Cardiovascular Research (2014) **104**, 388–398
doi:10.1093/cvr/cvu215

REVIEW

Id proteins in the vasculature: from molecular biology to cardiopulmonary medicine

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Received 7 January 2014; revised 14 September 2014; accepted 19 September 2014; online publish-ahead-of-print 1 October 2014

The inhibitors of differentiation (Id) proteins belong to the helix-loop-helix group of transcription factors and regulate cell differentiation and proliferation. Recent studies have reported that Id proteins play important roles in cardiogenesis and formation of the vasculature. We have also demonstrated that heritable pulmonary arterial hypertension (HPAH) patients have dysregulated Id gene expression in pulmonary artery smooth muscle cells. The interaction between bone morphogenetic proteins and other growth factors or cytokines regulates Id gene expression, which impacts on pulmonary vascular cell differentiation and proliferation. Exploration of the roles of Id proteins in vascular remodelling that occurs in PAH and atherosclerosis might provide new insights into the molecular basis of these diseases. In addition, current progress in identification of the interactors of Id proteins will further the understanding of the function of Ids in vascular cells and enable the identification of novel targets for therapy in PAH and other cardiovascular diseases.

Keywords Id • Inhibitor of DNA binding • Vasculature • PAH • Pulmonary arterial hypertension

1. Introduction

Impairment of vessel structure and function in response to pathophysiological factors contributes to numerous cardiovascular disorders. Endothelial dysfunction is an important early feature of vessel injury and promotes vasoconstriction leading to hypertension.[1] Abnormal remodelling of the vessel wall is common in atherosclerosis, systemic and pulmonary hypertension, and restenosis following vein grafting or angioplasty.[2,3] In addition, disordered angiogenesis is important in diseases such as pulmonary arterial hypertension (PAH) and also is an important contributor to tumour growth.[4]

Transcription factors are widely expressed in vascular cells and contribute to the maintenance of normal vessel homeostasis.[5] Several classes of transcription factors are known to be involved in the regulation of vascular cell differentiation and proliferation.[6] Recent studies implicate the Id family of proteins, which are helix-loop-helix (HLH) transcription factors, as important regulators of vascular cell function. Based on the dominant-negative effect of Id proteins on transcription factor binding, they promote wide-ranging effects on the expression of genes involved in vasculogenesis and angiogenesis.[7–9] Id proteins interact with tissue-specific transcription factors, but are also themselves regulated by key peptides and cytokines involved in vascular function, including angiotensin II, bone morphogenetic protein (BMP), and VEGF.[10–12] Id proteins act as orchestrators of multiple biological responses induced by these and other cytokines. Here, we will review the biological function of Id proteins in the cardiovascular system by examining their known protein-binding partners and gene targets, and then focus on the role of Id proteins in cardiogenesis, vascular cells, and cardiovascular diseases such as pulmonary hypertension and atherosclerosis. Finally, the potential of Id proteins as a new therapeutic target will be discussed.

1.1 Inhibitors of DNA binding and inhibitors of differentiation

Id proteins were first identified in 1990.[13] Since then four members of the Id family, Id1–Id4, have been identified in mammalian cells,[14–16] with differing expression patterns and protein structure. For example, Id4 is expressed at a much lower level in the vascular wall than the other three Id proteins.[17] Id2, Id3, and Id4 but not Id1 possess a consensus cyclin-dependent kinase 2 (CDK2) phosphorylation site in the N-terminus (*Figure 1*).[18,19] Further details of the specific differences between Id proteins are summarized in *Table 1*. These differences may have important functional consequences. For example, only Id2 binds specifically with retinoblastoma protein to induce cell cycle arrest, whereas Id3 induces apoptosis in vascular lesions by other mechanisms.[26,32]

Id proteins belong to the HLH family of transcription factors. Usually, basic HLH (bHLH) transcription factors possess a basic DNA-binding domain and a HLH region that mediates protein–protein interactions. bHLH factors activate transcription as homodimeric or heterodimeric complexes through binding to DNA where there is a specific recognition motif in the promoter region of the target gene, named E-box

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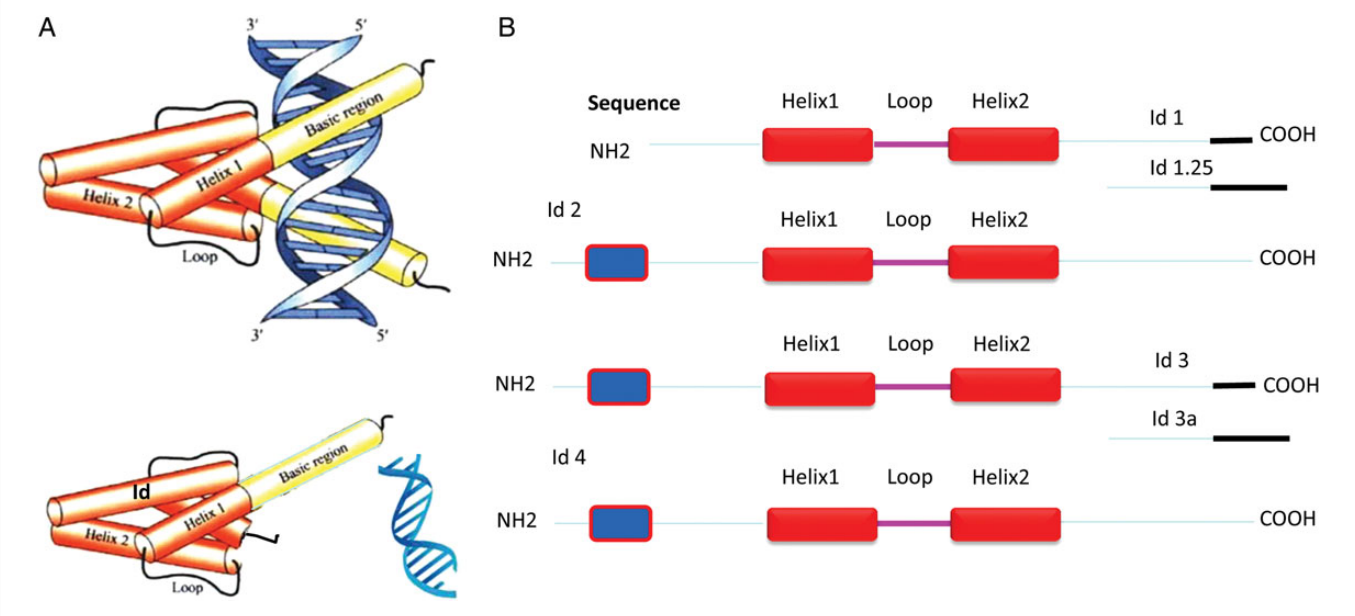

Figure 1 The structure and sequence homology of Id proteins. (*A*) bHLH proteins form dimers in order to bind to DNA (upper), and Id proteins have no basic region and inhibit the binding of bHLH protein to DNA through dimerization (lower). (*B*) The amino acid sequence differences between Id1, Id2, Id3, and Id4. The blue box represents the CDK2 phosphorylation site on the N-terminal regions of Id2, Id3, and Id4. The HLH conserved sequence is important for Id dimerization with other E proteins. Id1 and Id3 may exist as isoforms: Id1.25 and Id3a, resulting from intron retention at the C terminus.

