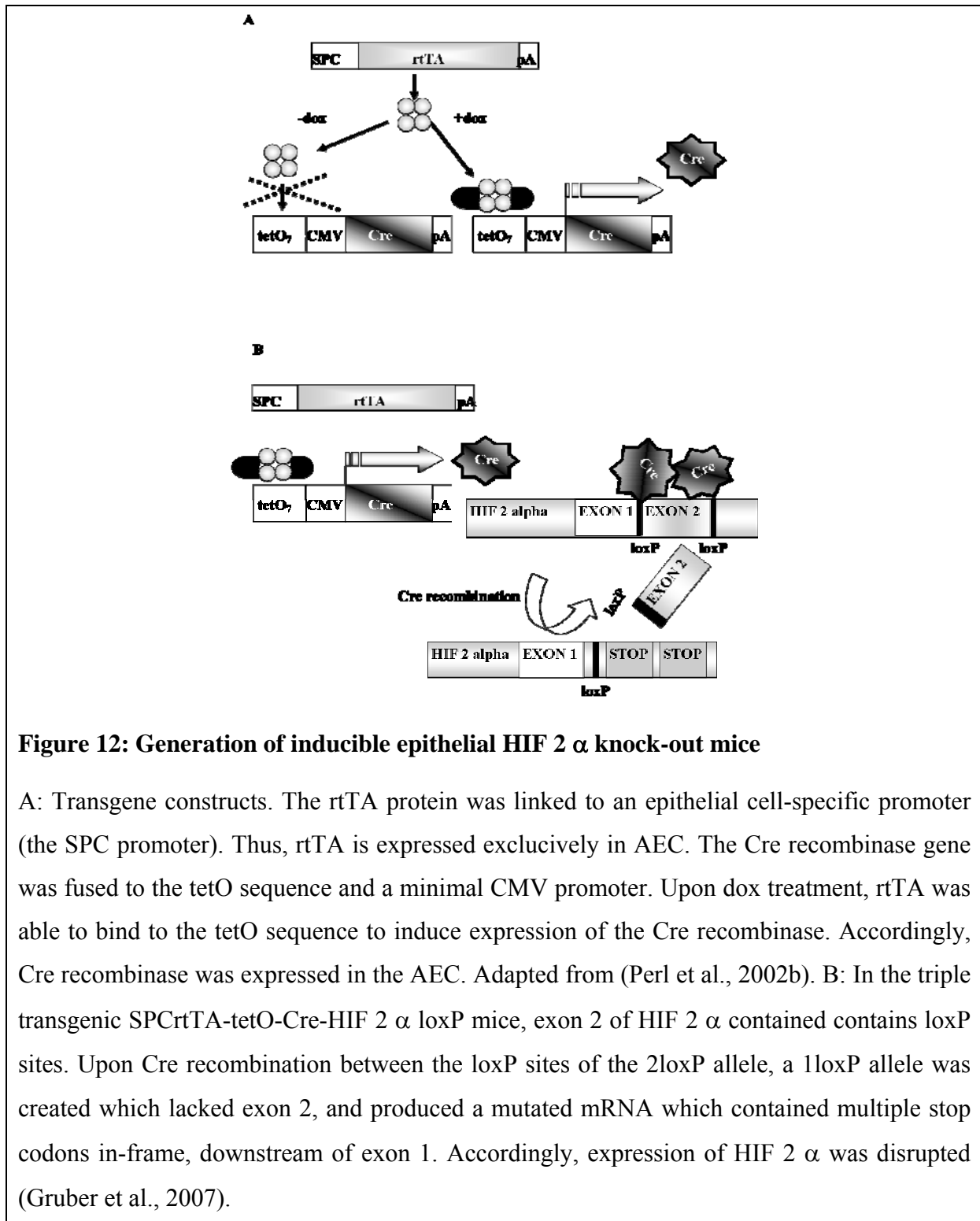


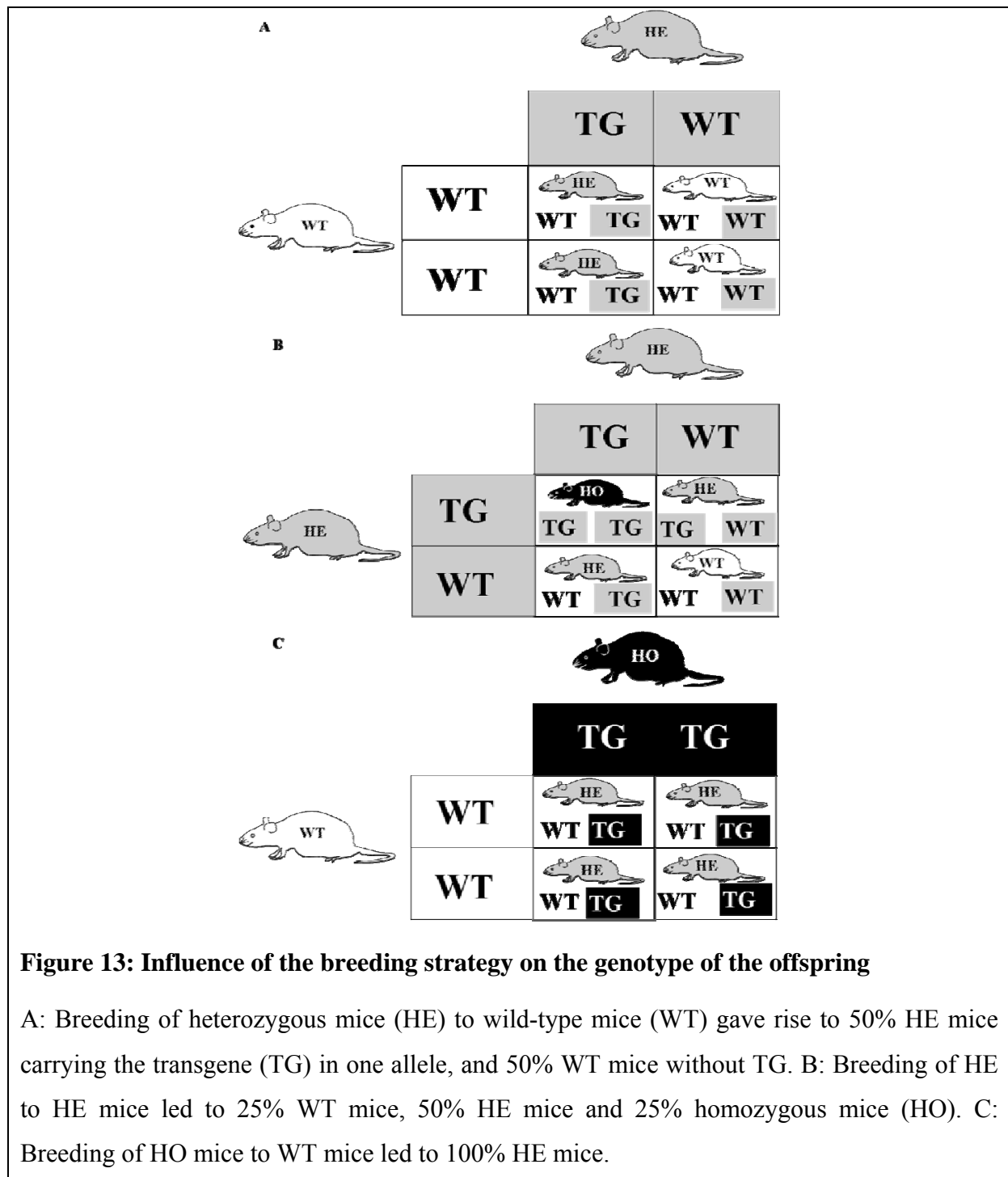
Figure 12: Generation of inducible epithelial HIF 2 α knock-out mice

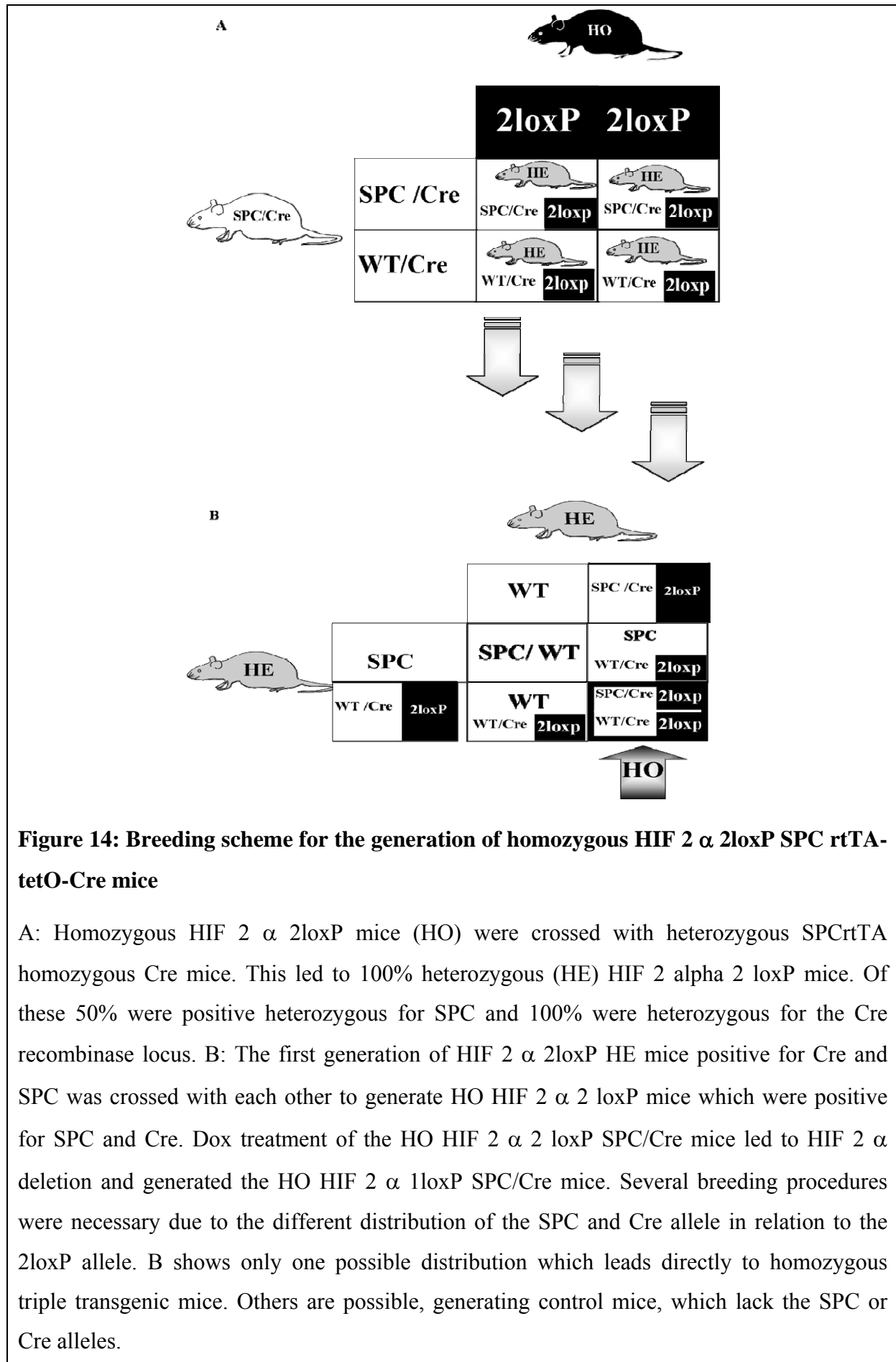
A: Transgene constructs. The rtTA protein was linked to an epithelial cell-specific promoter (the SPC promoter). Thus, rtTA is expressed exclusively in AEC. The Cre recombinase gene was fused to the tetO sequence and a minimal CMV promoter. Upon dox treatment, rtTA was able to bind to the tetO sequence to induce expression of the Cre recombinase. Accordingly, Cre recombinase was expressed in the AEC. Adapted from (Perl et al., 2002b). B: In the triple transgenic SPCrtTA-tetO-Cre-HIF 2 α loxP mice, exon 2 of HIF 2 α contained contains loxP sites. Upon Cre recombination between the loxP sites of the 2loxP allele, a 1loxP allele was created which lacked exon 2, and produced a mutated mRNA which contained multiple stop codons in-frame, downstream of exon 1. Accordingly, expression of HIF 2 α was disrupted (Gruber et al., 2007).

3.4.1. Generation of triple transgenic homozygous HIF 2 α 1loxP SPC rtTA tetO-Cre mice

To create an inducible pulmonary epithelial HIF 2 α deletion, triple transgenic homozygous HIF 2 α 2loxP SPCrtTA-tetO-Cre mice had to be generated. Upon dox treatment, Cre will be expressed in the pulmonary epithelial system and DNA recombination will lead to

homozygous HIF 2 α 1loxP SPCrtTA-tetO-Cre mice. HIF 2 α and one loxP site will be deleted after recombination (Figure 12 B). To generate these triple transgenic mice, double transgenic SPCrtTA-tetO-Cre mice (a kind gift from Anne Karina Perl and Jeffrey A. Whitsett, Cincinnati Children's Hospital Medical Center) (Perl et al., 2002b) were crossed with to single transgenic homozygous HIF 2 α loxP mice (a kind gift from Brian Keith and Celeste Simon Abramson Family Cancer Research Institute, Howard Hughes Medical Institute University of Pennsylvania School of Medicine) (Gruber et al., 2007). Offspring were genotyped for all the transgenes. The first generation comprised the HIF 2 α loxP locus in heterozygous manner or wild-type genotype without the locus. The SPCrtTA locus was found in 50% of the offspring because breeding mice were heterozygous for the locus. The tetO-Cre locus was present in 50% in case of heterozygous and in 100% in case of homozygous breeding mice (Figure 13). Heterozygous HIF 2 α loxP mice positive for at least Cre or SPC were bred to each other. Genotyping revealed 25% homozygous, 25% wild-type and 50% heterozygous HIF 2 α loxP mice. The outcome for SPC and Cre referred to the breeding outcome of heterozygous breeding pairs. Homozygous HIF 2 α loxP mice positive for tetO-Cre or SPCrtTA were intercrossed until a sufficient number of homozygous HIF 2 α 2loxP SPCrtTA tetO-Cre mice were born (Figure 14). Double transgenic homozygous HIF 2 α 2loxP mice positive for SPCrtTA or tetO-Cre were used as control animals. Dox treatment led to pulmonary epithelial HIF 2 α deletion in the homozygous HIF 2 α 2loxP SPCrtTA-tetO-Cre mice, but not in the control mice (Figure 14).





