Endothelial colony-forming cells and transforming growth factor-β superfamily signalling in idiopathic pulmonary arterial hypertension

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I declare that this thesis was conducted and written by myself and the work included within is original and my own unless otherwise stated.

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ABSTRACT

Circulating endothelial progenitor cells may be important in the pathogenesis of idiopathic pulmonary arterial hypertension (IPAH) and give rise to endothelial colony-forming cells (ECFCs) in culture. These cells represent an accessible surrogate population to investigate endothelial dysfunction in IPAH.

Peripheral blood mononuclear cells were cultured from healthy volunteers (n=25, 72% female; age range 23-57 yr) and IPAH patients (n=30, 60% female; age range 22-56 yr). Older IPAH patients (n=6, 50% female; age range 62-82 yr) were also sampled. Distinct colonies appeared after 13-35 days and exhibited a typical cobblestone morphology. The average frequency of colonies and clonal growth of isolated cells was similar in healthy volunteers and IPAH patients. Age-dependent differences were observed however in the number and frequency of colonies, which declines with age in the control but not in IPAH subjects. The endothelial phenotype was confirmed by immunostaining and flow cytometry, exhibiting endothelial and progenitor markers, but not hematopoietic markers. Endothelial cell functions, including proliferation, angiogenesis, migration and responses to apoptotic stimuli, were compared in cells between passages 4 to 7. IPAH cells showed enhanced angiogenic capacity (tube formation on Matrigel), significantly less apoptosis (lower caspase-3/7 activity) in response to serum and growth factor deprivation, and impaired migratory capacity compared with control ECFCs. Dysfunctional transforming growth factor (TGF)-β and bone morphogenetic protein receptor expression and signalling are implicated in IPAH and were assessed by RT-PCR and western blotting. IPAH and control ECFCs differed in their TGF-β type I (ALK5) receptor expression/signalling and in the expression of other regulatory proteins (e.g. chloride-like intracellular channel-4).

Blood-derived ECFCs display an endothelial lineage similar to that of mature endothelial cells. ECFCs from IPAH patients have a distinct functional phenotype and exhibit differences in TGF-β receptor superfamily expression/signalling, which may contribute to endothelial dysfunction and vascular remodelling in IPAH.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
5-HTT	5-hydroxytryptamine transporter
6MWD	6 minute walk distance
Ab	antibody
acLDL	acetylated low density lipoprotein
ActRIIA	activin receptor type IIA
ActRIIB	activin receptor type IIB
Ad	adenoviral
ADMA	asymmetric dimethylarginine
ADSC	adipose-derived stromal cells
AFS	amniotic fluid stem cells
ALK	activin receptor-like kinase
AM	adrenomedullin
Ang1	angiopoietin 1
ANOVA	analysis of variance
APAH	associated pulmonary arterial hypertension
APC	allophycocyanin
ARC	apoptosis repressor with caspase recruitment domain
ATP	adenosine triphosphate
AVMs	atreiovenous malformations
Bax	Bcl-2–associated X protein
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma extra large
BM	bone marrow
BMP	bone morphogenetic factor
BMPR2	bone morphogenetic factor receptor type 2
BNP	brain natriuretic peptide
BOECs	blood outgrowth endothelial cells
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CACs	circulating angiogenic cells
calcein AM	acetomethoxy derivate of calcein green
CCL-5	chemokine ligand 5
cDNA	complementary deoxyribonucleic acid
CF	carrier free
CFU-ECs	colony forming units-endothelial cells
CFU-Hill	colony-forming unit-Hill
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CHD	congenital heart disease
CI	cardiac index
CLIC4	chloride intracellular channel 4
CO	cardiac output
CO_2	carbon dioxide
Ct	cycle threshold
CTD	connective tissue disease
CTEPH	chronic thromboembolic pulmonary hypertension
CX	connexin
CXCR4	C-X-C chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole
DDAH	dimethylarginine dimethylaminohydrolase

DEPC	diethylpyrocarbonate	FC	functional class
DMSO	dimethyl sulfoxide	FGF	fibroblast growth factor
DNA	deoxyribonucleic acid	FITC	fluorescein isothiocyanate
DNase	deoxyribonuclease	Flk-1	fetal liver kinase-1
EBM-2	endothelial cell basal medium-2	FRAP	fluorescence recovery after photobleaching
ECFCs	endothelial colony-forming cells	G-CSF	granulocyte-colony stimulating factor
ECL	enhanced chemiluminescence	GAPDH	glyceraldehyde 3-phosphate dehydrogenase
ECM	extracellular matrix	GDFs	growth and differentiation factors
ECs	endothelial cells	GFP	green fluorescent protein
EDTA	ethylene diamine tetra-acetic acid	GFR	growth factor reduced
EGF	epidermal growth factor	GM-CSF	granulocyte-macrophage colony-stimulating factor
EGM-2	endothelial growth medium-2	GTPase	guanosine triphosphatase
EMS	Eisenmenger's syndrome	HA	heme agglutinin
eNOS	endothelial nitric oxide synthase	HC-Matrigel	Matrigel with high protein concentration
EOCs	endothelial outgrowth cells	HCl	hydrochloric acid
EPCs	endothelial progenitor cells	healthy-ECFCs	endothelial colony-forming cells derived from healthy volunteers
EPO	erythropoietin	HepG2	human hepatocellular carcinoma
ERA	endothelin receptor antagonist	hESC	human embryonic stem cells
ERK	extracellular signal-regulated kinases	HGF	hepatocyte growth factor
ET-1	endothelin-1	HHT	hereditary hemorrhagic telangiectasia
ETA/B	endothelin receptor type A/B	HIF-1α	hypoxia-inducible factor 1-α
FACS	fluorescence-activated cell sorting	HIV	human immunodeficiency virus
FBS	fetal bovine serum	HO-1	hemo oxygenase-1
Fc	fragment crystallisable	HPAECs	human pulmonary artery endothelial cells

HPAH	heritable pulmonary arterial hypertension	L-R shunt	left to right shunt
HPASMCs	human pulmonary artery smooth muscle cells	LDS	lithium dodecyl sulfate
HPCs	hematopoietic progenitor cells	LPP	low proliferative potential
HPLC	high-performance liquid chromatography	MAPK	mitogen-activated protein kinases
HPP	high proliferative potential	Mcl-1	myeloid leukemia cell differentiation protein
HRP	horseradish peroxidised	MCP-1	monocyte chemotactic protein-1
HSCs	hematopoietic stem cells	MCT	monocrotaline
HUVECs	human umbilical vein endothelial cells	MEECs	mouse embryonic endothelial cells
I-Smads	inhibitory Smads	MEM	minimum essential medium
i.v.	intravenous	miRNA/ miR	microRNA
ID	inhibitor of DNA binding protein	MIS	Müllerian inhibiting substance
$I_{K(V)}$	current amplitude of K+ channel	MMPs	matrix metalloproteinases
IL	interleukin	MNCs	mononuclear cells
Inh	inhaled	MOI	multiplicity of infection
IPAH	idiopathic pulmonary arterial hypertension	mPAP	mean pulmonary arterial pressure
IPAH-ECFCs	endothelial colony-forming cells drived from IPAH patients	mRNA	messenger ribonucleic acid
IPF	idiopathic pulmonary fibrosis	MSCs	mesenchymal stem cells
iPSCs	induced pluripotent stem cells	*MSH2*	MutS Homolog 2
iVPCs	induced vascular progenior cells	NAD+	nicotinamide adenine dinucleotide
JNK	c-jun N-terminal kinases	NIH	National Institutes of Health
KDR	kinase insert domain receptor	NO	nitric oxide
Kv	voltage-gated K+ channel	NOS	nitric oxide synthase
L-NMMA	NG-Monomethyl-L-arginine, monoacetate salt	NPPB	5-nitro-2-(3-phenylpropylamino)-benzoate

ns	no significanct difference	PH	pulmonary hypertension
NT-pro-BNP	N-terminal pro-brain natriuretic peptide	PMVECs	pulmonary microvascular endothelial cells
NYHA	New York Heart Association	PPIA	peptidylprolyl isomerase A
OD	optical densities	pSmad	phosphorylated Smad
PAECs	pulmonary artery endothelial cells	PVOD	pulmonary veno-occlusive disease
PAH	pulmonary arterial hypertension	PVR	pulmonary vascular resistance
PAI-1	plasminogen activated inhibitor-1	qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
PAP	pulmonary arterial pressure	R-Smads	receptor-activated Smads
PARP	polyadenosine diphosphate-ribose polymerase	RANTES	Regulated upon Activation, Normally T cell Experssed and Secreted
PASMCs	pulmonary artery smooth muscle cells	RIPA	radio-immuno-precipitation assay
PBMNCs	peripheral blood mononuclear cells	rlu/s	raw light units per second
PBS	phosphate buffered saline	RNA	ribonucleic acid
PBS-FBS	PBS containing 2% FBS and 0.05% EDTA	RNase	ribonuclease
PCH	pulmonary capillary hemangiomatosis	ROCK	RhoA/Rho kinase
PCR	polymerase chain reaction	ROIs	regions of interest
PDE-5-I	phosphodiesterase type 5 inhibitors	ROS	reactive oxygen species
PDE5	phosphodiesterase type 5	RT	reverse transcription
PDGF	platelet-derived growth factor	RV	right vetricular
PDs	population doublings	RVSP	right vetricular systolic pressure
PDT	population doubling time	SARA	SMAD anchor for receptor activation
PE	phycoerythrin	SC	subcuntaneous
PECAM-1	platelet endothelial cell adhesion molecule	SCD	sickle cell disease
PFA	paraformaldehyde	scram	scrambled controls
PGI_2	prostacyclin	SDF-1α	strom cell-derived factor-1

SDS	sodium dodecyl sulphate	TIMPs	tissue inhibitors of metalloproteinases
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis	TNF-α	tumour necrosis factor-α
SEM	standard error of the mean	Treg	regulatory T cells
shRNA	small hairpin RNA	TRPC	transient receptor potential channels
siRNA	small interfering RNA	UCB	umbilical cord blood
SMCs	smooth muscle cells	UEA-1	*Ulex europaeus* agglutinin-1
SMPCs	smooth muscle progenitor cells	uP	unilateral pneumonectomy
Smurf	Smad ubiquitin regulatory factor	UV	ultraviolet
STAT3	signal transducer and activator of transcription 3	UVB	umbilical venous blood
SVC-GFP	adipose tissue stromal vascular fraction GFP-positive cell	VCAM-1	vascular cell adhesion protein
TAE	Tris-acetate-EDTA	VEGF	vascular endothelial growth factor
TBS	tris-buffered saline	VEGFR2	vascular endothelial growth factor receptor type 2
TBS-T	TBS with 0.1% Tween-20, 5% BSA and 5% non-fat dry milk	vWF	von Willebrand factor
TGF-β	transforming growth factor-β	WHO	World Health Organisation
TGF-βRII	transforming growth factor-β receptor type II	ZO-1	tight junction protein-1
TGS	Tris-glycine-SDS	β2M	β2-microglobulin
THBS1	Thrombospondin-1		

SUMMARY OF THESIS STRUCTURE

Chapter 1 contains an overall introduction to the thesis, which concludes with the **hypothesis and objectives**.

Chapter 2 contains detailed **methodologies** with material sources covering the studies described in Chapters 3-7.

Chapter 3 focuses on comparing the appearance of blood-derived endothelial colony-forming cells (ECFCs), which shows that **IPAH patients possess different ECFC colony forming properties compared to healthy volunteers**. ECFCs phenotypes were confirmed by analysis of phenotypic expression and functional characteristics, and samples were then selected based on functional criteria for use as endothelial cell models for IPAH in further investigations in chapters 4 – 7.

Chapter 4 explores the functional characteristics of ECFCs from IPAH patients, with evidences to indicate that the **ECFCs from IPAH patients exhibit endothelial dysfunctions including resistant to apoptosis, enhanced angiogenesis, impaired migration and disrupted barrier functions**. These lead to investigation of molecular mechanisms underlying these dysfunctions in chapters 6 – 7.

Chapter 5 investigates the transforming growth factor-β (TGF-β) signalling receptors expression, and identifies **downregulated ALK5 expression and impaired TGF-β1 activation of ALK5/Smad2 signalling in ECFCs from IPAH patients**.

Chapter 6 investigates further the TGF-β signalling regulations of ECFCs functions, and establishes the **mechanism underlying the apoptosis-resistant dysfunctions in ECFCs from IPAH patients**, primarily due to the possible imbalance between ALK1 and ALK5 activations in IPAH cells.

Chapter 7 further explores **the credibility of ECFCs as endothelial cellular models for IPAH**. Two other novel mechanisms proposed to underlie endothelial pathogenesis in IPAH are investigated, through which the possible regulation of other dysfunctions such as dysregulated angiogenesis and disrupted barrier functions in IPAH cells are established.

Chapter 8 contains **conclusions from the main findings in this thesis** and **describes possible future works.**

Chapter 1:

Introduction

Chapter 1 – Introduction

1.1 The pulmonary vasculature – Structure, function and development

Anatomically, the human pulmonary circulation can be divided into distinct segments. The pulmonary trunk ascends from the right ventricle and branches into the right and left main pulmonary arteries. These arteries divide into lobar pulmonary arteries which supply individual lobes of the lung and sub-divide distally into muscular resistance pulmonary arteries, arterioles, capillary bed and pulmonary veins (Harris and Heath, 1986). Throughout the course of the pulmonary arterial tree, the vessel size and structure change according to their function, comprising elastic, muscular and non-muscular vessels (Wagenvoort and Wagenvoort, 1979). The walls of the pulmonary artery are composed of three layers – the tunica intima, the tunica media, and the tunica adventitia. In elastic and muscular arteries the tunica intima forms the innermost layer and comprises a single layer of endothelial cells (ECs) and associated connective tissue lying on an internal elastic lamina. The tunica media forms the middle layer of the vessel wall and comprises circumferentially-arranged pulmonary artery smooth muscle cells (PASMCs) and elastic laminae enclosed by an external elastic lamina. The tunica adventitia is the outermost layer and is primarily composed of connective tissue and matrix secreting fibroblasts that serves to anchor the vessels in place. Non-muscular vessels are made up of a single layer of ECs lying on an internal elastic lamina (Wagenvoort and Wagenvoort, 1979).

The large proximal vessels (pulmonary trunk, main arteries and lobar branches) are predominantly elastic in nature, consistent with their function as conduit and blood storage vessels following ventricular systole. Muscular pulmonary arteries continue imperceptibly from the elastic arteries and continue distally down to arteries 500-1000 µm in diameter (Brenner, 1935; Wagenvoort and Wagenvoort, 1979). Within the lung, branches of muscular pulmonary arteries run parallel to accompanying airways. The concentric arrangement of the smooth muscle and ability to contract is consistent with their role in the control of vascular resistance. Pulmonary arterioles are the terminal branches of the arterial tree and are also associated with the control of pulmonary vascular resistance. These pre-capillary vessels are approximately 70 µm in diameter and consist of a tube of endothelium surrounded by a spiral of elastic fibril, which is contiguous with the internal elastic lamina and, at their origin, may have a cuff of PASMCs or pericytes (Brenner, 1935). The pulmonary capillary bed, with its single cell lining, is the site of gaseous exchange and forms an extensive,

interdigitating network within the alveolar walls. The pulmonary veins lie in the interlobular connective tissue septae and receive blood from the capillary bed, serving as a conduit to return oxygenated blood to the left side of the heart (Wagenvoort and Wagenvoort, 1979).

The development of the pulmonary circulation begins in the first 4-16 weeks of gestation, with the formation of a recognisable blood gas barrier by 24 weeks of gestation (Hislop, 2005). The pulmonary arteries and veins are independently derived and initially form *de novo* as endothelial tubes in the mesechyme adjacent to airways by a process of vasculogenesis (Hall et al., 2000; Hall et al., 2002). The endothelial tubes combine to form the pulmonary circulation, which develops in synchrony with the airways of the lung and results in an increase in surface area of alveoli and its capillary supply by a process of angiogenesis (Hislop, 2002; Parera et al., 2005). Growth factors such as vascular endothelial growth factor (VEGF), angiopoietin, fibroblast growth factor (FGF) are important in stimulating and stabilising the growth of the new endothelium in a bed of extracellular matrix (Hislop, 2005). Large vessels are supported by the development of several layers of smooth muscle, separated by collagen that provides rigidity, and elastin that provides the vessels with distensibility (Hislop and Pierce, 2000).

1.2 Pulmonary hypertension

1.2.1 Definition and Classification

The healthy adult pulmonary circulation is a distensible, high-capacity and low-pressure system with relatively thin-walled blood vessels compared to the systemic circulation (Naeije and Rondelet, 2004). The normal mean pulmonary artery pressure (mPAP) is approximately 15 mmHg at rest. Pulmonary hypertension (PH) is a progressive and intractable disease of diverse origins that is clinically defined as an elevation of mean PAP above 25 mm Hg at rest (Badesch et al., 2009). PAP is a product of cardiac output (CO) and pulmonary vascular resistance (PVR). A sustained elevation in PVR has a harmful effect on the heart as the right heart is subjected to an increased work load in order to overcome the downstream resistance. This leads to right ventricular (RV) hypertrophy and eventual right heart failure, a common cause of death in patients with PH. PH has been classified into five main groups, according to pathological, physiological and therapeutic characteristics. The current classification was adopted at the 4th World symposium on PH held in 2008 at Dana Point, California (Simonneau et al., 2009) (Table 1.1).

Clinical Classification of Pulmonary Hypertension
1. Pulmonary arterial hypertension (PAH)
1.1. Idiopathic PAH
1.2. Heritable
1.2.1. BMPR2
1.2.2. ALK-1, endoglin (with or without hereditary hemorragic telangiectasia)
1.2.3. Unknown
1.3. Drug- and toxin-induced
1.4. Associated with
1.4.1. Connective tissue diseases
1.4.2. HIV infection
1.4.3. Portal hypertension
1.4.4. Congenital heart diseases
1.4.5. Schistosomiasis
1.4.6. Chronic haemolytic anemia
1.5. Persistent pulmonary hypertension of the newborn
1' Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)
2. Pulmonary hypertension owing to left heart disease
2.1. Systolic dysfunction
2.2. Diastolic dysfunction
2.3. Valvular disease
3. Pulmonary hypertension owing to lung disease and/or hypoxia
3.1. Chronic obstructive pulmonary disease
3.2. Interstitial lung disease
3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern
3.4. Sleep-disordered breathing
3.5. Alveolar hypoventilation disorders
3.6. Chronic exposure to high altitude
3.7. Developmental abnormalilites
4. Chronic thromboembolic pulmonary hypertension (CTEPH)
5. Pulmonary hypertension with unclear multifactorial mechanisms
5.1. Hematologic disorders: myeloproliferative disorders, splenectomy
5.2. Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis: lymphangloleiomyomatosis, neurofibromatosis, vasculitis
5.3. Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

Table 1.1 Updated clinical classification of pulmonary hypertension (Dana Point, 2008). ALK1, activin receptor-like kinase type 1; BMPR2, bone morphogenetic protein receptor type 2; HIV, human immunodeficiency virus. Adapted from Simmonneau *et al.* 2009.

1.2.2 Pulmonary arterial hypertension (PAH)

Pulmonary arterial hypertension (PAH) represents the first main group of PH and is further divided into 5 sub-categories (Table 1.1). These comprise: idiopathic PAH (IPAH) – formerly known as primary PH – for PAH with no identified cause or family history; heritable PAH (HPAH) – formerly known as familial PAH – when occurs in a familial context; drug- and toxin-induced PAH; PAH associated with various other diseases; and persistent pulmonary hypertension of the newborn. The definitive diagnosis of PAH is dependent on demonstrating presence of pre-capillary PH at cardiac catheterisation, with raised mPAP (>25 mmHg) and normal capillary wedge pressure (≤15 mmHg), and associates with substantial mortality and morbidity.

PAH is a rare disease that affects less than 5/100,000 in Europe and has a prevalence of 15-50 patients/million of the population (Humbert et al., 2006; Peacock et al., 2007; NAPH, 2011) (National Audit for Pulmonary Hypertension, 2011). The prognosis of PAH remains poor, being a progressive disease with high mortality rate. The estimated median survival time for untreated IPAH was historically 2.8 years, with 1- and 3-year survival rates of 68% and 48% respectively in a cohort of 194 patients with primary PH from 32 centres in the USA (D'Alonzo et al., 1991). Despite the advances in understanding and improved treatment options for PAH in recent years had improved the prognosis to a certain degree, a recent multi-centres report revealed 1- and 3-year survival rates of 83% and 58% respectively in PAH populations largely composed of IPAH (Humbert et al., 2010a). For end-stage PAH patients who failed to respond to the current therapeutics and interventions, there is no choice but to consider cardiopulmonary transplantation as the final option (Keogh et al., 2009).

While a normal level of the cardiac stress-marker brain natriuretic peptide (BNP) or its N-terminal peptide (NT-proBNP) may help rule out PH, there is no blood biomarker that is diagnostic of the condition. Risk stratification can inform not just prognosis but may help optimisation of treatment and the achievement of specific goals aimed at improving longer-term survival. Multicentre patient registries – National Institutes of Health (NIH), French PAH registry and US-based Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease Management (REVEAL) – have been very useful in identifying independent factors that are associated with prognosis of PAH, including age, sex, disease aetiology, World Health Organization (WHO) functional class, 6 minute walk distance (6MWD), and haemodynamic parameters (D'Alonzo et al., 1991; Rich et al., 2000; Benza et al., 2010; Humbert et al., 2010a). The WHO functional classification classifies PAH patients according to their physical capability and usually correlates with poor survival rate (McLaughlin et al., 2009b; Humbert et al., 2010a) (Table 1.2). Measurements of circulating BNP and NT-proBNP levels remain

the most commonly used blood biomarkers for stratifying PH patients and are recommended in the European guidelines for the diagnosis and treatment of PH (Galie et al., 2009b).

World Health Organisation Functional Classification of PH patients	
I	Patients with PH in whom there is no limitation of usual physical activity; ordinary physical activity does not cause increased dyspnea, fatigue, chest pain, or presyncope.
II	Patients with PH who have mild limitation of physical activity. There is no discomfort at rest, but normal physical activity causes increased dyspnea, fatigue, chest pain, or presyncope.
III	Patients with PH who have a marked limitation of physical activity. There is no discomfort at rest, but less than ordinary activity causes increased dyspnea, fatigue, chest pain, or presyncope.
IV	Patients with PH who are unable to perform any physical activity at rest and who may have signs of right ventricular failure. Dyspnea and/or fatigue may be present at rest, and symptoms are increased by almost any physical activity.

Table 1.2. World Health Organisation classification of functional status of patients with PH. Adapted from (McGoon et al., 2004).

PAH is characterised by increased PVR, mediated by vasoconstriction, endothelial dysfunction, vascular remodelling, inflammation and thrombosis (Archer et al., 2010; Schermuly et al., 2011), and these are discussed in further details in the following sections.

1.2.3 Pulmonary vasoconstriction

The increased vasoconstriction of the pulmonary arteries in patients with PAH reflects the abnormal regulation of vessel tone. The endothelium plays an essential role in regulating pulmonary vascular homeostasis and is the main source of vasodilators, such as prostacyclin (Christman et al., 1992) and nitric oxide, and the potent vasoconstrictors thromboxane A2 (Christman et al., 1992), endothelin-1 and serotonin (5-hydroxytryptamine) (Schermuly et al., 2011).

Prostacyclin (PGI_2) is a potent vasodilator that also has inhibitory effects on platelet aggregation, inflammation and vascular smooth muscle proliferation, all of which have been implicated in PAH. It is synthesised in ECs from prostaglandin H_2 by PGI_2 synthase and acts by stimulating the production of cyclic adenosine monophosphate (Clapp et al., 2002). PGI_2 synthase expression is lower in the pulmonary arteries of patients with PAH (Tuder et al., 1999) and circulating levels of PGI_2 are also reduced, while levels of the vasoconstrictor thromboxane A2 are raised (Christman et al., 1992).

Nitric oxide (NO) is a potent vasodilator and its activity is mediated via cyclic guanosine monophosphate (cGMP). The metabolism of cGMP is dependent on the activation of phosphodiesterases, in particular phosphodiesterase type 5 (PDE5) (Corbin and Francis, 1999). PDE5 is the most abundantly expressed isoform within the pulmonary circulation (Hanson et al., 1998) and levels are raised in the lungs and right ventricle of PAH patients (Murray et al., 2002; Corbin et al., 2005; Wharton et al., 2005). Reduced bioavailability of NO may reflect lower expression of endothelial NO synthase (eNOS) in the lung microvasculature (Giaid and Saleh, 1995) and/or increased levels of the endogenous eNOS inhibitor asymmetric dimethylarginine (ADMA). This in turn may be due to reduced expression of the enzymes – dimethylarginine dimethylaminohydrolase-I and -II (DDAH-I and DDAH-II) – responsible for the degradation of ADMA (Pullamsetti et al., 2005).

Endothelin-1 (ET-1) is a 21 amino acid peptide and potent endothelium-derived vasoconstrictor and SMC mitogen, which is thought to have an important pathogenic role in PAH (Giaid et al., 1993). It acts via two receptors (ET_A and ET_B) in the pulmonary vasculature, ET_A receptors being predominately expressed by PASMCs while ET_B receptors are mainly localised to the endothelium but also expressed by PASMCs in more distal regions of the arterial tree (Davie et al., 2002). Binding of ET-1 to receptors on PASMCs induces vasoconstriction and proliferation whereas binding to ET_B receptors on endothelial cells causes vasodilation via PGI_2 and NO production and mediates clearance of ET-1 (Hirata et al., 1993; Seo et al., 1994; Shao et al., 2011). The ET_B-mediated vasodilatation effect has also recently been shown to be reduced in animal models of PH, possibly due to increased ET-1 and aldosterone production and subsequent rise in reactive oxygen species (ROS) that inhibit ET_B-dependent NO production (Maron et al., 2012).

5-hydroxytryptamine (5-HT) levels are also raised in PAH (Herve et al., 1995) and the occurrence of PH in patients using anorexic agents has indicated a role for 5-HT in the pathogenesis of PAH (Abenhaim et al., 1996). The analysis of lung tissues from patients undergoing transplantation demonstrated increased expression of the 5-HT transporter (5-HTT), as well as enhanced proliferative response of isolated PASMCs to 5-HT (Eddahibi et al., 2001; Marcos et al., 2004). Inhibitors of 5-HTT reverse PH in monocrotaline (MCT)-induced PH in rats (Guignabert et al., 2005) and mice treated with targeted 5-HTT gene disruption or selective serotonin reuptake inhibitors develop less severe hypoxic PH than wild-type controls (Eddahibi et al., 2000; Marcos et al., 2003). But the benefits of this for patients with PAH are debatable (Kawut et al., 2006; Dhalla et al., 2012) and alternative strategies that target specific 5-HT receptors may be preferable (Dumitrascu et al., 2011).

The pathological pulmonary vasomotor tone in PAH has also been attributed to other abnormalities, including those in K^+ and Ca^{2+} channels (Yuan et al., 1998; Yu et al., 2004; Burg et al., 2008) and RhoA/Rho kinase (ROCK) signaling (Oka et al., 2007; Mouchaers et al., 2010). However, like the 5-HT pathway, these are still emerging targets for the treatment of PAH (as discussed in section 1.4).

1.2.4 Endothelial dysfunction in PAH

Pulmonary endothelial damage is widely considered to be an early event in the development of PAH and a critical step leading to adverse structural changes (remodelling) in the vascular wall. In addition to affect the production of vasodilators and vasoconstrictors, endothelial dysfunction has other important consequences in the pulmonary vasculature (Budhiraja et al., 2004; Humbert et al., 2004). Most endothelial vasoactive mediators affect the growth of SMCs and the imbalance may promote structural changes. For example, pulmonary microvascular ECs (PMVECs) from patients with IPAH exhibit excessive production of ET-1 and 5-HT, which can act as paracrine factors stimulating proliferation of PASMCs (Eddahibi et al., 2006). Injury to the endothelium and loss of barrier function also allows access to circulating factors that activate growth factor pathways and endogenous elastases, leading to cell proliferation and remodelling in the underlying medial and adventitial layers (Budhiraja et al., 2004; Humbert et al., 2004; Morrell et al., 2009). Enhanced production of chemokines such as monocyte chemotactic protein-1 (MCP-1) can promote the recruitment of circulating inflammatory cells (Sanchez et al., 2007). Another important function of the endothelium is the elaboration of factors (e.g. heparin sulphates, thrombomodulin, plasminogen activator inhibitors and von Willebrand factor) involved in the maintenance of normal coagulation. Dysfunction of the endothelium is likely to contribute to thrombotic process, which is a common feature of PAH, as discussed in section 1.2.7.

A paradigm of PAH has gained acceptance in which endothelial apoptosis is considered to be a key factor (Sakao et al., 2005; Michelakis, 2006). An inherited or acquired insult leads to the initial apoptosis of PAECs and subsequent appearance of apoptosis-resistant ECs, which may proliferate and give rise to the disorganised angiogenesis found in the plexiform lesions of advanced PAH (Yi et al., 2000). These lesions possess phenotypically distinct ECs that express markers of angiogenesis, such as vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR2) and hypoxia inducible factor subunits α and β (Cool et al., 1999; Geiger et al., 2000; Tuder et al., 2001). CD44 expression in plexiform lesions from IPAH patients has also been linked to increased adhesion and infiltration of T-cells (Ohta-Ogo et al., 2012). These lesions could lead to enormous pulmonary vascular luminal

obliteration. Endothelial dysfunction is now considered a hallmark of PAH (Budhiraja et al., 2004) and the restoration of endothelial function is a common goal of available treatments.

1.2.5 Pulmonary vascular remodelling in PAH

Vascular remodelling usually involves all three layers of the vessel wall and severely reduces the area of the lumen (Tuder et al., 2009) as well as the compliance of larger proximal pulmonary arteries (Fourie et al., 1992). Despite differences in the cause of progression in PAH patients with idiopathic and other forms of the disease, the structural abnormalities found in the pulmonary vasculature are remarkably similar (Strange et al., 2002). Structural changes occur in all three layers of the vessel wall and involve the proliferation or infiltration of ECs, SMCs, fibroblasts, platelets, inflammatory cells, and progenitor cells, as well as ECM components such as collagen, elastin and fibronectin (Tuder et al., 2009). Remodelling is observed in large elastic pulmonary arteries as well as the smaller muscular arteries. There is also distal muscularisation of normally non-muscular vessels, with differentiation of pericytes into SMCs and medial thickening due to SMC hyperplasia and hypertrophy (Rabinovitch, 2008). Occlusive lesions are often observed, with inward neointimal proliferation of SMCs and myofibroblasts that are embedded in a mucopolysaccharide matrix resulting from an imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Lepetit et al., 2005). Plexiform lesions are a feature of severe end-stage disease and thought to represent disorganised angiogenesis, with the clonal expansion of apoptosis-resistant ECs in IPAH patients (Lee et al., 1998). The vasculopathy in PAH leads to occlusion of the pulmonary artery lumen and obliteration of pulmonary vessels, thus the focus for therapy has now been directed towards preventing and reversing excessive vascular cell growth (see section 1.4).

1.2.6 Immune and inflammatory cell involvement

The relationship between inflammation and pulmonary vascular remodelling is well recognised, PAH being associated with a variety of connective tissue diseases as well as viral and parasitic infections. Increasing evidence indicates that inflammatory and/or immune processes are important both in PAH patients and experimental models of the disease (Hassoun et al., 2009). Several histological studies have described inflammatory cell infiltrates, composed of mast cells, monocytes, macrophages, dendritic cells and T lymphocytes, in perivascular regions as well as plexiform lesions in PAH (Tuder et al., 1994; Mitani et al., 1999; Perros et al., 2007). Most animal models of PH also

exhibit a perivascular infiltration of inflammatory cells associated with remodelled vessels (as reviewed by (Price et al., 2012)). Furthermore, Tuder and co-workers have now examined a large series of lungs explanted from PAH patients (n=62) and found significant correlations between the degree of perivascular inflammation and intima plus media remodeling, as well as with adventitial thickness (Stacher et al., 2012). The accumulation/infiltration of immunostained inflammatory and immune cells has also recently been described in lungs from IPAH patients, associated with vascular lesions and distributed in the adventitial layer of pulmonary arteries 20-50µm, 51—150µm and >150µm in diameter (Savai et al., 2012). These included mast cells, macrophages ($CD68^+$), monocytes/macrophages ($CD4^+$), dendritic cells ($CD209^+$), T cells ($CD3^+$), cytoxic ($CD8^+$) and T helper cells ($CD4^+$) and B cells ($CD20^+$). In contrast, regulatory T (Treg) cells ($FoxP3^+$) were found to be depleted in the IPAH pulmonary vasculature. This observation is interesting as it parallels the results of animal studies implicating T-cell deficiency and reduced Treg activity in the development of pulmonary hypertension (Taraseviciene-Stewart et al., 2007; Tamosiuniene et al., 2011).

Cytokines and chemokines are important mediators of inflammation and substantial evidence indicates they have a role in PAH. Circulating cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, and IL-12 are raised in PAH and may predict survival (Humbert et al., 1995; Soon et al., 2010). Levels of chemokines such as MCP-1, chemokine ligand 5 (CCL-5; also known as RANTES - Regulated upon Activation, Normally T cell Expressed and Secreted) and fractalkine (CX3CL1) are also elevated in PAH (Balabanian et al., 2002; Dorfmuller et al., 2002; Itoh et al., 2006; Sanchez et al., 2007). C-reactive protein, a circulating marker of inflammation and tissue damage, is also increased in patients with PAH and correlates with adverse outcomes (Quarck et al., 2009). Studies in experimental models support the role of cytokines/chemokines in the pathogenesis of PAH. For example, the injection or over expression of IL-6 has been shown to promote the development of pulmonary hypertension whereas mice deficient in IL-6 were protected (Miyata et al., 1995; Savale et al., 2009; Steiner et al., 2009). An interaction has also been found with BMPR2 signalling and increased IL-6 levels can suppress BMPR2 expression (Brock et al., 2009). Anti-inflammatory treatments targeting IL-1, MCP-1 and activation of nuclear factor activated T cells and the use of glucocorticoids has also been shown to prevent the development of experimental pulmonary hypertension (Voelkel et al., 1994; Ikeda et al., 2002; Suzuki et al., 2006; Bonnet et al., 2007; Lawrie et al., 2011).

1.2.7 Thrombosis

In situ thrombosis is a common feature of PAH (Fuster et al., 1984) and at least some patients with IPAH exhibit a hypercoagulable phenotype (Tournier et al., 2010). Endothelial dysfunction is likely to be an important cause, providing a pro- rather than anti-thrombotic environment, and the induction of tissue factor is a prominent feature of the pulmonary vasculature in IPAH patients as well as experimental models of pulmonary hypertension (White et al., 2007). Increased circulating levels of fibrinopeptide A (a marker of fibrin generation), plasminogen activator inhibitor 1 and von Willebrand factor (vWF), as well as reduced thrombomodulin, have led to the suggestion that abnormalities of blood coagulation, anti-thrombotic factors and fibrinolysis contribute to a pro-thrombotic state in PAH (Johnson et al., 2006). Platelet aggregation might be increased due to the imbalance in the production of pro-aggregatory (thromboxane A_2) and anti-aggregatory (NO and PGI_2) factors in patients with PAH (Schermuly et al., 2011). Increased platelet and leukocyte activation has been described in MCT-induced pulmonary hypertension (Hu et al., 2010). Anticoagulants such as warfarin are commonly prescribed for patients with PAH (see 1.3 below) and anticoagulants such as aspirin and Factor Xa inhibitor rivaroxaban have also been reported to prevent MCT-induced pulmonary hypertension (Delbeck et al., 2011; Shen et al., 2011).

1.2.8 Genetic mutation

It has been suggested that the development of PAH requires a permissive genotype, vulnerable cell phenotype (EC, SMC or both) and an additional exogenous trigger or "second hit", the latter accounting for reduced penetrance in families with PAH. The discovery of heterozygous germ-line mutations in BMPR2, a constitutively active serine/threonine kinase belonging to the transforming growth factor β (TGF-β) receptors superfamily, represents an important milestone in understanding the pathogenesis of PAH. *BMPR2* mutations are detected in about 70% of families with HPAH (Lane et al., 2000; Aldred et al., 2006; Cogan et al., 2006), and approximately 10 - 40% of IPAH patients (Thomson et al., 2000; Machado et al., 2006). Patients with *BMPR2* mutations present with PAH about 10 years earlier than those without mutations, and are more severely haemodynamically compromised (Sztrymf et al., 2008), and less likely to respond to vasodilator therapy (Rosenzweig et al., 2008). Nevertheless, disease penetrance in individuals with *BMPR2* mutation is only about 20%, and small animal models of heterozygous deletions of *BMPR2* fail to show a PAH phenotype (Long et al., 2006), suggesting that another genetic and/or environmental factor is necessary to develop the phenotype. Studying "second hits" in cultured cells may further our understanding of the differences

between pulmonary ECs in patients with PAH and healthy controls. Examples of potential additional hits include inflammatory insults and increased activity of vasoconstrictors such as 5-HT (Long et al., 2006).

More than 298 mutations identified in *BMPR2* in PAH patients to date (Machado et al., 2009). Many of the mutations (~70%) are either nonsense or frameshift mutations and are likely to result in degradation of the transcripts by nonsense-mediated mRNA decay and a state of haplo-insufficiency arises as only the non-mutant allele encodes protein production. About 30% of mutations lead to missense mutations in highly conserved cysteine residues in functional domains of the receptor. Some interrupt receptor trafficking to the cell surface (Rudarakanchana et al., 2002) and may be rescued with chemical chaperones (Sobolewski et al., 2008). In contrast, non-cysteine mutations within the kinase domain reach the cell surface but display dysfunctional downstream signalling. Mutations also occur in the long cytoplasmic tail of BMPR2. There is a general reduction in the capacity for Smad activation (phosphorylation) and reduced transcription of target genes in cells harbouring BMPR2 mutations (Yang et al., 2005).

Rarely, mutations in activin receptor-like kinase type 1 (ALK-1), another member of the TGF-β superfamily, have been linked with the development of severe PAH in families with hereditary hemorrhagic telangiectasia (HHT) (Trembath et al., 2001). More recently, mutation in *SMAD1, SMAD4* and *SMAD9* (gene symbol for SMAD8), the downstream target of receptors in TGF-β superfamily, were also discovered in HPAH patients (Shintani et al., 2009; Nasim et al., 2011), and mutation in TGF-β type I receptor *ALK-6* was also discovered in children with IPAH who are without mutation in *BMPR2*, *ALK-1* and *SMAD9* (Chida et al., 2012). The mechanism of the dysfunctional BMP and TGF-β signalling in relation to the pathogenesis of PAH will be discussed in more details in section 1.7.5. Other genetic mutations, including a polymorphic variant of *5-HTT* gene promoter have been associated with 5-HTT over-expression and increased 5-HT transport (Eddahibi et al., 2001). In addition, a frameshift mutation in caveolin-1 (*CAV1*) (Austin et al., 2012) has been found in IPAH and HPAH patients with no identified mutation in the TGF-β superfamily members (Maloney et al., 2012). However, these represent infrequent causes of the disease, and some of these mutations need adequately powered cohorts to be verified.

1.3 Current drug treatments for PAH

Most patients with PAH receive so-called 'background' or 'conventional' therapies, which comprise anticoagulants, diuretics, inotropic agents and oxygen supplementation. Anticoagulants such as warfarin might provide survival advantage and improve quality of life, but its contribution is difficult to be estimated and is based on retrospective analysis (Fuster et al., 1984; Frank et al., 1997). Analysis of clinical trials in PAH has shown that a majority of patients are taking diuretics and may offer symptomatic benefit by reducing volume overload and RV wall stress (Galie et al., 2009b). Inotropic agents such as digoxin may also provide some benefit in patients with RV dysfunction due to pulmonary hypertension (Rich et al., 1998), though its long-term effects have not been systemically examined.

Acute vasodilator testing is recommended for all patients with PAH in order to identify those with pulmonary vasoreactivity, defined as a reduction in mPAP of at least ≥ 10mm Hg to an absolute mPAP <35-40mm Hg with either no change or an increase in cardiac output (McLaughlin et al., 2009a). A small proportion (<10%) of patients exhibit pulmonary vasoreactivity when tested with short-acting vasodilators (e.g. adenosine or inhaled nitric oxide). Importantly, they respond to oral calcium-channel blockers such as nifedipine and diltiazem and show marked improvements in the quality of life and survival (Rich et al., 1992; Sitbon et al., 2005a). However, approximately half of these patients experience reduced benefit over time and required alternative PAH-targeted therapies.

Until the 1990s the only therapies available for PAH were those described above. With greater understanding of the pathophysiological mechanisms involved, in particular abnormalities in the endothelial prostacyclin, endothelin and nitric oxide pathways, a number of PAH-specific vasodilator therapies have been developed that target one of these three major pathways. These comprise prostanoids, endothelin receptor antagonists and phosphodiesterase type 5 (PDE5) inhibitors and the results of meta-analysis suggests that their introduction has led to significant improvement in patient's symptomatic status and clinical end-points, including exercise capacity, WHO functional class, pulmonary haemodynamics and survival (Galie et al., 2009c).

1.3.1 Prostanoids

Intravenous PGI_2 (epoprostenol) was the first targeted therapy for PAH and long-term use improves pulmonary haemodynamics and quality of life, delays lung transplantation and is the only therapy that has prospectively been shown to enhance survival (Rubin et al., 1990; Barst et al., 1996; Shapiro et al., 1997; McLaughlin et al., 2002; Sitbon et al., 2002). However, it has a short half life (< 5 min), and its intravenous administration via indwelling catheter and an infusion pump is complex and requires frequent monitoring. In common with all prostanoids, epoprostenol may cause adverse effects such as headache, flushing, jaw pain and gastrointestinal upset, but most problems arise from the route of delivery (Agarwal and Gomberg-Maitland, 2011). Other prostanoid analogues have since been developed and licensed that offer greater stability, longer half-life and alternatives routes of administration. These include a novel formulation of epoprostenol (veletri®) that is stable for up to 24 h and the PGI_2 analogue treprostinil, which can be administered by intravenous, subcutaneous, inhaled or oral routes (Barst et al., 1996; Simonneau et al., 2002; Channick et al., 2006; McLaughlin et al., 2010). Iloprost is a stable PGI_2 analogue developed for intravenous and inhaled administration and was approved in 2004 (Higenbottam et al., 1998; Olschewski et al., 2002; Opitz et al., 2005). Beraprost is an orally active prostanoid that exhibits modest efficacy in PAH patients (Galie et al., 2002; Barst et al., 2003). Data on the oral form of treprostinil has been mixed as it did not display significant benefit when examined in combination with PDE-5 inhibitors or endothelin receptor antagonists (Tapson et al., 2012a; Tapson et al., 2012b) but did display efficacy in PAH patients receiving it as a monotherapy (Rubin et al., 2011).

1.3.2 Endothelin receptor antagonists

Bosentan is an irreversible dual ET_A/ET_B antagonist that was approved in Europe in 2002 on the basis of the BREATHE-1 trial (Channick et al., 2001; Rubin et al., 2002). Subsequent studies indicated that long-term treatment was associated with sustained improvements in exercise capacity, functional class and pulmonary haemodynamics and possibly survival (McLaughlin et al., 2005; Sitbon et al., 2005b). Bosentan also conferred haemodynamic improvements and delayed time to clinical worsening in patients with mildly symptomatic PAH (Galie et al., 2008b) and may be effective in slowing disease progression in children with PAH (Hislop et al., 2011).

The selective ET_A antagonists sitaxentan and ambrisentan were subsequently licensed in 2006 and 2008 after showing favourable results in clinical trials (Barst et al., 2004; Barst et al., 2006; Galie et al., 2008a; Oudiz et al., 2009). However, sitaxentan was withdrawn worldwide in 2010 after a

number of deaths attributed to liver failure were reported (Lavelle et al., 2009; Lee et al., 2011). In the absence of head-to-head, adequately powered, randomised controlled trials, it is difficult to judge whether selective ET_A antagonism, which spares endothelial ET_B receptors and theoretically preserves ET-1-mediated vasodilatory and anti-mitogenic activity, offers advantages in efficacy over combined ET_A and ET_B antagonism, but their effects are similar in magnitude (Rhodes et al., 2009; O'Callaghan et al., 2011). Ambriesentan has however displayed less hepatotoxicity than bosentan, providing an alternative for patients that develop acute liver damage with other antagonists (McGoon et al., 2009). Macitentan, a novel and highly potent dual ET_A/ET_B antagonist, has recently completed a phase III placebo-controlled (SERAPHIN) trial, which showed favourable results with reduced risk of a morbidity/mortality event by 30% (3 mg dose) to 45% (10 mg dose) compared with those who received placebo and was well tolerated (Rubin et al., 2012).

1.3.3 Phosphodiesterase type 5 inhibitors

The NO-cGMP signalling pathway has become an important therapeutic target in PAH, with phosphodiesterase type 5 (PDE5) inhibitors exerting anti-proliferative as well as vasodilatory effects on PASMCs (Wharton et al., 2005). Sildenafil was the first PDE-5 inhibitor approved as a PAH therapy, based on the SUPER-1 trial (Galie et al., 2005). There are uncertainties however concerning its impact at approved doses on haemodynamics and long-term survival (Agarwal and Gomberg-Maitland, 2011) and in Europe it is only approved as a monotherapy for PAH patients with functional class II-III symptoms (Figure 1.1). Two other PDE5 inhibitors, tadalafil and vardenafil, have also been examined as treatments (Galie et al., 2009a; Jing et al., 2011) and tadalafil received approval for use in patients with PAH in 2009.

1.3.4 Combination treatment

Meta-analysis of randomised controlled trials in PAH suggests an improvement of survival in the patients treated with the targeted therapies (Galie et al., 2009c) and consensus evidence-based treatment algorithms have been developed (Figure 1.1). Continued disease progression in PAH has led however to consideration of the need for early aggressive intervention and combination therapy in patients who fail to show improvement or deteriorate with monotherapy (Galie et al., 2009b). Several studies support the efficacy of combination treatment but the long-term clinical benefits remain unclear (Barst et al., 2009). In this regard, ongoing trials such as AMBITION (ClinicalTrials.gov

Identifier NCT01178073), COMPASS-2 (ClinicalTrials.gov Identifier NCT00303459) and A1481243 (ClinicalTrials.gov Identifier NCT00323297) should provide additional data on how combination therapy and goal-oriented treatment strategies affect outcome in PAH.

While the present therapies represent a significant advance many PAH patients do not respond to the current vasodilator treatments and responsiveness declines as the disease progresses. The one year incident mortality rate remains high (12-15%) for many patients with PAH (Humbert et al., 2006; Thenappan et al., 2007) and contemporary registry data in France and the USA indicate that longer term survival rates are also poor (Benza et al., 2010; Humbert et al., 2010a; Humbert et al., 2010b).

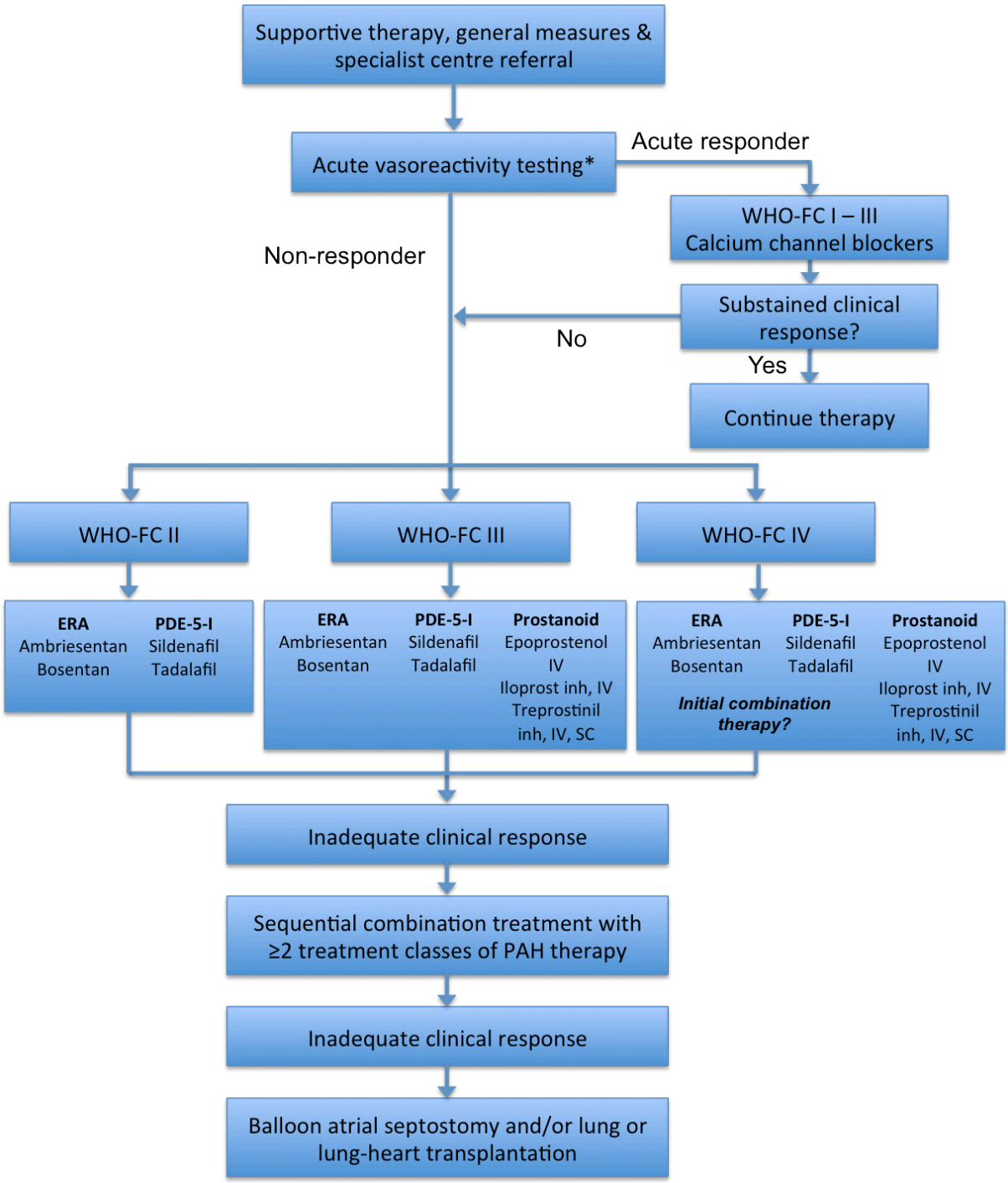


Figure 1.1. Treatment algorithm for pulmonary arterial hypertension. *Licensed indications for individual agents with respect to functional class vary between countries. ERA: endothelin receptor antagonists; Inh: inhaled; IV: intravenous; PAH: pulmonary arterial hypertension; PDE-5-I: phosphodiesterase type 5 inhibitors; SC: subcuntaneous; WHO-FC: World Health Organisation functional class. (Modified from O'Callaghan *et al.* 2011a)

1.4 Emerging therapeutics for PAH

1.4.1 Emerging drug treatments for PAH

There is an unmet need for drugs that can reverse pulmonary vascular remodelling, improve survival and inhibit disease progression in patients with PAH. This includes agents that can stop cell proliferation, inhibit cell migration and matrix deposition or promote apoptosis, as recently reviewed (Archer et al., 2010; Agarwal and Gomberg-Maitland, 2011; McLaughlin, 2011; Schermuly et al., 2011). In fact, there are now more than 30 drugs at different stages of development and although some represent variations/extensions of current therapies, the majority are novel 'first-in-class' drugs. They include growth factor receptor tyrosine kinase inhibitors (imatinib, nilotinib and sorafenib) (Moreno-Vinasco et al., 2008; Ghofrani et al., 2010b; Gomberg-Maitland et al., 2010; Hoeper et al., 2011; Chaumais et al., 2012; Hoeper et al., 2012); drugs that target changes in mitochondrial and metabolic function (e.g. dichloroacetate, a pyruvate dehydrogenase kinase inhibitor, and peroxisome proliferator-activator receptor agonists) (Michelakis et al., 2002; McMurtry et al., 2004; Guignabert et al., 2009); soluble guanylate cyclise stimulators and activators (e.g. riociguat) (Ghofrani et al., 2010a); agents that enhance coupling of eNOS (e.g. cicletanine) (Waxman et al., 2008); prostacylin receptor agonists (e.g. selexipag) (Simonneau et al., 2012); vasoactive intestinal peptide (Petkov et al., 2003; Leuchte et al., 2008); adrenomedullin (Harada-Shiba et al., 2009); apelin (Alastalo et al., 2011); serotonin inhibitors (e.g. terguride) (Ghofrani et al., 2012); inhibitors of Rho kinase signalling (Fujita et al., 2010); protease and elastase inhibitors; the oral late sodium current inhibitor ranolazine (Liles et al., 2011); and drugs that affect epigenetic targets such as histone deacetylase (HDAC) activity (Zhao et al., 2012). Studies with tyrosine kinase inhibitors (imatinib, nilotinib), soluble guanylate cyclise stimulator (riociguat), serotonin 5-HT_{2A} and 5-HT_{2B} receptor antagonist (terguride) and orally available prostacyclin receptor agonist (selexipag) are among the most advanced, having displayed encouraging results in animal models and reached phase II-III human trials (Schermuly et al., 2011). Not all drugs prove to be successful however and several have been withdrawn following human trials, recent examples being cicletanine and imatinib.

1.4.2 Gene- and microRNA-based therapies for PAH

Recent studies in experimental animal models have demonstrated the potential of gene- and microRNA-based therapies in PAH. Deficiencies in BMPR2 expression and signalling in PAH patients and animal models makes this an obvious potential target, although an initial attempt to over

express *BMPR2* was not successful in inhibiting MCT-induced pulmonary hypertension (McMurtry et al. 2007). In contrast, Reynolds and co-workers used an adenoviral *BMPR2* gene delivery vector to target the endothelium in the pulmonary hypertensive rat lung, restoring BMPR2 levels, diminishing up-regulation of TGF-β signalling, improving pulmonary haemodynamics and reducing cardiovascular remodelling (Reynolds et al., 2007; Reynolds et al., 2012). As most *BMPR2* mutations are thought to lead to haploinsuffiency, alternative approaches that increase BMPR2 mRNA or protein expression might also be successful. For example, it may be possible to rescue the failure of BMPR2 protein trafficking in PAH (Sobolewski et al., 2008) and inhibit the removal and degradation of the receptor from the cell surface (Durrington et al., 2010).

It is now recognised that non-protein coding microRNA (miRNA or miR) sequences have a critical role in regulating gene expression in the cardiovascular system (Small and Olson, 2011) and aberrant miRNA expression has been implicated in the pathogenesis of cardiovascular diseases such as PAH (McDonald et al., 2012). In particular, two miRNAs (miR-17 and miR-20a) have been found to regulate *BMPR2* expression and specific inhibitors (so-called 'antagomirs') used to block these miRNAs in experimental animals (Brock et al., 2012; Pullamsetti et al., 2012). Injection of antigomirs directed against miR-17 or miR-20a was found to improve pulmonary haemodynamics and reduce cardiovascular remodelling in hypoxia- and MCT-induced pulmonary hypertension.

1.4.3 Cell-based therapies for PAH

Cell-based therapies represent another approach for the treatment of pulmonary diseases. This strategy involves the use of stem or progenitor cells, which may be manipulated to inhibit disease progression or initiate repair and regeneration. This is further discussed in section 1.6.4.

1.5 Endothelial progenitor cells (EPCs)

1.5.1 Definition

The endothelium is considered to be central in maintaining vascular homeostasis and in the pathogenesis of vascular diseases such as PAH (Hirschi et al., 2008). New blood vessel formation occurs via three main processes: vasculogenesis, angiogenesis, or arteriogenesis. Vasculogenesis is the *de novo* formation of vascular structures, begins with local differentiation of mesodermal precursors-derived cluster of hematopoetic stem cells at the centre and angioblasts at the periphery that differentiate into ECs that coalesce into a primitive network, and is historically thought to be exclusively a phenomenum of embryonic development (Risau and Flamme, 1995). Angiogenesis comprises several morphogenic events that involve sprouting, branching, lumen formation, making new connections (anastomose) and rearrangements, a process that involves cell migration, proliferation and tubulogenesis. These result in a new vascular network from the endothelium of pre-existing vessels that can be regulated by proteases, growth factors and micro-environmental cues (Risau, 1997). Arterogenesis involves the specific remodelling of existing endothelial capillaries for greater size, elasticity, and reactivity by recruitment of and encompassed by SMCs and other mesenchymal cells that secrete specific ECM components (Carmeliet and Jain, 2011).

It has been known for several decades that circulating ECs exist and have some level of proliferative potential, as shown by the ability of EC monolayers to form on Dacron grafts in animal models (Stump et al., 1963; Gonzalez et al., 1969). However, a paradigm shift occurred when a circulating population of $CD34^+$ or $VEGFR2^+$ progenitor cells was identified in adult human peripheral blood, with the ability to differentiate into ECs *in vitro* and participate in postnatal vasculogenesis *in vivo* (Asahara et al., 1997; Shi et al., 1998). These so-call endothelial progenitor cells (EPCs) have since been recognised as a normal component of the blood and the term has been widely applied to describe a heterogeneous group of cells that have the potential to form mature ECs and/or participate in angiogenesis. Typically, they were thought to arise from mesodermal stem cells or so-called haemangioblasts (common progenitor of hematopoietic cells and ECs) in the bone marrow (BM) and home to sites of ischemia or endothelial injury, contributing to neovascularisation and tissue repair (Hristov et al., 2003; Hristov and Weber, 2004; Liew et al., 2006) (see Figure 1.2). Numerous studies have since attempted to define the circulating levels of EPCs in a variety of clinical disorders, but true circulating EPCs appear to be present in very low numbers and in order to study their functional activity and differentiation into ECs, *ex vivo* expansion is necessary (Hirschi et al., 2008).

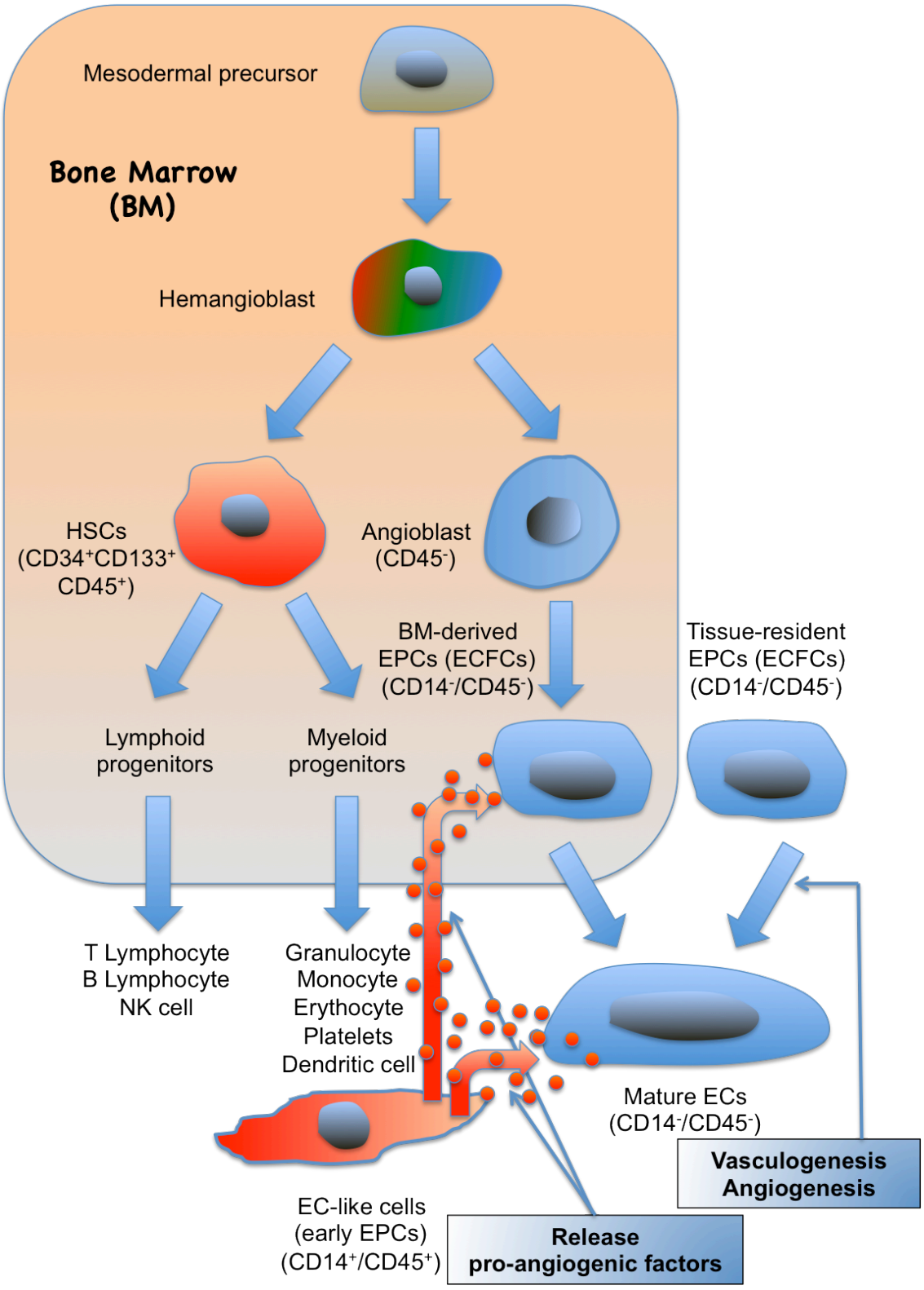


Figure 1.2. Putative origin and differentiation of endothelial progenitor cells (EPCs) and their relation to cells expressing CD45. The figure displays the possible origin and differentiation of EPCs and the interaction between the pro-angiogenic "early" EPCs and ECFCs in promoting angiogenesis and vasculogenesis. BM, bone marrow; ECs, endothelial cells; ECFCs, endothelial colony-forming cells; HSCs, hematopoietic stem cells.

1.5.2 Identification and growth of EPCs in vitro

Despite enormous interest in EPCs and their role in cardiovascular disease, there is a lack of consensus regarding their phenotype. In fact, a variety of cells types have been described as being EPCs, leading to considerable confusion in nomenclature (Hirschi et al., 2008; Timmermans et al., 2009). Essentially two methods have been used to assess EPCs: flow cytometry and cell culture.

Identification by flow cytometry

Many research groups have attempted to identify circulating EPCs, using flow cytometry to demonstrate surface proteins. This included different combinations of the three most commonly used markers – CD34 (hematopoietic stem cell – HSC – marker), CD133 (immature hematopoietic cell marker), and VEGFR2 (KDR in human, Flk-1 in mice) – with or without other markers such as CD31 and Tie2 (comprehensively reviewed by Timmermans et al. 2009). Various studies have reported statistically significant correlations between circulating putative EPCs and disease state, but relatively few also sought to establish the functional properties of these cells. A specific subset of $CD34^+$ ($CD34^+VEGFR2^+CD133^+$) mononuclear cells (MNCs) was widely considered to represent EPCs and were isolated from human BM, umbilical cord blood (UCB) and granulocyte-colony stimulating factor (G-CSF)-mobilised adult peripheral blood. However, they failed to establish EC colonies or Matrigel tubular networks *in vitro,* expressed the pan-leukocyte marker CD45 and displayed hematopoietic progenitor cell (HPC) properties in hematopoiesis assays (Case et al., 2007; Timmermans et al., 2007). In fact, $CD14^+$, $CD34^+/CD45^+$, $CD45^+$ or $CD133^+$ selected MNCs do not give rise to EPC colonies with typical cobble-stone morphology. Conversely, cells with this capacity lack the monocytic ($CD14^-$) and haematopoietic ($CD45^-$) markers (Gulati et al., 2003; Case et al., 2007; Timmermans et al., 2007). This led to doubt about the validity of using surface marker combinations involving $CD34^+$ subsets to identify circulating EPCs. Flow cytometry is currently limited by the lack of specific cell surface markers and standardised methodology for identifying EPCs (see also section 1.5.5).

Identification by cell culture

Cell culture methods enable the isolation and expansion of cells for analysis *in vitro* and *in vivo*. Two major cell sub-populations have been identified in human MNCs cultures derived from UCB and adult peripheral blood (Figure 1.3). These comprise (i) so-called **"early" EPCs** (also known as early

outgrowth EPCs, colony-forming unit-endothelial cells (CFU-EC), colony-forming unit-Hill (CFU-Hill), circulating angiogenic cells) that display a mixed endothelial/monocytic/haematopoietic phenotype (Asahara et al., 1997; Shi et al., 1998; Peichev et al., 2000; Romagnani et al., 2005; Diller et al., 2008; Sieveking et al., 2008), and (ii) proliferative **"late" EPCs** (also known as late outgrowth EPCs, endothelial colony-forming cells (ECFCs), blood outgrowth endothelial cells, endothelial outgrowth cells, endothelial progenitor-derived cells) that display typical endothelial morphology and characteristics and possesses an endothelial progenitor lineage (Lin et al., 2000; Ingram et al., 2004; Yoder et al., 2007; Sieveking et al., 2008). "Early" EPCs are identified after the short term (4 - 7 days) in culture of peripheral blood MNCs (PBMNCs) on fibronectin- or collagen-coated plates. They are typically spindle-shaped and have the capacity to form colonies (so-called CFU-ECs or CFU-Hill) when cultured in EndoCult™ medium. In contrast, "late" EPCs represent rare precursors (~ one cell in 20ml blood), they are generally identified only ~14 days after culturing PBMNCs, and give rise to a circumscribed colony of cells with a cobblestone morphology akin to the appearance of primary endothelial cells in culture (Lin et al., 2000; Ingram et al., 2004; Yoder et al., 2007). These cells were first identified as blood outgrowth endothelial cells (BOECs) or endothelial outgrowth cells (EOCs) (Lin et al., 2000), but are now commonly referred to as endothelial colony-forming cells (ECFCs) after Yoder and colleague refined the culture technique and demonstrated their ability to form colonies from a single cell (Ingram et al., 2004; Yoder et al., 2007). Throughout this thesis, I will use the term ECFCs. A diagrammatic summary of the common culture methods and cultured "EPCs" is provided in Figure 1.3.

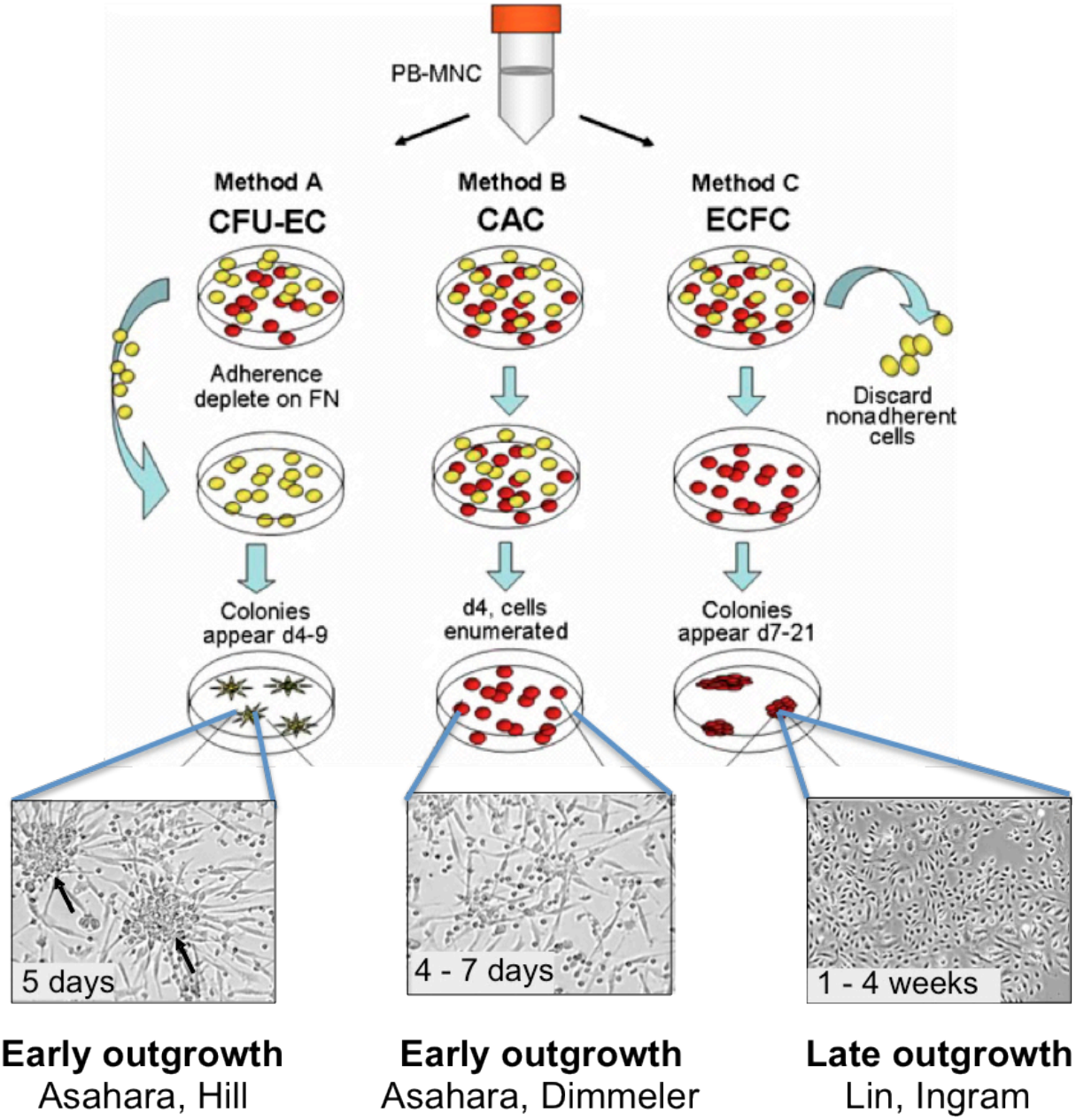


Figure 1.3. An overview of the most common methods used to isolate EPCs from peripheral blood mononuclear cells (PB-MNCs). Method A - Culture of colony-forming unit-endothelial cells (CFU-EC, also known as CFU-Hill) includes a 5 day process where non-adherent MNCs are recultured and give rise to the colony. **Metod B** – Culture of circulating angiogenic cells (CAC). Includes adherent cells after 4 to 7 days of culture, which do not typically display colony formation. **Method C** – Culture of endothelial colony-forming cells (ECFCs), derived from adherent MNCs cultured for 7 days (for MNCs derived from cord blood), or 13 – 28 days (for MNCs derived from peripheral blood). Colonies display a cobblestone morphology. Modified from (Prater et al., 2007; Diller et al., 2010).

1.5.3 Hematopoietic cells/CFU-EC characteristics

Both "early" and "late" populations of EPCs are positive for endothelial surface markers (e.g. CD31, CD105, VE-cadherin, eNOS and VEGFR2) and the intracellular protein markers (eNOS and vWF). They can also express the mesoderm progeny marker mucosialin (CD34), type I scavenger receptor (as demonstrated by uptake of acetylated low density lipoprotein; acLDL) and bind *Ulex europeasus* agglutinin-1 (UEA-1) lectin (Hirschi et al., 2008). "Early" EPCs can be distinguished by their ability to form colonies in culture using *in vitro* colony-forming assays, hence they are also known as "CFU-EC" or "CFU-Hill" (Hill et al., 2003). However, this capacity has been shown to be heavily dependent on T cells (particularly activated T cells) that produces cytokines and MMP-9, questioning the use of CFU-EC assay as a means of identifying EPCs (Rohde et al., 2006; Hur et al., 2007; van Beem et al., 2008). In addition, these cells are heterogeneous and comprise predominantly hematopoietic cells of myeloid lineage that display hematopoietic ($CD133^+$), pan-leukocyte ($CD45^+$) and monocyte/macrophage ($CD14^+$; $CD11c^+$; $CD163^+$) surface markers – which are not expressed by mature ECs – and possess the monocyte/macrophage characteristics phagocytosis, and form hematopoietic colonies in hematopoiesis assays (Rehman et al., 2003; Rohde et al., 2006; Zhang et al., 2006; Case et al., 2007; Prater et al., 2007; Yoder et al., 2007; Medina et al., 2010). Although these cells also take-up acLDL and bind UEA-1 lectin (historically considered to be endothelial properties), it is now known that monocytes and macrophages also take up ac-LDL and UEA-1 lectin binds to epithelial cells and a variety of other cell types (Hirschi et al., 2008). CFU-ECs, but not ECFCs, can also harbour mutations present in patients with myeloproliferative disorders, indicating the CFU-ECs belong to the hematopoetic lineage (Piaggio et al., 2009). Moreover, CFU-ECs lack the proliferative capacity of true progenitors and do not independently form *de novo* capillary-like structures *in vitro* (Hur et al., 2004; Sieveking et al., 2008) or contribute directly to neovessel formation via *in vivo* (Yoon et al., 2005; Cho et al., 2007; Yoder et al., 2007). Nonetheless, they may contribute indirectly in a paracrine fashion by secreting angiogenic substances such as VEGF, hepatocyte growth factor (HGF), IL-8, G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rehman et al., 2003; Hur et al., 2004) that promote vascular repair by resident ECs *in vivo* (Yoon et al., 2005; Cho et al., 2007; Yoder et al., 2007) and the incorporation of ECFCs in tubular networks *in vitro* (Rehman et al., 2003; Yoon et al., 2005; Cho et al., 2007; Yoder et al., 2007; Sieveking et al., 2008). Criticism of the culture method has also been raised due to prominent contamination by platelets and the indiscriminate transfer of platelet plasma membrane proteins to adherent cells attached to the culture matrix (Prokopi et al., 2009).

1.5.4 Endothelial colony-forming cells (ECFCs)

ECFCs display properties of true EPCs, having the ability to independently form *de novo* tubular networks *in vitro* (Hur et al., 2004; Case et al., 2007; Sieveking et al., 2008); structurally contribute to neovessels and inosculate with nearby vessels in subcutaneous gels implanted in immunodeficient mice (Shepherd et al., 2006a; Melero-Martin et al., 2007; Yoder et al., 2007); and exhibit high clonogenic and proliferative potential (Hur et al., 2004; Ingram et al., 2004; Yoder et al., 2007). Typical EC characteristics were also demonstrated through up-regulation of activated EC marker VCAM-1 upon TNF-α and IL-1 stimulation (Ingram et al., 2004). In addition, ECFCs do not express pan-leukocyte marker CD45, monocytic/macrophage markers CD14 or CD115 and do not ingest bacteria or give rise to HPCs colonies (Case et al., 2007; Yoder et al., 2007). Secretion of high level of MMP-2, but low level of MMP-9, and selective expression of BMP-2 and BMP-4 in ECFCs also distinguishes them from early EPCs, these features being considered to be important for their differentiation and regulation in angiogenesis (Yoon et al., 2005; Smadja et al., 2008). Other factors may influence the survival and proliferation of ECFCs. For example, activation of the thrombin receptor protease-activated receptor 1 promotes proliferation, migration, differentiation and IL-8 synthesis in these cells, leading to a pro-angiogenic effect *in vitro* (Smadja et al., 2005; Smadja et al., 2009a). The features that distinguish the “early” and “late” EPC populations in culture are summarised in Table 1.3.