Table 1 The known functions of individual Id proteins in vascular cells

Official human gene symbol	Alternative name	Human chromosome	Subtype	Effect on ECs	Effect on EPCs	Effect on VSMCs
Id1	bHLHb24	20q11	Id1.25[20]	Pro-angiogenesis[5] Activates ECs[21]	Controls EPCs formation[5,22] Promotes EPC migration and proliferation[23]	Mediates MAPK and angiotensin II-induced proliferation[6]
Id2	bHLHb26	2p25		Activate ECs[21] Proliferation and differentiation[24]		Modulates phenotype[25]
Id3	bHLHb25	1p36.13–p36.12	Id3a[26]	Pro-angiogenesis[5] Proliferation and differentiation[24]	Controls EPCs formation[5]	Proliferation[10,26–30] Modulates phenotype[25] Differentiation[31]
Id4	bHLHb27	6p22.3				

(CANNTG) or N-box (CACNAG), where *N* = any nucleotide.[33,34] While Id proteins lack the basic DNA-binding domain, they still form heterodimers with bHLH transcription factors. However, the resulting complex cannot bind with DNA and promote gene transcription. Thus, Id proteins function as dominant-negative transcriptional regulators and have been named 'inhibitors of DNA binding'. Soon after their identification, Id proteins were found to be associated with cell differentiation. In mammalian cell culture systems, Id expression was down-regulated during the differentiation of some cell lineages.[35–38] *In vivo* studies showed that targeted expression of Id genes could inhibit muscle cell differentiation.[39] Thus, Id proteins are also alternatively known as *i*nhibitors of *d*ifferentiation.

Id genes demonstrate characteristics of early response genes that respond to a multitude of ligand-receptor combinations at the cell surface. However, much less is known about the range of extracellular signals that trigger expression of Id gene. Members of the transforming growth factor (TGFβ) superfamily, including BMPs and TGF-β, regulate Id1 expression via Smad-dependant pathways in certain cell types, and the regulation occurs at the level of the Id promoter.[40] Id protein levels can also be regulated via the ubiquitin-proteasome degradation pathway or by phosphorylation during cell cycle progression. It has been reported that Id proteins turn over quickly after activation and have a short half-life of ~20 min in HEK293 cells. The half-life varies for individual Ids and is dependent on the cell type. It is noteworthy that when Id proteins exist in the heterodimeric state, they are less sensitive to degradation by the 26S proteasome. In addition, the localization of Id proteins from cytoplasm to nucleus only occurs following formation of the heterodimer.[41] These studies suggest the important relationship between Id protein stability, localization, and function, though the precise relevance of this, has not yet been revealed.

1.2 Id protein-binding partners and targets

The main targets of Id proteins are E-proteins (e.g. E12, E47, E2-2, and HEB), which belong to the class I of bHLH transcription factors.[42–44] E-proteins are ubiquitously expressed in many tissues including the vasculature. They also associate with class II bHLH factors (e.g. MyoD and NeuroD), forming heterodimers, and modulate cell type-specific gene transcription and cellular differentiation.[6] Id proteins can interact with class I and II bHLH factors, but individual Id proteins have distinct preferences for these targets.[13,45] Id proteins bind to class I bHLH factors (E12, E47, E2-2, and HEB) with high affinity. In contrast, a broad range of affinities exists for class II proteins, such as the myogenic regulatory factors (MyoD, myogenin, Myf-5, and MRF4/MYF-6). No interactions were observed between the Ids and the haematopoietic bHLH factors (Scl/Tal-1, Tal-2, and Lyl-1). The first helix of the HLH domain of the Id protein and the residues immediately adjacent to it are responsible for the dimerization selectivity.[46] In addition to bHLH factors, Id proteins can also interact with some non-bHLH factors and this function is probably as important as the Id–bHLH interactions. For example, non-bHLH-binding partners, such as Rb (retinoblastoma protein, a tumor suppressor protein), ETS (E26 transformation-specific or E-twenty-six, one of the largest families of transcription factors), and PAX (paired box transcription factors, the important regulators in early development), are known for their roles in cell fate determination; proteasome components were reported recently as another important group of non-bHLH interactors with Id proteins.[32]

Recently, new binding partners have been identified (*Table 2*), including hairy and enhancer of split-1 (Hes1) and dead ringer-like-1 (Dril1) that have a similar protein structure to Id1.[47,54] Bai *et al.* showed that Id proteins interact directly with Hes1 and release the negative feedback autoregulation of Hes1 without interfering with its ability to affect other target genes. These results indicate that Id proteins participate in the maintenance of neural stem cells through sustaining Hes1 expression in early embryos.[54] The interaction with Hes1 also facilitates the Ids to regulate tip- vs. stalk-cell selection and vessel plasticity.[8] Since Notch3 activation of Hes5 was found to be crucial for the proliferation of smooth muscle cells in animal models of PAH, the interaction of Ids with Hes family members warrants further studies in this setting.[61] Furthermore, Id1 interacts with Dril1 to interrupt its binding to DNA, providing a potential mechanism for suppression of fibrosis through inhibition of the profibrotic function of Dril1.[47]

Id1, Id2, and Id3 are subject to ubiquitination and subsequent degradation by the 26S proteasome. Ling *et al.*[49] reported that Id1 interacts with the Hepatitis-B virus-encoded protein HBX, and that Id1 binds to the proteasome subunit C8 to facilitate its interaction with the HBX protein. In addition, the proteasome subunit S5a was previously isolated as an Id1-interacting protein.[48] A yeast two hybrid screen identified that Id3 interacts with a mouse JAB1 homologue, which is related to factors thought be present in the 19S regulatory complex of the 26S proteasome.[48] The ubiquitously expressed APC/Cdh1 complex is an E3 ubiquitin ligase that governs Id2 stability and reprogrammes quiescent neurons into the axonal growth mode.[55] Ubiquitin-specific protease 1 (USP1) deubiquitinates and stabilizes Id1, Id2, and Id3. USP1 directly interacts with Id2 in HOS and U2-OS cells to preserve the stem cell state.[50]