3.4.2. Characterization of the phenotype of epithelial HIF 2 α knock-out mice in comparison to control mice

Epithelial HIF 2 α knock-out mice were characterized by analysis of isolated alveolar epithelial cells and whole lung sections. Isolated alveolar epithelial cells of epithelial HIF 2 α knock-out mice and control mice were analyzed for changes in protein levels of flk-1 and SPC. Lung morphometric analysis (section 4.2.11) was used to determine the impact of the epithelial HIF 2 α deletion on lung structure. The number of alveoli, alveolar wall thickness and total alveolar volume were measured. Alveolar epithelial cell morphology was investigated by electron microscopy on whole lung sections (section 4.2.12).

4. Materials and methods

4.1. Investigations on wild type C57bl 6 mice

4.1.1. Animals

C57 black 6 N mice (C57bl 6) were purchased from Charles River. Pregnant C57bl 6 mice were ordered at particular embryonic stages to investigate the different stages of lung development by *in situ* hybridization, immunohistology and AEC isolation.

4.1.2. Preparation of embryonic pups

Term pregnant C57bl6 female mice were anesthetized with inhaled Isofluran® (Baxter Deutschland GmbH, Unterschleißheim, Germany) at a lethal dose. The abdominal cavity was opened and embryonic pups were removed. The yolk sac was opened, the placenta was removed and embryonic pups were sacrificed on dry ice. Pups were embedded as described in section 4.1.3. for whole mount sections, or lungs were removed and embedded as described in section 4.1.3. for lung cryosections.

4.1.3. Preparation of the lung

Mice were anesthetized with inhaled Isofluran® (Baxter Deutschland GmbH, Unterschleißheim, Germany) at a lethal dose. The thoracic cavity was opened after inducing a pneumothorax to prevent lung disturbance. The right ventricle was cannulated and left atrium was opened by incision. The lung was perfused with 10 ml PBS, the trachea was prepared and intubated, Tissuetek® (Sakura Finetek, Europe, Zoeterwoude, Netherlands) 1:1 diluted in PBS was instilled to fill the airspaces. Filled lungs were taken out of the thoracic cavity, and embedded in Tissuetek®. A container filled with isopentane (Roth, Karlsruhe, Germany) was placed in a container filled with liquid nitrogen. Lungs were kept in the isopentane fluid to slow cool. Lungs were then transferred to liquid nitrogen, and finally stored at -80 °C. Frozen sections of the lung tissues and embryonic pups were prepared with a cryotome (Leica CM 3050, Leica, Wetzlar, Germany) at a thickness of 10 µm. Sections were then used for immunohistochemistry (section 4.1.4) and *in situ* hybridization (section 4.1.5).

4.1.4. Immunohistochemistry and immunocytology

Cells or cryostat section were fixed for 10 minutes using acetone or acetone/methanol. After blocking with pure goat serum for 1 hour, sections or cells were incubated with primary antibodies overnight (flk-1, BD Pharmingen, Danvers, USA; cytokeratin, Dako, USA;

proSPC, Chemicon, Temecula, USA). Secondary antibody (goat anti rat Alexa Fluor® 555 and goat anti rabbit Alexa Fluor®488, Molecular Probes, Invitrogen, UK) incubation and incubation with FITC-labeled CD31 antibody (Chemicon) was performed for 1 h. Final fixation in 4% paraformaldehyde for 10 min was done prior to nuclear staining (Topro-3®, Molecular Probes) for 10 min. Finally, sections and slides were coverslipped with Vectashield (Vector Labs Burlingame CA, USA) and analyzed by confocal laser scanning microscopy (Leica Type TCS SP 2, DMLFSA, Leica, Wetzlar, Germany) Staining with respective Isotype controls with rat and rabbit IgG respectively were carried out with purified rabbit IgG (Serotec, Düsseldorf, Germany) and rat IgG (clone 9B5) a kind gift of Georg Breier (Department of Pathology Dresden, University of Dresden, Germany).

4.1.5. *In situ* hybridization

Messenger RNA (mRNA) *in situ* hybridization for flk-1 was performed as described previously (Breier, 1999) on frozen sections using [³⁵S]α-UTP-labeled antisense mRNA probes. The probes were generated from the murine flk-1 cDNA which was integrated into a plasmid containing a promoter for bacteriophage RNA polymerase on each side of the integrated cDNA. The flk-1 cDNA-containing plasmid was a kind gift of Georg Breier (Department of Pathology, University of Dresden, Dresden, Germany). According to the protocol (Breier, 1999), RNA probes were generated by *in vitro* transcription of the linearized flk-1 cDNA containing-plasmid. [³⁵S]α-UTP-labeled sense and antisense probes were generated by using ³⁵S α UTP (Amersham, Freiburg, Germany) during the *in vitro* transcription from DNA to RNA. Template DNA was removed by DNase I (Boehringer, Mannheim, Germany) digestion. Probe purification was assessed by RNA extraction using the phenol-chloroform-isoamyl alcohol (PCI) method, and precipitation with tRNA (Sigma, Steinheim, Germany) and ammonium acetate (Sigma, Steinheim, Germany). Frozen sections were generated from frozen tissue (section 4.1.2 and 4.1.3) and pretreated according to the protocol (Breier, 1999). Probes were hybridized to prewarmed sections in a humid chamber (50% formamide, Sigma Aldrich, Steinheim, Germany) overnight. Unbound RNA was removed by RNase (Boehringer, Mannheim, Germany) digestion. After dehydration in ethanol and air drying, hybridized [³⁵S]α-UTP-labeled sense and antisense probes were visualized with a Kodak D 19 developer. Core staining was performed using 0.02% Toluidine Blue. Sections were analyzed using a Zeiss Microscope (Zeiss, Axioplan 2 Carl Zeiss, Jena, Germany) with light and dark fields.

4.2. Generation of triple transgenic inducible pulmonary epithelial HIF 2 α knock-out mice

4.2.1. Transgenic mouse strains

Double transgenic SPCrtTA-tetO-Cre mice were obtained as a kind gift from Anne Karina Perl and Jeffrey A. Whitsett, Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, USA (Perl et al., 2002b). Mice were raised on a FVBN background. Single transgenic homozygous HIF 2 α loxP mice were a kind gift from Brian Keith and Celeste Simon Abramson Family Cancer Research Institute Howard Hughes Medical Institute University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA (Gruber et al., 2007). Mice were raised on an agouti background. Upon Cre recombination between the two loxP sites of the 2loxP allele, a 1loxP allele was created which lacks exon 2 and produced a mutated mRNA which contained multiple stop codons in-frame, downstream of exon 1. Accordingly expression of HIF 2 α was disrupted (Gruber et al., 2007), (Figure 12).

4.2.2. DNA-extraction from tailcuts

DNA extraction was performed with a Qiagen DNeasy Blood and Tissue Kit (Nr: 69506, Qiagen, Hilden, Germany). Tail cuts were taken 0.5cm in length, and lysed in 180 μ l lysis buffer with 20 μ l Proteinase K overnight, according to the manufacturer's protocol.

4.2.3. PCR for genotyping

PCR was performed to differentiate the genotype of the different transgenic mice. Two transgenic systems were used to create an epithelial tissue-specific, inducible knock-out: 1) The rtTA tet on system and 2) The Cre-loxP system. The pulmonary epithelial cell specific SPC promoter was used to drive the expression of the rtTA protein. The tetO sequence was linked to a minimal CMV promoter upstream of the Cre recombinase. Therefore, three different genotypes had to be identified: SPCrtTA, tetO Cre and HIF 2loxP. Concerning the Cre-loxP system, loxP sites were integrated upstream of exon 2 of the endogenous HIF 2 α gene. Hence also in the loxP-system three different genotypes had to be identified: 1) the integrated two loxP sites (2loxP), 2) the wild-type (WT) and 3) the knock-out after recombination (1loxP). Specific primers and PCR product sizes are shown in Table 1. Specific primers were ordered from MWG Biotech (Ebersberg, Germany). PCR was carried

out using the AmpliTaq Gold system from Applied Biosystems (Nr: 4311816; Foster City, CA, USA) as detailed in Table 2.

Table 1: Primers for genotyping

Sequences of forward primers, reverse primers and the PCR product sizes are listed for the three transgene loci: SPCrtTA, tetO-Cre and HIF 2 loxP. Concerning the HIF 2 loxP loci, three different products are possible: WT (wild-type), 2-loxP (inserted locus) and 1-loxP (recombined locus)

Transgene	Forward primer	Reverse primer	PCR product size in bp
SPCrtTA	GAC ACA TAT AAG ACC CTG GTC	AAA ATC TTG CCA GCT TTC CCC	350 bp
tetO-Cre	TGC CAC GAC CAA GTG ACA GCA ATG	AGA GAC GGA AAT CCA TCG CTC G	374 bp
HIF 2 loxP	BK380: CAG GCA GTA TGC CTG GCT AAT TCC AGT BK430: CTA ACA CTG TAC TGT CTG AAA GAG TAG C	BK381: CTT CTT CCA TCA TCT GGG ATC TGG GAC T	BK380/BK381: WT: 430 bp 2-LoxP: 460 bp BK380/BK430 1-LoxP (KO): 360 bp

Table 2: PCR protocol for genotyping

Optimized conditions for the transgene detection by PCR of the three transgene loci: SPCrtTA, tetO-Cre and HIF 2 loxP.

	SPCrtTA	tetO-Cre	HIF 2 loxP
Initial denaturation	120 s 94 °C	120 s 94 °C	120 s 94 °C
Denaturation	30 s 94 °C	30 s 94 °C	30 s 94 °C
Annealing	30 s 60 °C	30 s 60 °C	30 s 58 °C
Elongation	40 s 72 °C	90 s 72 °C	45 s 72 °C
Final elongation	300 s 72 °C	300 s 72 °C	300 s 72 °C
Cycles	35	35	35

PCR products were separated by gel electrophoresis on 1.5% agarose (Invitrogen Nr: 15510-027, UK) gel, in TAE (1 l 50× TAE: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH 8). For visualization, ethidium bromide (Fluka Nr: 46067, Seelzen, Germany) was included in the electrophoresis run, and the gel was examined with the CHEMI GENIUS BIO IMAGING SYSTEM (Syngene, Cambridge, UK).

4.2.4. Induction of transgene expression

Due to the rtTA system, transgene expression could be induced by dox (doxycycline, Sigma Aldrich, Steinheim, Germany) a tetracycline analogue. For *in vivo* induction, dox was added to the drinking water at a concentration of 0.2 mg per liter with 0.2 mg Sodium Citrat per liter and 10 g glucose per liter. Expression was induced continuously from the day of conception (ED 0). *In vitro* dox was supplied to the culture medium in a concentration of 1 µg/ml for 48 h.

4.2.5. AEC isolation and culture

Alveolar epithelial cells were isolated as previously described in detail (Corti et al., 1996). The cells were cultured for three days in DMEM (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum. Cells were washed thoroughly every 24 h and supplied with fresh media. Before VEGF-A stimulation, supernatants were removed and subjected to a VEGF-A ELISA. Cells were starved for 4 h in serum-free medium before stimulation with 100 ng/ml VEGF-A (R&D Systems, Wiesbaden, Germany) for 1 min. The cells were washed after

incubation with cold phosphate-buffered saline and lysed in RIPA buffer supplemented with Complete™ protease inhibitor cocktail (Roche, Basel, Switzerland). For western blot analysis and immunocytochemistry for HIF 2 α , cells were kept in culture for 48 h under normoxic and hypoxic (1% of oxygen) conditions. DNA isolation for Southern blot analysis was performed after 48 h culture with dox, supplied in the medium at a concentration of 1 $\mu\text{g}/\text{ml}$.

4.2.6. Southern blot Analysis for genotyping

DNA from isolated AEC was extracted as described in section 4.2.2. The DNA was digested using 10 μl DNA (5 μg), 5.3 μl H₂O, 2 μl 10 \times buffer (as provided with enzyme), 0.2 μl BSA, 2.5 μl Nco I, (10 U/ μl) (Promega, Mannheim, Germany) overnight at 37 °C. The reaction was stopped by heating for 10 min to 65 °C. After addition of 6 \times loading dye, samples were loaded onto an 0.7 % agarose gel with ethidium bromide included. The gel was run at 90 V until the dye front had migrated approximately 6 cm. The samples were blotted on a Hybond N membrane (Amersham, Freiburg, Germany) by capillary transfer overnight. Following transfer, the membrane was oven baked at 80 °C for 2 h to fix DNA to the membrane.

The probe was derived from plasmid kindly provided by Brian Keith and Celeste Simon (Abramson Family Cancer Research Institute, Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, USA) by digestion with KpnI and ClaI (Promega, Mannheim, Germany). Following purification of the probe by electrophoresis and gel extraction, the probe was labeled using the Amersham Gene Images Alk Phos Direct Labeling and Detection System (Amersham, Freiburg, Germany). All subsequent procedures were carried out according to the manufacturer's protocol.

4.2.7. mRNA isolation from AEC

The mRNA was isolated from AEC after three days of culture in medium with 1 $\mu\text{g}/\text{ml}$ dox. For mRNA isolation the Qiagen mini kit® (Qiagen, Hilden, Germany) was used. The procedure was performed according to the manufacturer's protocol. The mRNA concentration was measured using a Nanodrop® (Thermo Scientific, Wilmington, USA) spectrophotometer.

4.2.8. RT PCR

RT-PCR was performed with reverse transcriptase from Applied Biosystems (Foster City, CA, USA) using 1 μg RNA as template. Samples were heated at 95 °C for 7 min, the reaction mixture was added, and transcription was performed for one hour at 55 °C. cDNA was used for PCR as described in section 4.2.3.

4.2.9. Immunoprecipitation and western blot analysis

Immunoprecipitation

The AECs were lysed in lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X 100, 2.5 mM Sodium-pyrophosphate and 1 mM glycerophosphate. Vanadate (1 mM) and the protease inhibitor Complete™ (Protease Inhibitor Cocktail, Roche; Nr. 11836170001; Roche, Mannheim, Germany) were added freshly before use. Antibody (2 µg) was added to each sample, and samples were incubated at 4 °C with constant mixing for 1 h. Protein-antibody complexes were precipitated by the addition of 25 µl of protein A-agarose and an additional hour of incubation. Samples were centrifuged, supernatant removed and the agarose pellet washed three times with lysis buffer. After a final wash, 30 µl of 2× sample buffer was added to each sample, and the samples were boiled for 5 min and resolved by gel electrophoresis.

