Emerging Molecular Targets for Anti-proliferative Strategies in Pulmonary Arterial Hypertension

Ly Tu and Christophe Guignabert

Abstract The combination of pulmonary vasoconstriction, in situ thrombosis, and pulmonary arterial wall remodeling is largely responsible for the rise in pulmonary vascular resistance (PVR) and pulmonary arterial pressure (PAP) in patients with pulmonary arterial hypertension (PAH). Even though several drugs have been developed over the past decades, at this time there is no cure for PAH. The overriding goals of the current therapeutic options seek to compensate for the defects in the relative balance of competing vasoconstrictor and vasodilator influences. Because the past decade has seen great strides in our understanding of the pathogenesis of PAH, interest has been growing in the potential use of anti-proliferative approaches in PAH. Indeed anti-proliferative strategies could offer ways not only to reinstate the homeostatic balance between cell proliferation and apoptosis but also to reverse the progressive pulmonary vascular obstruction in PAH. However, further efforts still need to be made in order to establish the long-term safety and efficacy of those anti-proliferative approaches in PAH and their potential additive benefit with other drugs.

Keywords Pulmonary hypertension • Pulmonary vascular remodeling • Anti-proliferative compounds • Signal transduction • Growth factors
1 Introduction

Pulmonary arterial hypertension (PAH) is a rapidly progressive disease characterized by sustained elevation of pulmonary vascular resistance (PVR) and pulmonary arterial pressure (PAP) leading to right heart failure and death. Although the exact mechanisms of remodeling of pulmonary arteries, leading to the onset and progression of the disease, are still largely unclear, many disease-predisposing factors and/or contributing factors have been identified, including inflammation, endothelial dysfunction, aberrant vascular wall cell proliferation, as well as mutations in the *bone morphogenetic protein receptor type 2* (*Bmpr2*) gene (Humbert et al. 2004; Rabinovitch 2005; Morrell et al. 2009) (Fig. 1).

Development of therapeutic agents that modulate abnormalities in three main pathobiologic pathways for PAH endothelin (ET)-1, prostacyclin (PGI2), and nitric oxide (NO) has revolutionized our approach to the treatment of PAH and has changed the course of this devastating disease (O’Callaghan et al. 2011). However, although the spectrum of therapeutic options for PAH has expanded in the last decade, available therapies remain essentially palliative.

Irreversible remodeling of the pulmonary vasculature is the cause of increased PAP in PAH and frequently leads to progressive functional decline in patients despite treatment with current available therapeutic options. This process is ascribed to the increased proliferation, migration, and survival of pulmonary vascular cells within the pulmonary artery wall, i.e., myofibroblasts, pulmonary vascular smooth muscle (SMCs), and endothelial cells (ECs). The increasing knowledge on PAH pathogenesis has revealed the complex nature of these structural and functional changes in the pulmonary arteries of patients with PAH and highlighted the need to elucidate the molecular mechanisms involved. Over recent years, special attention has been devoted to the disease-promoting roles of three different growth factors and their corresponding tyrosine kinase
receptors: platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) receptors. Altered expression and/or increased activity of these three signaling pathways as well as their contribution to the abnormal proliferation and migration of smooth muscle and endothelial cells have been demonstrated in human and experimental models of pulmonary hypertension. Furthermore, their inhibition by specific inhibitors, tyrosine kinase inhibitors (TKIs), has been shown to exert beneficial effects in rodent models (Merklinger et al. 2005; Schermuly et al. 2005; Perros et al. 2008; Izikki et al. 2009; Tu et al. 2011, 2012). Currently, imatinib (a TKI of PDGF receptors), which is widely used for the treatment of chronic myeloid leukemia, is being tested as a new potential therapy in PAH and ongoing studies seek to better evaluate the overall risk benefit ratio of this anti-proliferative molecule in PAH.

The last few years have seen the emergence of the concept that anti-proliferative strategies could offer a novel approach for the treatment of PAH, by downregulating the progression of the disease and reversal of pulmonary vascular remodeling. However, further studies are needed to better understand the mechanisms underlying this abnormal over-activation of some growth factor-stimulated signaling pathways in PAH and to identify novel pharmacological targets for the development of new, better-tolerated, and more powerful therapeutic tools. This chapter aims to provide an overview on current status and future perspectives of target-based anti-proliferative therapies in pulmonary hypertension.

Fig. 1 Conceptual framework for the development of pulmonary arterial hypertension (PAH) therapies. ECM extracellular matrix
2 Growth Factor Signaling Pathways as Targets for Anti-remodeling Therapies in Pulmonary Hypertension

Growth factors are diffusible proteins that act through activation of diverse signaling pathways to regulate a myriad of critical cellular functions for normal lung development and homeostasis. As growth factors dictate growth, proliferation, and survival, there has been an increasing interest in the cellular biology of growth factor signaling in PAH in recent years. Excessive release of growth factors that are encrypted in the extracellular matrix, and/or modification of growth factor production, and receptor expression, and/or alterations in the intracellular mitogenic signals, have been reported to have a critical role in the disease; however, it is unknown whether or not this imbalance is the cause or the consequence of PAH. Many molecular therapies that target aberrant growth factor signaling pathways are being investigated, with some agents in the late stages of clinical testing.

2.1 The Platelet-Derived Growth Factor Signaling System in PAH

The PDGF family consists of PDGF-A, -B, -C, and -D, which form either homo- or heterodimers (PDGF-AA, -AB, -BB, -CC, -DD). The four PDGFs are inactive in their monomeric forms. The PDGF-AA, -AB, and -BB dimers are processed intracellularly and secreted as active dimers. In contrast, PDGF-CC and PDGF-DD differ from the others in that they are secreted as an inactive form until their N-terminal complement C1r/C1s, Uegf, Bmp1 (CUB) domain is cleaved. There are two receptor subunits -α and -β that dimerize upon binding one PDGF dimer, leading to three possible receptor combinations, namely, PDGFR-αα, -ββ, and -αβ. PDGF-AA binds exclusively to PDGFR-αα, while PDGF-BB is the only PDGF that can bind all three receptor combinations with high affinity. PDGF-AB and -CC could assemble and activate PDGFR-αα and -αβ. PDGF-DD activates PDGFR-ββ with high affinity, and under certain conditions the PDGFR-αβ. The PDGFR autophosphorylation in the kinase insert region regulates interactions with different cell proteins involved in the initiation of the intracellular signaling pathway.