More recently, endogenous inhibitors of Ids have been discovered in neuroblastoma cells, including an inhibitor of Id2, known as 13I. 13I was isolated from a phage display library of HLH domains, harbouring amino acid substitutions in residues critical for dimerization.[62] 13I selectively binds to Id2, impairs complex formation with Rb, and relieves repression of E protein-activated transcription. A novel peptide aptamer, Id1/3-PA7, was found to specifically interact with Id1 and Id3. Id1/3-PA7 deregulated expression of Id1 and Id3, releasing E47 to activate the E-box promoter and increased the expression level of CDK inhibitors, p16 and p21. This non-toxic exogenous agent led to antiproliferative and apoptotic effects in ovarian cancer cells.[63] Although Id proteins have been found to be expressed at high level in most types of cancer, they can also act as tumour suppressors in certain lymphomas.[32] Thus, understanding the cell-specific and tumour-specific effects of Id modulators is of great importance before embarking on clinical trials.

1.3 Role of BMP type II receptor and Ids in cardiogenesis

BMPs are pleiotropic cytokines regulating growth, differentiation, and apoptosis in diverse cell types and act as instructive signals during embryogenesis. BMPRII, like other TGFβ superfamily type II receptors, is a constitutively active serine/threonine kinase. BMPs signal via three type II receptors (BMPRII, ActRIIA, and ActRIIB). Binding of ligand leads to the assembly of a hetero-oligomeric receptor complex in which the type II receptor phosphorylates and activates the type I receptor. The activated type I receptor then phosphorylates intercellular signalling molecules, termed Smads. Smad complexes translocate into the nucleus to regulate transcription of target genes. Id HLH proteins are among of the most highly regulated downstream targets of BMP/Smad signalling.[12,21,24]

It was reported in 2000 by two groups that heterozygous germ-line mutations in BMP type II receptor caused >70% of cases of familial PAH.[64–66] Homozygous *bmpr2* knockout (KO) mouse die during gastrulation without forming mesoderm. Heterozygous *bmpr2* KO mice (heterozygous mutation generated by deletion of exon 4, 5, 6 in C57BL/6J mice) recapitulate the major effect of BMPR2 mutations in man, which is haploinsufficiency. In response to an additional injury, such as inflammation, hypoxia, or serotonin, heterozygous *bmpr2* KO mice developed pulmonary hypertension and remodelling of the pulmonary vasculature.[67] Beppu *et al.* employed Mox2-Cre to delete *bmpr2* throughout the embryo during gastrulation (targeting the epiblast at E5.5). These investigators found cardiac defects including double-outlet right ventricle, ventricular septal defect (VSD), atrioventricular cushion defects, and thickened valve leaflets in these mice. BMPRII was also required for proper positioning of the aorta (*Figure 2*). Thus, although there was no obvious myocardial defect in these mice, endocardial BMPRII expression was required for septum formation and valvulogenesis.[68]

Similar defects were also found in compound Id KO mice lacking Id1, Id2, and Id3 ($Id1^{-/-}Id3^{-/-}$; $Id1^{-/-}Id2^{-/-}$; $Id1^{-/-}Id2^{+/-}Id3^{-/-}$; and $Id1^{+/-}Id2^{+/-}Id3^{-/-}$).[69,70] In the developing heart, Id1, Id2, and Id3 are detected in the endocardium and epicardium from embryonic day E10.5 through E16.5, but Id4 is absent. Id1 and Id3 expression persists in these regions at post-natal day P7. Id1, Id2, or Id3 single KO embryos do not exhibit developmental abnormalities, but double- and triple-Id KO embryos display severe cardiac defects and die at mid-gestation. Double KO embryos displayed VSDs associated with impaired ventricular trabeculation and thinning of the compact myocardium. Since the defect in the myocardium was not observed in $BMPRII^{flox/-}$ Mox2-Cre mice, the lack of expression of Ids in the myocardium provides support for the idea that the myocardial defect of Id KO mice may arise from dysregulated molecular signalling between the myocardium and the epicardium or endocardium. Compound Id KO mouse

Table 2 Binding partners of Id proteins and their biological function (©: common for Id1, Id2, Id3; 1&3: common for Id1 and Id3; 2&3: common for Id2 and Id3)

Id	Class I HLH-binding partner	Other class HLH	Non-HLH factors	Targeted gene and downstream effector	References
Id1	E47			Mediates BMP-induced EC migration	(12)
		Dril1		Suppression of fibrosis through inhibiting Dril1	(47)
			26S proteasome subunit S5a	Regulates terminal myogenic differentiation	(48)
			20S proteasome subunit C8	Regulates HBX protein stability in HCC cells	(49)
	E12, E47, E2-2, HEB	MyoD, Myf-5		Plays a role in myogenesis©	(46)
			USP1	Preserves a mesenchymal stem cell programme in osteosarcoma©	(50)
	E47			Suppresses Rap1GAP to synchronize stemness with adhesion©	(9)
		Hes1		Regulates tip- vs. stalk-cell selection and vessel plasticity©	(8)
	E47			Suppression of neural differentiation©	(51)
	E47			Regulates p21 to control NIH 3T3 cells growth (1&3)	(52)
	E2-2			Regulates p16 and VEGF-induced angiogenesis in ECs (1&3)	(11)
				Regulates p16/p21 in prostate cancer cells independent of E protein (1&3)	(53)
Id2		Hes1		Neural stem cells maintenance in early embryos	(54)
			APC/Cdh1 (E3ubiquitin ligase)	Inhibition of axonal growth	(55)
			Rb	Mediates cell cycle regulation by Myc	(56)
				Promotes cell death by increase Bax level independent of E protein	(57)
	E12, E47, E2-2, HEB	MyoD, Myf-5		Plays a role in myogenesis©	(46)
			USP1	Preserves a mesenchymal stem cell programme in osteosarcoma©	(50)
	E47			Suppresses Rap1GAP to synchronize stemness with adhesion©	(9)
		Hes1		Regulates tip- vs. stalk-cell selection and vessel plasticity©	(8)
	SRF/E12			Binding on SM-α-actin promoter to control SMC differentiation (2&3)	(25)
Id3	E47			SREBP-1c-mediated adiponectin expression	(58)
	E47			Regulates VSMCs through P21 within vascular lesion	(26)
			26S proteasome regulatory complex 19S	Degraded through the ubiquitin-proteasome pathway when E47 is absent	(48)
	E12, E47, E2-2, HEB	MyoD, Myf-5		Plays a role in myogenesis©	(46)
			USP1	Preserves a mesenchymal stem cell programme in osteosarcoma©	(50)
	E47			Suppresses Rap1GAP to synchronize stemness with adhesion©	(9)
		Hes1		Regulates tip- vs. stalk-cell selection and vessel plasticity©	(8)
	E12, E47, E2-2, HEB	MyoD		Negatively regulates E2A to inhibit fibroblasts growth by serum (3)	(59)
	SRF/E12			Binding on SM-α-actin promoter to control SMC differentiation (2&3)	(25)
Id4				Suppresses tumour growth through AR, p21, p27, and p53 in prostate cancer	(60)