Western blot analysis

Cell lysates from freshly isolated AEC and immunoprecipitations were separated on a 6 % sodium dodecyl sulphate polyacrylamide gel. Proteins were transferred to a polyvinyl difluoride (PVDF) membrane using a wet blotting chamber, and after blocking with 7.5 % milk, incubated with primary antibody overnight (anti-flk-1, Cell Signaling Technology, Boston, MA, USA dilution 1: 1000; or anti-phosphotyrosine; BD Transduction, Heidelberg, Germany; dilution 1: 1000, anti HIF 2 α antibody Novus, Littleton, USA, dilution 1:1000; anti proSPC; Chemicon, Hofheim, Germany, dilution 1:1000). Bound antibody was visualized with species-specific peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA; dilution 1: 3000) followed by detection with an incubation for 1 hour and ECL® system, (RPN 2132 Amersham, Freiburg, Germany).

4.2.10. Preparation of cytosolic and nuclear extracts

For nuclear extraction, all steps were performed on ice. Medium from cultured AEC was removed and cells were washed twice with ice-cold PBS, then 1 ml of PBS was added to the cell culture dish and cells were scraped using a rubber policeman. The cell suspension was transferred into a reaction tube and centrifuged. The supernatant was removed completely, and pellets were resuspended in low-salt buffer (three times the volume of pellet) containing 20 mM Hepes pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.2 % NP-40, 10 % glycerol. Vanadate (1 mM) and Complete™ (Proteinase Inhibitor Cocktail, Roche Nr:11836170001, Roche Mannheim, Germany) were added prior to use. Samples were incubated on ice for 10

min, vortexed and centrifuged. The supernatant was designated a cytosolic extract, the pellet was resuspended in high-salt buffer (2/3 of the volume used for cytosolic extraction) containing 20 mM Hepes pH 7.9, 420 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 20 % glycerol. Vanadate (1 mM) and Complete™ (Proteinase Inhibitor Cocktail, Roche Nr:11836170001, Roche Mannheim, Germany) were added prior to use. Samples were incubated on ice for 30 min, vortexed and centrifuged. The resulting supernatant was designated a nuclear extract. Cytosolic and nuclear extracts were aliquoted and frozen at -80 °C. The Bradford method was used to determine protein concentration (BioRad Quick Start Bradford Dye Reagent, Nr: 500-0205, BioRad, München, Germany). Samples were then used for western blot analysis as described in section 4.2.9.

4.2.11. Lung morphometry analysis

Lung morphometry analysis was kindly performed by Dipl. Ing. Msc. Biol. Sandeep Nikam.

Lung isolation and volume determination

The mice were heparinized by an intraperitoneal (i.p.) injection of 500 units of heparin, and then sacrificed as described in section 4.1.3. The thorax was opened and the trachea and pulmonary artery were cannulated. The lung and heart were flushed with PBS followed by perfusion through the pulmonary artery and through the trachea, respectively, with 4 % buffered paraformaldehyde (PFA) (Sigma-Aldrich, Seelzen, Germany) with a pressure of 20 cm and 10 cm H₂O, respectively. Afterwards, the trachea was ligated, and the heart and lung were resected *en bloc* from the thorax and fixed in fresh fixative overnight at 4 °C. The volume of lung was determined by water submersion method (Scherle, 1970).

Lung preparation for design-based stereology

The lungs were prepared for the estimation of various parameters using design-based stereology as described by Voswinckel et al., (Voswinckel et al., 2004). Systematic random sampling of the lung tissue was performed according to Michel and Cruz-Orive (Michel and Cruz-Orive, 1988). The organ was cut into 3 mm slices, dehydrated with a graded series of alcohol, and paraffin-embedded. The 3 µm sections were cut on microtome (Microm, Germany), and stained with Wiegert Elastin-Fuchselin and Nuclear-Fast-Red-Aluminium sulphate (Waldeck GmbH, München, Germany). Each section of every slice from each lung was used for quantitative estimation using stereology. Using meander sampling, a collection of fields was obtained from each lung slice, thus ensuring that the scanning was done over the

whole organ. A two-level procedure was performed for point and intersection counting to determine the volumetric parameters and total surface area as given by Howard and Reed (Howard, 1998). Lungs were divided in two compartments, non-parenchyma compartments (comprising of lumen, wall, and tunica adventitia of airways and vessels) and parenchyma compartments (alveolar duct airspace, alveolar airspace and alveolar septum). Furthermore, volume fractions of each compartment were estimated. Absolute volumes of the respective lung compartments were obtained by multiplication of the total lung volume with the respective volume fraction. Total alveolar surface area was calculated by multiplication of the surface density of the alveolar septum (surface per unit volume of parenchyma) and the absolute parenchymal volume. The surface density of the alveolar septum was estimated from the sum of intersections of the test lines (22.5 μm of length) with the alveolar septa and the sum of test points hitting parenchymal structures. All stereological analyses were performed by means of a computer-based system (connected to a Leica DM6000 microscope (Leica, Wetzlar, Germany)).

4.2.12. Electron microscopy

Analysis by electron microscopy was kindly performed by Prof. Dr. Wolfgang Kummer, Institute for Anatomy and Cell Biology, University of Giessen, Germany.

Lungs were fixed via tracheal instillation at a pressure of 10 cm H₂O from the right ventricle with 2.5 % glutardialdehyde, 1.5 % PFA in 0.1 M phosphate buffer, pH 7.4. Tissue blocks (< 2 mm) were cut from peripheral lung with a razor blade, washed in 0.1 M phosphate buffer, osmicated for 1 h in 0.1 OsO₄ in 0.1 M phosphate buffer, transferred to 0.05 M maleate buffer, pH 5.2, stained *en bloc* for 1 h with 1 % uranyl acetate in this buffer, washed again in maleate buffer, pH 5.2, and then dehydrated through a graded series of ethanol and embedded in Epon (Agar100; Plano, Wetzlar, Germany). Thin sections (70-80 nm) were cut with an ultramicrotome (Ultracut E; Reichert-Jung, Heidelberg, Germany), collected on nickel grids, and stained with lead citrate. Sections were evaluated with a Zeiss EM 902 transmission electron microscope (Zeiss, Jena, Germany).

5. Results

5.1. *Flk-1* expression during lung development and in isolated AEC of adult mice

5.1.1. *Flk-1* mRNA expression during lung development

To characterize *flk-1* mRNA expression during lung development, we analyzed embryonic mouse lungs of C57bl 6 mice at ED12.5 to ED18.5 as well as postnatal days 1 (P1) and 14 (P14) by radioactive [³⁵S]α-UTP-labeled mRNA *in situ* hybridization. On ED12.5, *flk-1* expression was restricted to the pulmonary vascular network (Figure 15, black and white arrowheads). From ED16.5 onwards *flk-1* mRNA was detected in the epithelial cells of the branching epithelial tubes (Figure 15, black and white arrows), persisted in the primordial bronchi at ED17.5 and spread to the emerging gas exchange regions at ED18.5. At postnatal days 1 and 14, *flk-1* expression was maintained in the bronchial epithelium, while in the alveolar septa expression was increasingly restricted to individual cells (Figure 15, black and white arrows). Along with maturation of the vascular system, endothelial *flk-1* mRNA expression regressed over time (Figure 15, ED 17.5-P14).

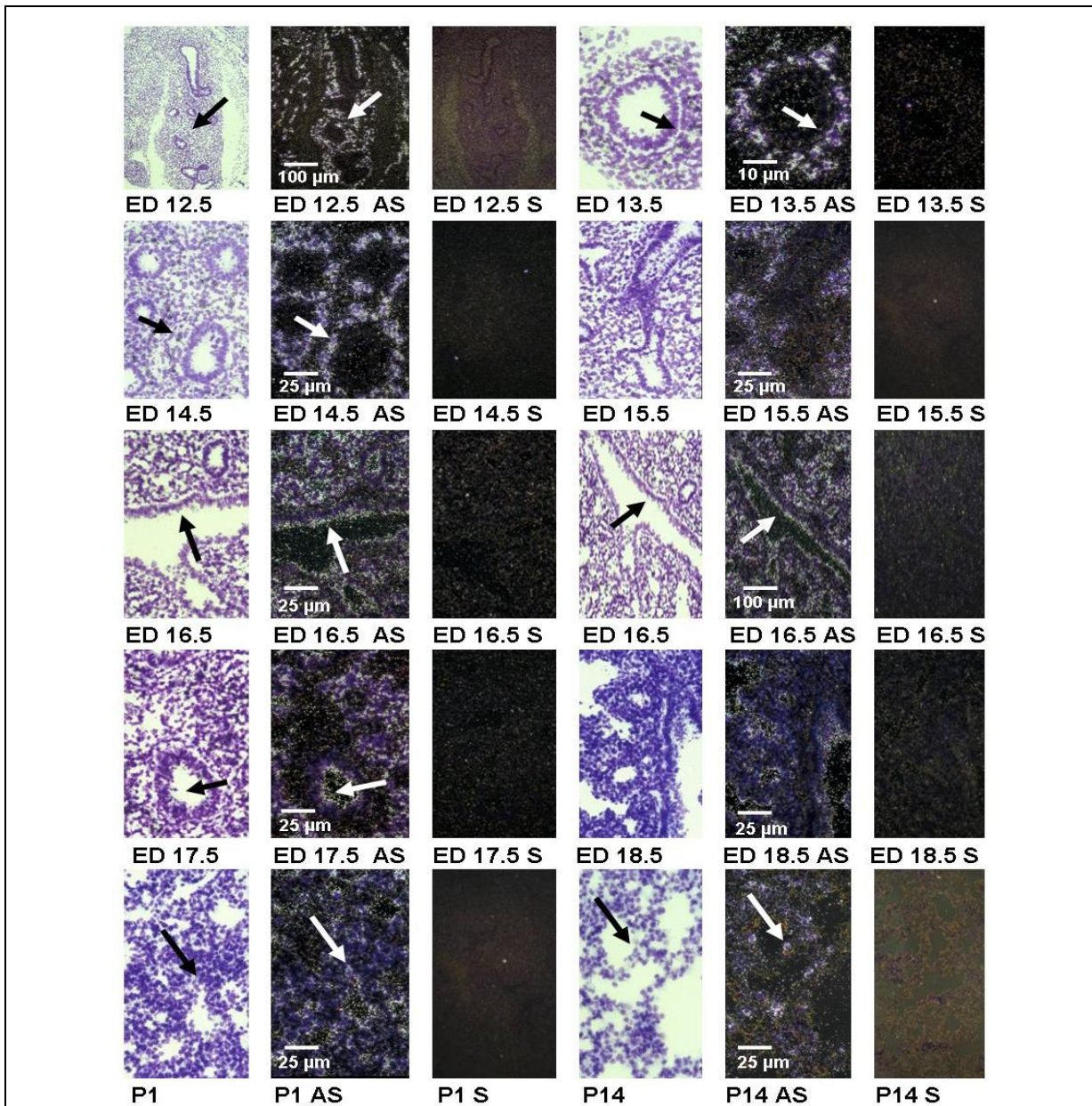


Figure 15: Spatiotemporal *flk-1* mRNA expression during lung development

Radioactive [^{35}S] α -UTP-labeled mRNA *in situ* hybridization for *flk-1* mRNA, on C57bl/6 mouse embryonic and postnatal tissues. Sections are shown in light field, with blue nuclear staining and dark field for mRNA probe signal for antisense (AS) and sense (S) probes from ED12.5 until P1 and P14. *Flk-1* mRNA is expressed from ED12.5 throughout ED16.5 in the vascular network (depicted by white and black arrowhead, white spots of AS probe). At ED16.5, *flk-1* expression was present in the epithelial cells of bronchial tubes (indicated by white and black arrows). The epithelial *flk-1* expression persisted until later stages and was restricted to single cells in the alveolar regions of postnatal day. (ED = embryonic day; P = postnatal day).

5.1.2. Flk-1 protein expression during lung development

The localization of flk-1 protein expression was assessed by immunohistochemistry and confocal laser scanning microscopy. Embryonic mouse lungs of C57bl 6 mice at ED14.5 and ED18.5 as well as adult mouse lung tissue (12 weeks of age) were investigated. Cellular phenotyping for specific endothelial and epithelial cell protein expression in combination with flk-1 detection confirmed the flk-1 mRNA expression pattern derived from the *in situ* hybridization experiments. During the early embryonic stages (ED14.5 to ED16.5) flk-1 protein expression was exclusively co-localized with the endothelial marker CD 31 in the vascular networks (Figure 16, A), whereas no flk-1 immunoreactivity could be detected in cytokeratin-positive epithelial cells (Figure 16, D-F). From ED17.5 onwards, co-expression of flk-1 and cytokeratin was localized to the bronchial tubules and primordial gas exchange regions (Figure 16, G-I). In adult lung tissue, flk-1 was predominantly expressed in airway epithelial cells (Figure 16, J-L). Staining with respective IgG controls did not show any immunoreactivity.