Since PDGF is an important autocrine and paracrine mitogen for vascular smooth muscle cells, mediating both hyperplasia and migration for pulmonary vascular remodeling, studies on animal models of PH as well as in human tissues have been undertaken (Tanabe et al. 2000; Berk 2001; Yamboliev and Gerthoffer 2001). Balasubramaniam et al. (2003) were the first to note a PDGF upregulation in a lamb model with chronic intrauterine PH and to suggest a pathogenic role of the PDGF signaling system for the disease. This hypothesis was tested and validated by a study from Schermuly et al. (2005), showing that the PDGF receptor antagonist imatinib reverses pulmonary vascular remodeling in the monocrotaline-induced PH in rats and the chronic hypoxia-induced PH in mice. They also reported a
substantial increase in the protein levels of PDGFR-β in total lung homogenates of patients with PAH as compared with controls. Finally, by investigating abnormalities within human tissue of patients with idiopathic PAH, Perros et al. (2008) have reported increased levels of mRNA encoding PDGF-A, PDGF-B, PDGFR-α, and PDGFR-β in microdissected small pulmonary arteries of patients with idiopathic PAH. In addition, they demonstrated a marked increase in the protein levels of PDGFR-β in total lung homogenates of patients with PAH as compared with controls. Consistent with these observations, intense immunoreactivity of the phosphorylated form of PDGFR-β has been shown in vascular lesions in lungs of idiopathic PAH patients. In addition, it has also been demonstrated that PDGFR, specifically PDGFR-β, is transactivated by sphingosine-1 phosphate (Baudhuin et al. 2004), G protein-coupled receptors (GPCRs) stimulated by lysophosphatidic acid (LPA) (Herrlich et al. 1998), angiotensin II (Saito and Berk 2001), and serotonin transporter (5-HTT or SERT) (Ren et al. 2011). Furthermore, Tie2-mediated loss of peroxisome proliferator-activated receptor (PPAR)-γ in mice causes PDGFR-β-dependent pulmonary arterial muscularization (Guignabert et al. 2009a).

2.2 The Fibroblast Growth Factor Signaling System in PAH

FGFs are a group of at least 23 structurally related heparin-binding polypeptide mitogens that are expressed in almost all tissues and constitute a signaling system conserved throughout animal evolution. FGFs interact with a family of four distinct, high-affinity RTKs, designated FGFR 1 to 4. The FGF system has a broad range of biological activities that not only stimulate cell proliferation, migration, and differentiation but also inhibit cell death. FGFRs are protein tyrosine kinase receptors, which consist of three extracellular immunoglobulin-type domains (D1–D3), a single-span transmembrane domain and an intracellular split tyrosine kinase domain. FGFRs 1, 2, and 3 undergo an alternative splicing event in which two alternative exons (IIIb and IIIc) can be used to encode the carboxy terminal portion of the third immunoglobulin-like loop. The splicing variant IIIa of FGFRs is a secreted FGF-binding protein. In addition, other types of alternate mRNA splicing events have been described. Each alternatively spliced variants binds to a specific subset of FGFs with variable affinities and have distinct tissue-specific expression patterns. Upon binding of FGF, FGFR monomers dimerize, and subsequently the TK domains autophosphorylate, which initiates the intracellular signaling pathway.

Since FGF2 is a known potent pro-angiogenic stimulator of endothelial and smooth muscle cell proliferation, migration, and synthesis of various extracellular components (Goncalves 1998), there is a strong interest for this growth factor in PAH. In addition, FGF2 is up regulated in response to hypoxia and shear stress in pulmonary vascular cells (Quinn et al. 2002; Li et al. 2003). Lung and circulating FGF2 levels are increased in both experimental and human PH. Abnormally high levels of FGF2 were found in the blood of 51 % and in the urine of 21 % of patients.
with idiopathic PAH (Benisty et al. 2004). FGF2 levels are increased in two animal models, a lamb model of PH developed by inserting an aorto-pulmonary vascular bypass graft (Wedgwood et al. 2007) and the rat model of monocrotaline-induced PH (Arcot et al. 1995; Izikki et al. 2009). In PAH patients, we have shown that FGF2 is markedly overproduced by pulmonary endothelial cells in walls of distal arteries and in isolated primary cells than in controls. Furthermore, we have demonstrated that this FGF2 overexpression contributes significantly not only to smooth muscle hyperplasia by a paracrine action, but also to the abnormal endothelial phenotype by an autocrine action (Izikki et al. 2009; Tu et al. 2011, 2012). In idiopathic PAH, dysregulation of the FGF2 signaling pathway was associated not only with FGF2 overproduction, but also with altered expression of the FGF receptor. We found increased FGFR2 expression in pulmonary endothelial cells derived from idiopathic PAH patients as compared to control cells. Interestingly, Matsunaga et al. (2009) recently demonstrated that overexpression of a constitutively active FGFR2 in endothelial cells in vitro enhanced migration and survival, and augmented autocrine FGF2 production. Selection of endothelial cells that naturally overexpress FGF2 in early stages of the disease and/or deficient activity of PPARγ may also explain the augmented FGF2 production by endothelial cells of idiopathic PAH patients (Tian et al. 2009). Moreover, FGF2 can be sequestered and stored as a complex in the extracellular matrix and then released by proteolytic processes to bind and activate cell targets, thereby promoting mitogenesis (Benezra et al. 1993; Thompson and Rabinovitch 1996; Buczek-Thomas and Nugent 1999; George et al. 2001). Recently, we reported that daily treatment with the FGFR inhibitor dovitinib started 2 weeks after a subcutaneous monocrotaline injection substantially attenuated the abnormal increase in p130Cas and ERK1/2 activation and regressed established PH (Tu et al. 2012).