demonstrates more severe septal defects and disrupted endocardial cell lining, which suggests that the Id genes are important target genes of a range of BMP receptors during cardiogenesis.

1.4 Id proteins in endothelial cells and angiogenesis

BMPs induce Id gene expression in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). Besides BMPs, growth and differentiation factor 5 (GDF5) and TGF-β also regulate the expression of Id proteins. GDF5 induces Id1 and Id3 expression in human umbilical vein smooth muscle cells.[71] Although, TGF-β induces the expression of Id genes, this tends to be transient in some cell types due to induction of Id1 that is then suppressed by cyclic AMP-dependent transcription factor 3.[72]

Although previous reviews described the role of Id proteins in angiogenesis during tumour formation, their effects on the disordered angiogenesis in PAH still remain unclear.[73] Here, we review updated information about the involvement of Id gene induction by diverse stimuli and their contribution to angiogenesis and vascular remodelling.

Proliferation and migration of ECs is necessary for angiogenesis. Id proteins are thought to act as pro-angiogenic factors by regulating migration, proliferation, and apoptosis of ECs. Id1, Id2, and Id3 are all expressed by human ECs derived from the microvasculature and pulmonary artery, and in smooth muscle cells derived from the aorta and pulmonary artery (PASMCs).[38,74] Human umbilical vein endothelial

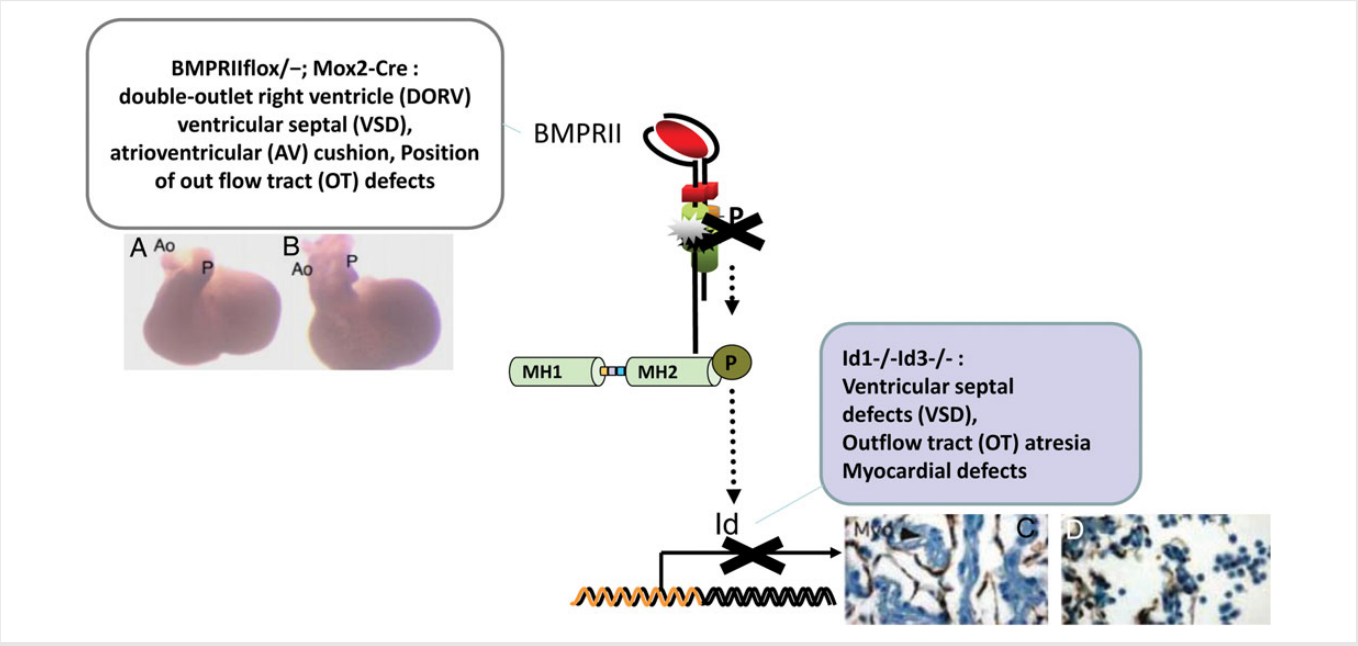

Figure 2 Id double KO mice and BMPRII-deficient mice exhibit similar cardiac defects. BMPs signal through the BMP type II receptor and the activated receptor in turn phosphorylate BMP-restricted Smads, which translocate to the nucleus and regulate Id gene transcription. BMPRII flox$^{/-}$; Mox2-Cre mice survived gastrulation, but demonstrated outflow tract (OT) defects at E12.5 (Ao: aorta; P: pulmonary artery). The normal rotation of the OT (*A*) was interrupted as shown in (*B*). Id1$^{-/-}$Id3$^{-/-}$ mice had VSDs and the myocardial defects (*D*), compared with wild type (WT) at E11.5 (*C*) (Myo: myocardium).

