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REVIEW

Regulation of lung development and regeneration by the vascular system

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Abstract Blood vessels have been described a long time ago as passive circuits providing sufficient blood supply to ensure proper distribution of oxygen and nutrition. Blood vessels are mainly formed during embryonic development and in the early postnatal period. In the adult, blood vessels are quiescent, but can be activated and subsequently induced under pathophysiological conditions, such as ischemia and tumor growth. Surprisingly, recent data have suggested an active function for blood vessels, named angiocrine signaling, releasing trophogens which regulate organ development and organ regeneration including in the pancreas, lung, tumor cells, liver and bone. Lung development is driven by hypoxia as well as an intense endothelial–epithelial interaction, and important mechanisms contributing to these processes have recently been identified. This review aims to summarize recent developments and concepts about embryonic pulmonary vascular development and lung regeneration. We discuss hypoxia-inducible factor HIF-2α and vascular endothelial growth factor VEGF as important mediators in lung development and focus on endothelial–epithelial interactions and angiocrine signaling mechanisms.

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Introduction

Preparation for birth after proper intrauterine development includes the major switch from placental oxygenation to breathing. More precisely, it is the transition from a fluid-filled to a functional air-filled lung which is essential for neonatal survival. A large clinical study reported that preterm delivery is associated with a significantly increased risk for severe breathing problems. In addition, this study has described that the preterm delivery rate increases in developed countries—12–13 % in the USA and 5–9 % in many others [1]. Respiratory distress develops in 80 % of the infants born before 27 weeks of gestation and in about 24,000 infants a year [2]. Major advances in perinatal care, including therapeutic treatment with glucocorticoids and postnatal application of surfactant, save many newborns from death. Thus, glucocorticoid treatment that promotes lung maturation is a subject of intensive discussion [3–6].

A better understanding of lung development may help to define new molecular targets that can potentially be used to develop new therapies. A newly identified process named "angiocrine signaling" points to an endothelial cell-driven active paracrine function of blood vessels on organ development and regeneration mediated by the organ-specific microvasculature. In lung, recent studies identified pulmonary endothelial–epithelial interactions revealing that pulmonary development and regeneration is driven by blood vessels derived growth factors that trigger epithelial differentiation (Fig. 1).