2.3 The Epidermal Growth Factor Signaling System in PAH

The EGF family consists of EGF, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), epiregulin, amphiregulin (AR), epigen, beta-cellulin (BTC), and neuregulin 1, 2, 3, and 4. Each ligand displays overlapping but distinct binding affinities toward ErbB receptors and subsequently induces the formation of homo- and heterodimers of these receptors. There are four types of EGF receptors (EGFR) including ErbB1, also referred to as EGFR or Her1, ErbB2 (Neu/Her2), ErbB3 (Her3), and ErbB4 (Her4). All EGF receptors have a common extracellular ligand-binding region, a single membrane-spanning region, and a cytoplasmic protein tyrosine kinase domain. EGF, HB-EGF, TGF-α, AR, BTC, and epiregulin all bind ErbB1. HB-EGF, epiregulin, and BTC are known to bind to ErbB4 as well as ErbB1. The other group includes the neuregulins, which are ligands for ErbB3 and ErbB4. Upon ligand binding, EGFR monomers dimerize, and subsequently the TK domains autophosphorylate, which leads to the activation of intracellular signaling pathways.
Multiple lines of evidence suggest that the EGF signaling system contributes to the SMC proliferative response and are involved in the initiation and/or progression of the pulmonary vascular remodeling in PAH. Several studies have demonstrated that EGF co-localizes with Tenascin C, a component of the extracellular matrix (ECM) that is overabundant in obstructive lesions of patients with PAH and thus leads to an EGF-dependent proliferation and migration of pulmonary vascular cells (Jones and Rabinovitch 1996; Jones et al. 1997a, b, 1999; Cowan et al. 1999, 2000b). Consistent with this pathogenic role of the EGF signaling system, transgenic mice that over-express TGF-α under the control of the human surfactant protein (SP)-C promoter (the TGF-α mice) developed severe PH and vascular remodeling characterized by abnormally extensive muscularization of small pulmonary arteries (Le Cras et al. 2003). This phenotype was prevented in bi-transgenic mice expressing both TGF-α and a dominant-negative mutant EGF receptor under the control of the SP-C promoter. In addition, Merklinger et al. (2005) have shown that inhibition of EGFR by PKI166, a dual EGFR/HER2 inhibitor, mediated PASMC apoptosis and improved survival of monocrotaline-injected rats. However, Dahal et al. (2010) reported no changes in the levels of mRNA encoding of BTC, AR, HB-EGF, ErbB1, ErbB2, ErbB3, and ErbB4 as well as in the protein levels of ErbB1 between lung homogenates from idiopathic PAH (late stage of the disease) and normal subjects. In the same study, Dahal et al. (2010) found that three clinically approved EGFR antagonists (gefitinib, erlotinib, and lapatinib) substantially reduced the EGF-induced proliferation of PASMCs isolated from healthy or monocrotaline-injected rats. In this rat monocrotaline model of PH, an upregulation of EGF, TGF-α, ErbB1, ErbB2, and ErbB3 mRNA levels has been noted in lung homogenates from monocrotaline-injected rats as compared with control rats. However, no differences were noted between the two groups regarding the levels of mRNA encoding for HB-EGF, epiregulin, AR, and ErbB4 in this rodent PH model. They also found in rats that daily treatment with gefitinib and erlotinib but not with lapatinib started 3 weeks after a subcutaneous monocrotaline injection substantially regressed established PH. In contrast to the beneficial effects of gefitinib and erlotinib treatments in the monocrotaline model, no substantial improvements were observed with these drugs in the chronic hypoxia mouse model of PH. The discordant findings may be attributable to differences in species, disease severity, mechanisms underlying PH induction and/or may suggest a much more complex regulatory network. Indeed, cooperative and synergistic signaling exists between EGF and other factors including TGF-β1 and FGF2 (Kelvin et al. 1989; Ciccolini and Svendsen 1998; Park et al. 2000; Murillo et al. 2005; Ding et al. 2007; Grouf et al. 2007; Uttamsingh et al. 2008).

### 2.4 The Serotonergic System in PAH

Serotonin (5-hydroxytryptamine or 5-HT) is an endogenous vasoactive indolamine found mainly in enterochromaffin tissue, brain, and blood platelets. 5-HT is
synthesized from amino acid tryptophan in two steps: the hydroxylation of tryptophan to form the 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (TPH), and then the decarboxylation of this intermediate by the aromatic L-amino acid decarboxylase. 5-HT is predominantly synthesized in the enterochromaffin cells of the intestine, representing more than 95% of total body 5-HT. In the circulation, 5-HT is actively incorporated into platelets and stored in platelet dense storage, keeping the free circulating 5-HT in low levels (Nilsson et al. 1985; Vanhoutte 1991; Brenner et al. 2007). 5-HT is also synthesized in the raphé nuclei of the brain, pineal gland, and in pulmonary vascular endothelial cells. In addition to SERT, 5-HT concentration is regulated by the mitochondrial enzyme monoamine oxidase (MAO) and by 5-HT storage. 5-HT is metabolized by monoamine oxidase (MAO) in 5-hydroxyindole acetic acid (5-HIAA). There is one 5-HT transporter (5-HTT or SERT) encoded by the \textit{SLC6A4} gene, and seven known families of serotonin receptors: 5-HT1A-E, P, 5-HT2A-C, 5-HT3, 5-HT4, 5-HT5, 5-HT6, and 5-HT7. The serotoninergic system is particularly important for promoting pulmonary arterial smooth muscle cell proliferation, pulmonary arterial vasoconstriction, and local microthrombosis.