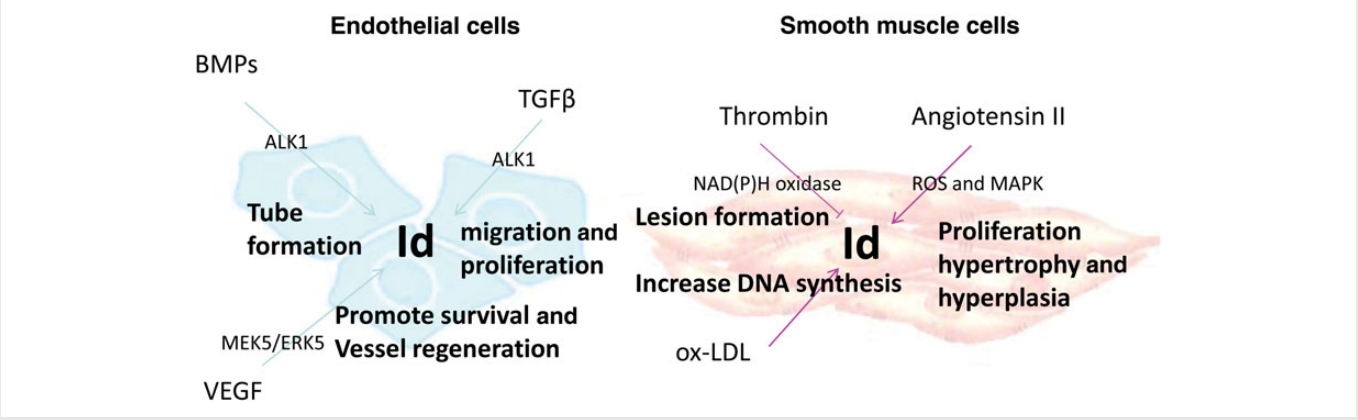

Figure 3 The regulation of Id proteins in ECs and smooth muscle cells affects vascular remodelling. In ECs, BMPs, TGF-β, and VEGF regulate angiogenesis by inducing the expression of Id proteins. BMPs and TGF-β promote EC migration and tube formation through induction of Id1 protein. VEGF induces activation of Id1 and Id3 to promote EC survival and vessel regeneration. In smooth muscle cells, thrombin regulates Id genes in an NAD(P)H oxidase-dependent manner during vascular lesion formation. Id3 is an atheroprotective factor that limits carotid intima-media thickness, whereas angiotension II-induced hyperplasia in ascending aortas is dependent on Id3 expression. Also, Id3 is essential for ox-LDL-induced VSMC growth.

cells (HUVECs) overexpressing Id1 and transplanted into the hind limbs of mice accelerated recovery of blood flow and increased capillary density in the ischaemia area.[75] Conversely, knockdown of Id1 abolished VEGF-induced angiogenic responses in HUVECs.[11] The expression of Id proteins are induced in response to several EC mitogens including BMPs, TGF-β, and VEGF (*Figure 3*). It has been suggested that Id genes are the main mediators of BMP effects on ECs.[76,77] For example, Id1 was found to mediate EC migration and tube formation induced by BMP6 and ectopic expression of Id1 in cultured ECs enhanced capillary-like tube formation *in vitro*.[12] It is of interest that Id1 and Id2 can only be induced by TGF-β through the activin receptor like kinase 1 (ALK1) receptor but not the ALK5 receptor in HUVECs.[78] High levels of TGF-β inhibit EC migration and proliferation through the induction of plasminogen activator inhibitor 1 (PAI-1) gene expression, whereas low levels of TGF-β stimulate EC migration and proliferation dependent on Id1 expression. Furthermore, endoglin activates ECs under hypoxic conditions through up-regulating the ALK1/Id1 pathway rather than ALK5.[79] It should be pointed out that the actions of VEGF and Id proteins

are closely linked in the vasculature. The angiogenic defect in compound Id-deficient mice is due to impaired VEGF-driven mobilization of VEGFR2 (vascular endothelial growth factor receptor 1, also known as Flk-1, KDR)-positive circulating endothelial precursor cells and impaired proliferation of VEGFR1 (vascular endothelial growth factor receptor 1, also known as Flt1)-positive cells.[7] Another study revealed that VEGF induces Id1 expression through VEGFR2 signalling in liver sinusoidal endothelial cells.[80] The use of VEGFR-specific inhibitors could lead to a better understanding of the roles of VEGF as a pro-angiogenic factor in the pathology of PAH. The first VEGF receptor 2 inhibitor, SU5416, was originally in clinical development for cancer therapy. In rodent models of PAH, the administration of SU5416 combined with chronic hypoxia results in severe angioproliferative PAH accompanied by neointimal thickening. It is believed that the inhibition of VEGFR2 increases the apoptosis of ECs, and favours the survival of apoptosis-resistant cells. It is hypothesized that hyperproliferation of these apoptosis-resistant clones of ECs gives rise to the characteristic plexiform lesions in human PAH. The up-regulation of BMP receptor signalling via the Smad/Id pathway has been shown to promote pulmonary arterial endothelial cells survival, prevent loss of vessels, and induce vessel regeneration in PAH.[81] It is thus likely that Id proteins contribute to the complex interplay between EC apoptosis and proliferation in PAH.

As to the mechanism of the angiogenic effects of Id proteins, it is known that inhibition of thrombospondin-1 by Id proteins is involved in the induction of angiogenesis.[82] Doebele *et al.*[83] found that VEGF up-regulated Id1 expression via the MEK5/ERK5 pathway in ECs. In addition, overexpression of Id1 enhanced the expression of intercellular cell adhesion molecule-1 and E-selectin, induced angiogenic processes such as EC migration, and increased activity of MMP-2 and -9.[11]

Id1 also promotes the generation, proliferation, and migration of endothelial progenitor cells (EPCs).[23] Recently, Id proteins were recognized as a biomarker of EPCs since Id1 can be used to track EPCs from the bone marrow (BM). Silencing of Id1 caused ablation of BM-derived EPCs and led to significant defects in angiogenesis-mediated tumour growth.[84] Id1 mutant mice demonstrate an absence of EPCs in peripheral blood. The generation of EPCs in BM appears to be dependent on the repression of p21 by Id1.[22]

1.5 Id proteins and VSMCs

Id proteins are abundantly expressed in VSMCs and regulate their proliferation and differentiation.[6] Id proteins are involved in the proliferative responses of VSMCs to thrombin, angiotensin II, and oxidized low-density lipoprotein (ox-LDL). Thrombin regulates the expression of BMP4 and Id proteins in VSMCs via the NAD(P)H oxidase. During wire-induced vascular stenosis in mice, levels of Id1 and Id3 are reduced in the vascular media. However, mice deficient in p47phox—an important component of NAD(P)H—were resistant to vascular stenosis following injury and exhibited increased expression of Id1 and Id3.[85] In contrast, the hypertrophic activity of angiotensin II appears to be partly mediated by Id3 downstream of increased reactive oxygen species and MAPK signalling.[27] Another *in vivo* study also suggested that angiotensin II infusion could cause aortic VSMC hypertrophy and hyperplasia via up-regulating Id3.[10] Therefore, the roles of Id proteins under these experimental conditions warrant further investigation to delineate the precise contribution to vascular homeostasis and pathology.