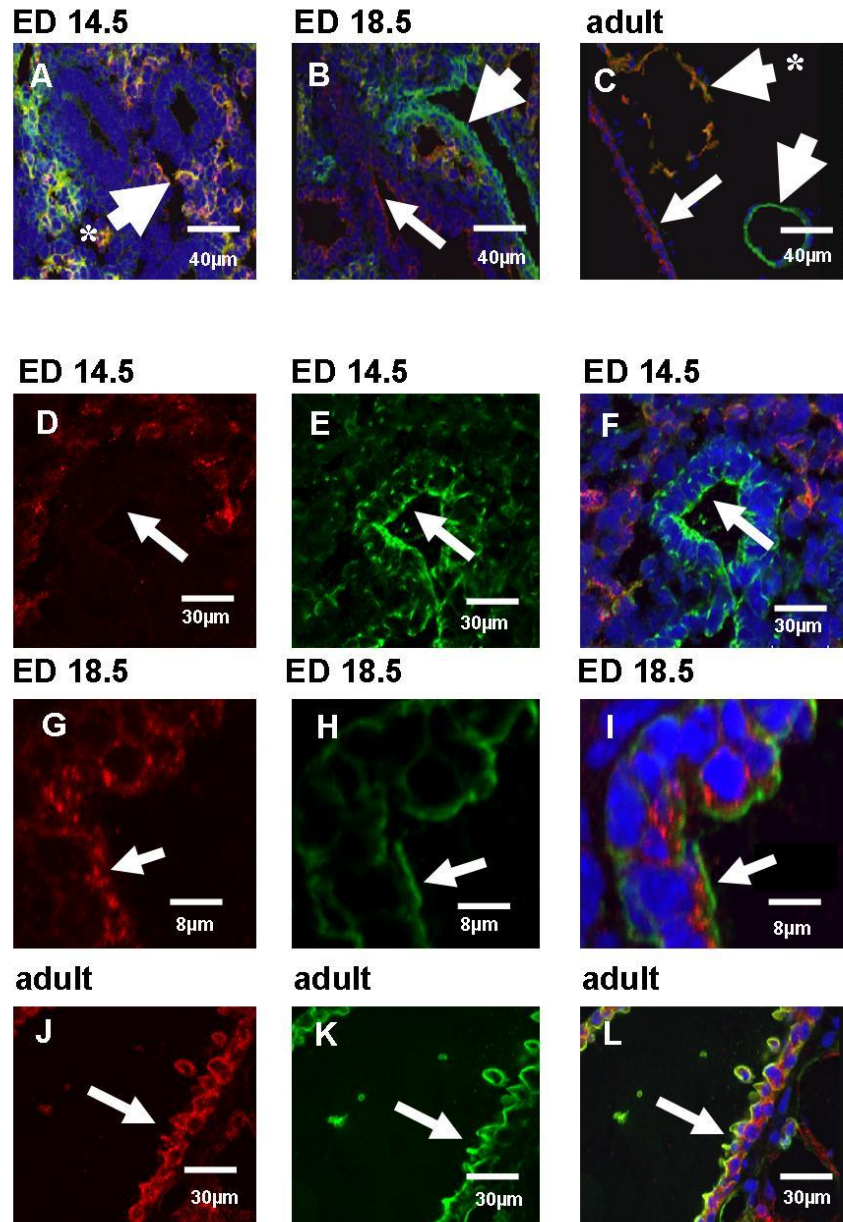


Figure 16: Epithelial and endothelial flk-1 protein expression in lung development

Confocal laser scanning microscopy of immunoreactivity for flk-1 (red) on C57bl 6 mouse embryonic (ED14.5, ED18.5) and adult tissue in combination with CD 31 (green; A, B, C), cytokeratin (green; E, F, H, I, K, L), nuclear staining with Topro-3 (blue) (A-C, F, I, L). Flk-1 co-localized with CD31 at ED14.5 in the developing vascular network (yellow, white arrowhead with asterisk in A, but not with cytokeratin in the early bronchial tube (white arrow in D-F). At ED18.5 and in the adult mouse lung, flk-1 was coexpressed with cytokeratin in the bronchial tubes (white arrow in G-L) but not with CD 31 in the larger vessels (arrowhead in B and C), whereas co-localization of flk-1 and CD 31 persisted in the capillaries in adult lung tissue (arrowhead with asterisk in C).

5.1.3. Flk-1 expression and receptor phosphorylation in isolated AEC

To elucidate the functional role of flk-1 expression, we isolated AEC from adult C57bl 6 mice. Endothelial cell contamination of the AEC culture was excluded by absence of cells immunoreactive for CD 31 (a specific endothelial cell marker) and revealed a purity of >95% (data not shown). Immunocytochemistry of AEC, 48 and 72 h post isolation, revealed co-expression of flk-1 with the epithelial cell markers proSPC and cytokeratin (Figure 17 A-F). Immunoreactivity of respective IgG controls was not detectable. Immunoprecipitation of flk-1 from isolated AEC lysates followed by western blot analysis for flk-1 further confirmed VEGFR-2 expression in these cells (Figure 17 G). To investigate the functional activity of flk-1 in AEC, isolated AEC were treated with 100 ng/ml VEGF-A. Immunoprecipitation of flk-1 from cell lysates and western blot analysis for phosphotyrosine showed a clear increase of the phosphorylation state of the receptor compared to the non stimulated control (Figure 17 H).

5.1.4. Freshly isolated alveolar epithelial cells constitutively secrete VEGF-A

To examine whether AEC also secrete VEGF-A, supernatants were collected from isolated AEC after 72 h of culture and subjected to VEGF-A ELISA. The VEGF-A concentration in the supernatant was 128.3 ± 5.6 pg/ml (Figure 17 I).

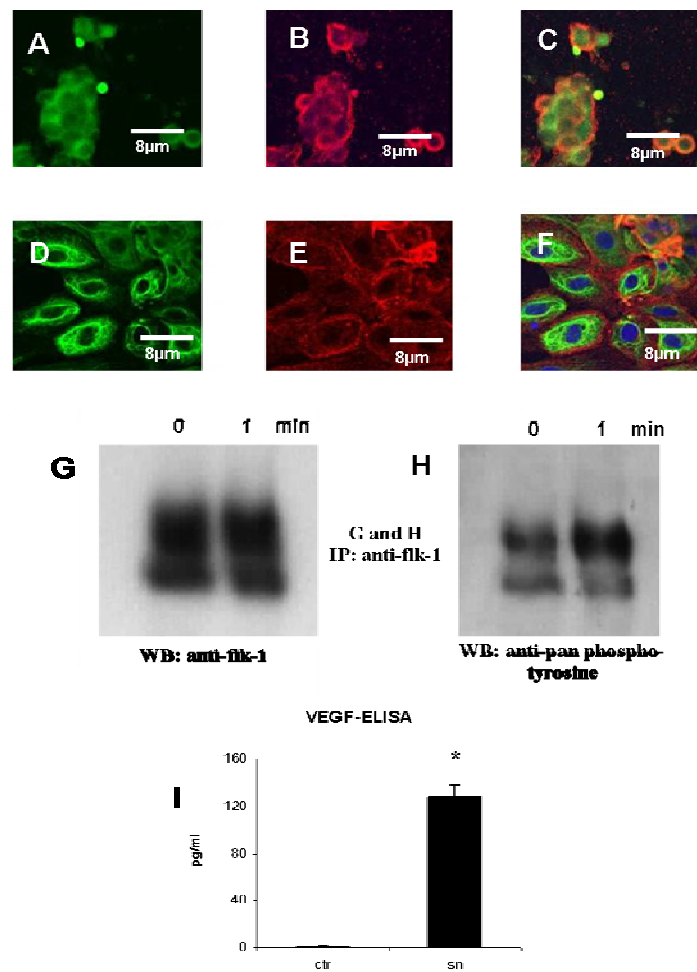


Figure 17: Flk-1 protein expression in AEC

A-F: Immunofluorescent staining of isolated alveolar epithelial cells from C57bl 6 mice at 48 (A-C) and 72 h (D-F) after isolation. Immunoreactivity is shown for flk-1 (red: B, C, E, F), prosurfactant protein C (proSPC, green; A, C) and cytokeratin (green; D, F). Flk-1 was present in proSPC- and cytokeratin-immunoreactive cells (overlays in C and F). G-I: Flk-1 expression and receptor phosphorylation in isolated alveolar epithelial cells (AEC). Immunoprecipitation of flk-1 protein from lysates of murine alveolar epithelial cells 72 h post isolation. Western blot of the precipitate for flk-1 shows the presence of flk-1 in AEC (G). Blotting the precipitate for phosphotyrosine with and without prior VEGF application (100 ng/ml VEGF-A for 1 min) revealed constitutive flk-1 receptor phosphorylation that was significantly enhanced by VEGF stimulation (H). The constitutive secretion of VEGF-A by AEC was shown by ELISA for murine VEGF-A from supernatants (sn) of isolated alveolar epithelial cells and unconditioned media (ctr) as control after 72 h of incubation (I). *, $p < 0.01$ (N=3).

5.2. HIF 2 α expression in isolated adult AEC

5.2.1. HIF 2 α expression in isolated AEC

To investigate the role of the HIF 2 α flk-1 system in AEC, we primarily wanted to examine HIF 2 α expression in these cells. Accordingly, we isolated AEC and examined HIF 2 α expression at the protein level. Immunocytological staining with a rabbit anti-HIF 2 α antibody and secondary Alexa 488®-labeled anti-rabbit antibody yielded a strong green signal in these isolated AEC (Figure 18 A and C). Staining with rabbit IgG control antibody and a secondary Alexa 488®-labeled anti-rabbit antibody did not give any signal (Figure 18 B and D).

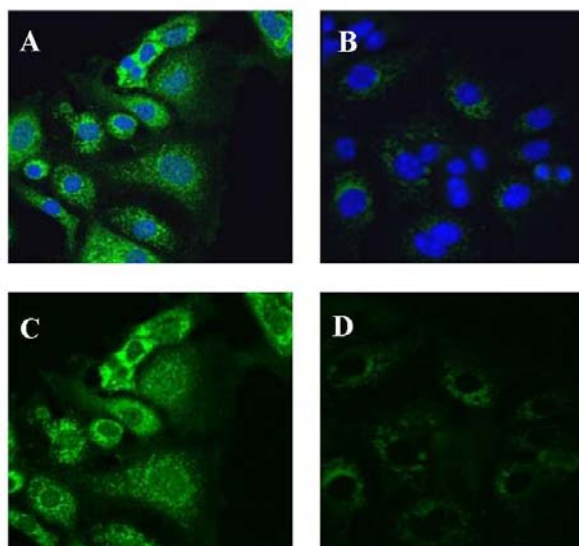


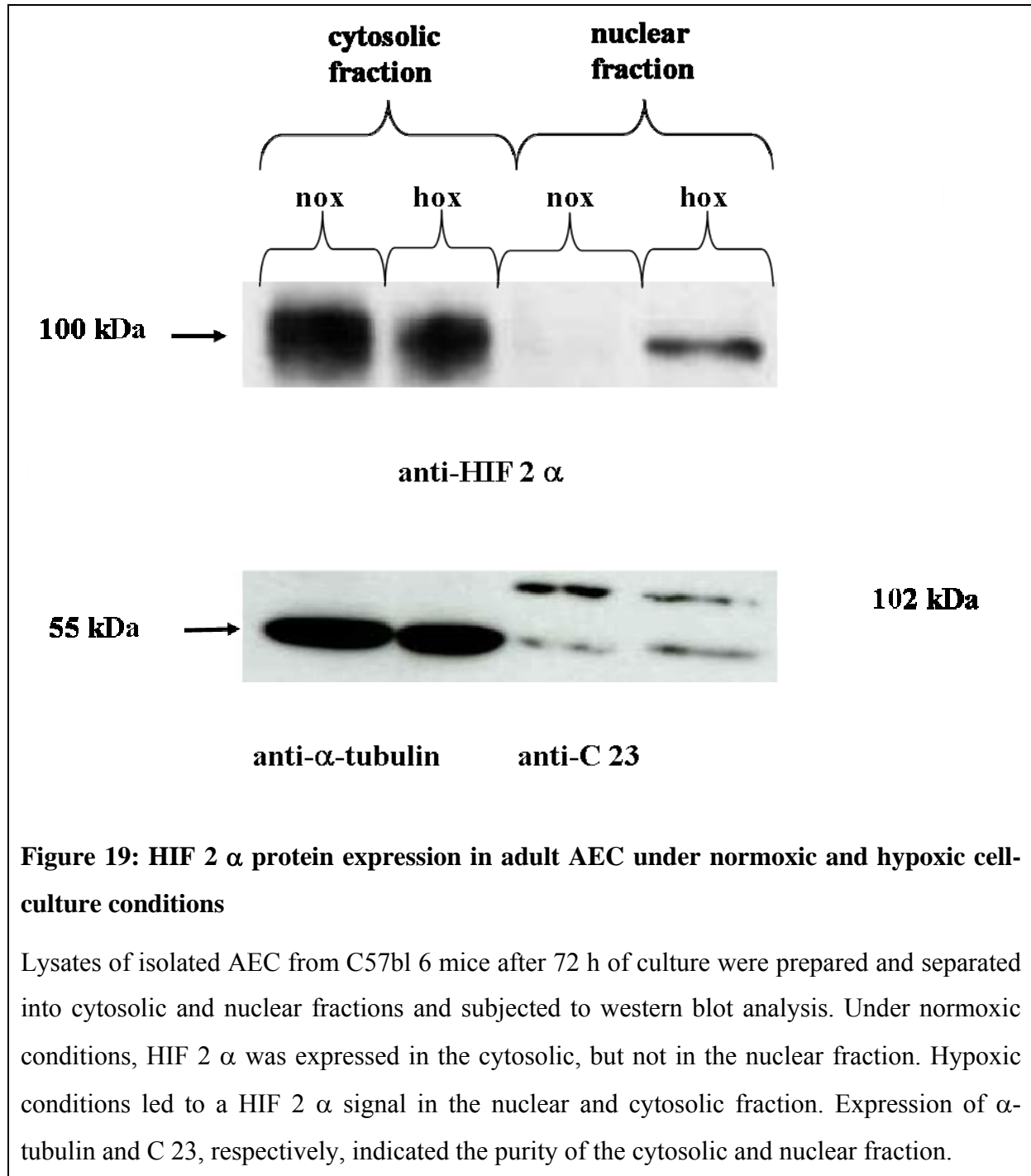
Figure 18: HIF 2 α protein expression in isolated AEC

Immunofluorescent staining of isolated alveolar epithelial cells from C57bl 6 mice 72 h (A-D) after isolation. Immunoreactivity is shown for anti-HIF 2 α (green, A, C) and rabbit IgG (green; B,D). HIF 2 α was present in these isolated AEC (A and C). Rabbit IgG did not give any signal (B, D). Topro-3 was used for nuclear staining (blue).

5.2.2. HIF 2 α protein expression in adult AEC and intracellular distribution under normoxic and hypoxic conditions

To confirm the expression of HIF 2 α in isolated AEC as shown by immunocytochemistry, lysates of AEC cultured under normoxic and hypoxic conditions were subjected to western blot analysis for HIF 2 α . Lysates were prepared and separated for nuclear and cytosolic fractions. Under normoxic conditions, HIF 2 α was highly expressed in the cytosolic fraction,

but not in the nuclear fraction (Figure 19). Hypoxic culture conditions led to an increase in HIF 2 α signal in the nuclear fraction with stable expression in the cytosolic fraction. α tubulin and C 23 expression, respectively, represented the purity of nuclear and cytosolic fractions (Figure 19).



5.3. Generation of an in vivo inducible pulmonary epithelial HIF 2 α knock-out mice

5.3.1. Genotyping of triple transgenic SPCrtTA-tetO-Cre-HIF 2 α loxP mice

To generate an inducible pulmonary epithelial knock-out of HIF 2 α , we generated a triple transgenic line combining SPCrtTA-tetO-Cre mice and HIF 2 α loxP mice. Homozygous HIF 2 α loxP mice were bred with SPCrtTA-tetO-Cre mice. The offspring were genotyped for all three transgenes (Figure 20 A-C). Triple transgenic heterozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice were intercrossed to generate homozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice. Offspring were genotyped (Figure 20 D-E) to choose homozygous HIF 2 α loxP SPCrtTA-tetO-Cre for further mating procedures with induction of Cre recombinase expression.