The serotoninergic system has long been suspected of playing important roles in the pathogenesis of idiopathic PAH. Plasma 5-HT levels are elevated in patients with PAH and remain high even after lung transplantation, indicating that this condition is not secondary to the disease (Herve et al. 1995). 5-HTT belongs to a large family of integral membrane proteins and is responsible for 5-HT uptake (e.g., by platelets, endothelial and vascular SMCs). Analysis of distal pulmonary arteries of patients with PAH and their cultured PA-SMCs indicates that 5-HTT is overexpressed and that the level of expression correlates with PAH severity (Eddahibi et al. 2001, 2002; Marcos et al. 2004, 2005). Tryptophan hydroxylase (TPH), the rate-limiting enzyme in 5-HT biosynthesis, is also expressed at abnormally high levels in pulmonary endothelial cells from patients with idiopathic PAH and therefore raises 5-HT levels locally (Eddahibi et al. 2006). There is evidence that alterations in platelet 5-HT storage and/or increased platelet consumption by the lung may trigger the development of PAH (Herve et al. 1990, 1995; Breuer et al. 1996; Eddahibi et al. 2000b; Kereveur et al. 2000; Morecroft et al. 2005). Furthermore, serotoninergic appetite suppressant drugs have been associated with an increased risk of developing PAH (Douglas et al. 1981; Gurtner 1985; Loogen et al. 1985; Brenot et al. 1993; Abenhaim et al. 1996; Perros et al. 2008). Serotonylation of RhoA was also proposed as a possible risk factor of pulmonary vascular remodeling in PAH (Guilluy et al. 2007, 2009). Similarly, findings from another recent study from Wei et al. (2012) indicate increased serotonylation of fibronectin in human and experimental PH. During the last few years, direct evidence for a molecular interplay between 5-HTT signaling and Kv1.5 expression/activity has emerged (Guignabert et al. 2006, 2009b; Guignabert 2011).

Additionally, studies on animal models of PH consolidate all these observations obtained from human subjects. Plasma 5-HT levels are elevated not only in rodents treated with the anorectic agent dexfenfluramine (Eddahibi et al. 1998), but also in
the progression of monocrotaline- and chronic hypoxia-induced PH. The chronic infusion of exogenous 5-HT via osmotic pumps can potentiate the development of PH in rats exposed to chronic hypoxia (Eddahibi et al. 1997). A BMPR-II deficiency increases susceptibility to PH induced by 5-HT in mice (Long et al. 2006). In the fawn-hooded rat, a strain with a genetic deficit in platelet 5-HT storage that causes elevated plasma 5-HT concentrations, PH develops when the animals are exposed to mild hypoxia but not in control rats (Sato et al. 1992). An abnormally high level of 5-HTT in the lungs was reported for fawn-hooded rats (Sato et al. 1992; Morecroft et al. 2005). Furthermore, rodents engineered to constitutively express angiopoietin 1 in the lung develop PH. This effect was found to be directly related to the elevated production and secretion of 5-HT by stimulated pulmonary endothelial cells (Sullivan et al. 2003). It has also been shown in the monocrotaline model that 5-HTT expression levels increased prior to the onset of PH, which strongly supports a role for 5-HTT overexpression in disease development (Guignabert et al. 2005). Treatment with selective serotonin reuptake inhibitors (e.g., fluoxetine) abrogates the disease in chronically hypoxic mice and rats with monocrotaline-induced PH (Wang et al. 2011; Marcos et al. 2003; Guignabert et al. 2005, 2009b; Jiang et al. 2007; Zhai et al. 2009; Zhu et al. 2009). Furthermore, mice carrying null mutations at the 5-HTT locus are protected from developing PH induced by prolonged hypoxia (Eddahibi et al. 2000a). Similarly, hypoxia-induced PH in mice lacking the tph1 gene, which exhibit marked reductions in 5-HT synthesis rates and contents in their peripheral organs, was less severe than in wild-type mice (Izikki et al. 2007). More recently, direct evidence that elevated levels of 5-HTT gene expression can promote pulmonary vascular remodeling and spontaneous PH was obtained with the creation of two different types of transgenic mice: (1) SM22 5-HTT+ mice that selectively express the human 5-HTT gene in smooth muscle at levels close to that found in human idiopathic PAH; (2) SERT+ mice that ubiquitously express high levels of the human 5-HTT gene from a yeast artificial chromosome (YAC) construct. SM22 5-HTT+ mice undergo pulmonary vascular remodeling, develop PH, and exhibit marked increases in right ventricular systolic pressures (RVSPs), right ventricular hypertrophy (RVH), and muscularization of pulmonary arterioles. One major point is that PH in these mice developed without any alterations in 5-HT bioavailability and therefore occurred as a sole consequence of the increased 5-HTT protein levels in SMCs. Compared to wild-type mice, SM22 5-HTT+ mice exhibited increases of three- to fourfold in lung 5-HTT mRNA and protein, together with increased lung 5-HT uptake activity. However, there were no changes in platelet 5-HTT activity or blood 5-HT levels. PH worsened as the SM22 5-HTT+ mice grew older (Guignabert et al. 2006). Consistent with these observations, female SERT+ mice housed in normoxic conditions developed a threefold increase in RVSP values compared to those of their wild-type controls (MacLean et al. 2004).