Nomenclature	**"Early" EPCs** Also known as CFU-EC, CFU-Hill, circulating angiogenic cells & hematopoietic progenitor cells	**"Late" EPCs or ECFCs** Also known as blood outgrowth endothelial cells, endothelial outgrowth cells & endothelial progenitor-derived cells
Origin	**$CD45^+$ hematopoietic lineage cells ($CD34^+$ $CD45^+$/$CD133^+$ $CD45^+$, $CD34^-$ $CD45^+$ $CD14^+$ $CD45^+$)**	**$CD45^-CD133^-CD34^+$ cells/ $CD34^+$ $CD146^+$ $CD31^+$ $CD105^+$ $CD45^-$ cells, from bone marrow or the vascular wall**
Appearance	**Round to spindle shape appearance**	**Typical polygonal cells in a confluent cobblestone monolayer**
Endothelial population	**Heterogeneous**	**Homogenous**
Functional properties:		
Clonal proliferative status	**Low**	**High**
Replating ability	**-**	**+**
In vitro tube formation	**-**	**+**
De novo vessel formation *in vivo*	**-**	**+**
NO generation	**Low**	**High**
Homing to ischemic sites *in vivo*	**+**	**+**
Proangiogenic factors release	**High**	**Low**
Paracrine augmentation of angiogenesis	**+**	**+/-**
Hematopoeitic colony-forming potential	**+**	**-**
Phagocytosis	**+**	**-**
MMPs release	**High MMP9 release**	**High MMP2 release**
Phenotype:		
Endothelial markers expression	**$CD31^+$, Flk-1^+, Tie-2^+, $VEGFR2^+$, Also VE-$cadherin^+$, $CD105^+$, vWF^+, $eNOS^+$**	**$CD31^+$, Flk-1^+, Tie-2^+, $VEGFR2^+$, VE-$cadherin^+$,$CD105^+$, vWF^+, $eNOS^+$, $CD146^+$, Caveolin-1^+**
Hematopoetic/monocytic markers expression	**$CD14^{+/-}$, $CD45^{+/-}$, $CD11c^+$, $CD163^+$, $CD115^+$**	**None**
Progenitor markers expression	**$CD34^{+/-}$, $CD133^+$**	**$CD34^{+/-}$, $CD133^-$, c-$kit^{+/-}$**
acLDL uptake	**+**	**+**
UEA-1 lectin binding	**+**	**+**
BMP-2 & BMP-4	**-**	**+**

Table 1.3. Characteristics of the two main "EPC" populations in culture. Represents a summary of the literature cited in this chapter. (+) adherent cells that display a function/expression; (-) cells that do not display a function/expression ; (+/-) the literature provides conflicting evidence. MMP, matrix metalloproteinase; NO, nitric oxide; eNOS, endothelial NO synthase; VEGFR2 , vascular endothelial growth factor receptor 2; vWF, von Willebrand factor; acLDL, acetylated low density lipoprotein; UEA-1, *Ulex europeasus* agglutinin-1; BMP, bone morphogentic protein.

Despite their detailed characterisation, it is still unknown whether (i) ECFCs expanded *ex vivo* represent the differentiated progeny of an undifferentiated precursor cell *in vivo*; (ii) ECFCs are unipotent or possess mutli-potent stem cell potential; and (iii) if ECFCs are fully differentiated ECs with high proliferative potential. Earlier studies indicated that ECFCs displayed greater proliferative potential, angiogenic cytokine release and stress resistance compared with HUVECs and other types of EC (Bompais et al., 2004; He et al., 2004). However, some of these studies compared UCB-derived ECFCs that are known to have enhanced clonogenic and proliferative potential (Ingram et al., 2004) and contradicting results have been reported elsewhere (Ingram et al., 2007). In fact, only one study to date has reported increased *in vitro* and *in vivo* vessel forming ability in UCB-ECFCs compared to HUVECs (Nagano et al., 2007). Like ECFCs, mature ECs also have the ability to independently form tubular networks in Matrigel implants (Melero-Martin et al., 2007; Yoder et al., 2007). More detailed molecular analysis of ECFCs has also served to emphasise their endothelial nature, possessing a transcriptome, proteome ultrastructure that closely resembles those of mature (human dermal microvascular) ECs (Medina et al., 2010). Despite exhibiting features consistent with an endothelial lineage, ECFCs may also express specific proteins (Medina et al., 2010) and possess functional characteristics that distinguish them from mature ECs. For example, ECFCs are reported to be more proliferative and resistant to serum-deprivation than mature ECs and may also differ in their *in vivo* vasculogenic potential (Hur et al., 2004; Finkenzeller et al., 2009). ECFCs seem to possess higher rate of spreading and higher expression of $\alpha_5\beta_1$ when compared to human aortic ECs (Stroncek et al., 2009). Furthermore, ECFCs and mature ECs in culture have recently been shown to vary in their response to hypoxia and deposition of extracellular matrix (Kusuma et al., 2012).

1.5.5 Origin of ECFCs

The origin of ECFCs remains elusive. Although proliferative ECFCs have been demonstrated to originate from BM in human BM transplants (Lin et al., 2000), human UCB and adult peripheral blood (Ingram et al., 2004), a similar hierarchy of ECFCs have also been identified among ECs isolated from the human umbilical vein, aorta (Ingram et al., 2005; Zhang et al., 2009) and pulmonary artery (Duong et al., 2011), as well as rat PMVECs and rat PAECs (Alvarez et al., 2008). Thus, the endothelium of the mature vessel wall contains ECs with a robust proliferative potential and maybe the origin of ECFCs. Indeed, a recent study has challenged the concept of a BM-derived circulating precursor for ECs, demonstrating that ECFCs from $CD34^+/CD133^-/CD146^+$ PBMNCs are similar, if not identical, to mature ECs and contribute to *de novo* vessel formation whereas BM-derived cells isolated under the same conditions do not (Tura et al., 2012).

However, no markers yet to date can convincingly discriminate ECFCs against mature ECs, impeding its progress as a therapeutic agent for cardiovascular disorders. Indeed, a common origin has been suggested for both the hematopoietic pro-angiogenic cells and ECFCs, as both cell populations share similar endothelial surface antigens (e.g. CD31, VEGFR2) and can both be derived from $CD34^+$ cells (Asahara et al., 1997; Bompais et al., 2004). They could be derived from the so-called hemangioblasts, a bipotent mesodermal stem cell that can differentiate into both hematopoietic cells and ECs (Timmermans et al., 2009) (see Figure 1.3). Although CD133, an immature progenitor cell marker, is not present in both ECFCs and mature ECs, CD133 expression can be detected earlier at day 14 of culture in ECFCs derived from UCB-$CD34^+$ cells and decrease gradually during maturation (low expression at day 28 and undetectable at day 40), while endothelial markers (CD144, KDR, Tie-1) gradually increases, suggesting possible CD133+ expression in the immature phase of ECFCs *in vivo* that might have been lost upon long term *ex vivo* expansion (Bompais et al., 2004).

Recent advances in flow cytometry instrumentation and analytical software have allowed the development of polychromatic flow cytometry that may permit the identification of putative EPCs and can distinguish pro-angiogenic "early" EPCs with hematopoietic lineage form and circulating ECFCs (Estes et al., 2010a; Estes et al., 2010b). Using this advanced technique, a rare subset of UCB MNCs was identified ($CD34^+CD146^+CD31^+CD105^+CD45^-$) that contained both highly proliferative ECFCs, which gave rise to ECFC but not HPC colonies, and circulating ECs with little or no clonogenic potential (Mund et al., 2012). In the future, the identification of ECFCs may also be aided by novel peptide ligands that specifically bind to these cells (Veleva et al., 2007). Nevertheless, blood-derived ECFCs closely resemble ECs of the vascular wall and represent a potentially important non-invasive means of obtaining patient-specific ECs for functional and biochemical investigation (Fadini and Avogaro, 2010).

1.6 Role of progenitor cells in PAH

1.6.1 Involvement of progenitor cells in pulmonary vascular remodelling in PAH

Several investigations have described mobilisation of BM-derived MNCs and recruitment of endogenous cells in the lung and pulmonary vasculature in experimental hypoxia-induced PH and pneumonectomy-induced PH, but it is unclear whether this represents a protective process (Satoh et al., 2006) or their involvement in the disease progression, advancing neointimal formation, adventitial thickening, perivascular fibrosis, and myofibroblast accumulation (Davie et al., 2004; Hayashida et al., 2005; Satoh et al., 2006; Sahara et al., 2007; Satoh et al., 2009; Angelini et al., 2011; Liang et al., 2011).

The transmembrane tyrosine kinase receptor for stem cell factor, c-kit, is considered to be a marker for BM-derived HSCs, although it is not exclusive to these cells (Lerner et al., 1991; Ogawa et al., 1991). Putative BM progenitor cells expressing c-kit, CD45 and CD133 have been found to accumulate among ECs in the expanded vasa vasorum of pulmonary arteries in chronically hypoxic bovine models of PH (Davie et al., 2004; Frid et al., 2009). An accumulation of similar cells has been observed in lung tissues from PAH patients and circulating soluble c-kit is increased in IPAH, but it is unclear whether these cells are derived locally or from the circulation (Toshner et al., 2009; Montani et al., 2011). Nevertheless, increased stromal cell-derived factor-1α (SDF-1α) and its respective C-X-C chemokine receptor type 4 (CXCR4) were also found in ECs within occlusive lesions in the IPAH lung (Toshner et al., 2009; Montani et al., 2011). This is considered to be a major homing pathway for progenitor and inflammatory cells and could be modulated by c-kit activity (Phillips et al., 2004; Satoh et al., 2009; Young et al., 2009; Cheng et al., 2010; Sharma et al., 2011). Conflicting arguments exist for using this pathway as a therapeutic target, as its inhibition has been associated with a reduction in BM-derived cell recruitment and reduced pulmonary vascular remodelling in hypoxia-induced PH (Satoh et al., 2009; Young et al., 2009; Yu and Hales, 2011), while it has also been reported to augment cell recruitment and neovascularisation in another study (Jujo et al., 2010). In addition to PAH, putative EPCs are postulated to contribute to neointimal proliferation and vascular remodelling in patients with CTEPH (Yao et al., 2009b).

1.6.2 Circulating progenitors in PAH

The number of circulating "EPCs" and activity of cultured cells isolated from peripheral blood may be affected in various pulmonary conditions (Weiss, 2008). An increase in circulating progenitors

(defined by Flk-1$^+$/CD133$^+$, CD45$^+$/VEGFR2$^+$, CD45$^+$/VEGFR2$^+$/c-kit$^+$ and CXCR4$^+$/VEGFR2$^+$/c-kit$^+$ cells) has been observed in hypoxia-induced PH in mice (Satoh et al., 2006; Marsboom et al., 2008; Satoh et al., 2009), whereas CD133$^+$/VEGFR2$^+$ cells were depleted in a canine model of MCT-induced PH (Xia et al., 2009b). Conflicting data has also been obtained when putative circulating progenitors were assessed in human PAH. A number of studies have described a reduction in circulating "early" EPCs in PAH patients (including patients with IPAH, HPAH and PAH associated with connective tissue disease, congenital heart disease, or sickle cell disease), when compared with healthy controls (Fadini et al., 2007; Diller et al., 2008; Junhui et al., 2008; Anjum et al., 2012), while others have reported an increase in circulating angiogenic cells in patients with PAH (Asosingh et al., 2008; Toshner et al., 2009; Farha et al., 2011; Hansmann et al., 2011; Montani et al., 2011) or found no difference (Smadja et al., 2009b) (see Table 1.4 for a summary). These discrepancies may be due to variation in the methodology and protein markers employed to identify and quantify putative progenitor cells, differences in flow cytometry, experimental design and cell preparation techniques, and differences in the stage of disease and therapy prior to blood sampling. It might be argued that a reduction in circulating progenitor cells reflects a depletion of BM pool due to ongoing endothelial damage and EPC recruitment (Fadini et al., 2007), while increased circulating progenitors might represent participation in the proliferative vascular remodelling in PAH. It remains difficult to identify and quantify EPCs without specific cells markers, and advancement in flow cytometry might help to elucidate these specific markers in future (discussed in section 1.5.5). The recent development of a microfluidic chip that measures circulating progenitor in only 200 µl of human blood may enable the development of bedside screening and monitoring of patients in the future (Hansmann et al., 2011).

A number of studies have shown that the number of CFU-ECs and CD14 monocytes are related to cardiovascular risk (Hill et al., 2003) and implicated in the genesis of vascular pathology in diseases such as diabetes (Loomans et al., 2004), COPD (Palange et al., 2006), congenital heart failure (Valgimigli et al., 2004) and rheumatoid arthritis (Grisar et al., 2005). In addition to alterations in the number of circulating cells, the *in vitro* colony forming ability of the CFU-ECs was also altered, with increased CFU-ECs or "early" adherent cells detected in IPAH patients (Asosingh et al., 2008; Toshner et al., 2009; Farha et al., 2011). All the studies have pointed to a dysfunctional phenotype, cells from IPAH patients exhibiting distinct dysfunctional responses to apoptotic stimuli and TGF-β1 or BMPs, an impaired ability to incorporate in tubular networks, as well as reduced migration, adherence and production of angiogenic factors (Teichert-Kuliszewska et al., 2006; Asosingh et al., 2008; Diller et al., 2008). A Janus faced paradigm has emerged, where EPCs recruited from the bone marrow or resident in the vessel wall, either proliferate to repair the damaged endothelium or contribute in the vascular remodelling and progression of pulmonary vasculopathy (Figure 1.4).

Author	Year	Type of PH	N	EPCs definition for quantification	Difference vs controls
Fadini et al.	2007	IPF-PH	5	CD34+	↓~50%
				CD133+/VEGFR2+	↓~90%
				CD34+/CD133+/KDR+	↓~90%
JunHui et al.	2008	IPAH	20	CD133+/KDR+	↓~30%
Diller et al.	2008	IPAH	55	CD34+	ns
				CD34+/CD133+	ns
				CD34+/CD133+/KDR+	↓~60%
				CD34+/CD45low	↓~50%
		APAH (EMS)	41	CD34+	↓~60%
				CD34+/CD133+	↓~60%
				CD34+/CD133+/KDR+	↓~80%
				CD34+/CD45low	↓~95%
Asosingh et al.	2008	IPAH	10	CD34+/CD133+	↑~3.0x
Toshner et al. (2 center study)	2009	IPAH	7	CD34+/CD133+/VEGFR2+	↑~5.0x
				CD34+/CD133+/VEGFR2-	ns
		HPAH	4	CD34+/CD133+/VEGFR2+	↑~3.0x
				CD34+/CD133+/VEGFR2-	ns
		IPAH	10	CD133+/VEGFR2+	↑~6.0x
		APAH	13	CD133+/VEGFR2+	↑~4.0x
Smadja et al.	2009	APAH (CHD)	26	CD34+/CD133+	ns
Farha et al.	2011	PAH (total)	52	CD34+/CD133+	↑~1.6x
				CD34+/CD133-	↑~1.5x
				CD34-/CD133+	↑~2.0x-~6.0x
				Total (of the 3 types)	
		(IPAH)	(24)	CD34+/CD133+	↑~5x
				CD34+/CD133-	ns
				CD34-/CD133+	ns
		(HPAH)	(13)	CD34+/CD133+	↑~3x
				CD34+/CD133-	↑~2.5x
				CD34-/CD133+	↑~4x
		(APAH)	(15)	CD34+/CD133+	ns
				CD34+/CD133-	ns
				CD34-/CD133+	ns
Montani et al.	2011	IPAH	9	lin-CD34high/CD133high/(c-kithigh/CXCR4low)	↑~2.4x
				lin-CD34low/CD133-/(c-kitlow/CXCR4high)	↑~2.3x
Hansmann et al.	2011	PAH (including IPAH, HPAH, drug-induced PAH, and APAH (CTD))	43	CD34+/VEGFR2+	↑~1.7x
				CD34+/CD31+/VEGFR2+/CD45-	↑~2.0x
Anjum et al.	2012	SCD-PH	14	CD34+/CD14-/CD106+	↓~80%
				CD31+/CD133+/CD146+	ns

Table 1.4. Summary of human studies investigating number of endothelial progenitor cells (EPCs) in patients with pulmonary hypertension. PAH, pulmonary arterial hypertension; APAH, PAH associated with other diseases; EMS, Eisenmenger's syndrome; CHD, congenital heart disease; IPAH, idiopathic PAH; HPAH, heritable PAH; IPF, idiopathic pulmonary fibrosis; CTD, connective tissue disease; SCD, sickle cell disease; ns, no significant difference. Modified from Diller *et al.* 2010.

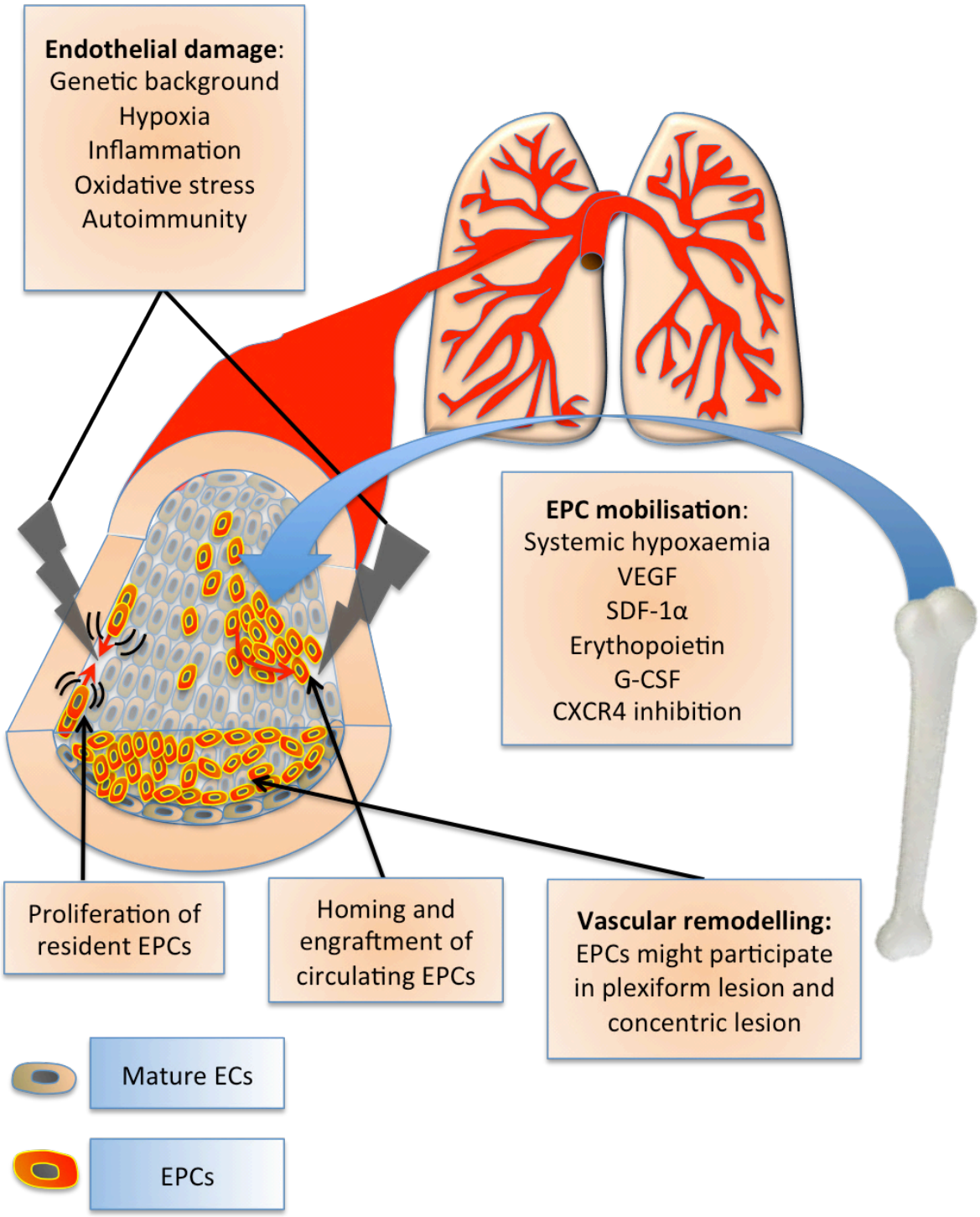


Figure 1.4. The EPC paradox in pulmonary hypertension – pulmonary vascular repair and/or contribution to disease progression development. Endothelial insults might (i) stimulate local EPCs in the pulmonary vessel wall to proliferate and contribute to vascular repair, or (ii) induce the mobilisation and homing of bone marrow-derived cells to the site of injury. Conversely, excessive proliferation and accumulation of EPCs might promote vascular remodelling and lead to the formation of plexiform lesions in PAH. CXCR4, C-X-C chemokine receptor type 4; G-CSF, granulocyte-colony stimulating factor; SDF-1α, stromal cell-derived factor-1α; VEGF, vascular endothelial growth factor.

1.6.3 ECFCs in cardiovascular disorders and PAH

Relatively few studies have examined ECFCs in patients with cardiovascular diseases. ECFC frequency have been used as biomarkers, demonstrating differences in the number of ECFCs derived in a variety of disease states, with higher ECFC frequency found in patients with age-related macular degeneration (Thill et al., 2008), acute myocardial infarction (Massa et al., 2009; Meneveau et al., 2011), coronary artery disease (CAD) (Guven et al., 2006), myeloproliferative disorders (Della Porta et al., 2008; Otten et al., 2008; Rosti et al., 2010), rheumatoid arthritis (Jodon de Villeroche et al., 2010) and proliferative diabetic retinopathy (Tan et al., 2010), while lower number of ECFCs are found in patients with chronic kidney disease (Krenning et al., 2009) and type 2 diabetes mellitus (Ingram et al., 2008; Leicht et al., 2011). Contradictory results exist in relation to pre-term infants (Javed et al., 2008; Baker et al., 2009; Borghesi et al., 2009; Ligi et al., 2011). ECFCs derived from peripheral blood also represent an attractive source of cells for protein expression and functional studies *in vitro*. Importantly, differences in growth and/or functional characteristics were maintained during subsequent cell culture. Increased angiogenic potentials for example have been observed in ECFCs from patients with macular degeneration (Thill et al., 2008) and von Willebrand disease (Starke et al., 2011), while impaired angiogenesis and endothelial functions were found in ECFCs from pre-term infants (Fujinaga et al., 2009; Ligi et al., 2011), patients with diabetes (Ingram et al., 2008; Tan et al., 2010), CAD (Wang et al., 2011) and HHT (Fernandez et al., 2005). More importantly, ECFCs have been used as surrogate endothelial cells to investigate novel mechanisms in disease pathogenesis (Fernandez et al., 2005; Fernandez et al., 2007; Avouac et al., 2008; Fujinaga et al., 2009; Enenstein et al., 2010; Ligi et al., 2011; Starke et al., 2011).

Heterogeneity of ECs along vessel walls with varying proliferative potential was first described more than three decades ago (Schwartz and Benditt, 1976). In fact, ECFCs exhibit a hierarchy of proliferative potential in the rat pulmonary circulation, cells with higher proliferative potential (HPP-ECFCs), higher telomerase activity, barrier properties and robust *de novo* vessel-forming properties being found in PMVECs than PAECs (Alvarez et al., 2008). A subset of highly proliferative resident progenitor cells can also be found in murine PMVECs (Schniedermann et al., 2010). More recently ECFCs were also isolated from human pulmonary artery endothelium (Asosingh et al., 2008; Duong et al., 2011). Interestingly, using a single cell colony assay, HPP-ECFCs were not found in rat kidney ECs (Basile et al., 2012), raising the possibility that ECFCs may be tissue-specific and reflect higher EC turnover in the pulmonary circulation. However, little is known about the phenotype and functional activity of ECFCs isolated from patients with PAH. There have been only three reports to date and in the first Asosingh *et al.* observed no difference in number of ECFC colonies isolated from the PAECs

of IPAH patients (Asosingh et al., 2008). Subsequently, the same group have published some evidence indicating that ECFCs derived from PAECs of IPAH patients may be more proliferative than those obtained from control subjects (Duong et al., 2011). A separate group found that ECFCs from three PAH patients with BMPR2 mutations displayed a hyperproliferative phenotype and an impaired ability to form vascular networks *in vitro* (Toshner et al., 2009). However, *BMPR2* mutants constitute a minor proportion of the IPAH population, and studying IPAH patients in general may provide a more comprehensive understanding of the ECFC phenotype in this disease.

1.6.4 Therapeutic potential of progenitor cells in PAH

Endothelial injury and apoptosis is thought to have a significant role in the pathogenesis of PAH, raising the possibility that novel treatments targeting vessel repair and regeneration of the vascular endothelium might have therapeutic potential in PAH. Pre-clinical studies have provided some evidences supporting the potential for cell-based therapies in preventing or even reversing experimental PH, using BM-derived cells and MNCs following short-term culture. These studies have described improvements in pulmonary hemodynamic parameters, right ventricular and pulmonary remodelling as well as survival benefits in rats treated with MCT (see Table 1.5). However, the rat model of MCT is characterised by a dramatic perivascular accumulation of inflammatory cells and endothelial damage, and does not represent all the histopathological changes associated with human PAH, such as occlusive intimal lesions (Stenmark et al., 2009). It is also important to note that the rat MCT model seems to be curable by almost every intervention tried, probably due to its acute nature, and is therefore quite unlike patients with PAH. Conversely, it was recently reported that injecting human early EPCs into MCT model in the rat did not confer to any clinical or histological improvements or survival benefits (Mirsky et al., 2011). These discrepancies between studies could be due to differences in (i) the severity of the model (MCT dosage), (ii) number of cells administered, (iii) difference in time of administration, (iv) route of administration, (v) the timing when end-points were assessed. Moreover, BM-derived progenitors are generally ineffective in hypoxia-induced PH (Raoul et al., 2007; Marsboom et al., 2008). Some studies have also reported that the injected BM-derived progenitor cells incorporated with pulmonary vessels in MCT-treated rats (Zhao et al., 2005; Spees et al., 2008), while others have found little evidence for a substantial incoporation of progenitor cells in the pulmonary arteries (Sahara et al., 2007; Mirsky et al., 2011).

Some investigators have found that survival and the restoration of pulmonary microcirculation in MCT-treated animals was markedly enhanced when progenitor cells were used to transfer

angiogenic genes such as eNOS (Zhao et al., 2005). Moreover, established hypoxia-induced PH can be reversed by MSCs expressing heme oxygenase-1, but not by early EPCs (Liang et al., 2011). Other studies have also reported improvements in pulmonary haemodynamics, using SMCs or fibroblasts as vehicles to transfer angiogenic genes (Table 1.5). These promising preclinical findings have led to an early-phase clinical study - The Pulmonary Hypertension And Cell Therapy (PHACet) (ClinicalTrials.gov Identifier: NCT00469027), to assess the tolerability of eNOS-enhanced autologous early EPCs delivered into the pulmonary circulation of patients with severe IPAH refractory to all available therapies. Further support for the therapeutic potential of cell-based therapy has been provided by two small pilot studies from a research group, indicating that intravenous infusion of cultured autologous MNCs is accompanied after 12 weeks by an augmented six-minute walk test distance and improvement in pulmonary vascular hemodynamics in IPAH patients (Wang et al., 2007; Zhu et al., 2008). Injection of BM-derived or peripheral blood-derived progenitors into patients are apparently safe, as they have been demonstrated in several larger clinical trials in patients with heart disease such as acute myocardial infarction and coronary heart disease (reviewed in Smadja et al. 2007). Although apparently safe, further blinded and placebo controlled trials are required to establish the potential of *ex-vivo* cell-based therapy in PAH using early outgrowth haematopoietic cells.

ECFCs have been shown to provide better vascular protection in rabbit carotid injury (Gulati et al., 2004) and improved symptoms in rat stroke model (Moubarik et al., 2011). The therapeutic potential of proliferating ECFCs in PAH is however less clear. Ormiston and colleagues recently compared the effects of administering "early" human EPCs and ECFCs in rats with MCT-induced PH and found that while the early cells attenuated PH through an immune-dependent mechanism (possibly involving stimulation of natural killer cells) the ECFC population was ineffective (Ormiston et al., 2010). Nevertheless, ECFCs were recently shown to be an effective carrier of angiogenic genes such as erythropoietin (EPO), conferring systemic release of EPO and the formation of functional anastomoses in the vasculature of mice with radiation-induced anemia (Lin et al., 2011). ECFCs have also been engineered to carry PGI_2 synthase, leading to the substantial release of PGI_2 and improved Matrigel tube formation *in vitro* (Liu et al., 2012). A summary of these studies is provided in Table 1.5.

Table 1.5

Author(s)	Year	Species	PH model	Cell type used	Days in culture	Gene therapy	Outcome
Animal studies							
(Nagaya et al., 2003)	2003	Rat	MCT	Human EPCs (UVB)	8	AM	PAP ↓, PVR ↓, survival ↑, remodelling ↓
(Takahashi et al., 2004)	2004	Dog	MCT	Dogs EPCs	14–18	-	PAP ↓, PVR ↓, CO ↑
(Zhao et al., 2005)	2005	Rat	MCT	Rat EPCs (BM)	7–10	eNOS	RVSP ↓, RV-hypertrophy ↓, perfusion ↑, survival ↑
(Kanki-Horimoto et al., 2006)	2006	Rat	MCT	Rat MSCs (BM)	*P3-5	eNOS	RVSP ↓, RV-hypertrophy ↓, survival ↑
(Sahara et al., 2007)	2007	Rat	MCT + uP	BM chimeric rats	-	-	limited cell incorporation, NS effects on PH
(Zhao et al., 2007)	2007	Rat	L-R-shunt	Human EPCs (PB)	7	CGRP	PAP↓, PVR↓, survival↑, remodelling↓
(Raoul et al., 2007)	2007	Mouse	MCT Hypoxia	Mouse BM Mouse BM	- -	- -	RVSP ↓, RV-hypertrophy ↓ RVSP -, RV-hypertrophy -
(Spees et al., 2008)	2008	Rat	MCT	BM chimeric rats	-	-	Incorporation of cells in lung and heart tissue
(Yip et al., 2008).	2008	Rat	MCT	Rat EPCs (BM)	21	-	RVSP ↓, RV-hypertrophy ↓, remodelling ↓
(Marsboom et al., 2008)	2008	Mouse	Hypoxia	Mouse EPCs	7	-	RVSP -, RV-hypertrophy -
(Yoshida et al., 2009)	2009	Mouse	MCT	Mouse MNCs (BM)	-	-	RV-hypertrophy ↓, remodelling ↓, VEGF ↑
(Sun et al., 2009a)	2009	Rat	MCT	Rat EPCs (BM) + cilostazol	21	-	RVSP ↓, remodelling ↓
(Xia et al., 2009a)	2009	Rat	MCT	Human eEPCs (PB)	7	-	RVSP ↓, RV-hypertrophy ↓, remodelling ↓
(Umar et al., 2009)	2009	Rat	MCT	Rat MSCs (BM) + MCT	-	-	RV-hypertrophy ↓, remodelling ↓
(Ormiston et al., 2010)	2009	Rat	MCT	Human eEPCs Human ECFCs	7 *P4	- -	RVSP ↓, RV-hypertrophy ↓ RVSP -, RV-hypertrophy -
(Mirsky et al., 2011)	2011	Rat	MCT	Human eEPCs (PB)	7	-	RVSP -, RV-hypertrophy -, remodelling -, survival -
(Sun et al., 2011)	2011	Rat	MCT	Rat EPCs (BM) +/- sildenafil	21	-	RVSP ↓, RV-hypertrophy ↓, combination therapy superior than monotherapy
(Liang et al., 2011)	2011	Mouse	Hypoxia	Mouse MSCs (BM)	-	HO-1	RVSP ↓, RV-hypertrophy ↓, remodelling ↓
(Zhang et al., 2012)	2012	Mouse	MCT	Human MSCs (BM) hESC-MSCs	-	-	RVSP ↓, RV-hypertrophy ↓, remodelling ↓, capillary density ↑, hESC-MSCs superior than BM-MSCs
(Jiang et al., 2012)	2012	Rat	MCT	Rat MSCs (BM)	*P1-3	-	RVSP ↓, RV-hypertrophy ↓, remodelling ↓, survival ↑
(Luan et al., 2012)	2012	Dog	MCT	Dog BM	-	-	PAP ↓, PVR ↓, CO ↑, RV-hypertrophy ↓, remodelling ↓, capillary density ↑

Table 1.5 (continued)

Author(s)	Year	Species	PH model	Cell type used	Days in culture	Gene therapy	Outcome
Human studies							
(Wang et al., 2007)	2007	Human	Adult IPAH	Autologous EPCs (PB)	5	-	PAP ↓, PVR ↓, CO ↑, 6MWD ↑
(Zhu et al., 2008)	2008	Human	Ped. IPAH	Autologous EPCs (PB)	5	-	PAP ↓, PVR ↓, CO ↑, NYHA class ↓, 6MWD ↑
PHACet	-	Human	Adult PAH	Autologous EPCs (PB)	-	eNOS	Safety & dosing study, ongoing
Non-EPC/BM-cell-based therapy in animals							
(Campbell et al., 1999)	1999	Rat	MCT	SMCs	-	eNOS	PAP ↓
(Campbell et al., 2001)	2001	Rat	MCT	SMCs	-	VEGF	RVSP ↓, RV-hypertrophy ↓
(Zhao et al., 2003)	2003	Rat	MCT	SMCs	-	Ang1	RVSP ↓, RV-hypertrophy ↓, survival↑
(Zhao et al., 2006)	2006	Rat	MCT	Fibroblasts	-	eNOS or VEGF	RVSP ↓, RV-hypertrophy ↓, remodelling ↓, perfusion ↑
(Angelini et al., 2011)	2011	Rat	MCT	Human AFS or rat SVC-GFP	-	-	RV-hypertrophy ↓, remodelling ↓, BNP ↓, inflammatory cytokines ↓
(Liu et al., 2011)	2011	Rat	Shunt	Rat ADSC	*P2-3	-	PAP ↓, RV-hypertrophy ↓, remodelling ↓, capillary density ↑

Table 1.5. Summary of studies investigating the effect of infusion of native or genetically modified progenitor cells in experimental models of PH and patients with severe PAH. ADSC, adipose-derived stromal cells; AFS, amniotic fluid stem cells; Ang1, angiopoietin 1; AM, adrenomedullin; BM, bone marrow; CGRP, calcitonin gene-related peptide; CO, cardiac output; hESC, human embryonic stem cells; HO-1, hemo oxygenase-1; L-R shunt = left to right shunt; MCT, monocrotaline; MSCs, mesenchymal stem cells; PAP, pulmonary arterial pressure; PVR, pulmonary vascular resistance; RV, right ventricle; RVSP, right ventricular systolic pressure; SMCs, smooth muscle cells; SVC-GFP, adipose tissue stromal vascular fraction green fluorescence protein-positive cell; uP, unilateral pneumonectomy; UVB, umbilical venous blood; *P, passage; 6MWD, 6-minuate walk distance; NYHA, New York Heart Association. Modified from Diller *et al.* 2010.

1.7 Transforming growth factor-β (TGF-β) signalling

1.7.1 Ligands and receptors of the TGF-β superfamily

TGF-β was first discovered more than three decades ago as a secreted dimeric peptide (25 kDa) that could induce a reversible transformed phenotype of fibroblasts in a soft agar colony-forming assay (De Larco and Tadaro, 1978; Moses et al., 1981; Roberts et al., 1981). Three different mammalian homodimeric isoforms – TGF-β1, TGF-β2 and TGF-β3 – were subsequently identified (Cheifetz et al., 1987; Derynck et al., 1988; ten Dijke et al., 1988). More than 30 other proteins that are structurally similar to TGF-β and functionally use the same family of receptors and signalling intermediates were since identified and makes up the large family of TGF-β superfamily ligands, including TGF-βs, BMPs, growth and differentiation factors (GDFs), activins, inhibins, Müllerian inhibiting substance, nodal and leftys (Mueller and Nickel, 2012) (Figure 1.5). The latent form of these secreted proteins all have a similar structure: an amino-terminal signal peptide, a larger precursor segment, and a 25-kDa carboxyl-terminal polypeptide that becomes the mature peptide following its release from the precursor by proteolytic cleavage (Khalil, 1999). The structural similarity of the carboxyl-terminal sequence defines this family of related proteins.

Members of the TGF-β family elicit their biological actions by binding to a family of structurally related cell-surface transmembrane serine-threonine kinases, which is known as the TGF-β receptor superfamily and consists of seven type I and five type II receptors (see Figure 1.6). Each receptor has a common structure, comprising a short extracellular cysteine-rich domain that is subjected to N-linked glycosylation, a single transmembrane domain, and an intracellular serine/threonine kinase domain (Wrana et al., 2008). The BMPR2 receptor represents an exception to this common structure as it contains a long extension downstream of the kinase domain, which is believed to serve as a scaffold for signalling molecules to the receptor (Wrana et al., 2008). The main difference between type I and type II receptors lies in a glycine-serine-rich sequence (the GS region) that is located upstream of the intracellular kinase domain in all type I receptors (Derynck and Miyazono, 2008). Each member of the TGF-β receptor family is thought to exist as a homodimer in the cell membrane, which assemble into heterotetramers of type II and type I receptors upon ligand binding (Yamashita et al., 1994). Ligand binding and signal transduction is also assisted by the type III receptors betaglycan and endoglin (Wrana et al., 2008).

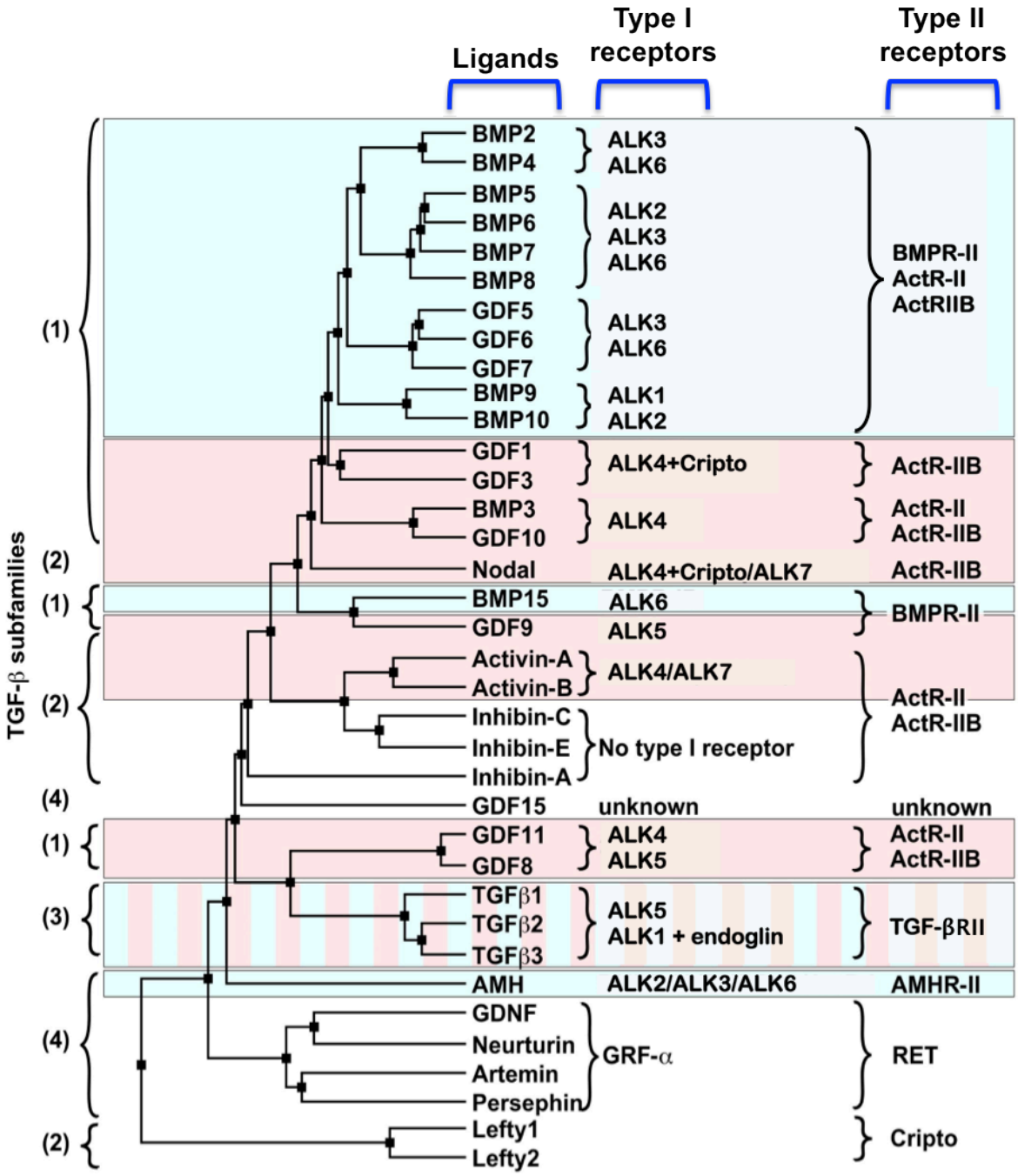


Figure 1.5. Phylogenetic tree of the TGF-β protein family in humans. There are four subfamilies of TGF-β ligands (numbers to the right of the branches) comprising: BMP/GDF (**1**), activin/inhibin/nodal (**2**), TGF-β (**3**) and others (**4**). Type I and type II receptor usages for each ligand are deduced from biophysical interaction or *in vitro* pulldown and crosslinking analyses. Light-blue shaded boxes emphasise SMAD 1/5/8, whereas light-red shaded boxes highlight SMAD 2/3 downstream signalling. Figure modified from Mueller & Nickel 2012, updated with findings from (Scharpfenecker et al., 2007; Upton et al., 2009; Luo et al., 2010; Townson et al., 2012).

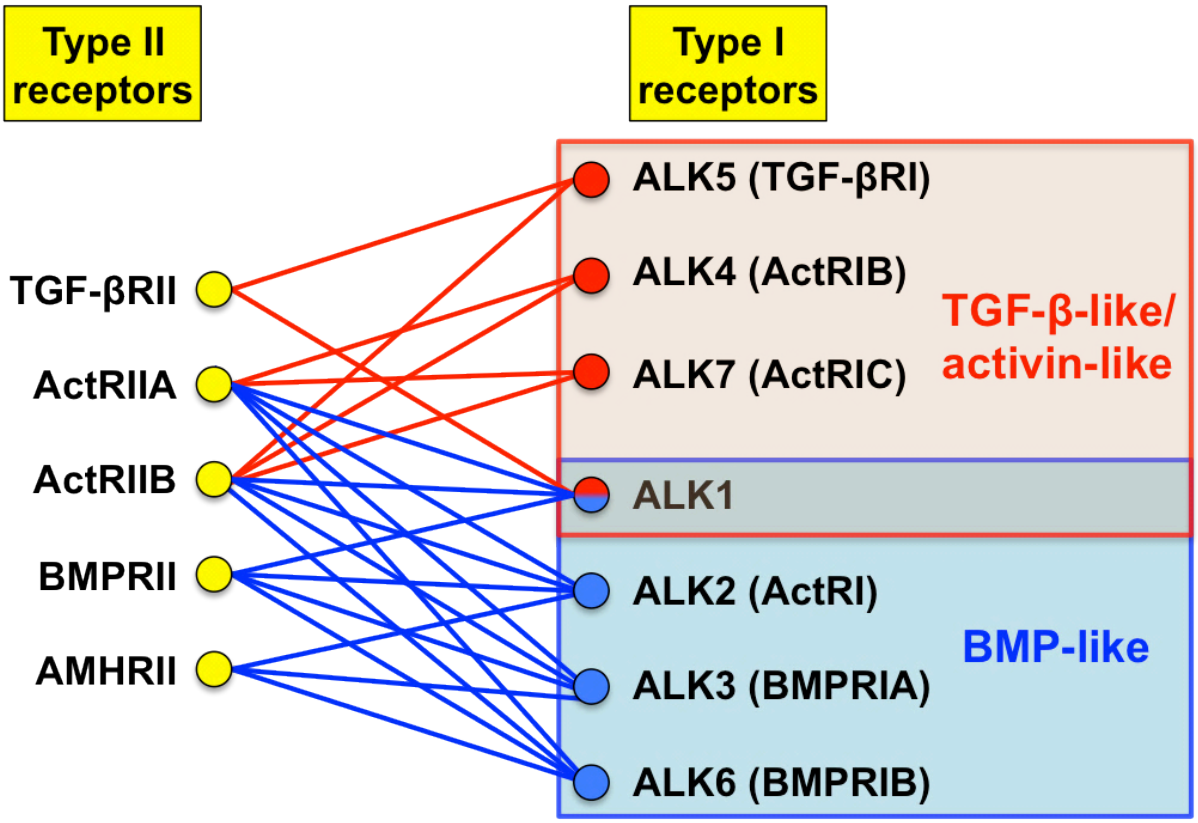


Figure 1.6. The interactions of the TGF-β receptor superfamily. Type I (red or blue) and type II receptor (yellow) subtypes are indicated by the coloured nodes. Alternative terms in parentheses are also widely used for type I receptors. The functional clustering of the type I receptors into either TGF-β-like/activin-like (light red box) or BMP-like (light blue box) is indicated, while ligands that induce formation of complexes between type I and type II receptors are indicated as coloured lines (red for TGF-β-like ligands and blue for BMP-like ligands). Figure modified from Wrana et al. 2008, "The TGF-β Family", p.153.

1.7.2 TGF-β and BMP signalling pathways

The TGF-β superfamily of ligands and serine-threonine kinase receptors represents a unique ligand-receptor system (Wrana et al., 2008). Multiple ligands are able to interact with selected combinations of type I and type II receptors (see Figures 1.5 and 1.6 for diagrammatic summaries of the ligand and receptor combinations). Typically, the signalling cascade is initiated when an active TGF-β ligand binds to the type II receptor homodimer, subsequently forming a heterotetramer complex with the type I receptor. However, the order of binding is reversed for some members of the BMP family, as BMP ligands contain independent type II and type I binding sites (Sebald et al., 2004; Miyazono et al., 2005). Ligands that initiate TGF-β signalling transcriptional responses in ECs have been shown to engage the TGF-βRII receptor together with either the ubiquitously expressed type I receptor ALK5 or the predominantly EC restricted type I receptor ALK1 (Oh et al., 2000; Goumans et al., 2002). Ligands that initiate BMP signalling transcriptional responses engage the type II receptors BMPRII, activin receptor type IIA (ActRIIA) or activin receptor type IIB (ActRIIB), and the type I receptors ALK1, ALK2, ALK3 or ALK6 (Cai et al., 2012). Formation of these ligand-bound heterotetrameric receptor complexes results in the type I receptor becoming phosphorylated in the GS rich region and a conformational change that induces full receptor activation. This in turn permits the binding of intracellular signalling receptor-activated Smads (R-Smads), which are directly phosphorylated by the type I receptor at two serine residues in their carboxyl termini. ALK1/2/3/6 activation induces phosphorylation of R-Smad1, 5 and 8 (pSmad1/5/8), while ALK5 activation mediates R-Smad2 and 3 phosphorylation (pSmad2/3). Activated R-Smads form a heteromeric complex with the common mediator Smad4, which then translocate to the nucleus to regulate the transcription of specific target genes together with other transcriptional modulators (see Figure 1.7 for a diagrammatic illustration of TGF-β signalling in ECs) (Goumans et al., 2009). These complex receptor interactions and intracellular pathways allow for fine-tuning of TGF-β/BMP signalling and regulation of cellular function. In addition to canonical signalling via Smad activation, TGF-β and BMP ligands can also elicit responses through Smad-independent (non-canonical) pathways. These include various MAP kinase pathways – p38, extracellular signal related kinase (ERK) and c-jun N-terminal kinase (JNK), Rho GTPases and the phosphatidylinositol-3-kinase/Akt pathway (Zhang, 2009). Crosstalk between these different pathways is also considered to be important in maintaining EC homeostasis (Kimura et al., 2000; Su et al., 2011).

TGF-β signalling is further modulated by several accessory proteins. Access of ligands to TGF-β receptors is regulated by the transmembrane accessory type III homodimers betaglycan and endoglin (ten Dijke and Arthur, 2007). These proteins are structurally similar, possessing long extracellular and

short intracellular serine/threonine rich domains, and lack a signalling enzymatic motif. Betaglycan (also known as TGF-βRIII) potentiates TGF-β and BMP signalling by facilitating ligand binding to type II receptors. Endoglin (also known as CD105) selectively facilitates binding of TGF-βs, BMP9 and BMP10 to type II receptor that form complexes with ALK1 but not ALK5 (Lee et al., 2008; Goumans et al., 2009). Endoglin may also bind directly with BMP9 and BMP10 and thereby regulate EC functions (Castonguay et al., 2011). Other accessory proteins, including Smad anchor for receptor activation (SARA) and Disabled-2 (Dab-2) also modulate TGF-β signalling at the Smad level (Tsukazaki et al., 1998; Hocevar et al., 2001). The inhibitory Smads (I-Smads) – Smad6 and Smad7 – oppose TGF-β signalling by competing with R-Smads for interaction with type I receptors (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997), recruiting the E3 ubiquitin ligase Smad ubiquitin regulatory factor (Smurf) to facilitate proteasomal degradation of the activated receptor complex (Ebisawa et al., 2001), and by interacting directly with TGF-β-related transcription factors (Schilling et al., 2008).

TGF-β1 can demonstrate opposing actions depending on its concentration, for example inducing pro-angiogenic effects at low concentration and anti-angiogenic effects at higher concentration (Pepper et al., 1993). These paradoxical effects may be mediated by the activation of different TGF-β receptors. Subsequent studies revealed that, depending on its concentration, TGF-β1 is capable of stimulating both ALK1/Smad1/5/8 and ALK5/Smad2/3 signalling pathways in mouse embryonic endothelial cells (MEECs) (Goumans et al., 2002). Smad2 phosphorylation occurs over a wide range of TGF-β concentrations and remains stable over time, whereas Smad1/5 phosphorylation is transient and only prominent at higher concentration. Furthermore, while ALK1 and endoglin activation of Smad1/5/8 directly antagonised ALK5 signalling at the transcriptional level (but not at the Smad level), the presence of ALK5 kinase activity was a requirement for efficient ALK1 signalling, as demonstrated by ALK5 deficient MEECs (Goumans et al., 2003; Lebrin et al., 2004). In contrast, Fernandez and colleagues showed that ALK1 and endoglin activations are essential for ALK5 promoter activity in ECFCs (Fernandez et al., 2005). Cunha and colleagues also showed that BMP9 activation of ALK1 had synergistic effects on TGF-β/ALK5-stimulated plasminogen activated inhibitor-1 (PAI-1) gene expression (Cunha et al., 2010). However, this crosstalk between ALK1 and ALK5 signalling has been questioned, as non-overlapping expression patterns of murine ALK1 and ALK5 suggest that these receptors may signal independently of each other (Seki et al., 2006). Indeed, ALK1 signalling has been reported to occur independently of ALK5 activity in both human and bovine aortic ECs (Shao et al., 2009). Nevertheless, the interdependency between TGF-β activation of ALK1 and ALK5 remains elusive in ECs.

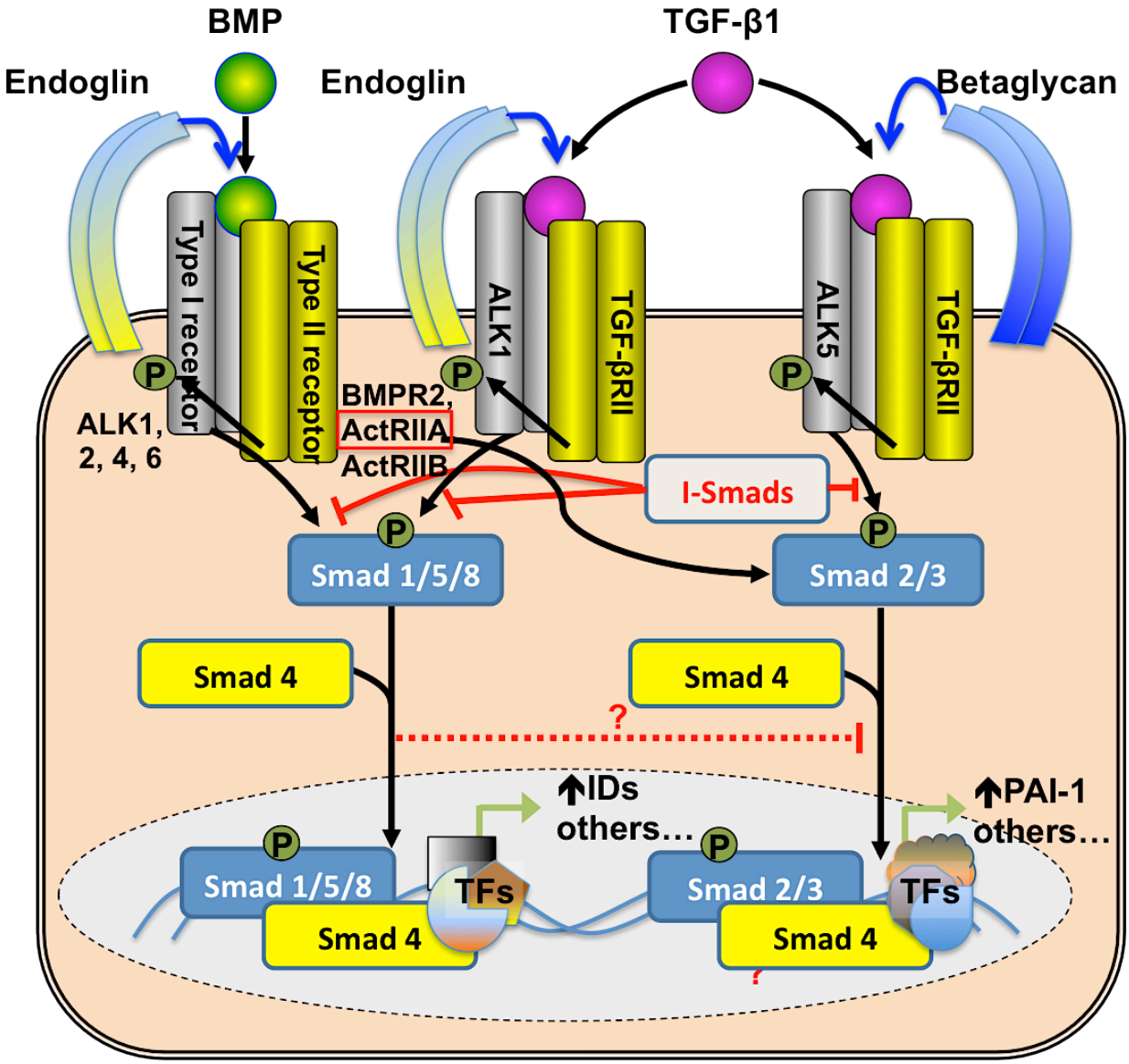


Figure 1.7. TGF-β/BMP canonical signalling in endothelial cells. TGF-β signalling occurs via TGF-βRII and both ALK1 and ALK5 type I receptors, whereas BMPs signal via three type II – BMPRII, ActRIIA and ActRIIB – and four type I receptors – ALK1, ALK2, ALK3 and ALK6. Accessory receptors betaglycan and endoglin can facilitate TGF-βRII/ALK5 and TGF-βRII/ALK1 signalling respectively. The activated receptor complexes bind with R-Smads, which are directly phosphorylated by the type I receptors (TGF-β/ALK5 activates Smad2/3, while TGF-β/ALK1 or BMP/ALKs activate Smad1/5/8). This results in their release from the receptor complex and association with Smad4. The Smads complexes then translocate to the nucleus where they regulate the transcription of specific target genes together with other transcription factors (e.g. pSmad1/5/8 stimulates transcription of inhibitor of differentiation (ID) while pSmad2/3 up regulates plasminogen activator inhibitor (PAI)-1). Activated ALK1/Smad1/5 signalling might inhibit the transcriptions by ALK5/Smad2/3 signalling (dotted line).

1.7.3 TGF-β and BMP signalling in endothelial cells

TGF-β signalling has important roles in vasculogenesis and angiogenesis during embryonic development. Inactivation of different components of the TGF-β signalling pathway, such as TGF-β1 (Dickson et al., 1995), ALK1 (Oh et al., 2000; Urness et al., 2000), ALK5 (Larsson et al., 2001), TGF-βRII (Oshima et al., 1996), endoglin (Bourdeau et al., 1999; Li et al., 1999), Smad1 (Lechleider et al., 2001) and Smad5 (Chang et al., 1999; Yang et al., 1999), are embryonically lethal due to abnormal yolk sac vasculogenesis and/or angiogenesis. TGF-β signalling is also essential for vascular homeostasis in the adult (ten Dijke and Arthur, 2007).

Substantial evidence indicates that TGF-β1-induced activation of ALK1 and ALK5 in ECs can elicit paradoxical effects on proliferation, migration and angiogenesis (Goumans et al., 2002; Shao et al., 2009). For example, some studies found that ALK1 activation promoted proliferation, migration, tube formation and sprouting of ECs (Goumans et al., 2002; Cunha et al., 2010; Mitchell et al., 2010), while others have found the opposite (Lamouille et al., 2002; Koleva et al., 2006; David et al., 2007b; Shao et al., 2009). Similar conflicting findings have also been described for ALK5 signalling, with some studies reporting that ALK5/Smad2 activation inhibits migration, proliferation and *in vitro* tube formation by ECs (Goumans et al., 2002; Lebrin et al., 2004; Froese et al., 2011) while others demonstrated pro-proliferative and pro-angiogenic effects (Shao et al., 2009; Tian et al., 2012a). Furthermore, most studies indicate that TGF-β1 has pro-apoptotic effects on ECs, either alone (Tsukada et al., 1995; Yan and Sage, 1998; Pollman et al., 1999; Emmanuel et al., 2002) or in combination with VEGF (Ferrari et al., 2006), but a few have also described pro-survival (anti-apoptotic) effects (Mauro et al., 2001; Vinals and Pouyssegur, 2001; Li et al., 2003). These opposing results are thought to reflect the context-specific nature of TGF-β signalling, which is dependent on factors such as ligand concentration, cell type, culture conditions and experimental design.

Pleotropic effects have also been observed following activation of ALK1 signalling by BMP9. On one hand, BMP9 is considered to contribute in the quiescence of the endothelium, as demonstrated by the inhibition of EC migration and proliferation *in vitro* and inhibition of angiogenesis in several *ex vivo* models (David et al., 2007a; Scharpfenecker et al., 2007; David et al., 2008; Shao et al., 2009; Larrivee et al., 2012; Yao et al., 2012). On the other, BMP9 has also been found to induce pro-angiogenic responses in several models (Suzuki et al., 2010; Lee et al., 2012; Park et al., 2012). One possible explanation is that BMP9 may activate different Smad pathways through its interaction with ActRIIA (Smad2) and BMPR2 (Smad1/5/8) receptors (Upton et al., 2009).

BMP2 and BMP4 have a similar structure and belong to the same sub-class of BMP ligands. Both promote EC proliferation, migration, tube formation and survival *in vitro* and tumour angiogenesis *in vivo* (Langenfeld and Langenfeld, 2004; Teichert-Kuliszewska et al., 2006; Rothhammer et al., 2007; Southwood et al., 2008; Alastalo et al., 2011; Gangopahyay et al., 2011; Cahill et al., 2012; Finkenzeller et al., 2012; Yao et al., 2012). Furthermore, BMP2, BMP4 and BMP7 have been shown to protect HPAECs against serum deprivation-induced apoptosis (Teichert-Kuliszewska et al., 2006; Southwood et al., 2008), while BMP4 induced apoptosis via the non-canonical (JNK and p38 MAPK) pathways in HUVECs and rat aortic ECs (Tian et al., 2012b). BMP6 also promotes proliferation, migration and tube formation in ECs, possibly acting through the stimulation of ALK3/ALK6 and ID1 expression (Valdimarsdottir et al., 2002). Collectively, these data point to the pleiotropic effects of both BMP and TGF-β signalling in ECs.

1.7.4 TGF-β signalling in cardiovascular disorders

Several cardiovascular disorders have been linked to alterations in TGF-β signalling. One of the most studied is hereditary haemorrhagic telangiectasia (HHT), which is an autosomal dominant vascular dysplasia, characterised by multisystemic mucocutaneous telangiectasia and arteriovenous malformations (AVMs) in the brain, lungs, liver and gastrointestinal tract (Govani and Shovlin, 2009). Mutations in three genes are casually related to HHT, comprising endoglin (HHT1), ALK1 (HHT2) and Smad4 (McAllister et al., 1994; Gallione et al., 2004). In fact, endothelial specific deletion of ALK1 and endoglin in mice leads to a phenotype resembling the clinical manifestation of HHT (Park et al., 2009; Mahmoud et al., 2010). Furthermore, blood-derived ECFCs from both HHT1 and HHT2 patients display impaired migration, proliferation and tube formation in culture (Fernandez et al., 2005; Fernandez et al., 2007).

Mutations in either ALK5 or TGF-βRII can lead to the autosomal dominant connective tissue disorder Loeys-Dietz syndrome (LDS) (Loeys et al., 2005) and mutations in Smad3 and TGF-β2 have also recently been reported in LDS (van de Laar et al., 2011; Lindsay et al., 2012). The syndrome is characterised by arterial aneurysms and/or dissections, with craniofacial or cutaneous manifestations (Loeys et al. 2006). The molecular mechanism underlying the disease is poorly understood. Despite the loss-of-function mutations in the TGF-β receptors, it is associated with increased TGF-β signalling and increased expression of TGF-β1 and TGF-β2 in affected tissues (Lindsay et al., 2012). The clinical features of LDS are similar to Marfan syndrome, which is another autosomal dominant connective tissue disorder that is associated with high circulating TGF-β levels

(Matt et al., 2009). This probably reflects either a loss-of-function mutation in fibrillin-1, leading to diminished sequestration of latent TGF-β in the ECM and increased TGF-β activation (Neptune et al., 2003). Marfan syndrome is also associated with mutation in the TGF-βRII locus (Mizuguchi et al., 2004). Overall, these findings serve to underline the importance of maintaining a balance in TGF-β signalling for vascular homeostasis.

1.7.5 TGF-β signalling in pulmonary arterial hypertension

Accumulating evidence shows that abnormalities in TGF-β signalling have a critical role in PAH. Heterozygous mutations in *BMPR2* and more rarely the genes encoding ALK1, ALK6, endoglin or Smad1, Smad4 and Smad8 are associated with PAH (as discussed in Chapter 1.2.4). Histological observations indicate that expression of BMPR2 (Atkinson et al., 2002), ALK3 (Du et al., 2003) and pSmad1 (Yang et al., 2005) is reduced in the pulmonary endothelium of IPAH lungs. Reduced expression of BMP receptors (ALK1, ALK3, ALK6, BMPR2), endoglin, and Smads, and decreased BMP signalling (pSmad1 and ID gene expression) has also been reported in rats with MCT-induced PH (Morty et al., 2007; Zakrzewicz et al., 2007; Long et al., 2009; Thomas et al., 2009), and reduced BMPR2 expression was described in rats with hypoxia-induced PH (Takahashi et al., 2006). Attempts have made to rectify deficiencies in gene expression and, while *BMPR2* gene therapy in the whole lung was found to be ineffective in MCT-induced PH (McMurtry et al., 2007), specifically targeting *BMPR2* gene delivery to the pulmonary endothelium led to improvements in RV and pulmonary vascular remodelling and pulmonary haemodynamics, both in hypoxia and MCT models of PH (Reynolds et al., 2012). Heterozygous mutation, as well as conditional ablation or mutation of *BMPR2* in the pulmonary endothelium predisposes mice to develop PH, as demonstrated by increases in RVSP, RV hypertrophy and remodelling of pulmonary vessels (Song et al., 2005; Hong et al., 2008; Majka et al., 2011). BMPR2 deficient mice also exhibit an increased pulmonary vasoconstrictor response (Long et al., 2006; Frank et al., 2008), disrupted pulmonary endothelial barrier function (Burton et al., 2011), and heightened susceptibility to inflammation (Burton et al., 2011; Soon et al., 2012). The observations that both *ALK1* and *BMPR2* mutations can lead to PAH argue in favour of defective endothelial BMP9/BMPR2/ALK1 signalling being critical in PAH (Upton et al., 2009). It is significant therefore that the ALK1 heterozygous mouse can develop PH, as characterised by increased RVSP, vascular remodelling, reduced pulmonary vascular density and endothelial dysfunction (Jerkic et al., 2011). Furthermore, this was associated with eNOS uncoupling and increased production of ROS, and the development of PH being prevented by treatment with anti-oxidant.

Histological examination of lung sections from IPAH patients has also provided evidence of dysregulated TGF-β signalling components, including ALK5, TGF-βRII and Smads (Richter et al., 2004), where were most apparent within plexiform lesions. These coincide with the somatic mutations and decreased expression of TGF-βRII found in the endothelial cells of plexiform lesions in IPAH patients (Yeager et al., 2001). Studies in MCT-treated rats have also provided evidence of reduced TGF-βRII, Smad3, and Smad4 expression, diminished TGF-β/ALK5 signalling (pSmad2) and dysfunctional TGF-β1 responses in cultured PASMCs (Zakrzewicz et al., 2007). Controversy exists however as other data indicates that TGF-β signalling is enhanced in PAH. Substantial expression of TGF-β ligands was found in the pulmonary arteries of patients with IPAH (Botney et al., 1994) as well as in the lungs and pulmonary arteries in MCT (Arcot et al., 1993), chronic hypoxia (Jiang et al., 2007) and shunt model (Mata-Greenwood et al., 2003) of PH. PASMCs isolated from IPAH patients also display an abnormal proliferative response to TGF-β1, compared with an inhibitory response in PASMCs from control and APAH patients (Morrell et al., 2001). Moreover, two studies described increased TGF-β1, TGF-βRII and pSmad3 expression in pulmonary arterial wall of rats with MCT-induced PH (Zaiman et al., 2008; Long et al., 2009), but not in rats exposed to chronic hypoxia (Long et al., 2009). Importantly, ALK5 and TGF-β ligands inhibitions have been found to prevent the development of PH and partially reverse established PH in MCT treated rats (Zaiman et al., 2008; Long et al., 2009; Thomas et al., 2009; Megalou et al., 2010), suggesting that ALK5/TGF-β signalling could be important at least in the initiation of experimental PH.

1.8 Dysfunctional phenotype of endothelial cells isolated from the lungs of IPAH patients

A hallmark of established end-stage IPAH is the existence of angioproliferative plexiform lesions within the pulmonary arteries (Cool et al., 1999; Tuder et al., 2001) and a model of PAH has emerged where initial endothelial apoptosis is considered to be a key factor (Sakao et al., 2005; Michelakis, 2006). Central to this is the proposition that an inherited or acquired insult leads to apoptosis of PAECs and loss of small capillaries. Increased flow and shear stress in the remaining vessels results in the emergence of apoptosis-resistant ECs that proliferate and give rise to plexogenic lesions (Sakao et al., 2005). At the same time, a loss of PAECs would allow the ingress for growth factors and proliferation of exposed PASMCs. Studies on pulmonary ECs isolated from the lungs of IPAH patients provide support for this model, as they display a hyperproliferative, apoptosis-resistant phenotype compared to control pulmonary ECs (Masri et al., 2007; Tu et al., 2011). The hyperproliferative potential may reflect increased activation of Janus kinase/signal transducer and activator of transcription 3 (STAT3) and ERK1/2, while their apoptosis-resistant phenotype was associated with increased expression of anti-apoptotic factors such as B-cell lymphoma (BCL)2, BCL extra long (BCL-xL), and myeloid leukemia cell differentiation protein (Mcl-1) (Masri et al., 2007; Tu et al., 2011). Furthermore, pulmonary ECs from IPAH patients have been found to produce greater amounts of 5-HT, ET-1, MCP-1 and FGF-2, stimulating the proliferation of PASMCs (Dewachter et al., 2006; Eddahibi et al., 2006; Sanchez et al., 2007; Izikki et al., 2009). The overproduction of FGF-2 might also act in an autocrine manner to increase proliferation and reduce sensitivity to apoptosis in pulmonary ECs (Tu et al., 2011).

The utility of pulmonary ECs, isolated *ex vivo* from patients with PAH, is further exemplified by the discovery of metabolic abnormalities, which are similar to those of cancer cells and characterised by reduced mitochondria and oxygen consumption, increased glycolytic rate (the Warburg effect) and an altered hypoxia sensing mechanism (Xu et al., 2007; Fijalkowska et al., 2010). In addition, the over production of hypoxia inducible factors by PAECs from PAH patients has been linked to intrinsic myeloid abnormalities and the recruitment of progenitor cells to their lungs (Farha et al., 2011). The inflammatory manifestation of IPAH has also been demonstrated by the increased secretion of inflammatory modulators and cytokines such as leptin (Huertas et al., 2012), IL-15 (Masri et al., 2007) and MCP-1 (Sanchez et al., 2007) by pulmonary ECs from IPAH patients. Most importantly, these cells have been used to identify and explore novel pathogenic mechanisms, such as dysregulated FGF-2 and apelin expression, leading to further studies in pre-clinical models and patients with IPAH (Izikki et al., 2009; Alastalo et al., 2011). Other studies have led to the recent identification of pathogenic

roles for $p130^{Cas}$ and adenomatous polyposis coli in IPAH (de Jesus Perez et al., 2012; Tu et al., 2012). Moreover, isolated pulmonary ECs have also been used to study disease mechanisms associated with mutations in *BMPR2* and *SMAD9*, leading to the discovery of dysfunctional microRNA processing in PAH (Drake et al., 2011), and cells from CTEPH patients have been shown to exhibit increased adhesion and secretion of pro-thrombotic factors such as ET-1 and vWF (Wynants et al., 2012).

These findings provide a powerful rationale for studying endothelial cells isolated from the lungs of PAH patients, both to understand the pathogenesis of the disease and develop novel therapeutics. The availability of suitable tissues is however very limited and a clear disadvantage of using cells isolated from explanted lungs is that they are only representative of endothelial dysfunction in patients with severe late-stage disease who require transplantation. Blood-derived ECFCs could offer a valuable non-invasive means of deriving endothelial cells from patients at various stages of disease progression and treatment, which offer great potentials in further understanding the development of IPAH.

This study intends to determine whether ECFCs derived from patients with IPAH can be distinguished from those produced by healthy volunteers and thus provide an *in vitro* model that can be used to explore the endothelial functions, secretion of vasoactive factors and expression of specific genes that are selectively affected in the disease. This model may be important in assessing the relative contribution of TGF-β signalling in the disease phenotype and identifying novel mechanisms underlying endothelial dysfunction.

1.9 Hypothesis

ECFCs isolated from adult peripheral blood possess a distinct cell phenotype and/or functional characteristics that distinguish patients with IPAH from healthy controls and are independent of known BMPR2 mutations. As such, ECFCs may represent an effective cell type for studying mechanisms underlying endothelial dysfunction in IPAH, testing drug responses and development of novel therapies *in vitro*.

1.10 Objectives

1. Isolate and grow ECFCs to a consistent standard and compare the phenotype and clonogenic potential of cells derived from well-characterised patients with IPAH and healthy volunteers.

2. To investigate the functional characteristics of ECFCs isolated from peripheral blood of IPAH patients and determine whether they replicate the endothelial dysfunction observed *in vivo* and differences found in endothelial cells isolated from the diseased pulmonary vasculature.

3. To investigate the dysfunctional TGF-β and BMP receptor expression and signalling in ECFCs from IPAH patients.

4. To determine whether the dysfunctional TGF-β receptors expression/signalling and expression of other regulatory proteins are responsible for the functional phenotypes presented in ECFCs from IPAH patients.

5. To determine whether blood-derived ECFCs can be used to investigate other novel aspects of signalling dysregulation that might contribute to the pathogenic dysfunction of endothelial cells in IPAH patients.

Chapter 2:

Materials and Methods

Chapter 2 – Materials and methods

2.1 Declaration of responsibilities

Blood samples were collected with the assistance of Dr Luke Howard and Dr Simon Gibbs, the nurses in the National Pulmonary Hypertension Service and the staff of the Sir John McMichael Centre for Clinical Research, Hammersmith Hospital, London, UK.

Data in most of the CLIC4 experiments (Fig. 7.1, 7.2 and 7.5) were collected and analysed in collaboration with Dr Vahitha Abdul-Salam. Fig. 4.8, 7.3 and data from the connexin 43 experiments (Fig. 7.6-7.9) were collected and analysed in collaboration with Dr Hilda Tsang. Asymmetric dimethylarginine (ADMA) measurement in Figure 7.6C was performed by Mr Lucio Iannone. Other data were collected, produced and analysed by myself.

2.2 Blood samples

This study was approved by the Charing Cross Hospital Research Ethics Committee (REC Ref 09/H0711/4) and all blood samples were obtained with written informed consent. Blood samples (up to 50ml) were drawn from the antecubital vein and collected in either Vacutainer™ tubes containing tripotassium ethylene diamine tetra-acetic acid (K3 EDTA, 10ml, cat. no. 368457, BD Diagnostics, Oxford, UK) or cell preparation tubes with sodium citrate (Vacutainers CPT™, 8ml, cat. no.362782, BD Biosciences). The blood was kept at room temperature and mononuclear cells (MNCs) isolated within 1 hour of collection. The diagnosis of IPAH was based on standard criteria with confirmation by right heart catheterisation and exclusion of other forms of PH as per recent guidelines at diagnosis (Galie et al., 2009b). MNCs were also isolated from healthy volunteers recruited within Imperial College London, UK.

2.3 ECFC isolation and cell culture

The method used was adapted from a protocol originally published by Ingram and co-workers in 2004 (Ingram et al., 2004), which has since been more fully described (Mead et al., 2008) (see Section 2.4). Venous blood collected in EDTA vacutainers was diluted 1:1 with PBS-FBS (phosphate buffered saline, Ca^{2+} and Mg^{2+} free (cat. no. 14190, Gibco, Invitrogen, Paisley, UK) containing 0.2% w/v EDTA (cat. no. 03664, Sigma-Aldrich, Dorset, UK] and 2% fetal bovine serum, FBS) and aliquots carefully layered onto an equal volume (~15 ml) of Ficoll (cat. no. 17-1440-03, Ficoll-Paque PLUS; GE Healthcare, Amersham, Buckinghamshire, UK) in 50 ml centrifugation tubes. Tubes were spun at 740 *g* for 30 min without brake at room temperature, and the separated MNC layer was aspirated with a sterile Pasteur pipette (Fig. 2.1). Blood in Vacutainer CPT tubes was separated according to the manufacturer instructions. Isolated MNCs were washed 3 times in PBS containing 2% FBS and 0.05% EDTA (PBS-FBS), stained with Türk's solution (cat no. 1092770500, Merck, Nottingham, UK) and counted using a haemocytometer (Bright-line Haemocytometer, cat no. # Z35, 962-9, Sigma-Aldrich). The cells were resuspended in complete endothelial growth medium-2 (EGM-2), comprising endothelial cell basal medium-2 (EBM-2; cat. No. CC-3156, Lonza Biologics, Slough, UK), EGM-2 SingleQuot supplement (cat no. CC-4176, but excluding the supplied FBS), 20% HyClone FBS (cat no. SH30070.03E, Thermo Scientific HyClone, Fisher Scientific UK Ltd, Leicestershire, UK) and 1% antibiotic/antimycotic solution (cat. no. 15240-062, Gibco, Invitrogen). The EGM-2 supplement contains hydrocortisone, epidermal growth factor, VEGF, basic FGF, insulin-like growth factor-I, ascorbic acid, heparin and gentamicin/amphotericin-B. The MNCs were seeded (3 to 5 x 10^7 cells/well in 4 ml of medium) into 6-well plates pre-coated with 50 µg/ml rat-tail collagen type 1 (cat no. 354236, BD Biosciences). Rat-tail collagen type 1 was prepared by diluting with sterile filtered 0.02N acetic acid. The cells were incubated in 5% CO_2, at 37°C, in a humidified incubator. The medium was replaced daily for the first week, by gently washing with 2ml of medium (removing non-adherent cells) before replacing gently with 4 ml of fresh medium. The medium was replaced every 2 days thereafter.

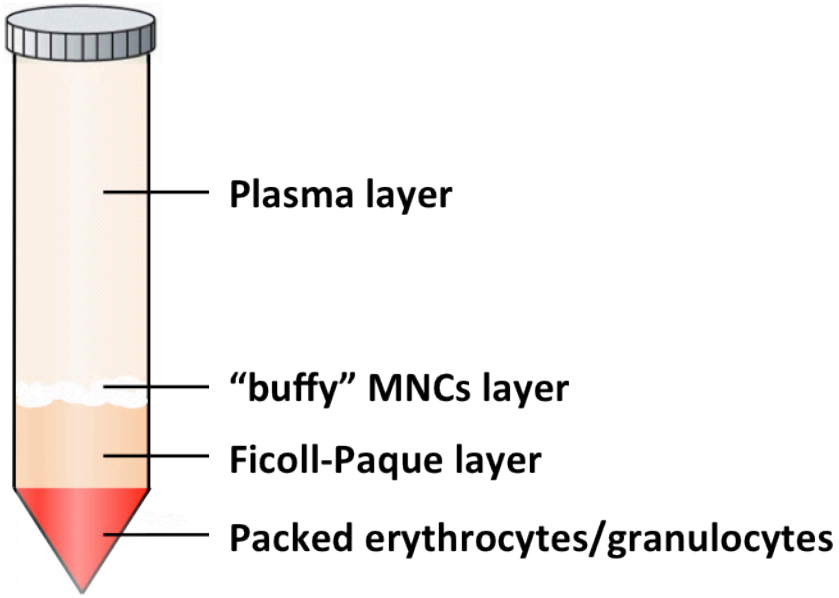


Figure 2.1. Ficoll-Paque separation of human peripheral blood mononuclear cells (MNCs). After gradient centrifugation, 4 layers are formed according to their relative density. Heavier blood components such as erythrocytes migrate to the bottom of the tube, while MNCs form a dense "buffy" layer above the Ficoll-Paque media. The MNC layer was carefully aspirated and washed to remove contaminating Ficoll.