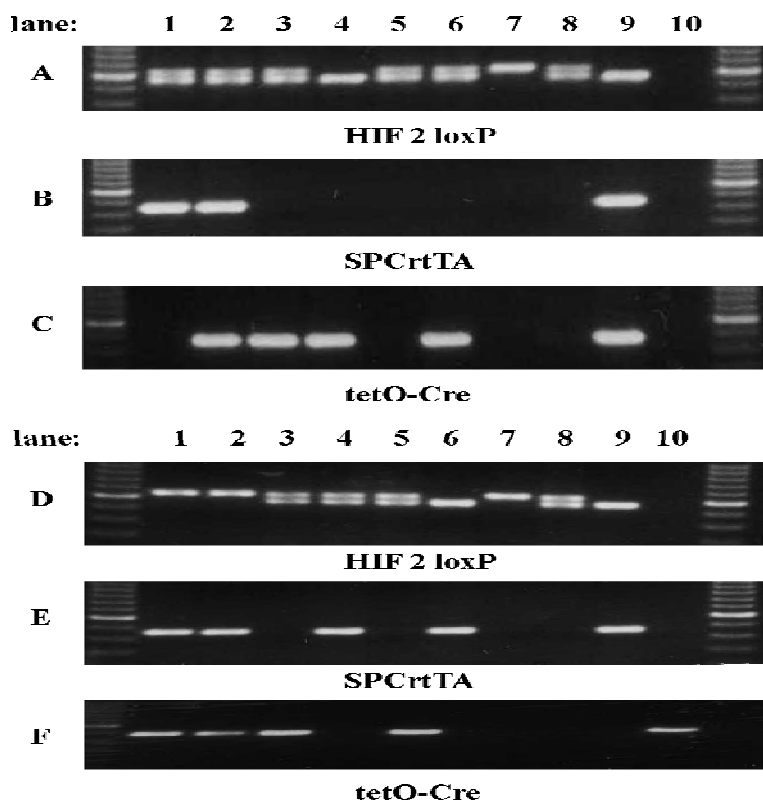


Figure 20: Generation of triple transgenic homozygous HIF 2 α SPCrtTA-tetO-Cre mice

A-C: Homozygous HIF 2 α loxP mice were crossed with SPCrtTA-tetO-Cre mice to generate heterozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice. The offspring were genotyped for all three genes: A: PCR of the HIF 2 α loxP locus, showing heterozygous mice (lanes 1-3, 5-6) and positive control of homozygous (lane 7) heterozygous (lane 8) and wild-type mice (lane 9) and negative control (lane 10). B: PCR for the SPCrtTA locus showing positive (lanes 1-2), negative mice (lanes 3-8), negative control and positive control (lane 9-10). C: PCR for the tetO-Cre locus showing positive (lanes 2-4, 6) negative (lane 1, 5, 7-8), negative and positive control (lane 9-10). D-F: Heterozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice (A-C lane 2) were intercrossed to generate homozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice. The offspring were genotyped for all three transgenes: D: PCR for the HIF 2 α loxP locus showing homozygous (lanes 1-2) and heterozygous (lanes 3-5) mice. Accordingly, positive control of homozygous (lane 7) heterozygous (lane 8) and wild-type mice (lane 9) and negative control (lane 10) were utilized as controls. E: PCR for the SPCrtTA locus showing positive (lanes 1-2, 4, 6) negative mice (lanes 3, 5, 7, 8), negative control and positive control (lanes 9-10). F: PCR for the tetO-Cre locus showing positive lane 1-3, 5 negative lane 4, 6-8, negative and positive control lane 9-10.

5.3.2. Generation of pulmonary epithelial HIF 2 α knock-out mice

To generate pulmonary epithelial HIF 2 α knock-out mice, homozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice were intercrossed. Cre recombinase expression was induced from embryonic day 0 (ED0) by feeding the breeding pair with dox. The offspring were genotyped for all transgenes (Figure 21 A-C). Homozygous HIF 2 α loxP SPCrtTA but tetO-Cre negative mice were utilized as control mice. At the age of 12 weeks, mice were sacrificed and AEC were isolated and cultured for 72 h hours. The DNA was isolated and PCR for the HIF 2 α loxP locus was performed to investigate Cre excision. Homozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice showed the one loxP band (1loxP) according to DNA recombination level and the double loxP band (2loxP) (Figure 21 D lane 1-3). Control mice did not show the one loxP band due to the lack of DNA recombination (Figure 21 D lane 4-5). To confirm the DNA recombination in the AEC of homozygous SPCrtTA-tetO-Cre mice, we isolated DNA and performed Southern blot analysis. Homozygous SPCrtTA-tetO-Cre mice showed the one loxP and double loxP band in the Southern blot analysis (Figure 21 E lane 2). Control mice exclusively showed the double loxP band (Figure 21 E lane 1).

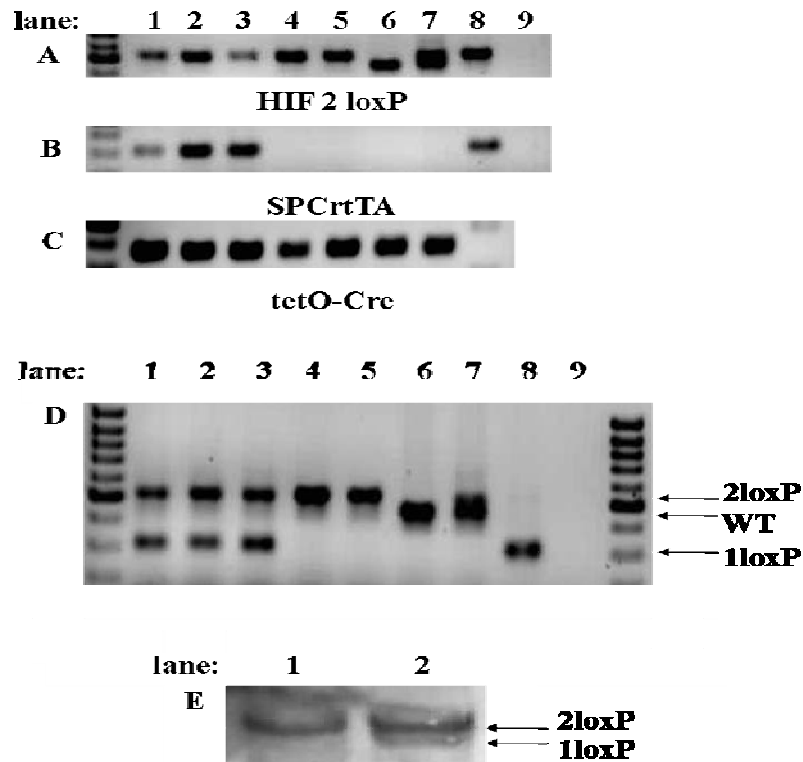


Figure 21: Generation of pulmonary epithelial HIF 2 α knock-out mice

A-C: Homozygous HIF 2 α loxP mice were intercrossed, and the breeding pairs were fed with dox to induce Cre expression from ED0. The offspring were genotyped for all three genes: A: PCR of the HIF 2 α loxP locus, showing homozygous mice (lanes 1-5) and positive controls: homozygous (lane 8) heterozygous (lane 7) and wild-type mice (lane 6) and negative control (lane 9). B: PCR for the SPCrtTA locus showing positive (lanes 1-3), negative (lanes 4-7), negative control and positive control (lanes 8, 9). C: PCR for the tetO-Cre locus showing positive (lanes 1-6), positive and negative control (lanes 7, 8). D, E: To investigate DNA recombination due to Cre expression, AEC from homozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice and control homozygous HIF 2 α SPCrtTA-tetO-Cre mice were isolated and cultured for 72 h in dox-containing media. DNA was isolated and PCR for the HIF 2 α loxP locus was performed. D: Homozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice showed the 1loxP band according to the DNA recombination (lanes 1-3) and the 2loxP band (lanes 1-3). Control mice did exclusively show the 2loxP band (lanes 4-5), positive and negative controls (lanes 6-9). E: To confirm this, Cre-induced DNA recombination, Southern blot analysis was utilized: Homozygous HIF 2 α loxP SPCrtTA-tetO-Cre mice showed the 1loxP band and the 2loxP band (lane 2), whereas control mice exclusively show the 2loxP band (lane 1).

5.3.3. Confirmation of HIF 2 α deletion at the protein level in pulmonary epithelial HIF 2 α knock-out mice

To confirm the pulmonary epithelial deletion of HIF 2 α in homozygous HIF 2 α 1loxP SPCrtTA-tetO-Cre mice AEC of adult homozygous HIF 2 α 1loxP SPCrtTA-tetO-Cre mice were isolated and cultured for 72 h in dox-containing media. Protein was isolated and a western blot for HIF 2 α was performed. Homozygous HIF 2 α 2loxP SPC-rtTA mice were chosen as control mice, which showed a strong signal for HIF 2 α at 100 kDa (Figure 22). Epithelial HIF 2 α knock-out mice did not give a signal for HIF 2 α compared to α -tubulin and to control mice (Figure 22).

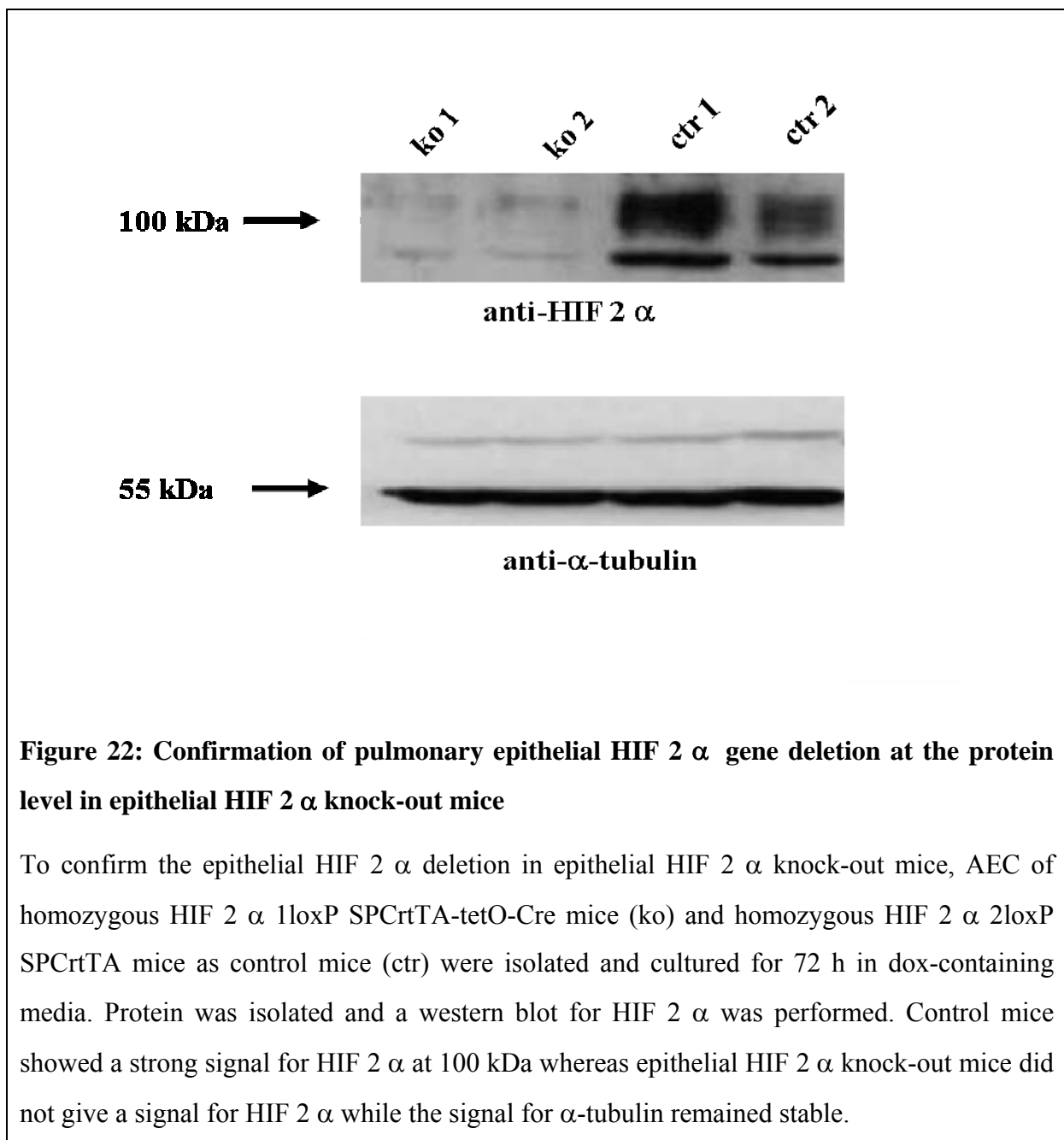


Figure 22: Confirmation of pulmonary epithelial HIF 2 α gene deletion at the protein level in epithelial HIF 2 α knock-out mice

To confirm the epithelial HIF 2 α deletion in epithelial HIF 2 α knock-out mice, AEC of homozygous HIF 2 α 1loxP SPCrtTA-tetO-Cre mice (ko) and homozygous HIF 2 α 2loxP SPCrtTA mice as control mice (ctr) were isolated and cultured for 72 h in dox-containing media. Protein was isolated and a western blot for HIF 2 α was performed. Control mice showed a strong signal for HIF 2 α at 100 kDa whereas epithelial HIF 2 α knock-out mice did not give a signal for HIF 2 α while the signal for α -tubulin remained stable.

5.4. Phenotype of pulmonary epithelial HIF 2 α knock-out mice

Induction of the deletion from the first day of embryonic development initially did not give rise to any specific phenotype. Pulmonary epithelial HIF 2 α knock-out mice were born at term and breathed normally, without any signs of cyanosis. To further analyze the effects of the pulmonary epithelial HIF 2 α deletion, AEC were further analyzed for differences in protein expression of flk-1 and SPC respectively. Furthermore, lung morphometric analysis and electron microscopy of epithelial HIF 2 α knock-out lungs compared to control lungs was performed.

5.4.1. Epithelial flk-1 expression in pulmonary epithelial HIF 2 α knock-out mice

To elucidate the impact of pulmonary epithelial HIF 2 α knock-out on flk-1 expression, AEC from adult homozygous HIF 2 α 1loxP SPC rtTA-tetO-Cre mice and homozygous HIF 2 α 2loxP SPCrtTA mice (as control mice) were isolated and cultured in dox-containing media. Protein was isolated and a western blot for flk-1 was performed. Control mice showed a strong signal for flk-1 at 200 kDa. In contrast, pulmonary epithelial HIF 2 α knock-out mice did show a strong reduction of flk-1 signal, while the signal for α -tubulin remained unchanged (Figure 23).