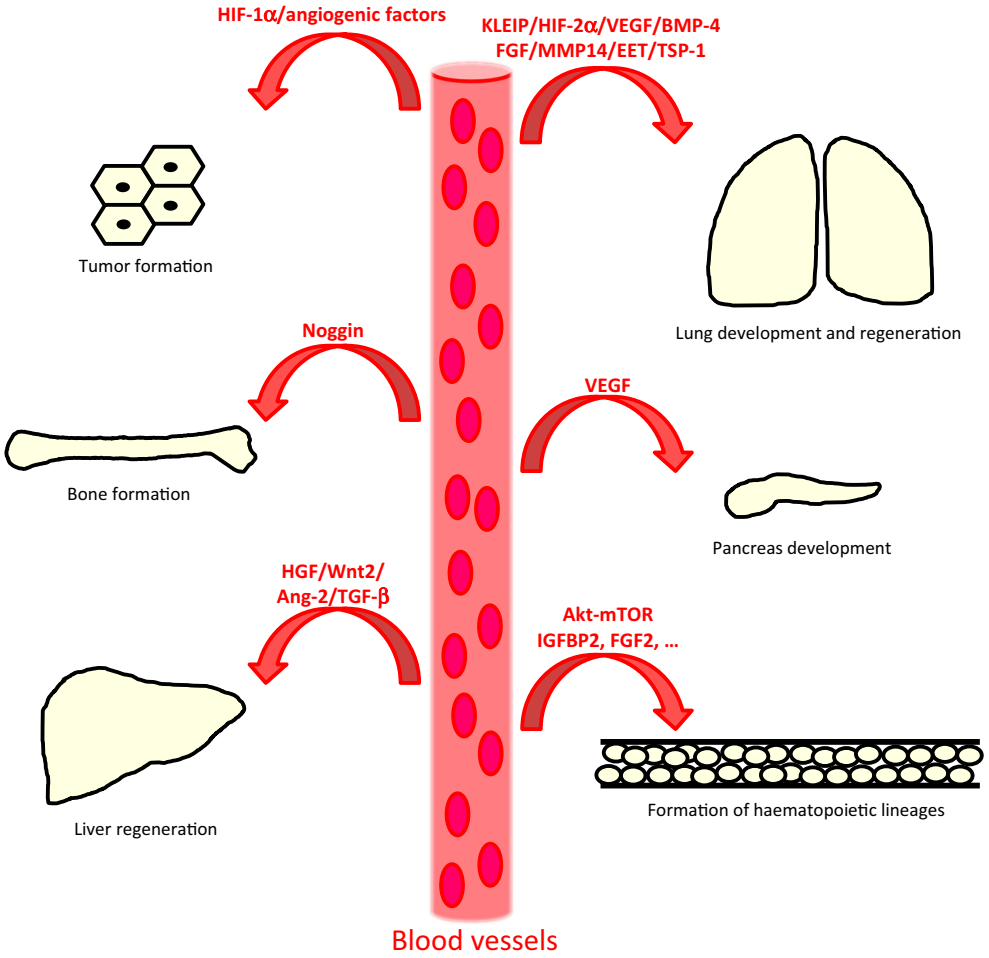

Fig. 1 Regulation of angiocrine organ development and regeneration in lung, pancreas, liver, hematopoietic system, tumors and bone. Blood vessels produce and release factors named "angiocrine molecules" which regulate organ growth and organ regeneration in a paracrine manner. For details see text and references

Regulation of lung development

Lung development is a well-regulated process that depends on complex interactions of the epithelium, mesenchyme and endothelium. It starts with the primary lung bud formation during the fourth gestational week in human—which corresponds to the embryonic days (E) 9.0–11.5 in mice—and is subdivided into five morphologically and biochemically defined stages: the embryonic stage, the pseudoglandular stage, the canalicular stage, the saccular stage and the alveolar stage (Fig. 2) as reviewed by Deutsch and Pinar [7] and Harding and colleagues [8].

During the embryonic stage, the primary lung buds form from the ventral foregut endoderm, and fuse in the midline to develop the tracheal primordia and two main bronchi. The lung bud grows into adjacent splanchnic mesoderm where it is induced to branch repeatedly, giving rise to the future respiratory tree. In parallel, the first blood vessels arise from condensed mesenchymal progenitors by vasculogenesis [9]. The pseudoglandular stage is defined by branching morphogenesis resulting in the primary bronchial tree including the bronchi and the terminal bronchioles. During this stage epithelial cells, lining the trachea, the bronchi and bronchioles, remain relatively undifferentiated [9]. During the canalicular stage, the conducting airways become lined by diverse cell types including squamous, basal, ciliated and secretory Clara cells. Morphological changes include bronchiole differentiation and formation of the respiratory ducts and sacs [9]. Furthermore, the vascular plexus enlarges by vasculogenesis and angiogenesis [9]. In the following saccular stage, each acinus supplied by a terminal bronchiole gets three to four respiratory bronchioles that end in a transitional duct from which saccules arise. In addition, the saccular stage is characterized by the differentiation of type I and type II pneumocytes and by capillary remodeling [9, 10]. This stage prepares the respiratory unit for the transition at birth. The subsequent alveolar stage occurs postnatally in mice