The number of colonies that appeared 11 to 35 days after plating was recorded, the colonies consisting of a well-circumscribed cobblestone monolayer of endothelial colony-forming cells or ECFCs (also known as late-outgrowth endothelial progenitor cells and blood outgrowth endothelial cells). Individual colonies were harvested either from a whole well (when colonies grew really quick to grow beyond the size of the cloning rings) or using a cloning cylinders attached with vacuum grease (8 mm or 10 mm in diameter; cat no. TR-1004 or TR-1005, Millipore, Watford, UK) (Javed et al., 2008; Mead et al., 2008). Cells were washed with Ca^{2+} and Mg^{2+} free PBS and detached with warm 0.05% Trypsin-EDTA (cat no. 25300-054, Invitrogen). The total number and proportion of viable cells was determined following staining with 0.4% Trypan Blue solution (cat no. T8154, Sigma-Aldrich), using a haemocytometer. After re-suspension in fresh EGM-2 medium containing 20% FBS, the cells were seeded (3000 to 5000 cells/cm^2) in culture vessels pre-coated with 1% gelatin (diluted to 1% with PBS from 2% stock, cat no. G1393, Sigma-Aldrich). The medium was replaced every 2 days and sub-confluent cells (i.e. ~70-80% confluent) were serially propagated in T25, T75 and T175 flasks. Frozen stocks of cells were prepared during early passages by suspending them ($0.5x10^6$ cells/ml) in freezing medium (95% FBS, 5% dimethyl sulfoxide (DMSO), immediately transferring 1 ml aliquots

into cryovials and leaving these overnight at -80°C in a freezing container (cat no. 5100-0001, Thermo Scientific Nalgene, Fisher). The cryovials were then transferred to the vapour phase of a liquid nitrogen tank for longer term storage.

2.4 Modification of ECFC culture protocol

While establishing the culture protocol for deriving ECFCs from adult peripheral blood I examined a number of steps that may influence the yield and growth of these cells (summarised in Table 2.1). **(1)** The vacutainer CPT tubes represent a convenient means of isolating MNCs but provided significantly fewer MNCs than the Ficoll density centrifugation method (see Figure 2.2). **(2)** I found that it was also preferable to wash the MNCs in PBS-FBS rather than EGM-2 medium, as this reduced the incidence of cell clumping. **(3)** Previous attempts to derive ECFCs in the Department used serum marketed for human endothelial cell culture and were unsuccessful. I found that the type/source of FBS was a critical factor in ECFC isolation and propagation of ECFCs from adult human blood, and have used defined FBS (HYC-001-330Y) and stem cell-screened FBS (HYC- 001-331G) from HyClone throughout these studies. Initially I used 20% FBS, as this was described in the original patent for ECFC isolation (WO 2005/078073 A2), and, having established the protocol, subsequently isolated and cultured all ECFC populations in the presence of 20% FBS. This concentration has also been adopted in a number of published studies (Guven et al., 2006; Asosingh et al., 2008; Moonen et al., 2010). **(4)** As described in other studies (Ingram et al., 2004; Mead et al., 2008), I used rat tail collagen Type I as the initial coating of plastic ware when plating MNCs. However, 1% gelatin was routinely used to coat plastics for endothelial cell culture and once the ECFC colonies were established I found that they could be propagated equally well with 1% gelatin or rat tail collagen coating. Recent studies have also described using 1% gelatin coating for the propagation of ECFCs (Starke et al., 2011). **(5)** ECFCs were successfully isolated and propagated after passing all the adherent cells in the well, not just those in the initial colony. Nonetheless, I routinely used cloning rings, as described by Mead and colleagues (Mead et al., 2008), in an attempt to isolate clonally proliferating cell populations and minimize contamination by other cell types. **(6)** During propagation of ECFCs, the cells were routinely passaged at 70 - 80% sub-confluence, as viability tended to be compromised when cultured to a higher density and was found to be a critical factor when using ECFCs in tube-formation assays.

Methodology	Tests	Results
1. Vacutainer CPT tubes as an alternative to Ficoll density centrifugation of MNCs	MNC yield per 50ml of blood	Vacutainer CPT tubes are convenient but provided fewer MNCs (up to 50% less) than Ficoll density separation of MNCs.
2. Washing MNCs during isolation	MNC clumping when washed with EGM-2 medium	Used PBS-FBS to wash MNCs, thereby avoiding problems with clumping.
3. Source/type of serum	Defined FBS (HYC-001-330Y) and stem cell-screened FBS (HYC- 001-331G) from HyClone, compared with other commercial sources of FBS	Colonies were routinely obtained using both defined and stem cell-screened FBS from Hyclone, but not with FBS from other sources.
4. Coating of culture vessels	Rat tail collagen type I versus 1% gelatin coating	Rat tail collagen type I was used to establish ECFC colonies whereas 1% gelatin and rat tail collagen type I provided equally effective coating solutions for propagating established ECFC cultures.
5. Isolating ECFC colonies	Propagating the contents of a whole well versus cells inside a cloning ring	Both methods were used successfully to obtain homogeneous and proliferative ECFC cultures, but cloning rings routinely used as they may help select clonally proliferating cells and reduce contamination by other cell types.
6. Degree of confluence before passing cells	70-80% versus 90-100% confluence	Sub-culturing ECFCs when ~70-80% confluent produced a higher yield of cells and prolonged their growth.

Table 2.1. Summary of modifications to the ECFC culture protocol. ECFC, endothelial-colony forming cells; EGM-2, endothelial growth medium-2; FBS, foetal bovine serum; PBS-FBS; phosphate buffered sulphate with 2% FBS and 0.05% EDTA; MNC, mononuclear cells.

An alternative method for deriving ECFCs from peripheral blood has been published which uses 10% pooled human platelet lysate instead of FBS (Reinisch et al., 2009). In addition to using culture conditions free of animal proteins, this protocol also offers other potential advantages as it requires smaller volumes of blood and does not require MNC isolation prior to plating of blood cells. I sought to use the protocol, exactly as described by Reinisch and co-workers.

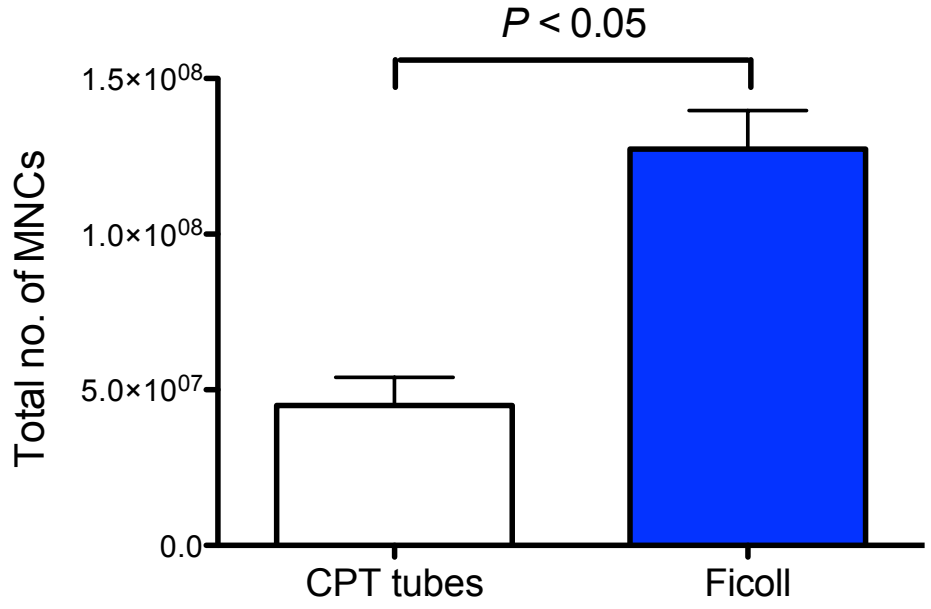


Figure 2.2. Number of mononuclear cells (MNCs) isolated using vacutainer CPT tubes and ficoll separation. Graph shows the number of MNCs isolated from 50 ml of peripheral blood obtained from four age- and sex-matched healthy volunteers. Total number of MNCs isolated by CPT tubes is less than half that obtained by the ficoll separation method. Data presented as mean±SEM, and compared using T-test.

2.5 Culture of other cell types

Human umbilical vein endothelial cells (cat. no. CC-2517, HUVECs, Lonza) and human pulmonary artery endothelial cells (cat. no. CC-2530, HPAECs, Lonza) were used as control mature endothelial cells. Both cell types were cultured in EBM-2 medium supplemented with EGM-2 SingleQuots (including the 2% FBS provided) and 1% antibiotic/antimycotic solution. Culture vessels were pre-coated with 1% gelatin and cells used at passages 3-7. Human pulmonary artery smooth muscle cells (cat no. CC-2581, PASMCs, Lonza) were used as negative controls for some of the protein and mRNA probed, and were cultured in SmBM Basal Medium (cat no. CC-3181, Lonza) supplemented with SmGM-2 SingleQuot (cat no. CC-4149, Lonza) and 1% antibiotic/antimycotic solution. Human hepatocellular carcinoma (HepG2) cells (cat. no. HB-8065, LGC Standards, Teddington, Middlesex UK) were cultured in minimum essential medium (MEM, cat no. M4655, Sigma-Aldrich) containing 10% FBS (cat no. 10437-028, Gibco, Invitrogen) and 1% antibiotic/antiomycotic solution. All cells were cultured in 5% CO_2, at 37°C, in a humidified incubator.

2.6 Population doubling time

From the 2nd passage onwards, the cells were counted during passaging to calculate the population doubling time (PDT). The number of population doublings (PDs) occurring between passages was determined according to the equation: PD = log2 (C_H/C_S), where C_H is the number of viable cells at harvest and C_S is the number of cells seeded. The PDT was derived using the time interval between cell seeding and harvest divided by the number of PDs, as previously described (Ingram et al., 2004).

2.7 Phenotyping of ECFCs

2.7.1 Immunostaining and lectin binding

ECFCs (5000 cells/cm^2) were grown to confluency on sterile glass coverslips (12 mm, cat. no. 631-0666, VWR International, Leicestershire, UK), etched with hydrochloric acid (1N HCl) overnight and coated with 1% gelatin. Prior to immunostaining, the cells were fixed in 4% w/v paraformaldehyde (PFA) in PBS for 20 min at 4°C. After washing twice in PBS, coverslips were immersed in 3% w/v bovine serum albumin (BSA; cat no. A9418, Sigma-Aldrich) in Tris-buffered saline (TBS; cat no. 94158, Sigma-Aldrich) for 30 min at room temperature. Cells were then incubated for 1 h at room temperature with either mouse monoclonal anti-human CD31; (1:30 dilution; clone JC70A, cat no. M0823, Dako, Glostrup, Denmark), mouse monoclonal anti-human CD144 (VE-Cadherin; 1:100 dilution, cat no. sc-9989, Santa Cruz, Insight Biotechnology, Middlesex, UK), rabbit polyclonal anti-human von Willebrand Factor (vWF; 1: 200 dilution; cat no. A0082, Dako), or CD34 (cat no. sc-9095, Santa Cruz). The coverslips were then washed again in TBS six times, and incubated for 1 h at room temperature with Alexa-488 green-conjugated goat anti-rabbit (1:500 dilution; cat no. A-11034, Invitrogen) or goat anti-mouse IgG (1:500 dilution; cat no. A-21121, Invitrogen). Controls included omission of the primary antisera.

Lectin binding, a well-known characteristic of endothelial cells (Holthofer et al., 1982), was assessed by incubating live cells on coverslips with fluorescein isothiocyanate (FITC)-conjugated EC-specific *Ulex europaeus* agglutinin-1 (UEA-1) lectin (1:30, cat no. L9006, Sigma-Aldrich) or FITC-conjugated microvascular EC-specific *Griffonia Simplicifolia* Lectin-1 (cat no. FL-1201, Vector Laboratories Ltd, Peterborough, UK) in complete EGM-2 medium for 1 h in a humidified incubator (5% CO_2, 37°C). These coverslips were then washed in TBS, and fixed in 4% w/v PFA in PBS for 20 min at 4°C. All coverslips were washed with TBS 6 times before being mounted in 4',6-diamidino-2-phenylindole

(DAPI)-containing Vectashield (cat no. H-1200, Vector Laboratories Ltd) in order to counter-stain the nucleus and reduced loss of fluorescence during microscopic examination. Stained cells were observed using confocal microscopy (TCS SP5, Leica Microsystems (UK) Ltd, Bucks, UK).

2.7.2 Flow cytometry

More than 10^6 ECFCs were detached with 0.05% trypsin-EDTA, resuspended in FACS buffer (PBS containing 2% FBS) and divided in 100 µl aliquots, containing at least 1 x 10^5 cells per tube. Cells were then suspended in Fc receptor blocker (1:50 dilution; cat no. 130-059-901, Miltenyl Biotec, Bergisch Gladbach, Germany) for 5 min in dark at room temperature, before being resuspended for 30 min at 4°C in the dark with antibodies against markers for endothelial cells, monocytes, hematopoetic stem cells or progenitor cells (see Table 2.2). Fluorochrome-matched isotype controls (mouse IgG1κ – FITC,-PE and –APC isotypes; BD Pharmingen) were used at the same dilution factor as the staining antibodies, as per the manufacturers recommendations. Cells were then washed with FACS buffer twice with centrifuging at 1000 rpm for 5 min each, before resuspending in 0.5 ml of FACS buffer. All flow cytometric analysis was performed using a three colour FACSCalibur flow cytometer and acquired by CellQuest Pro software (both from BD Biosciences). A gate was set to acquire events within the ECFC population on a forward and side scatter graph (Figure 2.3). Optimal compensation was set for green, red and far-red fluorescence by running the FITC-, PE- and APC-stained ECFCs. Samples were run at high flow rate with an acquisition cut-off of 20,000 to 30,000 gated events. Data were analysed using FCS Express (De Novo Software, Los Angeles, CA, USA) and presented as the number of antigen expressing (positive) cells, normalised by subtracting the amount of stained cells by the isotype controls, and expressed as percentage of the gated ECFC population (Figure 2.3).

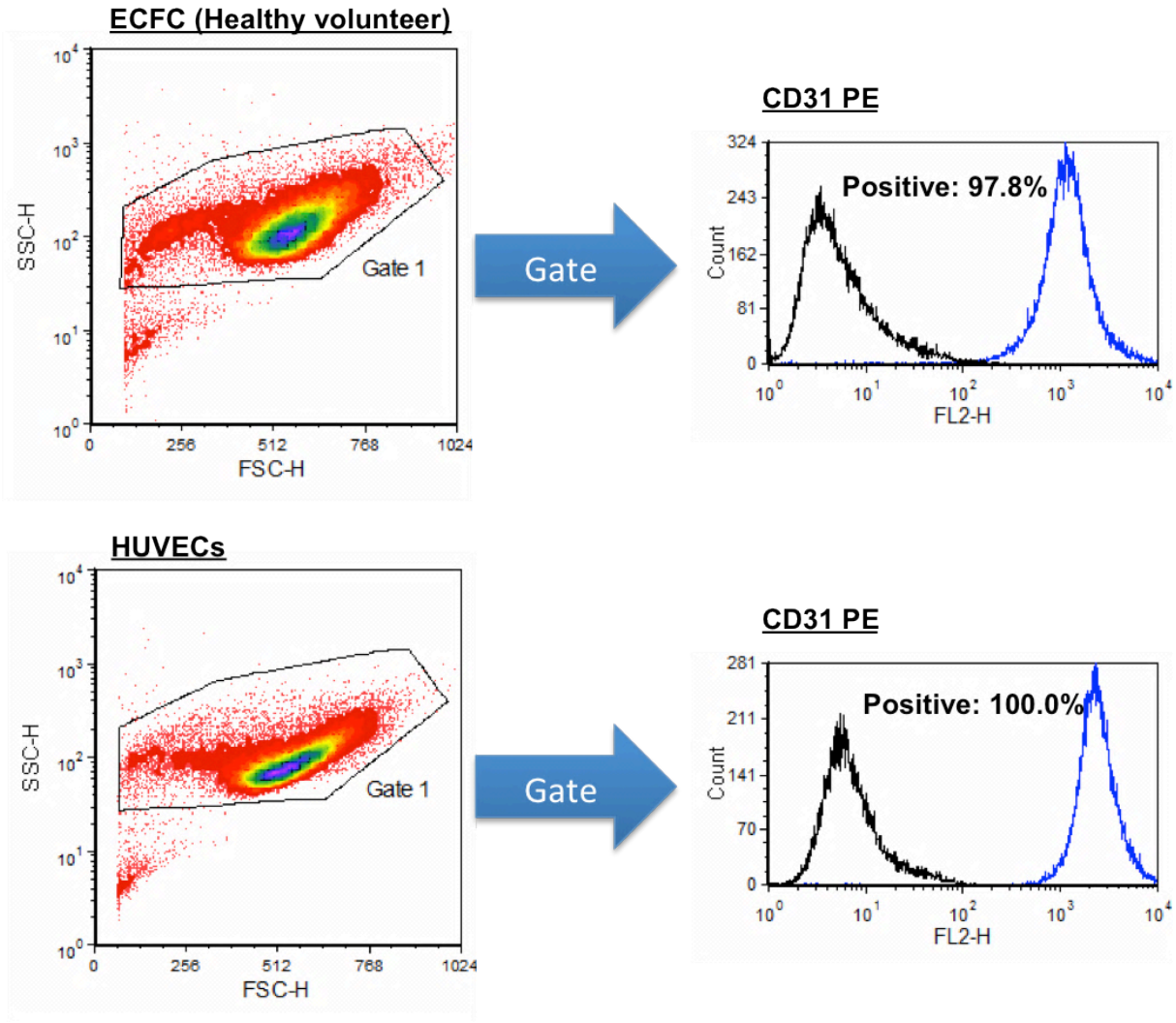


Figure 2.3. Gating strategy for flow cytometric analysis of ECFCs. Images on the left display a forward/side scatter density plots of acquired events from a ECFCs from healthy control (upper panel) and HUVECs (lower panel), with both showing similar pattern. The gated populations are then displayed in the histograms (right panel) with the CD31 staining (blue line) and its respective isotype control (black line) displayed. The expression of the protein is calculated by the FCS express software in subtracting the area covered by the isotype control from the area covered by the staining antibody, with the data presented as % expression of the gated population.

Anti-human antibody	Fluorochrome	Species of source	Source & Catalogue no.	Antibody dilution used	Cell type marker/ receptor type
CD31 (PECAM-1) (Clone WM59)	PE	Mouse mAb	BD Pharmingen #555446	1:10	Endothelial
VEGFR2/KDR (Clone 89106)	APC	Mouse mAb	R&D Systems #FAB357A	1:6.7	Endothelial
CD14 (Clone MΦP9)	PE	Mouse mAb	BD Pharmingen #345785	1:6.7	Monocyte
CD45 (Clone TU116)	PE	Mouse mAb	BD Pharmingen #557059	1:40	Pan-leukocyte
CD133 (Clone AC133)	PE	Mouse mAb	Miltenyi #130-080-801	1:10	Immature HSCs/HPCs
CD34 (Clone 581/CD34)	FITC	Mouse mAb	BD Pharmingen #555821	1:5	Progenitor
CD117 (c-kit) (Clone YB5.B8)	APC	Mouse mAb	BD Pharmingen #550412	1:6.6	Stem/progenitor cell
CXCR4 (Clone 13G5)	PE	Rabbit pAb	e-Bioscience #FAB170P	1:5	Chemokine receptor

Table 2.2. Antibodies used in flow cytometry for identification of ECFCs. APC, allophycocyanin; CXCR4, C-X-C chemokine receptor type 4; FITC, fluorescein isothiocyanate; HPCs, hematopoetic progenitor cells; HSCs, hematopoetic stem cells; KDR, kinase insert domain receptor; PE, phycoerythrin; PECAM-1, platelet endothelial cell adhesion molecule; VEGFR2, vascular endothelial growth factor receptor type 2.

2.8 Clonal growth of ECFCs

A key characteristic of progenitor cells is their ability to grow under limiting dilutions or clonal conditions and is one of the defining features that distinguishes ECFCs (also known as late outgrowth EPCs) from early outgrowth EPCs or haematopoietic cells (Ingram et al., 2004; Ingram et al., 2005). Early passage (passage 3) ECFCs, grown to ~80% confluence, were seeded at limiting dilution (equivalent to one cell per well in 200 µl of ECFC culture medium) in 96-well plates pre-coated with rat-tail collagen Type 1 (see Section 2.3) and incubated in 5% CO_2, at 37°C, in a humidified incubator. The medium was replaced every 4 days. After 14 days, the cells were washed twice with warm PBS

and stained with 100 µl/well (1:5000 in serum-free EBM-2) of Calcein-AM (cat no. C1359, Sigma-Aldrich) for 30 min at 37°C, before being washed three times with PBS and examined by fluorescence microscopy (Olympus IX70, Olympus, Essex, UK) within 2 h. Each well was examined for endothelial colonies, the colonies being scored as having high proliferative potential (HPP; >2000 cells/colony) and low proliferative potential (LPP; 50–2000 cells/colony) or representing endothelial clusters (<50 cells) (Coldwell et al., 2011). Images were taken of colonies comprising more than 50 cells by the CCD camera (F-view II, Olympus) attached to the microscope with its respective image software (analySIS, Olympus) and the number estimated using ImageJ software (NIH, http://rsbweb.nih.gov/ij/).

2.9 RNA extraction and cDNA synthesis

RNA was extracted using either Trizol (cat no. 15596-026, Invitrogen) or RNeasy kit (cat no. 74104, QIAGEN, Crawley, UK) according to manufacturers' instructions. To remove possible DNA contamination, samples were treated with DNase I (Amplification grade; cat no. AMPD1, Sigma-Aldrich) and the amount of RNA was quantified by absorption at 260 nm using NanoDrop® (ND-1000; NanoDrop Technologies, Willington, DE, USA). First strand complementary DNA (cDNA) was synthesised by reverse transcription (RT)- polymerase chain reaction (PCR). 2 µg of RNA in up to 10 µl diethylpyrocarbonate (DEPC)-treated RNase/DNase-free water (cat no. 750024, Invitrogen) were mixed with 4 µl 5x cDNA synthesis buffer, 1 µl 0.1M DTT, 1 µl RNaseOUT™, 1 µl ThermoScript™ reverse transcriptase with 1 µl of 50 ng/µl random hexamers, and 2 µl 10 mM dNTP mix (ThermoScript™ RT-PCR System; cat. no. 11146-016, Invitrogen). The 20 µl mixture was then placed into a thermal cycler (Peltier Thermal Cycler PTC-200; MJ Research, Watertown, MI, USA) with profiles as follows: 25^{o}C for 10 min, followed by 50 min at 50^{o}C, terminated at 85^{o}C for 5 min. 1 µl of RNase-H (ThermoScript™ RT-PCR System) was then added and the solutions were incubated for 20 min at 37^{o}C to digest any remaining RNA. The samples were finally diluted in DEPC-treated RNase/DNase-free water (100 µl final volume) to create a 20 ng/µl cDNA solution. Controls included: (1) omission of the RT step in addition to the use of DNase-treated RNA and (2) the use of cDNA from HUVECs and HPAECs as positive controls for EC markers, or cDNA from HPASMCs as controls for SMCs markers.

2.10 Semi-quantitative and quantitative RT-PCR (qRT-PCR) analysis

Appropriate PCR primers (Invitrogen) were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) unless otherwise stated, with the primers amplifying a sequence that spanned two exons to avoid amplification of genomic DNA. Primers for qRT-PCR were either designed by Primer-Blast and manufactured by Invitrogen or purchased from QIAGEN. Primer sets used and their thermal profiles are shown in Table 2.3 for semi-quantitative RT-PCR and in Table 2.4 for qRT-PCR.

2.10.1 Semi-quantitative RT-PCR

Semi-quantitative PCR was performed using 40 ng of cDNA mixed with 1 µM of forward (F) and reverese (R) primers (Table 2.3), 10 µl of PCR Master Mix (M750B, Promega, Southampton, UK), together with RNase/DNase-free water in a 20 µl reaction. The PCR amplification was performed with the following thermal profile: 94^{o}C for 2 min for denaturation, then 94^{o}C for 30 s, annealing temperature for 1 min, and 72^{o}C for 1 min (underlined repeated for each cycle), with a final extension of 72^{o}C for 10 min. 5 µl of the PCR product was mixed with 2 µl of 6x loading dye (cat no. R0611, Fermentas, York, UK) and separated in a 1% agarose Tris-acetate-EDTA (TAE) gel (both from Sigma-Aldrich) and stained with GelRed^{TM} Nucleic Acid Gel Stain (in 1:20,000 dilution; cat no. 41003, Biotium, Hayward, CA, USA). GeneRuler™ 1kb Plus DNA Ladder (0.25 µg; cat no. SM1331, Fermentas) was run alongside the samples in TAE running buffer at 10 V/cm for 20 – 50 min, and visualised under the UV light. The intensity of each band was quantified with ImageJ. To ensure PCR reactions were not saturated or components exhausted, preliminary runs were conducted over a range of different cycles, showing linear increases in reaction products, and the optimal cycle number selected that lie within the linear phase (see Figure 2.4A-C and Table 2.3). Control blanks were run with the no-RT template to ensure genomic DNA was not carried forward and being replicated in the cDNA solution (Figure 2.4D). Gene expressions were expressed as fold change relative to the reference gene β2-microglobulin. β2-microglobulin was the chosen reference gene because its expression was constant throughout all the ECFC samples with the equal initial amount of cDNA (Figure 2.4D). Conversely, β-actin was not expressed equally with equal starting cDNA of different ECFC samples in semi-quantitative PCR (Figure 2.4E) and was hence not used as the reference gene.

Target Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')	Product size (bp)	Annealing temp. (°C)	Cycles	Source
β2-microglobulin	GGGTTTCATCCATCCGACATTG	TGGTTCACACGGCAGGCATAC	167	64	26	(Atlasi et al., 2008)
β-Actin	ACAGAGCCTCGCCTTTGCCG	ACCATCACGCCCTGGTGCCT	189	55	24	Designed on Primer-Blast
ID1	CTGCTCTACGACATGAACGGCT	TGACGTGCTGGAGAATCTCCA	103	50	34	(Southwood et al., 2008)
ID2	GACCCGATGAGCCTGCTATACA	GGTGCTGCAGGATTTCCATCT	112	50	34	(Upton et al., 2009)
ID3	CTACGAGGCGGTGTGCTGCC	TCCGGCAGGAGAGGTTCCCG	411	55	27	Designed on Primer-Blast
ALK1	GGACCATGACCTTGGGCTCC	AGCTCCCTGTGCAAGTTCCC	232	53	27	Designed on Primer-Blast
ALK2	ACGATGGCTTCCACGTCTACCAG	ACAGTGTAATCTGGCGAGCCAC	453	56	28	Designed on Primer-Blast
ALK3	CAAGCAGACGTCGTTACAATCG	TACATCTTGGGATTCAACCATC	1040	56.2	40	(Upton et al., 2008)
ALK4	TCCAAAGACAAGACGCTCC	ATCATCTTCCCCATCACCC	898	57.5	32	(Upton et al., 2008)
ALK5	CGTGCTGACATCTATGCAAT	AGCTGCTCCATTGGCATAC	250	57.5	34	(Upton et al., 2008)
ALK6	TCTCAGAGCTCAGGAAGTGGATC	CTGTCAGCCTTGATGCAGGATTG	904	58.9	35	(Upton et al., 2008)
ActRIIA	GCAAAATGAATACGAAGTCTA	GCACCCTCTAATACCTCTGGA	435	56.5	33	(Upton et al., 2008)
ActRIIB	ACACGGGAGTGCATCTACTAC	GGCAAATGAGTGAAGCGCTCG	274	56.5	33	(Upton et al., 2008)
BMPR2	CTGCGGCTGCTTCGCAGAAT	TGGTGTTGTGTCAGGAGGTGG	347	53	27	Designed on Primer-Blast
TGF-βRII	CCAACAACATCAACCACAACAC	TCATTTCCCAGAGCACCAG	677	61.8	28	(Upton et al., 2008)
Endoglin	TGGAGCTGACTCTCCAGGCA	CAGGACCCTCAGGATGTGCG	440	53	27	Designed on Primer-Blast
Caveolin	AGACGAGCTGAGCGAGAAGC	TGGAATAGACACGGCTGGTGC	350	53	27	Designed on Primer-Blast
CLIC4	GTCAAGGCTGGCAGTGATGG	CAGTGCTTCATTAGCCTCTGGC	354	50	27	Designed on Primer-Blast

Table 2.3. Primer sequences for semi-quantitative RT-PCR.

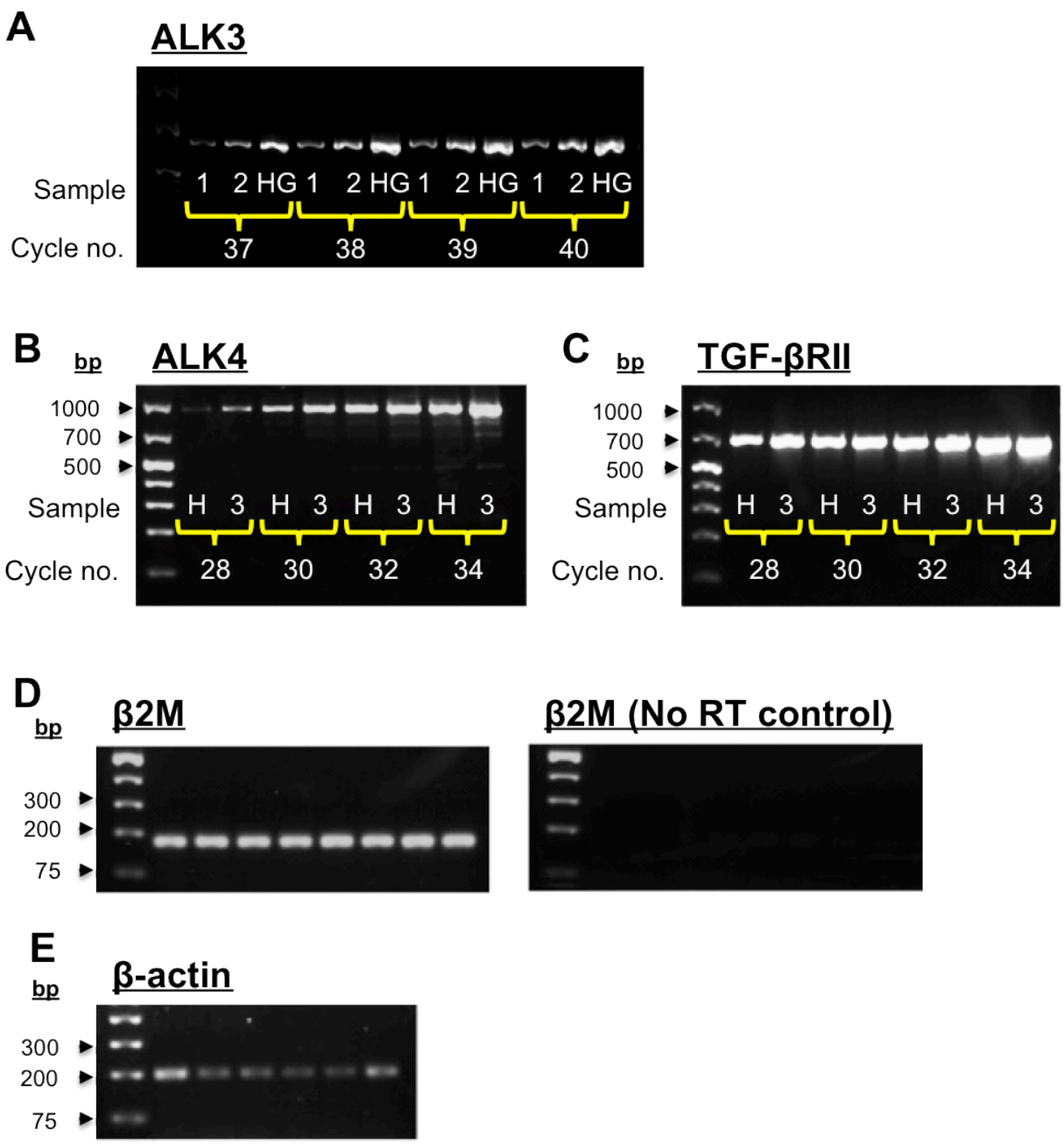


Figure 2.4. Optimisation of the PCR cycles for semi-quantitative analysis of gene expression. cDNA reverse-transcribed from RNA were run on a thermal cycler for an increasing number of cycles for each sets of primers and the end-products separated by 1% agarose gel electropheresis. As an example, the amplification of different cDNA samples are shown with primers for **(A)** ALK3 (37 – 40 cycles), **(B)** ALK4 (28 – 34 cycles) and **(C)** TGF-βRII (28 – 34 cycles). Bands in both **A** and **B** display amplification in the linear phase, while in **C** the products were nearly saturated after 32 cycles. **(D)** A batch of different cDNA samples were amplified for 26 cycles (the optimised cycle no.) with primers for the reference gene β2-microglobulin (β2M) on the left image, with the cDNA samples without the RT transcriptase during the cDNA production shown on the right. **(E)** A batch of different cDNA samples were amplified for 24 cycles with primers for β-actin. H, HPAECs; HG, HepG2 cells; RT, reverse transcriptase. ▶, number of base pairs (bp).

2.10.2 qRT-PCR

qRT-PCR was prepared with 20 ng of cDNA in a 10 µl reaction volume using 5 µl of Platinum SYBR Green qPCR SuperMix-UDG with 0.2 µl ROX reference dye (catalogue No. 11733-046; Invitrogen), 0.5 µM of primers (Table 2.4) and 1.8 µl DEPC water. qPCR was performed using a 7500 Fast Real-Time PCR System (catalogue No. 4351106; Applied Biosystem, Paisley, UK) with the following thermal profile: 95^{o}C for 20 s for denaturation, then 95^{o}C for 3 s and 56°C for 25 s (underlined repeated for each cycle, for 45 cycles), and the dissociation stage 95^{o}C for 15 s, 56^{o}C for 1 min and 95^{o}C for 15 s for assessing the melting curves to check for possible primer-dimer. The efficiency of each primer set was confirmed to be above 90%. The relative expression of target mRNAs was determined using the standard curve method and was normalised by the normalisation factors calculated from peptidylprolyl isomerase A (PPIA) and α-tublin expression using the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/). The standard curve method is depicted in Figure 2.5A. Standards were produced from serial dilutions of pooled samples and a standard curve constructed from the cycle threshold (Ct) value (Figure 2.5). This was then used to obtain the relative quantity of cDNA (i.e. mRNA) for individual samples. The fewer the cycles required to surpass the threshold, the more of the specific mRNA is present in the sample. The standard curve in Figure 2.5 indicates the primer sets for α-tubulin (Figure 2.5A) and PPIA were highly efficient (Figure 2.5B). The close position of the amplification curves for each sample, using both α-tubulin and PPIA primers, indicates similar levels of expression (i.e. RNA) for both housekeeping genes in each of the samples, making them both effective as reference genes. However, primers for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) provided a poor standard fit in the same set of samples (Figure 2.5B), indicating that GAPDH (at least for the set of primers used) was not an ideal reference gene for ECFCs.

Target Gene	Forward primer sequence (5' – 3') or Primer Cat. no.	Reverse primer sequence (5' – 3')	Product size (bp)	Source
GAPDH	TCATTTCCTGGTATGACAACGA	TCTACATGGCAACTGTGAGG	205	Gift from Dr Mahul-Mellier (Impeial College London)
PPIA	CTGCACTGCCAAGACTGA	GCCATTCCTGGACCCAAA	109	(Stronach et al., 2011)
α-tubulin	GCCAAGCGTGCCTTTGTTC	CACACCAACCTCCTCATAATCC	114	(Stronach et al., 2011)
ID1	QT00230650			QuantiTect® Primer
ID2	GACCCGATGAGCCTGCTATACA	GGTGCTGCAGGATTTCCATCT	112	(Upton et al., 2009)
ALK1	QT00050351			QuantiTect® Primer
ALK5	QT00083412			QuantiTect® Primer
ActRIIA	QT00077749			QuantiTect® Primer
BMPR2	CAAATCTGTGAGCCCAACAGTCAA	GAGGAAGAATAATCTGGATAAGGACCAAT	114	(Upton et al., 2009)
TGF-βRII	QT00014350			QuantiTect® Primer
Endoglin	QT00013335			QuantiTect® Primer
CLIC4	QT02312569			QuantiTect® Primer
THBS1	QT00028497			QuantiTect® Primer
Survivin	QT00081186			QuantiTect® Primer
ARC	QT00234794			QuantiTect® Primer

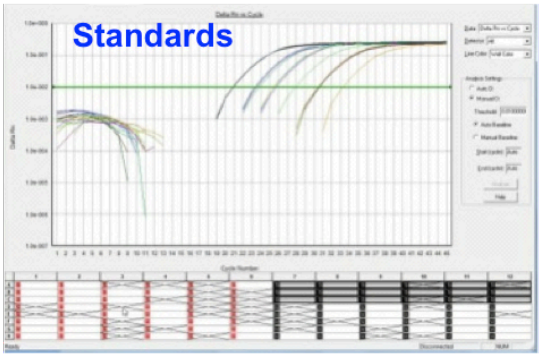
Table 2.4. Primer sequences or product details for quantitative RT-PCR. QuantiTect® Primer are pre-designed primers manufactured by QIAGEN specifically for qRT-PCR.

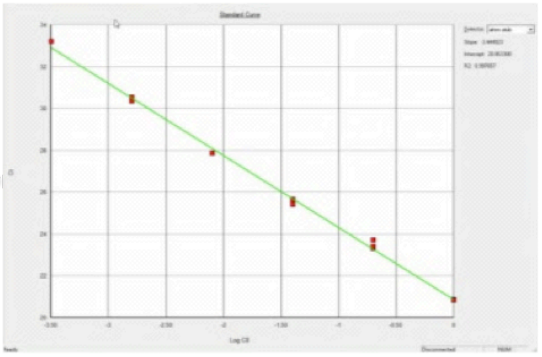
A

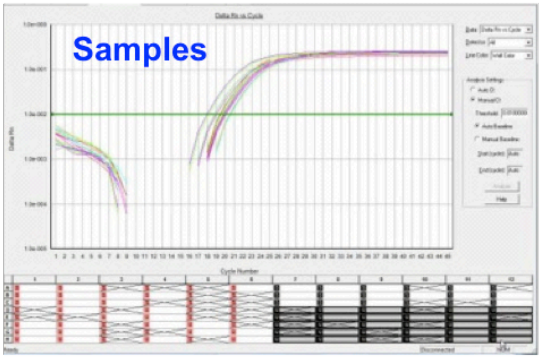
1. Standard curve is constructed by amplification of serially diluted (5x) cDNA pooled from all the samples on the plate.

2. Ct values are plotted against the relative quantity of each standard (all relative to the starting quantity: 1, 0.5, 0.1, 0.02, 0.004, 0.0008) in log scale by the software.

α-tubulin



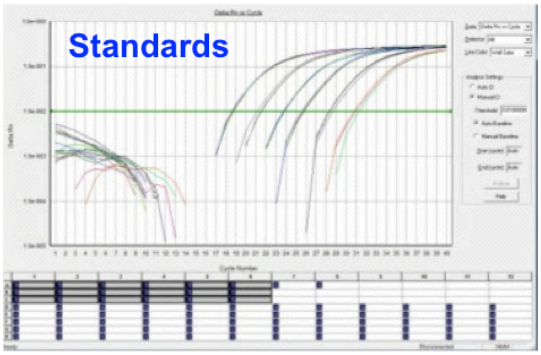


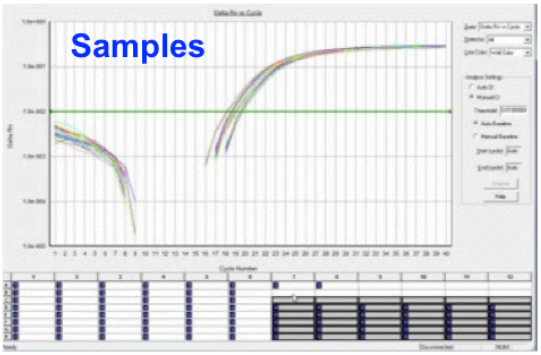


3. Ct value from each cDNA sample is matched on the standard curve by the software to determine the relative quantity that are used for anaylsis

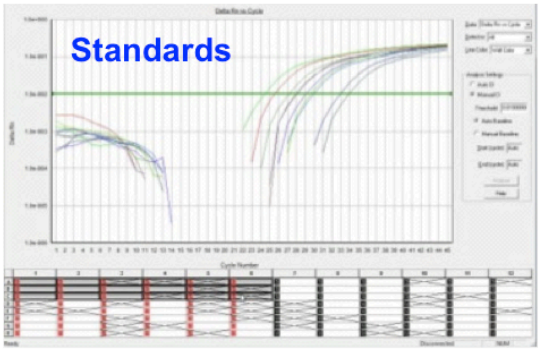
B

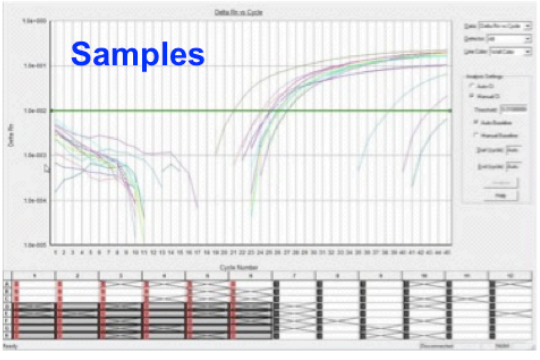
PPIA





GAPDH





← **Figure 2.5**. **qRT-PCR analysis using standard curve method. (A)** cDNA samples were subjected to amplification over 45 cycles with SYBR green qPCR mix and primers for α-tubulin, with the fluorescence intensity captured by the qPCR machine in every cycle. The threshold line is set within the linear kinetic phase of all the samples, with the cycle no. of each samples on the threshold line called threshold cycle (Ct) deduced. **(B)** The same set of samples was amplified with primers for PPIA and GAPDH. Both the α-tubulin (**A**) and PPIA (**B**, upper panel) primer sets amplify with high efficiency, indicated by the good fit of standard curve, while the primers for GAPDH do not provide a linear relationship between the standards and the Ct values (**B**, lower panel).

2.11 Western blotting

For protein extraction, cells were washed twice with ice-cold PBS and incubated in radio-immuno-precipitation assay (RIPA) buffer (180 µl 6-cm dish; 350 µl in 10-cm dish; cat no. R0278, Sigma-Aldrich) with protease inhibitor (1:100 with RIPA buffer; cat no. R8346, Sigma-Aldrich) on ice for at least 15 min, before being harvested using a cell scraper. Protein concentration was determined by Bradford assay (Bio-Rad, Hertfordshire, UK) according to manufacturer instruction. Additional phosphatase inhibitors (10 mM sodium fluoride and 1 mM sodium orthovanadate) (all ingredients from Sigma-Aldrich) were added into the lysis buffer when phosphorylated proteins were being analysed. Equal amounts of protein were denatured in lithium dodecyl sulphate (LDS) loading buffer and reducing agent (both from Invitrogen) by heating at 70°C for 10 min and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% or 4 – 12% NuPAGE® Novex Bis-Tris Mini Gel 1.5 mm; Invitrogen) at 120V for 2h. A mixture of 5 µl Novex® Sharp Pre-stained Protein Standard (cat no. LC5800, Invitrogen) and 5 µl MagicMark™ XP Western Standard (cat no. LC5602, Invitrogen) was also run on each gel to show migration of marker proteins for estimation of protein molecular weights. Proteins were electrophonically transferred to nitrocellulose membranes (Amersham Hybond™ ECL™, 0.2 µm pore size; cat no. RPN30320, GE Healthcare) using the wet transfer method (BioRad). Briefly, bis-tris gel and nitrocellulose membrane were sandwiched in between sponges and filter papers in a transfer cassette soaked with transfer buffer (20% methanol (Sigma) in 1x Tris-glycine-SDS (TGS) (cat no. 161-0772, BioRad)), and was placed in a transfer tank (BioRad) filled with transfer buffer and run at 80 V for 1 h with an ice pack.

Successful protein transfer was confirmed with protein staining on the membrane with Ponceau S (cat no. P7170, Sigma-Aldrich). Membrane was then washed with TBS and blocked in TBS-T (TBS with

0.1% Tween-20 (cat no. 94158, Sigma-Aldrich) with 5% w/v BSA and 5% w/v non-fat dry milk for 1h (all from Sigma-Aldrich). Membranes were then either incubated for 1 h at room temperature or overnight at 4°C with primary antibodies in TBS-T containing 5% w/v BSA or 5% w/v non-fat milk. After washing five times with TBS-T, 5 min each, membranes were incubated for 1 - 2 h at room temperature with respective, species-specific horseradish peroxidised (HRP)-conjugated secondary antibodies (HRP-conjugated sheep anti-mouse IgG, cat no. NA9310, GE Healthcare; HRP-conjugated goat anti-rabbit IgG, cat no A0545, Sigma-Aldrich), washed five times with TBS-T and visualised by chemiluminescence, using Novex® ECL chemiluminescent substract kit (cat no. WP20005, Invitrogen) for strongly-expressed protein bands or Luminata™ Forte Western HRP substrate (cat no. WBLUF0500, Millipore) for weakly-expressed bands. Following exposure to radiography film (FujiFilm X-ray film; cat no. AUT-300-040D, Fisher), the membrane was reprobed for β-actin expression to confirm equal protein loadings without stripping (as the molecular weight of all the proteins probed did not overlap with β-actin). Semi-quantitative analysis of protein expression was performed using densitometric measurement by Quantity One software (BioRad) and proteins of interest were normalised to the loading control protein β-actin. The antibodies used for Western blotting and the optimal incubation conditions are provided in Table 2.5.

Anti-human primary antibody	Species of source	Source & Catalogue no.	Primary antibody dilution used & condition	Secondary antibody dilution used & condition	Optimal protein loading (µg)
ALK5	Rabbit pAb	Cell signalling #3712	1:500 overnight 4°C	1:10000 2h RT	50
BMPR2	Mouse mAb	BD Transduction #612292	1:400 overnight 4°C	1:2000 2h RT	80
Caspase 3	Rabbit pAb	Cell signalling #9662	1:200 overnight 4°C	1:10000 2h RT	40
CLIC4	Mouse mAb	Santa Cruz # sc-135739	1:1000 1h RT	1:2500 1h RT	5 - 10
Connexin-40	Rabbit pAb	Sigma #AV36635	1:1000 overnight 4°C	1:10000 1h RT	40
Connexin-43	Mouse mAb	Sigma #C8093	1:1000 overnight 4°C	1:2000 1h RT	43
DDAHI	Goat pAb	(Leiper et al., 2007)	1:1000 overnight 4°C	1:5000 1h RT	30
HIF-1α	Mouse mAb	Enzo Life Sciences, #OSA-602	1:1000 overnight 4°C	1:2000 1h RT	120
PARP	Rabbit pAb	Cell signalling #9542	1:1000 overnight 4°C	1:15000 1h RT	40
pSmad1 (Ser463/465)/Smad5 (Ser 463/465)/Smad 8 (Ser426/428)	Rabbit pAb	Cell signalling #9511	1:500 overnight 4°C	1:10000 2h RT	40
Smad1	Rabbit pAb	Cell signalling #9743	1:1000 overnight 4°C	1:10000 1h RT	(same loading as pSmad1/5/8)
pSmad2 (ser465/467)	Rabbit mAb	Cell signalling #3108	1:500 overnight 4°C	1:15000 2h RT	70
Smad2 (L16D3)	Mouse mAb	Cell signalling #3103	1:1000 overnight 4°C	1:2500 1h RT	(same loading as pSmad2)
β-actin (AC15)	Mouse mAb	Sigma-Aldrich #A1978	1:2500 1h RT	1:2500 1h RT	(Reprobing each blot as loading control)

Table 2.5. Primary and secondary antibodies used in Western blotting. Secondary antibodies were used for respective, species specific primary antibodies, and they included horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich, Cat no. A0545), HRP-conjugated sheep anti-mouse IgG (GE Healthcare, Cat no. NA9310) and HRP-conjugated rabbit anti-goat IgG (Sigma-Aldrich, Cat no. A5420). DDAH-1, dimethylarginine dimethylaminohydrolase; HIF-1α, hypoxia-inducible factor 1-α; mAb, monoclonal antibody; pAb, polyclonal antibody; PARP, polyadenosine diphosphate-ribose polymerase; pSmad1/5/8, phospho-Smad1/5/8; pSmad2, phospho-Smad2; RT, room temperature.

2.12 Endothelial functional assays

2.12.1 Proliferation assays

In addition to determining the PDT from each ECFC line, cell proliferation was examined by counting cells (as described above) and using a chemiluminecent 5-bromo-2'-deoxyuridine (BrdU) incorporation assay to assess DNA synthesis. BrdU is a thymidine analogue that is incorporated into newly synthesised DNA during the S phase of the cell cycle and correlates well with the traditional [^{3}H]-thymidine incorporation assay (Waldman et al., 1991).

The BrdU assay (cat no. 11669915001; Roche, West Sussex, UK) was optimised by comparing BrdU incorporation in ECFCs plated at two different seeding densities (1500 and 3000 cells per well in a flat black-walled 96-wells optical bottom plates (Fisher)) at a final volume of 100 µl/well of EBM-2 (100 µl/well) containing 5% FBS. After overnight incubation, the cells were washed twice with warm sterile PBS and cultured in serum-deprived EBM-2 medium for 6 h. The medium was then replaced with EBM-2 containing either 5% or 10% FBS or EGM-2 containing 20% FBS. After 24 h, 10 µl of BrdU (according to manufacturers recommendation) was added to each well and the cells incubated for a further 24 h. The medium was then removed and the cells immersed in fixation solution at room temperature. Cells were incubated in secondary antibody (anti-BrdU conjugated with peroxidise, 100 µl/well) for 90 min at room temperature, before being washed 3x (5 min each) with PBS. A blank control (no cells) and background control (BrdU staining omitted) was included in all experiments. The optical bottom of the plate was then covered with a black adhesive foil (PerkinElmer, Beaconsfield, Bucks, UK) and luminescent substrate solution added (100 µl/well), incubated for 3 min on a shaker at room temperature and luminescence measured using a GloMax microplate luminometer (GloMax®-Multi Microplate Multimode Reader, Promega). BrdU incorporation was presented as raw light units/s (rlu/s) following subtraction of blank values. The values obtained in the background control did not exceed 2% of the respective value in the presence of BrdU.

The incorporation of BrdU was more than doubled when ECFCs were seeded at 3000 versus 1500 cells/well and cultured in EBM-2 containing 5% FBS (Figure 2.6). The presence of 10% FBS further increased BrdU incorporation. Maximum stimulation was observed in the presence of EGM-2 and 20% FBS, but exhibited a ceiling effect as cultures seeded at 3000 cells/well were confluent after 3 days (Figure 2.6). A seeding density of 1500 cells/well and cultures in EBM-2 (5% FBS) or EGM-2 (20% FBS) were therefore selected as the conditions to be used when assessing possible differences in DNA synthesis.

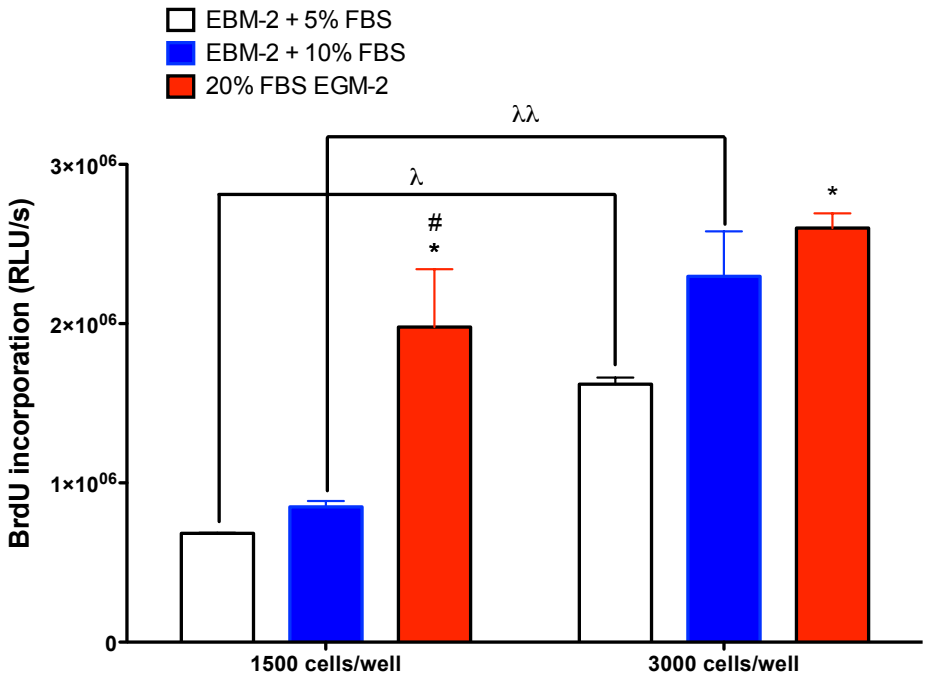


Figure 2.6. Optimisation of BrdU assay. Sub-confluent ECFCs, derived from healthy volunteers (n=2), were seeded in triplicate at 1500 or 3000 cells per well in 96-wells plates and cultured in EBM-2 containing 5% or 10% FBS, or EGM-2 containing 20% FBS for 48h. Statistics shown are from two-way ANOVA with Bonferroni post-hoc analysis, with data presented as mean±SEM of triplicate measurements. *, $P < 0.05$, compared with cells cultured in EBM-2 and 5% FBS. $^{\#}$, $P < 0.05$, compared with cells cultured in EBM-2 and 10% FBS. λ, $P < 0.05$, λλ, $P < 0.01$, comparison between indicated groups.

2.12.2 Apoptosis assay

A well-established model of serum deprivation-induced endothelial apoptosis (Karsan et al., 1997; Gerber et al., 1998) was used to examine the sensitivity of cells to apoptosis. Sub-confluent ECFCs and HPAECs (passage 4 to 7) were seeded (7000 cells/well) in their respective complete medium (100 µl/well), in 1% gelatin-coated, white/clear bottom 96-well plates (cat no. PMX-096-125S, Thermo Scientific Nunc, Fisher). After 24h, the cells were washed twice with warm PBS, before being incubated in control medium (EGM-2 with 2% FBS) or serum-deprived EBM-2 for a further 24 – 48 h. Staurosporine (cat no. S4400, Sigma-Aldrich), a potent apoptotic inducer, was used as a positive control. Apoptosis was assessed in duplicate using a Caspase-Glo®-3/7 assay (cat no. G8091, Promega), with minor modification from the manufacturer's protocol. Two dilutions of the luminogenic substrate were tested, corresponding to a 1:1 (100 µl, the suggested dilution) and 1:2 (50 µl) mixture with the medium, 10 min after the cell had acclimatised to room temperature. The plates were gently agitated on a plate shaker (300 – 500 rpm for 30 s) and luminescence measured at 15 min intervals for 2 h, at constant room temperature, using a microplate luminometer (GloMax®-Multi Microplate Multimode Reader, Promega). The blank value was subtracted from the measured rlu/s for each sample and the optimal incubation period (highest normalised values; typically 45 min to 60 min, see Figure 2.7A) determined. Substrate:medium ratios of 1:2 and 1:1 provided similar proportional difference in rlu/s in the positive controls compared to the negative controls (Figure 2.7B&C), and the 1:2 dilution was hence used for all further apoptosis assays.

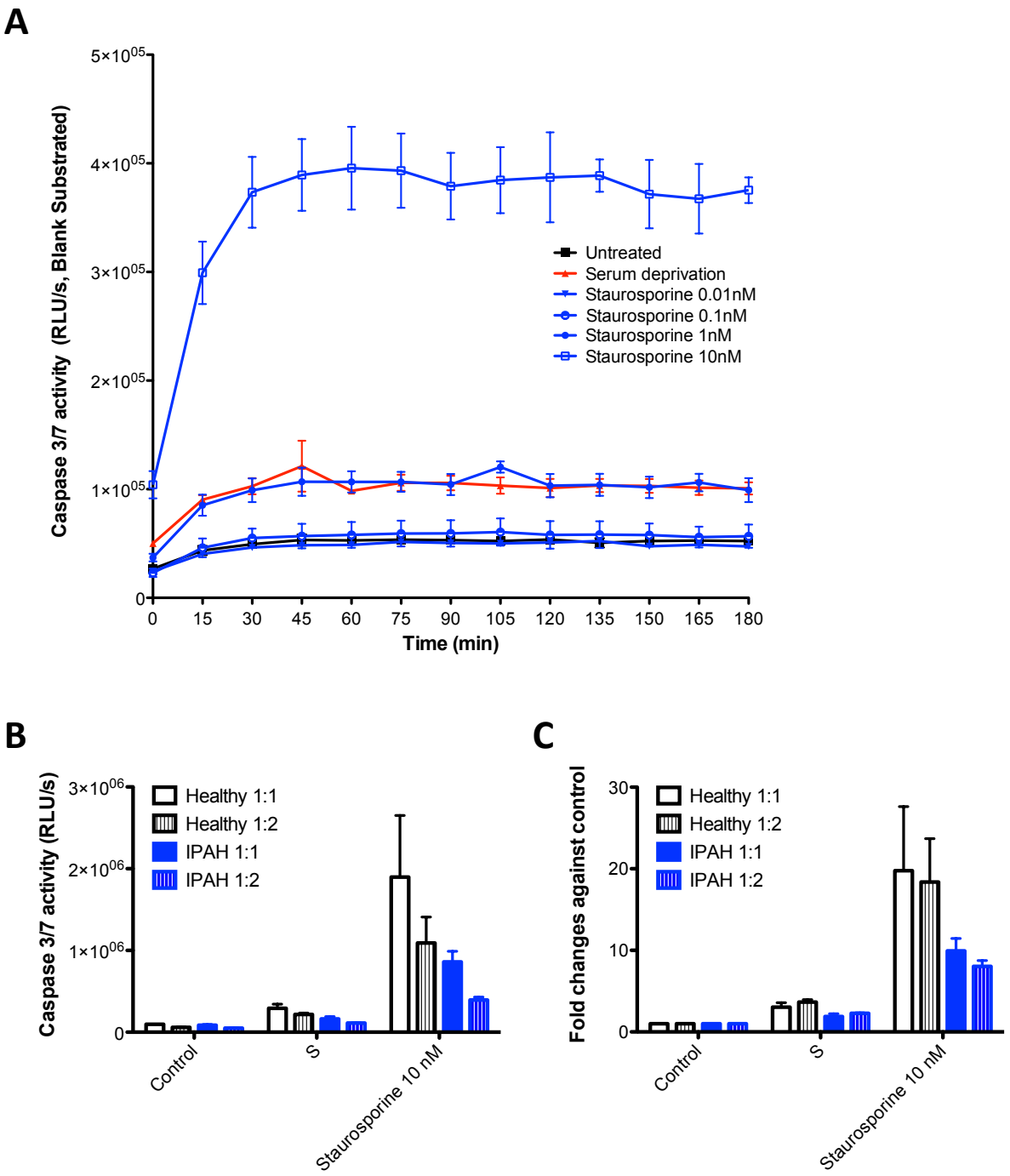


Figure 2.7. Modification and validation of Caspase-Glo 3/7 assay protocol. (A) Caspase-Glo 3/7 assay substrate was added in a ratio of 1:2 to ECFCs from a healthy volunteer up to 180 min. **(B)** Measurements taken after 60 min to compare the sensitivity of the assay with using substrate:medium ratios of 1:1 and 1:2. **(C)** Values normalised against control conditions, showing that the effects of staurosporine (10 nM) and serum deprivation are equally well detected by substrate:medium ratios of 1:2 and 1:1.

2.12.3 Matrigel tube formation assay

The ability of ECFCs to form capillary-like tubes, both in *vitro* and *in vivo,* is another critical characteristic that distinguishes ECFCs from early (haematopoietic) progenitor cells (Ingram et al., 2004; Timmermans et al., 2007; Sieveking et al., 2008). The angiogenic capacity of cultured cells was studied using the Matrigel tube formation assay. Matrigel is a solubilised basement membrane preparation that is rich in extracellular matrix proteins extracted from the Engelbreth-Holm-Swarm mouse sarcoma cells and is commonly used in assessing the *in vitro* angiogenic capacity of endothelial cells (Arnaoutova and Kleinman, 2010). Endothelial cell tube formation on Matrigel involves attachment, differentiation, migration and alignment of cells, but not proliferation (Kubota et al., 1988; Grove et al., 2002).

ECFCs were maintained either in complete EGM-2 medium or subjected to serum-free EBM-2 medium for 24 h before use. Growth factor reduced (GFR) Matrigel (BD Matrigel Basement Membrane Matrix Growth Factor Reduced, Cat no. 354230, BD Biosciecnes) was thawed on ice overnight, and 30 µl/well was added into cooled sterile transparent 96-well plate on ice with cooled sterile tip and incubated at 37°C for 30 min to solidify. Cells, at less than 70 - 80% confluence were detached with 0.05% Trypsin-EDTA, resuspended in EBM-2 (serum-deprived) and seeded at 7000 cells/well in triplicate onto Matrigel. Plates were then incubated in the humidified incubator (5% CO_2 at 37°C) for 16 h, prior to fixation in 4% formaldehyde (Sigma-Aldrich) in PBS and microscopic examination. In each well, two phase-contrast images, one from each side of the well, were captured. Total tube length in each image was determined using ImageJ software. Matrigel enriched with high protein concentration and standard growth factor (HC-Matrigel, BD Matrigel Matrix High Concentration, cat no. 354248, BD Bioscience) was also used with cells incubating with EBM-2 containing 1% FBS in order to test the consistency of the angiogenic capacity of ECFCs in different conditions. It is important to use sub-confluent ECFCs for seeding on Matrigel, as confluent cells failed to form tubular structures (Figure 2.8).

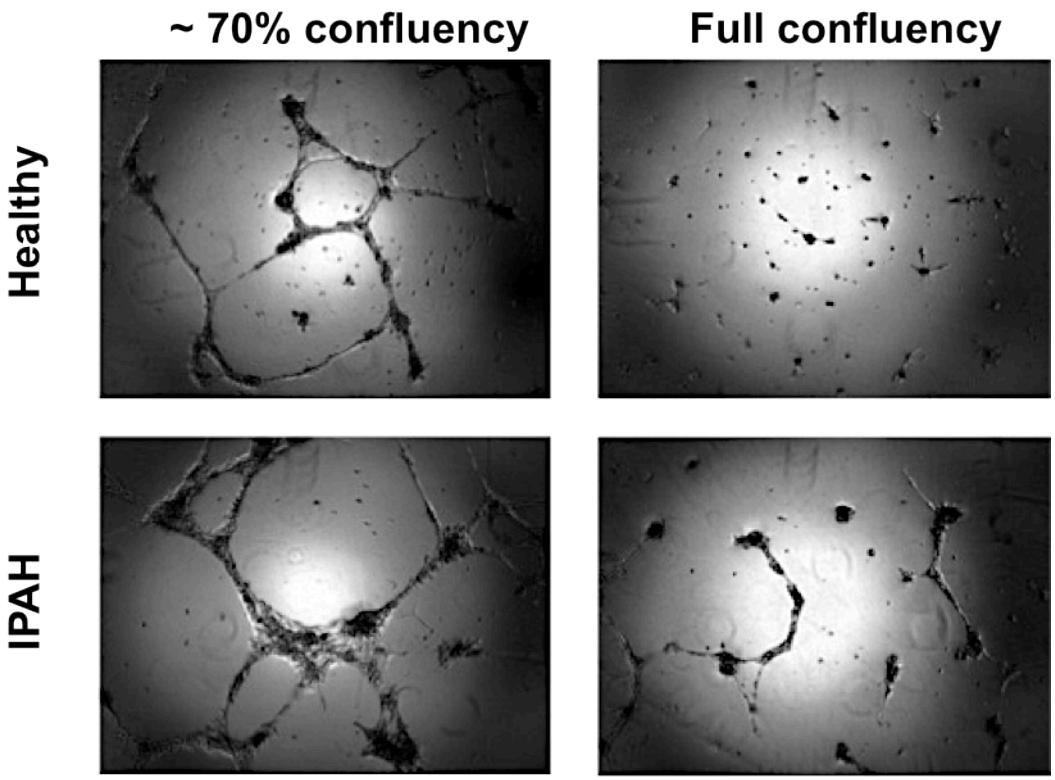


Figure 2.8. Effect of ECFC confluency on Matrigel tube formation. Examples of ECFCs from a healthy control and an IPAH patient that were cultured until ~70% or ~100% confluent before seeding on Matrigel for 16 h.

2.12.4 Wound healing assay

A so-called wound healing assay was used to assess the migratory properties of cultured endothelial cells (Liang et al., 2007). Sub-confluent ECFCs or HPAECs were seeded on to 1% gelatin-coated 24-wells plate at a density of 54,000/well (equivalent to 30,000/cm^2) in triplicate, and allowed to form a confluent monolayer, which typically took 2 days. A horizontal line was drawn across the centre on the bottom of each well as a marker, and a single vertical scratch was applied to each well using a sterile 200 µl pipette tip. Cells were then washed twice with warm sterile PBS and rocked gently to smoothen the edges of the wound, and 500 µl of EBM-2 containing 1% or 5% FBS, or EGM-2 containing 20% FBS, was added in each well. Images were captured at baseline (0 h) and at 6 h, 18 h and 24 h intervals thereafter at the same position just above or below the marked line. The area of the wound was measured using ImageJ software, and data presented as percentage of the area of the wound recovery from 0 h.

The wound healing assay represents restitution or chemokinesis (as occurs during the closure of a cell monolayer) and is distinct from chemotaxis (movement of cells along a chemical gradient), the other form of migration that is commonly measured in Boyden chamber experiments. The two forms of migration may also require different signalling pathways (Yamaoka et al., 2011; Costello et al., 2012). The advantage of the wound healing assay is that it is simple and economical to perform, although the scratches can be difficult to standardise and may damage the underlying extracellular matrix as well as cells at the edge of the scratch, possibly releasing unidentified factors into the media.

2.12.5 Measurement of intercellular gap junctional communication

The cells were labelled with acetomethoxy derivate of calcein green (calcein AM; Molecular Probe) and the dynamics of cell-to-cell communication was assessed by Fluorescence Recovery After Photobleaching (FRAP) (modified from (Nickel et al., 2006)). Confluent cells grown on black-walled 96-well optical bottom plates were incubated with calcein AM (0.5 µM, 30 min) and washed 3 times in sterile PBS. After the last wash, PBS was replaced by fresh culture medium and plates were placed on a heated stage of a confocal laser scanning microscope (Leica TCS SP5) in an atmosphere containing 5% CO_2. A small area of about 200 μm^2 of calcein-labelled cell monolayer was bleached at maximum laser power for 60 s. Recovery of fluorescence in the bleached area was monitored for 4 minutes at intervals of 10 s. An area of 388 μm^2 was recorded for pre-bleach and post-bleach scans at low laser power. Analysis was carried out using the Leica LAS-AF Lite 2.0 software. Fluorescence intensity in three selected regions of interest (ROIs) was expressed as a function of time. ROIs in the unbleached region served as control for an overall decrease in fluorescence attributable to successive scanning. The relative fluorescence recovery at time *t* was displayed as the percentage of pre-bleach levels (PR(*t*)):

$$PR(t) = ([(I_t - I_o) + (I_{pre} - I_{control})] / I_{pre}) * 100$$

Where I_{pre}, I_o, and I_t were the measured fluorescence intensities of the ROI before (pre-bleach), at the first post-bleach scan, and at the time *t* after the first scan, respectively. $I_{control}$ was measured in unbleached cells and the fluorescence recovery was corrected for the overall bleaching ($I_{pre} - I_{control}$) attributable to successive scanning.

2.12.6 In vitro assessment of endothelial cell permeability

Cells were plated in Transwell-Clear chambers (1-µm pore size, 12-mm diameter; Costar Corning, Costar, High Wycombe, United Kingdom) at cell density of 1 x 10^4 cells/well, and grown till confluence. FITC-dextran (MW 42,000, 1 g/L, Sigma) was added to the upper chamber of Transwell dishes, and samples were taken from the lower compartment after 1 h incubation, as previously described (Wojciak-Stothard et al., 2001; Wojciak-Stothard et al., 2005). The amount of FITC-dextran was determined with the GloMax Multi$^+$ plate reader (Promega), using an excitation wavelength of 490 nm and emission at 510 – 570 nm.

2.13 Manipulation of gene expression and activity in cultured cells

2.13.1 Small interference RNA (siRNA) transfection

siRNA transfection in HPAECs and ECFCs was performed according to the manufacturers guidelines for transfecting HUVECs. Cells were seeded in 6-well plates (200,000 cells per well) in 2 ml of growth medium without antibiotics per well, so cells should be about 85 – 95% confluent at the time of transfection. The following day, 375 pmol of siRNA duplexes (20 µM) (i.e. 18.75 µl) were diluted and mixed gently with 181.25 µl Opti-MEM reduced serum medium (cat no. 31985-062, Invitrogen) per transfection sample. siRNA specific for target genes or a scrambled sequence of similar GC content (catalogue No. 12935-200; Invitrogen) were used. In a separate RNase-free tube, 10 µl of Oligofectamine (cat no. 12252-011, Invitrogen) was mixed gently with 40 µl OptiMEM. To reduce well-to-well variability when transfecting multiple wells, all reagent volumes were proportionally scaled up in a RNase-free tube as mastermix. Both mixtures were incubated for 15 min at room temperature, and were then mixed together gently and incubated for a further 15 min period at room temperature to allow siRNA-Oligofectamine complexes to form. While complexes were forming, growth medium from the cultured cells were removed and washed once with 2 ml per well of pre-warmed Opti-MEM, and added 1 ml of Opti-MEM to each well. After the 15 min incubation, mixed the complexes gently and added 250 µl of the complexes dropwise to the cells in each well (total volume per well = 1.25 ml) and incubated in a 37°C in a humidified CO2 incubator for 5 h. 625 µl of EGM-2 (without the antibiotic/antimycotic) containing 3X the normal concentration of serum were added to each well without removing the transfection mixture. The cells were then incubated in the incubator for 24 h before changing for fresh complete culture medium. The efficiency of the

knockdown was determined 2 and 3 days after transfection by measuring protein and mRNA levels in cell extracts, using Western blotting and RT-PCR respectively.

2.13.2 Adenoviral transfection

The recombinant adenoviruses were constructed using the Ad-Easy-1 system, where the adenoviral construct is generated in bacteria BJ-5183 cells. These recombinant adenoviral vectors were linearized with PacI and used to infect 911 cells. All adenoviruses were amplified in HEK293 cells and subsequently purified on 2 sequential cesium chloride gradients and then passed through PD10 column (GE Healthcare) to reduce the salt concentration. To establish numbers of active viral particles, adenoviral titre was measured in plaque assay.

For manipulation of gene expression, cells were plated overnight and were washed twice with low serum (0.5%) EGM-2 before adding adenovirus with the recombinant constructs. After incubation for 4 h, cells were washed twice with culture medium and maintained in humidified incubator for 48 h before assays. The efficiency of the overexpression or knockdown was determined by measuring protein in cell extracts using Western blotting.

2.14 Additional reagents

Recombinant human BMP4 carrier free (cat no. 310-BP-010/CF; R & D Systems, Abingdon, Oxford, UK) reconstituted in sterile PBS; recombinant human BMP9 CF (cat no. 3209-BP/CF, R & D Systems) reconstituted in sterile PBS; recombinant human TGF-β1 CF (cat no. 240-B-002/CF, R & D Systems) reconstituted in filtered sterile 4 mM HCl containing 0.1% BSA; recombinant human ALK1-Fc chimera (cat no. 370-AL, R & D Systems) reconstituted in sterile PBS; SB 431542 hydrate (cat. no. S4317, Sigma-Aldrich), a selective ALK5 inhibitor, reconstituted in sterile DMSO; SD208 (cat. no. S7071, Sigma-Aldrich), a selective ALK5 inhibitor, reconstituted in sterile DMSO; SU1498 (cat no. 572888, Merck) a reversible VEGFR2 inhibitor, reconstituted in sterile DMSO; staurosporine (cat no. S4400, Sigma-Aldrich), an apoptosis inducer, was reconstituted in sterile DMSO; 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) (Sigma-Aldrich, Cat no. N4779) reconstituted in DMSO; ADMA was reconstituted in sterile water (cat no. 311203, Calbiochem, Millipore); L-257 was reconstituted in nuclease-free water (a kind gift from Dr James Leiper, MRC Clinical Sciences Centre, Imperial College London); Gap26 (VCYDKSFPISHVR) corresponding to residue 63-75 of the first extracellular loop of

Cx43 was custom synthesised (Peptide Protein Research Ltd, Hampshire, UK) and was reconstituted in nuclease-free water; rotigaptide was reconstituted in nuclease-free water (a kind gift from Dr Ninian Lang, University of Edinburgh).

During experiments with inhibitors for ALK5 (SB431542 or SD208), VEGFR2 (SU1498), CLIC (NPPB), DDAHI (L-257) or Cx43 (Gap26) were added 30 min before proceeding to assays with treatments with agonists. ALK1 binding sites of the agonists in the treatment medium and any additional cytokine treatments were blocked using recombinant human ALK1-Fc chimera for 30 min in prior to treating cells.

2.15 Data presentation and statistical analysis

Results are presented in scatter plots or bar charts where appropriate, and horizontal lines indicated means with standard error of the mean (SEM), while 'n' refers to the number of ECFCs samples per group.

All numerical data are tested for normal distribution using D'Agostino & Pearson omnibus normality test. Data were analysed by unpaired t-test (for normally distributed data) or Mann-Whitney test (for non-normally distributed data) for two sample groups. One-way ANOVA was applied for data of more than 2 sample groups, or one-way repeated measures ANOVA for data of the same group in multiple experimental conditions, with Bonferroni post-hoc analysis performed if $P < 0.05$. Two-way repeated measures ANOVA was applied for comparing data of multiple groups with multiple experimental conditions, with Bonferroni post-hoc analysis performed if $P < 0.05$. Statistical significance was regarded as $P < 0.05$ for all presented data. All graphs and calculations were performed with GraphPad Prism5 (GraphPad Software Inc, La Jolla, CA, USA).

Chapter 3:

Blood-derived endothelial cell forming colonies in IPAH

Chapter 3 - Blood-derived endothelial cell forming colonies in IPAH

3.1 Introduction

Circulating endothelial colony forming cells (ECFCs) are considered to be true EPCs, due to their ability to independently form tube-like networks *in vitro* and contribute structurally to angiogenesis *in vivo*, as well as exhibit high clonogenic and proliferative potential. The frequency of circulating ECFCs has also been considered a potential biomarker in a number of cardiovascular diseases (as summarised in Section 1.6.3), but relatively very little is known about ECFCs in PAH. In an early study by Asosingh and colleagues, no difference was observed in the number of ECFCs derived from IPAH patients and healthy volunteers (Asosingh et al., 2008). Conversely, the same research group has recently reported using a single cell clonogenic assay to identify ECFCs among PAECs cultured from pulmonary arteries in explanted lung tissues and suggested that those derived from IPAH patients were more proliferative than the cells obtained from healthy controls (Duong et al., 2011). Nevertheless, it is unclear whether the number of circulating ECFCs and/or the *in vitro* characteristics of their progeny differs in IPAH patients and correlates with disease progression.

Endothelial dysfunction is typically thought to play critical role in the pathogenesis of IPAH (Budhiraja et al., 2004), but studies of endothelial dysfunction at the cellular level have been limited by the difficulty in obtaining pulmonary ECs from patients. ECs may be examined in biopsy samples (Fadini and Avogaro, 2010), but open lung biopsy is invasive, associated with significant morbidity and mortality and rarely performed in PAH (Nicod and Moser, 1989). Another way to obtain pulmonary vascular ECs is through the isolation and *ex vivo* expansion of cells from the explanted lungs of patients undergoing pulmonary/cardio-pulmonary transplantation. However, these operations are increasingly uncommon in PAH and the cells obtained at end-stage disease may provide little insight into the mechanisms that initiate endothelial dysfunction and vascular remodelling in PAH. A non-invasive method of isolating ECs could therefore be immensely useful. In fact, the *ex vivo* expansion of blood-derived ECFCs has been used as a source of surrogate ECs in HPAH patients with *BMPR2* mutations (Toshner et al., 2009) as well as a variety of other diseases, including HHT (Fernandez et al., 2005), systemic sclerosis (Avouac et al., 2008), von Willebrand disease (Starke et al., 2011), macular degeneration (Thill et al., 2008), diabetes (Ingram et al., 2008; Tan et al., 2010) and coronary artery diseases (Wang et al., 2011).