Of the 14 distinct 5-HT receptors, the 5-HT-2A, -2B, and -1B receptors are particularly relevant to the pathogenesis of PAH. High levels of 5-HT-1B, -2A, and -2B receptor immunoreactivity were reported in remodeled pulmonary arteries
from patients with various forms of pulmonary hypertension, but only the 5-HTT was found to be overexpressed in pulmonary artery smooth muscle cells (Marcos et al. 2005). Several lines of evidence support the notion that functional interactions exist between some of these 5-HT receptors and 5-HTT and thus have encouraged studies to better understand these complex relationships (Lawrie et al. 2005; Launay et al. 2006). Antagonism of the 5-HT-2A receptor inhibits not only monocrotaline-induced pulmonary hypertension in mice (Hironaka et al. 2003) but also the 5-HT-induced pulmonary vasoconstriction in vessels from normoxic and hypoxic rats (Morecroft et al. 2005; Cogolludo et al. 2006). However, the 5HT-2A receptor antagonist ketanserin is not specific for pulmonary circulation, and systemic effects have limited its use in PAH (Frishman et al. 1995). 5-HT-2B knockout mice are resistant to hypoxia-induced pulmonary hypertension and administration of the specific 5-HT-2B receptor antagonist RS-127445 prevented an increase in pulmonary arterial pressure in mice challenged with hypoxia (Launay et al. 2002). Furthermore, the 5-HT-2B receptor may control 5-HT plasma levels in vivo (Callebert et al. 2006), and its functional loss may predispose humans to fenfluramine-associated PAH (Blanpain et al. 2003). A very recent study showed that terguride, a potent 5-HT-2A/5-HT-2B receptor antagonist, inhibits the proliferative effects of 5-HT on PA-SMCs and prevents the development and progression of monocrotaline-induced PH in rats (Dumitrascu et al. 2011). The 5-HT-1B receptor mediates 5-HT-induced constriction in human pulmonary arteries (Morecroft et al. 1999) and has been shown to be involved in the development of PH in rodents exposed to chronic hypoxia (Keegan et al. 2001). Recently, Morecroft et al. have reported that co-inhibition of the 5-HT-1B receptor and 5-HTT with a combined 5-HT-1B receptor/5-HTT antagonist (LY393558) is effective at preventing and reversing experimental PH in animal models and 5-HT-induced proliferation in PA-SMCs derived from idiopathic PAH patients.

### 2.5 Other Growth Factor Signaling Systems in PAH

Many other growth factor signaling pathways are suspected to be involved in the pathogenesis of the disease and may serve to identify potential target candidates for anti-proliferative strategies in PH; however, further studies on their pathogenic roles are important.

#### 2.5.1 The Vascular Endothelial Growth Factor Signaling System

The vascular endothelial growth factor (VEGF) signaling system is a potent and selective endothelial cell mitogen implicated in vascularization and angiogenesis and has been shown to be abnormal in PAH. VEGF family consists of five members, VEGFA, B, C, D, and placenta growth factor (PIGF) that show different binding specificities for two tyrosine kinase receptors, VEGFR1 (also called Flt-1)
and VEGFR2 (also called KDR or Flk-1). The binding of VEGF to its receptors induces receptor dimerization and autophosphorylation, which activates several downstream kinases, including protein kinases C and D (PKC and PKD), phosphatidylinositol 3-kinase (PI3K), and MAPK. (Hirose et al. 2000; Voelkel et al. 2006). The VEGF signaling system plays a crucial role for endothelial cell proliferation and promotes the release of endothelial mediators including nitric oxide and prostacyclin (Wheeler-Jones et al. 1997; Dimmeler and Zeiher 1999; He et al. 1999; Nagy et al. 2012). In the lungs of PH patients, VEGF and its receptors are upregulated with a close correlation to the plexiform lesions, suggesting a potential association with the severity of the remodeling process (Cool et al. 1997; Geiger et al. 2000; Hirose et al. 2000; Tuder et al. 2001; Voelkel et al. 2006). Furthermore, patients with PH have an increase in platelet VEGF content (Eddahibi et al. 2000b). The results from the animal models of PH are less clear with differences with the human data and also between the different models studied. On the one hand, Partovian et al. (1998) have reported reduced VEGF mRNA levels in lungs and right ventricles of monocrotaline-injected rats. On the other hand, although no changes in VEGF mRNA levels were noted in lungs from chronically hypoxic rats, an increase in VEGF mRNA levels in right ventricles was observed. Increased VEGF production in the lungs of rats with hypoxia-induced PH was also noted by other investigators (Christou et al. 1998; Laudi et al. 2007). Finally, it has been reported that the VEGF gene transfer in the monocrotaline model (Campbell et al. 2001) and in the chronic hypoxia model (Partovian et al. 2000; Louzier et al. 2003) is beneficial. The VEGFR2 is also increased in two other models of PH: in a canine experimental heart failure (Ray et al. 2008) and in overcirculation-induced PH in piglets (Rondelet et al. 2003). However, it is well known that VEGF signaling is required for maintenance of the alveolar structures (Kasahara et al. 2000) and VEGFR-2 blockade with SU5416 in combination with chronic hypoxia causes PH in rats (Taraseviciene-Stewart et al. 2001) and in mice (Ciuclan et al. 2011). In conclusion, further studies are required to identify the precise function of the pathogenic role of VEGF in early stages and during disease progression.

2.5.2 The Insulin-Like Growth Factor Signaling System

The insulin-like growth factor (IGF) signaling system consists of three different ligands (insulin, IGF1 and IGF2), two receptors (IGF1R, IGF2R), and from at least six high-affinity IGF-binding proteins (IGFBP 1-6). IGF1 is mainly secreted by the liver and binds with high affinity to IGF1R and with low affinity to the insulin receptor and IGF2R. IGF2 binds with high affinity to IGF2R and with low affinity to IGF1R and insulin receptor. IGFR1 and IGFR2 share 70 % homology in their protein sequences, but they present differences in signaling and functions. The binding of a ligand to IGFR1 leads to activation of distinct downstream signaling pathways such as the MAPK signaling and the phosphatidylinositol 3-kinase-Akt (PI3K-Akt) pathway. Since IGFR2 does not have an intra-cytoplasmic signaling
domain, the binding of a ligand to IGFR2 doesn’t initiate downstream signaling events. Different subtype of IGFBPs can result in enhancement or inhibition of IGF-mediated cellular effects. The IGF signaling system plays a pleiotropic role in normal cell metabolism, growth, proliferation, differentiation, cell–cell and cell–matrix adhesion, and survival. Recently, Guo et al. (2012) have found a substantial increase in IGFR1 in pulmonary arteries from rats exposed 9 days to hypoxic environments, suggesting a potential involvement of the IGF signaling system in the disease.