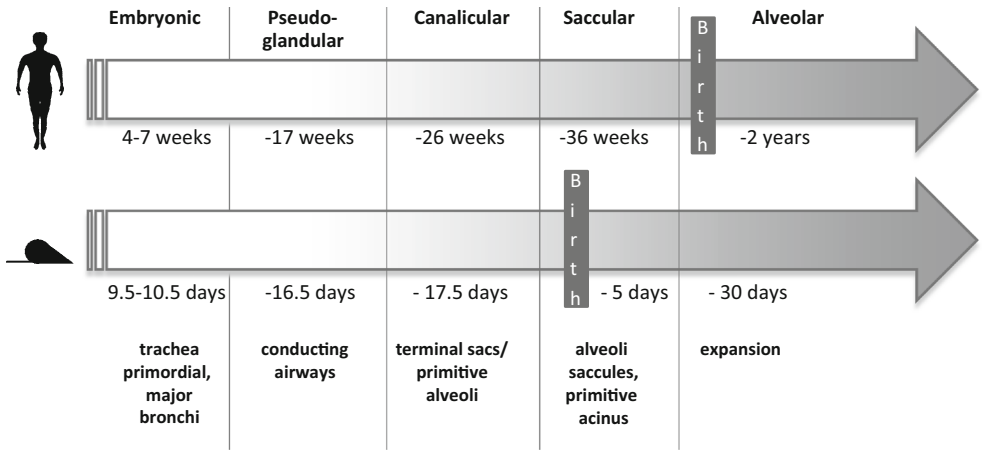

Fig. 2 Stages of lung development in human and mice

but starts within the last prenatal weeks in human and continues into adolescence. Herein, septation of the saccules gives rise to 300 million alveoli resulting in an immense surface extension that reaches 75–100 m^2 in adult human lung [11]. In addition, there is substantial capillary and epithelial proliferation and remodeling before and after birth that ensures a close proximity of the vascular and alveolar bed [7]. In the end, the transition to breathing dependents on alveolar epithelial cell differentiation but is also regulated by capillary endothelial differentiation [12].

Surfactant production

Breathing at birth requires prenatal surfactant production that is indispensable for respiration at birth because it reduces alveolar surface tension at the air–liquid interface and prevents alveolar collapse [13]. Thus, birth before the end of saccular stage is often accompanied by severe respiratory distress based on insufficient surfactant production. Surfactant is produced by type II pneumocytes. Surfactant produced by type II pneumocytes consists of ~10 % protein, ~10 % cholesterol and ~80 % phospholipids [14]. To synthesize surfactant at birth, type II pneumocytes further accumulate prenatal glycogen that gets transferred into phospholipids upon delivery [15]. Interestingly, surfactant production is also strongly regulated by hypoxia [16].

Surfactant proteins (SPs) include SP-A, SP-B, SP-C and SP-D. SP-C expression is unique to type II pneumocytes while SP-A, SP-B and SP-D may also be expressed by Clara cells [17–19]. Mutation of the *sp-b* gene alone inhibits surfactant protein and phospholipid synthesis, storage and function causing neonatal respiratory failure in mice [20] and neonatal respiratory disease in humans [21]. Disruption of the murine *sp-c* gene is characterized by severe lung damage—emphysema, epithelial cell dysplasia and monocytic cell infiltration [22]—and is related to interstitial lung disease in human [23]. SP-A and SP-D primarily function in innate immunity [24], thus, being of less importance for lung transition upon birth. However, specific *sp-a* alleles are associated with increased risk for respiratory distress in humans but not in mice [25–27].

Pulmonary vascular development

The cardiovascular system mediates gas transport and nutrition supply. Abnormal vascular development as recognized for VEGF and HIF-1α deficient mice is embryonically lethal [28–30]. During lung development, a vascular plexus is present as soon as the first lung buds arise from the foregut endoderm [31, 32]. The pulmonary vascular plexus is part of the vascular system which consists of arteries, veins and of the microvasculature. The development of the functional vascular plexus can be subdivided into two processes: vasculogenesis and angiogenesis [33–35].