I hypothesised that the frequency of circulating ECFCs might be higher in IPAH patients and correlate with the severity of the disease. The aims of this chapter are to:

1. Establish a working protocol to isolate and grow ECFCs from the peripheral blood of IPAH patients and healthy volunteers.

2. Investigate whether there is a relationship between the frequency of ECFCs and patient demographics or clinical parameters in IPAH patients.

3. Confirm the endothelial phenotype of blood-derived ECFCs and identify cell populations that can be expanded for functional/mechanistic investigations.

4. Identify potential phenotypic differences between ECFCs derived from IPAH patients and healthy volunteers.

3.2 Methods

See Chapter 2 for detailed materials and protocol.

25 healthy volunteers, 36 IPAH patients and 2 HPAH patients consented to provide blood samples (Table 3.1). The IPAH cohort included 6 patients who were more than 60 years of age, forming a distinct group of older patients. MNCs were isolated from ~50 ml of peripheral blood, counted using a haemocytometer and cultured in EGM-2 with 20% FBS for up to 35 days, as described in section 2.3. Colonies of ECFCs were identified as a well-circumscribed monolayer of cobblestone-appearing cells, and were recorded as they appeared, with the total number and frequency of colonies being determined for each subject.

The colonies were expanded (see section 2.3), with cells re-seeded at a density of 3000-5000 cells/cm^2) on each passage, and the population doubling time (PDT) calculated. ECFC populations were carefully phenotyped, by immunostaining and flow cytometry (see sections 2.7.1-2.7.2) and were subjected to serial passaging (i.e. continual growth). Only cells that grew beyond passage 8 and exhibited stable PDTs were used for further experiments, these being conducted with cells between passages 3-8. The ECFC phenotype was also demonstrated by their ability to grow under limiting dilutions (equivalent to one cell per well in a 96-wells plate), this being considered a defining feature that distinguishes ECFCs from early outgrowth EPCs or haematopoietic cells (Yoder et al., 2007).

		Control (n=25)	IPAH (n=30)	IPAH (n=6)	HPAH (n = 2)
Gender (male/female)		7/18	12/18	4/2	0/2
Age (years)		27.0 (33.0 to 57.0)	38.5 (22.0 to 56.0)	69.5 (62.0 to 82.0)	36 (35.0 to 37.0)
Time from diagnosis (months)		-	48.0 (0.5 to 122.0)	21 (0.1 to 61.0)	36.6 (0.2 to 73.0)
mPAP* (mm Hg)		-	56.0 (39.0 to 79.0)	49.5 (36.0 to 68.0)	79.0 (79.0)
PVR* (dynes.s.cm^5)		-	865.0 (364.0 to 1730.0)	737.5 (270.0 to 905.0)	2510 (2510)
CI* (l/min/m^2)		-	2.22 (1.25 to 4.10)	2.54 (1.96 to 3.86)	1.15 (1.15)
6MWD$^{\Psi}$ (m)		-	396.0 (0 to 570.0)	274.5 (120.0 to 420.0)	N/A
WHO class$^{\Psi}$	I	-	3	0	0
	II	-	10	0	1
	III	-	13	6	0
	IV	-	4	0	1
Warfarin		-	26	5	0
PAH therapies					
Naïve		-	1	1	1
Calcium channel blocker		-	3	0	0
ERA		-	16	4	1
PDE5 inhibitor		-	25	2	1
Prostanoid		-	10	0	0
Other therapies					
Statin		-	4	1	0

Table 3.1. Demographics and clinical information. Data presented as median (range). 6MWD, 6 minute walk distance; CI, cardiac index; ERA, endothelin receptor antagonist; mPAP, mean pulmonary arterial pressure; PDE5, phosphodiesterase type 5; PVR, pulmonary vascular resistance. *, obtained at baseline cardiac catheter; $^{\Psi}$, concurrent with blood sample collection.

3.3 Results

3.3.1 Establishing ECFC culture

ECFCs grew as well-circumscribed colonies of cobblestone-shaped cells (Figure 3.1A & B) and this typical endothelial-like morphology was maintained during serial passages (Figure 3.1C & D), until they became senescent (Figure 3.1E). Despite the use of cloning cylinders to isolate and pass individual colonies, a distinct mesenchymal-like phenotype occasionally (<4% of colonies) predominated as the cultures were expanded (Figure 3.1F). Others have noted a similar phenomenon (Mead et al., 2008). These elongated, fibroblast-like cells were highly proliferative (PDTs from two samples at P2 are 0.71 and 1.06 days).

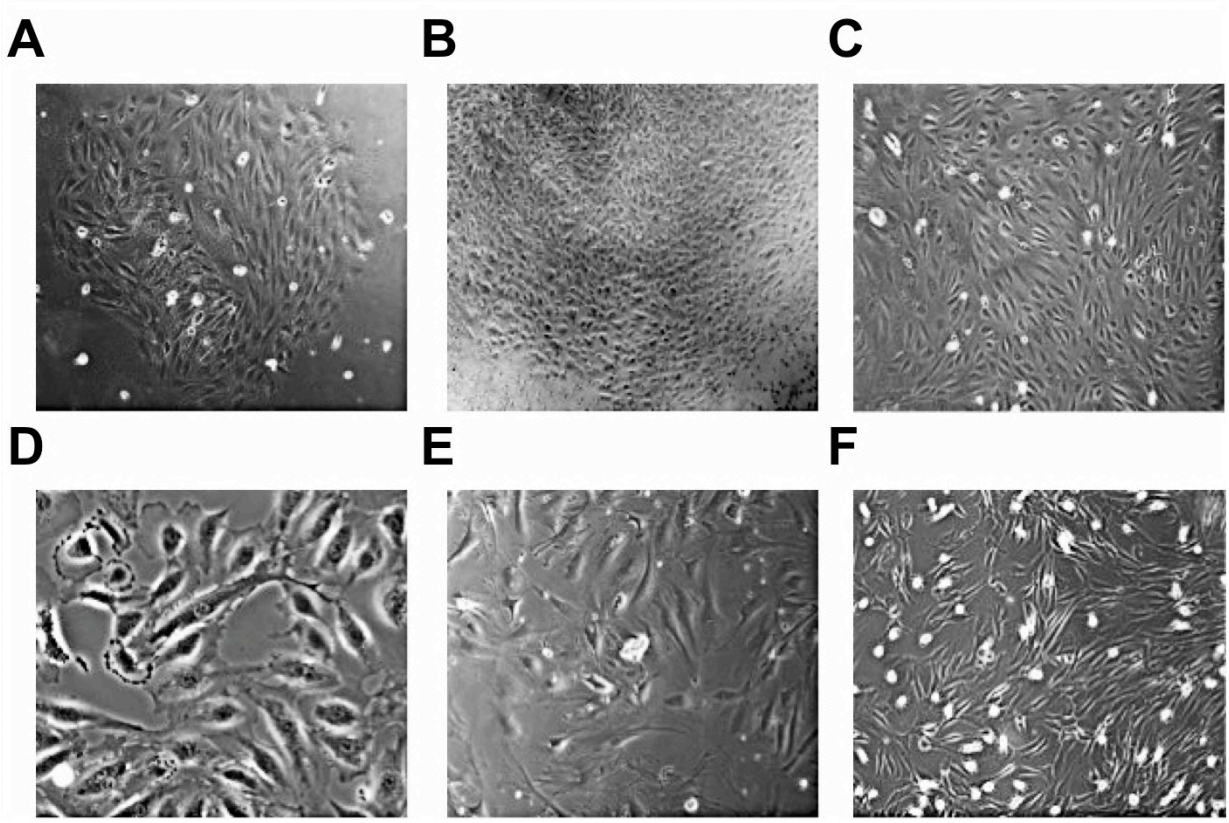


Figure 3.1. Morphology of ECFCs. Representative phase contrast photomicrographs of ECFCs from healthy volunteers (**A**, **D** & **E**) and IPAH patients (**B** & **C**). **(A)** A well-circumscribed colony of cobblestone-shaped cell at day 13, 10X magnification. **(B)** An ECFC colony at day 15, 4X magnification. **(C)** A confluent ECFC culture at passage 1, 10X magnification. **(D)** Subconfluent ECFCs at passage 4, 20X magnification. **(E)** ECFCs showing a larger and irregularly shape as they approach senescence at passage 5, 10X magnification. **(F)** A distinct population of fibroblast-like cells occasionally predominated following the expansion of a colony, 10X magnification.

Figure 3.2 outlines the ECFCs samples that were proceed to different stages of culture expansions, which were used subsequently in flow cytometric analysis, PDT determination, and functional studies. Demographics of the healthy volunteers and IPAH patients are detailed in Appendix 1 and 2.

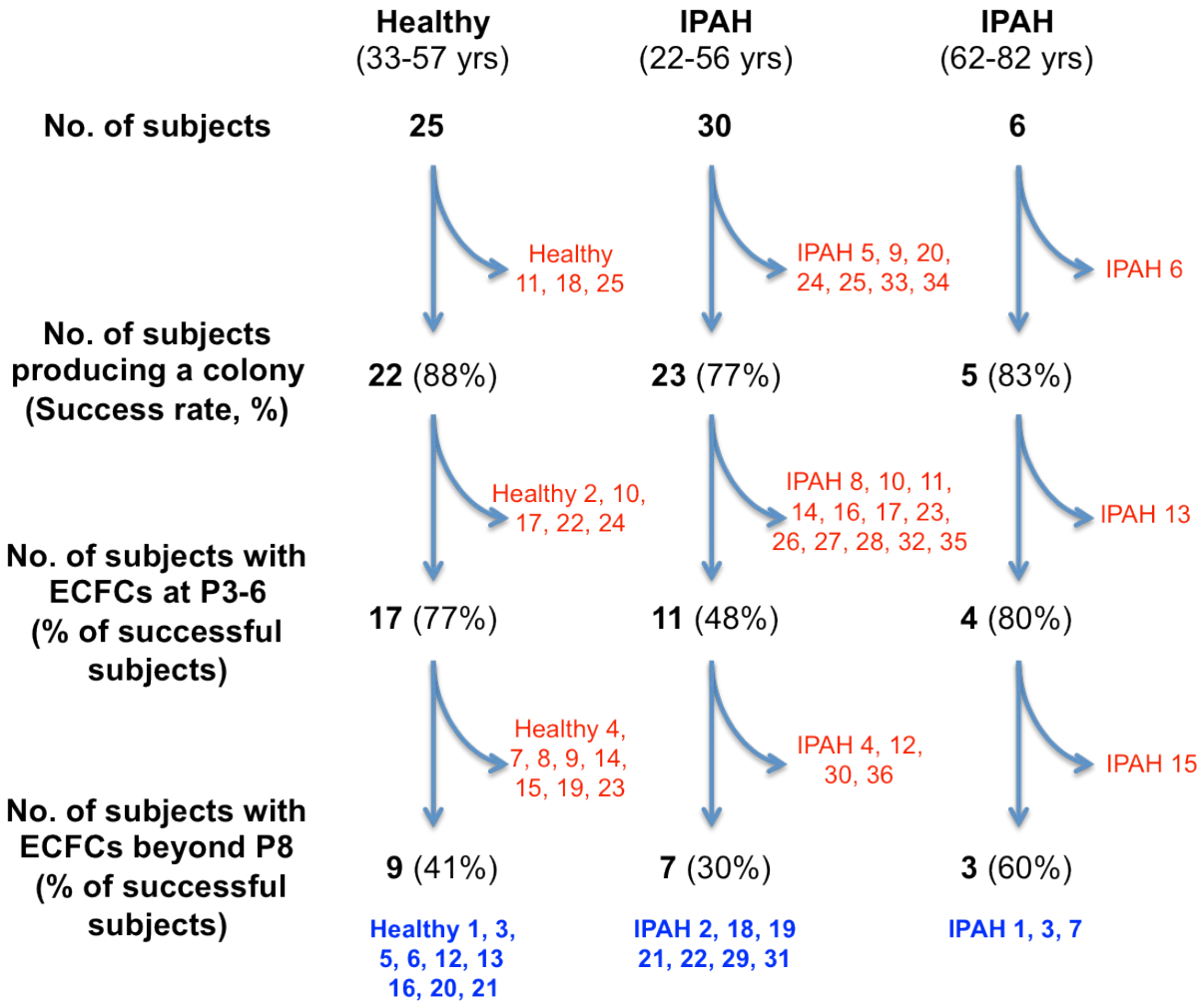


Figure 3.2. Overview of success in establishing ECFC colonies and subsequent expansion in culture. ECFCs at P3- P6 were used for flow cytometry and determination of population doubling times. ECFCs that grew beyond P8 (subject codes in blue) were used for functional studies. Subject codes in red indicate those that did not produce a colony or did not reach the next stage of colony expansion.

I also investigated an alternative method for deriving ECFCs from peripheral blood, which uses 10% pooled human platelet lysate instead of fetal bovine serum (Reinisch et al., 2009). This was repeated using blood from six healthy volunteers (Healthy 1, 2, 3, 4, 9, 10), all of whom produced ECFCs following the established protocol, and in two cases (Healthy 9 and 10) the two methods were performed concurrently. However, despite closely following the method of Reinisch and colleagues and obtaining pooled human platelet lysate directly from this research group, no colonies were observed after 35 days of culture. The reason for this was not apparent, but one confounding factor may have been the clotting potential of the platelet lysate and need for repeated filtering in order to remove aggregates. In addition, very few adherent cells could be observed on the culture flasks after 10 days of culture. It is also noteworthy that this alternative method has not been adopted by other groups to date.

3.3.2 Differences in colony formation

An average of 2-3 colonies were obtained from 50 ml of blood, with a frequency of $0.19\pm0.03/10^7$ MNCs (median 0.17; range 0 to 0.57) and $0.25\pm0.05/10^7$ MNCs (median 0.19; range 0 to 1.16) from healthy controls and IPAH patients respectively. Colonies of ECFCs were successfully grown from 88% (22/25) of healthy volunteers and 77% (23/30) of patients with IPAH (Figures 3.3A-B), the two groups having a similar gender and age distribution (Table 3.1). Colonies were successfully derived from 2 HPAH patients, but these were not analysed further due to the small sample size (Table 3.1). Colonies were also successfully derived from 83% (5/6) of the more elderly IPAH patients (Figures 3.3A-B). No difference was apparent in the total number or frequency of colonies derived from control subjects and patients with IPAH (Figure 3.3A & B). Similarly, no significant differences were observed when only the individuals that provided colonies were considered, although the number and frequency of colonies tended to be greater for IPAH patients versus controls (Figures 3.3C-D). Blood samples from the elderly IPAH patients provided a similar frequency of colonies when compared with their younger counterparts (Figure 3.3C-D). The time when ECFC colonies first appeared was similar in all three groups of subjects (Figure 3.3E).

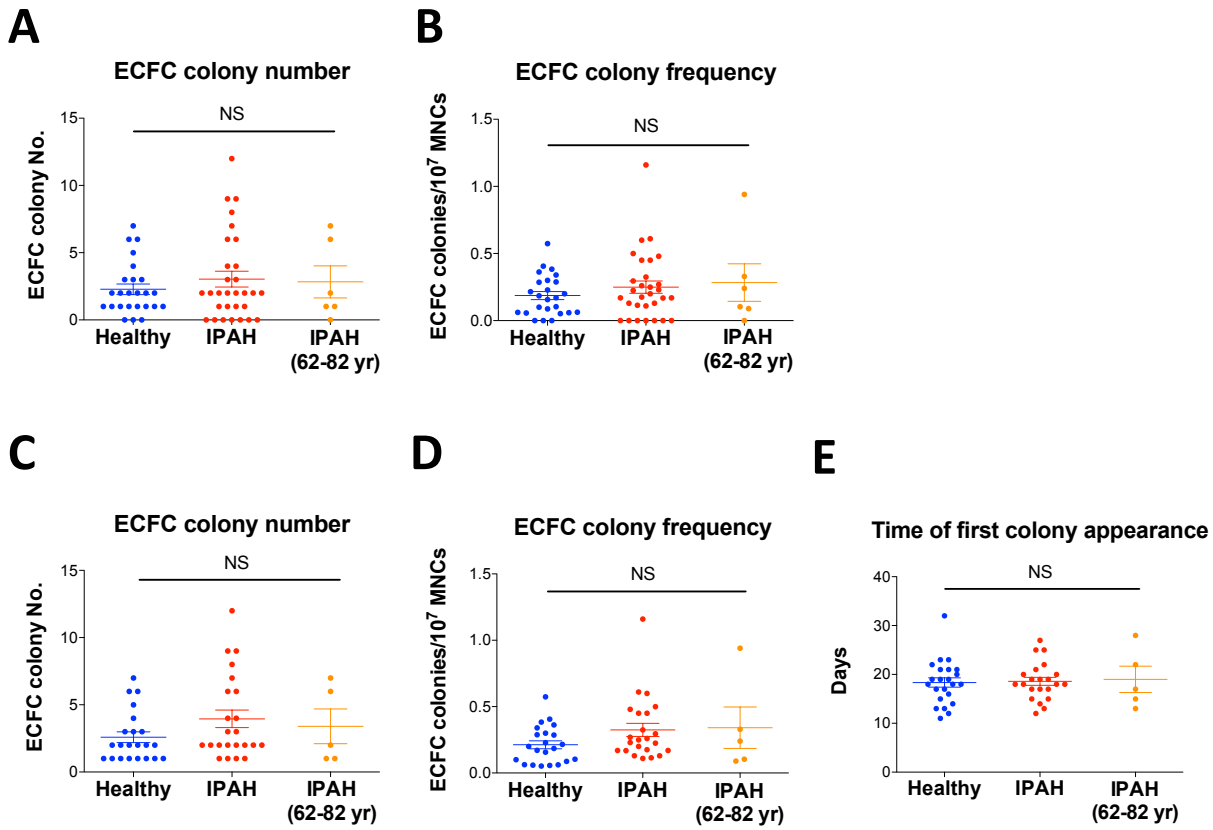


Figure 3.3. ECFC colonies derived from peripheral venous blood. Number **(A)** and frequency of colonies **(B)** obtained from all healthy volunteers (n=25), gender- and age-matched IPAH patients (n=30) and elderly (62-82 yr) IPAH patients (n=6). The number **(C)** and frequency of colonies **(D)** in subjects that gave rise to ECFCs. **(E)** Time when the first colony was observed, following the initial seeding of mononuclear cells. Data are presented as mean±SEM. Statistics shown are from one-way ANOVA.

The total number and frequency of colonies derived from healthy volunteers displayed an age-dependent decline, with Pearson's correlation coefficient (r) of -0.52 (P = 0.007, 95% CI -0.76 to -0.16) and -0.5 (P = 0.01, 95% CI -0.75 to -0.12) for colony number and frequency respectively (Figure 3.4A & C). In contrast, the number and frequency of colonies derived from IPAH patients tended to rise with increasing age and did not decline in the more elderly patients (Figure 3.4B & D). Using multivariate ANOVA, there was a significant difference between the age-dependent correlations for ECFC colony number (P = 0.02) and frequency (P = 0.03) between the two groups. Following continuous culture of ECFCs, it was also apparent that the cells that grew beyond passage 8 were generally from a broad age-range of IPAH patients (mean 46 yr, range 20-68 yr) and a younger cohort (mean 27 yr, range 20-35 yr) of healthy volunteers (Figure 3.4E). No colonies were obtained from healthy volunteers >50 yr of age whereas they were regularly derived from older IPAH patients (>60 yr of age).

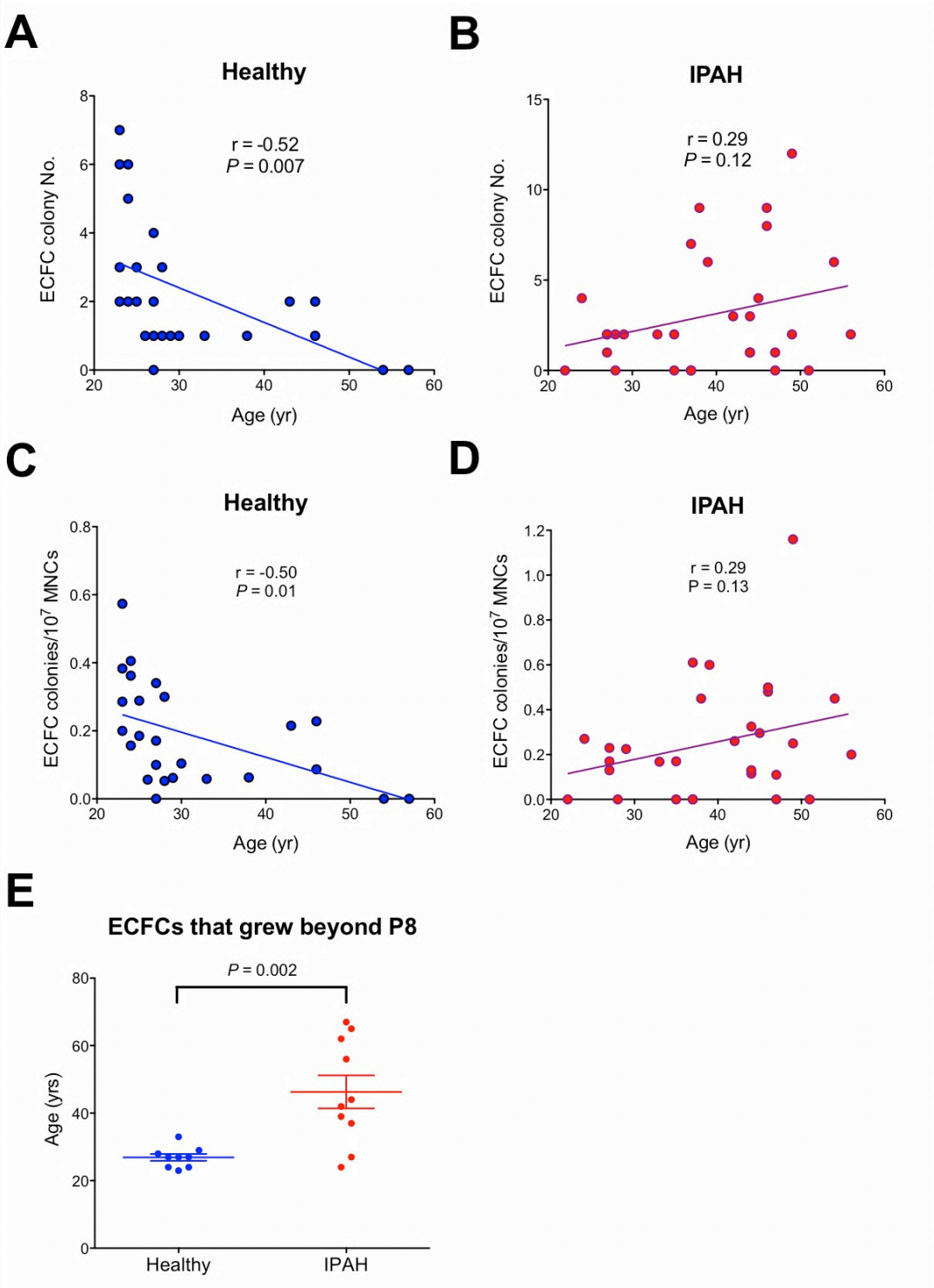


Figure 3.4. Age-related differences in ECFC colonies derived from healthy volunteers and patients with IPAH. ECFC colony number (**A-B**) and frequency (**C-D**) versus the age of 25 healthy volunteers (**A & C**) and 30 patients with IPAH (**B & D**). Statistics shown are from Pearson's correlation test. **(E)** The age distribution of 9 healthy volunteers and 10 IPAH patients, producing ECFCs that grew beyond passage 8. Data are presented as mean±SEM of ECFCs. N = 9 – 10. Statistic is from t-test.

3.3.3 ECFC colony formation and the functional capacity of IPAH patients

Assessment of ECFC colony formation in the IPAH patient cohort indicated that the frequency of colonies was not influenced by the patients' gender, duration of disease or treatment with therapies approved for PAH (Figure 3.5A-F). There was also no significant association with baseline haemodynamic parameters, although the frequency of colonies tended to be greater in patients with higher baseline PVR and lower CI values (Figure 3.6A-D). The number (P = 0.02) and frequency (P = 0.03) of ECFC colonies was however significantly greater in MNC cultures from IPAH patients in WHO functional classes III & IV versus I & II (Figure 3.6E & F). Patients in poorer functional classes III & IV, at the time of blood collection, also exhibited significantly lower 6MWDs (P = 0.0005) when compared with those in functional classes I & II (Figure 3.6G).

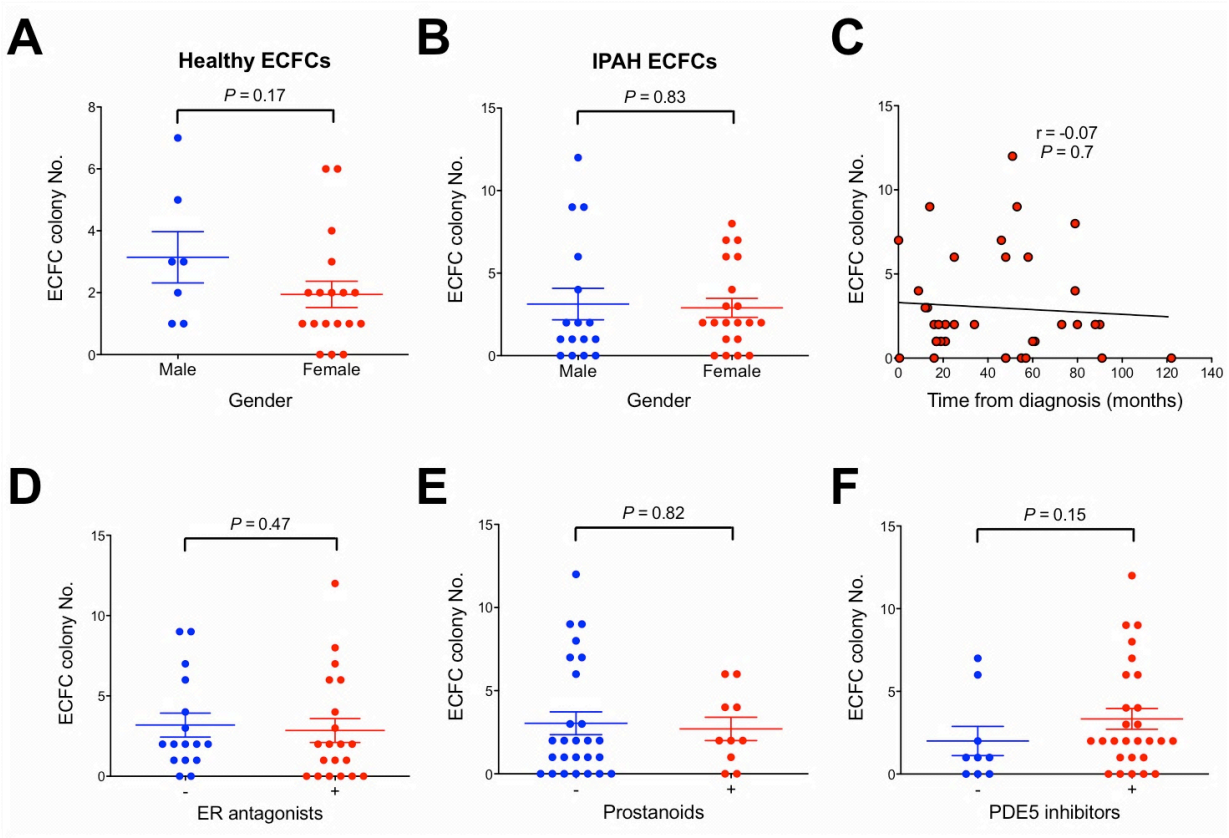


Figure 3.5. ECFC colony formation from patients with IPAH. Colony number according to gender of healthy volunteer (n = 25) **(A)** and IPAH patients (n = 36) **(B)**. Relationship of colony number with the disease duration **(C)**, and treatment with either endothelin receptor (ER) antagonists **(D)**, prostanoids **(E)** or phosphodiesterase type 5 (PDE5) inhibitors **(F)** within IPAH are also compared. Data are presented as mean±SEM. All statistics are from t-tests, except for Pearson's correlation test in **(C)**.

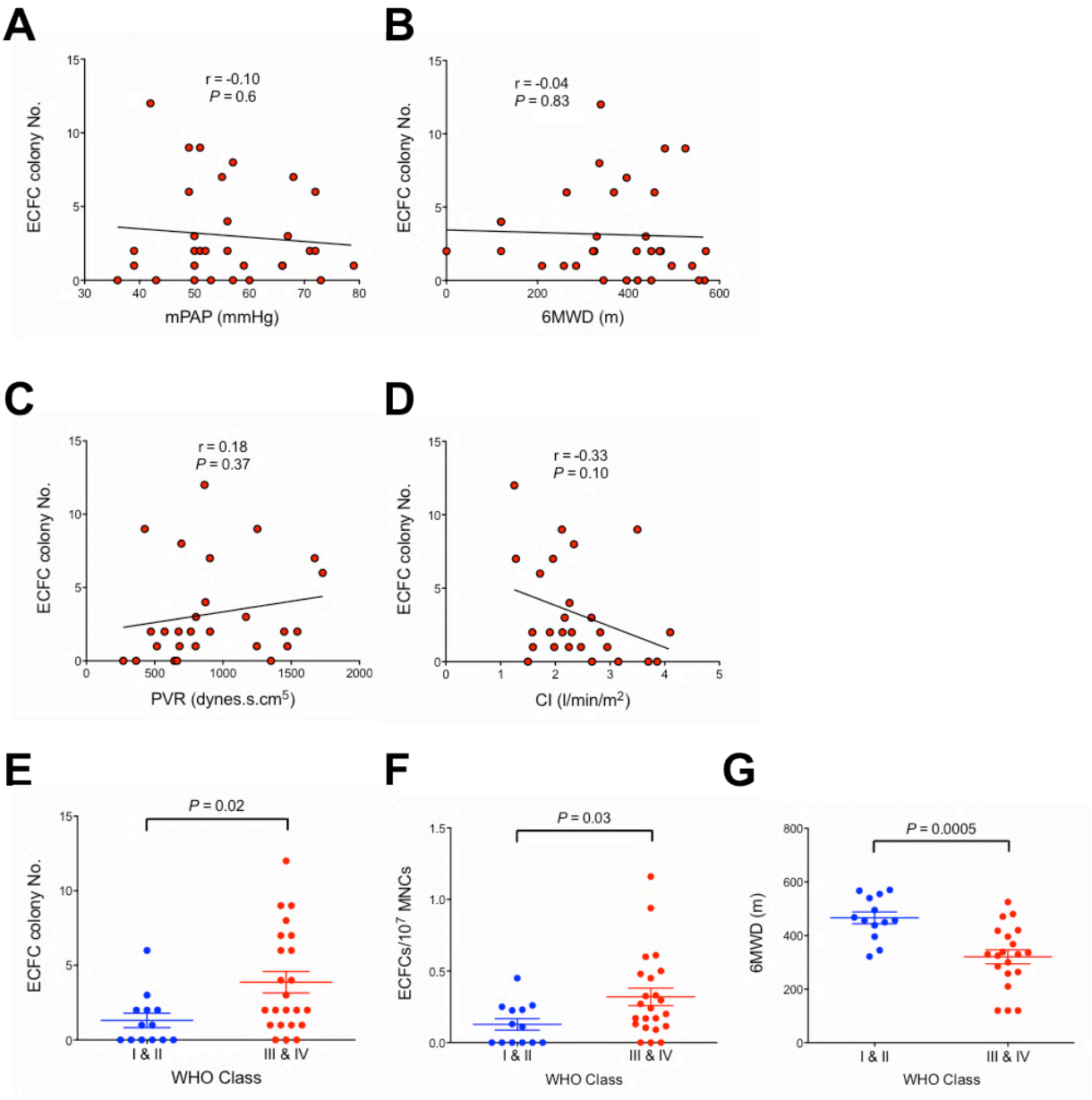


Figure 3.6. Relationship of ECFC colony formation and IPAH patient characteristics. ECFC colony number according to mean pulmonary arterial pressure (mPAP) **(A)**, 6 minute walk distance (6MWD) **(B)**, pulmonary vascular resistance (PVR) **(C)** and cardiac index (CI) **(D)** of IPAH patients (n = 36). ECFC colony number **(E)** and frequency **(F)** from IPAH patients in WHO functional classes I & II (n = 13) versus III & IV (n = 23). 6MWD concurrent with WHO functional class **(G)**. Data in **(E – G)** are presented as mean±SEM. Statistics shown are Pearson's correlation test **(A – D)** and t-tests **(E – G)**. Data for 6MWD were obtained in the same day as the peripheral blood sample collection, while the haemodynamic data used were from right-to-left heart catheterisation when the disease was first diagnosed.

3.3.4 Characterisation and flow cytometric analysis of ECFCs

Phenotyping was generally conducted using ECFCs at passages 2 - 6. In addition to their morphology, the endothelial-like phenotype was verified by positive immunostaining for endothelial surface proteins CD31 and CD144, the presence of von Willebrand factor (vWF) in peri-nuclear Weibal-Palade bodies and the ability of ECFCs to bind UEA-1 lectin (Figure 3.7A - D). Some immunostaining for the progenitor marker CD34 was also observed (Figure 3.7E). There were no apparent differences in the morphology or staining of ECFCs derived from healthy volunteers (healthy-ECFCs) and IPAH patients (IPAH-ECFCs) and no immunostaining was observed when primary antisera were omitted or cells were incubated with the microvascular ECs-specific FITC-conjugated *Griffonia simplicifolia* lectin (Figure 3.7F - H).

ECFCs were also characterised by flow cytometry, with human pulmonary artery endothelial cells (HPAECs) and human umbilical vein endothelia cells (HUVECs) being used as positive controls. All three cell types were positive for the endothelial cells markers CD31 and VEGFR2, but negative (<1% positive) for the pan-leukocyte marker CD45 and immature cell marker CD133 (Figure 3.8 & 3.9). A minority of ECFCs (14-20%) were considered to be CD34-positive and a variable proportion (~17%), as well as some HPAECs and HUVECs, appeared to be positive for the monocytic marker CD14. More than half of ECFCs, HPAECs and HUVECs were c-kit positive and ~25% exhibited CXCR4 expression (Figure 3.8 & 3.9). Overall, the ECFCs profile was very similar to that exhibited by mature endothelial cells (HUVECs and HPAECs) in culture. The phenotype was however markedly different from that of the mesenchymal-like cells, which occasionally predominated when ECFC cultures were expanded. More than 50% of these cells expressed CD14 and CD45, acting as a convenient positive control for both proteins, whereas the proportion displaying either CD31 or VEGFR2 was reduced compared with ECFCs (Figure 3.9B).

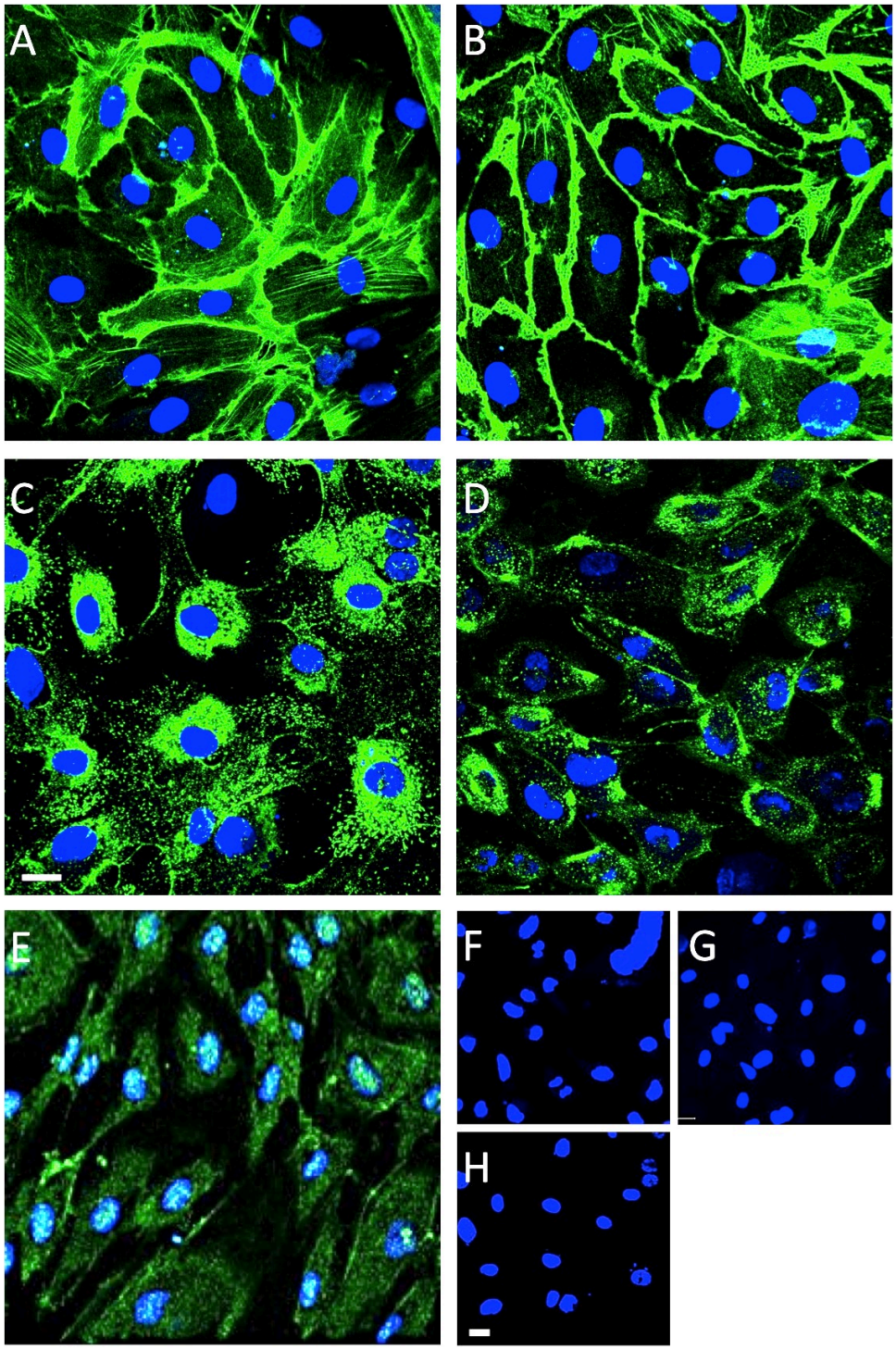


Figure 3.7. Immunostaining and lectin binding of ECFCs. Representative images of ECFCs, derived from the peripheral blood of IPAH patients, showing fluorescence staining for the endothelial markers CD31 **(A)**, CD144 **(B)**, von Willebrand factor **(C)** and UEA-1 lectin binding **(D)**, and immunostaining for the progenitor cell surface marker CD34 **(E)**. No staining observed when primary antibodies were omitted and cells incubated with either FITC-conjugated anti-mouse **(F)** or Alexa488-conjugated anti-rabbit secondary antibodies **(G)**, or FITC-conjugated *Griffonia simplicifolia* lectin **(H)**. Bar = 20 µm.

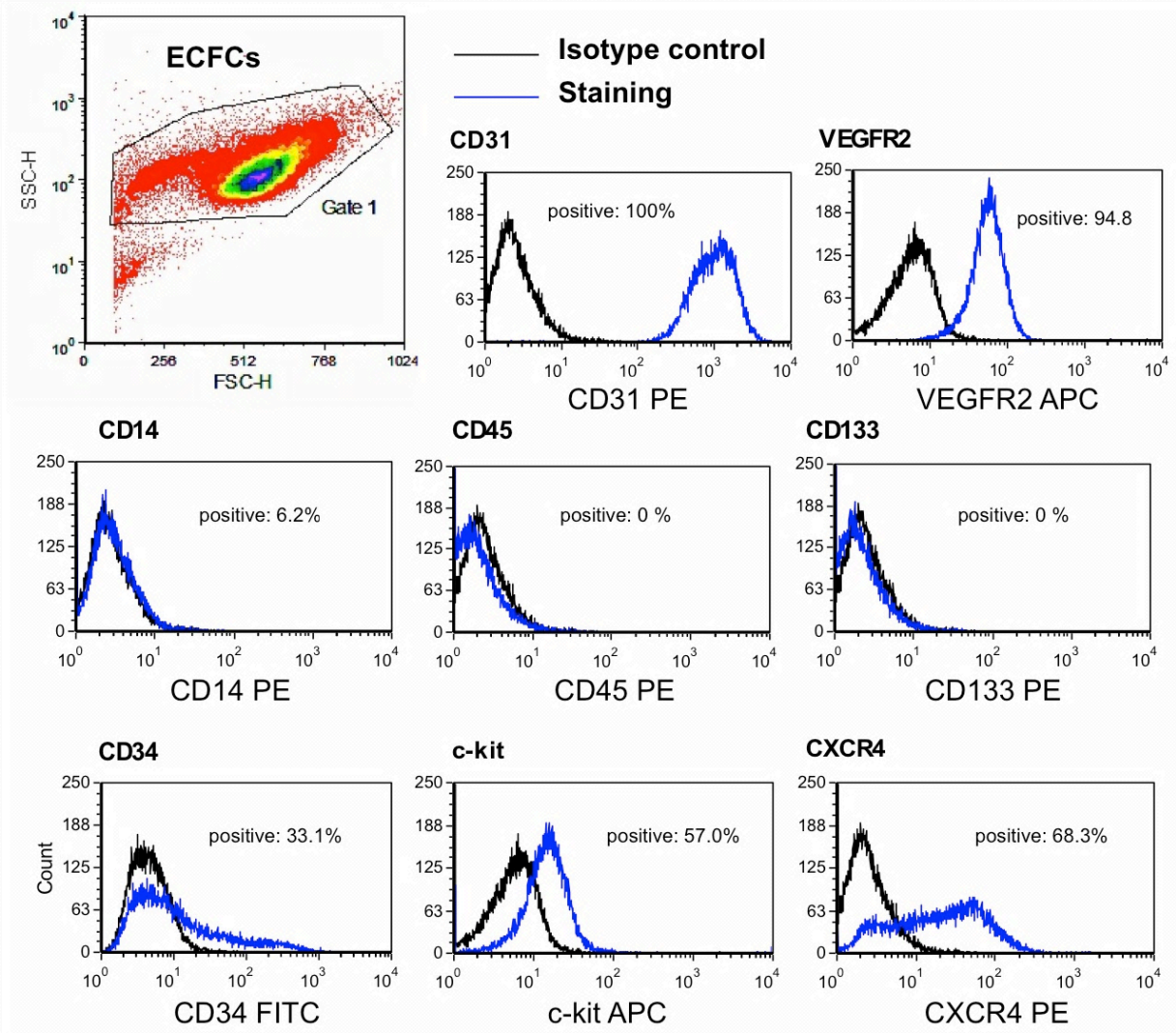


Figure 3.8. Flow cytometric analysis of ECFCs. Representative data showing flow cytometric analysis of ECFCs from an IPAH patient at passage 4. Cells were selected using a standard gating for all ECFC populations, following forward scatter/side scatter analysis, and the proportion of stained cells (blue line) determined after subtraction of the isotype control value (black line).

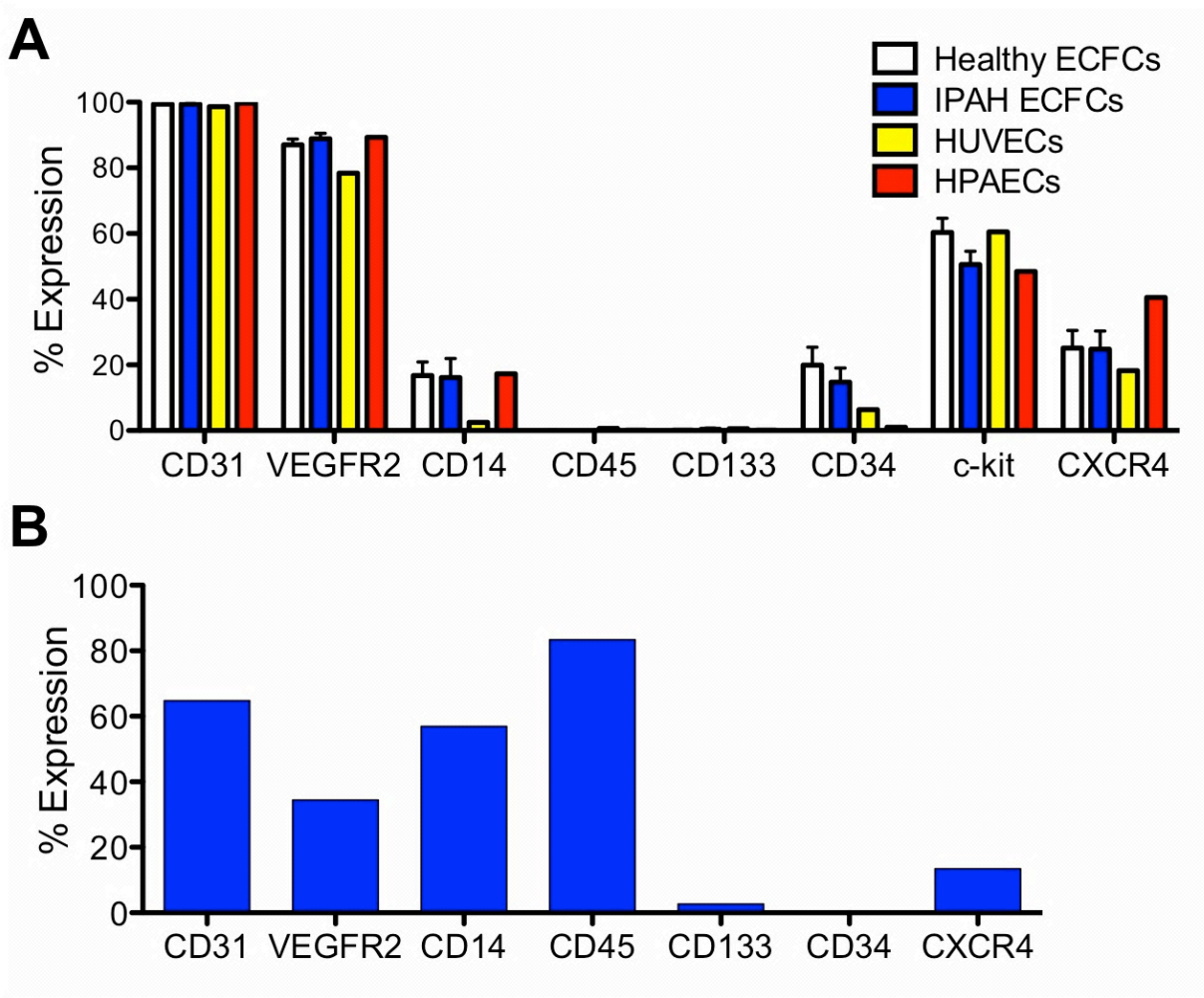


Figure 3.9. Flow cytometric analysis of ECFCs from IPAH patients and healthy volunteers. (A) Proportion of cells (P3 – P6) in separate ECFC populations, derived from healthy volunteers (n=10-16) and IPAH patients (n=13), showing expression of endothelial (CD31 and VEGFR2), hematopoietic/monocytic (CD14, CD45 and CD133) and progenitor markers (CD34, c-kit and CXCR4). Human pulmonary artery endothelial cells (HPAECs) and human umbilical vein endothelia cells (HUVECs) were included (n=2) as mature endothelial cell controls. Data are presented as mean±SEM of each analysed ECFC sample. **(B)** Representative data showing distinct phenotyping profile of a mesenchymal-like cell population (P3), displaying expression of monocytic/hematopoietic (CD14 and CD45) and endothelial markers (CD31 and VEGFR2).

Some variability was observed between different ECFC cultures in the expression of VEGFR2, CD14, CD34 and c-kit, but this did not correlate with the number/frequency of the primary colonies (Figure 3.10A). On the other hand, the number/frequency of colonies showed a positive association with the proportion of $CXCR4^+$ ECFCs (Figure 3.10A). Further analysis of this relationship indicated that $CXCR4^+$ expression correlated with number of ECFC colonies derived from healthy volunteers ($r = 0.56$, $P = 0.03$, 95% CI 0.06 to 0.83) but not IPAH patients, although the $CXCR4^+$ expression did not correlate with the age of the subjects (Figure 3.10B).

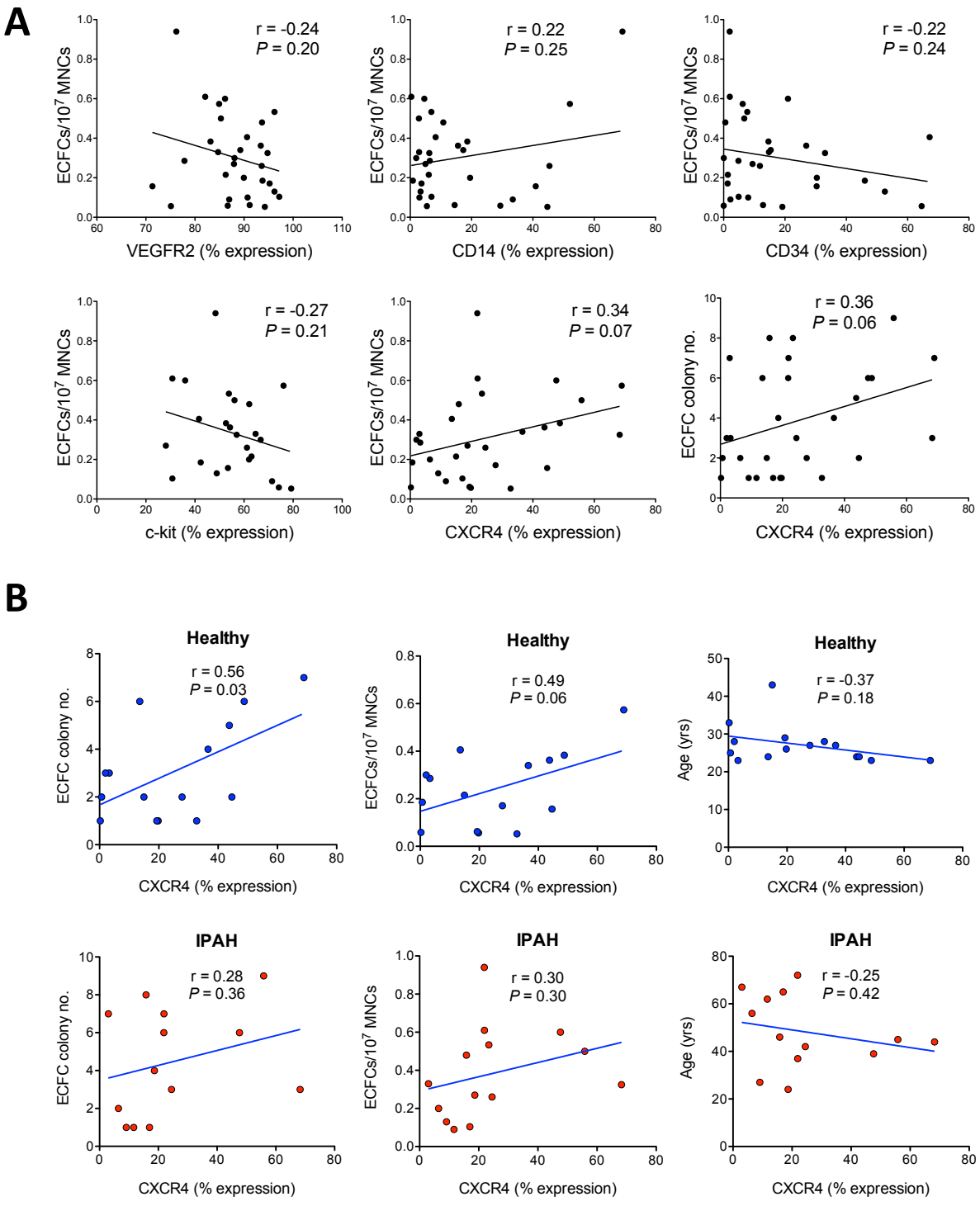


Figure 3.10. Relationship between cell surface markers and ECFC frequency and number. **(A)** Separate ECFCs from both control and IPAH subjects (n = 23-29; P3-6) were assessed by flow cytometry. Graphs show the association between the number/frequency of ECFC colonies and the proportion of ECFCs exhibiting endothelial (VEGFR2), monocyte (CD14), progenitor (CD34 and c-kit) and chemotactic (CXCR4) markers. **(B)** Positive correlation between number/frequency of colonies and subsequent CXCR4 expression in ECFCs from healthy volunteers (n = 15) and IPAH patients (n = 13). Statistics are from Pearson's correlation test.

Assessment of surface marker expression over serial passages suggested that CD31 expression was stable, whereas the proportion of cells identified as VEGFR2-positive tended to decline beyond passages 4-6 (Table 3.2). Some variability was also observed in the proportion of cells displaying CD14, CD34 and CXCR4 labelling between individual ECFC isolates and cultures at different passages. In contrast, the majority (>99%) of cells, studied between passages 2-7, lacked both CD45 and CD133 proteins (Table 3.2).

		Proportion of ECFCs exhibiting markers							
Sample	**Passage**	**Endothelial**		**Hematopoietic/Monocytic**			**Progenitor**		
		CD31	**VEGFR2**	**CD14**	**CD45**	**CD133**	**CD34**	**c-kit**	**CXCR4**
Healthy	2	99.6	95.7	22.6	0.1	0.3	0.9	-	18.9
	3	99.8	96.1	34.8	0.1	0.2	1.01	-	19.9
	4	99.9	95.9	6.8	0.5	0.1	2.8	-	25.1
	5	99.6	94.9	31.7	0.4	3.5	28.6	-	72.0
	6	96.9	76.5	12.4	0.0	0.3	0.0	-	83.2
Healthy	2	99.3	89.3	14.0	0.0	0.0	0.8	-	36.8
	3	99.7	91.9	4.0	0.2	0.9	0.1	-	21.3
	4	99.2	84.4	36.5	0.0	0.2	0.2	-	49.4
Healthy	2	98.6	90.7	18.6	0.9	0.1	39.5	-	14.6
	3	99.3	83.9	5.6	0.4	0.1	38.6	-	45.6
IPAH	4	99.9	92.1	45.4	0.0	15.9	6.2	69.4	24.2
	5	99.4	94.4	23.4	0.0	0.1	1.9	73.9	4.0
	6	99.7	87.5	36.1	0.2	0.3	0.2	69.0	10.9
	7	98.4	73.9	29.0	0.1	0.1	0.5	73.6	7.5

Table 3.2. Flow cytometric analysis of separate ECFC populations (derived from 3 healthy volunteers and 1 IPAH patient) over sequential passages.

3.3.5 Proliferative capacity of ECFCs from IPAH patients and healthy volunteers

Colonies were serially expanded in T25, T75 and T175 flasks, producing more than 10^7 cells within the first four passages. Variation was observed in the proliferative potential of ECFC populations, with some reaching senescence at an early stage of expansion whereas others continued to grow beyond 14 to 17 passages (~115 days) in culture (Table 3.3; Figure 3.11A). Cells that were frozen down during their early passages all recovered from cryopreservation and exhibited a similar proliferative capacity to that observed before freezing (Figure 3.11B).

After serially propagating each ECFC population for at least 9 passages, distinct cultures were identified from 9 healthy volunteers and 10 IPAH patients that grew beyond passage 8 and exhibited stable PDTs (Table 3.3). These cell populations represented 9/22 (41%) of the healthy volunteers and 10/28 (36%) of the IPAH patients that produced at least one colony. The PDT tended to increase between the 2^{nd}/3^{rd} and 6^{th} passage and generally increased markedly (PDT ≥3-4 days) prior to senescence (Table 3.3). Other than at the 2^{nd} passage, no significant difference was observed in the PDT of ECFCs from healthy volunteers and IPAH patients (Table 3.3). Similarly, little difference was apparent in the PDTs of those ECFCs that grew beyond the 8^{th} passage (Figure 3.12).

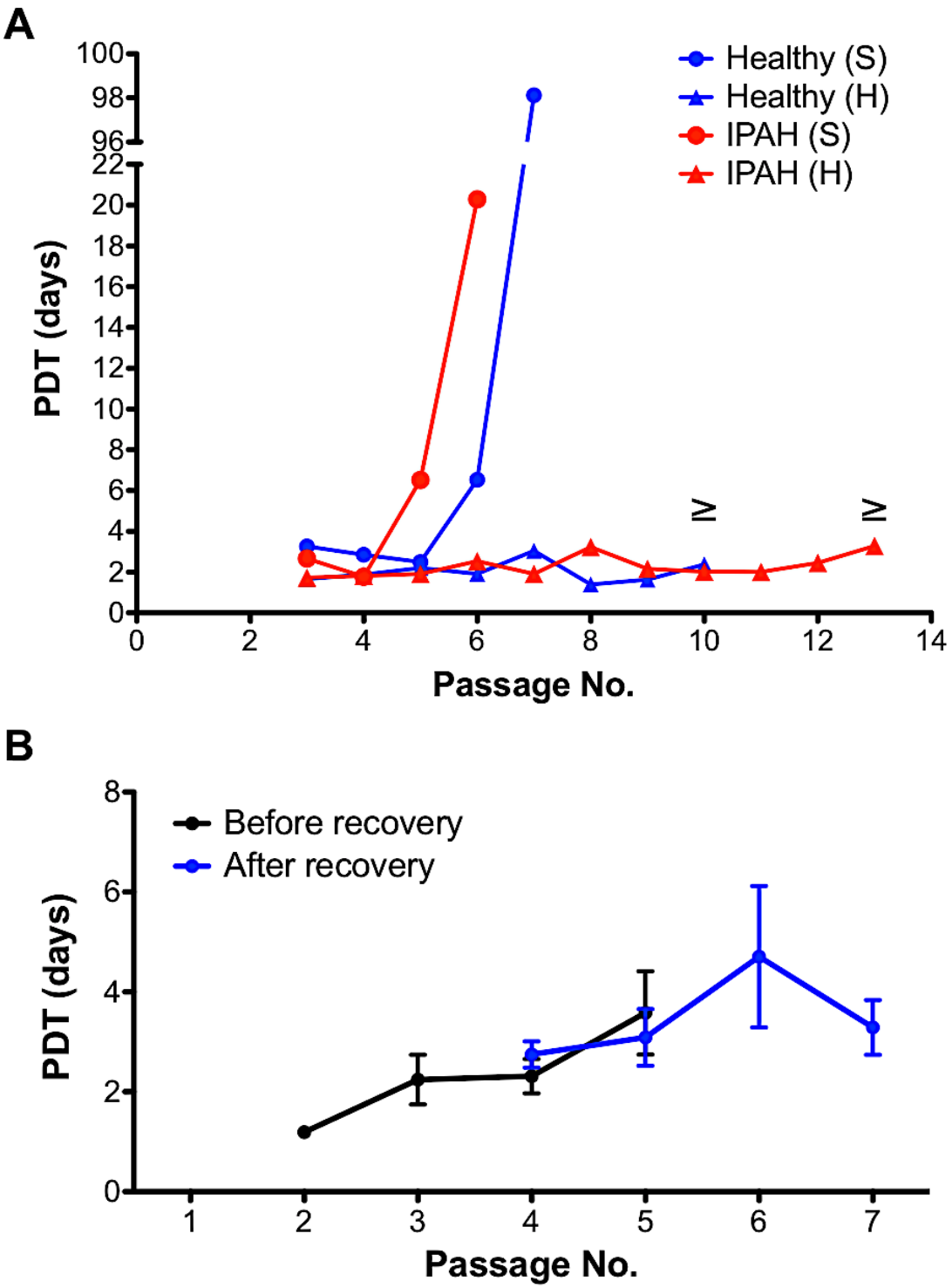


Figure 3.11. Proliferative potential of ECFCs. (A) Population doubling time (PDT) of representative ECFC populations that displayed either early senescence (n=2) or grew beyond eight passages (n=2) in continuous culture. Some isolates could potentially have grown further than the last PDT estimation (≥). **(B)** PDT of ECFCs (n=3-8) exhibiting no significant difference in PDT before and after recovery from liquid nitrogen as assessed by two-way ANOVA. Data points are presented as mean±SEM.

Sample	Population doubling time (days)					Highest passage No.
	P2	P3	P4	P5	P6	
Healthy 1			2.09	2.50	2.14	9
Healthy 3			3.60	2.44	3.05	≥9
Healthy 4		1.23	4.12	2.54	2.96	7
Healthy 5		1.10	2.78	2.53	3.12	≥8
Healthy 6	0.96	1.29	2.42	1.79	1.28	≥10
Healthy 7	2.12	4.36	3.26	**S**		4
Healthy 8	2.09	2.82	1.34	**S**		4
Healthy 9		3.43	2.90	**S**		4
Healthy 12		1.70	2.93	4.00	1.96	≥11
Healthy 13		0.97	0.96	0.95	1.13	10
Healthy 14		3.27	2.86	2.50	6.53	7
Healthy 15		1.31	1.67	3.63	**≥**	6
Healthy 16		1.48	1.78	1.79	2.13	9
Healthy 19		3.11	3.11	NA	2.95	6
Healthy 20	2.46	1.80	3.88	1.75	1.34	≥ 10
Healthy 21	1.39	1.48	2.10	1.24	3.28	9
Healthy 23	2.07	2.64	4.67	**S**		
Healthy 24	2.32	2.23	**S**			
Mean±SEM	**1.91±0.20**	**2.14±0.25**	**2.73±0.24**	**2.31±0.26**	**2.66±0.42**	
n	*7*	*16*	*17*	*12*	*12*	
IPAH 1		1.95	1.12	1.94	1.69	9
IPAH 2		1.14	1.07	2.08	1.94	≥17
IPAH 3		1.39	1.17	1.89	9.93	10
IPAH 4		1.33	2.84	3.09	4.03	7
IPAH 7		1.73	1.82	1.92	1.42	≥14
IPAH 12		4.34	1.94	5.97	**S**	5
IPAH 15		3.21	5.06	**S**		4
IPAH 16		3.43	**S**			3
IPAH 18	1.19	1.29	0.87	1.19	1.16	≥10
IPAH 19	1.37	1.79	2.23	2.20	2.95	9
IPAH 21	1.02	1.27	1.17	2.12	3.05	11
IPAH 22		3.84	1.94	1.29	2.77	≥10
IPAH 29	1.23	1.02	1.41	1.55	2.22	≥10
IPAH 30	1.20	1.72	3.21	2.56	7.77	6
IPAH 31		1.32	1.54	1.62	3.54	≥10
Mean±SEM	**1.20±0.06**	**2.10±0.30**	**2.02±0.32**	**2.30±0.38**	**2.96±0.57**	
N	*5*	*15*	*14*	*13*	*12*	
P-value	0.048*	0.82	0.12	0.92	0.54	
HUVEC	0.95	1.35	0.95	3.76	1.69±0.31	
N	*1*	*1*	*1*	*1*	*3*	

Table 3.3. Population doubling time (PDT) for ECFCs. PDTs between passages 2-6 in ECFCs from healthy controls (n=15) and IPAH patients (n=15). **S**, represents senescent cells; **≥,** represents cells that could potentially have grown further than the last recorded PDT. HUVEC, human umbilical vein endothelial cell PDT data included for comparison. Average data presented as mean±SEM. Statistics shown are from T-test.

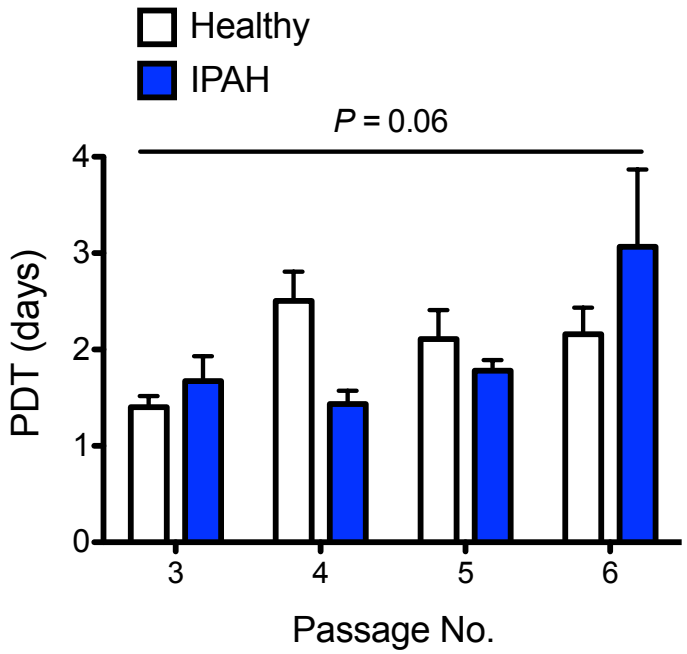


Figure 3.12. Population doubling time (PDT) for ECFCs that were serially propagated and grew beyond eight passages. ECFCs from healthy volunteers (n=9) and IPAH patients (n=10), at passages 3-6. Data are presented as mean±SEM. Statistic shown is from 2-way ANOVA.

3.3.6 Proliferative potentials of ECFCs measured by clonogenic growth assay

A key feature of ECFCs is their ability to grow under limiting dilutions and this also distinguishes ECFCs from so-called early outgrowth EPCs or haematopoietic cells. ECFCs were compared at passage 4 from healthy volunteers (n=3) and IPAH patients (n=3) of similar age and gender. After plating at limiting dilution (equivalent to one cell per well in a 96-well plate) the appearance and size of colonies was assessed after 14 days, as previously described (Ingram et al. 2004; International Patent WO 2005/078073 A2). A similar proportion of wells (~ 37%) in the two groups contained cells that had undergone at least one cell division and were distributed in clusters or colonies (Figure 3.13B). 40-60% contained clusters of 2-50 cells and 35-60% colonies of 51-2000 cells, the latter being considered to represent ECFCs of low proliferative potential (LPP-ECFC) (Coldwell et al., 2011). A small percentage (5-7%) showed more extensive proliferation, producing colonies that contained in excess of 2000 cells/well, and are considered to represent ECFCs of high proliferative potential (HPP-ECFC) (Coldwell et al., 2011). No significant difference was observed between healthy volunteers and IPAH patients in the relative proportions of either cell clusters or colonies (Figure 3.13C).

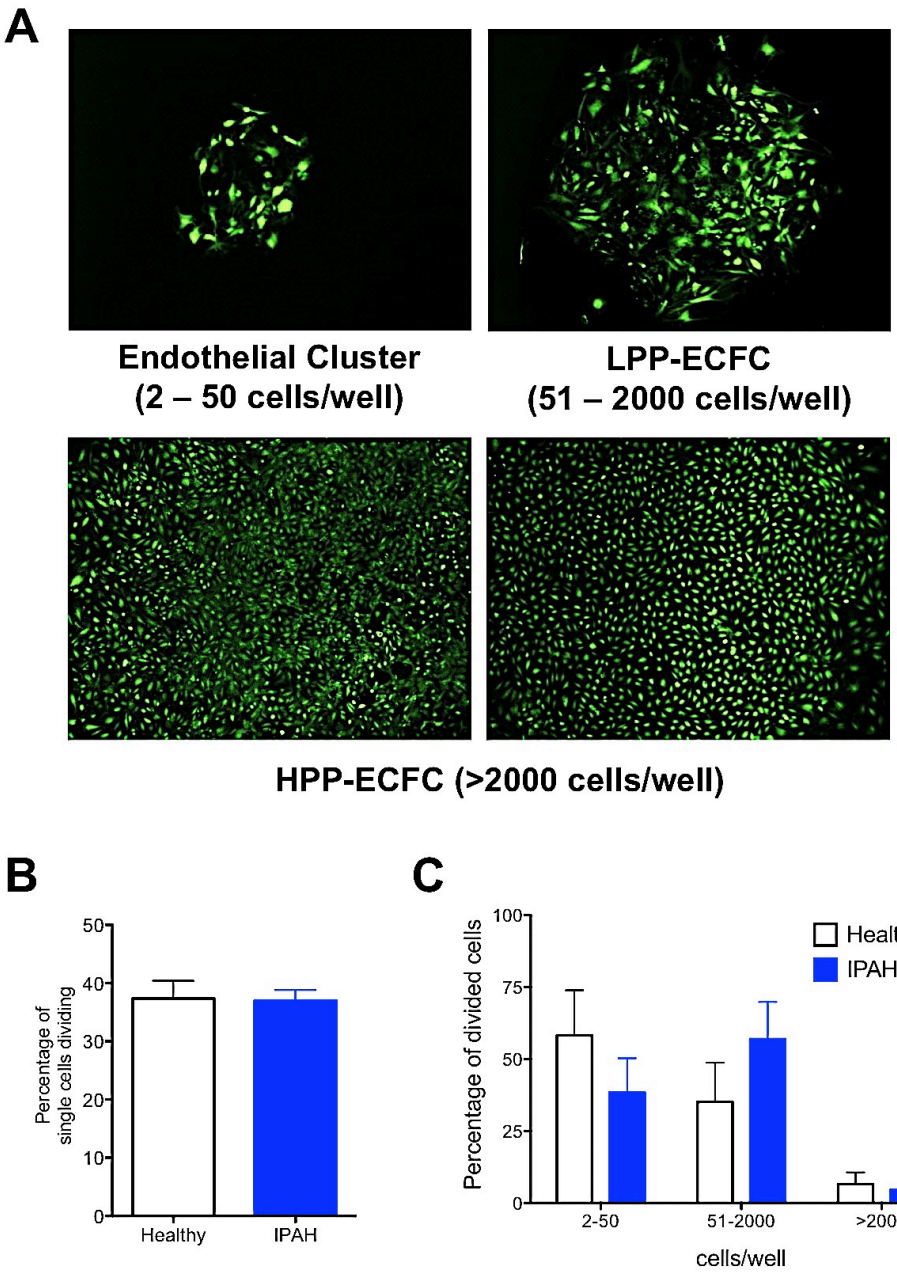


Figure 3.13. Clonogenic and proliferative potential of ECFCs. (A) Representative images of cells stained with Calcein-AM, 14 days after plating at limiting dilution. Images show endothelial cluster (2-50 cells/well) and colonies of low proliferative potential-ECFCs (LPP-ECFCs; 51-2000 cells/well; 20x magnification) and high proliferative potential-ECFCs (HPP-ECFCs; >2000 cells/well; 10x magnification). **(B)** Percentage of ECFCs from healthy volunteers (n=3) and IPAH patients (n=3) having undergone at least one cell division after 14 days of culture. **(C)** Approximate number of cell progeny derived from a single ECFC in each well, expressed as percentage of the divided cells. Data are presented as mean±SEM. Statistics are from Mann-Whitney test **(B)** and 2-way ANOVA **(C)**.

3.4 Discussion

In this chapter I describe the successful isolation, culture and characterisation of ECFCs from adult peripheral blood and obtained the following key findings:

- ECFC colony formation declined with age in healthy volunteers, but not in IPAH patients.
- Colony formation correlated with the WHO functional class of IPAH patients, but not with other clinical indices and prescribed treatments.
- ECFCs from IPAH patients and healthy volunteers exhibited a similar endothelial phenotype, proliferative potential and clonogenic capacity.

Using the protocol as described, I achieved a success rate of 77-88% in establishing at least one ECFC colony per subject. This is greater than the 54-70% success rate reported by some groups (Asosingh et al., 2008; Thill et al., 2008; Piaggio et al., 2009; Stroncek et al., 2009; Rosti et al., 2010; Teofili et al., 2011) and comparable with that achieved by others (Thill et al., 2008; Martin-Ramirez et al., 2012). Variation in success rates may reflect technical differences in the protocols used. For example, putting the blood sample on ice or in a refrigerator prior to MNC isolation and culture may have an adverse effect on establishing ECFCs (Coldwell et al., 2011; Martin-Ramirez et al., 2012). An average of 2-3 colonies were obtained from 50 ml of blood, with a median frequency of $0.17/10^7$ MNCs (range 0 to 0.57) and $0.19/10^7$ MNCs (range 0 to 1.16) from healthy controls and IPAH patients respectively. This is in agreement with the values reported for healthy volunteers in recent studies (Piaggio et al., 2009; Rosti et al., 2010; Teofili et al., 2011) and confirms that circulating endothelial progenitor cells are rare in human adult peripheral blood, with an estimated prevalence of one per million nucleated cells (Ingram et al., 2004; Reinisch et al., 2009).

Once formed, some ECFC colonies did not survive the first passage or exhibited early senescence between passages 3 to 6, this being consistent with the experience of other groups using a similar culture protocol (Otten et al., 2008; Stroncek et al., 2009). I therefore expanded all ECFC cultures for at least 8 passages in order to identify isolates that were stable between passages 3-8 and suitable for functional and mechanistic studies. In fact, many cultures could be expanded beyond 10 passages and some achieved 14–17 passages without showing signs of senescence or an increase in PDTs. Cryopreservation is necessary in order to maintain a store of early passage cells. Importantly, there

was no apparent difference in the proliferative capacity or PDTs of individual ECFC isolates when compared before and after cryopreservation.

A number of factors influence the ability to establish cultures of ECFCs from peripheral blood. In addition to the laboratory protocol, the source of the blood sample may affect the ability to derive colonies and factors such as the donor's age and disease status are likely to be important. Numerous studies have for example demonstrated a negative correlation between the number of circulating hematopoietic progenitor cells and increasing age (Thijssen et al., 2006; Hoetzer et al., 2007; Thum et al., 2007; Umemura et al., 2008; Sun et al., 2009b) and others have pointed to an age-related functional impairment rather than a decline in number (Scheubel et al., 2003; Heiss et al., 2005). Aging has also been associated with a pro-apoptotic phenotype and reduced telomere length, which may contribute to differences in the number and/or function of these cells (Kushner et al., 2009; Kushner et al., 2011). Recently, it has been reported that older healthy rhesus monkey produces lower frequency of ECFC colonies (Shelley et al., 2012) that supports the findings in this study in human, demonstrating an age-related reduction in the colony-forming capacity of ECFCs from healthy adults. This may also reflect an age-related reduction in the number of circulating endothelial progenitor cells, suggesting that larger volumes of blood (>50ml) might be required in order to establish colonies from donors over 50 years of age. The average number (2.28 colonies per 50 ml of blood) and median frequency of colonies ($0.17/10^7$ MNCs) obtained from healthy volunteers (median age 27 years, range 23-57 years) in this study is comparable with the values reported in other investigations involving volunteers of a similar age (Ingram et al., 2004; Shepherd et al., 2006b; Yoder et al., 2007; Thill et al., 2008). In keeping with my results, studies of older subjects with median ages of 67 years (range 42-81 years) and 52 years (range 32-68 years) obtained markedly fewer colonies, with median frequencies of 0 and $0.05/10^7$ MNCs respectively (Massa et al., 2009; Rosti et al., 2010). In addition, Xia and colleagues recently described impaired functions of ECFCs from elderly healthy volunteers (mean age 68.4±2.5 years), displaying migration and adhesion activities that were significantly lower than those from younger subjects (Xia et al., 2012b). It is therefore noteworthy that I found that the ECFCs capable of long-term culture, with expansion over at least 8 passages, were only derived from the younger volunteers whereas they were obtained from a broader age-range of patients with IPAH. In fact, the frequency and growth of ECFCs is known to change with gestational age (Baker et al., 2009; Borghesi et al., 2009) and a substantially higher frequency of ECFC colonies has been obtained from umbilical cord blood versus adult peripheral blood (Ingram et al., 2004; Ligi et al., 2011). Cells from cord blood also form colonies quicker, display a much higher proliferative capacity and, unlike adult cells, have the ability to form secondary and tertiary colonies in clonogenic assays (Ingram et al., 2004). Importantly, the enhanced growth characteristics of cord

blood ECFCs, compared with adult ECFCs, were associated with relatively high levels of telomerase activity (Ingram et al., 2004). In keeping with my results, this growing body of evidence indicates that age has a negative impact on the frequency and proliferative capacity of ECFCs. While children with IPAH may also produce more ECFC colonies than adult patients (Smadja et al., 2011b), I did not find any correlation between the number/frequency of ECFC colonies and the age of the IPAH patients in this study. I did not determine the telomerase activity of the cells, but observed that the average time before the appearance of colonies, the rate of growth and clonogenic capacity of ECFCs from IPAH patients was similar to that of cells derived from healthy volunteers. Duong and colleagues recently reported the isolation and characterisation of ECFCs from the pulmonary artery endothelium of patients with end-stage PAH (Duong et al., 2011). They also observed no difference in the number of ECFC colonies from PAH patients and control subjects.