2.5.3 The Protein Kinase B/Mammalian Target of Rapamycin Signaling Pathway

Mammalian target of rapamycin (mTOR) is a 289-kDa serine/threonine protein kinase and a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family. mTOR consists of several conserved functional domains: a catalytic kinase domain, a FKBP12-rapamycin-binding (FRB) domain, a putative autoinhibitory domain (repressor domain) near the C-terminus and up to 20 tandemly repeated HEAT (Huntington, EF3, A subunit of PP2A, TOR1) motifs at the amino terminus, and FAT (FRAP-ATM-TRRAP) and FATC (FAT C-terminus) domains. In fact, mTOR is found to associate with different cofactors and form two distinct multiprotein complexes, mTORC1 and mTORC2 (Wullschleger et al. 2006; Dann et al. 2007). mTORC1 is sensitive to rapamycin and regulates ribosome biogenesis, autophagy, translation, transcription, and mitochondrial metabolism. mTORC1 is activated by mitogens via the PI3K-Akt and ERK1/2 signaling pathways that promote activation of S6 kinase 1 (S6K1), cell growth, and proliferation (Krymskaya and Goncharova 2009). In contrast, mTORC2 is insensitive to rapamycin and functions as an important regulator of actin cytoskeleton through stimulation of F-actin stress fibers. mTORC2 is activated by insulin in a PI3K-dependent manner and phosphorylates protein kinase B (Akt) at Ser-473, which promotes cell cycle progression and increases cell survival (Laplante and Sabatini 2009). The development of monocrotaline- and hypoxia-induced PH in rats has been found to be associated with marked activation of the Akt/glycogen synthase kinase (GSK)-3 axis (Gary-Bobo et al. 2010). In addition to being a central regulator of cell growth, proliferation, apoptosis, and metabolism, mTOR is also linked to the phosphatidylinositol 3 kinase (PI3K)/phosphatase and tensin homolog (PTEN)/Akt/tumor suppressor complex (TSC) signaling pathway, where genetic mutations of many components in this pathway result in the development of a wide variety of cancers (Fig. 2).

Recent evidence demonstrated that mTOR activation in both mTORC1 and mTORC2 due to chronic hypoxia exposure is required for pulmonary arterial smooth muscle cell proliferation (Krymskaya et al. 2011). Similarly, Gerasimovskaya et al. (2005) showed that hypoxia-induced adventitial fibroblast proliferation requires activation and interaction of PI3K, Akt, mTOR, p70S6K, and ERK1/2 and provided evidence for hypoxic regulation of protein translational
pathways in cells exhibiting the capability to proliferate under hypoxic conditions. Furthermore, Ogawa et al. (2012) reported that PDGF-induced phosphorylation of Akt/mTOR signaling pathway in normal pulmonary arterial smooth muscle cells and that inhibition of Akt/mTOR signaling pathway by either rapamycin or Akt inhibitor attenuates PDGF-induced increase in store-operated calcium entry in these cells (Ogawa et al. 2012). Further studies into the role of Akt signaling are important, and the Akt/mTOR pathway may be a potential target for the anti-proliferative strategy in PH.

**Fig. 2** Schematic representation of the mTOR-signaling pathway. Akt protein kinase B; Erk extracellular signal regulated kinase; FoxO Forkhead box, class O; HIF1α hypoxia-inducible factor 1 α; IKKβ inhibitor of nuclear factor kappa β; JAK the Janus kinase; Mek mitogen-activated protein kinase; PDK1 phosphoinositide-dependent kinase-1; P13K phosphoinositide 3-kinase; PIP2 phosphatidylinositol 4,5-bisphosphate; PIP3 phosphatidylinositol 3,4,5-trisphosphate; PKCα protein kinase C α; pras40 proline-rich AKT substrate of 40 kDa; PTEN phosphatase and tensin homolog; RTK receptor tyrosine kinase; SCK1/2, suppressor of loss of cAMP-dependent protein kinase 1/2; S6K1 S6 kinase 1; SREBP1 sterol regulatory element-binding protein 1; TSC1/2 tuberous sclerosis complex 1/2

Emerging Molecular Targets for Anti-proliferative Strategies in Pulmonary...
2.5.4 The Janus Kinase/Signal Transducers and Activators of Transcription Signaling System

Four JAK family kinases, including JAK1, JAK2, JAK3, and TYK2, and seven STAT family members, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, have been identified. JAK1, JAK2, and TYK2 appear to be ubiquitously expressed, while JAK3 expression is normally limited to lymphoid cells. In addition, different isoforms of several STATs have been identified. STATs are latent cytoplasmic transcription factors that become activated after recruitment to an activated receptor complex. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is activated by a wide variety of cytokines, growth factors, interferons, and some hormones. Following the binding of cytokines to their cognate receptor, STATs are activated by members of the JAK family of tyrosine kinases. Once activated, they dimerize and translocate to the nucleus and modulate the expression of target genes. STATs are not only activated by cytokine receptors that associate with JAKs but also by RTKs and G protein-coupled receptors.