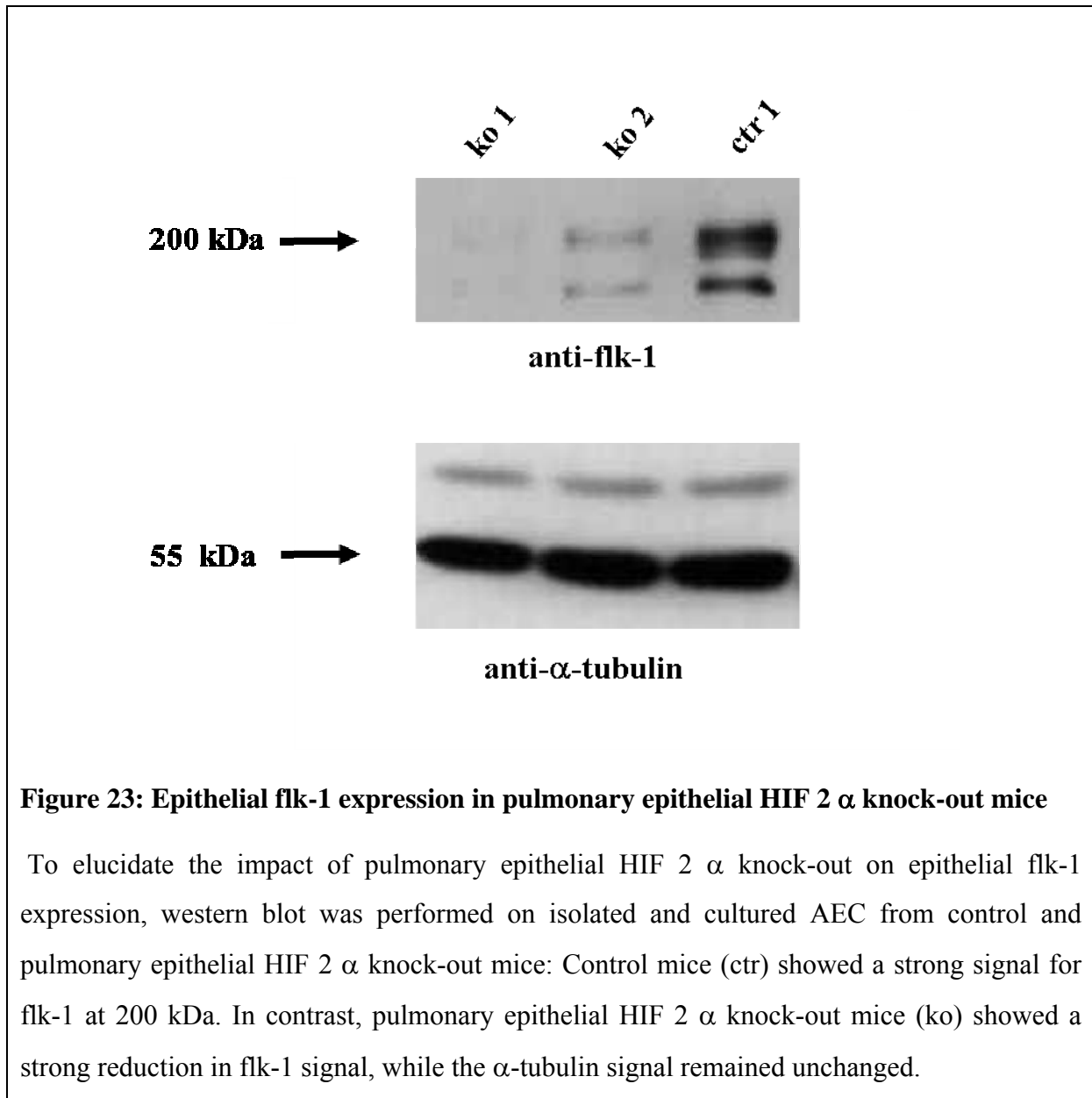
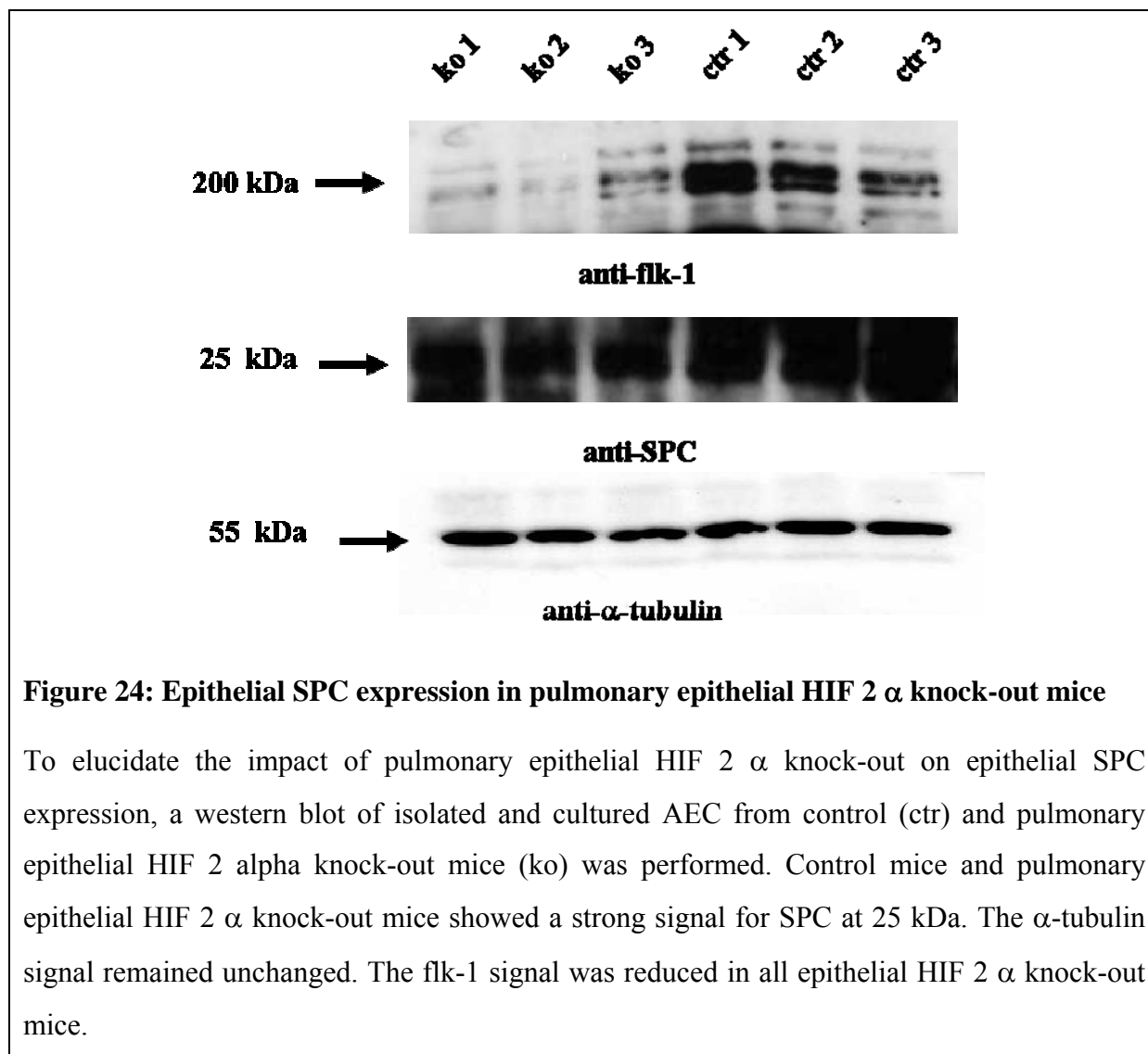


Figure 23: Epithelial flk-1 expression in pulmonary epithelial HIF 2 α knock-out mice

To elucidate the impact of pulmonary epithelial HIF 2 α knock-out on epithelial flk-1 expression, western blot was performed on isolated and cultured AEC from control and pulmonary epithelial HIF 2 α knock-out mice: Control mice (ctr) showed a strong signal for flk-1 at 200 kDa. In contrast, pulmonary epithelial HIF 2 α knock-out mice (ko) showed a strong reduction in flk-1 signal, while the α -tubulin signal remained unchanged.

5.4.2. Epithelial SPC expression in pulmonary epithelial HIF 2 α knock-out mice

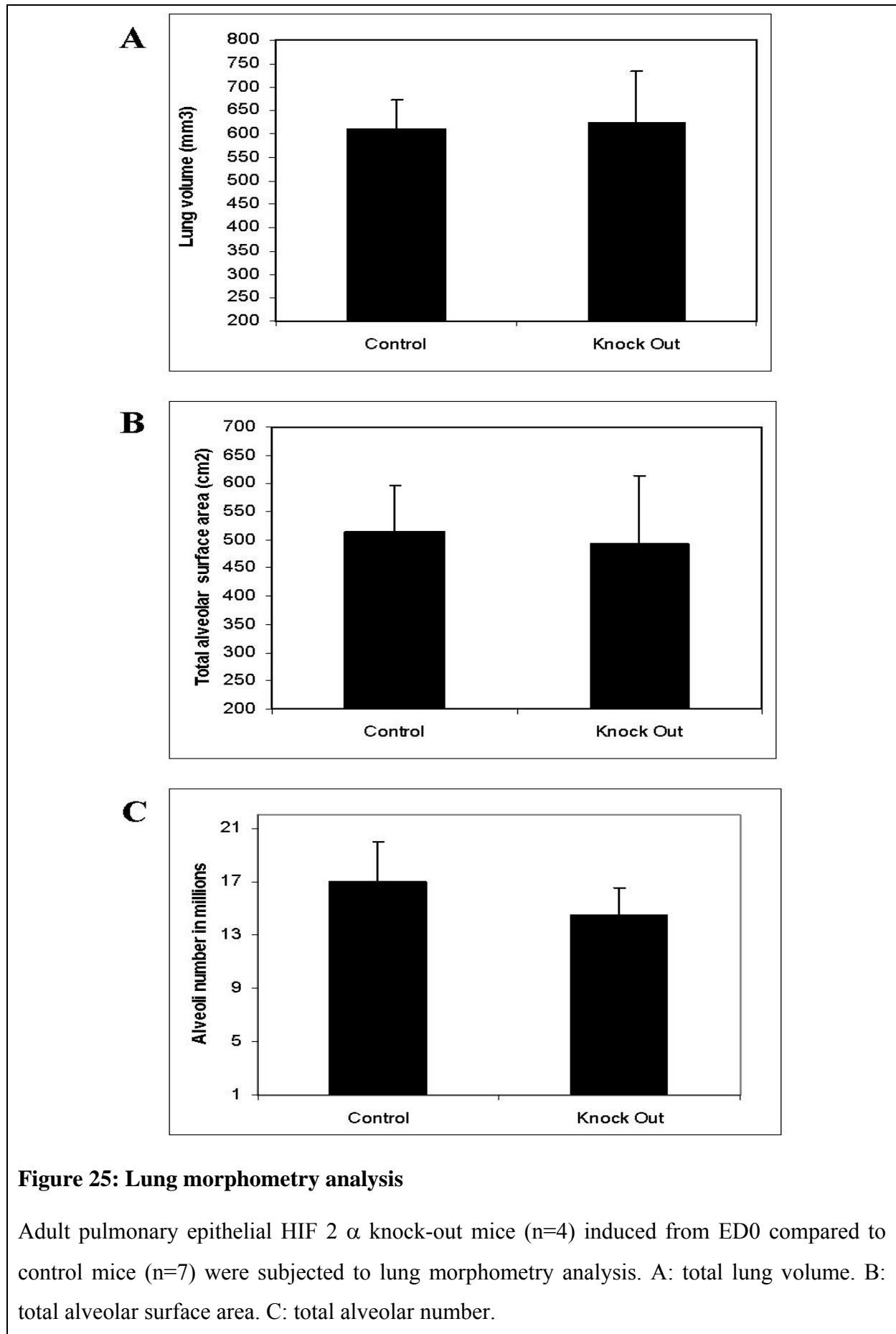
To elucidate the influence of pulmonary epithelial HIF 2 α knock-out on SPC expression, AEC from adult homozygous HIF 2 α 1loxP SPC rtTA-tetO-Cre mice and homozygous HIF 2 α 2loxP SPC rtTA mice as (control mice) were isolated and cultured in dox-containing media. Protein was isolated and a western blot for SPC was performed. Control mice and epithelial HIF 2 α knock-out mice showed a strong signal for SPC at 25 kDa. The signal for α -tubulin remained unchanged (Figure 24).



5.4.3. Lung morphometry analysis in adult pulmonary epithelial HIF 2 α knock-out mice induced from ED0 compared to control mice

Lung morphometry analysis were kindly performed by Dipl. Ing. Msc. Biol. Sandeep Nikam.