Id proteins are also involved in lipid-induced VSMC growth. The growth-promoting effects of 12/15-lipoxygenase are partially mediated through induction of Id3 transcription.[28] In these studies, Id3 mediated the mitogenic effect of hyperlipaemic sera and ox-LDL in VSMC via inhibition of p21cip1 expression, subsequently increasing DNA synthesis and proliferation.[29] These studies reveal an important role for Id proteins in lipid metabolism and atherosclerosis, which will be discussed further below. The interaction with Gut-enriched Kruppel-like factor may also explain the regulatory role of Id3 on VSMC proliferation in the pathogenesis of atherosclerosis.[30]

Id proteins play additional roles in the modulation of VSMC phenotype. Binding of class I bHLH proteins to the two E-boxes on the promoter region of the smooth muscle α-actin (α-SMA) gene, a differentiation marker of VSMCs, is required for the expression of α-SMA *in vivo* and can be inhibited by Id proteins.[25] Id proteins are also involved in SMC differentiation from BM-derived cells where individual Id proteins show unique effects.[31] For example, Id2 mRNA expression was up-regulated in parallel with increased expression of the smooth muscle cell markers, myosin heavy chain, calponin, and α-SMA, without a change in Id1 expression. However, in these cells, BMP4 induced Id1 expression along with a reduction of markers in smooth muscle cell differentiation.

1.6 Id proteins and atherosclerosis

In atherosclerosis, intimal plaques form as a consequence of endothelial dysfunction and the accumulation of lipid laden macrophages. Id proteins act as negative regulators of cellular differentiation and modulation of VSMC phenotype, which is a hallmark of the dedifferentiated phenotype of certain populations of SMCs observed in lesion formation in atherosclerosis and vasculoproliferative disorders (*Figure 4*). The role of the immune system in atherosclerosis is increasingly recognized. Recently, Doran *et al.*[86] revealed that Id3-mediated B cell homing is an important mechanism by which Id3 affords protection in atherosclerosis. As mentioned above, Id proteins are involved in lipid metabolism and Id3 has been shown to mediate VSMC growth induced by hyperlipaemia and ox-LDL.[29] One study found that Id3 and its partner E47 were novel regulators of adiponectin expression in differentiating adipocytes,[58] which might influence the process of atherosclerosis.

Matsumura *et al.*[87] found that Id3 was involved in the growth regulation of VSMCs in atherosclerotic plaques. Also, alternative splicing of Id3 protein is induced during vascular lesion formation, resulting in the appearance of the splice variant, Id3a. Id3a induces smooth muscle cell apoptosis and functions as a negative regulator to limit pathological vascular lesion formation in balloon-injured rat carotid arteries.[26] A single-nucleotide polymorphism variant at rs11574 in the human Id3 gene was independently associated with carotid intima-media thickness in patients. The fact that $Id3^{-/-}ApoE^{-/-}$ (apolipoprotein E-deficient mice as an atherosclerosis model) mice developed significantly more atherosclerosis than $Id3^{+/+}ApoE^{-/-}$ mice revealed a direct and robust relationship between Id proteins and atherosclerosis.[88] Furthermore, aortic VSMC proliferation was induced by angiotensin II via up-regulated Id3. These apparently conflicting reports of the role of Id proteins likely reflect the complex roles played by Id proteins induced by BMP signalling or other mediators in the cellular context. To highlight this complexity, recent studies have shown that inhibition of BMP signalling with the selective small molecule BMP type I receptor inhibitor, LDN-193189, protects against atherosclerosis and associated vascular calcification.[89] In contrast, specific endothelial deficiency of BMPRII induced endothelial inflammation and contributed to the development of atherosclerosis.[90]

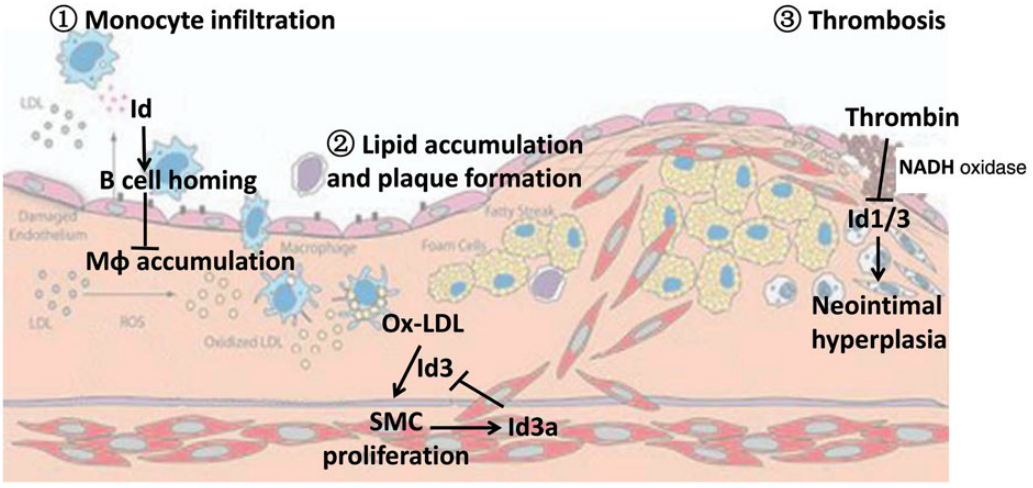

Figure 4 Id proteins are involved in the development of atherosclerosis. When monocyte infiltration occurs initially, Id3-mediated B cell homing plays a role in limiting macrophage accumulation. Later, during lipid accumulation and plaque formation, ox-LDL-driven proliferation of smooth muscle cells is mediated by Id3. The intron retention isoform of Id3, Id3a, functions in a negative feedback loop to down-regulate the expression of Id3. Id proteins also participate in thrombin-induced neointimal hyperplasia.