Several studies have suggested that the number/frequency of ECFC colonies cultured from PBMNCs is raised in a variety of inflammatory and vascular-related disease states including acute myocardial infarction (Massa et al., 2009; Meneveau et al., 2011), coronary artery disease (Guven et al., 2006) and rheumatoid arthritis (Jodon de Villeroche et al., 2010) when compared with healthy controls, while only a few studies have reported lower ECFC numbers and growth in disorder including chronic kidney disease (Krenning et al., 2009) and type 2 diabetes mellitus (Ingram et al., 2008; Leicht et al., 2011) (further discussed in section 1.6.3). Although the number and frequency of ECFC colonies was similar between healthy volunteers and IPAH patients, these were significantly greater in the IPAH patients who were more severely functionally impaired (WHO functional classes III/IV versus I/II) and also displayed lower exercise capacity (6MWD). Otherwise there was no correlation between establishment of ECFCs in culture and baseline haemodynamic measurements or targeted therapies for PAH. Interestingly, Smadja and colleagues have also found that the number of ECFC colonies was not affected by treatment with endothelin receptor antagonists and/or PDE5 inhibitors in children with PAH, but was increased when subcutaneous treprostinil treatment was combined with the other two therapies (Smadja et al., 2011b). I cannot exclude the possibility that a similar effect may occur in adult IPAH patients as only two subjects in the present study were prescribed the same combination of drugs (see Appendix 2, Table A2.2).

The endothelial phenotype of ECFCs was verified by flow cytometry and immunocytochemistry on cultures derived from up to 16 healthy volunteers and 13 patients with IPAH. Cells from both groups exhibited a typical endothelial-like phenotype, showing cobblestone morphology, staining positively for the typical endothelial surface markers CD31 (PECAM-1), CD144 (VE-cadherin) and CD309 (VEGFR2), and displaying vWF-immunoreactivity localised to endothelial cell-specific Weibel-Palade

bodies. Varying amounts of CD34 labelling was observed (average 14.7 – 20.0% of ECFCs) whereas ≥99% of cells were negative for the immature marker CD133 and haematopoietic marker CD45. This is consistent with the view that ECFCs do not originate from $CD133^+$ cells or $CD45^+$ hematopoietic precursors (Case et al., 2007; Timmermans et al., 2007; Mund et al., 2012). Importantly, the $CD31^+$, $VEGFR2^+$, $CD45^-$, $CD133^-$ phenotype of ECFCs in this study corresponds with that of other investigations on ECFCs from adult peripheral and umbilical cord blood (Ingram et al., 2004; Ingram et al., 2005; Yoon et al., 2005; Thill et al., 2008; Baker et al., 2009; Fujinaga et al., 2009; Stroncek et al., 2009; van Beem et al., 2009; Ligi et al., 2011).

Despite the fact that ECFCs lacked the haematopoietic marker CD45, I found that a sub-population (~17%) of ECFCs and HPAECs and a minority of HUVECs were positive for the monocytic marker CD14. In fact, examination of the representative flow cytometry plots published in other studies of human ECFCs and mature ECs (Ingram et al., 2005; Thill et al., 2008; Stroncek et al., 2009) suggests that this may not be an unusual phenomenon. However, the apparent expression of CD14 on mature ECs raises doubt about the specificity of the CD14 antibodies and flow cytometry analysis. Cloning cylinders were generally used to isolate individual ECFC colonies but I cannot exclude the possibility that the cultures were contaminated by cells with a monocytic lineage, particularly as distinct $CD14^+$ mesenchymal-like cells were found to occasionally overrun some colonies (<4%) when they were expanded.

Binding of the lectin UEA-1 is a typical characteristic of ECs and has been used to characterise ECFCs in this as well as other studies (Yoder et al., 2007; Otten et al., 2008; Ligi et al., 2011). UEA-1 can bind to ECs from proximal blood vessels (Conrad-Lapostolle et al., 1996) as well as distal microvessels (Gargett et al., 2000; Garrafa et al., 2006). Other lectins may distinguish between ECs from different regions of the vascular bed and thereby provide further insight into the phenotype and possible origins of circulating ECFCs. The lectin *Griffonia simplicifolia* is considered a specific marker of microvascular ECs, including human pulmonary microvascular ECs (Comhair et al., 2012). I observed no apparent immunostaining of ECFCs using this lectin, but the observation is uncertain due to the lack of a positive control. Ideally, this should be examined together with other markers, such as the macrovascular EC-specific *Helix Pomatia* (Comhair et al., 2012) and microvascular EC marker CD36 (Swerlick et al., 1992), and staining of appropriate mature ECs as controls.

The EC surface marker CD31 appeared to be stably expressed in >99% of cells when examined over 2-5 consecutive passages in a subgroup of ECFC cultures. The proportion of $VEGFR2^+$ ECFCs was relatively high (>90% of cells) during earlier passages, but tended to decline (74-84% of cells) at higher passage. Another group has recently reported a similar observation, albeit with far fewer cells

exhibiting VEGFR2 expression (Wang et al., 2011). This may be significant as an association has been found between VEGFR2 expression and the number of colonies or ECFCs obtained in culture (Thill et al., 2008). A decline in VEGFR2 expression might also be accompanied by a reduction in cell survival and the mitogenic effects of VEGF (Gerber et al., 1998). Nonetheless, there was no apparent correlation between the number or frequency of ECFC colonies and the proportion of $VEGFR2^+$ cells determined during the subsequent expansion of ECFCs in culture. The receptor tyrosine kinase c-kit was demonstrated on ≥50% of ECFCs. It is considered to be important in the recruitment of progenitor cells from the bone marrow in to the circulation (Heissig et al., 2002) and is also expressed by mature ECs, influencing for example the survival, migration and tube formation of HUVECs in culture (Matsui et al., 2004). But in the present study no association was found between the frequency of establishing ECFC colonies and proportion of c-kit^+ ECFCs.

The proportion of ECFCs exhibiting CXCR4 expression correlated with the number of primary ECFC colonies from healthy volunteers, but not from IPAH patients. CXCR4 expression modulates the recruitment, migration and homing of progenitor cells to the injured endothelium via a stromal derived factor-1α (SDF-1α, also known as CXCL12) gradient (Ceradini et al., 2004). Injection of the CXCR4 antagonist AMD3100 induces a potent and rapid increase in the number of ECFC colonies obtained from mice and healthy humans, reflecting the mobilisation of bone marrow cells (Shepherd et al., 2006b; Pitchford et al., 2009). CXCR4 is also important in regulating the function of ECFCs, as inhibition with CXCR4-small hairpin RNA (shRNA) or specific monoclonal antibodies cause a reduction in SDF-1α-induced cell migration and adhesion, and attenuates reendothelisation by ECFCs in a murine carotid artery injury model (Smadja et al., 2005; Xia et al., 2012a). Conversely, the induction of CXCR4 expression is accompanied by an increase in the proliferative and angiogenic capacity of ECFCs (Smadja et al., 2010; Smadja et al., 2011a). The variation of CXCR4 expression in ECFCs from different cord blood samples also correlates with their migratory ability and neovascularisation capacity in a hind limb ischemia model and lead to the suggestion that CXCR4 expression may be used as criteria to select ECFCs possessing superior proliferative and functional abilities (Oh et al., 2010). It is tempting to speculate that CXCR4 expression could be correlated to the impaired colony forming ability in older healthy volunteers, but subjects' age did not correlate with the CXCR4 expression of the isolated ECFCs. CXCR4 expression do not correlate with the ECFC colony formation in IPAH patients, possibly indicate that ECFCs mobilise into the circulation irrespective of their CXCR4 expression in IPAH patients. However, CXCR4 expression was not concurrently determined with the functional analysis in this study, therefore it is not possible to determine the exact role of CXCR4 expression in the IPAH-ECFCs. As antagonising CXCR4 has attenuated disease progression in hypoxic

model of PH (Young et al., 2009; Yu and Hales, 2011), these provide scope for future investigation in the role of CXCR4 expression in ECFCs in the development of IPAH.

There was no apparent difference between the expression of endothelial markers by ECFCs from healthy volunteers and IPAH patients or ECFCs and mature ECs. A number of studies have used flow cytometry and immunocytochemistry and described the comparable characteristics of ECFCs from healthy subjects and patients with various diseases (Avouac et al., 2008; Stroncek et al., 2009) as well as their similarity to mature ECs, including those from the umbilical vein, aorta and dermal microvasculature (Ingram et al., 2005; Avouac et al., 2008; Thill et al., 2008; Stroncek et al., 2009). A study has also reported similarities in molecular markers between mature human (dermal microvascular) ECs and ECFCs (Medina et al., 2010), but other studies have reported differences in functional characteristics that distinguish ECFCs from mature ECs (as discussed in section 1.5.4).

A number of studies have also described disease-related differences in gene and/or protein expression (Tan et al., 2010; Avouac et al., 2011) as well as the ability of ECFCs to proliferate, form tubes and migrate *in vitro* (Krenning et al., 2009; Toshner et al., 2009; Tan et al., 2010; Ligi et al., 2011; Starke et al., 2011), but few studies to date have examined ECFCs from patients with PAH. Asosingh *et al.* derived similar numbers of ECFCs from the peripheral blood MNCs of patients with IPAH (3 out of 5) and healthy controls (5 out of 7), but did not explore colony frequency and ECFC function *in vitro* (Asosingh et al., 2008). In another study, Toshner and co-workers provided evidence of dysfunctional ECFCs cultured from three patients with known BMPR2 mutations, the cells being more proliferative and less able to form tubes in Matrigel than ECFCs derived from control subjects (Toshner et al., 2009). In children with PH associated with congenital heart disease, ECFCs were found to be more frequent in those with surgically reversible PAH than irreversible PAH or IPAH (Smadja et al., 2011b). Finally, it has recently been shown that ECFCs are present in the pulmonary artery endothelium as well as peripheral blood of PAH patients, with and without BMPR2 mutation and mosaic deletion of the X-chromosome, and these cells may be more proliferative than those derived from donor lung tissues (Duong et al., 2011). It is important to investigate whether ECFCs derived from the peripheral blood of IPAH patients exhibit differences in endothelial functions that can distinguish them from healthy volunteers, which could improve our understanding in the endothelial dysfunctions underlying the pulmonary vascular remodelling in IPAH patients.

In the next chapter I determine ***the functional phenotype of ECFCs (as assessed by proliferation, apoptosis, Matrigel tube formation, migration and barrier function) and explore possible differences between cells derived from healthy volunteers and IPAH patients, using selected stable populations of ECFCs.***

Chapter 4:

Distinct phenotype of ECFCs from IPAH patients

Chapter 4 - Distinct phenotype of ECFCs from IPAH patient

4.1 Introduction

One of the histological hallmarks of end-stage PAH is the focal proliferation of ECs in plexiform lesions. It has been postulated that an initial apoptosis-inducing insult gives rise to ECs that are proliferative and resistant to apoptosis (Sakao et al., 2005) and, in the plexiform lesions of IPAH patients, a monoclonal proliferation of cells (Lee et al., 1998). Vascular lesions in PAH also exhibit inflammatory/immune cell infiltration and cells displaying progenitor cell markers such as CD133 (Majka et al., 2008; Toshner et al., 2009), but it is not known if the latter are derived from the tissue or circulation. The isolation and culture of pulmonary ECs from explanted IPAH lungs has revealed functional differences in several *in vitro* assays compared with control cells, including higher proliferation and transwell migration and reduced sensitivity to apoptosis (Masri et al., 2007; Tu et al., 2011; Tu et al., 2012). It is plausible that the dysfunctional characteristics of these pulmonary ECs might also reflect the angioproliferative state of plexiform lesions.

Blood-derived ECFCs from IPAH patients might manifest the dysfunctional characteristics of diseased pulmonary ECs *in vitro*. In fact, functions of ECFCs have been examined in variety of vascular diseases (Fernandez et al., 2005; Fernandez et al., 2007; Ingram et al., 2008; Thill et al., 2008; Fujinaga et al., 2009; Tan et al., 2010; Ligi et al., 2011; Starke et al., 2011), but few studies have explored the functional characteristics in PAH. Toshner and colleagues found that ECFCs from 3 patients with BMPR2 gene mutations proliferated significantly more over the course of 7 days than cells derived from healthy controls (Toshner et al., 2009). It is doubtful however whether BMPR2 mutations are a prerequisite as studies on ECs derived form explanted lung tissues indicate that IPAH patients may exhibit a proliferative EC phenotype irrespective of whether they carry a known BMPR2 mutation (Masri et al., 2007; Tu et al., 2011). This is also in keeping with the suggestion that hyperproliferative ECFCs may reside in the pulmonary artery wall of patients with various forms of PAH (Duong et al., 2011).

Therefore I hypothesised that blood-derived ECFCs from IPAH patients are functionally distinct from healthy volunteer cells and display characteristics similar to those reported for ECs cultured directly from the diseased lung.

The objectives of this chapter are:

- To confirm findings of Chapter 3 in regard to the proliferative capacity of ECFCs, as assessed by direct cell counting and measurement of DNA synthesis.

- To investigate the functional differences between ECFCs from IPAH patients and healthy volunteers. These include their angiogenic and migratory capacities, response to apoptosis and barrier functions.

4.2 Methods

See Chapter 2 for detailed experimental materials and protocol.

All cell populations (passages 4-7) for functional assays were selected according to the following criteria:

- Endothelial phenotype confirmed by flow cytometry
- Consistent population doubling times
- Continual growth beyond at least 8 passages

All data presented are representative of at least 2 repeated experiments.

4.2.1 Cell proliferation and DNA synthesis

Proliferation was assessed by viable cell counts and measuring DNA synthesis, as determined by the BrdU incorporation assay. Cell counting was performed in triplicate, with ECFCs from healthy volunteers (n=4) and IPAH patients (n=6) seeded in 24-well plates (7600 cells/well) in EGM-2 and 20% FBS. The medium was changed every 48h and trypsinised cells were counted on days 4 and 7 with a haemocytometer. BrdU assay was performed with ECFCs from each groups (n=6–7) using the optimised protocol as described in Chapter 2.12.1.

4.2.2 Apoptosis assays

Caspase-3/7 activation/cleavage and subsequent cleavage of its substrate poly(ADP-ribose) polymerase (PARP) are hallmarks of apoptosis. Optimisation of the caspase-3/7 assay is detailed in Chapter 2.12.2. For the assessment of caspase 3 and PARP cleavage, protein was harvested in ice cold RIPA buffer and equal amounts were (40 μg/lane) separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed using specific antibodies, as detailed in Chapter 2.11.

Serum deprivation-induced apoptosis was assessed in ECFCs from the healthy volunteers (n=7 in total) and IPAH patients (n=8 in total) and HPAECs (measured in quadruplicate), following incubation in serum-free EBM-2 medium for 24h and 48h. The same treatments were also applied to cells in

additional adjacent wells in clear-bottomed 96-well plate to those used for determining caspase activity and western blotting, enabling concurrent microscopic visualisation and the capture of images showing of cell morphology.

4.2.3 Matrigel tube formation assay

Matrigel tube formation was examined using ECFCs from healthy volunteers (n=3-5) and IPAH patients (n=3-6) and HPAECs (measured in triplicate). Tube formation was assessed in different conditions, using either high concentration (HC)- or growth factor reduced (GFR)-basement membrane matrix (Matrigel) and cells suspended in EBM-2 containing 1% FBS or no serum. In some experiments, cells were also serum-deprived for 24h before seeding on GFR-Matrigel. Data is presented as the average total tube length per field of view of values from 2 random fields in triplicate wells.

4.2.4 Wound healing assay

A wound healing (scratch) assay was used to assess the planar migratory capacity of ECFCs from healthy volunteers (n=3-6) and IPAH patients (n=4-6) and HPAECs (measured in triplicate). Wounds were created in confluent monolayers of cells, with triplicate wells being used for each experimental condition. Cells were incubated in either EBM-2 with 1% FBS or EGM-2 (with endothelial growth factors) containing 20% FBS, and images were captured at baseline (0h), 6h, 18h and 24h (EBM-2 and 1% FBS) or 3h and 21h (EGM-2 and 20% FBS). Data is presented as a percentage of the area of the wound recovered (closed) compared to baseline.

4.2.5 Assessment of endothelial barrier functions

Method for measuring gap junctional communication in ECFCs by FRAP is detailed in Chapter 2.12.5. Endothelial cell permeability measured by FITC-dextran passage over endothelial monolayers is detailed in Chapter 2.12.6.

4.3 Results

4.3.1 ECFCs from IPAH patients and healthy volunteers exhibit similar proliferation

As previously noted in Chapter 3, ECFCs from IPAH patients grew at a comparable rate to cells derived from healthy volunteers (Figure 3.11 and Table 3.3). Similarly, no significant difference was found between the two groups when ECFCs were counted after 4 and 7 days in culture (Figure 4.1A). The incorporation of BrdU varied between different ECFC populations, particularly when cultured in EGM-2 and 20% FBS, but again no significant difference was found between BrdU incorporation in ECFCs from healthy volunteers and IPAH patients, and equivalent results were also obtained with HPAECs (Figure 4.1B). Comparison of DNA synthesis and cell counts in the same ECFC populations, 3 - 4 days after seeding, indicated that there was a significant association between the two parameters ($r = 0.73$, $P = 0.02$, 95% CI 0.27 to 0.98; Figure 4.1C), validating both BrdU assay and viable cell counts as reliable assays for measuring proliferation of these cells.

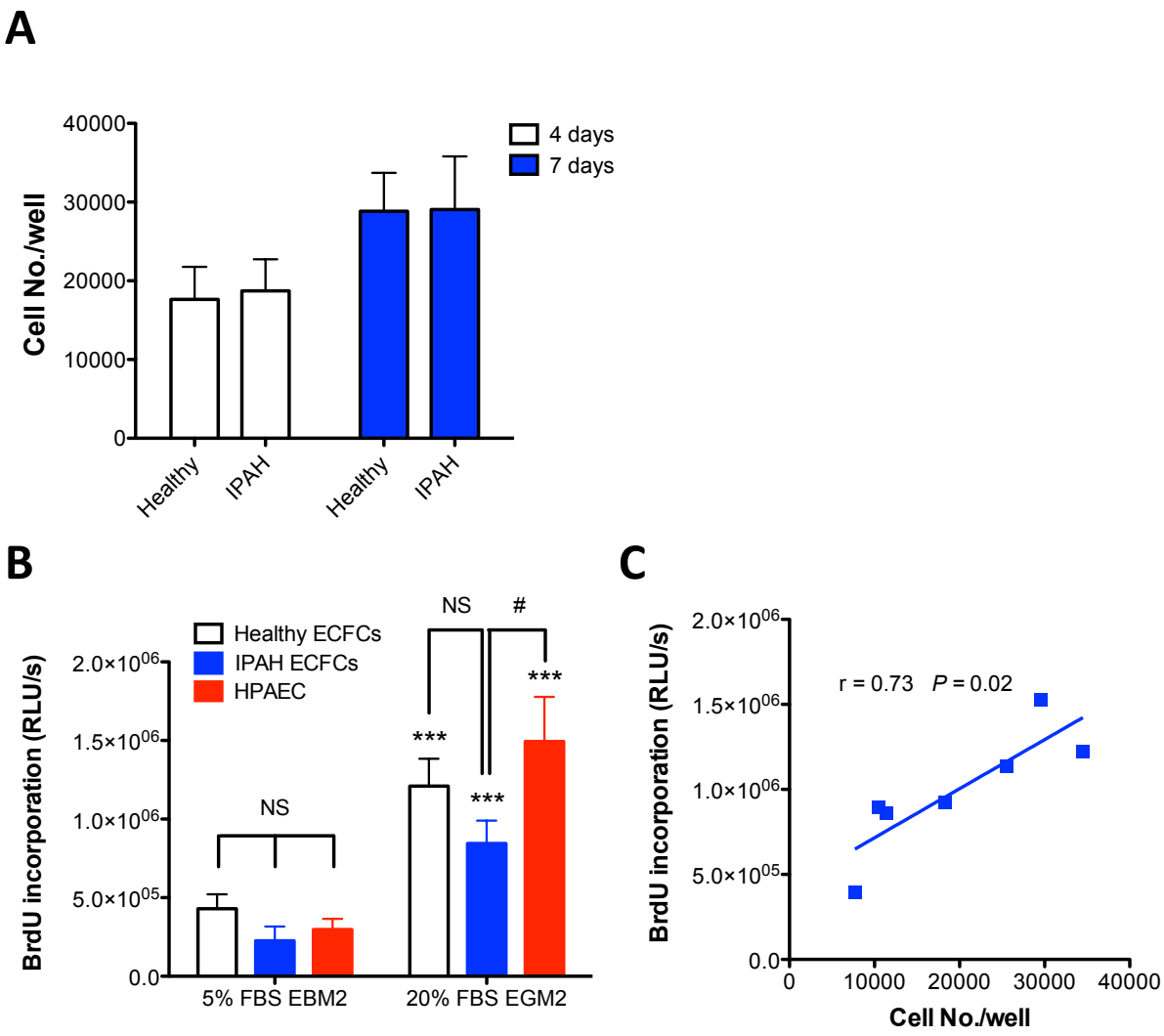


Figure 4.1. ECFC proliferation and DNA synthesis. (A) Number of cells from healthy volunteers (n=4) and IPAH patients (n=6), counted after 4 and 7 days in culture. **(B)** DNA synthesis measured by BrdU incorporation (expressed as relative light units per second – RLU/s) in ECFCs from healthy volunteers (n=6) and IPAH patients (n=7) and in HPAECs (n=3), following incubation in either EBM-2 and 5% FBS or EGM-2 and 20% FBS for 48h. ***, $P < 0.001$, compared with cells cultured in EBM-2 and 5% FBS. Similar findings were observed in repeated experiments. **(C)** Correlation between BrdU incorporation and cell number, determined 3-4 days after the seeding of ECFCs from healthy volunteers (n=3) and IPAH patients (n=4). Data presented as the mean±SEM of triplicate values from one sample. Statistics in (A) and (B) are from Bonferroni post-hoc analysis following two-way repeated-measures ANOVA. Statistic shown in (C) are from Pearson's correlation test.

4.3.2 ECFCs from IPAH patients are apoptosis-resistant

Serum deprivation-induced apoptosis was assessed by examining caspase-3/7 activity/cleavage and PARP cleavage in distinct ECFC populations from a total of 7 healthy volunteers and 8 patients with IPAH, as well as in HPAECs. Caspase 3/7 activity increased by 24h and was significantly raised after 48h serum deprivation, compared with cells incubated in the control condition in the presence of 2% FBS (Figure 4.2A-B). Most importantly, ECFCs from IPAH patients exhibited a significantly smaller increase ($P < 0.01$) in caspase-3/7 activity when compared with either ECFCs from healthy volunteers or HPAECs (Figure 4.2A-B).

The activity of the caspase-3/7 substrate declines once it is reconstituted and following storage for 4 weeks at 4°C the signal it provides is approximately 75% of that obtained with fresh reagent. Caspase-3/7 activity can therefore only be directly compared within each experiment, differences in signal intensity between experiments (Figure 4.2A-B) reflecting variation in substrate activity. Nevertheless, it is apparent that, in addition to displaying significantly less caspase-3/7 activity, serum-deprived ECFCs from IPAH patients also exhibit markedly less cleavage of caspase-3 and PARP when compared with ECFCs from healthy volunteers (Figure 4.2C). Interestingly, IPAH-ECFCs under basal conditions may also display less caspase-3/7 activity (Figure 4.2A-B) and cleavage of caspase-3 or PARP than cells from healthy volunteers or HPAECs (Figure 4.2C). In addition, adherent viable cells were more frequently observed following serum-deprivation of ECFCs from IPAH patients than ECFCs from healthy volunteers or HPAECs (Figure 4.3). Expression of the anti-apoptotic modulators survivin and apoptosis repressor with caspase recruitment domain (ARC) was assessed in ECFCs. No significant differences were observed between control and IPAH cells under basal conditions. However, serum deprivation for 48h was associated with a marked reduction in survivin expression in ECFCs from healthy volunteers but not in ECFCs from IPAH patients (Figure 4.4).

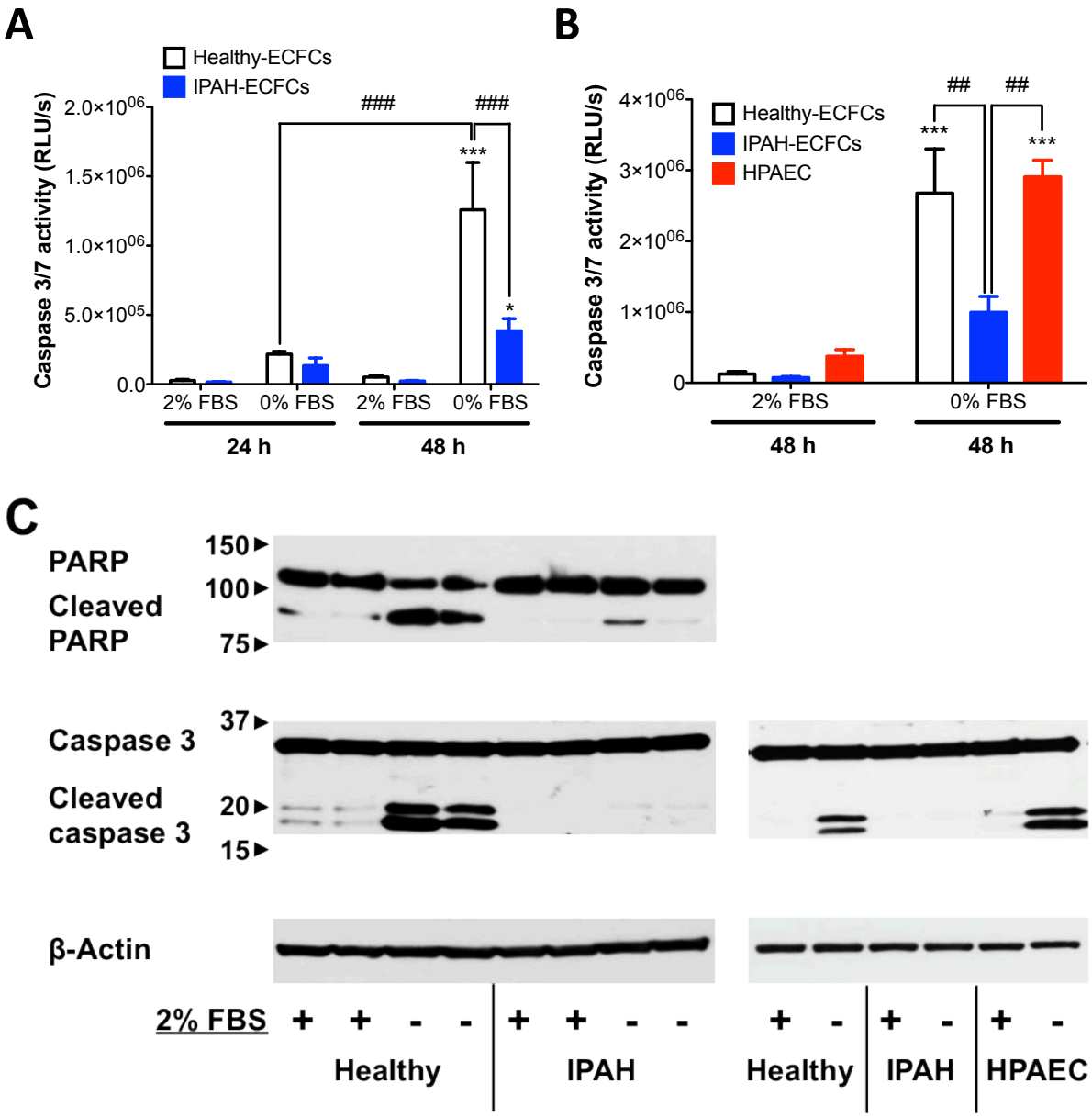


Figure 4.2. Serum deprivation-induced apoptosis. (A) Caspase-3/7 activity in ECFCs (passage 7) from healthy volunteers (n=3) and IPAH patients (n=4), measured after 24h and 48h incubation in control (EGM-2 with 2% FBS) and serum-deprived medium (EBM-2 with 0% FBS). **(B)** Caspase-3/7 activity in ECFCs (passage 4), from a distinct set of healthy volunteers (n=4) and IPAH patients (n=4), and HPAECs (triplicate) following serum-deprivation for 48h. Bars represent mean±SEM. Similar findings observed in repeated experiments. Statistics shown from two-way repeated measures ANOVA with Bonferroni post-hoc analysis. *, $P < 0.05$, ***, $P < 0.001$, versus their respected control condition. ##, $P < 0.01$, ###, $P < 0.001$, comparison between indicated groups. **(C)** Representative western blots showing the cleavage of poly(ADP-ribose) polymerase (PARP; upper blot) and caspase-3 (middle blots) following serum-deprivation for 48h in ECFCs from a total of 3 distinct healthy volunteers, 3 IPAH patients and HPAECs. β-actin (lower blots) was used to demonstrate equal protein loading. ►, molecular weight (kDa) markers.

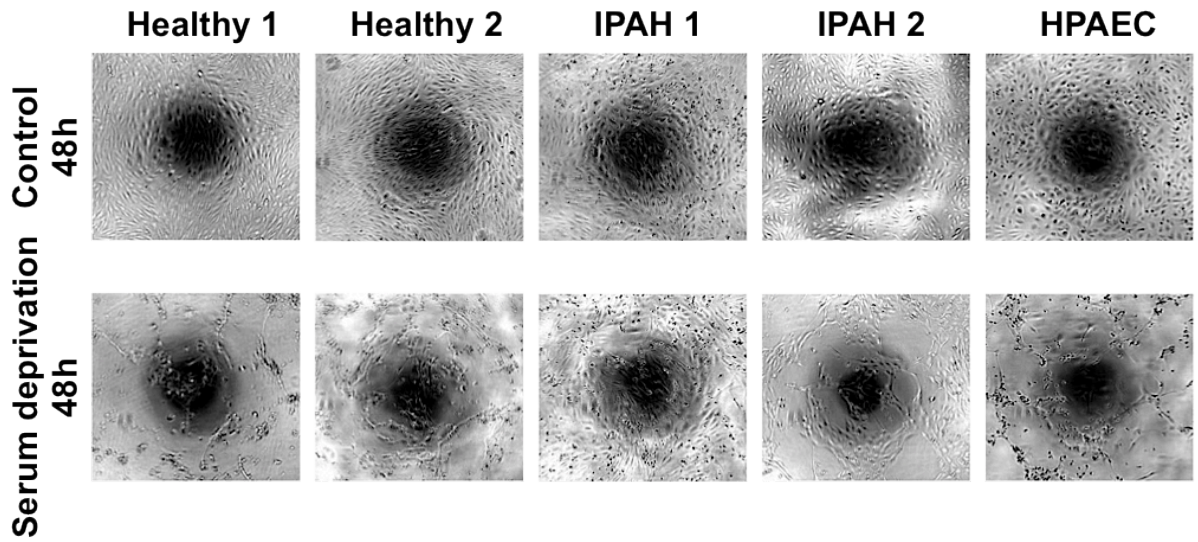


Figure 4.3. Effect of serum-deprivation on ECFCs. Representative phase contrast photomicrographs (4x magnification) showing cultures of ECFCs from distinct healthy volunteers and IPAH patients and HPAECs. Decline in number of adherent viable cells, after serum-deprivation for 48h, is less marked in ECFCs from IPAH patients versus those in healthy volunteers and HPAECs. Control cells cultured in EGM-2 medium containing 2% FBS.

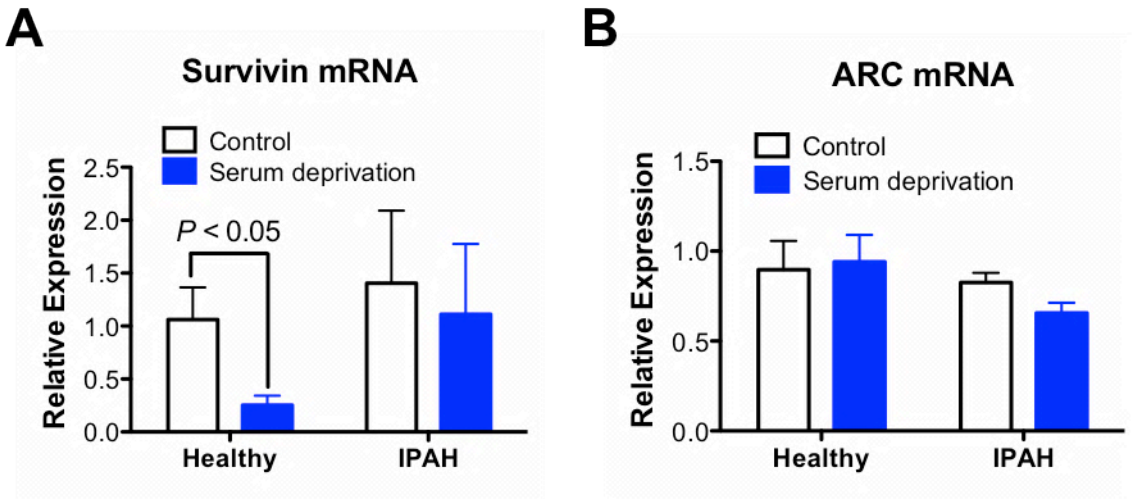


Figure 4.4. ECFCs survivin and ARC mRNA expression upon serum-deprivation. qRT-PCR analysis of **(A)** survivin (n=7) and **(B)** apoptosis repressor with caspase recruitment domain (ARC) mRNA expression (n=3) in ECFCs in both control (C) and serum-deprived conditions (S) for 48h. Data is presented as mean±SEM expression, normalised to the expression of housekeeping reference genes. Statistics shown from two-way repeated measures ANOVA and Bonferroni post-hoc analysis.

4.3.3 Enhanced angiogenic capacity of ECFCs from IPAH patients

ECFCs from IPAH patients exhibited a greater angiogenic capacity compared with ECFCs derived from healthy volunteers, this being apparent in each of the three conditions examined in the Matrigel tube formation assay (Figure 4.5A). The total tube length was significantly greater when IPAH-ECFCs were seeded on either HC-Matrigel (in EBM-2 with 1% FBS) or GFR-Matrigel (in EBM-2 without serum) and when the cells were subjected to 24h serum-deprivation prior to seeding (Figure 4.5B-C). Interestingly, the tubes appeared thicker and more cellular on HC-Matrigel (in the presence of 1% FBS) than under growth factor reduced and serum-free conditions. HPAECs exhibited a superior capacity to form tubular networks on Matrigel and, unlike ECFCs from IPAH patients, this was significantly reduced following serum-deprivation for 24h prior to seeding on GFR-Matrigel (Figure 4.5A & C). No significant difference was found in the expression of thrombospondin-1 (THBS1) in ECFCs from healthy volunteers and IPAH patients (Figure 4.6), suggesting that this is probably not an important factor in determining the functional phenotype of ECFCs from IPAH patients.

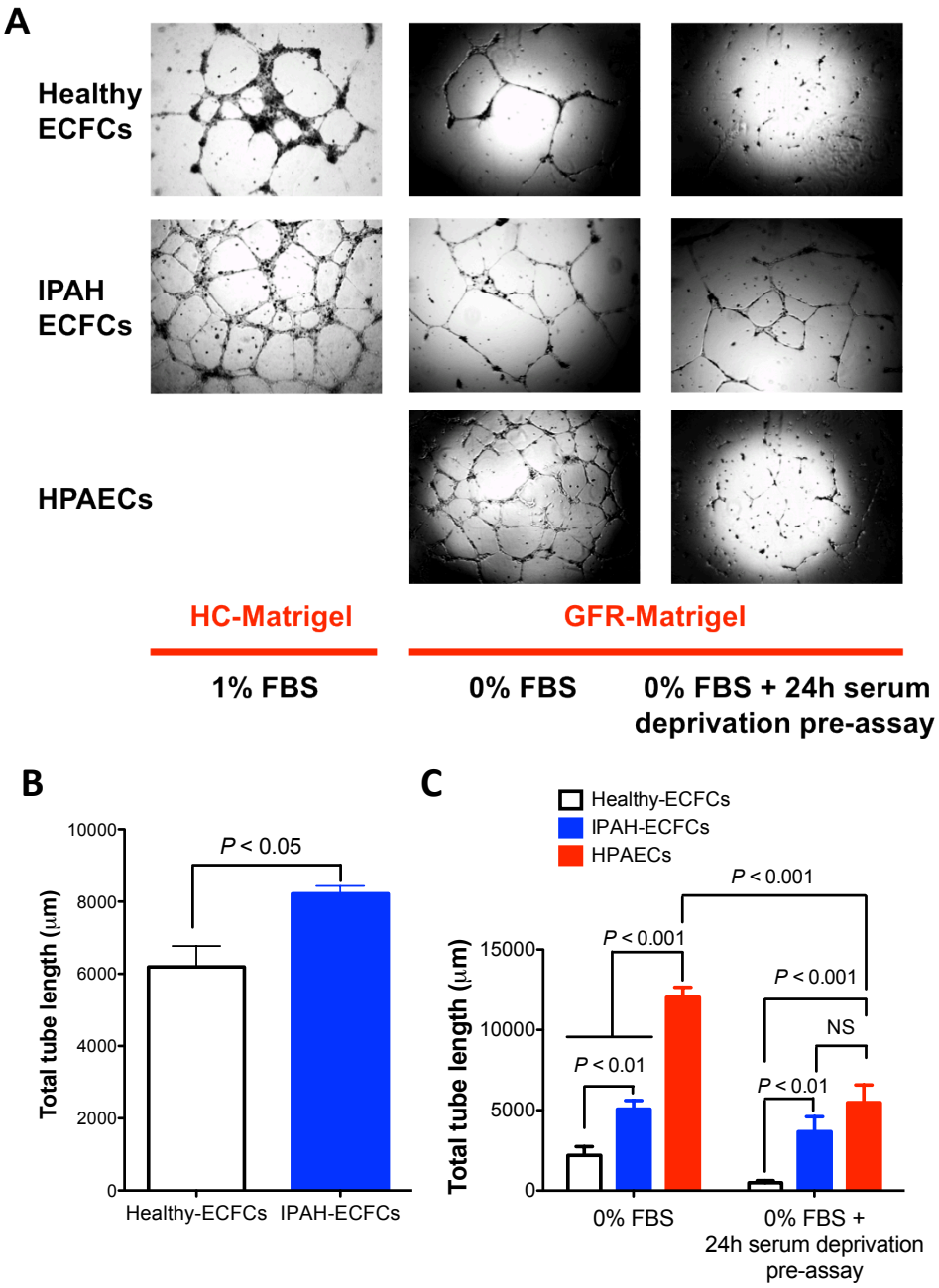


Figure 4.5. Matrigel tube formation by ECFCs and HPAECs. (A) Representative images captured with an inverted microscope, showing tube formation by ECFCs from healthy volunteers (top row), IPAH patients (middle row) and HPAECs (bottom row). Cells were seeded either on high protein concentration (HC)-Matrigel (EBM-2 & 1% FBS) or growth factor reduced (GFR)-Matrigel in the absence of serum (EBM-2 & 0% FBS) and some were also serum-deprived for 24h before seeding on GFR-Matrigel. 4x magnification. **(B)** Quantification of tube formation by ECFCs from healthy volunteers (n=3) and IPAH patients (n=3), seeded on HC-Matrigel in EBM-2 medium with 1% FBS. **(C)** Quantification of tube formation by ECFCs from healthy volunteers (n=5) and IPAH patients (n=6), and HPAECs (n=3), seeded on GFR-Matrigel in the absence of serum and -/+ 24h serum-deprivation prior to seeding. Data presented as mean±SEM total tube length for each group of cell isolates, using measurements from images of 2 random fields in triplicates wells, representative of at least two experiments. Statistics obtained by T-test (B) and two-way repeated measures ANOVA with Bonferroni post-hoc analysis (C).

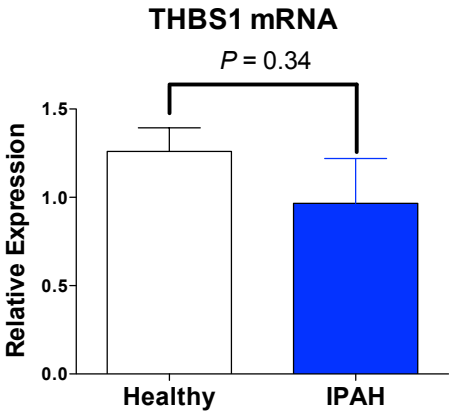
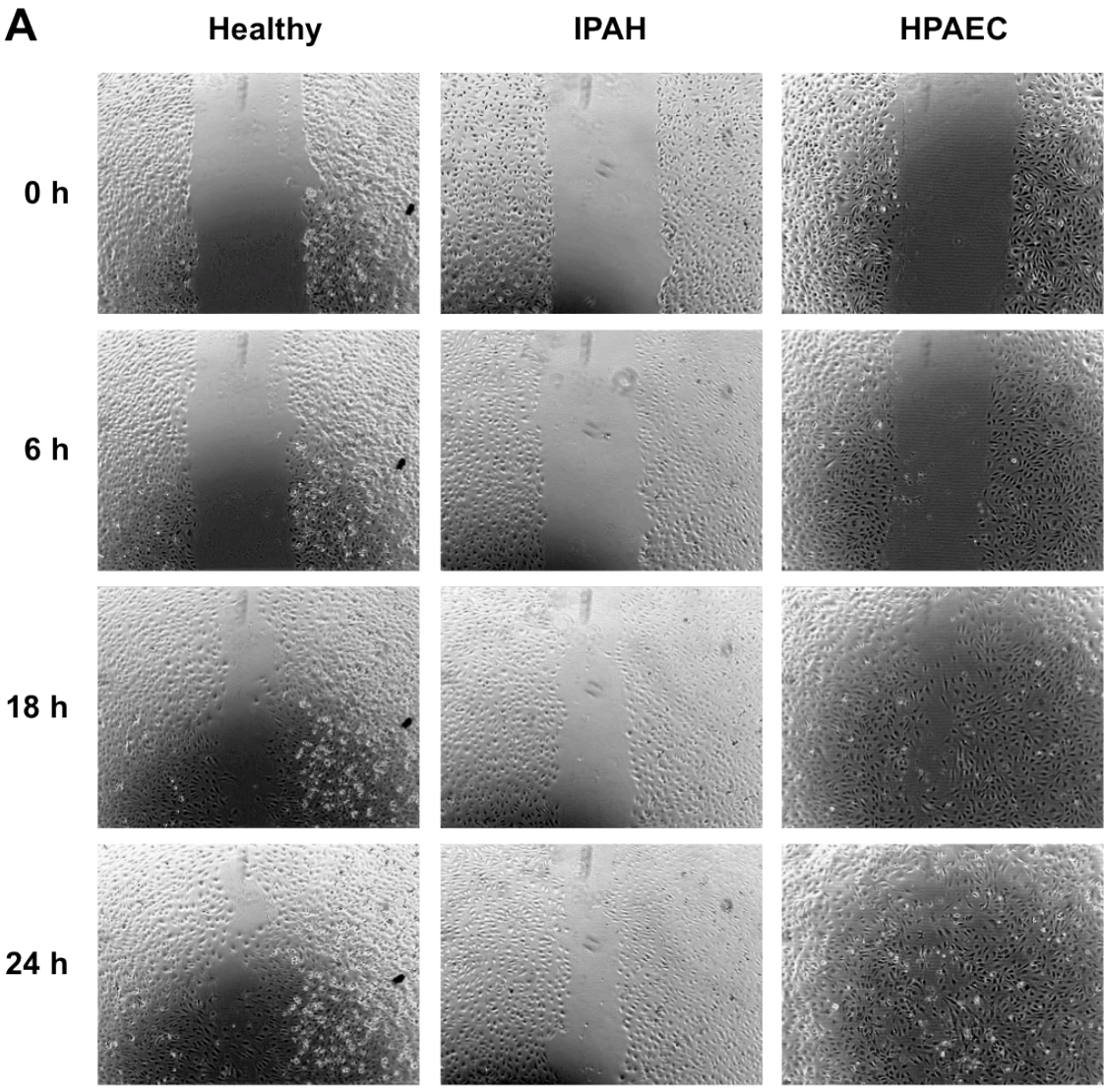
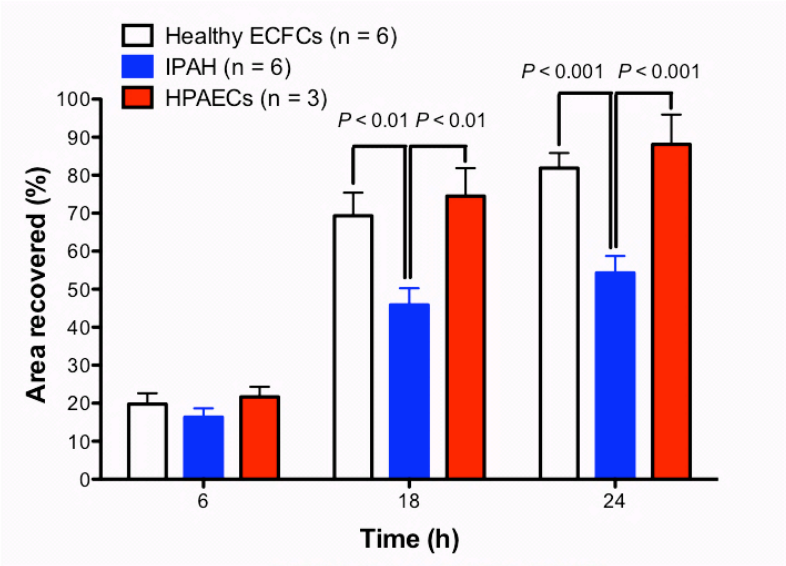


Figure 4.6. ECFCs Thrombospondin-1 mRNA expression. qRT-PCR analysis of Thrombospondin-1 (THBS1) mRNA expression in ECFCs from healthy volunteers (n = 8) and IPAH patients (n = 9). Data is presented as mean±SEM expression after normalised by expression of the reference genes. Statistics was from student T-test.

4.3.4 Impaired capacity in wound healing by ECFCs from IPAH patients

The migratory capacity of the ECFCs was assessed by wound healing assay. ECFCs from IPAH patients consistently displayed a slower rate of wound recovery, when compared with healthy ECFCs and HPAECs, and cultured either in EBM-2 with 1% FBS or EGM-2 and 20% FBS (Figure 4.7). HPAECs exhibited a superior rate of wound recovery, compared to both groups of ECFCs, when assessed in EGM-2 and 20% FBS (Figure 4.7B).





EBM-2 and 1% FBS

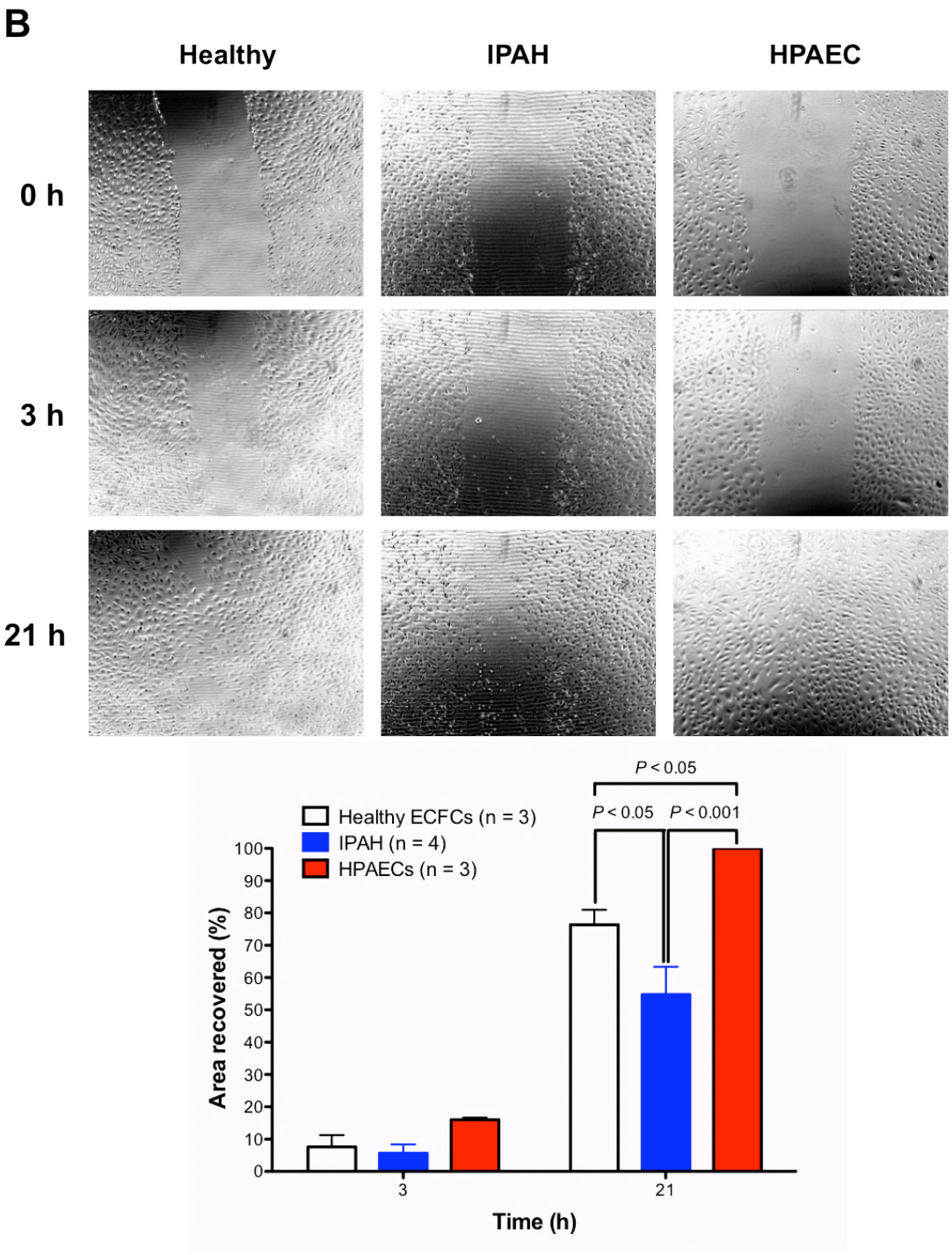


Figure 4.7. Wound recovery analysis of ECFCs. (A) Representative microscopic images and area of wound recovery (expressed as % of 0h) by ECFCs from healthy volunteers (left panel; n=6) and IPAH patients (middle panel; n=6) and HPAECs (right panel; n=3) after 6h, 18h and 24h in EBM-2 with 1% FBS. 4x magnification. **(B)** Representative images and area of wound recovery by healthy-ECFCs (n=3), IPAH-ECFCs (n=4) HPAECs (n=3) after 0h, 3h and 21h in EGM-2 containing 20% FBS. Data points represent the mean±SEM for distinct ECFC populations or triplicate values for HPAECs. Statistics from two way ANOVA and Bonferroni post-hoc test. P4 – P5 ECFCs and P7 HPAECs were used in these experiments.

4.3.5 ECFCs from IPAH patients display reduced barrier functions

FRAP analysis was performed to assess gap junctional communication in ECFCs (Figure 4.8A). Gap junctional communication was impaired in ECFCs from IPAH patients, displaying only about half the fluorescence recovery rate of ECFCs from healthy volunteers (Figure 4.8B). IPAH cells also formed leaky monolayers that displayed 4-fold higher permeability, compared with cells from healthy volunteers (Figure 4.8C).

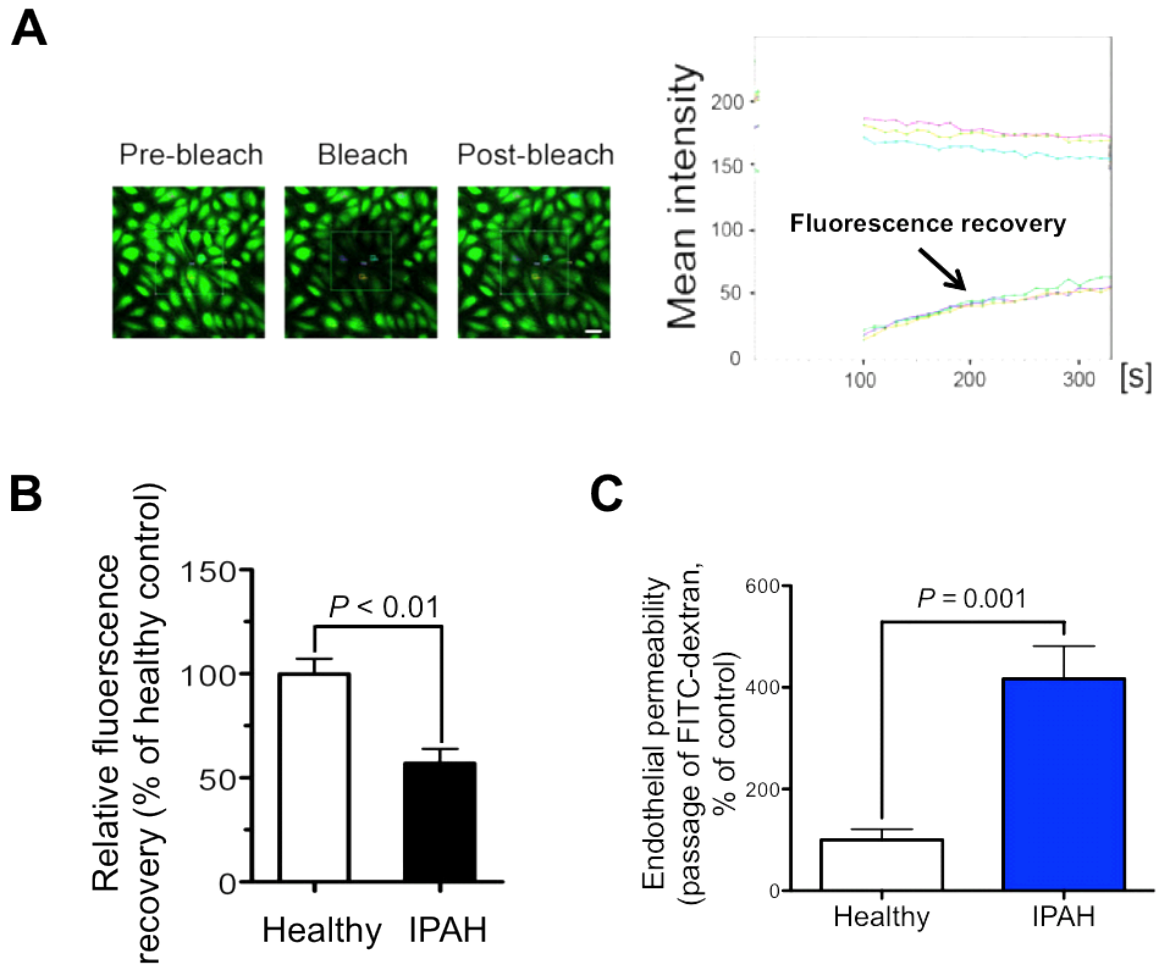


Figure 4.8. ECFCs from IPAH patients display impaired gap junctional communication and increased permeability. (A) Representative images of fluorescent recovery after photobleaching (FRAP) analysis. Confocal images show recovery of fluorescence in a laser-bleached area (marked with a square) in a monolayer of calcein-labelled HPAECs. The coloured traces in the corresponding graph show the kinetics of fluorescence recovery in three regions of interest as indicated by the arrow. Scale bar = 50µm. **(B)** FRAP data, expressed as a proportion of control, showing the relative levels of gap junctional communication in cultures of ECFCs from healthy volunteers (n=6) and IPAH patients (n=6). **(C)** Permeability of ECFC monolayers from healthy volunteers (n=6) and IPAH patients (n=6), as assessed by the flux of FITC-labelled dextran and expressed as a proportion that observed in ECFCs from healthy volunteers. Data presented as mean±SEM. Statistics was from t-test.

4.4 Discussion

The main findings of this chapter are:

- ECFCs from IPAH patients and healthy volunteers have a similar proliferative potential.
- ECFCs from IPAH patients are resistant to serum deprivation-induced apoptosis.
- ECFCs from IPAH patients possess a greater tube forming capacity than those from healthy volunteers.
- ECFCs from IPAH patients display a lower migratory capacity than those from healthy volunteers.
- ECFCs from IPAH patients also display impaired barrier function compared to ECFCs from healthy volunteers.

Endothelial dysfunction is considered to be critical factor in the initiation and progression of PAH (Budhiraja et al., 2004) and endothelial damage is postulated to initiate pulmonary vascular remodelling, contributing to intimal, medial and adventitial hypertrophy (Rabinovitch, 2008). The vascular remodelling includes the development of angioproliferative plexiform lesions, which have recently been found to be a relatively common feature in the explanted lungs of IPAH patients (Stacher et al., 2012). These lesions are found predominantly at sites immediately distal to the bifurcation of pulmonary vessels and severely reduce the cross-sectional area for blood flow (Cool et al., 1999). ECs within plexiform lesions are known to be monoclonal in primary (idiopathic) PAH, with distinct clones occurring in different regions of the same lung, but polyclonal in patients with secondary (associated) forms of the disease (Lee et al., 1998). ECs within IPAH plexiform lesions have also been found to display microsatellite instability within the *MSH2*, and microsatellite mutation and reduced expressions of *TGF*-β*RII* and pro-apoptotic *BAX* genes (Yeager et al., 2001). Indeed, acquired somatic mutations appear to occur relatively frequent in the PAECs of patients with PAH and may predate the development of plexiform lesions (Aldred et al., 2010). These data suggest that pulmonary ECs can acquire somatic mutations in genes regulating cell growth and apoptosis, which may lead to a selective advantage and monoclonal expansion of individual cells. Furthermore, ECs isolated from the pulmonary arteries of PAH patients are reported to be hyperproliferative and

apoptosis-resistant and display a predominantly glycolytic phenotype compared with control cells (Masri et al., 2007; Xu et al., 2007; Tu et al., 2011; Tu et al., 2012).

The origin of circulating ECFCs in IPAH remains elusive, there being no markers that distinguish them from mature ECs (Ingram et al., 2005). It was originally thought that while circulating endothelial cells may be derived form the vessel wall, ECFCs or blood outgrowth endothelial cells arose from bone-marrow derived angioblasts (Lin et al., 2000). But subsequent studies identified ECFCs in a 'vasculogenic zone' in blood vessels (Zengin et al., 2006) and the endothelium of various vascular beds (Ingram et al., 2005; Alvarez et al., 2008; Yoder, 2010). In fact, it has recently been suggested that ECFCs occur relatively frequently in pulmonary arteries, comprising 5-30% of ECs cultured from pulmonary arteries in explanted human lungs (Duong et al., 2011). It remains to be established if the rare ECFCs present in the circulation are derived from the vascular endothelium (Yoder, 2012), but blood-derived ECFCs from IPAH patients do appear to exhibit a disease-related phenotype and have at least some of the characteristics assigned to ECs in plexiform lesions and those cultured from the explanted lung tissues of patients with end-stage PAH.

Previous reports show that ECFCs isolated from patients with *BMPR2* gene mutations are more proliferative (Toshner et al., 2009), while ECFCs and PAECs derived from explanted lung tissues from IPAH patients also exhibit a hyperproliferative EC phenotype irrespective of whether they carry a known *BMPR2* mutation (Masri et al., 2007; Duong et al., 2011; Tu et al., 2011). On the other hand, Drake and colleagues recently demonstrated that PAECs isolated from explanted lungs of HPAH patients (n=4) with heterozygous mutations of *BMPR2* or *SMAD9* proliferated faster in culture than other forms of PAH (n=2) or controls (Drake et al., 2011). Interestingly, the rate of EC proliferation appeared to correlate not only with the extent of BMPR2 haploinsufficiency but also with an impaired response to the ALK1-selective agonist BMP9 and reduction in the BMP9-induced production of the anti-proliferative microRNAs miR-21 and miR-27a. I cannot exclude the possibility that at least some of the IPAH patients in the present study also possessed a mutation in the *BMPR2* gene as they were not genotyped and *BMPR2* mutations have been found to occur in ~20% of IPAH patients with no relevant family history of the disease (Thomson et al. 2000). I found a wide range of proliferative capacities among ECFCs, both from within and between different individuals, but no significant difference overall in either cell number, PDT or the incorporation of BrdU by ECFCs from IPAH patients and healthy volunteers.

ECFCs from IPAH patients possessed a number of other functional features that distinguished them from control cells. In particular, I found that IPAH-ECFCs were less sensitive to apoptosis induced by serum deprivation, exhibiting significantly lower levels of caspase-3/7 activity as well as less cleavage

in caspase-3 and its downstream target PARP compared to ECFCs from healthy volunteers and HPAECs. This is consistent with the finding of decreased numbers of apoptotic (TUNEL positive) ECs and strong immunostaining for the anti-apoptotic factors BCL-2 and BCL-xL in the endothelium of distal pulmonary arteries of patients with IPAH (Tu et al., 2011). Pulmonary ECs isolated from lungs of IPAH patients also displayed increased BCL-2 and BCL-xL expression and a reduced sensitivity to apoptosis induced by serum deprivation than ECs isolated from control lungs (Masri et al., 2007; Tu et al., 2011). The anti-apoptotic proteins survivin and apoptosis repressor with caspase recruitment domain (ARC) have both been implicated in the pulmonary vascular remodelling of IPAH and experimental PH, promoting PASMC survival and proliferation (McMurtry et al., 2005; Zaiman et al., 2011). In this study, I found no difference in ARC mRNA expression between the two groups of ECFCs, either at base level or upon apoptosis induction, indicating that ARC is probably more important as an anti-apoptotic factor in PASMCs than ECs (Zaiman et al., 2011). While survivin expression was also similar in the two groups of ECFCs under basal conditions, the expression of this anti-apoptotic factor was selectively decreased upon serum deprivation in ECFCs from healthy volunteers but not in cells derived from IPAH patients. Survivin is an important inhibitor of apoptosis, maintaining the hyperpolarisation of the mitochondrial membrane or preventing caspase activation in response to an apoptotic signal (McMurtry et al., 2005). In fact, the selection of apoptotic-resistant human pulmonary microvascular ECs in an *in vitro* artificial capillary (CELLMAX) system is accompanied by expression of survivin (Sakao et al., 2005) and the autocrine anti-apoptotic effect of VEGF is dependent, at least in part, on increased survivin expression (Farahani et al., 2005). It is possible that the reduction of survivin expression observed in control ECFCs following serum deprivation reflects the normal cellular response to apoptotic stimuli, whereas ECFCs from IPAH patients maintain their survivin expression in order to resist apoptosis.

Matrigel tube formation is traditionally used as an assay to measure the capacity of ECs to form capillary-like structures and is the preferred method for assessing angiogeneic capacity *in vitro* (Arnaoutova and Kleinman, 2010). Laminin is a major component of Matrigel and an important factor stimulating the attachment, migration and differentiation of endothelial cells into capillary-like structures (Kubota et al., 1988). I have demonstrated that ECFCs from IPAH patients have an enhanced capacity to form capillary-like structures in the Matrigel assay under a variety of conditions, involving both the manipulation of growth factors and serum content of the culture medium. The apparent decline in tube formation after 40h serum deprivation by both ECFCs from healthy volunteers and HPAECs but not IPAH-ECFCs, may also reflect the apoptosis-resistant phenotype of ECFCs from IPAH patients. Nonetheless, it is not known whether these cells also possess a superior angiogenic capacity *in vivo*.

Despite their superior capacity to form capillary-like structures, ECFCs from IPAH patients were consistently found to migrate slower in the wound healing assay compared with cells from healthy volunteers. These observations may seem contradictory, but it has recently been shown that EC tube formation in Matrigel is dependent more on the protrusion of filopodia, forming connections between adjacent endothelial cells that subsequently coalesce and differentiate into a tubule structure, than the proliferation or migration of cells (Logie et al., 2010). In fact, cell proliferation and migration appear to play little role in the *in vitro* development of EC tube-like structures and cortisol-mediated inhibition of tube formation was shown to occur without a measurable reduction in either EC migration (transwell migration assay), proliferation (BrdU assay) or viability (assessed by ATP production). Indeed, ECFCs from PAH patients with *BMPR2* mutations are reported to have impaired Matrigel tube formation *in vitro* while exhibiting no difference in transwell migration and enhanced proliferation compared to control cells (Toshner et al., 2009). The present results should therefore serve to emphasise functional differences between ECFCs derived from IPAH patients and healthy volunteers, rather than necessarily be extrapolated from one assay to another. Differences in extracellular matrix (ECM) may also confound comparisons between the migration and tube forming assays. Matrigel is mainly composed of laminin and collagen type IV, whereas I used gelatin-coated plasticware for the wound healing assay. Gelatin coating has also been used in other studies to assess difference in the migration of ECFCs from patients or infants with vascular disorders (Ligi et al., 2011; Starke et al., 2011). Growth factors and their receptors are known to co-ordinate with the ECM and ECM receptors (e.g. integrins) to regulate angiogenesis (Eliceiri, 2001). While laminin and collagen predominate in the ECM of mature vessels, fibronectin and its primary receptor (integrin α5β1) are predominantly expressed in developing vessels and considered to be essential in vascular physiology (Tian et al., 2012a). In fact, Matrigel does not contain fibronectin but recent data indicate that the fibronectin/integrin α5β1 pathway is intimately involved in the regulation of TGF-β superfamily signalling in ECs, affecting cell migration, apoptosis and capillary stability (Tian et al., 2012a). Other studies have also revealed that ECFCs are capable of depositing collagen IV, fibronectin, and laminin, whereas mature ECs only express these ECM proteins intracellularly, and ECFC-derived ECM is abrogated in response to the inhibition (SB431542) of TGF-β signalling and actin cytoskeleton disruption (Kusuma et al., 2012).

Thrombospondin-1 (THBS1) regulates the activation of TGF-β and inhibits EC and SMC proliferation, and loss-of-function mutations in the *THBS1* gene have been identified in heritable PAH (Maloney et al., 2012). Examination of gene expression in laser microdissected regions of the explanted IPAH and APAH lung also indicates that THBS1 expression is lower in the plexiform lesions and adjacent remodelled pulmonary vasculature, compared with arteries from control lung sections (Jonigk et al.,

2011). Plexiform lesions were also reported to show up-regulation of other "remodelling-associated genes", such as HIF-1α, VEGF-α, VEGFR-1/2 and Tie-2, and c-kit expression, and decreased apoptosis factor caspase 9. I found no apparent difference between THBS1 mRNA expression in ECFCs from IPAH patients and healthy volunteers, although HIF-1α protein levels were greater in ECFCs from IPAH patients than control cells (see Chapter 7).

One of the most important functions of the endothelium is to maintain a semi-permeable barrier, controlling the passage of blood-borne factors, inflammatory cells and fluid between the blood and tissue interstitial compartments. It is recognised that endothelial barrier disruption is likely to have a critical role in the development of PAH (Budhiraja et al., 2004). The loss of endothelial integrity enables the infiltration of inflammatory cells and activation of growth factor pathways and elastases in the underlying media, leading to the PASMC proliferation and vascular remodelling (Morrell et al., 2009). Furthermore, BMPR2 expression has been linked to pulmonary endothelial integrity. HPAECs with reduced BMPR2 expression display increased permeability, cytokine production and leukocyte recruitment, and the lungs of *BMPR2* deficient mice exhibit increased pulmonary vascular leakage and leukocyte infiltration *in vivo* (Burton et al., 2011). In the present study I also found evidence of disrupted barrier function, including impaired gap junctional communication and abnormal endothelial permeability, in ECFCs from IPAH patients.

Finally, apart from Matrigel tube formation, no apparent differences were observed between HPAECs and ECFCs from healthy volunteers. This is consistent with the endothelial lineage of ECFCs and corresponds with the results of other studies indicating that ECFCs and HUVECs display a similar sensitivity to apoptosis, migration, proliferation and tube forming capacity (Liu et al., 2005). It is unclear why HPAECs displayed superior tube formation to ECFCs, but it is possible that this reflects differences in sensitivity to serum depletion. HPAECs were routinely cultured in medium containing 2% FBS whereas the normal serum content for ECFC cultures was 20% FBS. HPAECs might therefore be more resilient than control ECFCs to the depletion of growth factors and serum in the Matrigel assay.

There are a number of limitations to the studies in this chapter. Ideally, the patients providing blood samples would have been genotyped for known mutations in *BMPR2* and for rare mutations in *ALK1*, *ENG* (gene for endoglin), *SMAD1* and *SMAD9* (gene for Smad8). I would have liked to also include ECFCs derived from patients with other forms of PAH, as well as types of PH that do not develop plexiform lesions, and compare blood-derived ECFCs with those directly derived from explanted lung tissues. In addition, it would have been interesting to determine whether differences observed between ECFCs *in vitro* were also apparent *in vivo,* using for example a Matrigel plug assay in mice,

and if cells derived from IPAH patients exhibited the chromosomal abnormalities that have been found in PAECs from PAH patients.

In this chapter, I have demonstrated that ECFCs from IPAH patients exhibit a distinct functional phenotype, but the underlying molecular mechanisms are not clear. BMPs have been shown to be critical in the differentiation, regulation and angiogenic capacity of ECFCs (Smadja et al., 2008). In addition to mutations in the *BMPR2* gene, PAH can also arise from mutations in *ALK1* (Trembath et al., 2001; Harrison et al., 2003), an endothelial-selective receptor that responds to the circulating quiescent factor BMP9 (David et al., 2007a; Scharpfenecker et al., 2007). Given the important roles that BMP and TGF-β signalling play in the pathogenesis of IPAH and regulation of EC functions (Morrell, 2010), I will focus on exploring the possible dysfunction of these pathways in ECFCs.

In Chapter 5, I will investigate if members of the TGF-β receptor superfamily are differentially expressed in ECFCs from IPAH patients and healthy volunteers and determine whether downstream BMP and TGF-β signalling pathways are affected.

In Chapter 6, I will investigate whether differences in BMP and TGF-β signalling contribute to the distinct functional phenotype of ECFCs from IPAH patients.

Chapter 5:

Expression and signalling of the transforming growth factor-β (TGF-β) receptor superfamily in ECFCs

Chapter 5 – Expression and signalling of the transforming growth factor-β (TGF-β) receptor superfamily in ECFCs

5.1 Introduction

Abnormalities in TGF-β/BMP signalling pathways are associated with PAH. As discussed in Chapter 1.2.8, *BMPR2* mutations have been identified in most cases of HPAH and 10 – 40% of IPAH patients. The dysregulation of TGF-β signalling may differ however in IPAH patients without mutations of genes encoding BMP receptors and canonical signalling molecules, as differences in the expression pattern of the TGF-β/BMP superfamily have been found that could differentiate these groups of patients (Geraci et al., 2001). Indeed, as discussed in Chapter 1.7.5, dysregulation of both BMP and TGF-β signalling pathways has been reported in IPAH patients and pre-clinical models of PH. Nevertheless, the role of TGF-β signalling in regulating pulmonary EC function and the pathogenesis of the disease is still unclear. The pulmonary endothelium of IPAH patients displays several changes in gene expression and activation, which persist *in vitro* and are associated by the dysregulation of endothelial functions (Xu et al., 2004; Dewachter et al., 2006; Masri et al., 2007; Xu et al., 2007; Fijalkowska et al., 2010). The expression of TGF-β receptors has been poorly assessed in ECs isolated from the explanted lungs of IPAH patients, possibly reflecting the limited nature of this resource. ECFCs represent a readily accessible autologous patient cell type of endothelial lineage, which can be obtained throughout the course of the disease and may provide further insights in the dysregulation of TGF-β receptors and signalling in the pathogenesis of IPAH.

In this chapter I will use PCR and Western blotting techniques to conduct a comprehensive analysis of members of the TGF-β receptor superfamily in ECFCs derived from IPAH patients and healthy volunteers. Possible dysregulation of endothelial TGF-β signalling will be determined both by examining expression of ALK1, ALK5 and TGF-βRII receptors and the accessory membrane protein endoglin, and assessing TGF-β1-induced activation of Smad2/3 (Goumans et al., 2002). BMP9 and BMP10 are specific agonists for ALK1, which is almost exclusively expressed by ECs and forms a receptor complex with BMPR2 and other type 2 receptors, ActRIIA and ActRIIB (David et al., 2007a; Townson et al., 2012). In addition to the expression of these receptors, signalling will be assessed by stimulating the cells with BMP9 and determining the activation of the downstream targets pSmad1/5/8 and ID gene expression. Further details on receptor combination and their specific agonists can be found in Figure 1.5 in Chapter 1.7.

The main objectives of this chapter are to:

- Determine mRNA expression of members of the TGF-β receptor superfamily in ECFCs from IPAH patients and healthy volunteers, using semi-quantitative PCR and qPCR.
- Confirm differences in mRNA expression at the protein level, using Western blotting.
- Investigate dysregulation of ALK5/TGF-βRII signalling by assessing TGF-β1-stimulated Smad2 phosphorylation in ECFCs.
- Investigate dysregulation of ALK1/BMPR2 signalling by assessing BMP9-stimulated Smad1/5/8 phosphorylation and *ID* gene expression.

5.2 Methods

See Chapter 2 for detailed experimental materials and protocols.

5.2.1 Expression of TGF-β receptor superfamily

Expression of members of the TGF-β receptor superfamily was examined in ECFC populations with the capacity to grow beyond passage 8. This included expression of both TGF-β type 1 (ALK1, ALK2, ALK3, ALK4, ALK5, ALK6) and type 2 receptors (ActRIIA, ActRIIB, BMPR2, TGF-βRII), the accessory membrane protein endoglin (CD105) and a related protein caveolin-1. Cells were seeded (5000/cm^2 in EGM-2 containing 20% FBS) in 10-cm petri dishes and grown to confluency, prior to the extraction of mRNA or protein. In parallel studies, ECFCs were cultured to sub-confluency (typically ~70% confluent) in normal culture medium, or confluent cells serum-deprived for 13-18 h in EBM-2 containing 0.1% FBS before assessing receptor expression. RNA was extracted from cultured cells using either Trizol (Sigma) or RNeasy kits (QIAGEN) and proteins extracted in RIPA buffer, as described in Chapter 2. The expression level of selected receptors was also compared in surgical samples of lung tissue from IPAH patients and lobectomy controls, as described below.

5.2.2 BMP and TGF-β signalling

Potential differences between the canonical BMP and TGF-β signalling pathways, in ECFCs from IPAH patients and healthy volunteers, were determined by examining the effects of (1) TGF-β1 on Smad2/3 activation (phosphorylation – pSmad2/3), and (2) BMP9 (an ALK1-selective ligand) on Smad1/5/8 activation (phosphorylation – pSmad1/5/8) and downstream transcription of *ID* genes. As junctional clustering between confluent ECs is critical for optimal TGF-β signalling (Rudini et al., 2008), all ECFCs were seeded (5000/cm^2) in gelatin-coated 6-cm dish and cultured to confluence before proceeding to the following signalling experiments. For Western blot analysis of TGF-β signalling, ECFCs were serum-deprived for 12 h in EBM-2 with 0.1% FBS, before stimulation with TGF-β1 (0.001 – 2.5 ng/ml) for 1h. Following SDS-PAGE separation, membranes were probed first for pSmad2 and then re-probed for Smad2, the latter typically being detected within a few seconds of exposure to autoradiography film. The optimal ligand concentration and duration of stimulation was established before comparing Smad phosphorylation in ECFCs from healthy volunteers and IPAH patients. The specificity of TGF-β1-induced pSmad2 was also established using the ALK5 inhibitors

SD208 and SB431542. Cells were treated with 0–15 μM SD208 or SB431542, 30 min before stimulation by TGF-β1. For Western blot analysis of BMP signalling, ECFCs were seeded at 5000 cells/cm^2 in 6-cm dishes and grown to full confluency (typically 4 days). Cells were then serum-deprived for 12 h in EBM-2 with 0.1% FBS, and stimulated with 0.01–10 ng/ml of BMP9 for 1 h in fresh serum-deprived medium. Equal amounts of protein were probed in separate gels for pSmad1/5/8 and total Smad1 protein expression, the latter typically being demonstrated after 30 min exposure to radiography film. The specificity of BMP9-induced pSmad1/5/8 was also established using the ALK1 inhibitors ALK1-Fc chimera (ALK1-Fc). BMP9-containing medium were reconstituted with 0–10 μg/ml ALK1-Fc, or cells treated with 15 μM SD208, 30 min before stimulation by BMP9. All blots were reprobed for β-actin to act as loading control. Further details of the Western blotting methods and antibodies used are provided in Chapter 2.11 and Table 2.5.

The downstream effects of BMP9 stimulation on the transcription of *ID* genes was examined in confluent ECFCs, following serum-deprivation (EBM-2 and 0.1% FBS) for 16h and stimulation with 0.01–10 ng/ml BMP9 (in fresh EBM-2 and 0.1% FBS) for 2, 4, and 8h. RNA was extracted using Trizol (Invitrogen) as this provided a higher concentration of RNA from a smaller culture surface area when compared with extraction by RNeasy kit**.** ID1, ID2 and ID3 mRNA expression was first analysed by semi-quantitative RT-PCR and then validated in separate experiments using qRT-PCR, as described in Chapter 2.