The term vasculogenesis describes the *de novo* formation of blood vessels out of endothelial precursor cells (EPCs or angioblasts) that form blood islands and afterwards differentiate into endothelial and hematopoietic cells [36, 37]. In comparison to vasculogenesis, angiogenesis describes the vessel formation from a pre-existing network through vascular remodeling and expansion [38]. Thus, angiogenesis is not only essential for the formation of a mature vascular network out of a primitive vascular plexus arising from vasculogenesis, but also for adult vascular homeostasis, regeneration and adaption.

Hypoxia, the main trigger of late stage lung development, is stimulating sprouting angiogenesis via VEGF and other angiogenic factors [31]. During sprouting

angiogenesis endothelial cells functionally separate into tip cells that sense VEGF (vascular endothelial growth factor), thereby, directing the developing sprout towards hypoxic areas; stalk cells that build up and elongate the new capillary following the tip cell and phalanx cells that stay tightly aligned to the parental vessel building the sprout origin [34]. Finally, the newly formed vessels fuse and build a functional network that gets covered by pericytes or smooth muscle cells (mural cells) [39].

VEGF-A, also referred to as VEGF, is the key regulator of angiogenesis [34]. VEGF belongs to a family that further consists of VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, PlGF-1 and P1GF-2 which are secreted proteins with complementary and distinct functions [33]. VEGF molecules activate the tyrosine kinase transmembrane receptors VEGFR-1, VEGFR-2 and VEGFR-3 which are partially specific for the different VEGF-variants [40]. The total loss of VEGF caused embryonic lethality due to vascular failure. Strikingly, already VEGF heterozygosity was lethal underlining the importance of VEGF in vascular development [28, 29]. Apart from its function in vascular guidance, VEGF has been shown to promote endothelial viability, vessel enlargement and branching in health and disease [41, 42].

The mechanisms of pulmonary vascular development are still under debate. Based on histological sections and transgenic mouse reporter lines, both vasculogenesis and angiogenesis may contribute to the developing vasculature in lung [43]. Yet, factors which regulate vascular development in other organs also act in the lungs. For example, overexpression of VEGF as well as the disruption of the VEGF pathway leads to aberrantly formed airways [44–46]. Conditional VEGF expression in distal and proximal airway epithelial cells identified VEGF's contribution to proximal and distal pulmonary development [47, 48]. VEGF stimulation of whole lung culture caused increased branching and gene expression including SP-C [49]. Likewise, VEGF is expressed in bronchial epithelium and alveolar macrophages in humans [50], interstitial VEGF concentrations in mice are strongly hypoxia dependent [51] and HIF-2α expression is known to increase in the beginning of the saccular stage [52]. Interestingly, even though HIF-2α is known to be one major trigger for pulmonary VEGF expression, in the conditional endothelial specific HIF-2α mouse, minor lung vascular defects did not affect Mendelian ratios of born mice [53]. Nevertheless, it has recently been shown, that the HIF-2α/VEGF axis determines neonatal respiratory failure in Kelch-like ECT-2 interacting protein (KLEIP) deficient mice [54–57]. Yet, the role of HIF-2a in lung development needs to be further elucidated.

Interdependence of bronchiolar branching and lung vascularization

Pulmonary branching pattern has extensively been studied since the first description of the developing bronchial tree in 1961 [58], and the three-dimensional branching pattern has now been summarized by Metzger and colleagues [59].

Airway and vascular branching proceed interdependently in the lung [60]. The subsequent molecular regulation is the result of epithelial–mesenchymal interactions that is driven by the close proximity of the vasculature and the airways within all branching states/generations and the close proximity within the alveolar-capillary unit that enables gas exchange. Thus, the vascular plexus is initiated as soon as the first foregut invaginations form and originates from vasculogenesis and angiogenesis [32, 61]. Indeed, co-culturing experiments predicted that the vascular plexus forms interdependent of the developing epithelium showing that vascular cells fail to proliferate in the absence of lung epithelium [61]. Just in time, interventional in vivo studies using angiogenesis inhibitors against the VEGF pathway showed a reduced alveolarization and lung hypoplasia [12]. Hence, an exogenous VEGF-A replacement therapy seemed promising to treat the respiratory distress syndrome. However, conditional activation of VEGF-A in murine bronchial epithelial cells caused neonatal lethality due to epithelial and endothelial failure and thus, conflicted with the idea of VEGF therapy [47]. Yet, these experiments gave the first evidence that during alveolar stage, vascular plexus formation depends on pulmonary alveolarization and vice versa.