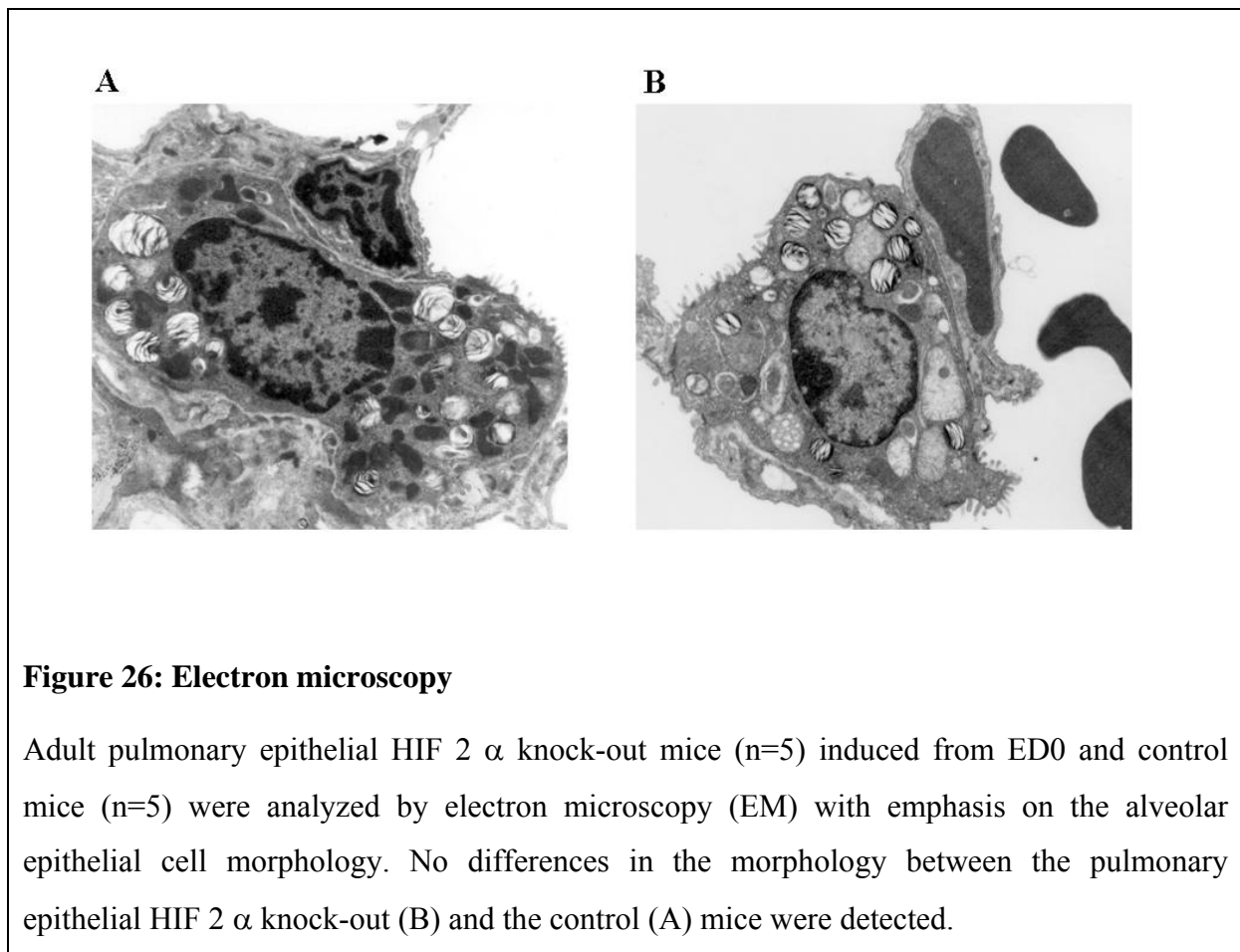
To investigate the possible impact of the pulmonary epithelial HIF 2 α deletion on lung morphology, lungs of the pulmonary epithelial HIF 2 α knock-out mice and control mice were subjected to lung morphometry analysis. Analysis of total lung volume, alveolar surface area and the number of alveoli revealed no differences between pulmonary epithelial HIF 2 α knock-out and control mice (Figure 25 A-C).



5.4.4. Electron microscopy of AEC of adult pulmonary epithelial HIF 2 α knock-out mice induced from ED0 compared to control mice

Analysis by electron microscopy was kindly performed by Prof. Dr. Wolfgang Kummer, Institute for Anatomy and Cell Biology, University of Giessen, Germany.

Lungs of pulmonary epithelial HIF 2 α knock-out mice were subjected to electron microscopy to analyze alveolar type two cell morphology. Control mice (n=5) showed a regular alveolar type two cell structure with regular type two cell features (Figure 26 A). The cell organelles, such as lamellar bodies and mitochondria, showed a normal physiological structure. The same characteristic features were observed in alveolar type two cells of pulmonary epithelial HIF 2 α knock-out mice (Figure 26 B). No changes could be observed upon HIF 2 α deletion.



6. Discussion

In the present study we investigated the possible role of the pulmonary epithelial HIF 2 α flk-1 system during lung development. In the first part we characterized flk-1 expression at daily intervals from ED12.5 throughout lung development until postnatal stages, and in isolated alveolar epithelial cells from adult mice. As a result we demonstrated widespread epithelial flk-1 expression in mice commencing around ED17.5 and persisting postnatally (Ahlbrecht et al., 2008). To our knowledge, this is the first murine study characterizing epithelial flk-1 expression at daily intervals throughout lung organogenesis, until birth, and at later postnatal stages. In comparison with the early endothelial flk-1 expression which has been described previously (Flamme et al., 1995), the epithelial expression appears in the later stages of lung development. Since it has been shown that flk-1 is highly regulated by HIF 2 α (Elvert et al., 2003) we looked at HIF 2 α expression in AEC. We found strong expression at the protein level in isolated alveolar epithelial cells (Figure 18 and Figure 19). Accordingly, we hypothesized that pulmonary epithelial HIF 2 α knock-out would lead to a severe phenotype by a consequent reduction in flk-1 expression. Thus, in the second part of our study, we generated inducible pulmonary epithelial-specific HIF 2 α knock-out mice to elucidate epithelial HIF 2 α function. Induction of the deletion from the first day of embryonic development did not yield any specific phenotype. Pulmonary epithelial HIF 2 α knock-out mice were born at term, and were breathed normally. This was unexpected, because Compernelle et al reported a severe respiratory distress syndrome upon homozygous HIF 2 α deletion (Compernelle et al., 2002). Analysis of lung morphometry revealed no change in lung structure upon pulmonary epithelial HIF 2 α deletion, even though flk-1 expression in isolated alveolar epithelial cells was highly downregulated.

6.1. Flk-1 expression during lung development

The development of the vasculature is a key process in lung development. Angiogenesis and vasculogenesis are detected at early stages of lung development (Gebb and Shannon, 2000), and, VEGF-A has been shown to be a central growth factor regulating physiological and pathological vascular development (Carmeliet, 2005; Mustonen and Alitalo, 1995; Risau and Flamme, 1995). Accordingly, flk-1 has been shown to be expressed in the early primitive vascular network as a key receptor mediating these angiogenic signals (Flamme et al., 1995; Yamaguchi et al., 1993). In the present study, we characterized flk-1 expression from ED12.5 throughout lung development at daily intervals and at postnatal stages. We found flk-1

expression throughout the early stages restricted to the early endothelial system (Figure 15). This is in line with the previous reported *flk-1* expression pattern (Flamme et al., 1995; Yamaguchi et al., 1993). Furthermore, our data demonstrate epithelial *flk-1* expression commencing around ED16.5 (Figure 15). Interestingly, this epithelial *flk-1* expression appears around the transition from the pseudoglandular to the canalicular stage. This period has been described as marked by the birth of the acinus (Burri in: McDonald, 1997 p.4-6). Furthermore, epithelial cells which are loaded with glycogen, which is the key substance required for further cell differentiation (Burri in: McDonald, 1997 p. 4-6), appear at this stage. Thus, epithelial *flk-1* expression as demonstrated by our data might have impact on epithelial differentiation. Some reports show *flk-1* expression in isolated ED19 rat alveolar epithelial type two cells (Raoul et al., 2004) and in epithelial cells of human fetal lung explants (Brown et al., 2001), however, our data are the first murine data characterizing the epithelial *flk-1* expression at daily intervals throughout lung development and until postnatal stages (Ahlbrecht et al., 2008). Due to this epithelial expression, we suggested a further role of *flk-1* during lung development, which is not restricted to the endothelial system. Accordingly, there is recent discussion about the role of *flk-1* in branching morphogenesis. Del Moral et al. have demonstrated increased epithelial branching morphogenesis in whole lung culture in response to VEGF-A treatment at ED11. In contrast, isolated endodermal cells did not respond to VEGF-A treatment, whereas differentiation of isolated *flk-1*-positive cells was increased upon VEGF-A treatment (Del Moral et al., 2006). While this provides evidence for an indirect effect of endothelial *flk-1* on the epithelial system, our data suggest a more direct role of *flk-1* on the epithelial system. Another role of *flk-1* was postulated by Gebb and Shannon, who suggested that due to spatial juxtaposition of mesenchymal *flk-1*-positive and epithelial cell, signals from the epithelium may be directing pulmonary vascularization. Furthermore, they demonstrated *flk-1* mRNA expression postnatally in the alveoli of rats (Gebb and Shannon, 2000). There are further studies focusing on the role of *flk-1* in the crosstalk between the vascular and the epithelial system. Tuyl et al. inhibited vascular development by antisense oligonucleotides directed against VEGF, which lead to reduced epithelial branching (van Tuyl et al., 2005), and Yamamoto et al suggested that primary septa formation is dependent on reciprocal interactions between respiratory epithelium and the underlying vasculature. They selectively inactivated the VEGF gene in the pulmonary epithelial system, which resulted in a complete absence of pulmonary capillaries. This further led to a disruption of primary septa formation (Yamamoto et al., 2007). Similar effects were described by Zhao et al., using a soluble receptor in lung renal capsule graft, they inhibited VEGF-A activity which led to

inhibition of vascular development with secondary alterations to epithelial proliferation and sacculation (Zhao et al., 2005). There are further studies focusing on the role of VEGF in lung development (Akeson et al., 2005; Akeson et al., 2003; Le Cras et al., 2004; Zeng et al., 1998). However, the majority of these studies point out the importance of epithelial VEGF and endothelial flk-1 for branching morphogenesis and septa formation, but do not focus on the direct epithelial role of flk-1 which is suggested by our data. In contrast, investigations by Raoul et al. revealed an increase in surfactant protein B transcripts in isolated rat fetal alveolar epithelial cells in response to VEGF-A stimulation, and demonstrated epithelial flk-1 expression in these cells (Raoul et al., 2004). This supports our hypothesis of a unique epithelial function of flk-1. Furthermore, Brown et al. showed immunoreactivity for flk-1 in the distal epithelial cells of human fetal lung explants, and an increase in SPA and SPC expression in these cells upon VEGF-A treatment (Brown et al., 2001). Thus, this is another hint supporting our hypothesis of epithelial flk-1 function. To further follow this hypothesis, we analyzed isolated adult alveolar epithelial cells for flk-1 expression.

6.2. Flk-1 expression and VEGF-A secretion in isolated AEC of adult C57bl 6 mice

To further investigate our hypothesis of a unique role of epithelial flk-1 expression we isolated adult alveolar epithelial cells of C57bl 6 mice. As a result, we found strong immunoreactivity for flk-1 in the immunocytology. This expression could be confirmed by western blot analysis. These results are in line with findings by Raoul et al. and Brown et al., where both groups showed immunoreactivity for flk-1 in isolated fetal rat alveolar epithelial cells and human fetal lung explants (Brown et al., 2001; Raoul et al., 2004). But these studies did not confirm flk-1 expression by western blot analysis. To further prove regulation integrity of epithelial flk-1 expression, we analyzed flk-1 activation by VEGF-A treatment of these cells. VEGF-A treatment resulted in an increase of flk-1 phosphorylation 1 min after stimulation. Taken together, we demonstrated flk-1 expression and regulation which has not been demonstrated for the pulmonary epithelial system before. Since flk-1 could be activated in these cells, we suggested an autocrine loop of the VEGF-A flk-1 system in these cells. Thus, we investigated VEGF-A secretion of these isolated AEC. We found significantly higher VEGF-A concentrations in the supernatant of these cells, compared to unconditioned media as a control. Since we characterized an intact VEGF-A/flk-1 ligand receptor system in the murine alveolar epithelial system, we hypothesized a functional impact of this system on lung development. Data by Compennolle et al. support this hypothesis, where the development

of a severe respiratory distress syndrome upon HIF 2 α deletion was demonstrated, which could be prevented by VEGF treatment. Furthermore, they demonstrated that intrauterine delivery of anti flk-1 antibodies caused respiratory distress syndrome (Compernelle et al., 2002). Since flk-1 has been shown to be regulated by HIF 2 α (Elvert et al., 2003) the severe phenotype of these HIF 2 α knock-out mice might be caused by reduction of flk-1. In deed in the study of Compernelle et al. the respiratory distress syndrome was accompanied by severe epithelial dysfunction. Thus, this is a further hint supporting epithelial function of flk-1. To analyze this, we investigated HIF 2 α expression in isolated AEC as a key regulator of the VEGF-A flk-1 system.

6.3. HIF 2 α expression in isolated AEC of C57bl 6 mice

Due to our data on epithelial flk-1 expression, we hypothesized that the pulmonary phenotype of global HIF 2 α knock-out mice (Compernelle et al., 2002) was strongly influenced by the loss of the epithelial HIF 2 α flk-1 system. Supporting this hypothesis, Ema et al. showed epithelial Hif 2 alpha expression (Ema et al., 1997). Furthermore Wagner et al. 2004 showed Hif 2 α expression in the canalicular phase in the bronchial epithelium and in septal cells (Wagner et al., 2004). Accordingly, we analyzed isolated AEC of C57bl 6 mice for HIF 2 α expression. We found strong immunoreactivity for HIF 2 α in these isolated cells. Investigations on hypoxic regulation of HIF 2 α showed that Hif 2 α was expressed in the AEC and nuclear translocation was induced by hypoxia as described for hypoxia-inducible factors (Semenza et al., 1991). Taken together, we demonstrated alveolar epithelial expression of all three components of the HIF 2 α /flk-1/VEGF system. To elucidate the functional impact of this system on lung development, we generated an *in vivo* inducible epithelial deletion of HIF 2 α using a triple transgenic approach combining the rtTA and the Cre loxP sytem.

6.4. Pulmonary epithelial HIF 2 α knock-out

To investigate the role of the pulmonary epithelial HIF 2 α /flk-1 system we generated an *in vivo* pulmonary epithelial HIF 2 α deletion in a triple transgenic murine approach. The deletion was caused by DNA recombination. Even partial DNA recombination as demonstrated by PCR (Figure 21 D) and Southern blot (Figure 21 E) led to a strong reduction of HIF 2 α at the protein level (Figure 22) in isolated AEC of induced triple transgenic mice compared to control mice. HIF 2 α deletion induced from ED0 on did not cause a specific

disorder or phenotype. This was unexpected, because we hypothesized a key function of the epithelial HIF 2 α flk-1 system during lung development. Thus, we expected a severe disorder in phenotype upon epithelial HIF 2 α deletion. Global HIF 2 α deletion *in vivo* as shown by Compennolle et al. led to a severe respiratory distress syndrome (Compennolle et al., 2002). Since the respiratory distress syndrome was accompanied by severe epithelial defect in that study, we hypothesized that this phenotype was mainly induced by the epithelial HIF 2 α flk-1 system. But our data demonstrated contradictory effects: even during further postnatal development, no developmental disorders could be detected. Taken together, we succeeded in generating an *in vivo* inducible epithelial HIF 2 α deletion using a transgenic approach combining the rtTA and Cre-loxP system. Surprisingly, this pulmonary epithelial HIF 2 α deletion did not impact regular lung development. To further investigate the influence of pulmonary epithelial HIF 2 α deletion we looked at flk-1 and SPC expression in isolated AEC of pulmonary epithelial HIF 2 α knock-out mice. Furthermore we subjected pulmonary epithelial HIF 2 α knock-out lungs to morphometric analysis and electron microscopy.