5.2.3 Lung tissue samples

Lung tissue samples were previously obtained with the informed consent of patients and the approval of the Brompton, Harefield & NHLI and Hammersmith and Queen Charlotte's & Chelsea Hospitals Research Ethics Committees (Ref No. 01-210 and 2001/6003). Fresh surgical specimens of explanted lung tissue were collected, either at transplantation or at lobectomy/pneumonectomy for carcinoma of the bronchus, snap frozen within 4 hours of surgery and stored at -80°C. RNA was extracted from comparable frozen samples of peripheral lung tissue (200-500mg each) from patients with IPAH (n=12) and controls (n=7), using RNeasy kits (QIAGEN).

5.2.4 Reagents

Details on the reagents used were described in Chapter 2.14.

Where receptor inhibitors (e.g. SB 431542 and SD208) were used, cells were treated with inhibitors or DMSO solution (vehicle) 30 min in prior to treatments with TGF-β1. ALK1 binding sites were blocked by incubating treating medium with recombinant human ALK1 antibodies for 30 min in prior to treatment.

5.2.5 Data presentation

Western blotting and RT-PCR data are presented as mean±SEM. Data from semi-quantitative PCR analysis are presented as a ratio relative to the reference gene β2-microglobulin. Data from qPCR analysis are presented as ratio following normalisation to PPIA and α-tubulin expression, as described in Chapter 2.10.2. Data of protein expression from Western blotting are presented as a ratio relative to β-actin. Levels of pSmad1/5/8 were assessed following normalisation against Smad1 and β-actin, enabling cross-comparison between different blots. pSmad2 levels were assessed following normalisation against Smad2 in the same blot. All Smad phosphorylation and ID gene expression data are presented as fold changes against vehicle controls.

5.3 Results

5.3.1 Differential expression of the TGF-β receptor superfamily in ECFCs from IPAH patients

The integrity of RNA extracted from ECFCs was confirmed by demonstrating two clear 28S and 18S ribosomal bands (Figure 5.1A). The expression of the TGF-β receptor superfamily was examined with semi-quantitative RT-PCR, using optimal PCR conditions to ensure amplified products were within their linear phase of amplification and was not over-saturated.

Semi-quantitative RT-PCR examination of mRNA expression showed that ECFCs expressed the TGF-β type 1 receptors ALK1, ALK2, ALK3, ALK4, ALK5, and ALK6, and type 2 receptors ActRIIA, ActRIIB, BMPR2, and TGF-βRII, as well as the accessory membrane protein endoglin (CD105) and caveolin-1 (Figures 5.1 & 5.2). Except for ALK3 and ALK6 transcripts, which were generally in low abundance, expression of the various receptors was demonstrated in all ECFC populations examined and in HPAECs. As expected, human PASMCs exhibited relatively high levels of ALK5 and lacked expression of the endothelial specific ALK1 receptor (Figure 5.1). Interestingly, the relative expression of ALK5 and ActRIIA receptors was significantly lower in ECFCs from IPAH patients, when compared with cells derived from healthy volunteers (Figure 5.1 & 5.2).

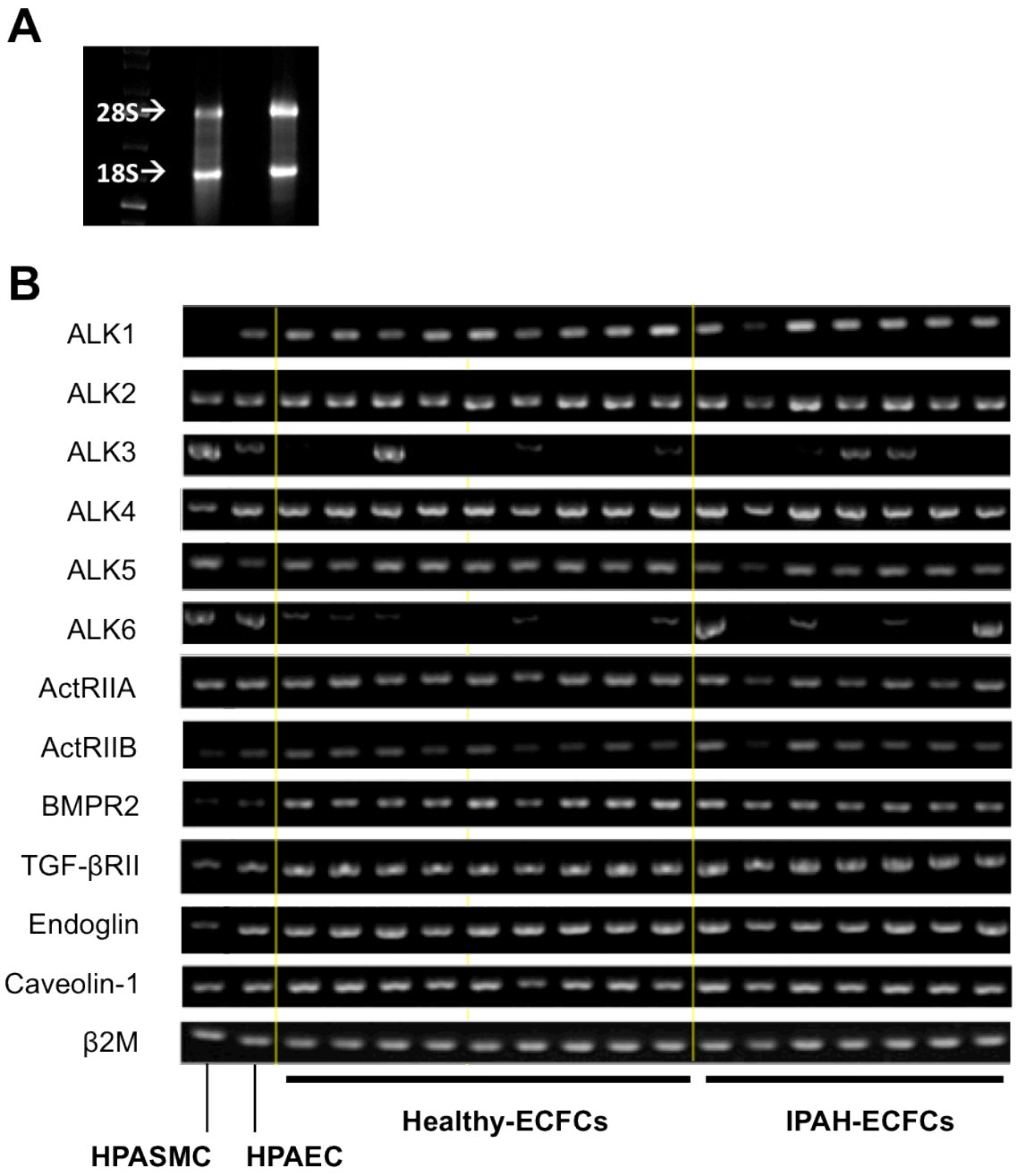


Figure 5.1. Expression of the TGF-β receptor superfamily in ECFCs, HPAECs and HPASMCs. (A) RNA integrity in two ECFC extracts, as demonstrated by distinct 18S and 28S rRNA bands following GelRed-stained agarose gel electrophoresis. **(B)** Expression of TGF-β type 1 receptors ALK1, ALK2, ALK3, ALK4, ALK5, and ALK6; type 2 receptors ActRIIA, ActRIIB, BMPR2, and TGF-βRII; endoglin; caveolin-1 and β2-microglobulin (β2M) mRNA in ECFCs from healthy volunteers (n=9) and IPAH patients (n=7). HPAECs were included as a mature endothelial cell control, while HPASMCs were used as negative control for ALK1 expression. mRNA transcripts were reverse-transcribed into cDNA and subjected to PCR amplification before being separated by GelRed-stained agarose gel electrophoresis.

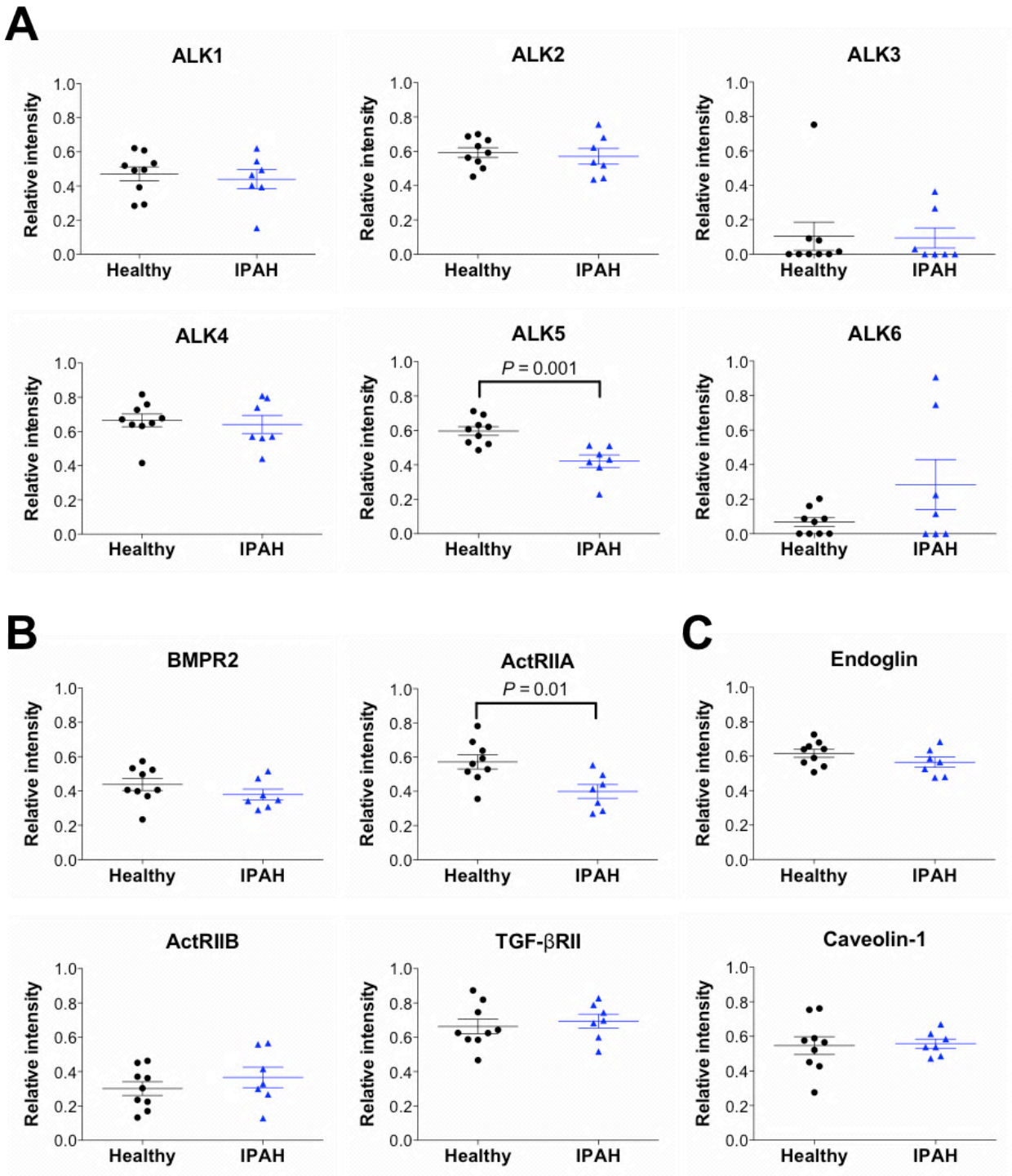


Figure 5.2. Semi-quantitative analysis of TGF-β receptor superfamily mRNA expression in ECFCs. The intensity of the bands in Figure 5.1 was measured using ImageJ and normalised against the reference gene β2-microglobulin for each cell population. Relative expression of **(A)** TGF-β type 1 receptors ALK1, ALK2, ALK3, ALK4, ALK5, and ALK6; **(B)** type 2 receptors ActRIIA, ActRIIB, BMPR2, and TGFβRII; **(C)** endoglin and caveolin-1 in ECFCs from healthy volunteers (n=9) and patients with IPAH (n=7). Data are presented as mean±SEM. Statistics shown are obtained by t-test.

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In subsequent studies, quantitative RT-PCR was used to examine the expression of ALK5 and ActRIIA, as well as four other genes (ALK1, endoglin, BMPR2, TGF-βRII) implicated in regulating endothelial function and the pathophysiology of PAH. Following culture in complete EGM-2 medium and 20% FBS, the expression levels of ALK5, ActRIIA and endoglin were all found to be significantly lower in confluent ECFCs from IPAH patients versus healthy volunteers. Interestingly, the levels of ALK5, TGF-βRII and endoglin expression were also greater in ECFCs from healthy controls than HPAECs (Figure 5.3A). In ECFCs that were serum-deprived in EBM-2 and 0.1% FBS for up to 18h, ALK5 expression was again significantly lower in cells derived from IPAH patients compared with healthy volunteers (Figure 5.3A-B). Decreased ALK5 expression was also confirmed in ECFCs cultured in EGM-2 with 2% FBS for 48 h (Figure 5.3C). In addition to ECFCs, lower ALK5 mRNA levels were observed in lung tissues from end-stage IPAH patients versus control lobectomy samples (Figure 5.4). BMPR2 expression was also lower in lung homogenates from IPAH patients compared with control tissues, whereas no significant difference was found in ALK1 or endoglin expression (Figure 5.4).

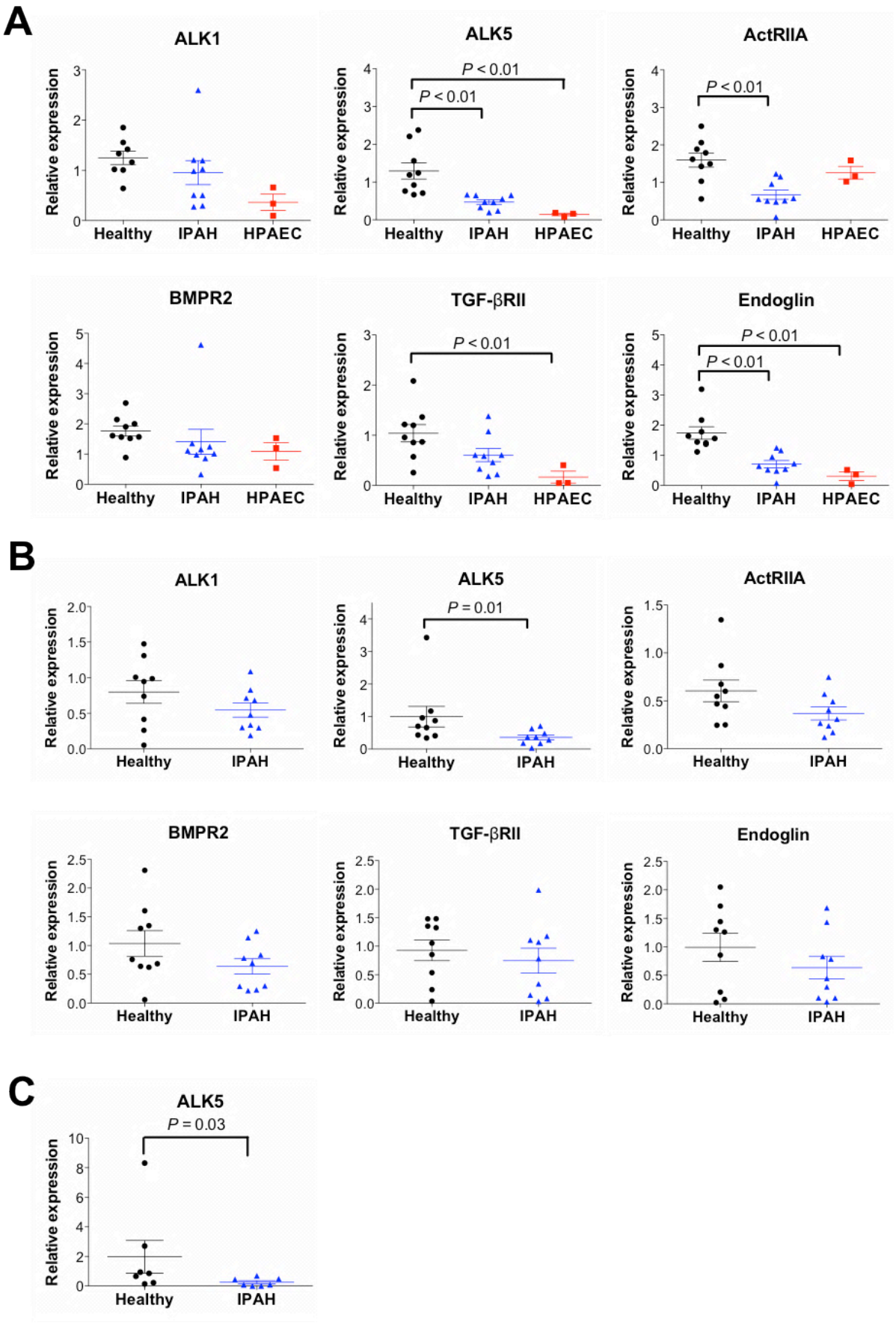

A
ALK1
ALK5
ActRIIA
BMPR2
TGF-βRII
Endoglin
Relative expression
P < 0.01
Healthy
IPAH
HPAEC
B
P = 0.01
C
P = 0.03

Figure 5.3. Relative expression of ALK1, ALK5, ActRIIA, BMPR2, TGFBRII and endoglin in ECFCs and HPAECs. Quantitative RT-PCR analysis of mRNA expression in confluent ECFCs from healthy volunteers (n=9) and IPAH patients (n=9) and HPAECs (n=3). **(A)** ECFCs cultured in EGM-2 with 20% FBS and HAPECs in EGM-2 with 2% FBS. **(B)** ECFCs serum-deprived in EBM-2 and 0.1% FBS for 18 h prior to RNA extraction. **(C)** Decreased ALK5 expression is confirmed in ECFCs cultured in EGM-2 with 2% FBS for 48 h. Data are presented as mean±SEM. Statistics obtained following (A) one-way ANOVA and Bonferroni post-hoc analysis and (B-C) t-test or Mann-Whitney test where appropriate. The data presented is representative of at least 2 independent experiments.

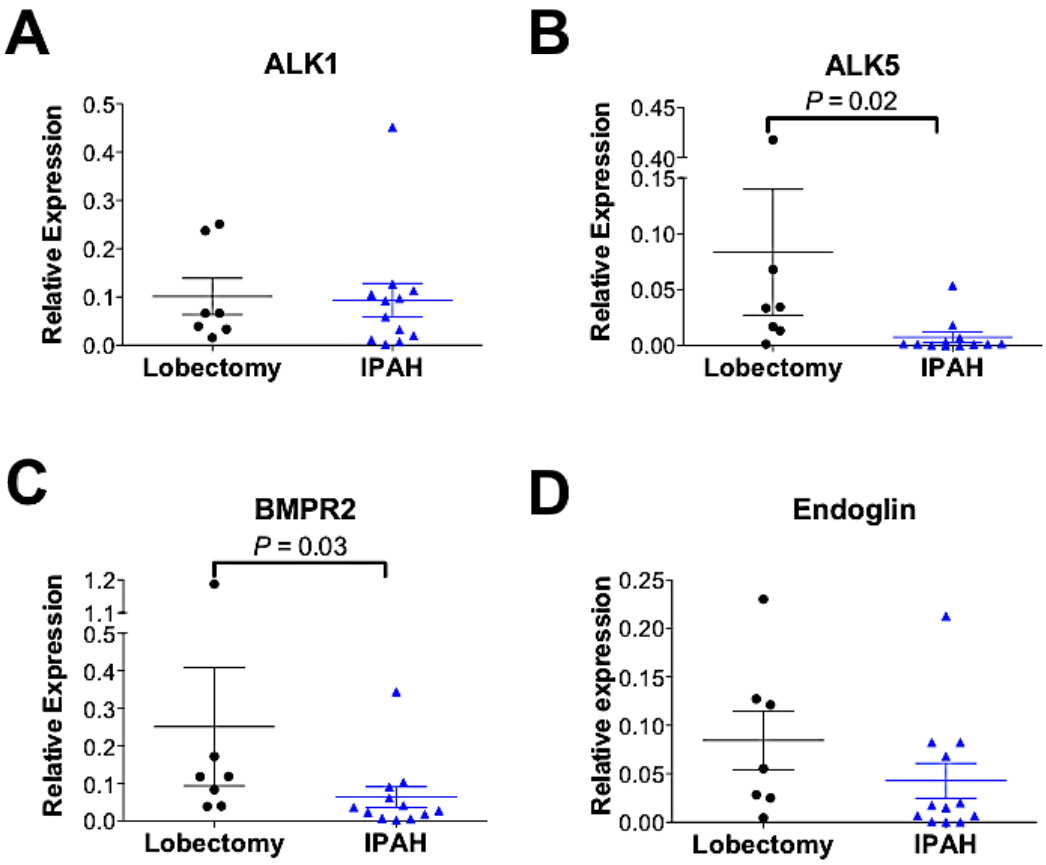


Figure 5.4. Relative expression of (A) ALK1, (B) ALK5, (C) BMPR2 and (D) endoglin mRNA in human peripheral lung. Surgical samples obtained at lobectomy (n=7) and transplantation (IPAH, n=12). Data are presented as mean±SEM. Statistics obtained from Mann-Whitney test.

5.3.2 ALK5 protein expression and Smad2 signalling in ECFCs

Confluent ECFCs from IPAH patients exhibited lower (~40% less) ALK5 protein levels compared with ECFCs from healthy volunteers (Figure 5.5A). This difference was not apparent however in an additional experiment, which compared ALK5 protein expression in sub-confluent cultures of ECFCs (Figure 5.5B).

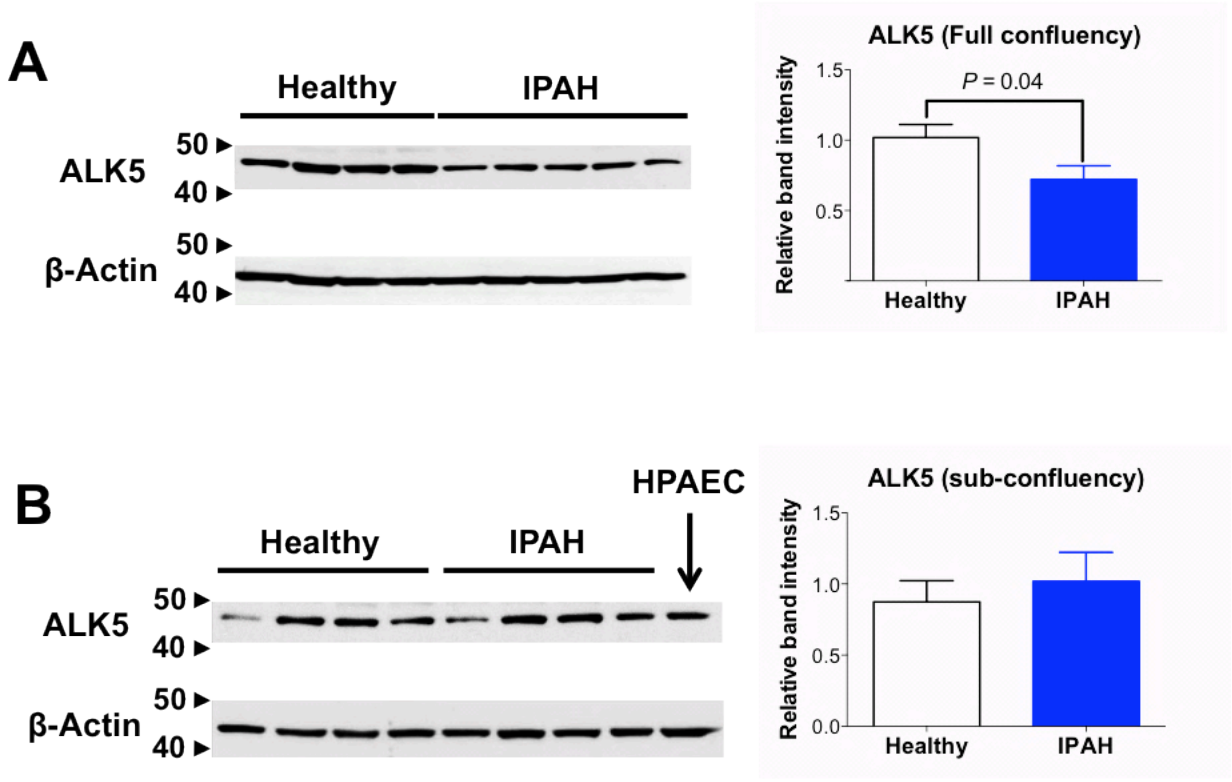


Figure 5.5. ALK5 protein expression in confluent and sub-confluent ECFCs. Representative Western blots and quantitative assessment of ALK5 protein expression in **(A)** confluent ECFCs from healthy volunteers (n=8) and IPAH patients (n=9) and **(B)** sub-confluent ECFCs from healthy volunteers (n=7) and IPAH patients (n=6). Data are presented as mean±SEM. Statistics obtained from t-test.

To explore whether intracellular signalling was affected in ECFCs exhibiting reduced ALK5 receptor expression, the effect of TGF-β1 (an ALK5 agonist) on downstream Smad2 phosphorylation was examined. Firstly, the selective effect of TGF-β1 on Smad activation was assessed by examining its ability to stimulate pSmad2 but not pSmad1/5/8 in ECFCs (Figure 5.6A-B).

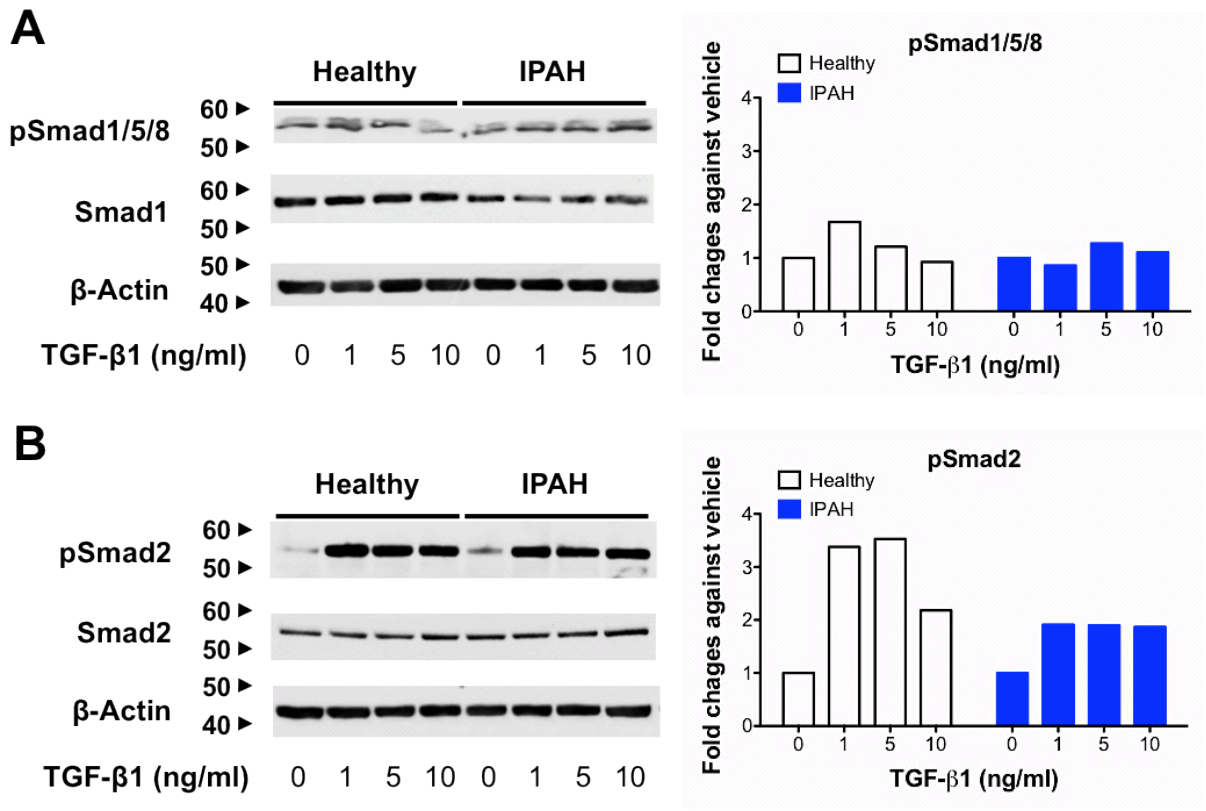


Figure 5.6. TGF-β1-stimulated phosphorylation of Smad1/5/8 (pSmad1/5/8) and Smad2 (pSmad2). Representative Western blots from ECFCs from a healthy volunteers and a patient with IPAH, showing expression of **(A)** pSmad1/5/8 and total Smad1, and **(B)** pSmad2 and total Smad2 after serum-deprived (12 h in 0.1% FBS) and treated with TGF-β1 (1–10 ng/ml) for 1 h, with desitometric anaylsis of the bands displayed on the right.

It was apparent that 1–10 ng/ml TGF-β1 over saturated the activation of pSmad2, hence ECFCs were examined using a wider range of TGF-β1 concentrations (0.001-2.5 ng/ml). TGF-β1 induced a concentration- and time-dependent stimulation of pSmad2, while having no effect on total Smad2 protein levels (Figure 5.7A-B). These initial experiments indicated that TGF-β1 had a biphasic influence on confluent ECFCs, pSmad2 levels rising to a maximum 1 h after stimulation with 0.05 ng/ml TGF-β1 and declining in the presence of increasing concentrations of TGF-β1. The stimulation of pSmad2 was attenuated with the addition of the selective ALK5 inhibitor SD208 in a concentration-dependent manner, and was abolished in the presence of 15 µM SD208 (Figure 5.7C). The addition of SB431542, another ALK5 inhibitor, also abolished pSmad2 in TGF-β1 stimulated ECFCs (Figure 5.7D).

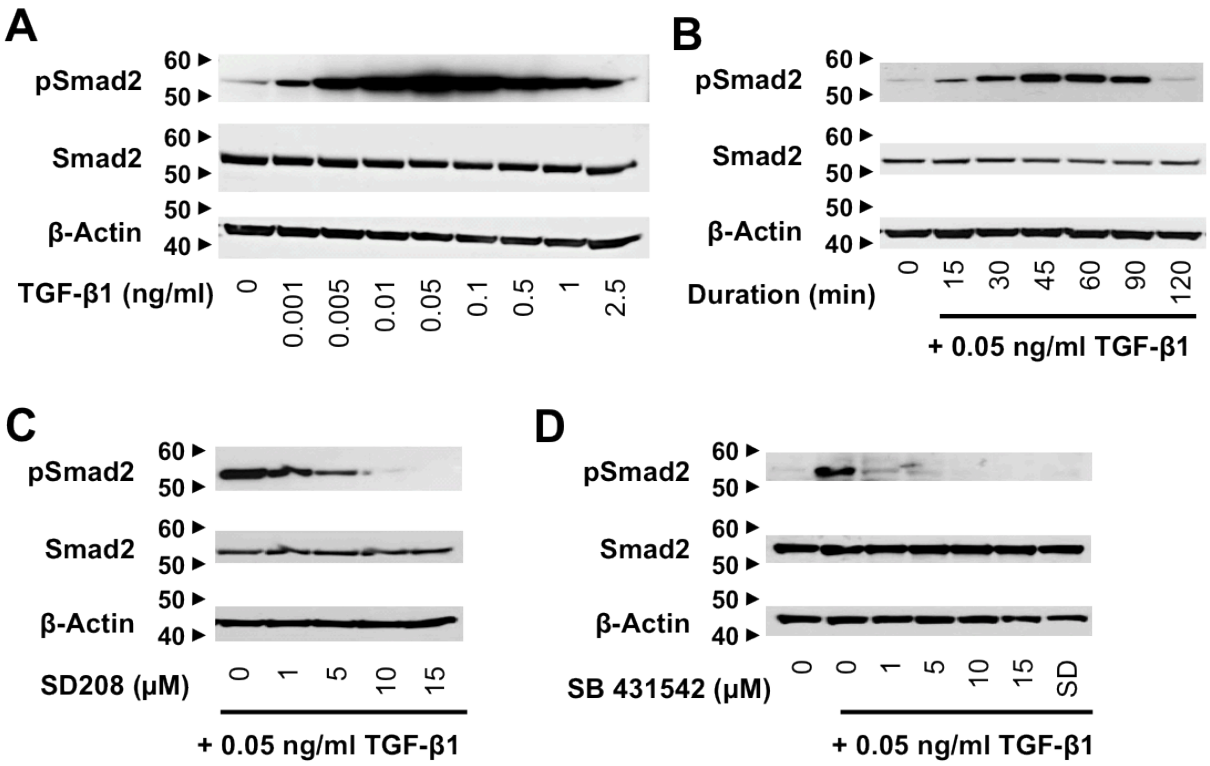


Figure 5.7. TGF-β1-stimulated phosphorylation of Smad2 (pSmad2). Representative Western blots showing expression of pSmad2 and total Smad2 in serum-deprived ECFCs. **(A)** pSmad2 and total Smad2 in the presence of vehicle alone (0) or increasing concentrations of TGF-β1 (0.001 – 2.5 ng/ml) for 1 h. **(B)** pSmad2 and Smad2 expression in ECFCs after stimulation with 0.05 ng/ml TGF-β1 for various times (0 – 120 min). **(C-D)** TGF-β1 (0.05 ng/ml for 1 h) stimulated pSmad2 in the presence of the ALK5 inhibitors SD208 and SB431542 (1 – 15 μM). Treatment with SD208 (SD, 15 μM) was used as control (D). All blots were reprobed for β-actin to ensure equal protein loadings. Results shown are representative of at least 1 healthy-ECFC sample, and similar effective range of concentrations and durations of treatment with TGF-β1 and ALK5 inhibitors were seen in IPAH-ECFC.

Stimulation with 0.005, 0.05 and 0.5 ng/ml TGF-β1 for 1 h induced similar levels of Smad2 phosphorylation in ECFCs from IPAH patients and healthy volunteers (Figure 5.8A-C). HPASMC were used as a control (Figure 5.8B) as they exhibit high ALK5 expression and low ALK1 expression (Seki et al., 2006) and can be efficiently stimulated by TGF-β1 (Upton et al., 2009). A differential response was observed however when ECFCs were stimulated with 1.0 ng/ml TGF-β1, pSmad2 levels being lower in cells from IPAH patients compared with those derived from healthy volunteers (Figure 5.8D-E).

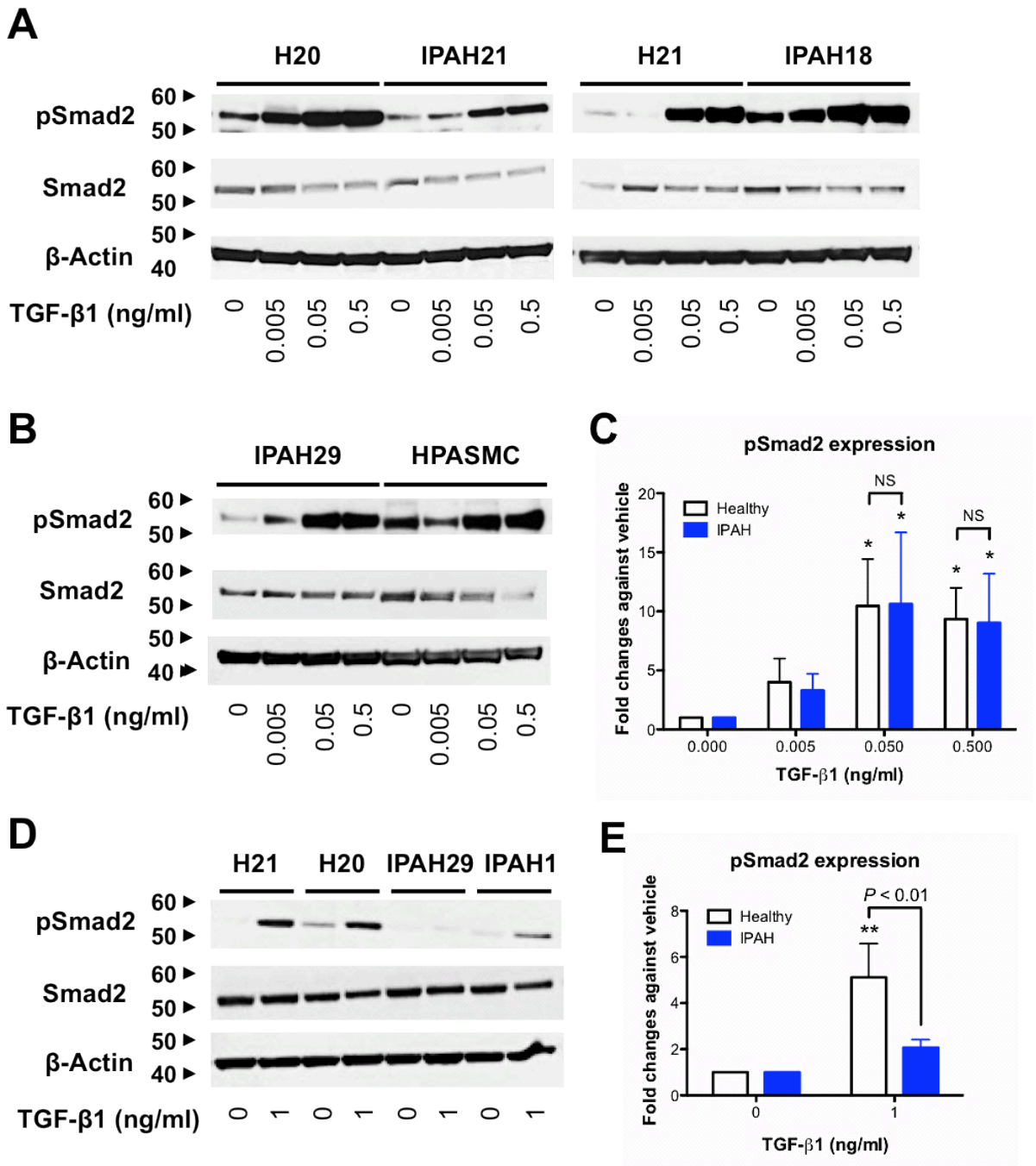


Figure 5.8. TGF-β1-stimualated phosphorylation of Smad2 in ECFCs from healthy volunteers and IPAH patients. Confluent ECFCs were serum-deprived in 0.1% FBS for 12h before being treated with TGF-β1 or vehicle alone (0) for 1 h. **(A-C)** Representative Western blots and quantitative assessment of pSmad2 in ECFCs from IPAH patients (n=6) and healthy volunteers (n=6), with HPASMCs used here as controls. Blots were reprobed for β-actin to ensure equal protein loadings. **(D)** Representative Western blots of pSmad2 and Smad2 in two healthy (H20 and H21) and IPAH-derived ECFC populations (IPAH1 and IPAH29) after treatment with vehicle (0) or 1 ng/ml TGF-β1 for 1 h. **(E)** Quantitative assessment of pSmad2, following treatment with 1 ng/ml TGF-β1, in ECFCs from IPAH patients (n=6) and healthy volunteers (n=6). Data presented as fold changes compared with vehicle controls. Bars represent mean±SEM. Statistics obtained from two-way ANOVA, with Bonferroni post-hoc analysis. *, $P < 0.05$; **, $P < 0.01$, compared with vehicle controls.

5.3.3 BMPR2 protein expression and Smad1/5/8 signalling in ECFCs

Previous studies have indicated that pulmonary BMPR2 expression is affected in patients with IPAH, with a reduction in BMPR2 protein rather than mRNA levels being observed in diseased lung tissues and isolated pulmonary vascular cells (Atkinson et al., 2002; Dewachter et al., 2009; Alastalo et al., 2011). I therefore used Western blotting and a widely employed antibody against human BMPR2 to compare BMPR2 protein levels in ECFCs. Three distinct molecular weight bands were identified in proteins extracted from confluent ECFCs and HPAECs, corresponding to ~110, ~130 and ~150 kDa (Figure 5.9A, 5.10A and 5.11F), and the intensity of all three bands decreased upon knockdown by siRNA targeting BMPR2 (Figure 5.11F). The intensity of the three BMPR2 molecular weight bands showed marked variation between different ECFC populations and no significant difference between the two groups of ECFCs, this being reproducible in several different experiments as well as in cells that had been deprived of serum prior to the extraction of proteins (Figure 5.9B-E & 5.10B-E). Human BMPR2 transcripts are alternatively spliced to produce at least two primary transcripts and BMPR2 protein isoforms (Liu et al., 1995; Rosenzweig et al., 1995), the ratio between the two having recently been used to distinguish BMPR2 mutant PAH patients from unaffected carriers (Cogan et al., 2012). I therefore compared the ratio of the bottom band versus the top two bands and found that this was significantly lower in confluent ECFCs from IPAH patients cultured compared with cells from healthy volunteers (Figure 5.9F & 5.10F).

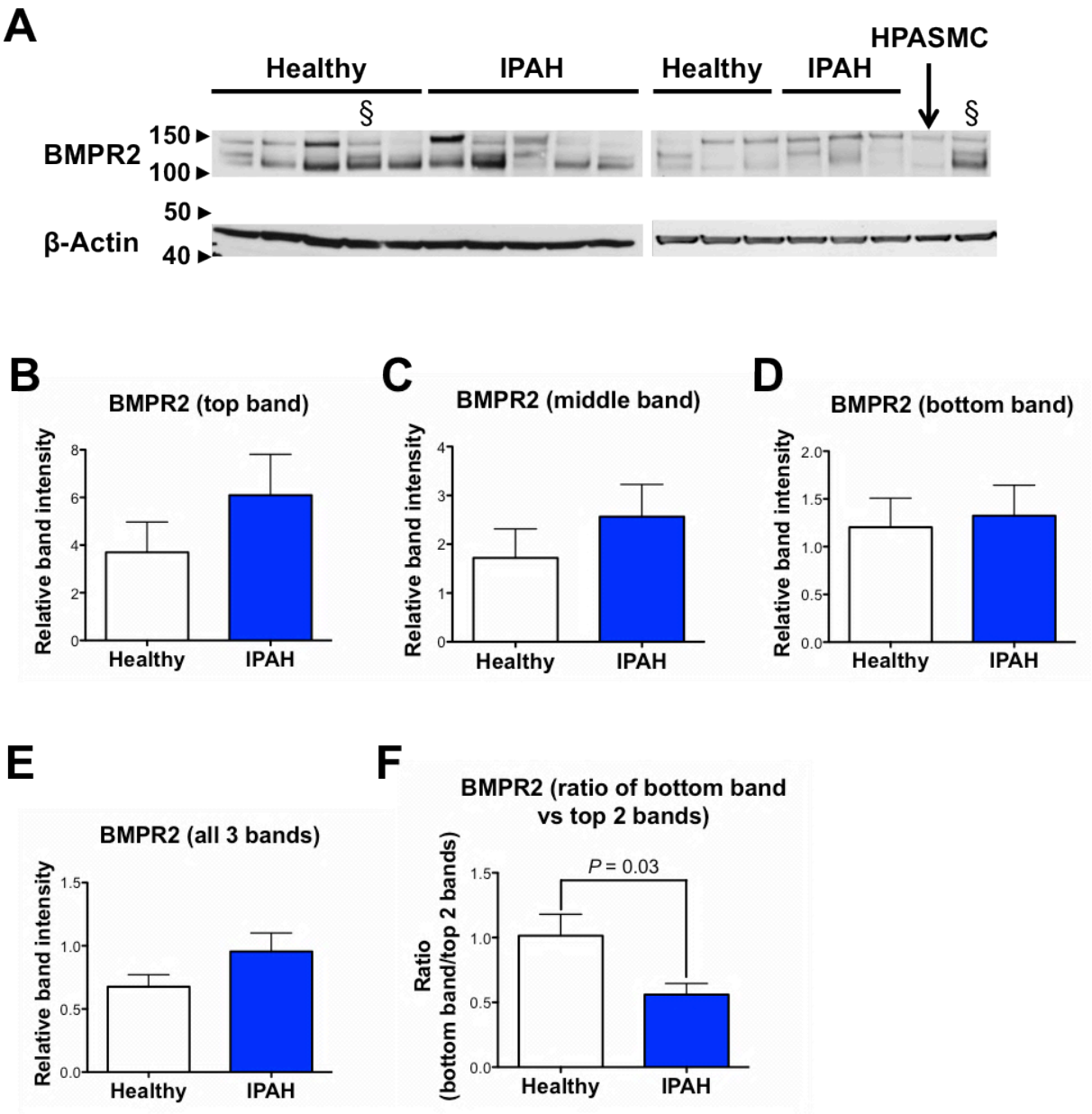


Figure 5.9. BMPR2 protein expression in confluent ECFCs cultured in the presence of 20% FBS. **(A)** Representative Western blots showing varying expression of BMPR2 molecular weight bands in two distinct groups of confluent ECFCs from healthy volunteers and IPAH patients (n=8 in each group). Confluent HPASMCs extract was used as control. § indicates the same ECFC sample run on both gels for comparison. Graphs display the quantitative assessment of the relative intensity of the **(B)** top band, **(C)** middle band, **(D)** bottom band, and **(E)** the total intensity of all three bands of the detected BMPR2 expression. **(F)** The relative intensity of the bottom band versus the top two bands. Bars represent mean±SEM. Data are representative of 2 independent experiments. Statistics shown was from t-test.

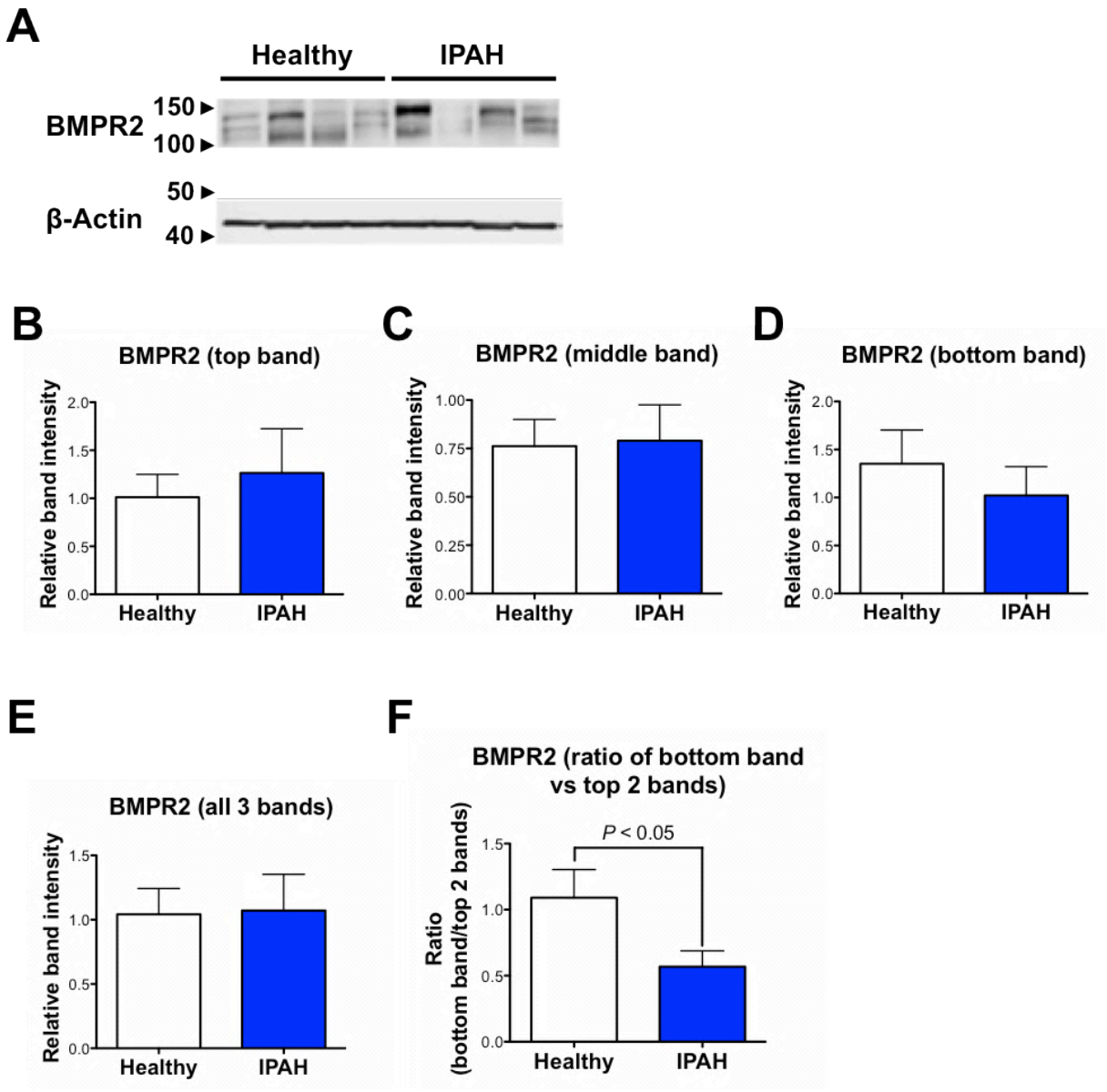


Figure 5.10. BMPR2 protein expression in confluent ECFCs following serum-deprivation. **(A)** Representative Western blots showing varying expression of BMPR2 molecular weight bands in confluent ECFCs from healthy volunteers and IPAH patients (n=6-7), harvested after 13 h serum deprivation in 0.1% FBS. Graphs display the quantitative assessment of the relative intensity of the **(B)** top band, **(C)** middle band, **(D)** bottom band, and **(E)** the total intensity of all three bands of the detected BMPR2 expression. **(F)** The relative intensity of the bottom band versus the top two bands were analysed. Bars represent mean±SEM. Data are representative of 2 independent experiments. Statistics shown was from t-test.

In contrast to confluent ECFCs, two molecular weight bands (~110 and ~150 kDa) dominated the Western blots of sub-confluent cells and HPAECs (Figure 5.11A), but no significant difference were observed between the two groups of ECFCs (Figure 5.11B-E). Two similar protein bands were found in extracts of confluent HPASMCs (Figure 5.9A), whereas two distinct BMPR2 isoforms appeared to predominate in HPAECs (~110 kDa) and HepG2 cells (~150 kDa) respectively (Figure 5.11F).

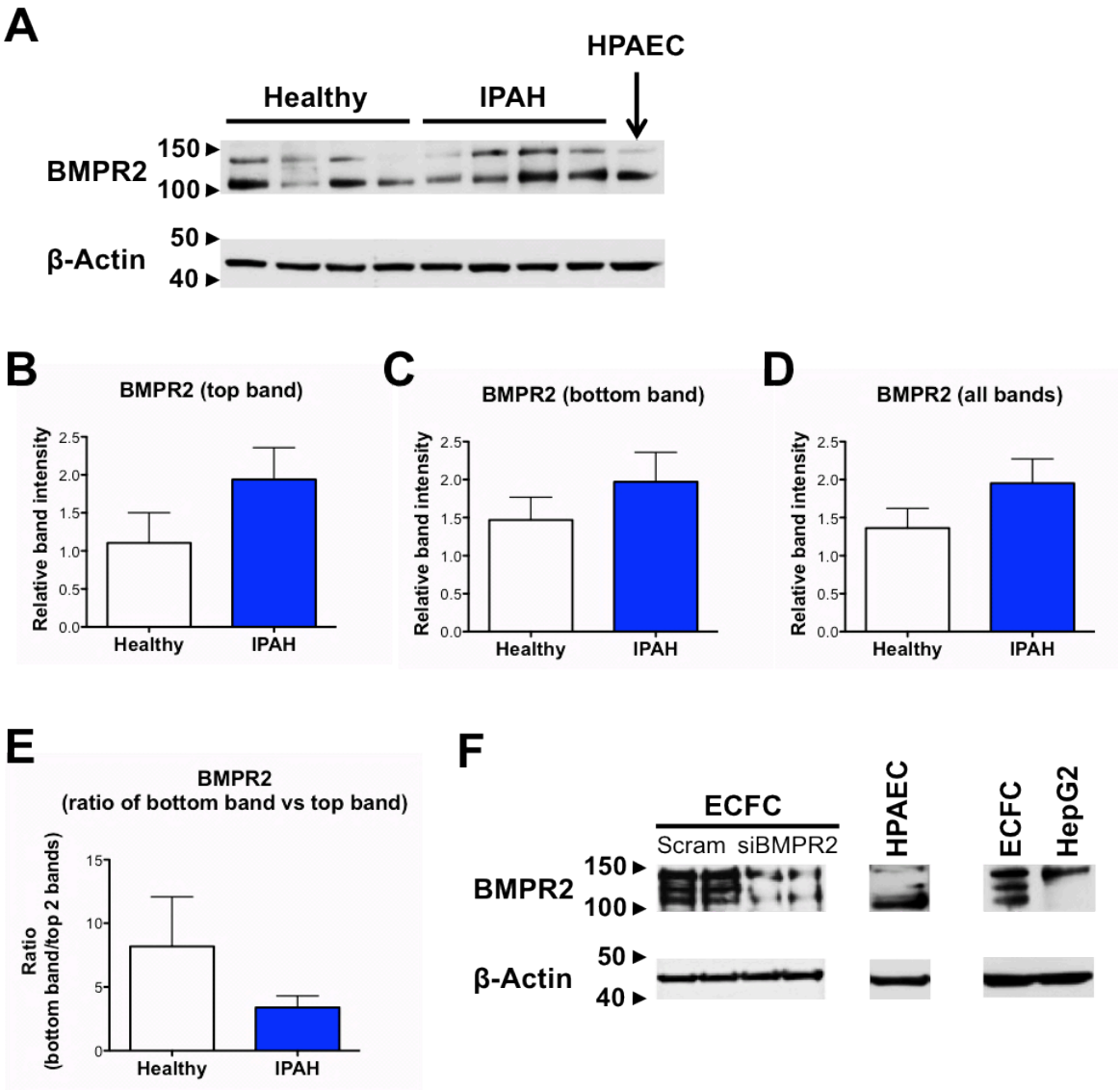


Figure 5.11. BMPR2 protein expression in sub-confluent ECFCs cultured in the presence of 20% FBS. (A) Representative Western blots showing varying expression of BMPR2 molecular weight bands in two distinct groups of sub-confluent ECFCs from healthy volunteers and IPAH patients (n=6-7). A sub-confluent HPAEC extract was used as control. Graphs display the quantitative assessment of the relative intensity of the **(B)** top band, **(C)** bottom band, **(D)** total intensity for both bands and **(E)** the ratio of the bottom versus the top band. Bars represent mean±SEM. Statistics shown was from t-test. **(F)** BMPR2 expression is reduced in ECFCs following transfection with siRNA targeting BMPR2 (siBMPR2) compared to transfection with scrambled controls (Scram). Two distinct BMPR2 isoforms predominated in confluent HPAECs and HepG2 cells.

Given the finding that BMPR2 forms complexes with the type I receptor ALK1 on endothelial cells, selectively mediating the effects of BMP9 (David et al., 2007a; Scharpfenecker et al., 2007), I sought to explore whether the downstream signalling effects of BMP9 differed in ECFCs derived from IPAH patients and healthy volunteers. Initial experiments were conducted to determine the concentration-dependent effects of BMP9 on Smad1/5/8 phosphorylation, with pSmad1/5/8 levels reaching a plateau after 1h treatment with 1-10.0 ng/ml BMP9 (Figure 5.12A). The addition of the ALK1-Fc chimera (1-10 μg/ml) inhibited BMP9-induced pSmad1/5/8 in a concentration-dependent manner, whereas the ALK5 inhibitor SD208 had no effect (Figure 5.12B), confirming the specific affinity of BMP9 for ALK1. No significant difference was found between BMP9-induced pSmad1/5/8 levels in ECFCs from healthy volunteers and IPAH patients (Figure 5.12C-D).

The downstream effect of BMP9 stimulation on the transcription of *ID* genes was also examined. Serum-deprived ECFCs exhibited a concentration-dependent increase in ID1, ID2 and ID3 expression after 2-4h treatment with 0.01-10.0 ng/ml BMP9, although the response appeared to be attenuated by 8h (Figure 5.13A). Subsequent studies, using qPCR techniques, showed that both ID1 and ID2 expression was induced in ECFCs derived from IPAH patients and healthy volunteers (Figure 5.13B-C). No significant difference in BMP9-induced ID1 and ID2 expression was found between the two groups, although after 4h the ECFCs from IPAH patients treated with 0.1 & 1 ng/ml BMP9 tended to show lower ID2 expression (P = 0.09 & P = 0.08 respectively) when compared with cells from healthy volunteers (Figure 5.13B-C).

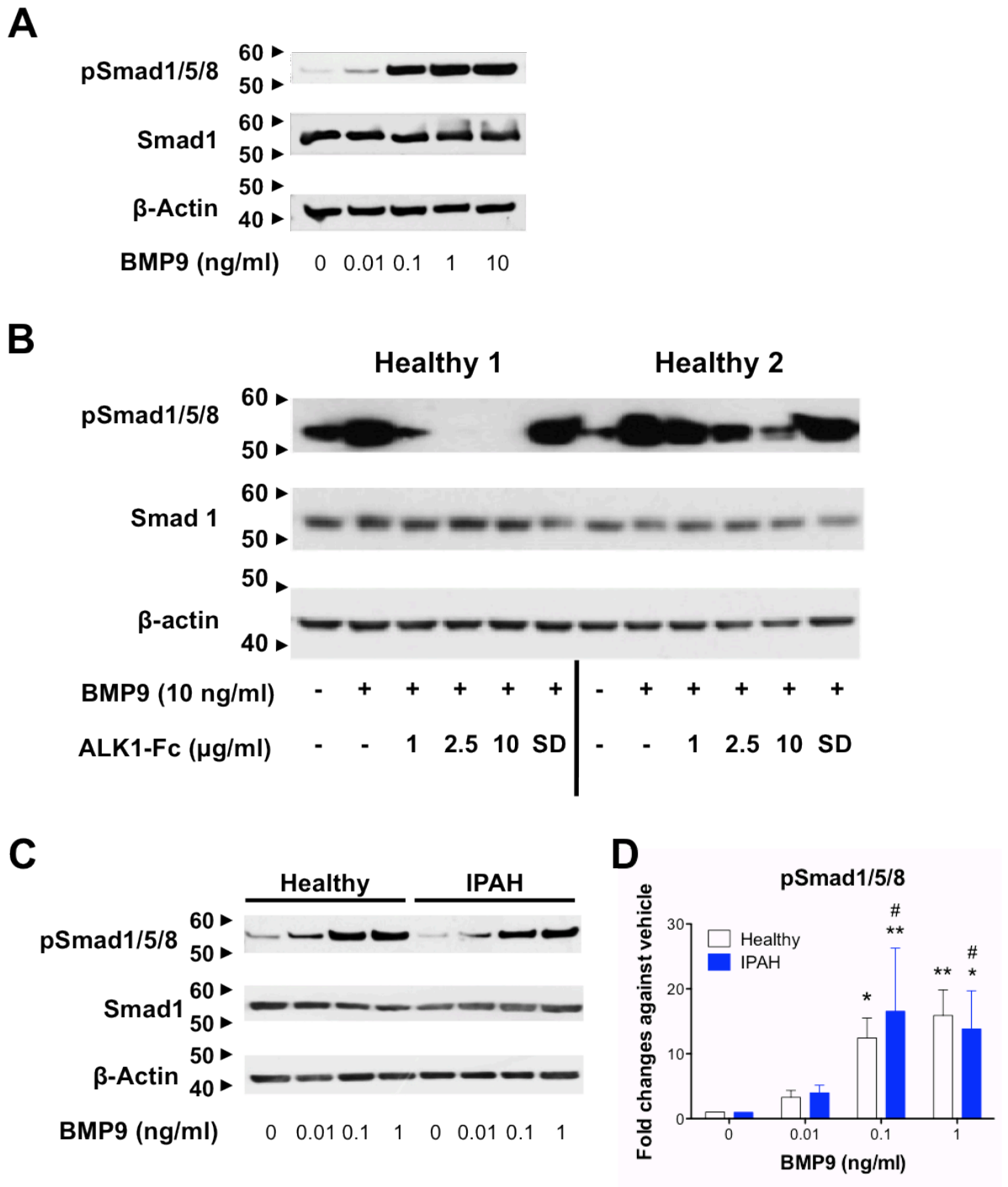


Figure 5.12. BMP9-stimulated Smad1/5/8 phosphorylation (pSmad1/5/8). ECFCs were serum-deprived for 12 h in 0.1% FBS before being treated with various concentration of BMP9 (0.01 – 10 ng/ml) or vehicles for 1 h. **(A-B)** Representative western blots showing stimulation of pSmad1/5/8 by BMP9 in serum-deprived ECFCs from healthy volunteers and inhibition in the presence of ALK1-Fc chimera (1 – 10 μg/ml). **(C-D)** Representative western blots and quantitative assessment of BMP9-stimulated pSmad1/5/8 in ECFCs from healthy volunteers (n=6) and IPAH patients (n=5). All blots were reprobed for β-actin to ensure equal loading. Data presented as fold increases of pSmad1/5/8 against vehicle controls. Bars represent mean±SEM. Statistics shown from two-way ANOVA, with Bonferroni post-hoc analysis. *, $P < 0.05$; **, $P < 0.01$; versus respective vehicle controls. #, $P < 0.05$; versus treatment with 0.01 ng/ml BMP9.

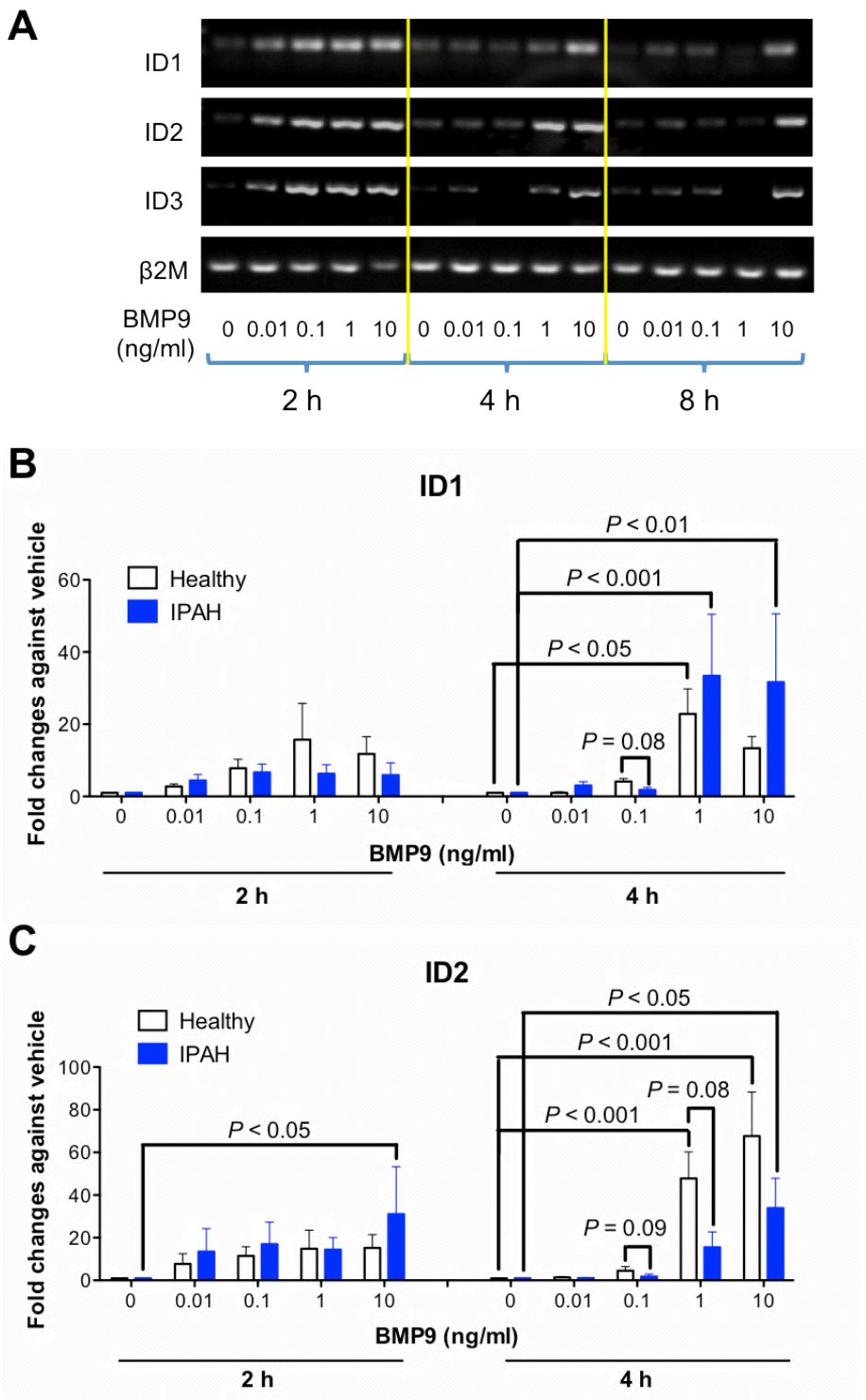


Figure 5.13. BMP9-stimulated *ID* gene expression in ECFCs. ECFC were serum-deprived for 16h (EBM-2 with 0.1% FBS) before being treated with various concentrations of BMP9 (0.01 – 10 ng/ml) or vehicle (0) for 2 h, 4 h and 8 h. **(A)** Representative agarose gel images of PCR amplified ID1, ID2 and ID3 transcripts. β2-microglobulin (β2M) was included as a reference housekeeping gene. **(B-C)** Quantitative PCR analysis of ID1 and ID2 expression in ECFCs from healthy volunteers (n = 7) and IPAH patients (n = 5 – 6) after treatment with BMP9 for 2 h and 4 h. Data presented as fold change compared to vehicle controls. Statistics shown from two-way ANOVA and Bonferroni post-hoc analysis.

5.4 Discussion

The main findings in this chapter are:

- ALK5 expression is reduced at both the mRNA and protein level in ECFCs from IPAH patients.
- ECFCs from IPAH patients display an impaired response to 1ng/ml TGF-β1-stimulated Smad2 phosphorylation.
- BMP9 stimulation of BMPR2/ALK1 induced similar downstream activation (phosphorylation) of Smad1/5/8 in ECFCs from IPAH patients and healthy volunteers.
- There is marked heterogeneity in the expression of BMPR2 protein isoforms among ECFCs.

In this chapter I have analysed members of the TGF-β receptor superfamily that are implicated in PAH (Richter et al., 2004). Throughout these studies, I found reduced ALK5, ActRIIA and endoglin mRNA expression levels in ECFCs from IPAH patients. In particular, ALK5 expression was consistently reduced in confluent ECFCs from IPAH patients, both in the presence of serum and under serum-deprived conditions, although no difference was apparent in sub-confluent cultures of cells. This is perhaps not surprising as TGF-β/ALK/Smad signalling in ECs is known to be context dependent and may vary according to cell confluence and the organisation of intercellular junctional proteins such as VE-cadherin (Rudini et al., 2008). Interestingly, lung tissues from IPAH patients also displayed lower levels of ALK5 mRNA expression, compared with control lobectomy tissues. Furthermore, other groups have described a decrease in ALK5 protein expression in the lungs of IPAH patients, notably localised to ECs in the core of plexiform lesions (Richter et al., 2004; Gore et al., 2010). In keeping with lower ALK5 expression, TGF-β1-induced Smad2 phosphorylation was also reduced in ECFCs from IPAH patients. The impaired response was apparent following stimulation with 1ng/ml TGF-β1, rather than at lower concentrations (0.005-0.5 ng/ml). This suggests that the second part of the biphasic response to TGF-β1 may be selectively affected in IPAH cells and indicates the need to further compare the effects of TGF-β1 at physiological concentrations; the normal venous level of TGF-β1 has been reported to be 2.6-12.5 ng/ml or 1.0-4.7 ng/ml (Selimovic et al., 2009; Evrard et al., 2012). Nevertheless, it remains to be established whether a 30-40% reduction in ALK5 expression alone leads to the observed reduction in Smad2 phosphorylation or if the altered expression of other receptors is also required.

A substantial body of evidence indicates that TGF-β signalling has an important role in the pathogenesis of PAH and, despite differences between studies, it is generally considered that there is dysregulation of TGF-β signalling in the lungs and vascular cells of patients with IPAH. Earlier immunohistochemical studies indicated that endothelial BMPR2 expression was reduced in the lungs of PAH patient irrespective of whether *BMPR2* mutations were present, while the levels of TGF-βRII appeared to be similar to that of controls (Atkinson et al., 2002). In contrast to the high level of expression and potential TGF-β signalling in endothelial cells of pulmonary arteries, Richter and colleagues found a marked reduction of TGF-β receptors and signalling proteins (ALK5, TGF-βRII, pSmad1/5/8 and pSmad2/3) in the core ECs of plexiform lesions in patients with IPAH, regardless of their *BMPR2* mutation status (Richter et al., 2004). Decreased pulmonary ALK5 expression has also been reported in the lungs of IPAH patients, which was accompanied by increases in TGF-β production and endoglin and ALK1 protein levels (Gore et al., 2010). In addition, a reduction in TGF-β signalling proteins expression has been found in the lungs and PASMCs of MCT-treated rats with severe PH (Zakrzewicz et al., 2007), although most studies have described increased TGF-β signalling in the MCT model and attenuation of PH in animals treated with ALK5 inhibitors (Zaiman et al., 2008; Long et al., 2009; Thomas et al., 2009). The reduction of BMP signalling in the MCT model of PH may also contribute to a gain in TGF-β signalling (Long et al., 2009). At the cellular level, PASMCs from patients with IPAH exhibit a heightened sensitivity to TGF-β1 *in vitro*, the abnormal proliferative effect being abolished by pre-incubation with an ALK5 inhibitor (Morrell et al., 2001; Thomas et al., 2009). It is uncertain how TGF-β signalling may be affected in ECs from IPAH patients, but the response could differ from that of PASMCs and may vary between cells from different regions of the pulmonary arterial tree (Lu, 2008; Lu et al., 2009). PAECs harbouring BMPR2 mutations can also produce more TGF-β1 than control cells, with conditioned medium causing an ALK5-dependent proliferation of PASMCs (Yang et al., 2011), indicating the therapeutic effects of ALK5 inhibitions in the MCT models of PH could be due to the effects on PASMCs. In the present study, I postulated that decreased ALK5 expression and impaired TGF-β/Smad2 signalling might contribute to the dysfunctional (apoptosis-resistant and angiogenic) phenotype displayed by ECFCs from IPAH patients, similar to those observed in ECs within plexiform lesions.

Immunohistochemical studies have described decreased endothelial BMPR2 protein expression in sections of lung and isolated pulmonary microvascular ECs from IPAH patients without *BMPR2* mutations (Atkinson et al., 2002; Alastalo et al., 2011). Others have found no difference in BMPR2 protein levels in lung tissues and isolated ECs from controls and IPAH patients, using Western blotting to identify single bands of 90 kDa and 75 kDa respectively (Du et al., 2003; Dewachter et al., 2009). Another recent study also indicated that the mRNA expression of BMPR2 in ECs within the

plexiform lesions of IPAH lungs was similar compared to controls (Jonigk et al., 2011). Overall, I observed no difference in BMPR2 mRNA or protein expression between ECFCs from IPAH patients and healthy controls, despite having analysed cells that were cultured in a variety of conditions. In fact, there was considerable heterogeneity in the BMPR2 isoforms expressed by different ECFC populations. Three molecular isoforms were expressed by confluent ECFCs (~110 kDa, ~130 kDa and ~150 kDa) and two bands (~110 kDa and ~150 kDa) predominated in sub-confluent ECFCs, the intensity of all three bands being significantly reduced following BMPR2 siRNA knockdown. Interestingly, three bands of BMPR2 protein expression were also seen in human aortic ECs (Shao et al., 2009). Cultures of HPAECs also produced two bands (~110 kDa and ~150 kDa), with the lower molecular isoform predominating. This is consistent with published reports of BMPR2 protein expression in HPAECs and HPMVECs that used the same source of BMPR2 antibody (from BD Transduction), with the majority demonstrating either single or double bands in the 110-150 kDa range (de Jesus Perez et al., 2009; Upton et al., 2009; Alastalo et al., 2011; Burton et al., 2011; Drake et al., 2011; Gangopahyay et al., 2011; Park et al., 2012; Star et al., 2012). Two similar bands were also detected in confluent human PASMCs in this study and others (Upton et al., 2008) and in HeLa cells (Durrington et al., 2010), whereas HepG2 exhibited a single ~150 kDa isoform. Taken together, these data suggest that the processing of BMPR2 isoforms may be influenced by cell-cell contact in ECFCs and can vary between different cell types.

The *BMPR2* gene contains 19 exons and, out of 6 alternatively spliced potential variants, two primary transcripts and BMPR2 protein isoforms have been identified (Liu et al., 1995; Rosenzweig et al., 1995) that occur in most normal and HPAH patient tissues (Cogan et al., 2012). These comprise a 'long' 13-exon isoform A (NCBI ref: NM_001204) and a 'short' 12-exon isoform B (NCBI ref: HSU25110) in which exon 12 is spliced out. The latter sequence encodes most of the intracellular cytoplasmic tail of the receptor, which is important for interactions between BMPR2 and β-actin, and mutations that lack exon 12 are thought to cause HPAH through a dominant negative effect (Cogan et al., 2012). Interestingly, lung and placental tissues were found to have the highest levels of expression of both isoforms of the receptor and, in cultured lymphocytes, HPAH patients with BMPR2 mutation exhibited a higher B/A isoform ratio than healthy/unaffected *BMPR2* mutation carriers (Cogan et al., 2012). Importantly, this difference in ratio also had a measurable effect on BMP signalling, cells with a high B/A ratio dephosphorylating phospho-cofilin less efficiently than those from non-affected carriers with a lower B/A ratio. It is unclear whether isoforms A and B correspond with the two protein bands that were found to predominate in ECFCs and PAECs by Western blotting, the lower molecular weight band (~110 kDa) being larger than that predicted for

isoform B. Nevertheless, I consistently found the ratio of the bottom band intensity over the top two bands was lower in confluent ECFCs from IPAH patients, compared with control cells.

Endoglin mRNA expression was found to be lower in confluent ECFCs from IPAH patients, cultured in the presence of 20% FBS, although this was not consistently observed in other conditions. Endoglin expression is upregulated by TGF-β and BMP9 in bovine aortic ECs and HPAECs (Scharpfenecker et al., 2007; Upton et al., 2009), TGF-β-stimulated endoglin expression being mediated through Smad3 (Sanchez-Elsner et al., 2002) and the effect of BMP9 via ALK1, BMPR2 and Smad4 (Upton et al., 2009). It is conceivable that reduced endoglin expression in ECFCs from IPAH patients reflects impaired stimulation of the endoglin transcription promoter by TGF-β/Smad2/3 signalling, but this would require further investigation to establish.

BMP9 is a potent ALK1 agonist in various types of endothelial cells, which typically induces downstream signalling via pSmad1/5/8 and ID gene expression (David et al., 2007a; Scharpfenecker et al., 2007; Upton et al., 2009). I have shown that BMP9 also stimulates pSmad1/5/8 and induces ID1 and ID2 gene expression in ECFCs, the responses being similar in cells derived from IPAH patients and healthy volunteers. This is consistent with the recent analysis of BMP9-induced ID1 expression in PAECs isolated from HPAH, IPAH and APAH patients, demonstrating that the response was unchanged in IPAH and APAH patients and only affected in cells isolated from HPAH cases with *BMPR2* or *SMAD9* mutations (Drake et al., 2011). In addition, Toshner and co-workers have reported that ECFCs from HPAH patients with *BMPR2* mutations exhibited impaired BMP9/pSmad1/5 signalling and induction of downstream *ID1* gene expression (Toshner et al., 2011). These differences in activation of BMP canonical signalling suggest possible variation in the molecular mechanisms of ECFC dysfunction between HPAH patients with BMPR2 mutations and IPAH patients. Conversely, another recent study indicated that the expression of genes-associated with remodelling (e.g. HIF-1α, TGF-β1, VEGF-α, VEGFR-1/2, Ang-1, Tie-2 and THBS1) did not differ between plexiform lesions from a patient with BMPR2 mutation and others with idiopathic or associated forms of PAH (Jonigk et al., 2011). However, TGF-β/BMP signalling can also occur via Smad-independent (non-canonical) pathways, including activation of Rho GTPases and the MAP kinases p38, ERK and JNK, and involve cross-talk with other pathways (Zhang, 2009; Akhurst and Hata, 2012) as well as alterations in microRNA processing (Drake et al., 2011). I cannot exclude possible dysregulation of these alternative Smad-independent pathways in ECFCs from IPAH patients, especially as the expression of other receptors and accessory proteins (e.g. ActRIIA and endoglin) may also be affected.