Over the last few years, increased evidence has supported the role of inflammation as well as the involvement of immunologic disorders in idiopathic PAH (Humbert et al. 1995; Dorfmuller et al. 2003; Tamby et al. 2005; Terrier et al. 2008; Kherbeck et al. 2013; Tamosiuniene et al. 2011; Huertas et al. 2012; Perros et al. 2012; Price et al. 2012). An abnormal activation of the JAK/STAT signaling system was firstly noted by Mathew et al. (2004) in the monocrotaline-induced PH and by Masri et al. (2007) in human tissues of patients with idiopathic PAH. Besides its central role in chronic inflammation, dysregulated activation of the JAK/STAT3 signaling pathway was shown to play an important role in pulmonary arterial endothelial cell proliferation and survival in response to growth factors as demonstrated by pharmacological inhibition of STAT3 phosphorylation by AG-490 (Masri et al. 2007). More recent evidences of the pathogenic role of the JAK/STAT signaling system were documented by the group of Bonnet et al. (Courboulin et al. 2011, 2012; Paulin et al. 2011a, b) showing close interrelationships with Pim1 (proviral integration site for Moloney murine leukemia virus) kinase and NFATc2 (nuclear factor of activated T-cells). It is now well established that among inflammatory cytokines interleukin-6 (IL-6) plays an important role in the development of PH. Overexpression of IL-6 promotes PH in mice (Steiner et al. 2009), while IL-6-deficient mice are protected from hypoxia-induced PH (Savale et al. 2009). In addition, elevated serum IL-6 levels have been reported in patients with idiopathic PAH or PH associated with inflammatory diseases such as scleroderma and lupus (Nishimaki et al. 1999; Pendergrass et al. 2010; Soon et al. 2010). All known members of the IL-6 cytokine family (IL-6, IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukemia inhibitory factor (LIF), and oncostatin M (OSM)) through their receptors comprised of the signal transducer gp130 in combination with IL-6R, IL-11R, LIF-R, or OSM-R are known to potently activate STAT3 (Fischer and Hilfiker-Kleiner 2007). A better knowledge of the molecular processes by which JAK-STAT...
signaling can be turned off will certainly help to identify new targets for drug treatment in PH.

2.5.5 The RhoA/Rho-Kinase Signaling System

RhoA, a small GTPase protein, and its immediate downstream target, RhoA/Rho-kinase (ROCK), control a wide variety of signal transduction pathways. ROCKs are kinases belonging to the AGC (PKA/PKG/PKC) family of serine–threonine kinases that exist in two isoforms: ROCK1 and ROCK2 (Nakagawa et al. 1996). ROCK is comprised of an amino-terminal kinase domain, followed by a coiled-coil region that contains the ρ-binding domain. The carboxy-terminal consists of a plexstrin-homology domain, which contains an internal cysteine-rich domain. ROCK1 and ROCK2 are highly homologous, sharing an identity of 65 % in their overall amino acid sequences and 92 % in their kinase domains. In addition to their effect on actin organization, or through this effect, ROCKs have been found to regulate a wide range of fundamental cell functions such as contraction, motility, proliferation, and apoptosis.

Recent pharmacological studies suggest that activation of RhoA/ROCK signaling system is an important event in the pathogenesis of PH. In vivo, beneficial effects of treatment with Rho kinase inhibitor fasudil have been demonstrated in several animal models of PH (Nakagawa et al. 1996; Abe et al. 2004; Fagan et al. 2004; Nagaoka et al. 2004, 2005; Guilluy et al. 2009). In addition, the beneficial effect of sildenafil on PH is mediated, at least in part, by the inhibition of the RhoA/Rho kinase pathway (Guilluy et al. 2005). Serotonylation of RhoA by intracellular type 2 transglutaminase (TG2), leading to constitutive RhoA activation was also proposed as a possible risk factor of pulmonary vascular remodeling in PAH (Guilluy et al. 2007, 2009). Similarly, findings from another recent study from Wei et al. (2012) indicates increased serotonylation of fibronectin in human and experimental PH.

2.5.6 Other Growth Factor Signaling Systems

Several other studies have similarly found that other growth factor signaling might be involved in the pathogenesis of PAH such as angiotensin II (AngII) (de Man et al. 2012), connective tissue growth factor (CTGF) (Lee et al. 2005), hepatocyte growth factor (HGF) (Ono et al. 2004a, b; Hiramune et al. 2011), nerve growth factor (NGF) (Ieda et al. 2004; Kimura et al. 2007), and placenta growth factor (PIGF) (Sundaram et al. 2010; Sands et al. 2011).
3 Restitution of the Aberrant Extracellular Matrix Remodeling in PH

The proteolytic ECM remodeling not only causes qualitative and quantitative changes in the ECM, but is also actively involved in the creation of a permissive pericellular/extracellular environment for cell proliferation, survival and migration. Indeed, an aberrant ECM remodeling can (1) generate the excessive releases of growth factors and various molecules that are encrypted in the ECM; (2) expose functionally important cryptic sites in collagens, laminins, elastin, or fibronectin; (3) generate fragments of various ECM components (Giannelli et al. 1997; Rabinovitch 2001; Shang et al. 2001; Xu et al. 2001; Ma et al. 2011; Wei et al. 2012).

Various studies demonstrated an imbalance between proteases and protease inhibitors in PH which, among others, include defects in several proteolytic enzymes: elastases (Rabinovitch 1999; Kim et al. 2011), matrix metalloproteinases (Lepetit et al. 2005; George et al. 2012), chymase (Mitani et al. 1999), and tryptase (Kwapiszewska et al. 2012). In addition, impairments of both the urokinase-type plasminogen activator (uPA)—plasmin or the tissue-type plasminogen activator (tPA)—plasmin systems have also been reported in PH (Huber et al. 1994; Christ et al. 2001; Katta et al. 2008; Kouri et al. 2008). Beneficial effects of serine elastase inhibitors in several experimental models of PH have been obtained (Ilkiw et al. 1989; Maruyama et al. 1991; Cowan et al. 2000a; Zaidi et al. 2002). Similar results were found with MMP inhibitors in the monocrotaline model (Vieillard-Baron et al. 2003), but deleterious effects were found with these MMP inhibitors in the chronic hypoxia model (Vieillard-Baron et al. 2000). Differences between both animal models might partially explain the different outcome obtained with MMP inhibitors. Collectively, these findings support that restitution of the aberrant ECM remodeling in PAH may represent another strategy for inhibition of pro-migratory and pro-proliferative signaling pathways.