Later on, the model was expanded by the finding that pulmonary vascular development is rate limiting for prenatal epithelial branching and that the involved pulmonary VEGF levels are strongly hypoxia dependent [51]. In addition, in vitro and in vivo transgenic experiments proved the essential role of VEGF signaling in both, epithelial and endothelial branching morphogenesis [48, 49, 62]. This is supported by observational studies in mice that identified differential VEGFR-2 regulation in endothelial and epithelial cells [63]. Furthermore, in vivo 3D experiments provided information for a perfusion-independent determination of branching stereotypy of proximal lung airways by blood vessels [64, 65]. Altogether, the mechanisms underlying proximal vascular network formation are still poorly understood, but point to a reciprocal interaction of airway and vascular growth that coordinates lung development. Predicted triggers of this cross-talk are hypoxia and subsequently VEGF.

Hypoxia and hypoxia-inducible factors in lung development

The ability to sense and respond to acute and prolonged changes in oxygen concentration is a fundamental requirement to avoid hypoxia and ensure survival. The main factors that act in hypoxic conditions are the hypoxia-inducible factors (*HIFs*). HIF-1 and HIF-2 are α,β-heterodimers composed of two basic helix-loop-helix (bHLH) subunits of the PAS family (*P*ER-period circadian protein, *A*RNT-aryl-hydrocarbon-receptor nuclear translocator, *S*IM-single minded protein)—HIF-α and HIF-β. The HIF-dimer binds to transcriptional regulatory DNA sequences, known as hypoxia response elements (HREs) [66]. Nowadays, three *hif-α* genes—*hif-1α, hif-2α* and *hif-3α*—have been identified. *Hif-1α* and *hif-2α* possess a similar functional structure with 48 % sequence homology, including three hydroxylation sites, revealing that both are oxygen regulated in a similar manner [67].

HIF-1α was first identified as a DNA-binding protein bound to the erythropoietin (EPO) gene [68], but is meanwhile known to be a highly conserved, ubiquitously expressed and tightly regulated transcription factor that under hypoxic conditions controls the expression of hundreds of genes [16]. In contrast, HIF-2α—so far detected in hepatocytes, cardiomyocytes, glial cells, type II pneumocytes and vascular endothelial cells—exhibits a much more tissue-specific restricted expression [69]. HIF-1α and HIF-2α regulate distinct, but overlapping batteries of target genes. One of the most prominent genes regulated by HIF-1α and HIF-2α is *vegf*, the key regulator of angiogenesis [70].

Global deficiency of HIF-1α resulted in a developmental arrest at E8.0 and lethality by E11 [71] [72]. HIF-1$\beta^{-/-}$ embryos are not viable beyond E10.5 [73]. Absence of HIF-2α leads to only 12.5 % fetal lethality [74, 75]. In comparison to the HIF-1α and HIF-1β knockout, in the HIF-2α knockout model, the surviving embryos suffer from impaired lung maturation which provokes neonatal lethality of 50 % of the newborns [10]. This points to an important role of HIF-2α in perinatal lung development; yet, it should be mentioned that Hif-2α phenotypes in mice may vary between different mouse strains [10, 73–76].