In the present study, ECFCs exhibited most members of TGF-β receptor superfamily, except for ALK3 and ALK6, which were generally expressed only at low level. Previously, Upton and colleagues found

that ALK3 and ALK6 were also expressed at a low level in HPAECs and linked this to poor binding and stimulation by the selective ligands BMP2 and BMP4 (Upton et al., 2008; Upton et al., 2009). However, BMP2/4 proteins are reported to be selectively expressed in ECFCs and involved in regulating their proliferation, migration, and tube formation (Smadja et al., 2008). The efficiency of BMP9 in stimulating pSmad1/5/8 and ID gene expression in ECFCs is comparable to that described in HPAECs (Upton et al., 2009), these studies having been performed under very similar experimental conditions. This is perhaps not surprising as we and others (Evrard et al., 2012) have found comparable levels of ALK1 and BMPR2 expression in ECFCs and HPAECs.

There are a number of limitations to the studies in this chapter. I was unable to explore further the differential effect of TGF-β1 stimulation (at higher concentration) on pSmad2/3 or the impact of intercellular contact and other regulatory mechanisms on the expression of BMPR2 isoforms and downstream signalling in ECFCs. I did not consider possible differences in the expression of accessory proteins such as Smurf and SARA or explore potential differences in other non-canonical TGF-β/BMP/Smad signalling pathways in ECFCs. In ECs, TGF-β1 can signal via both ALK5-Smad2/3 and ALK1-Smad1/5/8, with possible cross-talk between the two, and stimulation of these pathways can have quite distinct effects on EC quiescence, proliferation, migration and tube formation (Goumans et al., 2002; Goumans et al., 2003). Given the necessary additional time, I would have examined the effect of knocking down the expression of ALK5 and ALK1 receptors, as well as BMPR2, in order to gain further insight into the contribution and interaction of the two pathways in ECFCs. Despite these limitations, I have demonstrated impaired ALK5 expression and TGF-β signalling in ECFCs from IPAH patients. This might contribute to endothelial dysfunction, possibly shifting the balance towards activation of the alternative endothelial TGF-β/ALK1 pathway as described in ECs (Goumans et al., 2002), and contribute to the phenotypic differences found between ECFCs from IPAH patients and healthy controls.

In chapter 6 ***I will investigate the role of ALK1 and ALK5 receptor signalling in regulating ECFC functions, using selective TGF-β/BMP stimulation and pharmacological inhibition to examine the phenotypic differences that exist between ECFCs from IPAH patients and healthy controls.***

Chapter 6:

TGF-β receptor signalling and phenotypic differences in ECFCs from IPAH patients

Chapter 6 – TGF-β receptor signalling and phenotypic differences in ECFCs from IPAH patients

6.1 Introduction

In Chapter 5, I demonstrated reduced expression of the TGF-β type 1 receptor ALK5 and impaired TGF-β1 activation of Smad2 in ECFCs from IPAH patients. Due to the importance of the TGF-β receptor superfamily in the regulation of the endothelium, it is possible that deficiencies in the ALK5/Smad2 pathway might contribute to a dysfunctional ECFC phenotype. In order to establish this link, it is necessary to understand the role of the endothelial ALK1 and ALK5 signalling pathways in regulating cell function, but few studies have examined the effects of TGF-β/BMP signalling in ECFCs. An early investigation of ECFCs from patients with hereditary hemorrhagic telangiectasia (HHT), caused by mutations in *ENG* (*HHT1*) or *ALK1* (*HHT2*), revealed deficiencies in both ALK5- and ALK1-dependent signalling that were associated with a disorganised cytoskeleton and disruption of proliferation, migration and angiogenesis (inability to form a tubular-like network) *in vitro* (Fernandez et al., 2005; Fernandez et al., 2007). BMP2 and BMP4 have been shown to induce proliferation, migration and angiogenesis of ECFCs obtained from umbilical cord blood (Smadja et al., 2008). A subsequent study found that ECFCs derived from the peripheral blood of HPAH patients with *BMPR2* gene mutations were more proliferative and less angiogenic *in vitro* than cells derived from healthy controls (Toshner et al., 2009). More recently, TGF-β1 has been found to promote the migration of ECFCs *in vitro* as well as their angiogenic capacity *in vivo* (Evrard et al., 2012).

Endothelin-1 (ET-1) is a potent endothelium-derived vasoconstrictor and SMC mitogen (Hassoun et al., 1992) and increased ET-1 levels are implicated in the pathogenesis of PAH (Stewart et al., 1991; Giaid et al., 1993). The endothelium is postulated to be the main source of increased ET-1 in the circulation and lung tissues in PAH (Langleben et al., 2006). It is known that TGF-β1 stimulates ET-1 synthesis in ECs from several vascular origins (Kurihara et al., 1989; Rodriguez-Pascual et al., 2004), including human PMVECs (Star et al., 2009). More recent studies have shown that BMP9 and BMP7 regulate ET-1 production, acting via several pathways to induce the release of ET-1 from human PMVECs (Star et al., 2009; Star et al., 2010; Star et al., 2012). Studies on human PAECs also indicate that BMP9-stimulated ET-1 production may have a role in angiogenesis, ALK1 or BMPR2 knockdown attenuating this while also increasing ET-1 expression and release (Park et al., 2012). In addition to

investigating potential deficiencies in TGF-β/BMP signalling I therefore also sought to examine the regulation of ET-1 production by ECFCs.

The objectives of this chapter are to:

- Determine the contribution of ALK1 and ALK5 signalling in the survival of serum-deprived ECFCs, using protein ligands (BMP4, BMP9, TGF-β1) and selective inhibitors for these receptors.

- Investigate the role of ALK1 and ALK5 signalling in regulating the proliferation, migration and tube forming capacity of ECFCs *in vitro*.

- Compare the production of ET-1 by ECFCs from IPAH patients and healthy controls and examine the role of ALK1 and ALK5 signalling in regulating ET-1 release.

6.2 Methods

See Chapter 2 for detailed experimental materials and protocol.

6.2.1 Apoptosis assay

Serum deprivation-induced apoptosis was assessed by determining caspase-3/7 activity. Optimisation of apoptosis assay is detailed in Chapter 2.12.2. Sub-confluent ECFCs and HPAECs were examined 48h, 64h and 72h after culture in EBM-2 medium containing 2% FBS (control) or under serum deprived conditions (apoptosis stimulus), either in the absence of serum or presence of 0.5% FBS. Cells were treated with either 10 ng/ml BMP4, 10 ng/ml BMP9 or 0.01-50 ng/ml TGF-β1 and, in some experiments, were also co-treated with the ALK5 inhibitors SD208 (15 μM) and SB431542 (15 μM), the ALK1 inhibitor ALK1-Fc (2.5 or 10 μg/ml) or vehicle alone.

6.2.2 BrdU incorporation assay

Optimisation of BrdU assay is detailed in Chapter 2.12.1. Sub-confluent cultures of ECFCs or HPAECs were seeded (3000 cells/well in EBM-2 containing 5% FBS) in black-walled optical-bottomed 96-well plates and incubated for 18 h before being serum-deprived for 6h. Cells were then incubated for a total of 48h in EBM-2 medium (containing 5% FBS) and treated (in triplicate) with either vehicle, 10 ng/ml BMP4, 10 ng/ml BMP9 or 0.1-10 ng/ml TGF-β1, both in the absence and presence of 15 μM SD208, 15 μM SB431542, or 10 μg/ml ALK1-Fc. DNA synthesis was assessed by measuring BrdU incorporation and the luminescence signal was not saturated under these conditions (Chapter 2.12.1; Figure 2.6).

6.2.3 Matrigel tube formation assay

The effects of BMP4, BMP9 and TGF-β1 on ECFC and HPAEC tube formation were assessed using GFR-Matrigel and EBM-2 medium without serum. Sub-confluent (~70%) ECFCs or HPAECs were trypsinised and resuspended in medium containing either 10 ng/ml BMP4, 10 ng/ml BMP9 or 0.1-10 ng/ml TGF-β1, both in the absence and presence of 15 μM SD208, 15 μM SB431542 and 10 μg/ml ALK1-Fc. Control experiments included the addition of the VEGFR2 inhibitor SU1498 (10 μM). Cells

were seeded onto GFR-Matrigel (7000 cells/well in a 96-wells plate) and incubated in a humidified atmosphere at 37°C for 16h, before being fixed with 4% formaldehyde. Measurements of total tube length are presented as total tube lengths, each data point being the mean of triplicate values derived from images of 2 random fields per well.

6.2.4 Wound healing assay

Confluent ECFCs and HPAECs, cultured in EBM-2 and 1% FBS on gelatin-coated 24-wells plates, were treated with either 15 μM SD208, 15μM SB 431542 or vehicle for 30 min before creating the wound. Afterwards, 10 ng/ml BMP4, 10 ng/ml BMP9 or 0.1-10 ng/ml TGF-β1 was added. Some cells were treated with the VEGFR2 inhibitor SU1498 (10 μM) as negative control. Images were captured at baseline (0 h), 18 h and 24 h and data presented as a percentage of the area of the wound recovered (closed) compared to baseline.

6.2.5 Measurement of endothelin-1 (ET-1) release

Sub-confluent cells were resuspended in EGM-2 containing 20% FBS and seeded at 40,000 cells per well (equivalent to 7000 cells/well in a 96-wells plate) on gelatin-coated 24-well plates. Following overnight incubation, cells were washed twice with warm sterile PBS and incubated (200 μl/well) in either EGM-2 with 2% FBS (control), EBM-2 with 0.5% FBS or EBM-2 with no serum. Cells were also treated with vehicle or 10 ng/ml BMP9 or 15 μM SD208. Conditioned medium was collected after 24h and stored frozen at -80°C until used. Cells were washed with PBS and protein harvested in RIPA buffer for protein measurement. ET-1 levels were assayed with a commercial ELISA kit (Human Endothelin-1 QuantiGlo ELISA kit, Cat no. QET00B, R & D Systems), according to the manufacturer's instructions.

6.3 Results

6.3.1 BMP4 and BMP9 attenuate apoptosis in ECFCs from healthy volunteers and in HPAECs

The effects of BMP4 and BMP9 on serum deprivation-induced apoptosis were examined in ECFCs and HPAECs. As demonstrated in Chapter 4, ECFCs from IPAH patients were again found to be protected from serum deprivation-induced apoptosis, exhibiting significantly less caspase-3/7 activity compared to ECFCs from healthy volunteers and HPAECs (Figure 6.1A-B, Figure 6.2A-B). BMP4 (10 ng/ml) induced a small, but nonetheless significant reduction in caspase-3/7 activity in ECFCs from healthy volunteers and in HPAECs, whereas no difference was apparent in ECFCs from IPAH patients (Figure 6.1A and C-D). BMP9 (10 ng/ml) exhibited a more marked inhibitory effect on apoptosis in ECFCs from healthy volunteers and HPAECs, both cell types displaying a homogeneous (~50%) reduction in caspase-3/7 activity (Figure 6.1B and E). When considered together, BMP9 had no overall effect on caspase-3/7 activity in ECFCs from all the IPAH patients studied. The effect was heterogeneous however, with cells from four out of the seven patients examined displaying an inhibitory response whereas an equivocal or positive effect was observed in the other three (Patient code: IPAH 18, IPAH 19 and IPAH 31, see Appendix 1 for patient demographics) (Figure 6.1B & F).

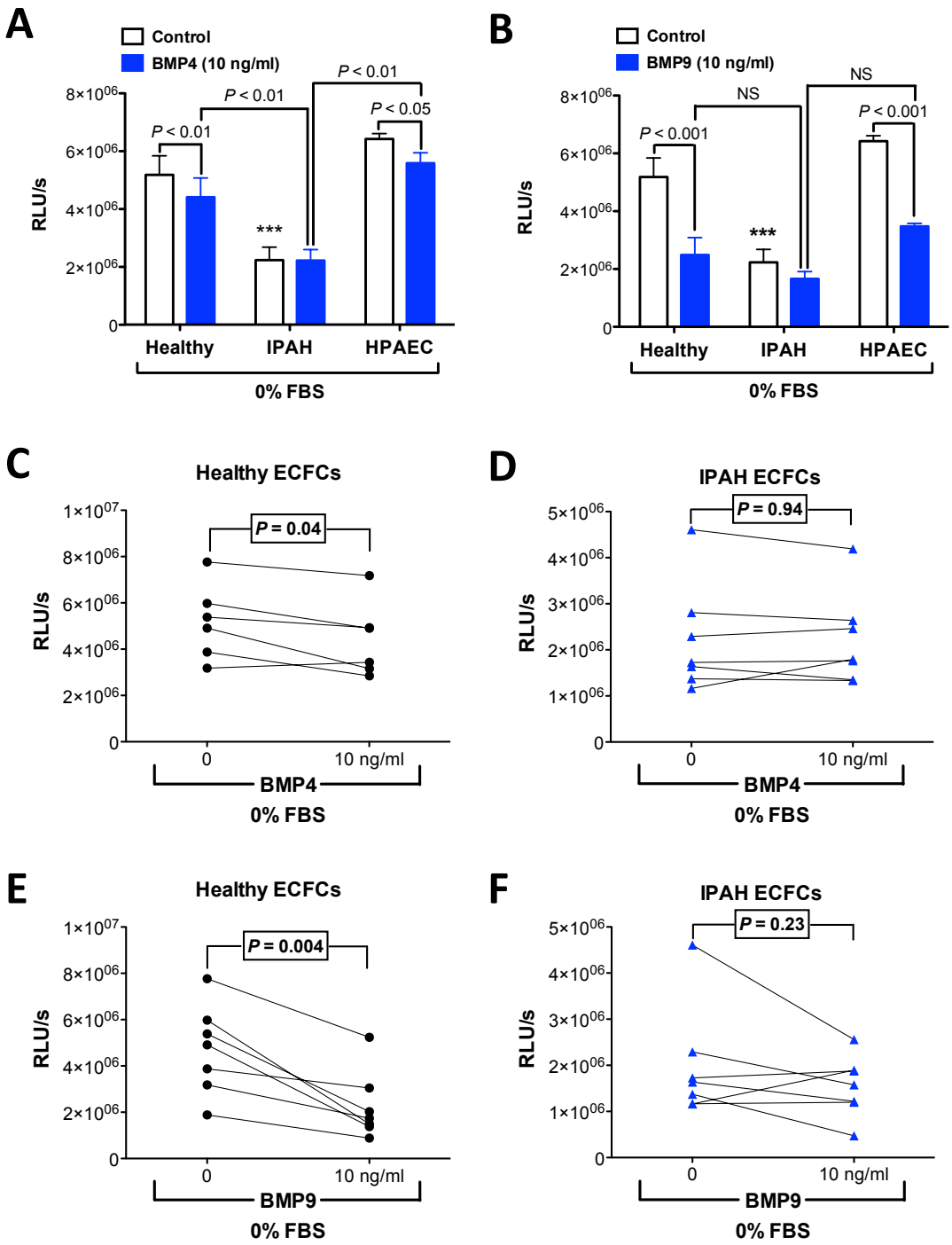


Figure 6.1. Effects of BMP4 and BMP9 on serum deprivation-induced apoptosis. Serum-starved (0% FBS) ECFCs and HPAECs were incubated for 48 h in the presence of vehicle (control), BMP4 (10 ng/ml) or BMP9 (10 ng/ml). **(A-B)** Caspase-3/7 activity (expressed as RLU/s) in ECFCs derived from healthy volunteers (n=6-7) and IPAH patients (n=7) and HPAECs (n=3). Bars represent mean±SEM. **(C-D)** The effect of BMP4 on caspase-3/7 activity in ECFCs from individual healthy volunteers and IPAH patients. **(E-F)** The effect of BMP9 on caspase-3/7 activity in ECFCs from individual healthy volunteers and IPAH patients. Statistics obtained from Bonferroni post-hoc analysis following two-way repeated measures ANOVA (A-B) or paired t-test (C-F). ***, $P < 0.001$ (caspase-3/7 activity significantly lower in ECFCs from IPAH patients compared with other cell types).

6.3.2 BMP9 attenuates apoptosis through ALK1 receptor signalling

In agreement with previous experiments, BMP9 had a significant inhibitory effect on serum deprivation-induced apoptosis in ECFCs from healthy volunteers and HPAECs, but not in ECFCs from IPAH patients (Figure 6.2A-B). Interestingly, the impaired response to BMP9 was most evident in cells from the same subset of IPAH patients (Patient code: IPAH 18, IPAH 19 and IPAH 31) that were distinguished above in section 6.3.1 (Figure 6.2B). The inhibitory effect of BMP9 on caspase-3/7 activity, both in ECFCs from healthy volunteers and HPAECs, was unaffected by the addition of 2.5 µg/ml ALK1-Fc but abolished in the presence of a higher concentration (10 µg/ml) of ALK1-Fc (Figures 6.2A-B and 6.3). In contrast, the addition of 15 µM SD208 (a concentration that effectively blocks ALK5 signalling, see Figure 5.7 in Chapter 5) had no apparent effect on the responses of these cells to BMP9 (Figures 6.2A-B and 6.3).

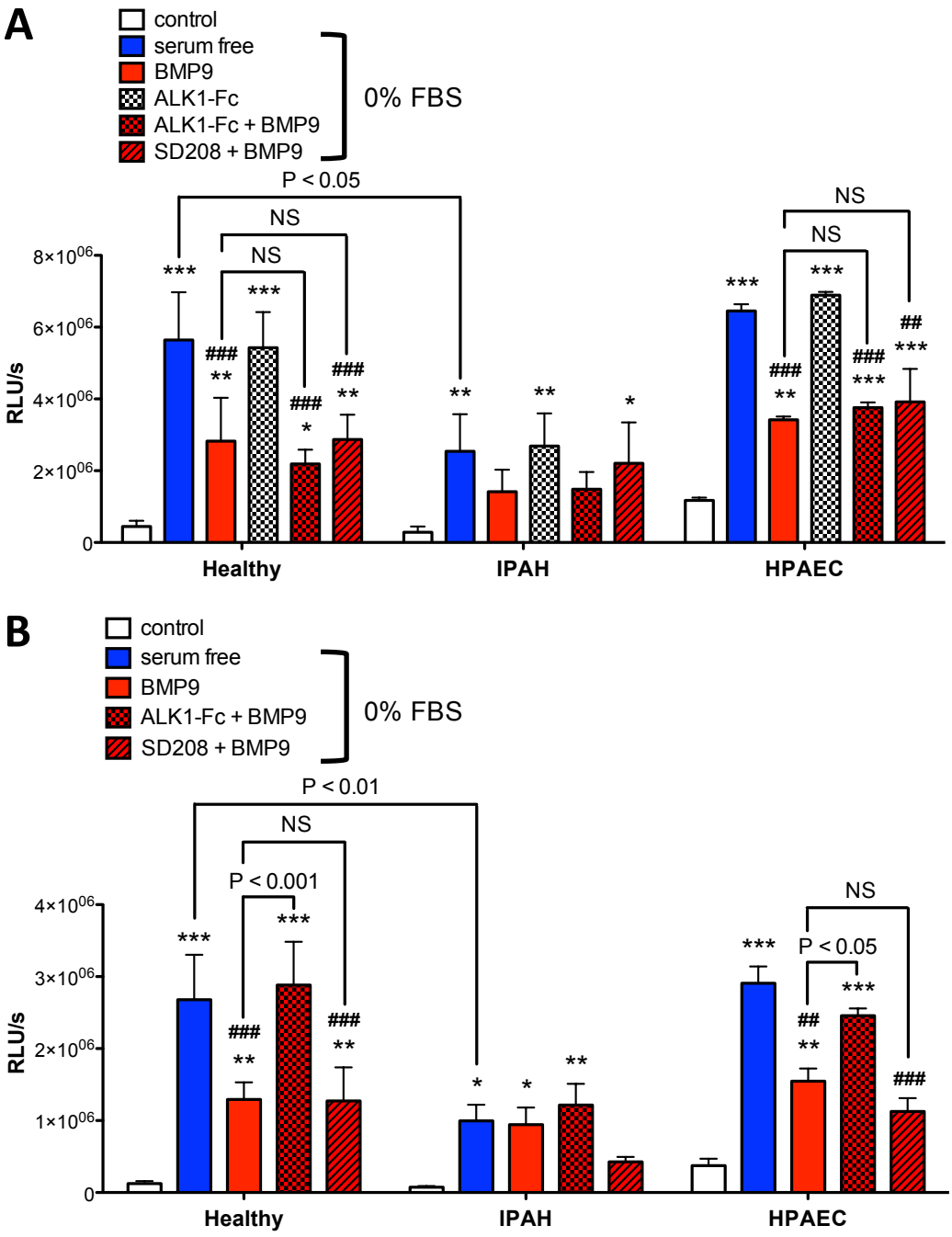


Figure 6.2. BMP9 attenuates apoptosis through ALK1 receptor signalling. (A) Caspase-3/7 activity (expressed as RLU/s) in ECFCs from healthy volunteers and IPAH patients and HPAECs (n=3 in all groups), measured 48h after culture in EBM-2 medium containing either 2% FBS + EGM-2 growth factors (control) or no serum, together with 10 ng/ml BMP9, 2.5 µg/ml ALK1-Fc chimera and 15µM SD208 or a combination of these compounds. **(B)** Caspase-3/7 activity in ECFCs from healthy volunteers (n=4) and IPAH patients (n=4) and HPAECs (n=3), measured 48h after culture in EBM-2 medium containing either 2% FBS + EGM-2 growth factors (control) or no serum, in the presence of 10 ng/ml BMP9 or BMP9 together with either 10 µg/ml ALK1-Fc chimera or 15µM SD208. Data are presented as mean±SEM. Statistics obtained from Bonferroni post-hoc analysis following two-way repeated measures ANOVA; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, compares to 2% FBS within the group; ##, $P < 0.01$, ###, $P < 0.001$, compares to serum-deprived cells in each group.

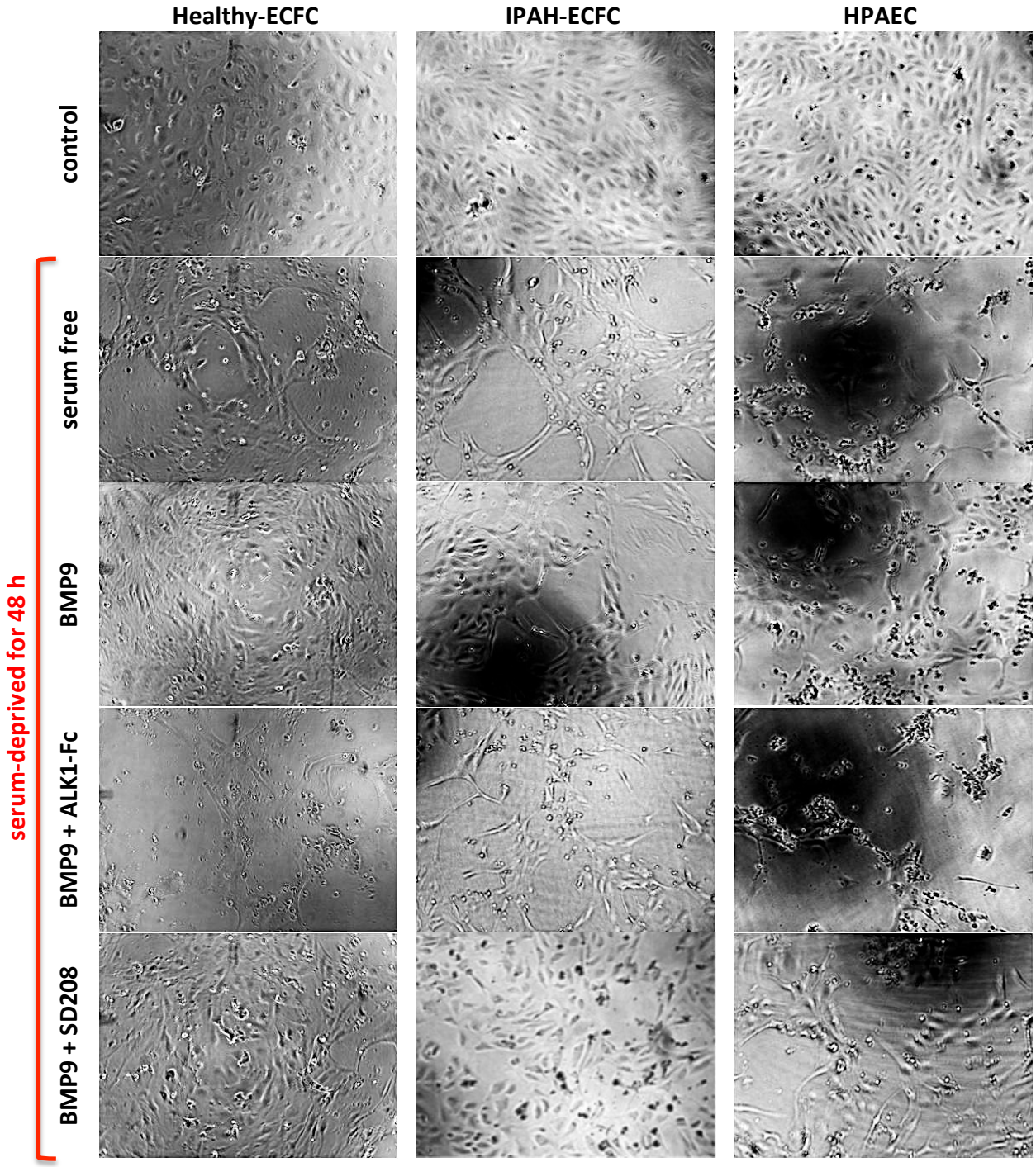


Figure 6.3. Protective effect of BMP9 is mediated through ALK1 receptor. Representative phase-contrast photomicrographs of data in **Figure 6.2B**, showing ECFCs and HPAECs cultured for 48h in EGM-2 (control) or serum-free EBM-2 medium in the presence of BMP9 (10 ng/ml), both alone and in combination with either 10 µg/ml ALK1-Fc or 15 µM SD208. 10x magnification.

6.3.3 Effect of exogenous TGF-β1 on apoptosis

Initial experiments indicated that the addition of TGF-β1 (0.01-10 ng/ml) had relatively little effect on serum deprivation-induced apoptosis in ECFCs, and a moderate pro-apoptotic effect in HPAECs at the highest concentration (Figure 6.4A). When examined again in a larger cohort of cells, TGF-β1 (10 ng/ml) appeared to selectively enhance caspase-3/7 activity in ECFCs from IPAH patients and HPAECs, but not in ECFCs from healthy volunteers (Figure 6.4B). However, this was not reproduced when the experiment was repeated in the presence of both 10 and 50 ng/ml TGF-β1 (Figure 6.4C).

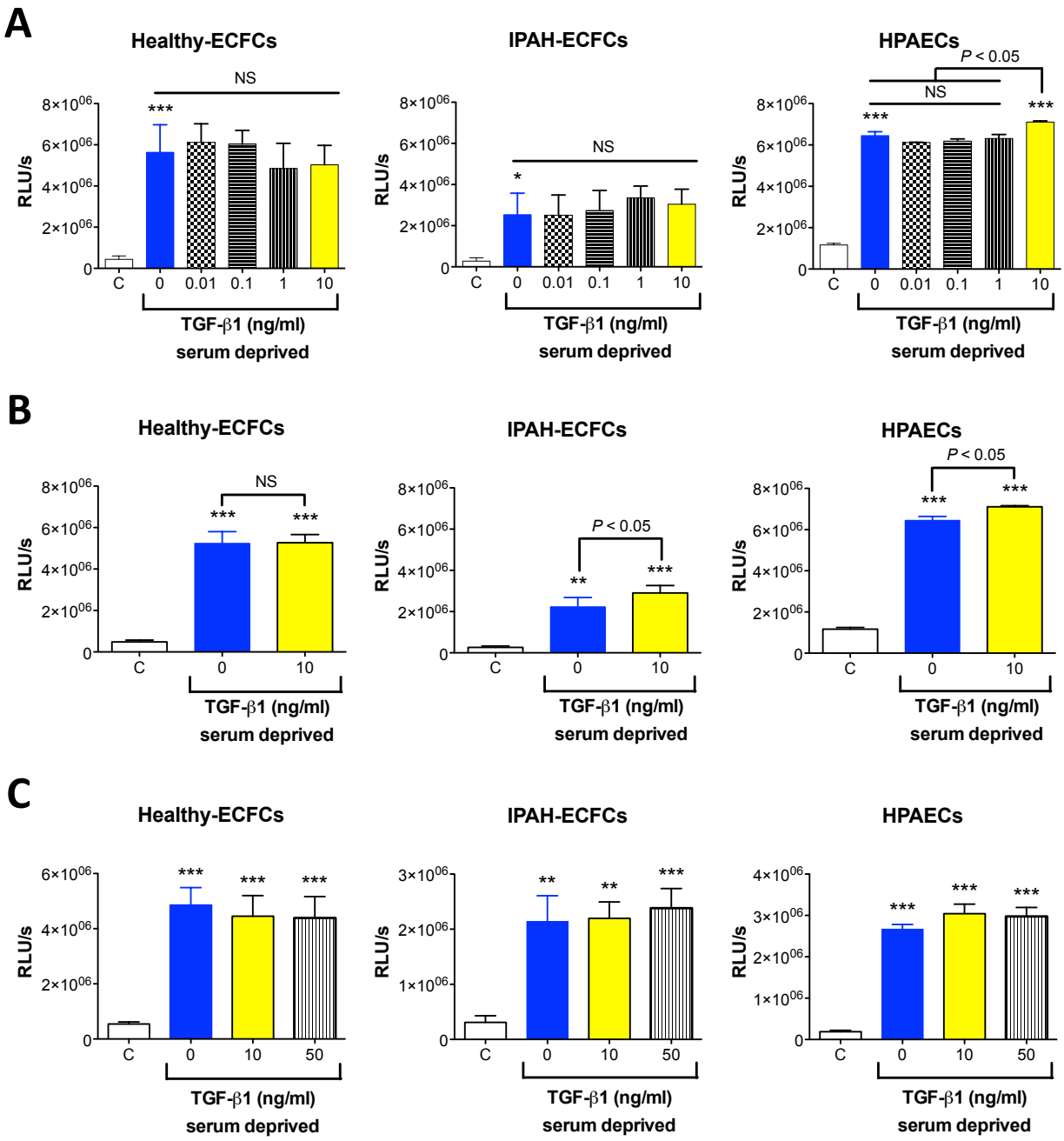


Figure 6.4. Effect of TGF-β1 on serum deprivation-induced apoptosis. (A) Caspase-3/7 activity (expressed as RLU/s) in ECFCs from healthy volunteers and IPAH patients and HPAECs (n=3 in all groups) after 48h in EBM-2 with 2% FBS (control) or no serum, together with 0-10 ng/ml TGF-β1. **(B)** Caspase-3/7 activity in ECFCs from healthy volunteers (n=7) and IPAH patients (n=7) and HPAECs (n=3) after 48h in EBM-2 with 2% FBS (control) or no serum, together with 10 ng/ml TGF-β1. **(C)** Caspase-3/7 activity in ECFCs from healthy volunteers (n=4) and IPAH patients (n=4) and HPAECs (n=3) after 48h in EBM-2 with 2% FBS (control) or no serum, together with either 10 or 50 ng/ml TGF-β1. Data are presented as mean±SEM. Statistics derived from Bonferroni post-hoc analysis following one-way repeated measures ANOVA; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, compared with control (C).

6.3.4 ALK5 inhibition attenuates apoptosis in ECFCs from IPAH patients

During the course of my studies it became apparent that ECFCs from IPAH patients were less sensitive to the apoptotic effect of serum-deprivation, when compared with ECFCs from healthy volunteers and HPAECs (see Chapter 4). Moreover, I fortuitously found that, in the presence of an ALK5 inhibitor, these cells were also able to survive for prolonged periods (at least 11 days) in continuous culture without a change in medium (EBM-2 and 1% FBS; Figure 6.5). Further experiments were therefore conducted to explore the effects of ALK5 inhibition on caspase-3/7 activity in ECFCs cultured either in serum-free medium for 48h or in the presence of low level serum (0.5% FBS) for 72h. Taking the results as a whole, ALK5 inhibitors had a tendency but insignificant effect in ECFCs from healthy volunteers and HPAECs, but reduced caspase-3/7 activity by up to ~50% in ECFCs from IPAH patients. This effect was most marked under less severe serum-deprivation conditions, in the presence of 0.5% FBS (Figure 6.6A-B).

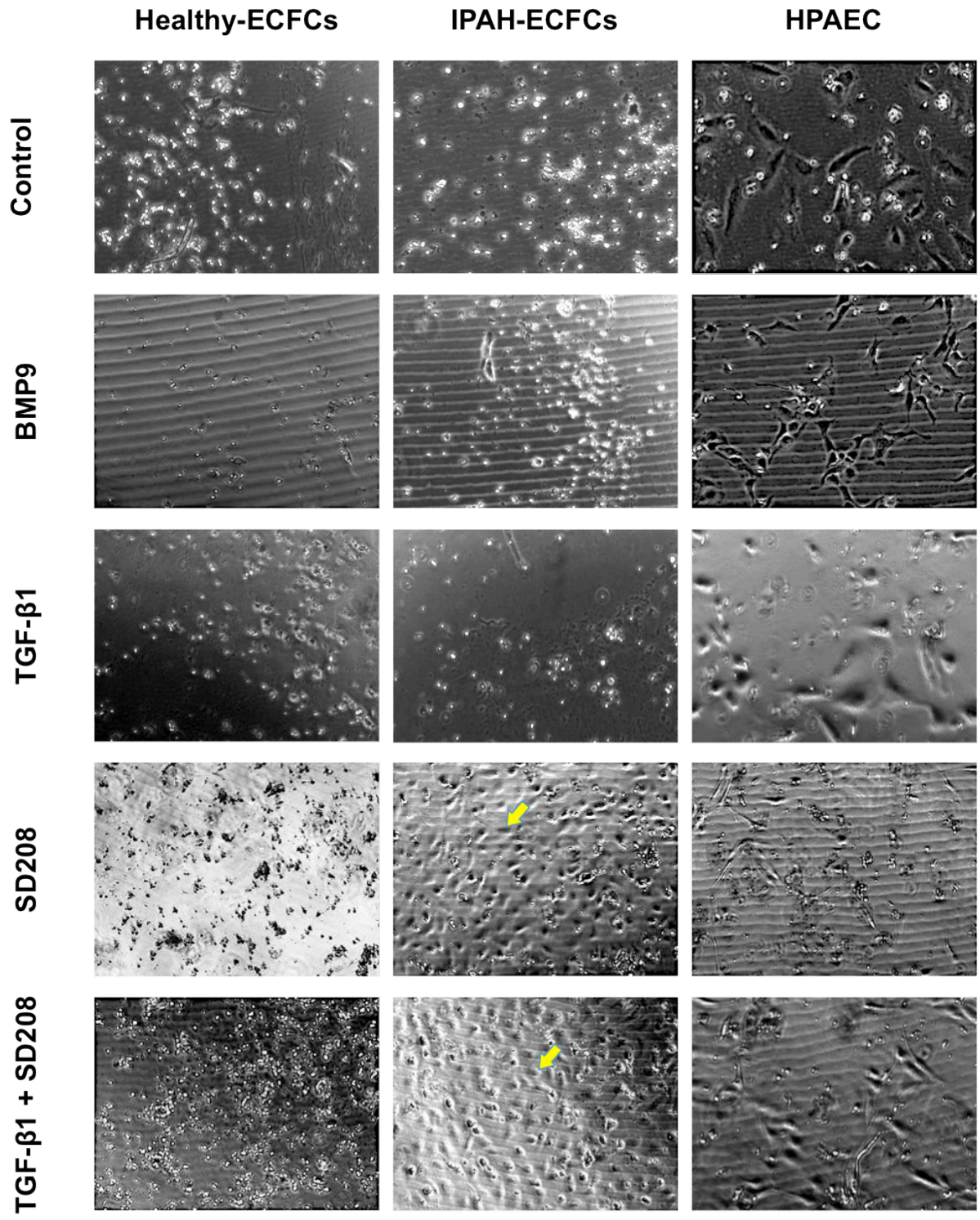


Figure 6.5. Protective effect of ALK5 inhibition in ECFCs from IPAH patients. Representative phase-contrast photomicrographs of ECFCs from healthy volunteers and IPAH patients and HPAECs, 11 days after continuous culture in EBM-2 and 1% FBS supplemented with either 10 ng/ml BMP9, 10 ng/ml TGF-β1 or 15 μM SD208 and TGF-β1 in combination with SD208. 10x magnification. Arrow, refractile viable cells.

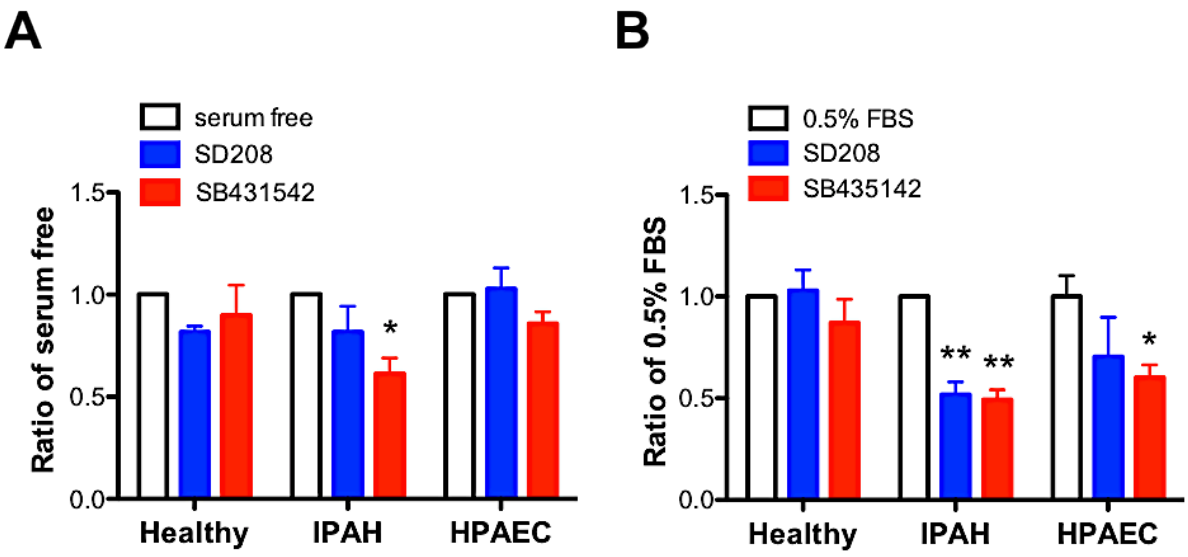


Figure 6.6. ALK5 inhibitors attenuate serum deprivation-induced apoptosis. Caspase-3/7 activity (expressed as a ratio of control) in ECFCs from healthy volunteers (n=4), IPAH patients (n=4), and HPAECs (n = 3-6) incubated in **(A)** serum-free conditions (EBM-2 without FBS) for 48h or **(B)** serum-deprived medium (EBM-2 and 0.5% FBS) for 72h. Cells were incubated in the presence of either vehicle (control), 15 µM SD208 or 15 µM SB431542. Data are presented as mean±SEM. Statistics derived from Bonferroni post-hoc analysis following one-way repeated measures ANOVA, *, $P < 0.05$, **, $P < 0.01$, compared with serum free (A) or 0.5% FBS (B).

6.3.5 Differential effects of ALK1 and ALK5 signalling on caspase-3/7 activity

In the presence of low concentrations of serum (0.5% FBS), apoptosis is induced in all ECFCs, and moderately increased in HPAECs (Figure 6.7A-C). Prolonged incubation (up to 64h) with ALK5 and ALK1 inhibitors had opposing effects on caspase-3/7 activity. In fact, the ALK5 inhibitor SB431542 (15 μM) had a significant negative effect, reducing caspase-3/7 activity significantly by about 1.8-fold in ECFCs from healthy volunteers, 2.8-fold in ECFCs from IPAH patients and 2.6-fold in HPAECs after 64 h incubation (Figure 6.7A-C & Figure 6.8A), which effect is somehow more prominent than that observed in healthy-ECFCs after longer incubation for 72 h (Figure 6.6B). In contrast, the ALK1-Fc chimera (10 μg/ml) was found to exacerbate apoptosis and significantly increased caspase-3/7 activity more than 1.6-fold in all three cell types. When added together, the respective effects of the ALK5 and ALK1 inhibitors were negated in ECFCs from IPAH patients and HPAECs whereas the effect of ALK1 inhibition still predominated in ECFCs from healthy volunteers (Figure 6.7A-C & Figure 6.8A). The addition of TGF-β1 (at 10 and 50 ng/ml) had a modest but insignificant positive effect on caspase-3/7 activity in all the three cell types and was attenuated in the presence of SB431542, this effect being most prominent in IPAH-ECFCs co-treated with 50 ng/ml TGF-β1 (Figure 6.7B). In contrast, caspase-3/7 activity was either unaffected or enhanced when TGF-β1 was combined with the ALK1-Fc chimera (Figure 6.7A-C). Taken altogether, these results suggest an antagonism between ALK5 and ALK1 signalling, reflecting the respective activation and inhibition of apoptosis through ALK5 and ALK1 receptors. In keeping with this proposal, ALK5 inhibition appeared to enhance the longer-term survival of ECFCs from IPAH patients. The addition of SB431542 was associated with the presence of more adherent 'viable' cells after 7 days (168 h) of continuous culture in serum-deprived conditions compared to culture in the control medium (2% FBS in EGM-2), and this effect was abolished following co-treatment with the ALK1-Fc chimera or TGF-β1 (Figure 6.8B).

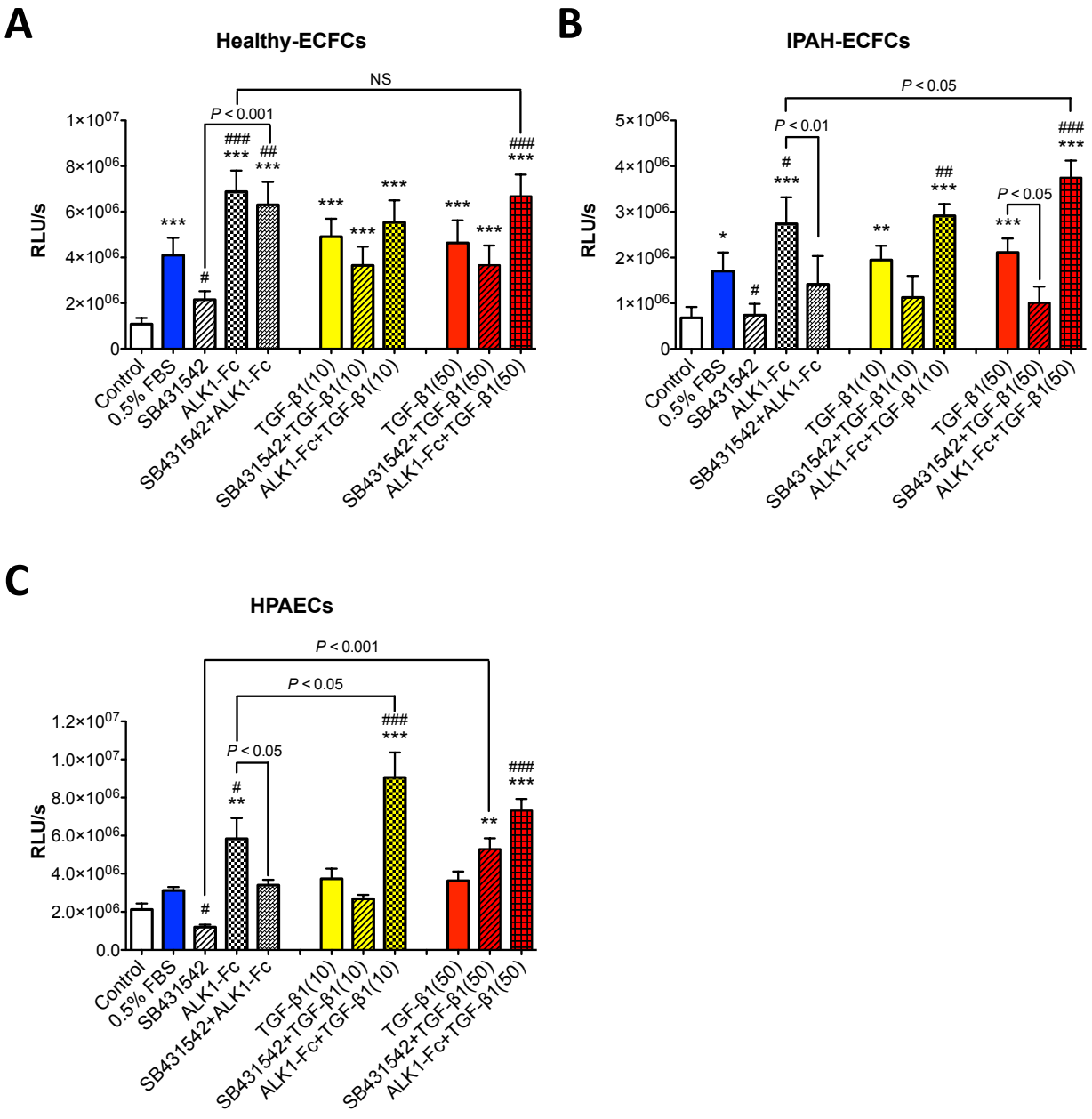


Figure 6.7. Differential effects of ALK1 and ALK5 signalling on caspase-3/7 activity. Caspase 3/7 activity (expressed as RLU/s) in ECFCs from **(A)** healthy volunteers (n=4), **(B)** IPAH patients (n=4) and **(C)** HPAECs (n=3), 64h after incubation in EBM-2 medium containing either 2.0% FBS and EGM-2 growth factors (control) or 0.5% FBS. Serum-deprived cells were treated with TGF-β1 (10 or 50 ng/ml), SB431542 (15 μM) and ALK1-Fc (10 μg/ml) as well as a combination of these compounds. Data are presented as mean±SEM. Statistics obtained from Bonferroni post-hoc analysis following one-way repeated measures ANOVA. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, compared with C; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$, compared with 0.5% FBS.

Figure 6.8A

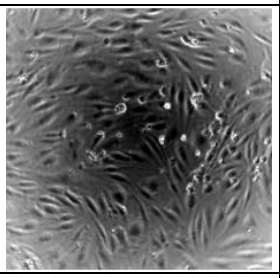
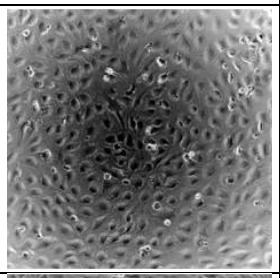
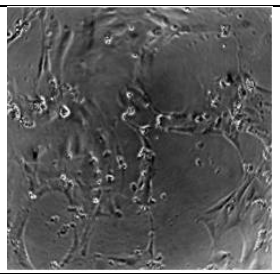
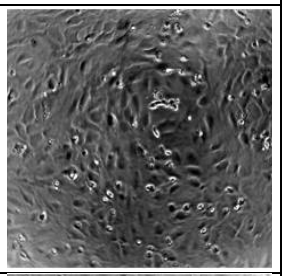
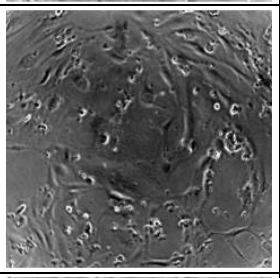
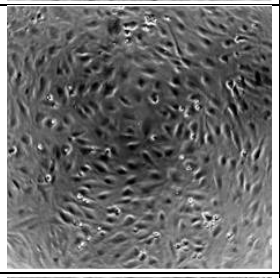
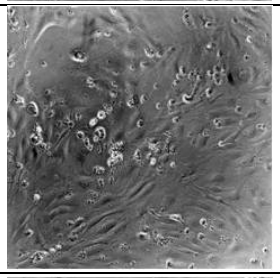
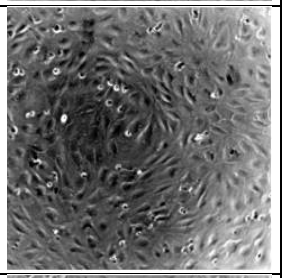
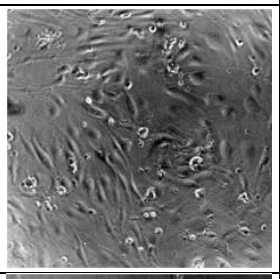
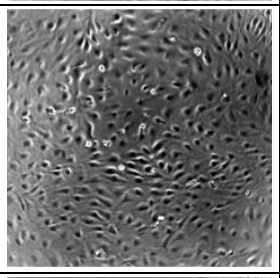
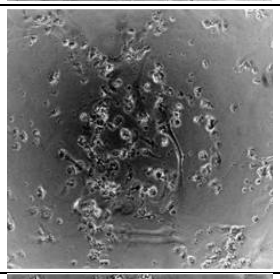
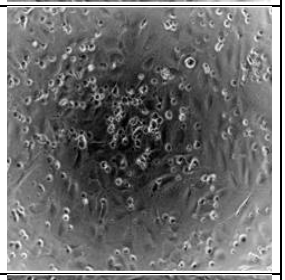
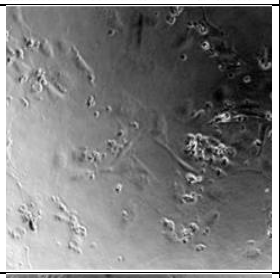
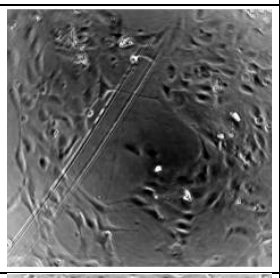
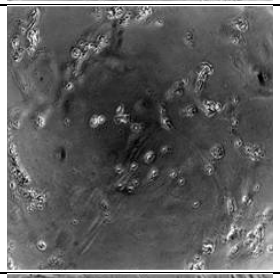
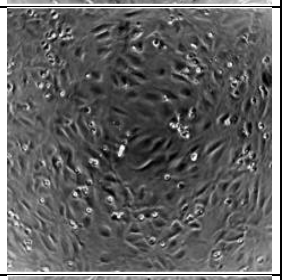
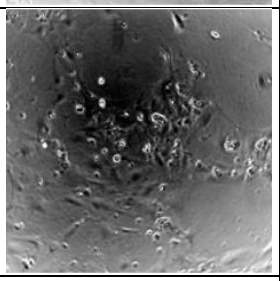
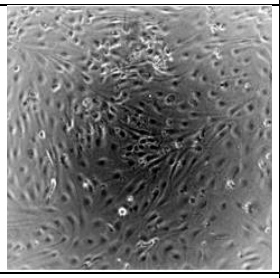
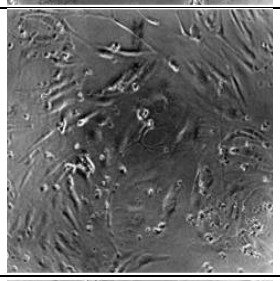
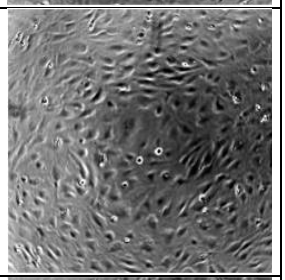
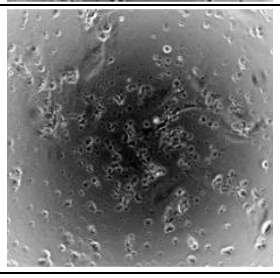
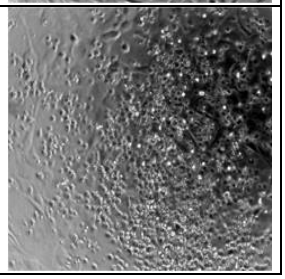
	Healthy-ECFCs	IPAH-ECFCs		Healthy-ECFCs	IPAH-ECFCs
Control			TGF-β1 (10)		
0.5% FBS			SB431542 + TGF-β1 (10)		
SB431542			ALK1-Fc + TGF-β1 (10)		
ALK1-Fc			TGF-β1 (50)		
SB431542 + ALK1-Fc			SB431542 + TGF-β1 (50)		
			ALK1-Fc + TGF-β1 (50)		

Figure 6.8B

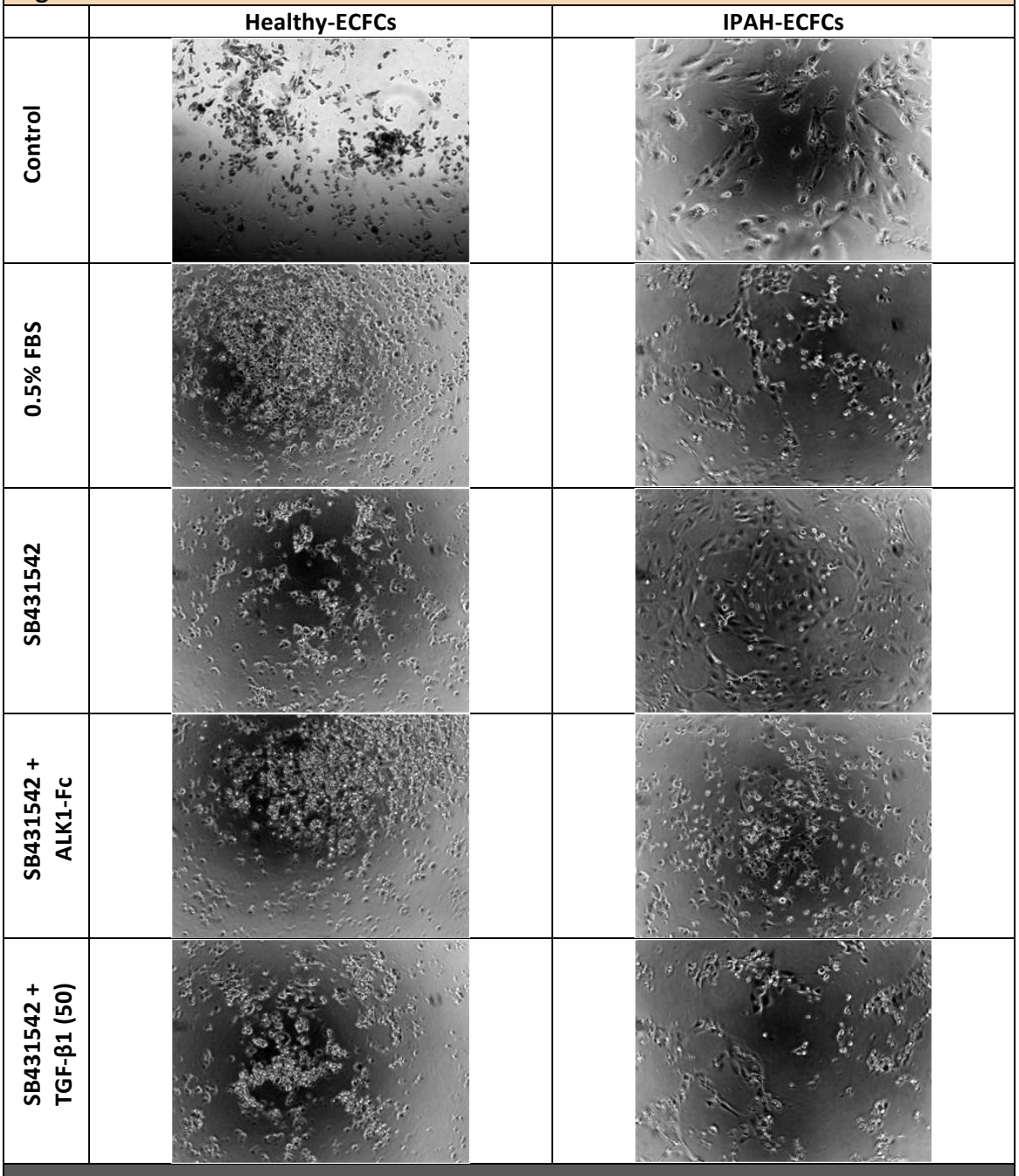


Figure 6.8. Differential effects of ALK1 and ALK5 inhibtion on caspase-3/7 activity. Representative phase-contrast photomicrographs showing: **(A)** the differential effects of TGF-β1 (10 or 50 ng/ml), SB431542 (15 μM) and ALK1-Fc (10 μg/ml) on the number of adherent, viable-looking ECFCs from a healthy volunteer and IPAH patient, 64h after incubation in serum-deprived (0.5% FBS) EBM-2 medium; **(B)** the differential effect of SB431542 and co-treatment with ALK1-Fc or TGF-β1 (50 ng/ml) on the ECFCs from the same subjects as (A), 168h after incubation in serum-deprived (0.5% FBS) EBM-2 medium. Control medium is EGM-2. 10x magnification in all images.

6.3.6 Effects of BMP4, BMP9 and TGF-β1 on DNA synthesis

The incorporation of BrdU again varied between different ECFC populations, but no significant difference was found between BrdU incorporation in ECFCs from healthy volunteers and IPAH patients (Figure 6.9A-D). Treatment with either BMP4 or BMP9 (10 ng/ml) had no apparent effect on the synthesis of DNA in ECFCs from IPAH patients (Figure 6.9A). BMP4 enhanced BrdU incorporation in both ECFCs from healthy volunteers and HPAECs, while BMP9 selectively inhibited DNA synthesis in HPAECS (Figure 6.9A). ECFCs from IPAH patients also appeared to be unresponsive to TGF-β1 stimulation, whereas ECFCs from healthy volunteers and HPAECs exhibited a concentration-dependent increase in BrdU incorporation (Figure 6.9B). Treatment of cells with an ALK5 inhibitor (SD208 or SB431542) tended to promote DNA synthesis when added alone in both healthy-ECFCs and HPAECs (Figure 6.9C-D), and in HPAECs, ALK5 inhibitor further enhanced the effect of TGF-β1 stimulation (Figure 6.9C-D). ALK1 inhibition using 10 μg/ml ALK1-Fc had tended to lower BrdU incorporation of ECFCs in healthy-ECFCs, and this was not rescued by the addition of TGF-β1 (Figure 6.9E), while no effects could be observed in IPAH-ECFCs (Figure 6.9F). In contrast, ALK1-Fc significantly increased BrdU incorporation in HPAECs (Figure 6.9G), suggesting there might be different receptor activation mechanisms regulating DNA synthesis in these cells.

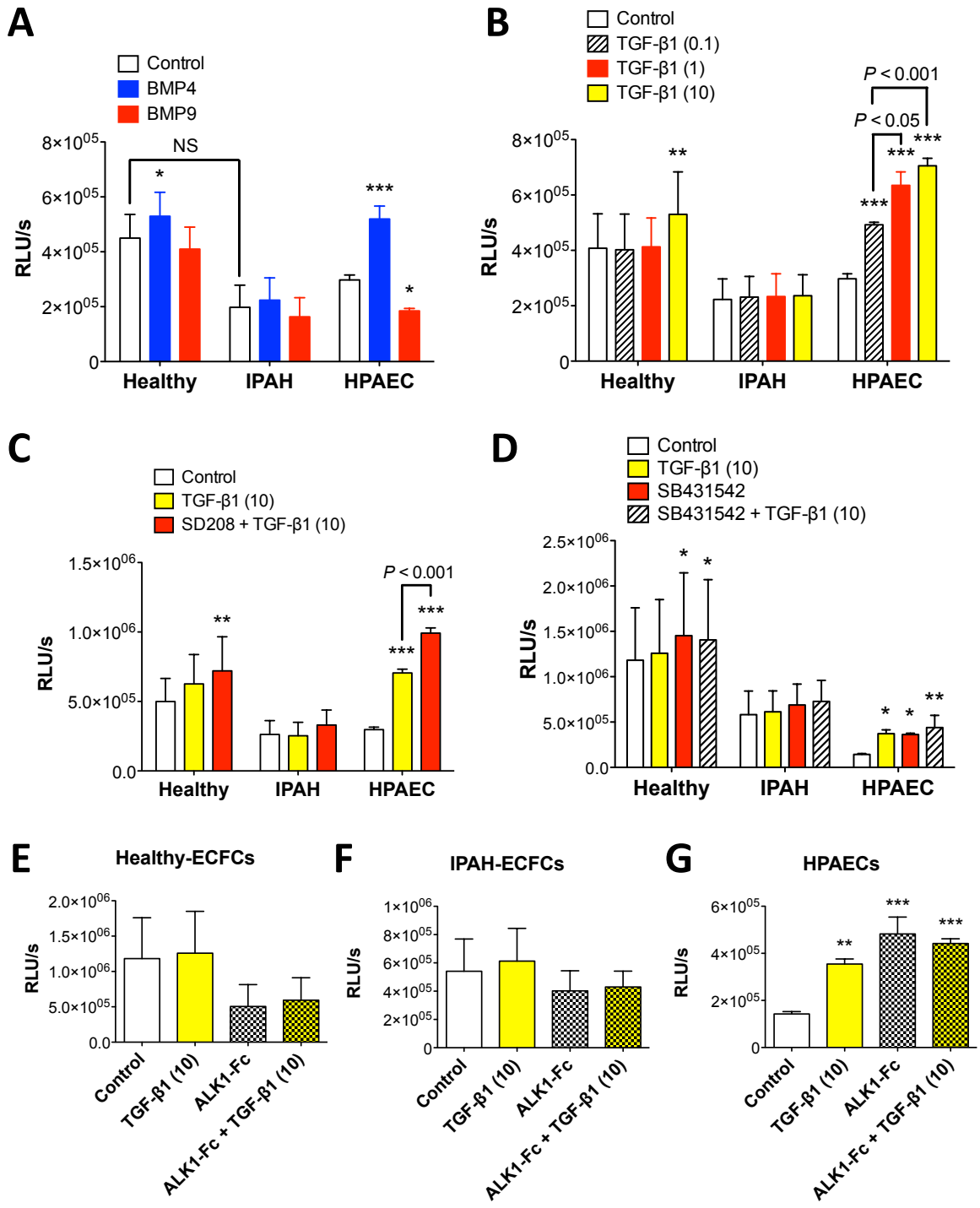


Figure 6.9. Effects of BMP4, BMP9 and TGF-β1 on DNA synthesis. BrdU incorporation in ECFCs from healthy volunteers (n = 4-6), IPAH-ECFCs (n = 4-7) and HPAECs (n = 3-4) cultured in EBM-2 and 5% FBS with vehicle controls or **A)** BMP4 and BMP9, **(B)** TGF-β1 (0.1 – 10 ng/ml), **(C-D)** TGF-β1 (10 ng/ml) alone or co-treated with ALK5 inhibitor SD208 (15 μM. BrdU incorporation in response to treaetmnet with ALK1-Fc (10 ng/ml), TGF-β1 (10 ng/ml), or both together in **(E)** healthy control ECFCs, **(F)** ECFCs from IPAH patients and **(G)** HPAECs. Data presented as mean±SEM. Statistics from Bonferroni post-hoc analysis following either two-way repeated measures ANOVA (A-D) or one-way repeated measures ANOVA (E-G). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, compared with C; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$, comparisons as indicated.

6.3.7 Effects of BMP4, BMP9 and TGF-β1 on Matrigel tube formation

ECFCs from IPAH patients again displayed increased level of Matrigel tube formation compared to ECFCs from healthy volunteers (Figure 6.10A-B). Tube formation was attenuated, albeit non-significantly, in the presence of BMP4 (10 ng/ml) and significantly inhibited by BMP9 (10 ng/ml) in ECFCs from healthy volunteers and IPAH patients and HPAECs (Figure 6.10A-C). Additional studies indicated that TGF-β1 had little or no effect on tube formation by ECFCs and induced a concentration-dependent increase in the tube formation of HPAECs, these cells also being sensitive to TGF-β1 at low concentration (0.01 ng/ml) (Figure 6.11A). The addition of the ALK5 inhibitor SD208 (15 μM) had a negative effect, inhibiting tube formation in all three groups of cells (Figure 6.11A). The VEGFR2 inhibitor SU1498 also exerted a significant inhibitory effect on tube formation by both ECFCs and HPAECs (Figure 6.11B).

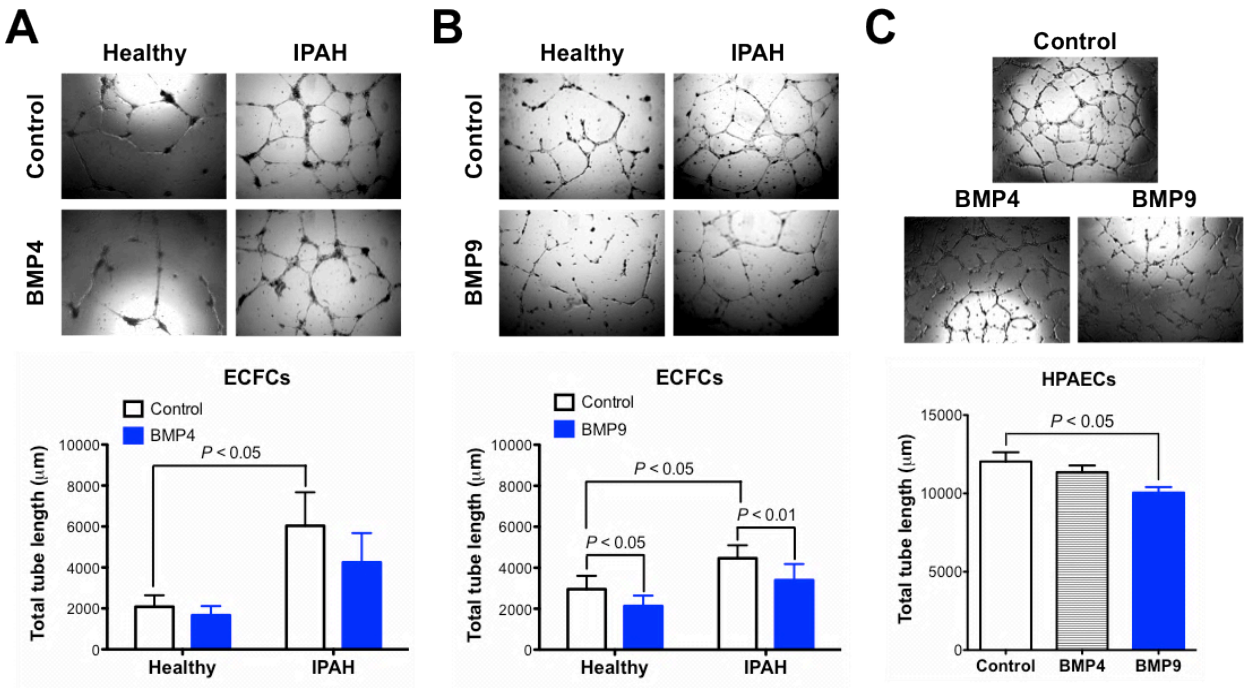


Figure 6.10. Effects of BMP4 and BMP9 on Matrigel tube formation. Total tube length formed on growth factor reduced Matrigel in response to EBM-2 with vehicle (control) or **(A)** BMP4 (10 ng/ml) by ECFCs from healthy volunteers (n=4) and IPAH patients (n=3); **(B)** BMP9 (10 ng/ml) by ECFCs from healthy volunteers (n=7) and IPAH patients (n=6); **(C)** BMP4 (10 ng/ml) or BMP9 (10 ng/ml) by HPAECs (n=3). Representative phase-contrast photomicrographs of tube formation by each sample groups are shown to demonstrate the effects of each condition. Data are presented as mean±SEM. Statistics obtained from Bonferroni post-hoc analysis following either two-way repeated measures ANOVA (A-B) or one-way ANOVA (C).

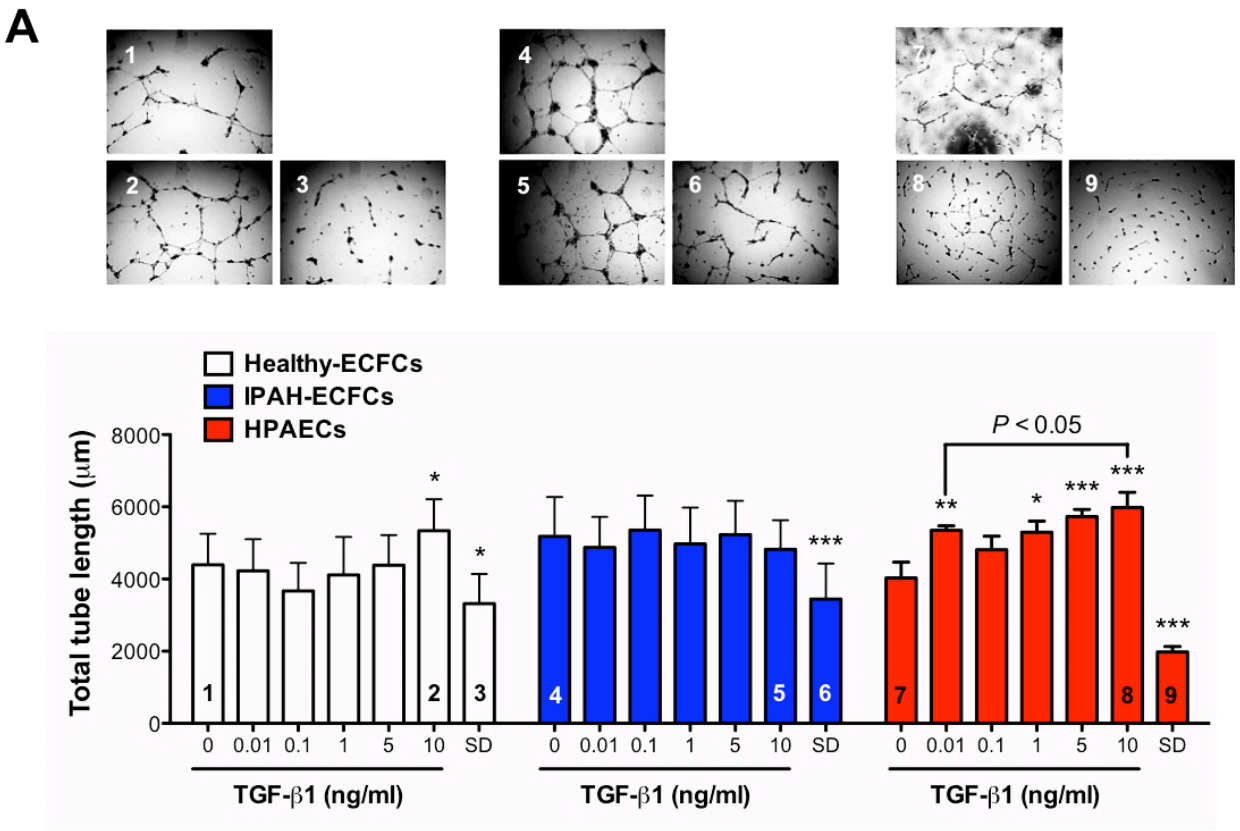


Figure 6.11. Effects of TGF-β1 and VEGFR2 inhibitor on Matrigel tube formation. Total tube length, formed on growth factor reduced Matrigel, in response to EBM-2 with vehicle (0, control) and **(A)** TGF-β1 (0.01–10 ng/ml) or ALK5 inhibitor SD208 (SD; 15 μM) by ECFCs from healthy volunteers (n=5), IPAH patients (n=5) and HPAECs (n=4) or **(B)** VEGFR2 inhibitor SU1498 (10 μM) by ECFCs from healthy volunteers (n=6), IPAH patients (n=6) and HPAECs (n=3). Representative phase-contrast photomicrographs of each corresponding numbered condition in the bar chart in (A) or effects of SU1498 in (B) are displayed. Data are presented as mean±SEM. Statistics obtained from Bonferroni post-hoc analysis following one-way repeated measures ANOVA. *, $P < 0.05$, **, $P < 0.01$, versus control; ###, $P < 0.001$, compared with control.

6.3.8 Effects of BMP4, BMP9 and TGF-β1 on cell migration in wound healing assay

ECFCs from IPAH patients exhibited an impaired capacity to recover and repopulate the area made by a wound (scratch), when compared with ECFCs derived from healthy volunteers (Chapter 4.3.4). This difference was reproduced in the current experiments (Figure 6.12A-C). The addition of BMP4 or TGF-β1 had little influence on wound recovery in ECFCs from healthy volunteers and IPAH patients (Figure 6.12A-C), while BMP9 appeared to selectively reduce migration in IPAH cells (Figure 6.12A). ALK5 inhibition was accompanied by a reduction in the rate of wound recovery, both in ECFCs and HPAECs, with SD208 tending to be more effective than SB431542 (Figure 6.12B), and co-treatment of cells with SD208 and TGF-β1 had a similar inhibitory effect to SD208 alone (Figure 6.12C). As in the tube formation assay above, the VEGFR2 inhibitor SU1498 had a negative effect on the migration of all three cell types (Figure 6.12C).

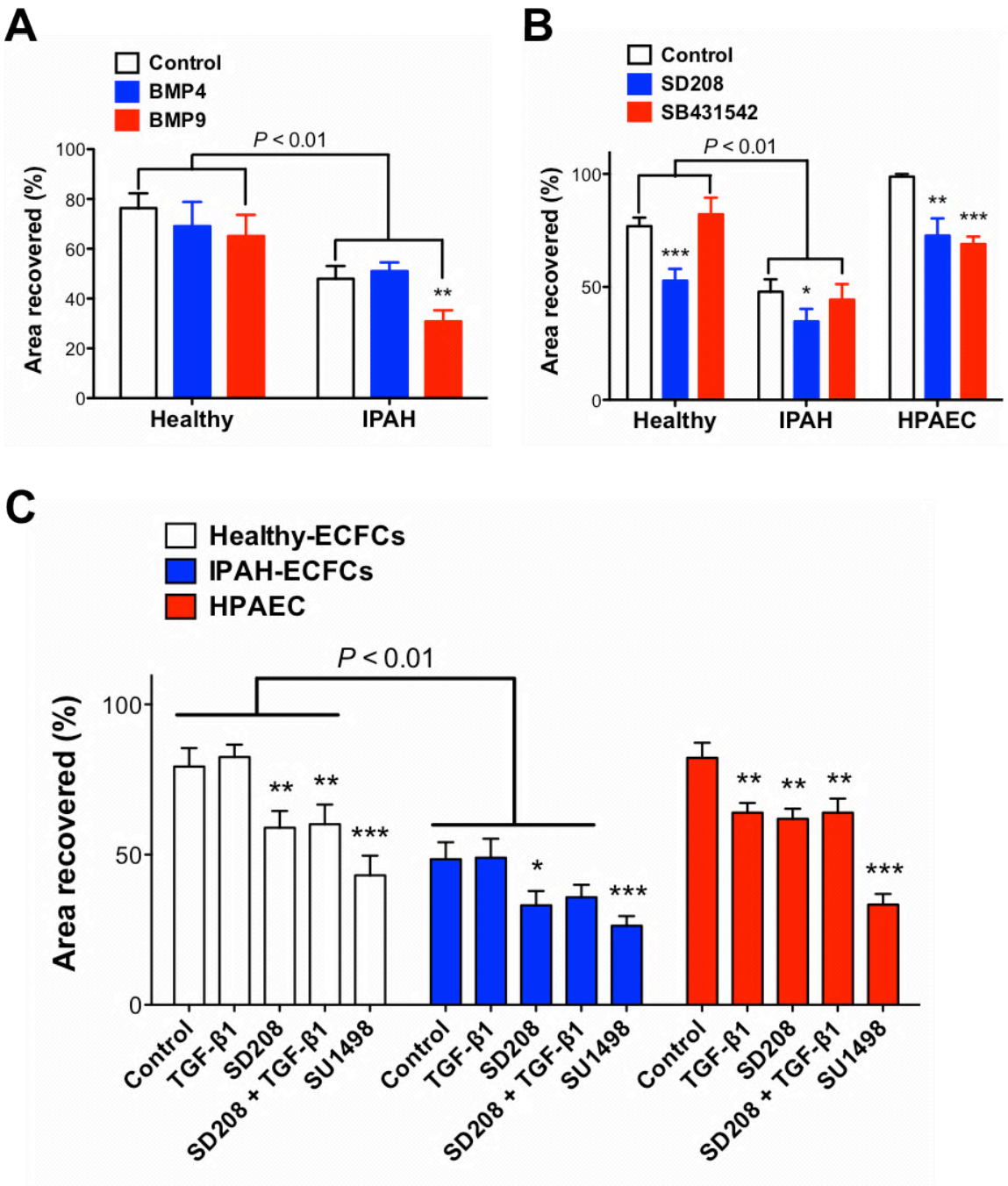


Figure 6.12. Effects of BMP4, BMP9 and TGF-β1 on cell migration in wound (scratch) assay. (A) Area recovered by ECFCs from healthy volunteers (n=6) and IPAH patients (n=7) after 18 h treatment with vehicle (control), BMP4 (10 ng/ml) or BMP9 (10 ng/ml). **(B)** Area recovered by ECFCs from healthy volunteers (n=5), IPAH patients (n=7) and HPAECs (n=3) in response to pre-treatment with the ALK5 inhibitors SD208 (15 μM) or SB431542 (15 μM). **(C)** Area recovered by ECFCs from healthy volunteers (n=6), IPAH patients (n=7) and HPAECs (n=6) after 18 h treatment with TGF-β1 (10 ng/ml), SD208 (15 μM) or a combination of the two, and in cells pre-treated with the VEGFR2 inhibitor SU1498 (10 μM). Data are presented as mean±SEM. Statistics from Bonferroni post-hoc analysis following two-way repeated measures ANOVA. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, compared to vehicle controls in each group.