4 Restitution of the Dysfunctional BMPR-II Signaling System in PAH

Bone morphogenetic proteins (BMPs) are a large family of secreted molecules that belongs to the transforming growth factor (TGF) β family. To date, over 20 BMP family members and 10 antagonists have been identified and characterized. They operate with varied duration, distance, and affinity. There are two classes of transmembrane receptors, type I receptors (ACVRL-I, ACVR-I, BMPR-IA, and BMPR-IB), and the type II receptors (BMPR-II, ActR-IIA, and ActR-IIB). Following ligand binding, the kinase domain in BMPR-II phosphorylates the type 1 receptor, which then phosphorylates Smad proteins 1, 5, and 8. Following activation of Smad 1, 5, and 8, a complex with the common partner Smad4 is generated and it migrates into the nucleus and transactivates specific target genes involved in cell
proliferation, survival, migration, and differentiation. Bmpr2 gene mutations confer a reduction in the BMPR-II signaling activity (Foletta et al. 2003) resulting from a dose-dependent modulation of BMPR-II oligomerization with its co-receptor, most commonly, BMPR-IA (Gilboa et al. 2000). BMPR-II expression is also substantially reduced in patients with various form of PH without a mutation, as well as in experimental animal models (Takahashi et al. 2006; Reynolds et al. 2012). Steady-state levels of BMPR-IA are also reduced in the pulmonary vasculature of patients with pulmonary hypertension (Du et al. 2003), suggesting that disrupted BMP signaling contribute to the pathogenesis of PAH and/or represent a genetic susceptibility of developing the disease. Recently, a study by Reynolds et al. (2012) suggested a therapeutic potential for upregulation of the BMPR-II axis in PAH.

It has been shown that the BMPR-II signaling system plays pleiotropic roles, depending on the cell types: on the one hand, BMPs inhibit proliferation of smooth muscle cells; on the other hand, they promote pulmonary arterial endothelial cell survival (Teichert-Kuliszewska et al. 2006; Nasim et al. 2012). In addition, a constitutive activation of p38MAPK has been shown in primary cultured pulmonary arterial smooth muscle cells harboring a mutation in BMPR-I, a phenomenon that could contribute partly to the failure to suppress cell proliferation (Yang et al. 2005). Although further studies are required to determine the importance of these abnormalities for the initiation/progression/reversal of the disease, restitution of the dysfunctional BMPR-II signaling system may represent another anti-proliferative strategy.

5 Recovery of Oxidative Metabolism in PAH

In the presence of oxygen, normal cells completely oxidize glucose to CO₂ and H₂O, and generate ATP through aerobic oxidation. The Warburg effect is defined as an increased dependence on glycolysis for ATP synthesis, even in the presence of abundant oxygen. The Warburg effect has been found in a wide spectrum of human cancers as well as in PH (Xu et al. 2007), however the underlying mechanisms are still unclear. In cancer cells, this metabolic change has been found to be regulated by both oncogenes and tumor suppressor genes including hypoxia-inducible factors (Goda and Kanai 2012), p53 (Puzio-Kuter 2011), E2F transcription factor-1 (Puzio-Kuter 2011), and phosphatase and tensin homolog (PTEN) (Garcia-Cao et al. 2012). Interestingly, several groups have shown abnormalities in these different signaling pathways in PAH (Bonnet et al. 2006; Natali et al. 2011; Ravi et al. 2011). Restitution of oxidative metabolism with the use of dichloroacetate has been shown to be efficient in several animal models of PH (Michelakis et al. 2002; McMurtry et al. 2004; Guignabert et al. 2009b). Inhibition of pyruvate dehydrogenase kinase (PDK) by dichloroacetate frees up the mitochondrial gate-keeping enzyme pyruvate dehydrogenase (PDH), which is then able to convert pyruvate to acetyl-CoA and initiate normal oxidative phosphorylation via the Krebs cycle.
Since mitochondrial fatty acid oxidation (FAO) contributes to the “Randle cycle” inhibition of glucose utilization, the FAO inhibition prevents also this metabolic shift and limits the proliferative and anti-apoptotic cell phenotype observed in PH (Sutendra et al. 2010).

6 Conclusions and Challenges

In this chapter, we summarize several different emerging molecular targets for anti-proliferative strategies in pulmonary hypertension (Fig. 3).

However, growth factors, RTKs, BMPR-II, energetic and metabolic adaptation, as well as the proteolytic ECM remodeling control various aspects of normal cellular physiology, including cell growth, differentiation, motility, and death. Both the potency and selectivity of TKIs as well as of other anti-proliferative molecules are therefore important considerations, particularly as these agents are...
being tested as a potential therapeutic approach to PAH. Another important aspect concerns the potential impacts of these anti-proliferative molecules on the adaptive response of myocardial hypertrophy that need to be further evaluated. Therefore, a better understanding of how these factors act in the lung as well as in the heart under normal and pathologic conditions will provide a stronger rationale for their use in specific therapeutic interventions and minimize the adverse effects of less focused treatments.

Substantial work remains to be done to discover and/or develop a new, better-tolerated, and more powerful therapeutic tool for PAH that combines promotion of vasorelaxation, suppression of cellular proliferation, and activation of apoptosis within the pulmonary-artery wall. Therefore, further studies are needed to better evaluate the overall risk benefit ratio of the available and future anti-proliferative molecules in PAH as well as their efficacies in experimental models of PH.

Acknowledgments The authors thank Pr. Marc Humbert, Pr. Elie Fadel, Pr. Philippe Dartevelle and Pr. Gérald Simonneau for valuable discussions and suggestions.

References


Jones PL, Rabinovitch M (1996) Tenascin-C is induced with progressive pulmonary vascular disease in rats and is functionally related to increased smooth muscle cell proliferation. Circ Res 79(6):1131–1142
Emerging Molecular Targets for Anti-proliferative Strategies in Pulmonary...


436 L. Tu and C. Guignabert