Lung development is driven by hypoxia [77]. During lung development, both hypoxia-inducible transcription factors HIF-1α and HIF-2α act as key regulators in epithelial, mesenchymal and vascular lung morphogenesis [78, 79]. Herein, expression of HIF proteins underlies a strict spatiotemporal regulation. While HIF-1α expression dominates early lung development till the saccular stage and decreases quickly after birth; HIF-2α up-regulation starts at saccular stage and remains until adulthood [52]. Genetic loss-of-function models elucidate the importance of HIF-2α but not HIF-1α in saccular stage lung development [10, 78, 80]. Deletion of HIF-2α leads to impaired lung development, reduced surfactant production, postnatal respiratory distress and neonatal lethality [10] because it controls type II pneumocyte maturation and thus surfactant production and type I pneumocyte differentiation during late stage lung development [80].

Elucidating the function of HIF-2α in pulmonary capillary formation remained challenging due to the following reasons. At first, capillary bleedings were observed for HIF-2α deficient mice but the function of HIF-2α for endothelial maturation and resulting bleedings remained unclear [10]. Second, conditional endothelial HIF-2α deficient mice displayed physiological lung maturation [53]. Third, a recent study proposed reduced embryonic HIF-2α levels as a cause for respiratory failure after birth [54]. Finally, a function of HIF-2α for pulmonary vascular remodeling is likely because HIF-2α deficient mice display defective vascular remodeling in the yolk sac [75] and HIF-2α is a potent activator of VEGF transcription. Together, to date, HIF-2α but not HIF-1α seems to specifically promote late stage lung development but its function in context of pulmonary vascular network formation and pneumocyte maturation remains poorly understood.

Angiocrine signaling

The term “angiocrine” has recently been introduced by Butler and colleagues [81] and describes the observation that endothelial cells promote tumor growth by producing stem and progenitor cell-active trophogens (Fig. 1). Angiocrine signaling during organ development has already been described 10 years before by Lammert and colleagues [82] and has recently been reviewed [83]. It refers to a series of experiments that propose endothelial cells to control organ growth, morphogenesis and differentiation in the pancreas, liver and kidney [82, 84–87]. Subsequently, angiocrine signaling was linked to stem cell behavior in haematopoiesis [88–91] and osteogenesis [92]. Recently, angiocrine signaling was also observed during liver regeneration [93–95], in the heart [96], during inflammatory spinal cord injury [97], in the lung [54, 98] and in tumor development and metastasis [99–102] (Fig. 1). In common, all these findings suggest microvascular endothelial cells—capillary endothelial cells and sinusoidal endothelial cells—as a source of angiocrine factors that regulate organ growth and organ regeneration. Today, the term angiocrine factors refer to the entire set of paracrine factors secreted by the endothelium that promote organ differentiation, regeneration, tumor growth and metastasis (Fig. 1).

Angiocrine signaling in embryonic lung development

Recently, angiocrine signaling was linked to distal lung development (Fig. 1). A study that aimed to identify the molecular mechanisms involved in preterm birth showed that loss of the Kelch-like ECT2-interacting protein (KLEIP/KLHL20) causes neonatal respiratory failure characterized by reduced ventilated airspace and reduced septal thinning in neonatal KLEIP$^{-/-}$ lungs [54]. As a consequence, half of the KLEIP$^{-/-}$ neonates die within the first few hours after birth. KLEIP was identified to be selectively expressed within lung capillaries and embryonic KLEIP$^{-/-}$ lungs showed reduced surfactant proteins, HIF-2α and VEGF expression. This led to the model that embryonic surfactant production is at least partially triggered by the endothelial derived factors HIF-2α and VEGF. Moreover, reduced VEGF-levels in KLEIP$^{-/-}$ lungs led to pulmonary endothelial apoptosis and subsequently to degeneration of the initially intact microvascular network. Remarkably, the lung phenotype in KLEIP$^{-/-}$ mice was rescued by prenatal betamethasone application which increased perinatal pulmonary HIF-2α expression and normalized lung development [54]. Together, the data showed that the pulmonary microvasculature contributes to distal pulmonary maturation and that distal pulmonary development is controlled by an epithelial–endothelial cross-talk that is regulated by KLEIP and HIF-2α.