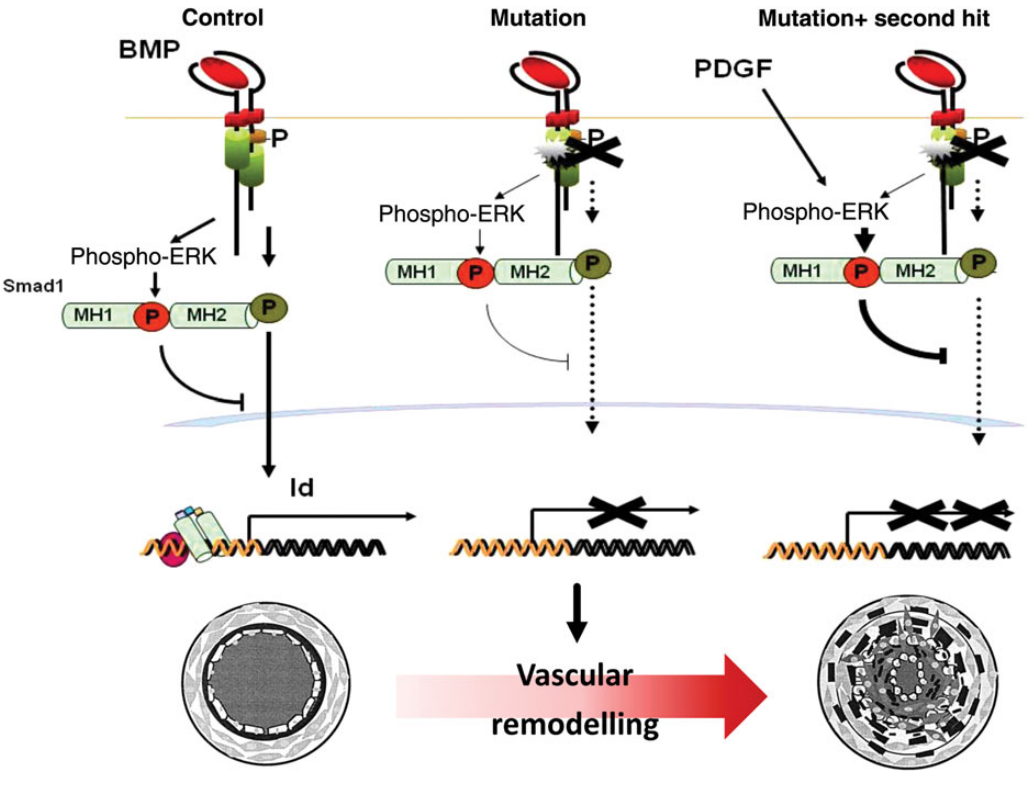

Figure 5 The deficient BMP signalling and second 'hit' hypothesis in PAH. In control human pulmonary arterial smooth muscle cells (hPASMCs), BMPs activate BMPRII and signal through Smad1 activation, whereby C-terminal phosphorylated Smad1 forms a transcriptional complex to bind the promoter region of Id genes. BMP signalling is important to inhibit proliferation of hPASMCs. At the same time, BMPRII activates ERK to phosphorylate Smad1 at its linker region to restrict the C terminal phosphorylation. When the BMP type II receptor is mutated, there is a reduction in Id gene transcription. In the presence of PDGF or other environment stimulation, constituting the 'second hit', the enhanced Smad1 linker region phosphorylation by ERK further exaggerates the existing deficiency of BMP signalling, which leads to the uncontrolled proliferation of hPASMCs and pulmonary vascular remodelling.

1.7 Id proteins and PAH

PAH is characterized by a sustained elevation of pulmonary arterial pressure. The increased pressure usually results from a complex process of vascular remodelling and occlusion involving the endothelial, smooth muscle, and fibroblast components of the pre-capillary pulmonary arteries.[91] It is well recognized that BMPRII mutations are responsible for the majority of heritable PAH.[92] In addition, dysfunction of BMP signalling is found in idiopathic PAH and experimental models of PAH induced by chronic hypoxia, monocrotaline (MCT) exposure, and high flow.[92] The fact that *bmpr2*$^{+/-}$ mice do not spontaneously develop PAH suggests that additional 'second hits' or further dysfunction of BMP signalling are necessary to cause initiation and progression of PAH.[67,93] The restoration of BMP signalling has proved effective as a therapeutic strategy in preclinical models of disease.[81]

In PASMCs, the most consistent transcriptional targets of BMP/Smad signalling are the Id proteins (*Figure 5*). Our group has shown that cells harbouring a mutation in BMPRII failed to induce Id gene expression in response to BMPs, though this can be partly overcome by higher concentrations of BMPs. We also showed that loss of BMPRII function or knockdown of Id1 leads to loss of the growth suppressive effects of BMPs in PASMCs.[40,74] Hypoxia is a well-recognized stimulus for pulmonary hypertension. In a report that apparently contradicts some of these findings, de Caestecker and colleagues recently reported elevated Id1 and Id3 expression in hypoxic pulmonary VSMCs *in vivo* in a BMP-dependent manner. In response to chronic hypoxia, Id1 null mice did not develop more PH than wild types. An increase in Id3, but not Id2, expression in pulmonary VSMCs of Id1 null mice suggests that Id1/Id3 may play a compensatory role in regulating VSMC responses to chronic hypoxia.[94] Since Id1 and Id3 are known to compensate for each other in VSMCs, studies in VSMC-specific Id1/3 double KO mice are required to further explore their role in hypoxic pulmonary hypertension. A marked reduction in the expression of Id proteins was also observed in MCT-treated and hypoxia-induced PAH models in rats.[94,95] All these studies point towards a major role of Id proteins in the complex process of PAH. However, the precise mechanisms by which the Id proteins contribute to the development of PAH are still not known.

1.7.1 Crosstalk with other signalling pathways

Generally, Ids are thought to mediate mitogen-induced growth and inhibit systemic VSMC differentiation. However, in PASMCs, we observed minimal Id gene and protein induction by the mitogen platelet-derived growth factor (PDGF)-BB, and other angiogenesis-related growth factors.[40,96] BMP4-induced Id1 expression was negatively regulated by extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation. The mechanism involved ERK1/2-dependent phosphorylation of the Smad1 linker region (serine 206), which inhibits C-terminal serine 463/465 phosphorylation and Smad nuclear accumulation. Taken together, these findings indicate an important interaction between ERK1/2 and Smad1/5 in the regulation of Id genes in PASMCs (*Figure 5*).[74]

1.7.2 The potential therapeutic target for PAH

Prostanoids are one of the most effective clinical treatments for PAH. Although predominantly vasodilators, prostanoids may exert antiproliferative effects during long-term administration, which beneficially affect pulmonary vascular remodelling. We investigated the potential interaction between prostacyclin analogues and BMP signalling in PASMCs (*Figure 6*). We demonstrated that prostacyclin analogues enhance the growth suppressive effects of BMPs in human PASMCs through up-regulation of Id1 and provided direct evidence that overexpression of Id1 leads to growth suppression of PASMCs. We also provided