6.5. Phenotype of pulmonary epithelial HIF 2 α knock-out mice

Triple transgenic mice in which pulmonary epithelial HIF 2 α had been ablated mice did not show a distinct disorder or phenotype at birth. Even further postnatal development was regular, and the animals grew without any obvious defects. This was an unexpected result, because due to the pulmonary phenotype of global HIF 2 α knock-out mice reported by Compennolle et al. (Compennolle et al., 2002), we postulated a key role of the epithelial HIF 2 α flk-1 system in lung development. We demonstrated the epithelial expression of the system, and we succeeded in generating a complete epithelial HIF 2 α deletion. But the expected disorder in pulmonary phenotype was not observed in the epithelial knock-out animals. Even though western blot analysis of the isolated AEC did reveal a consequent reduction of flk-1 protein upon HIF 2 α deletion, the expected changes in epithelial function were absent. In contrast to data presented by Raoul et al. and Brown et al., who showed regulation of surfactant proteins upon VEGF-A treatment in AEC from rats and humans (Brown et al., 2001; Raoul et al., 2004), our data showed no change in SPC expression levels upon HIF 2 α deletion in the isolated AEC isolated from pulmonary epithelial HIF 2 α knock-out mice compared to control mice. These differences might be due to interspecies differences, but this is highly speculative. A further possible explanation might be a strong influence of the different background of the triple transgenic mice. The SPC/Cre mice were raised on a FVBN

background whereas the HIF 2 α loxP mice were raised on a agouti and C57bl 6 background. The influence of the murine background has been discussed previously to be the reason for the observed differences in the different existing HIF 2 α knock-out strains (Scortegagna et al., 2003a). Furthermore, our data on lung morphometry of the pulmonary epithelial HIF 2 α knock-out mice revealed no single parameter changed, compared to control mice. Finally, we analyzed AEC of these epithelial HIF 2 α knock-out mice by electron microscopy. Again, no differences between control and pulmonary epithelial HIF 2 α knock-out mice were observed (Figure 26) in particular the lamellar bodies appeared regular and unaltered. This again is an unexpected result because several reports suggested an impact of the epithelial HIF 2 α /flk-1 system particular on alveolar type two cell function and surfactant production (Brown et al., 2001; Compennolle et al., 2002; Raoul et al., 2004). We hypothesized that compensatory mechanisms might take over the HIF 2 α function. Thus, we initiated transgene induction later at ED17.5 to reduce the time, to limit the possible induction of long term compensatory mechanisms, but this did not have a different effect from that at the induction from ED0 on (data not shown). Taking together, we characterized the epithelial HIF 2 α /flk-1 system in the pulmonary epithelial system. Furthermore we were able to generate a complete pulmonary epithelial HIF 2 α deletion which led to consequent reduction in flk-1 expression. But this epithelial deletion did not lead to a severe epithelial or developmental defects. This suggests that the HIF 2 α /flk-1 system is present in the epithelial system, but its functional impact is supported by further factors which were not addressed in the present study. A further possibility might be related to technical concerns. As demonstrated by PCR and Southern blot, DNA recombination was not completed. However, HIF 2 α and flk-1 protein levels were strongly reduced. It might be possible that the remaining epithelial cells without recombination were sufficient to recover the HIF 2 α and flk-1 deletion. But this suggestion seems to be unlikely because the remaining Hif 2 alpha and flk-1 levels were undetectable, as demonstrated by western blot analysis. Thus, it might be suggested that the development of the epithelial system is such a pivotal process of lung development that different systems are working in parallel to guarantee undisrupted development. Thus, loss of one system can be compensated by another system, which has not been discovered. Furthermore, the differentiation of the epithelial system appears during a later period of lung development than does the differentiation of the early primitive vascular network. Thus, the endothelial deletion of HIF 2 α and flk-1 results in a more severe and lethal phenotype during early development (Duan et al., 2005; Shalaby et al., 1995). It might be postulated that the possibilities of repair

and compensatory mechanisms increase with the age of the developing embryo to protect the growing organism, whereas early defects are more likely to result in severe and lethal phenotype. Thus the described pulmonary phenotype of the global HIF 2 α knock-out mice as demonstrated by Compernelle et al. (Compernelle et al., 2002) appear to be dominated by the vascular loss of the HIF 2 α /flk-1 system.

7. Summary

In the present study, we hypothesized that the HIF 2 α /flk-1 system has a unique role in the pulmonary epithelial system, with further impact on lung development. This hypothesis was based on data by Compennolle et al. showing a severe respiratory distress syndrome upon global HIF 2 α deletion which could be overcome by the application of VEGF (Compennolle et al., 2002). However, key functions of the HIF 2 α /flk-1 system have been demonstrated to be restricted to the vascular system, to regulate physiological and pathological blood vessel formation. In contrast, reports by Brown et al. and Raoul et al. suggest a direct influence of the flk-1/VEGF system on pulmonary epithelial cell function (Brown et al., 2001; Raoul et al., 2004). Since flk-1 has been shown to be regulated by HIF 2 α (Elvert et al., 2003) it represents the functional target molecule of the HIF 2 α /flk-1 system. Accordingly, we characterized flk-1 expression from ED12.5 at daily intervals throughout lung development until postnatal stages. We found flk-1 expression, as previously described, in the early primitive vascular network. At the end of the pseudoglandular stage, at ED16.5 we found widespread epithelial expression of flk-1 which persisted until postnatal stages. Furthermore, we analyzed flk-1 expression and functional integrity in isolated adult (Ahlbrecht et al., 2008). To test our hypothesis of a direct functional impact of the HIF 2 α /flk-1 system on the pulmonary epithelial system, we analyzed isolated AEC for HIF 2 α expression, and found strong expression of HIF 2 α at the protein level. Thus, we demonstrated that the HIF 2 α /flk-1 system is not restricted to the endothelial system, and is present in the pulmonary alveolar epithelial system. To analyze the functional impact of this newly characterized pulmonary epithelial system, we generated an inducible *in vivo* pulmonary epithelial HIF 2 α deletion combining the SPCrtTA and Cre-loxP system. We succeeded in generating the epithelial HIF 2 α deletion which could be demonstrated at the protein level. But, in contrast to our hypothesis that the epithelial HIF 2 α /flk-1 system might have functional impact on lung development, epithelial HIF 2 α deletion did not lead to a disorder or phenotype. Even though flk-1 was strongly downregulated, no changes were observed regarding lung structure or AEC morphology and SPC expression respectively. This was unexpected. Thus we postulate that the epithelial HIF 2 α system works within a complex network. Upon HIF 2 α deletion, a compensatory system takes over the HIF 2 α -related functions to rescue the deletion to protect the process of lung development. This is highly speculative and further investigations are needed to characterize the different factors involved which were not addressed in the present study.

Taken together our study demonstrates:

Characterization of flk-1 expression at daily intervals throughout lung development with epithelial expression of flk-1 rising from ED16.5 and persisting at postnatal stages (Ahnbrecht et al., 2008). Furthermore HIF 2 α and flk-1 expression could be demonstrated in isolated AEC. Thus, we were able to demonstrate the existence of the epithelial HIF 2 α /flk-1 system.

Analysis of the direct epithelial function of the newly characterized pulmonary epithelial system could be addressed by successful generation of an pulmonary epithelial-restricted HIF 2 α *in vivo* deletion using the SPCrtTA-tetO-Cre system.

Surprisingly, characterization of the phenotype of pulmonary epithelial HIF 2 α knock-out mice did not show any disorders or phenotype, or changes in lung structure and epithelial cell development. These data may be basis for future studies elucidating the network of cooperating factors regulating pulmonary epithelial development.

8. Zusammenfassung

In der vorliegenden Arbeit wird die Hypothese untersucht, dass das HIF 2 α /flk-1 System eine eigenständige funktionelle Rolle in dem pulmonalen epithelialen System spielt und darüber Einfluss auf die Lungenentwicklung nimmt. Diese Hypothese basiert auf Untersuchungen von Compennolle et al. die ein akutes Lungenversagen als Folge einer globalen HIF 2 α Deletion beschreiben, welches durch Applikation von VEGF überwunden werden konnte (Compennolle et al., 2002). Bisher wurde die Regulation der physiologischen und pathologischen Blutgefäßentwicklung als die zentrale Funktion des HIF 2 α /flk-1 Systems beschrieben. Die Wirkungsweise schien somit auf das vasculäre System begrenzt zu sein. Demgegenüber deuten jedoch Daten von Brown et al. und Raoul et al. darauf hin, dass das flk-1/VEGF System auch direkten Einfluss auf die Funktion des pulmonalen epithelialen Systems zu nehmen scheint (Brown et al., 2001; Raoul et al., 2004). Flk-1 wird durch HIF 2 α reguliert (Elvert et al., 2003) und stellt somit das funktionelle Zielmolekül des HIF 2 α /flk-1 Systems dar. Diesbezüglich charakterisierten wir in dieser Arbeit zunächst die flk-1 Expression während der Lungenentwicklung täglich von ED12.5 bis hin zu postnatalen Stadien. Als Ergebnis zeigte sich in den frühen Stadien die flk-1 Expression wie bereits beschrieben in dem frühen primitiven vasculären Netzwerk. Am Ende der pseudoglandulären Phase zeigte sich eine großflächige epitheliale flk-1 Expression, die bis in die postnatalen Stadien zu finden war. Des Weiteren konnten wir in murinen isolierten alveolären Epithelzellen die flk-1 Expression und Aktivierung nachweisen (Ahlbrecht et al., 2008). Darüber hinaus zeigte sich eine starke Expression von HIF 2 α in diesen Zellen. Um die Funktion des nun neu charakterisierten pulmonalen epithelialen Systems zu analysieren, generierten wir eine *in vivo* pulmonal epithelzellspezifisches HIF 2 α Deletion mittels SPCrtTA- und tetO-Cre System in einem dreifach transgenen Mausmodell. Die erfolgreiche Deletion zeigte sich auf Proteinebene. Gegensätzlich zu der zu Grunde liegenden Hypothese einer spezifischen Funktion des epithelialen HIF 2 α /flk-1 Systems, zeigte sich jedoch kein pathologischer Phenotyp. Es zeigte sich eine stark reduzierte epitheliale flk-1 Expression, die sich jedoch nicht auf die Lungenstruktur, Epithelzellmorphologie oder SPC Expression auswirkte. Auf Grund dieser unerwarteten Ergebnisse postulieren wir, dass das HIF 2 α /flk-1 System in ein komplexes Netzwerk von Faktoren integriert ist, welches bei Deletion eines Faktors die Funktion übernehmen kann, um eine ungestörte Entwicklung zu ermöglichen. Das ist eine spekulative Vermutung, zu deren Klärung weitere Studien notwendig sind, um die

unbekannten Faktoren des Netzwerkes zu untersuchen, die in der vorliegenden Arbeit nicht untersucht wurden.

Zusammenfassend konnte die vorliegende Arbeit folgendes zeigen:

Charakterisierung der flk-1 Expression während der Lungenentwicklung in täglichen Intervallen. Es zeigte sich eine epitheliale flk-1 Expression von ED16.5 beginnend und persistierend bis zu postnatalen Stadien (Ahlbrecht et al., 2008). Darüber hinaus konnte die epitheliale HIF 2 α Expression in isolierten alveolaren Epithelzellen (AEC) gezeigt werden, und so die Existenz eines epithelialen HIF 2 α /flk-1 Systems demonstriert werden.

Als *in vivo* Model zur funktionellen Analyse des neu charakterisierten pulmonal epithelialen Systems konnte eine pulmonal epithelzellspezifische HIF 2 α Deletion mittels SPCrtTA-tetO-Cre Systems in einem dreifach transgenen Ansatz erzielt werden.

Entgegen der Hypothese einer speziellen Funktion des epithelialen HIF 2 α /flk-1 Systems zeigte sich kein pathologischer Phenotyp unter der epithelspezifischen HIF 2 α Deletion bezüglich Lungenstruktur und Epithelzellentwicklung. Diese Daten können eine Basis für weitere Studien darstellen, die das Netzwerk der involvierten Faktoren der Lungenentwicklung und speziell der pulmonalen Epithelzellentwicklung weiter aufklären.

9. Abbreviations

AEC	alveolar epithelial cells
AP	alkaline phosphatase
ARNT	aryl hydrocarbon receptor
bHLH/PAS	Basic helix-loop-helix/Per Arnt Sim domain
bp	base pair
BSA	bovine serum albumin
C57bl 6	C57 black 6
CD 31	cluster of differentiation 31
Cre	Cre recombinase
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dox	doxycycline
ED	embryonic day
EDTA	ethylenediaminetetraacetic
EPO	erythropoietin
ES	embryonic stem
FIH	Factor inhibiting HIF
flk-1	Fetal liver kinase-1/VEGFR-2/KDR
flt-1	Fms-like tyrosine kinase-1/VEGFR-1
GFP	green fluorescent protein
h	hour
HE	heterozygous
HLF	HIF like factor
HO	homozygous
HRE	hypoxia-responsive element

HGF	hepatoocyte growth factor
HIF	hypoxia-inducible factor
i.p.	intraperitoneal
kb	kilo base
kDa	kilodalton
ko	knock out
mg	milligram
µg	microgram
min	minute
mRNA	messenger ribonucleic acid
neo	neomycin
l	liter
loxP	recognition site for Cre recombinase
ODN	oligodeoxynucleotide
orf	open reading frame
Orf Virus	Parapox virus
P	post natal
pA	polyadenylation site
PBS	phosphate-buffered saline
PCI	phenol-chlorophorm-isoamyl alcohol
PECAM-1	Platelet endothelial cell adhesion molecule-1
PFA	paraformaldehyde
proSPC	pro surfactant protein C
PVDF	polyvinylidene difluoride
RFP	red fluorescent protein
RIPA	modified radioimmunoprecipitation
ROS	reactive oxygen species

rtTA	reverse tetracycline transactivator
s	seconds
SPC	surfactant protein C
SPCp	surfactant protein C promoter
TATA	minimal promoter containing TATA box
TEA	triethanolamine
tetO	<i>tet</i> operon
tetO ₇	Seven tandemly repeated 19-bp tetO sequence
tetR	tetracycline repressor
tk	thymidine kinase
TSP	tissue-specific promoter
tTA	tetracycline transactivator
V	Volt
VEGF	vascular endothelial growth factor
VP 16	Transactivation domain of the herpes simplex virus Protein VP 16

10. Citations

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12. Declaration

I declare that I have completed single handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or based on the content of published or unpublished work of others and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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14. List of publications

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