Angiocrine signaling in lung regeneration

Lung regeneration can be induced upon injury and disease; yet, the underlying mechanisms are not well known. Two major hypotheses suggest that stem or progenitor cell lineages may contribute to lung regeneration. Alternatively, upon injury or disease, lung epithelial cells start to proliferate and replace lost cells [103]. Angiocrine signaling in lung regeneration was investigated after H1N1 influenza virus infection, clearly demonstrating a link between capillary growth and alveolar regeneration from the transformation-related protein 63 (*p63*) positive basal-like stem cells [104]. In addition, another study showed that lung stem cell differentiation is directed by lung endothelial cells and is mediated by endothelial thrombospondin-1 [105]. The angiocrine triggers identified after pneumonectomy are VEGF and FGF that regulate expression of the matrix metalloproteinase 14 (MMP14) in pulmonary capillary endothelial cells which resulted in the unmasking of extracellular epidermal growth factor (EGF)-like ectodomains that stimulated epithelial stem cell proliferation (Fig. 1) [98, 106]. This is further demonstrated by the observation that deficiency for MMP14 in endothelial cells resulted in reduced alveolar expansion while endothelial proliferation stayed unaltered. Thus, the proposed model predicts an accumulation of epithelial cells induced by endothelial-derived angiocrine factors (MMP14). This caused reconstitution of physiologically functional alveolar-capillary sacs. On the other hand, proliferation of endothelial cells prompted by an epithelial feedback via VEGFR-2 and FGF receptor 1 (FGFR-1) activation vascularizes the regenerating lung tissue to restore the blood supply and gas exchange function [98]. Recently, this mechanism was further elucidated by showing that activated platelets release SDF-1 upon pneumonectomy and activate pulmonary capillary endothelial cells via CXCR4 and CXCR7 leading to Akt/PKB phosphorylation. Subsequently, endothelial cells activate MMP14 which releases HB-EGF (heparin-binding EGF-like growth factor) and finally stimulates lung regeneration [107]. Another study connects angiocrine pathways to endothelial Epoxyeicosatrienoic acids (EETs) signaling in lung, liver and kidney (Fig. 1). The study characterized mice that endothelium specifically overexpressed EET producing enzymes or soluble epoxide hydrolase (SEH) null mice during keratectomy, pneumonectomy or nephrectomy. Herein, unilateral pneumonectomies resulted in a 23 % increase in contralateral lung growth in the EET overproduction model compared with WT mice revealing EETs to be of importance for regeneration. This is further supported by the findings that administration of EET increased lung regeneration, accelerated wound healing, stimulated neonatal retinal vessel formation and tissue vascularization [108].

Summary and clinical perspective

As summarized in this article, recent evidence in the literature suggests an important paracrine function of blood vessels in the formation, regeneration and pathological alterations of different organs, including lung, liver, pancreas, bones, hematopoietic system and heart (Fig. 1). In these studies, blood vessel produced angiogenic molecules have been identified which directly affect organ growth, regeneration and tumor growth. Furthermore, some reports already suggested clinically relevant drugs, such as betamethasone, that increase expression of angiogenic factors and subsequently compensate for developmental lung alterations [54]. Based on these observations two experimental and translational strategies need to be further developed. First: identification and characterization of so far unknown angiogenic molecules that are produced by vascular cells and that act on surrounding organs in development and disease. In addition, it needs to be further determined whether activation or inhibition of blood

vessels derived angiocrine factors is able to improve organ development and organ regeneration. Likewise, it is poorly understood whether application of purified angiocrine factors will improve organ development and regeneration or whether application of endothelial (progenitor) cells producing selected angiogenic factors are more beneficial. Second: The availability and mechanisms of drugs that may increase or decrease expression and activity of blood vessel derived angiocrine factors need to be further tested in development, regeneration and in malignancies to explore their potential to modulate these processes. Finally, established anti-angiogenic therapies in the clinic should be tested for their benefits or pitfalls in angiocrine-based organ growth and organ regeneration.

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