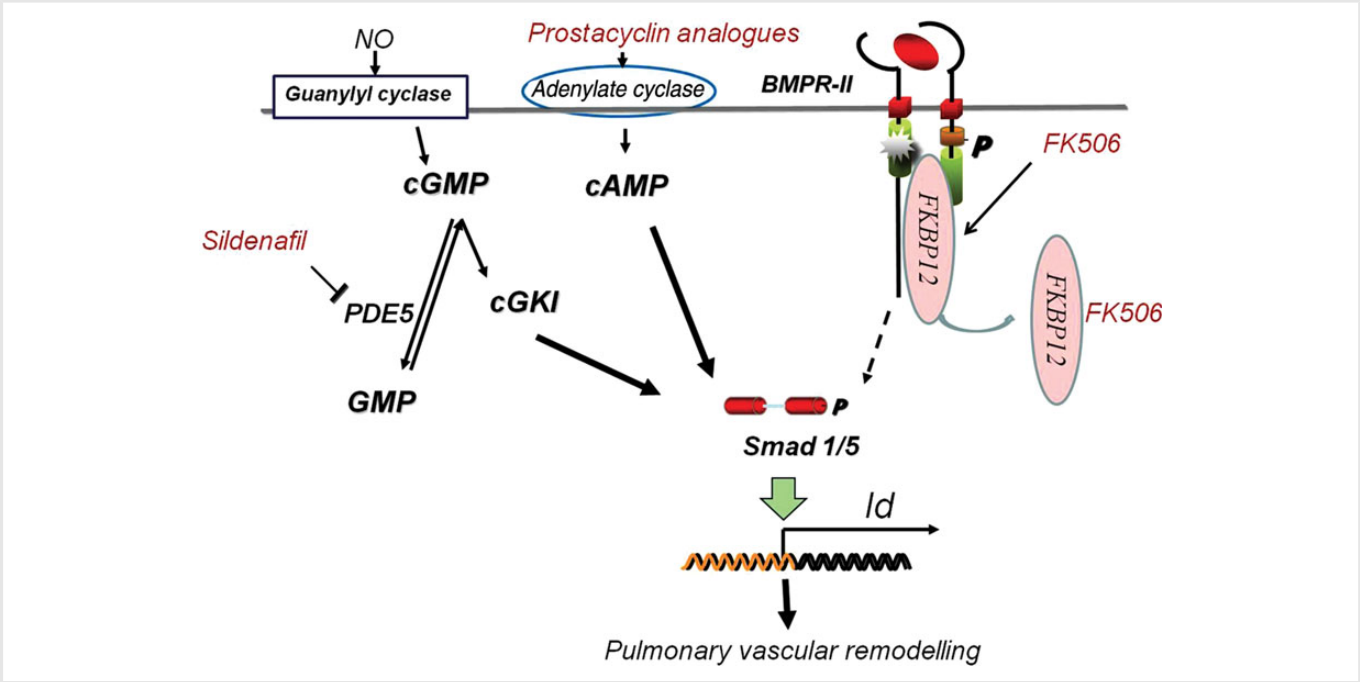

Figure 6 The crosstalk between BMP/Id and other pathways currently targeted in the treatment of PAH. Prostacyclin analogues enhance intracellular cAMP levels to regulate BMP signalling in a Smad-dependent and Smad-independent manner. Sildenafil promotes BMP signalling through the interaction of cGKI with BMPRII and the formation of cGKI–Smad transcription factor complex on the Id gene promoter. Recently, FK506 was found to release BMPRI from FKBP12, thereby promoting BMP signalling to inhibit pulmonary vascular remodelling.

in vivo evidence in the MCT rat model of PAH that treprostinil infusion prevented progression of PAH and reduced muscularization of intra-acinar pulmonary arteries while increasing phosphorylation of Smad1/5 and Id1 gene expression in the lung without altering BMPRII expression.[40]

The interaction between cGMP and BMP receptors was also recently demonstrated in vascular cells.[97] We found that sildenafil enhances canonical BMP signalling via cyclic GMP and cGKI [cyclic nucleotide-dependent protein kinase G (PKG or cGKI), is activated by cGMP] *in vitro* and *in vivo* and partly restores deficient BMP signalling in BMPRII mutant PASMCs. Our findings demonstrate a novel mechanism of action of sildenafil in the treatment of PAH and suggest that targeting BMP signalling may be beneficial in this disease. Based on the knowledge that Ids are the major functional targets of BMP signalling in pulmonary vascular cells, an elegant study used the BMP response element from the Id1 promoter as a reporter to screen for compounds capable of inducing the expression of Id genes. The compounds screened included 3756 FDA approved drugs. Of these, Spiekerkoetter *et al.* identified the immune suppressant tacrolimus (FK506) as the most potent activator of the BMP response element. The mechanism involves FK506 binding to FK-binding protein-12 (FKBP12) to activate the BMP type I receptor, while also acting as an inhibitor of the phosphatase, calcineurin. *In vivo* FK506 proved beneficial in three different animal models of PAH, including the reversal of PAH in the sugen/hypoxia rat model. Experimental medicine studies are now underway in patients. Thus, the strategy of screening for compounds that up-regulate Id genes with high selectivity may prove a promising strategy for the treatment of PAH.

2. Conclusions

In summary, although Id proteins were initially identified as controllers of terminal myogenic differentiation, they play important roles in development including neural stem cell differentiation, osteoblast differentiation, and lymphocyte maturation. In the cardiovascular system, Id proteins play major roles in cardiogenesis. The major function of Id proteins in cancer appears to be to promote proliferation and inhibition of differentiation.[32,98] Although BMPs are critical regulators on Id protein expression, additional growth factors and cytokines regulate Id gene expression in a highly cell- and tissue-specific context to impact on vascular cell proliferation, differentiation, and function. Research in atherosclerosis has revealed that Id protein expression is important in the multistep process of disease development. Furthermore, targeting Id proteins have proved to be an effective therapeutic strategy in PAH where they impact on PASMC proliferation and EC survival. However, the role of the individual Id proteins in the complex process of vascular disease remains to be fully elucidated. Determining the regulation and function of Id proteins during vascular development and disease will contribute to our understanding of cardiovascular disease, particularly PAH, and will be essential to develop approaches with tissue selectivity for targeted therapies.

Acknowledgements

We thank Rafia Al-Lamki and Neda Farahi for comments on the manuscript.

Conflict of interest: none declared.

Funding

This work were supported by a grant from the British Heart Foundation (RG/08/002/24718) to N.W.M and a grant from China 1000 young talents plan to J.Y.

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