6.3.9 Endothelin-1 release in ECFCs and HPAECs

One of the hallmarks of IPAH is raised circulating levels of ET-1 (Stewart et al., 1991; Langleben et al., 2006), with ECs in the remodelled pulmonary vasculature being considered a likely source of production (Giaid et al., 1993). I compared ET-1 levels in the conditioned medium of ECFCs from healthy volunteer and IPAH patients and found no difference, although both groups of cells produced more ET-1 than HPAECs after 24h in either medium with no serum or EGM-2 containing 2% FBS (Figure 6.13A-C). ET-1 has been reported to act as a pro-survival factor in ECs (Dong et al., 2005) and BMP9 stimulates ET-1 release from human pulmonary microvascular ECs and HPAECs (Star et al., 2010; Park et al., 2012). I therefore sought to investigate whether the pro-survival effect of BMP9 on ECFCs might also be linked to ET-1 release. Cells were seeded in 24-well plates, at the same density used for the caspase 3/7 assay, and treated with 10 ng/ml BMP9 in serum-free EBM-2 medium. This concentration of BMP9 is within the normal range (9-41 pmol/ml or 2-12 ng/ml) reported for circulating BMP9 (David et al., 2008). Treatment with BMP9 significantly increased ET-1 release in both ECFCs from healthy volunteers and HPAECs, but not in ECFCs from IPAH patients (Figure 6.13D–F). However, the latter response was heterogeneous and two cell populations (IPAH 18 and IPAH 31) showed a negative effect (Figure 6.13E), these corresponding with two of the three ECFC lines that also did not respond to BMP9 in the apoptosis assay (Figure 6.1F). The protein content of ECFC cultures was not affected following changes in either the serum content of the medium or treatment with BMP9 for 24h (Figure 6.14), suggesting that differences in ET-1 levels did not reflect variation in cell number. Although the protein level was lower in BMP9-treated HPAECs, the production of ET-1 was still significantly increased after normalising the values for the protein content (Figure 6.14G).

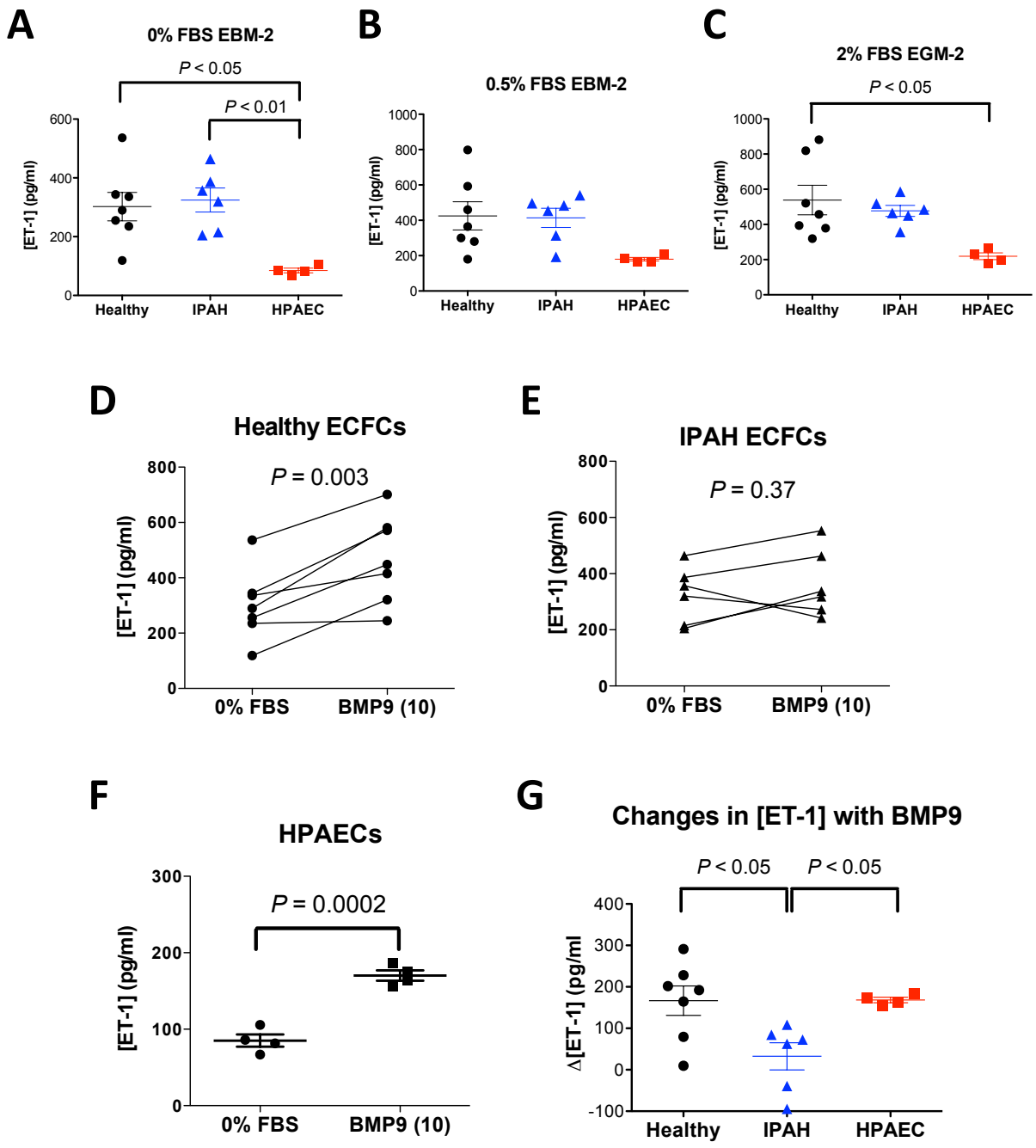


Figure 6.13. Endothelin-1 production by ECFCs and PAECs. Endothelin-1 (ET-1) levels, measured by ELISA, in conditioned medium collected after 24h. The upper panel shows ET-1 release by cells incubated in **(A)** EBM-2 with no serum, **(B)** EBM-2 with 0.5% FBS and **(C)** EGM-2 with 2% FBS. The lower panel shows ET-1 release by ECFCs from **(D)** healthy volunteers and **(E)** IPAH patients and **(F)** HPAECs, cultured in EBM-2 medium with no serum and in the presence of BMP9 (10 ng/ml) for 24 h. **(G)** The incremental change in ET-1 release upon treatment with BMP9 (10 ng/ml), relative to the vehicle control, for each ECFC population. Data are presented as mean±SEM (A-C, F, G). Statistics obtained from Bonferroni post-hoc analysis following one-way ANOVA (A, B, C, G) or t-test (D-F).

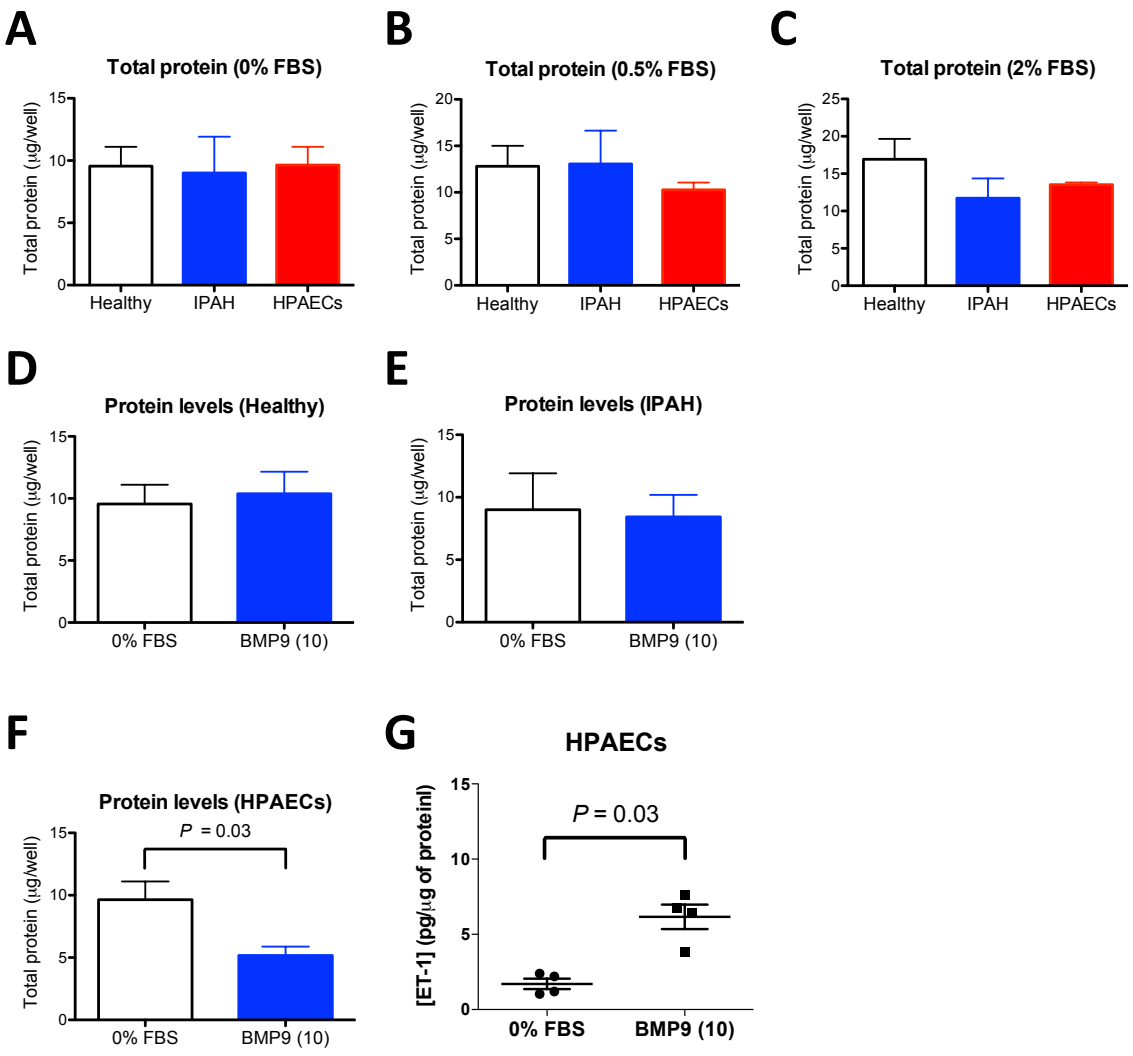


Figure 6.14. Protein levels in cell cultures used for ET-1 measurements. (A-F) Cell protein levels were measured after removal of the 24 h conditioned medium (see Figure 6.13). **(G)** Total ET-1 release by HPAECs after normalisation for the content of each well. Data are presented as mean±SEM. Statistics from Bonferroni post-hoc test following one-way ANOVA (A-C) or t-test (D-G).

TGF-β1 is a known stimulator of ET-1 release from HMVECs and HPAECs (Star et al., 2009). Interestingly, the ALK5 inhibitor SD208 had dramatic effect, abolishing ET-1 release in all cells while having no significant effect on cell protein level (Figure 6.15), indicating that ALK5 receptors and TGF-β signalling are important in regulating ET-1 release.

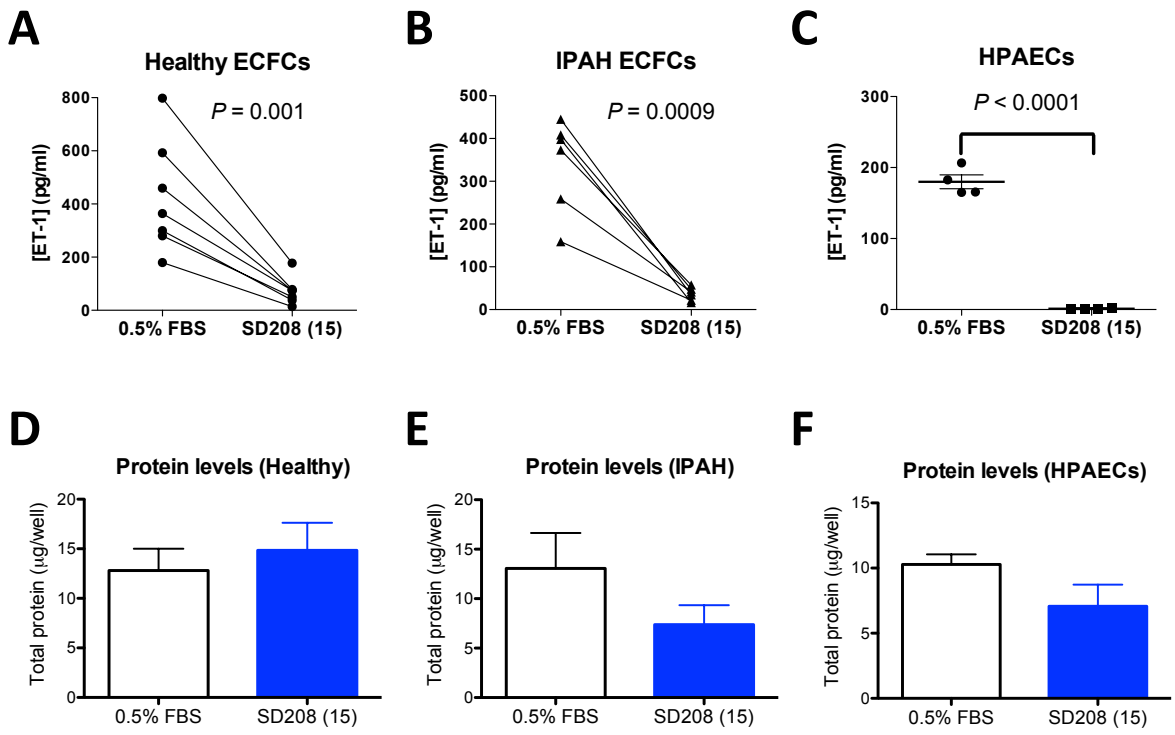


Figure 6.15. Effect of ALK5 inhibition ET-1 release by ECFCs and HPAECs. ET-1 production by ECFCs, derived from **(A)** healthy volunteers (n = 7) and **(B)** IPAH patients (n = 6), and **(C)** HPAECs (n = 4). Conditioned medium collected 24h after incubation in EBM-2 with 0.5% FBS (control) and in the presence of 15 µM SD208. **(D-F)** Proteins levels were also measured in each of the three groups of cells. Data are presented as mean±SEM (D-F). Statistics from t-test.

6.4 Discussion

The main findings in this chapter are:

- ECFCs from healthy volunteers and HPAECs exhibited comparable functional responses to BMP4, BMP9, TGF-β1 and selective ALK1 or ALK5 inhibitors.
- ECFCs from IPAH patients were generally less responsive than the other two groups of cells to stimulation by BMP4, BMP9 and TGF-β1.
- ALK1 and ALK5 receptor signalling exerted opposing effects on apoptosis (caspase-3/7 activation) induced by serum-deprivation.
- An imbalance in ALK1 and ALK5 signalling might contribute to the apoptosis-resistance and slower migration of ECFCs from IPAH patients.
- Equivalent amounts of ET-1 were produced by ECFCs from IPAH patients and healthy volunteers and its release was markedly sensitive to ALK5 inhibition.

Regulation of apoptosis by TGF-β/BMP signalling

Normal BMP signalling is important for the survival of HPAECs and disruption of BMPR2 expression promotes serum deprivation-induced apoptosis (Teichert-Kuliszewska et al., 2006; de Jesus Perez et al., 2009; Yang et al., 2011). I explored signalling through BMPR2 by stimulating cells with BMP4 and BMP9. The addition of BMP4 reduced the apoptotic response in HPAECs and ECFCs from healthy volunteers, similar to that reported by others (Southwood et al., 2008), but the effect was relatively modest. This may reflect the longer exposure to serum deprivation (48 h), compared to other studies (24 h) with BMP4 (Southwood et al., 2008) and the structurally related ligand BMP2 (Teichert-Kuliszewska et al., 2006; de Jesus Perez et al., 2009). The endothelial ALK1/BMPR2 receptor complex is selectively stimulated by BMP9 (Upton et al., 2009) and the addition of BMP9 induced a marked reduction in caspase-3/7 activation in HPAECs and ECFCs from healthy volunteers but not in ECFCs from IPAH patients. Interestingly, only 3 out of the 7 IPAH cell lines tested failed to display an inhibitory response to BMP9, suggesting that cohort of ECFCs from IPAH patients was heterogeneous. Further evidence of the importance of ALK1 activation in modulating EC survival is provided by the pro-apoptotic effect that the ALK1 inhibitor ALK1-Fc had induced on all three groups of cells.

TGF-β1 is typically considered to be pro-apoptotic agent in ECs, although the underlying mechanism is not fully clear. Most studies have described pro-apoptotic effects of TGF-β1 in ECs derived from the umbilical vein, aorta and microvasculature of various species (Tsukada et al., 1995; Ferrari et al., 2006; Lu et al., 2009; Tian et al., 2012a). But other studies found that TGF-β1 was also capable of promoting survival in bovine PAECs and mouse capillary ECs (Vinals and Pouyssegur, 2001; Lu, 2008). These disparate responses have been attributed to endothelial heterogeneity in the vasculature, with TGF-β1 exerting pro- and anti-apoptotic effects (through the ALK5 receptor) in ECs derived from proximal and distal regions of the bovine pulmonary artery bed respectively (Lu, 2008; Lu et al., 2009). I found that two different pharmacological inhibitors of ALK5 (SD208 and SB431542) also attenuated serum deprivation-induced apoptosis in all three cell types, and selectively promoted the longer-term survival of ECFCs from IPAH patients (beyond 3 – 11 days). This suggests that ALK5 signalling is an important determinant of apoptosis/survival and, at least in longer-term culture, may differ in IPAH. Further studies are required to explore the autocrine/paracrine activation of ALK5 signalling during serum-deprived conditions and the potential contribution of serum as a source of BMP9 and TGF-β1 in the culture experiments. It is recognised that the effects of TGF-β signalling through ALK1 and ALK5 receptors are both cell type and context dependent. Studies with ECFCs from normal subjects have shown for example that incubation with growth factor-free, low serum medium containing high glucose and glycated albumin induces ALK5 expression and TGF-β1 production without affecting ALK1 or endoglin protein levels (Wang and Hirschberg, 2009). The 'diabetic milieu' induced ECFC proliferation as well as caspase-3 activity and the effects were respectively enhanced and attenuated by the ALK5 inhibitor SB431542.

Despite using a wide concentration range (0.01-50 ng/ml), the pro-apoptotic effects of TGF-β1 were not prominent in ECFCs. However, TGF-β1 has been shown to induce survival as well as apoptotsis in ECs, by shifting VEGF signalling between different p38 MAPK isoforms (Ferrari et al., 2009; Ferrari et al., 2012). This results in the transient induction of apoptosis, within 6-12 h of stimulation, followed by a long period when the cells are refractory to the pro-apoptotic effects of TGF-β1. So it is possible that the apoptotic effect of TGF-β1 in ECFCs had waned after 48-64 h incubation and the results were confounded by its dual activity. Conversely, sustained ALK5 activation may impair the permeability and contact of ECs and lead to apoptosis over a longer time course (42 h) (Antonov et al., 2012).

Nevertheless, when considered together, the present data support the concept that ALK1 and ALK5 signalling have opposing actions on EC apoptosis: activation of ALK1 being pro-survival (anti-apoptotic) and activation of ALK5 pro-apoptotic (see Figure 6.16A). It is unclear if the reduced expression of ALK5 and impaired ALK5/Smad2 activation contributed to the apoptosis-resistant

phenotype of ECFCs from IPAH patients, but ALK5 inhibition in serum-deprived healthy-ECFCs have attenuated the apoptosis down to similar level as those observed in serum-deprived IPAH-ECFCs without treatment, while co-treatment of ALK1 inhibition and TGF-β1 in IPAH-ECFCs has raised the apoptosis level close to those displayed by serum-deprived healthy-ECFCs (Figure 6.7A & B). It is conceivable to suggest that normal TGF-β signalling allows ECFCs to undergo normal programmed cell death, whereas dysregulated TGF-β signalling in ECFCs from IPAH patients could lead to the impaired sensitivity of these cells to apoptosis (Figure 6.16A-B). This might play an important role especially in the formation of apoptosis-resistant ECs lining the plexiform lesions in IPAH lungs where ALK5 and pSmad2 expression are both significantly reduced (Richter et al., 2004). Ideally, I would like to have confirmed the differential effects of ALK1 and ALK5 signalling using another apoptotic stimulus, such as the treatment of ECs with the combination of TNF-α and cycloheximide (Sakao et al., 2005), and explored the effect of manipulating ALK5 expression on the apoptosis of ECFCs from healthy volunteers. Additional experiments might also benefit from the use of luiferase reporter gene constructs that are responsive to ALK1 (e.g. BRE-lux) and ALK5 (e.g. CAGA-lux) activation (Shao et al., 2009). Interestingly, caspase-3/7 activation was modulated by selective ALK1 and ALK5 inhibitors on their own, indicating signalling through these receptors in the absence of exogenous ligands. Whether the receptors are constitutively active, stimulated by endogenously produced proteins and/or BMP9 (David et al., 2008) and TGF-β1 (Selimovic et al., 2009) present in serum is not clear.

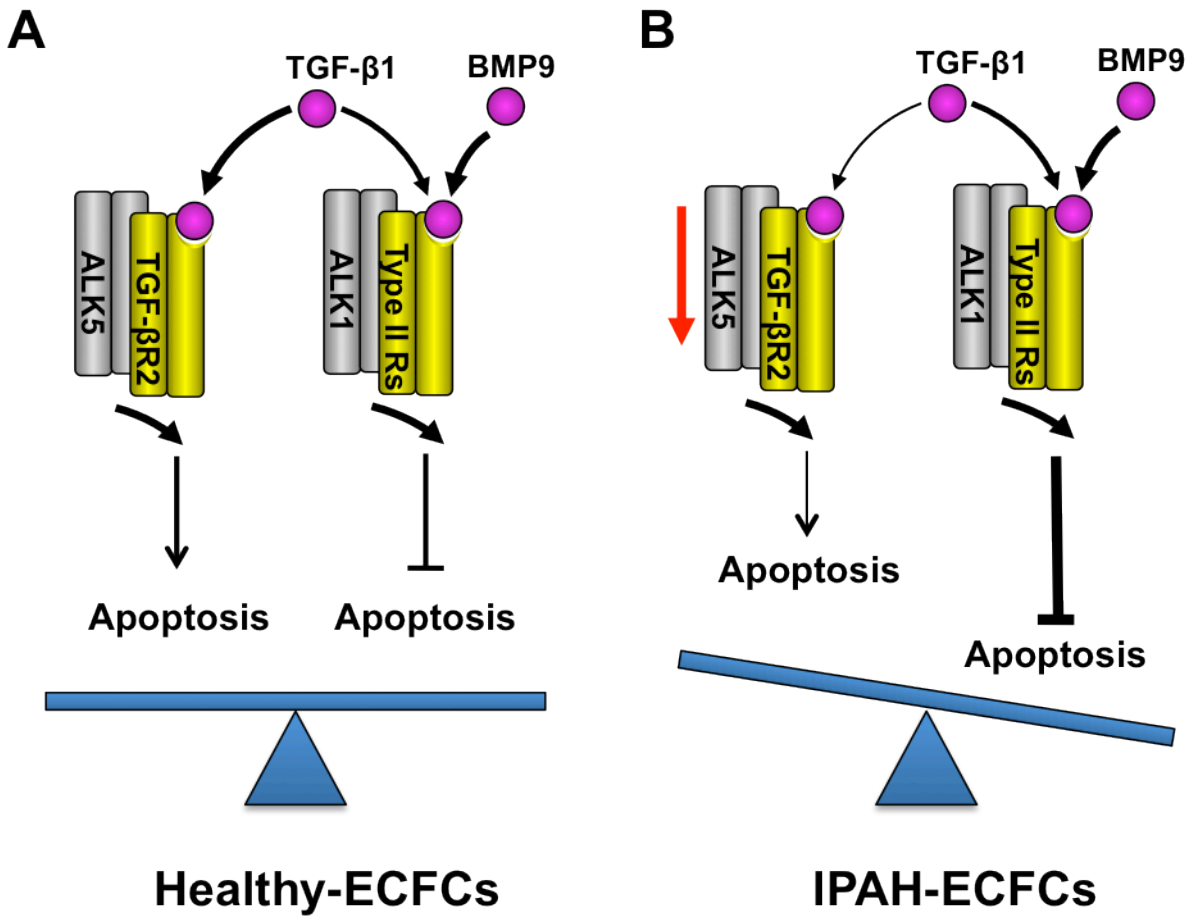


Figure 6.16. ALK1 and ALK5 regulations of apoptosis in ECFCs. (A) TGF-β signalling regulation of ECFCs apoptosis rely on the balance of the ALK1 and ALK5 signalling. ALK5 activation promotes apoptosis, while ALK1 activation paradoxically inhibits apoptosis and promotes survival of ECFCs. In healthy-ECFCs with normal ALK5 expression, TGF-β1 preferentially activates ALK5 while BMP9 activates ALK1 to provide a fine-tuned balance that allows ECFCs to undergo normal programmed cell death. **(B)** In IPAH-ECFCs, ALK5 expression is significantly lower. This could significantly impair ALK5 signalling, shifting the balance of functional regulation to ALK1, resulting in substantial resistance to apoptosis in these cells that could be important in the pulmonary vascular remodelling in IPAH.

Regulation of proliferation, migration and angiogenesis by TGF-β/BMP signalling

Exploring the role of ALK1 and ALK5 signalling in other ECFC functions may provide further insight into the significance of impaired ALK5 expression/signalling in the disease-related phenotype of ECFCs from IPAH patients. Some understanding of the importance of these receptors and their interaction has been provided by studies of ECFCs isolated from HHT patients with endoglin (*HHT1*) and ALK1 (*HHT2*) mutations (Fernandez et al., 2005). Notably, reduced expression of endoglin and ALK5 occurred in both types of mutation and was accompanied by impaired ALK5 signalling and Matrigel tube formation. Furthermore, it was proposed that the down regulation of ALK5 expression/signalling represents an adaptive response that aims to maintain the balance between endothelial TGF-β/ALK1 and TGF-β/ALK5 signalling (Fernandez et al., 2005). ECFCs derived from HPAH patients with BMPR2 mutations have also been found to exhibit increased proliferation as well as reduced tube formation *in vitro* (Toshner et al., 2009). These observations suggest that disruption of key components in the BMPR2/ALK1/endoglin receptor complex may affect the ALK5 pathway and functions of ECFCs in culture.

In accordance with previous findings (Smadja et al., 2008), BMP4 had a positive effect on proliferation (DNA synthesis) in ECFCs from healthy controls. However, the effects of BMP9 and TGF-β1 on DNA synthesis were inconclusive and ECFCs from IPAH patients appeared unresponsive to all three proteins under the same experimental conditions. The results with HPAECs were more informative; BMP9 causing a marked reduction in DNA synthesis while TGF-β1 had the opposite effect and this was further enhanced in the presence of an ALK5 inhibitor. While most studies to date have also shown that ALK1 signalling inhibits EC proliferation (Lamouille et al., 2002; Koleva et al., 2006; David et al., 2007a; Shao et al., 2009; Upton et al., 2009) several have reported that ALK5 signalling has this effect as well (Goumans et al., 2002; Castanares et al., 2007; Froese et al., 2011). This serves to underline the complexity of TGF-β receptor signalling in the endothelium and the need for further work to dissect the role of ALK1 and ALK5 pathways in regulating the proliferation of mature ECs as well as ECFCs.

BMP9/ALK1 signalling has consistently been shown to inhibit EC migration (Lamouille et al., 2002; David et al., 2007a; David et al., 2007b; Scharpfenecker et al., 2007; Lee et al., 2009; Park et al., 2012). In this study, BMP9 inhibited the migration of ECFCs, significantly in cells derived form IPAH patients. ALK5 inhibition also attenuated the migration of ECFCs. This is in agreement with other studies describing the opposing effects of ALK1 and ALK5 signalling on the migratory capacity of ECs (Shao et al., 2009; Tian et al., 2012a). Dysregulation of ALK5 in ECFCs from IPAH patients might therefore contribute to their impaired migration phenotype.

In contrast to the marked inhibitory effect of BMP9 on Matrigel tube formation, TGF-β1 exerted a modest positive effect on ECFCs from healthy volunteers and stimulated angiogenesis by HPAECs. The observations are consistent with the opposing angiogenic effects of TGF-β1 and BMP9 in other EC studies (Shao et al., 2009; Ferrari et al., 2012), but are limited by the absence of experiments using selective ALK1 and ALK5 receptor blockade. While BMP9-stimulation of ALK1 has been described to inhibit EC proliferation and angiogenesis (David et al., 2007a; Scharpfenecker et al., 2007; Lee et al., 2009; Shao et al., 2009), some studies have reported that angiogenesis was promoted by BMP9 *in vitro* (Park et al., 2012) and *in vivo* (Suzuki et al., 2010). A possible explanation is that the distinct effects of BMP9 are concentration-dependent and the BMP9/ALK1 pathway is essential for endothelial sprouting (van Meeteren et al., 2012). Thus ALK1 has been considered a potential target in anti-angiogenesis therapy, with clinical phase I studies being conducted to block ALK1 signalling using ALK1-Fc chimera (ClinicalTrials.gov Identifier NCT00996957) and an anti-human ALK1 antibody (ClinicalTrials.gov Identifier NCT00557856).

Regulation of ET-1 release by TGF-β/BMP signalling

One of the hallmarks of IPAH is raised circulating levels of ET-1 (Stewart et al., 1991; Langleben et al., 2006), with ECs in the remodelled pulmonary vasculature being considered a likely source of production (Giaid et al., 1993). Serum-deprived pulmonary ECs from IPAH patients release higher level of ET-1 compared to the controls (Eddahibi et al., 2006). However, the results presented here demonstrate that basal ET-1 production by ECFCs from IPAH patients and healthy volunteers is comparable in serum-deprived condition as well as cultured in various levels of FBS. A few discrepancies might explain these disparate findings. Firstly, it is not known whether the isolated pulmonary ECs in the earlier study also constitute a large proportion of ECs from the remodelled plexiform lesions. Secondly, it is unclear whether ECs from these lesions also release excessive levels of ET-1 in IPAH lungs, as dysfunctional ECFCs might specifically contribute to the plexiform lesions rather than exist as ECs elsewhere in the pulmonary circulation. In fact, increased ET-1 levels might not be exclusively contributed by the dysregulated pulmonary endothelium, as other pulmonary cells such as PASMCs (Wort et al., 2001) and lung fibroblasts (Shi-Wen et al., 2004) are also capable of producing considerable amount of ET-1.

ET-1 is reported to be a survival factor for ECs (Dong et al., 2005) and it would be interesting to know whether the anti-apoptotic effects of BMP9 are dependent on the paracrine/autocrine action of ET-1. As expected from recent studies on ECs by other groups (Star et al., 2010; Park et al., 2012),

BMP9 induced a significant increase in ET-1 release from healthy ECFCs and HPAECs. But I did not have time to explore the contribution of ET-1 to the downstream effects of BMP9/ALK1 signalling, using either inhibitors or mature ET-1 cleavage or selective ET_A/ET_B receptor antagonists. In contrast to ECFCs from healthy controls, those derived from IPAH patients displayed a heterogeneous response to BMP9 stimulation, with no significant change overall. It is notable that three of the ECFC lines derived from IPAH patients did not exhibit either BMP9-induced ET-1 secretion or BMP9-induced inhibition of apoptosis. Future studies might explore whether ALK1, the type II receptors BMPR2, ActRIIA, and ActRIIB or ALK1 co-receptor endoglin are selectively affected in these cells.

ET-1 is also a target gene for the action of TGF-β1, which stimulates endothelial expression and release of ET-1 through activation of the ALK5/Smad3 pathway (Castanares et al., 2007). In addition, ET-1 was shown to mediate some of the anti-migratory and anti-proliferative actions of TGF-β1 in cultured ECs (Castanares et al., 2007). I did not directly demonstrate the effect of TGF-β1 on ET-1 production, but did establish that basal ET-1 release from both ECFCs and HPAECs was almost abolished in the presence of the ALK5 inhibitor SD208. It would seem doubtful if this general inhibitory effect on ET-1 production contributes to the apparently selective effect of ALK5 inhibitors, attenuating serum-deprivation induced apoptosis in ECFCs from IPAH patients versus healthy controls. A diagrammatic summary of TGF-β/BMP signalling in the regulation of ECFC proliferation, tube formation, migration and ET-1 production is shown in Figure 6.17A-C.

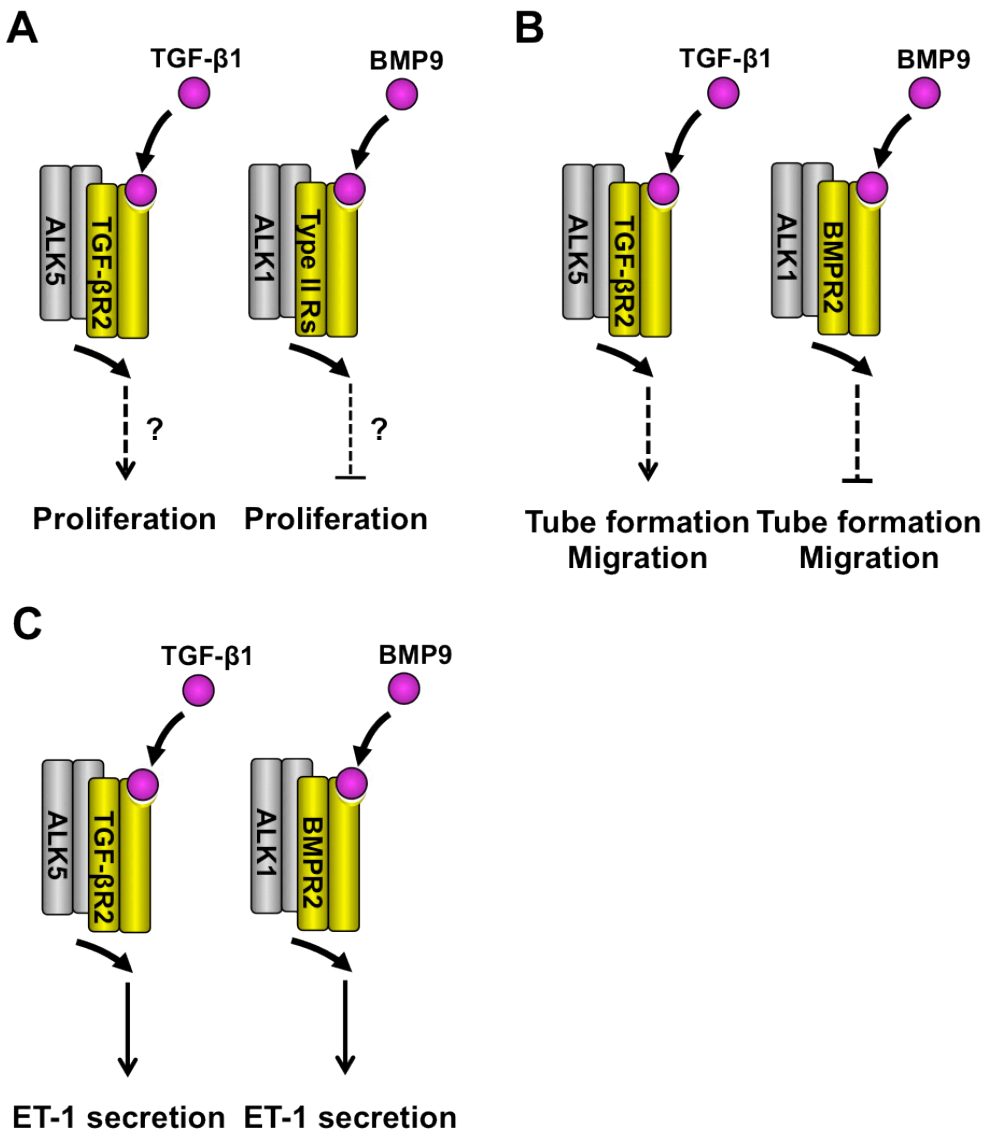


Figure 6.17. TGF-β and BMP signalling in functional regulations of ECFCs. (A) Hypothetical mechanism showing the regulation of proliferation by ALK1 and ALK5 activation based on the literature and data collected in this study. **(B)** TGF-β1-activated ALK5 signalling promotes tube formation and migration, while BMP9-activated ALK1 signalling paradoxically inhibits both of these functions. **(C)** Both ALK5 and BMP9-activated ALK1 signalling induces ET-1 secretion from ECFCs. Full arrow represents confirmed observations, while dashed arrow shows mechanism that has not been completely validated in this and other studies.

Limitations and conclusions

There are a number of limitations to the studies in this chapter, several of which have been highlighted above. In particular, I sought to expose potential differences at the receptor level and did

not consider downstream Smad-dependent and -independent intracellular pathways. I used only pharmacological tools whereas molecular interventions, designed to manipulate expression of specific genes and introduce reporter constructs, may have been more informative. The concentrations of proteins/reagents used were determined by their ability to acutely affect Smad phosphorylation, as shown in Chapter 5, and the results of published studies, and I may not have adequately considered the biphasic and time-dependent nature of the functional effects. I used two ALK5 inhibitors, with SB431542 being considered one of most specific and effective ALK5 inhibitors commonly available, but cannot exclude possible inhibitory effects on other receptors (e.g. ALK4, ALK7) (Vogt et al., 2011). ALK1 has a very discriminating ligand binding profile, binding both BMP9 and BMP10 with high affinity and these ligands are considered to signal exclusively via the ALK1 receptor in ECs (David et al., 2007a; Scharpfenecker et al., 2007; Upton et al., 2009). I used BMP9 to explore ALK1 mediated responses in ECFCs, but it has recently been shown that BMP9 and BMP10 differ in their interactions with ALK1 and the type II receptors BMPR2, ActRIIA and ActRIIB, the relative affinity of BMP9 being ActRIIB > BMPR2 >> ActRIIA whereas BMP10 exhibited a similar high affinity for all three receptors (Townson et al., 2012). Future studies may need to use both ligands and selective knockdowns or mutants in order to explore which type II receptors are involved in complexes mediating effects in ECFCs and ECs. Although there was no difference in ET-1 production between the two groups of ECFCs, this observation could be limited by the controlled cell environment used in this study. As alterations in shear stress and levels of angiotensin II, NO, growth factors and inflammatory cytokines can modulate the release of ET-1 (Kuchan and Frangos, 1993; Marasciulo et al., 2006), it is important in future to analyse the ability of ET-1 release by the ECFCs under more physiological conditions to determine whether alterations in those parameters could contribute to the excessive endothelial production of ET-1 in IPAH patients.

In conclusion, ALK1 and ALK5 signalling pathways exert distinct effects on ECFC apoptosis, migration and tube formation. ECFCs from healthy volunteers and HPAECs exhibited comparable functional responses when treated with BMP4, BMP9, TGF-β1 and selective ALK1 or ALK5 inhibitors. ECFCs from IPAH patients were generally found to be less reactive and displayed heterogeneity in specific responses to BMP9 stimulation. An imbalance in ALK1 and ALK5 signalling may be significant in the apoptosis-resistance and slower migration of ECFCs from IPAH patients, but the contribution of these pathways in the enhanced tube forming capacity of these cells seems less likely. Equivalent amounts of ET-1 were produced by ECFCs, with no difference being found between cells derived from IPAH patients and healthy volunteers. ***In the next chapter I will use ECFCs to examine two other targets/pathways implicated in the pathogenesis of PAH: chloride intracellular channel 4 and asymmetric dimethylarginine.***

Chapter 7:

Use of ECFCs to investigate endothelial dysfunction in IPAH

Chapter 7 - Use of ECFCs to investigate endothelial dysfunction in IPAH

7.1 Introduction

To further demonstrate that ECFCs represent an effective cellular model for investigating endothelial dysfunction in IPAH, I have used these cells to examine two novel targets/pathways implicated in the pathogenesis of PAH: chloride intracellular channel 4 and asymmetric dimethylarginine.

Chloride intracellular channel 4 (CLIC4) belongs to a family of six highly conserved redox-sensitive proteins (CLIC1-6) that are homologous to glutathione transferases (Littler et al., 2010). Expression of CLIC4 is affected by reactive oxygen species, DNA damage and cytokines such as tumour necrosis factor-α (TNF-α) and TGF-β (Suh et al., 2007; Yao et al., 2009a). Studies on the systemic vasculature *in vitro* and *in vivo* have shown that, unlike other CLIC proteins, CLIC4 is required for endothelial cell network formation and morphogenesis (Chalothorn et al., 2009; Tung et al., 2009; Littler et al., 2010). Knock out of the murine CLIC4 gene also results in reduced collateral vessel formation *in vivo* and a reduction in the ischemia-induced expression of hypoxia inducible factor-1α (HIF-1α) and downstream targets such as vascular endothelial growth factor (VEGF) and angiopoietin-2 (Chalothorn et al., 2009). Furthermore, CLIC4 contributes to several signalling pathways that have been implicated in the pathogenesis of PAH, including Rho, VEGF, BMP, TGF-β and TNF-α signalling (Suh et al., 2005; Shukla et al., 2009; Spiekerkoetter et al., 2009).

The level of CLIC4 expression is greater in the explanted lungs of patients with PAH, compared with control lung tissues (Abdul-Salam et al., 2010). In the PAH lung, CLIC4 is localised predominantly to the endothelium and is prominent in occlusive and plexiform lesions, which are characterised by the proliferation of apoptotic-resistant endothelial cells and considered to reflect disorganised angiogenesis (Tuder and Voelkel, 2002). Pulmonary CLIC4 levels are also raised in rat models of PH, where CLIC4 is also localised predominantly to the vascular endothelium (Abdul Salam et al., 2012). Furthermore, CLIC4 expression promoted the stabilisation of HIF-1α (Abdul Salam et al., 2012), and this in turn has been associated with increased tube formation by HPAECs (Yamakawa et al., 2003). I therefore sought to determine whether ECFCs from IPAH patients also exhibited increased expression of CLIC4 and explore the impact this may have on their tube forming capacity.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) production, inhibiting all three isoforms of NO synthase, and is a cardiovascular risk factor (Vallance and Leiper, 2004). While reduced NO signalling in PAH is well described (Budhiraja et al., 2004) and increased plasma and tissue levels of ADMA are evident in patients with IPAH (Pullamsetti et al., 2005), the mechanisms leading to impaired endothelial function in IPAH are not fully understood.

The lung has a critical role in determining circulating levels of ADMA (Bulau et al., 2007) and two key enzymes, dimethylarginine dimethylaminohydrolase I and II (DDAHI and DDAHII), are responsible for ADMA metabolism (Leiper et al., 2007). DDAHI heterozygous knockout mice have high plasma ADMA levels compared to controls and show remodelling of small pulmonary arteries, increased pulmonary vascular permeability and right ventricular pressure (Leiper et al., 2007; Wojciak-Stothard et al., 2009). Increased ADMA levels have also been associated with reduced DDAH expression and activity in lung tissues and pulmonary endothelium of patients with IPAH and established animal models of PH (Millatt et al., 2003; Pullamsetti et al., 2005).

ADMA has been shown to induce pulmonary endothelial leakage and inhibit VEGF-induced angiogenesis *in vitro,* these effects being prevented by over expression of DDAHI (Fiedler et al., 2009; Wojciak-Stothard et al., 2009). Gap junctions connect the cytoplasm of adjacent cells and allow exchange of small signalling molecules such as cyclic nucleotides, Ca^{2+}, adenosine triphosphate (ATP) and inositol 1,4,5-trisphosphate between cells. They are formed by two hemi-channels, each composed of six connexin subunits, and in the vascular system, the core proteins of these channels are connexins (Cx) 37, 40 and 43 (van Kempen and Jongsma, 1999). Deletion of these connexin genes causes severe cardiovascular abnormalities (Reaume et al., 1995; Liao et al., 2001; Simon and McWhorter, 2002; de Wit et al., 2003). NO signalling and gap junctional communication are also essential for normal vascular function (Looft-Wilson et al., 2012). My colleagues have recently demonstrated that Cx43 has a role in mediating the effects of ADMA on HPAECs, where ADMA disrupts endothelial barrier function and angiogenesis by inhibiting the expression and membrane localisation of Cx43 (Tsang et al., 2011). I therefore sought to explore whether ADMA metabolism and Cx43 expression are affected in ECFCs from IPAH patients, and explore the impact these might have on their permeability and tube forming abilities.

I hypothesised that CLIC4 expression is increased in ECFCs from IPAH patients and contributes to the dysregulation of tube formation by these cells. I also postulated that ECFCs from IPAH patients would produce more ADMA and exhibit corresponding differences in DDAHI and Cx43 expression compared to control cells. Finally, disruption of Cx43 expression might contribute to the impaired endothelial barrier function exhibited by ECFCs from IPAH patients.

The objectives of this chapter are:

- Investigate the expression of CLIC4 and HIF-1α in ECFCs from healthy volunteers and IPAH patients.
- Examine the role of CLIC4 in the excessive tube formation exhibited by ECFCs from IPAH patients.
- Determine the effects of BMPR2 knockdown on CLIC4 expression in ECFCs.
- Investigate ADMA production and the expression of connexins, DDAHI and DDAHII by ECFCs from healthy volunteers and IPAH patients.
- Examine the effects of manipulating Cx43 and DDAHI expression on endothelial permeability and *in vitro* tube formation in ECFCs from healthy volunteers and IPAH patients.

7.2 Methods

See Chapter 2 for detailed experimental materials and protocols for cell culture, Matrigel tube assay, Western blotting, adenoviral transfection, siRNA transfection and permeability assessment.

7.2.1 Declaration of responsibilities

Data from the CLIC4 experiments (Figures 7.1, 7.2 and 7.5) were collected and analysed in collaboration with Dr Vahitha Abdul-Salam. Data in Figure 7.3 and data from the connexin 43 and DDAHI experiments (Figures 7.6-7.9) were collected and analysed in collaboration with Dr Hilda Tsang. The measurement of ADMA (Figure 7.6C) was performed by Lucio Iannone. Other data were produced and analysed by myself.

7.2.2 Protein expression

Cells were seeded (5000/cm^2 in EGM-2 medium containing 20% FBS) in 10 cm gelatin-coated petri dishes, grown to full confluency and extracted in ice-cold RIPA buffer. Expression of CLIC4, HIF-1α, Cx37, Cx40, Cx43, DDAHI and DDAHII proteins was assessed by Western blotting. Protocols and antibody characteristics are detailed in Chapter 2.11 and Table 2.5 respectively.

7.2.3 Manipulation of gene expression and activity in cultured cells

Manipulation of CLIC4

Over expression of CLIC4, predominantly cytoplasmic, was induced by adenoviral (AdCLIC4) gene transfer (Suh et al., 2004). AdCLIC4 was heme agglutinin (HA)- and myc-tagged and expressed under tetracycline control, allowing precise regulation of CLIC4 expression and localization. Cells were infected with adenoviruses at a multiplicity of infection (MOI) of 100 and left for 24 h prior to further treatment or analysis. Cells infected with AdGFP or AdCLIC4 without AdTet-off were treated as adenoviral controls.

Inhibition of CLIC4 expression was achieved with adenoviral gene transfer of CLIC4 small hairpin RNA (shRNA) (pEQU6-Clic4-shRNA-GFP; 5'-GCCGTAATGTTGAACAGAATT-3', Welgen Inc.). pEQU6-controlshRNA-GFP (5'-CAACAAGATGAAGAGCACC-3') was used as control and shRNA constructs were transfected 24 h before CLIC4 adenoviral gene transfer of AdCLIC4 (i.e. total expression time 48 h). The effects of CLIC4 gene silencing were verified by Western blotting. Inhibition of CLIC4 activity was

also achieved by treatment with the non-selective CLIC inhibitor 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) (Sigma-Aldrich, catalogue No. N4779).

Knockdown of BMPR2 and ALK5

Knockdown of BMPR2 and ALK5 expression in ECFCs and HPAECs was performed using siRNA specific for ALK5 (catalogue No. SI00301903; Qiagen, Crawley, UK), BMPR2 (catalogue No. VHS41350; Invitrogen, Paisley, UK) or a scrambled sequence of similar GC content (catalogue No. 12935-200; Invitrogen). The efficiency of the knockdown was determined by measuring protein and mRNA levels in cell extracts, using Western blotting and RT-PCR respectively.

Manipulation of Cx43 and DDAHI

To modulate Cx43 and DDAHI expression, AdGFP (Wojciak-Stothard and Ridley, 2003), AdCx43-GFP and AdDDAHI-GFP (all from Welgen Inc, Worcester, MA) were generated using Ad5 ΔE1ΔE3 backbone vector. The cDNA of wildtype Cx43 (a kind gift from Professor David Becker, University College London) (Becker et al., 2001) was cloned into KpnI and XbaI restriction sites of pAdTrackCMV vector. The recombinant adenoviruses were constructed using the Ad-Easy-1 system as described in Chapter 2.13.2. The titre of Ad-GFP was 1 x 10^{11} pfu/ml, and AdCx43-GFP was 8.5 x 10^{10}. Adenoviral transient infections of cells using MOI of 100 lead to a maximal rate of infection efficiency (~90 – 95%) without obvious cytopathic effects. Manipulation of Cx43 and DDAH1 expression was confirmed by Western blotting for both proteins. Activation of Cx43 gap junction was achieved by treatment with rotigaptide (100 nM) (a kind gift from Dr Ninian Lang, University of Edinburgh), whereas inhibition of Cx43 gap junction and DDAHI was achieved by treatment with Gap26 (VCYDKSFPISHVR, 200 μM, Peptide Protein Research Ltd) and L-257 (a kind gift from Dr James Leiper, MRC Clinical Sciences Centre, Imperial College London) respectively.

7.2.4 Matrigel tube formation assay

Cells were plated on gelatin-coated 6-well plates at a density of 1 x 10^5 cells/well, incubated overnight, and either left untreated, infected with recombinant adenoviruses to induce over expression of GFP, DDAHI and Cx43 or inhibition of CLIC4 expression. Two hours after infection, adenovirus was removed and cells were incubated with fresh medium for a further 24h before being trypsinised and reseeded for the Matrigel assay (HC-Matrigel was used in the CLIC4 experiment, while GFR-Matrigel was used in the DDAHI/Cx43 experiment) together with relevant treatments for

24 h. Changes in total endothelial tube length were expressed as a proportion of the control, each data point representing the mean of triplicate values derived from images of 2 random fields per well.

7.2.5 In vitro assessment of endothelial cell permeability

Cells were plated in Transwell-Clear chambers (1µm pore size, 12mm diameter; Costar Corning, Costar) at a cell density of 1 x 10^4 cells/well and grown to confluency. The cells were left untreated or infected with recombinant adenoviruses to induce over expression of GFP, DDAHI or Cx43. 2 h post-infection, adenovirus was removed and cells were incubated with fresh medium for a further 24 h before adding FITC-dextran for assessment of endothelial permeability.

7.2.6 ADMA measurement

Levels of ADMA in conditioned medium were quantified by high-performance liquid chromatography (HPLC) as previously described (Leiper et al., 2007). Briefly, ECFCs were seeded on 24-well plate and grown to confluence in EGM-2 and 20% FBS. The medium was replaced with fresh medium (containing 5% FBS) and incubated for a further 48h. The conditioned medium was centrifuged to remove cell debris and 100 µl used for ADMA measurement. 2,3,3,4,4,5,5-D7-asymmetric dimethylarginine (D7-ADMA , 5 µmol/L) was added to each sample as an internal standard. Methanol protein precipitation was performed followed by desiccation in a vacuum centrifuge, and the resultant pellets were re-suspended in mobile phase (0.1% formic acid in ultra-pure water). The ADMA concentration was determined by HPLC with an ASI-100 auto sampler (model P680, gradient pump model RF-2000). Protein precipitation efficiency was estimated from the concentration of D7-ADMA and the ADMA concentration corrected accordingly.

7.3 Results

7.3.1 CLIC4 and HIF-1α protein expression is increased in ECFCs from IPAH patients

ECFCs from IPAH patients displayed significantly higher levels of CLIC4 and HIF-1α protein expression, compared with cells isolated from healthy volunteers (Figure 7.1A-B).

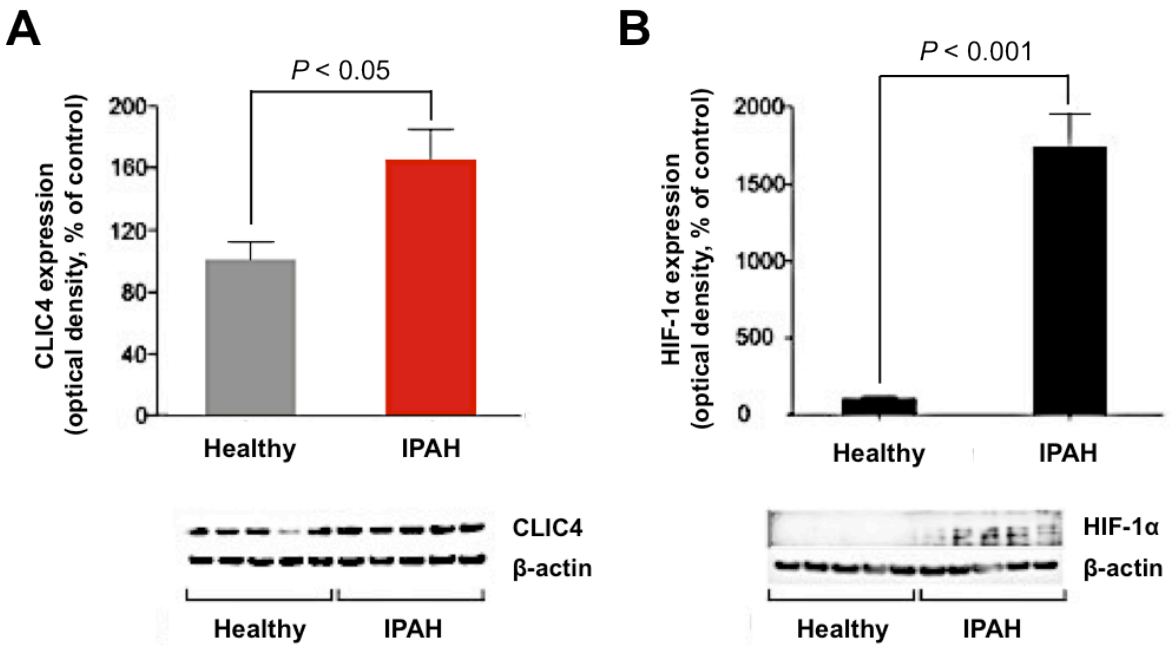


Figure 7.1. CLIC4 and HIF-1α expression in ECFCs. Quantitative assessment and representative Western blots showing **(A)** CLIC4 and **(B)** HIF-1α expression in ECFCs from healthy volunteers (n=5) and IPAH patients (n=5). Statistics obtained from t-test.

7.3.2 Increased CLIC4 expression in IPAH-ECFCs mediates increased tube formation

Manipulation of CLIC4 expression by AdCLIC4 and CLIC4 shRNA were confirmed by Western blotting (Figure 7.2A). Hypoxia induced a 3.2-fold increase in Matrigel tube formation in HPAECs, compared with normoxic controls (Figure 7.2B-C). Overexpression of CLIC4 significantly increased tube formation (2.0-fold in normoxia and 5.8-fold in hypoxia) compared to adenoviral controls (Figure 7.2B-C). Treatment with CLIC4 shRNA inhibited the stimulatory effects of hypoxia and CLIC4 overexpression (in both normoxic and hypoxic conditions), while the chloride channel inhibitor NPPB markedly reduced tube formation under both conditions (Figure 7.2C).

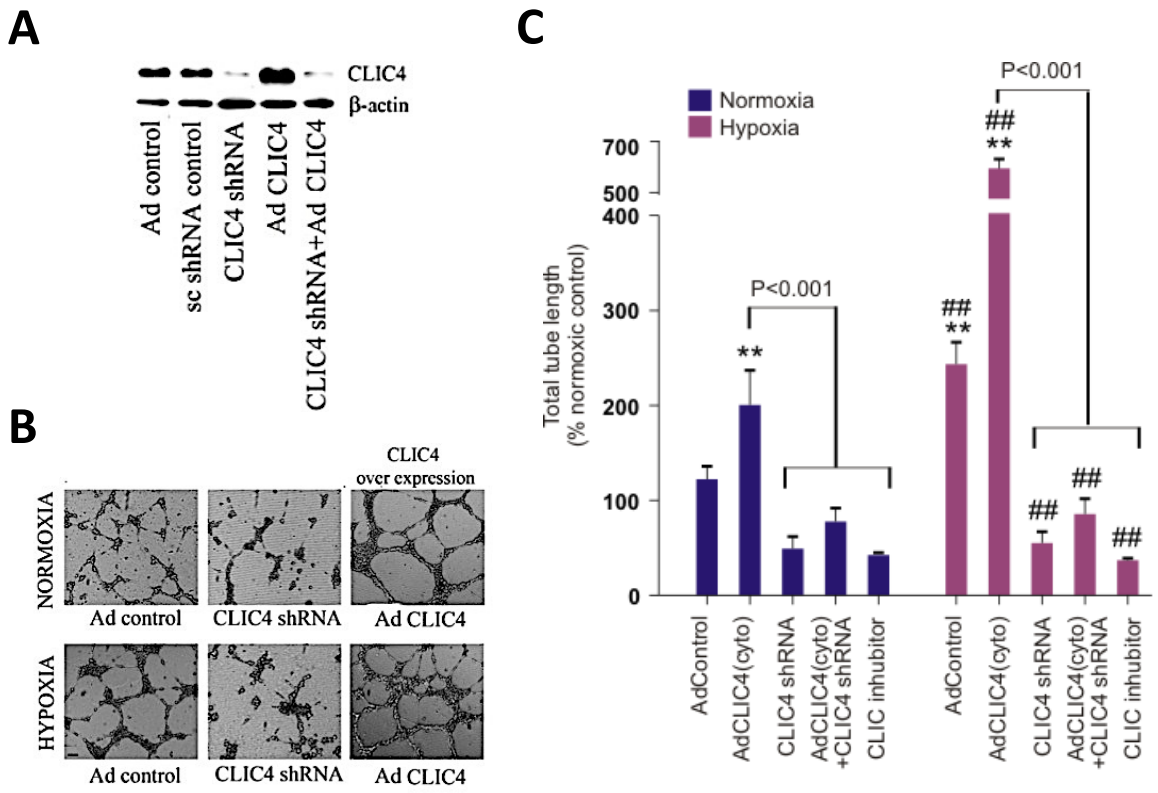


Figure 7.2. CLIC4 modulates *in vitro* Matrigel tube formation by HPAECs. (A) Representative Western blots showing the over expression of CLIC4 following transfection with AdCLIC4 and inhibition of CLIC4 expression by CLIC4 shRNA. **(B)** Representative images showing the effects on HPAEC tube formation following exposure to hypoxia, overexpression of CLIC4 and inhibition of CLIC4 expression. **(C)** Changes in total endothelial tube length (expressed as a proportion of the control) following over expression of CLIC4, inhibition of CLIC4 by CLIC4 shRNA or the CLIC inhibitor NPBB (25 µmol/L) and exposure to hypoxia for 24 h. Each bar represents mean±SEM (n=5). Statistics from two-way ANOVA and Bonferroni post-hoc analysis. *, P <0.05; **, P <0.01, compared with normoxic Ad control; ##, P <0.01, compared with hypoxic Ad control.

In keeping with the proposed pro-angiogenic role of CLIC4, the enhanced Matrigel tube forming capacity of ECFCs from IPAH patients was attenuated following the inhibition of CLIC4 expression with CLIC4 shRNA. CLIC4 protein levels were reduced to the level found in ECFCs derived from healthy volunteers (Figure 7.3A-B).

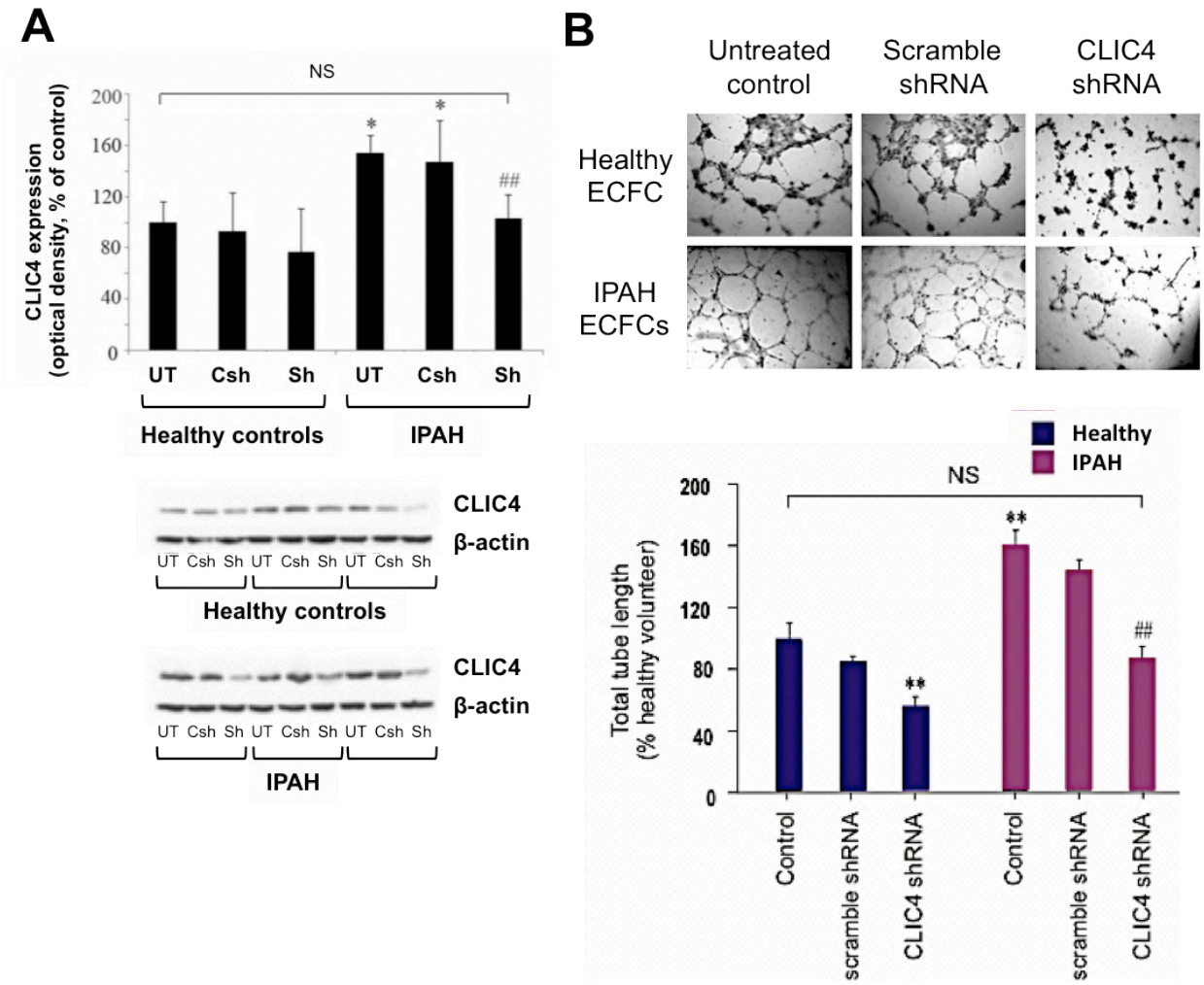


Figure 7.3. Inhibition of CLIC4 expression in ECFCs and effects on tube formation. CLIC4 protein expression was selectively attenuated by adenoviral mediated expression of CLIC4 shRNA (CSh), with scramble shRNA (Ssh) and untreated (UT) samples being used as controls. **(A)** Representative Western blots and quantitative demonstration of CLIC4 expression. **(B)** Representative phase-contrast photomicrographs and quantitative demonstration of Matrigel tube formation by ECFCs from healthy volunteers (n=3-4) and IPAH patients (n=3-4). Each bar represents mean±SEM. Statistics obtained from two-way ANOVA with Bonferroni post-tests. *, $P < 0.05$; **, $P < 0.01$, compared with untreated controls. ##, $P < 0.01$, compared with untreated ECFCs from IPAH patients.

7.3.3 CLIC4 expression is not affected by BMPR2 knockdown

BMPR2 siRNA was used to inhibit expression of the receptor in both ECFCs from healthy volunteers and HPAECs, BMPR2 mRNA and protein levels being reduced by ~80% compared to controls (Figures 7.4 & 7.5). Despite this marked reduction in BMPR2 expression, no significant change was found in CLIC4 mRNA or protein expression in either cell type (Figures 7.4 & 7.5).

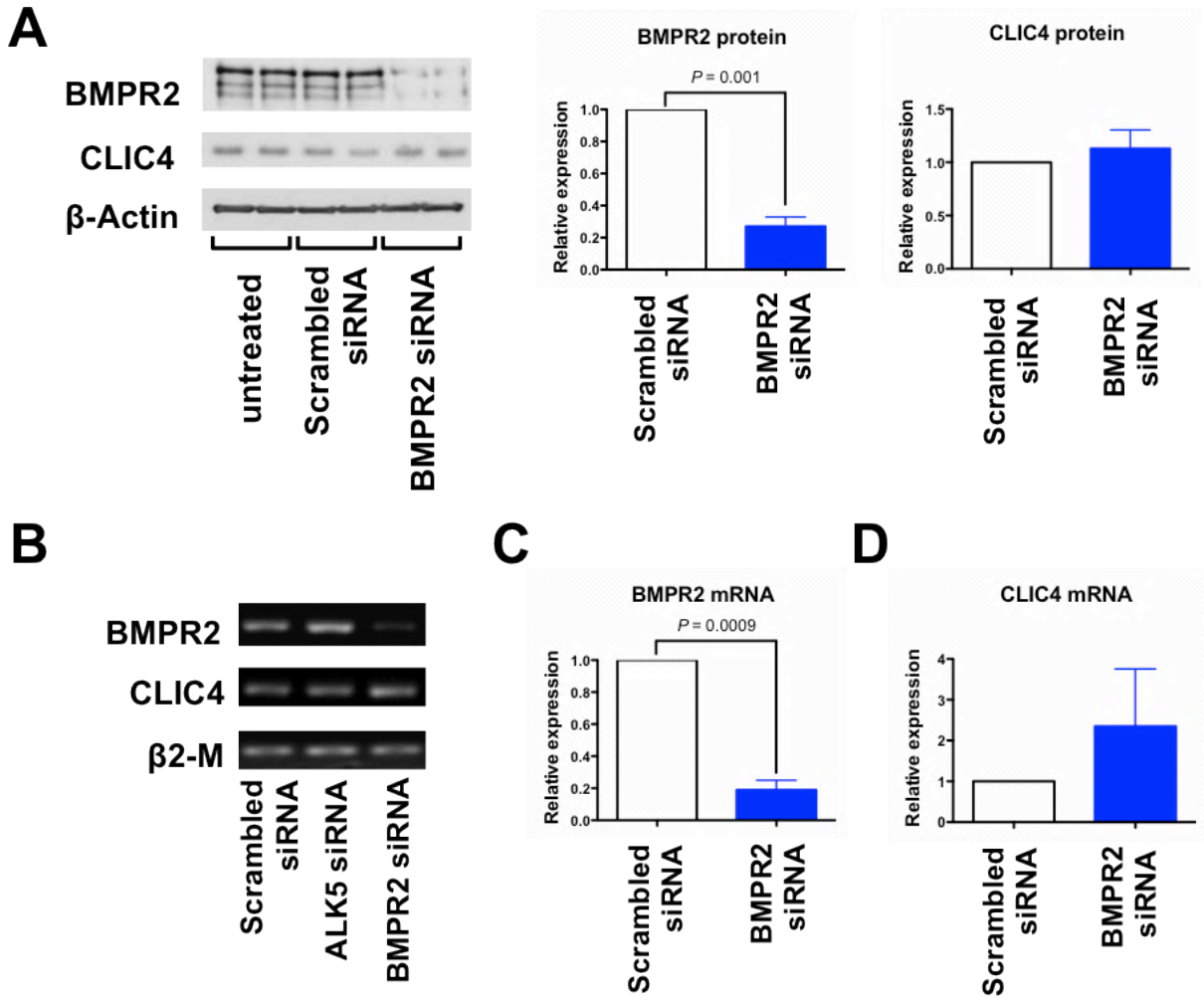


Figure 7.4. Effect of BMPR2 knockdown on CLIC4 expression in ECFCs from healthy volunteers. Cells were subjected to transfection with scrambled siRNA as controls or BMPR2 siRNA and expression of BMPR2 and CLIC4 analysed 2 days later. **(A)** Representative Western blots showing BMPR2 and CLIC4 protein expression, with densitometric analysis normalised to the β-actin loading control. **(B)** Representative images of RT-PCR anaylsis showing BMPR2 and CLIC4 mRNA expression. Transfection of ALK5 siRNA served as an additional negative control, not targeting BMPR2 expression. β2 microglobulin (β2M) served as a reference gene. **(C)** Quantitative RT-PCR analysis of **(C)** BMPR2 and **(D)** CLIC4 mRNA expression normalised to PPIA and α-tubulin (n=3-4). Each bar represents mean±SEM. Statistics from paired t-test.

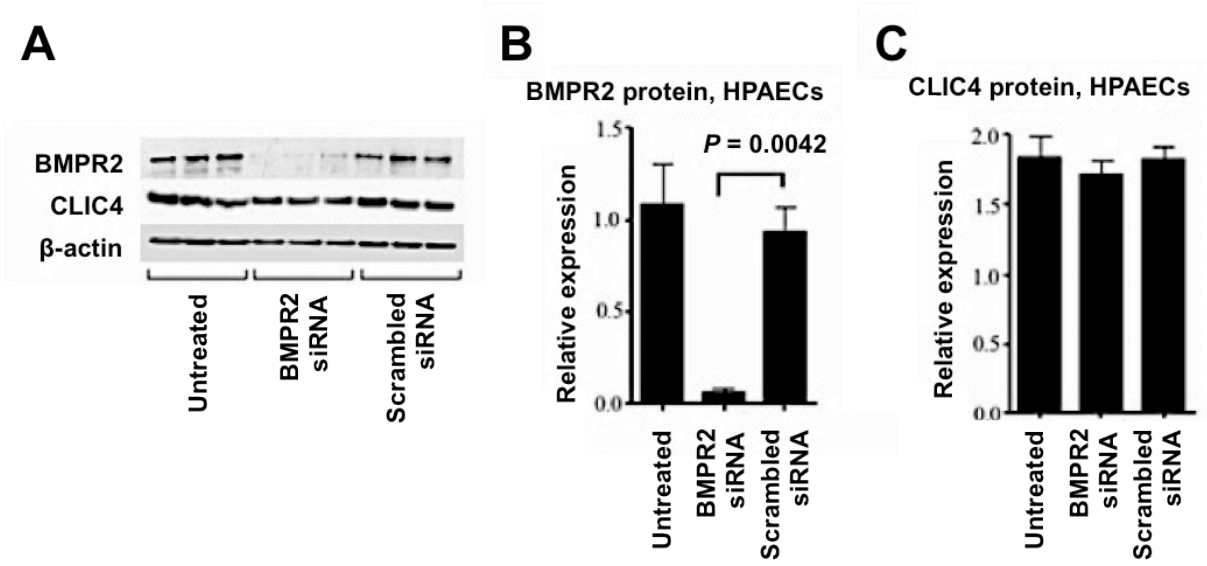


Figure 7.5. Effect of BMPR2 knockdown on CLIC4 expression in HPAECs. (A) Representative Western blot images showing BMPR2 and CLIC4 expression in HPAECs at 2 days post-transfection. Scrambled siRNA served as a control for BMPR2 siRNA and β-actin acted as a loading control. Densitometric analysis of **(B)** BMPR2 and **(C)** CLIC4 protein expression normalised to β-actin (n=3). Each bar represents mean±SEM. Statistics obtained from t-test.

7.3.4 ECFCs from IPAH patients have lower DDAHI and Cx43 protein expression and greater ADMA production

ECFCs from IPAH patients exhibited significantly lower levels (≥50% less) of Cx43 and DDAHI proteins compared to ECFCs from healthy volunteers (Figure 7.6A-B). In contrast, there was no significant difference in the expression of Cx40 between the two groups of cells. The expression of DDAHII and Cx37 was also probed, but neither protein was detectable. ECFCs from IPAH patients produced significantly more ADMA (2-folds) compared to ECFCs from healthy volunteers (Figure 7.6C).

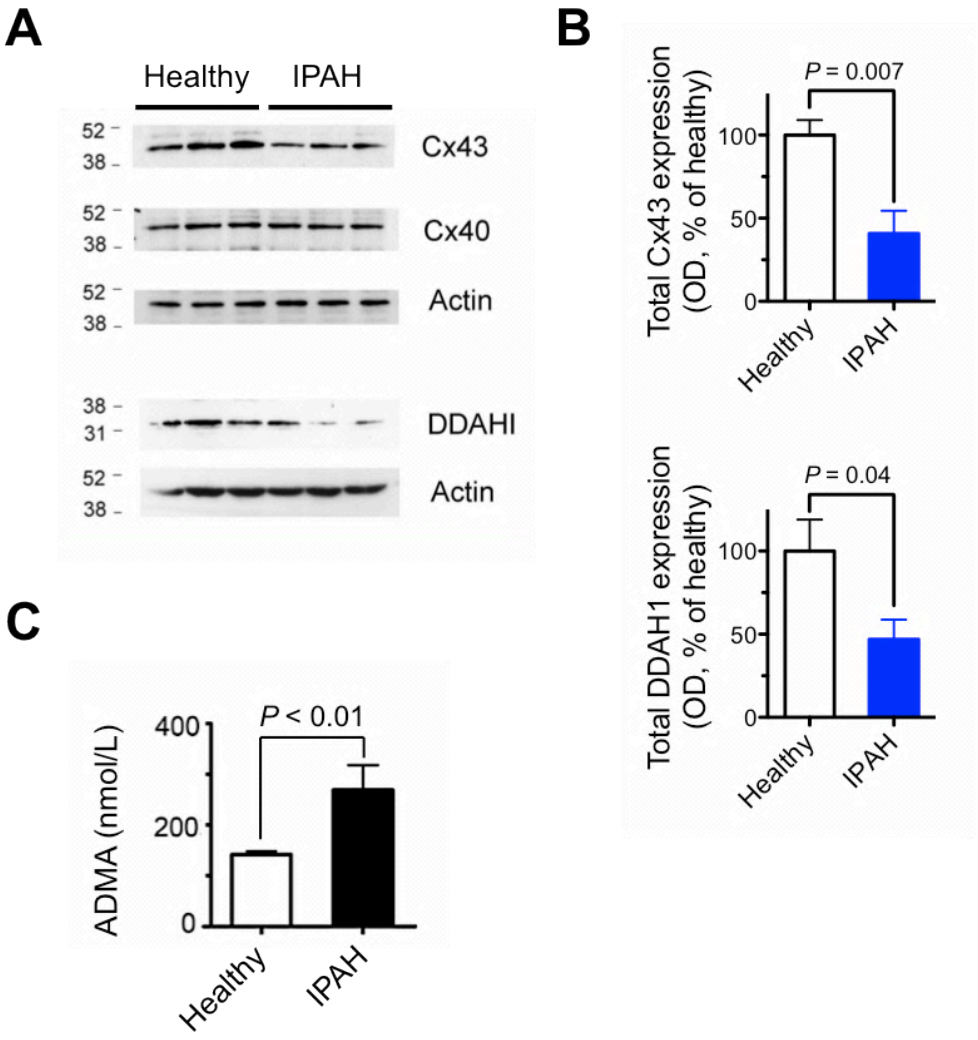


Figure 7.6. Expression of Cx43 and DDAHI proteins and production of ADMA by ECFCs. **(A)** Representative Western blots showing Cx43, Cx40 and DDAHI levels in ECFCs from healthy volunteers and IPAH patients. Numbers on the left indicate molecular weight (kDa). β-actin was used to demonstrate equal protein loading. **(B)** Quantitative analysis of Cx43 and DDAHI protein expression, showing the optical densities (OD) of the respective bands expressed as a proportion of control values. **(C)** ADMA production measured in conditioned medium from ECFCs cultures. Each bar represents mean±SEM (n=6). Statistics obtained from t-test.

7.3.5 DDAHI and Cx43 overexpression reduces endothelial permeability

ECFCs from IPAH patients were found to form leaky monolayers that exhibited higher endothelial permeability than cells from healthy volunteers (Figure 4.8 in Chapter 4). Here, over expression of DDAHI or Cx43 expression partially improved endothelial barrier function, reducing the levels of FITC-dextran flux to approximately half of that measured in untreated cells (Figure 7.7).

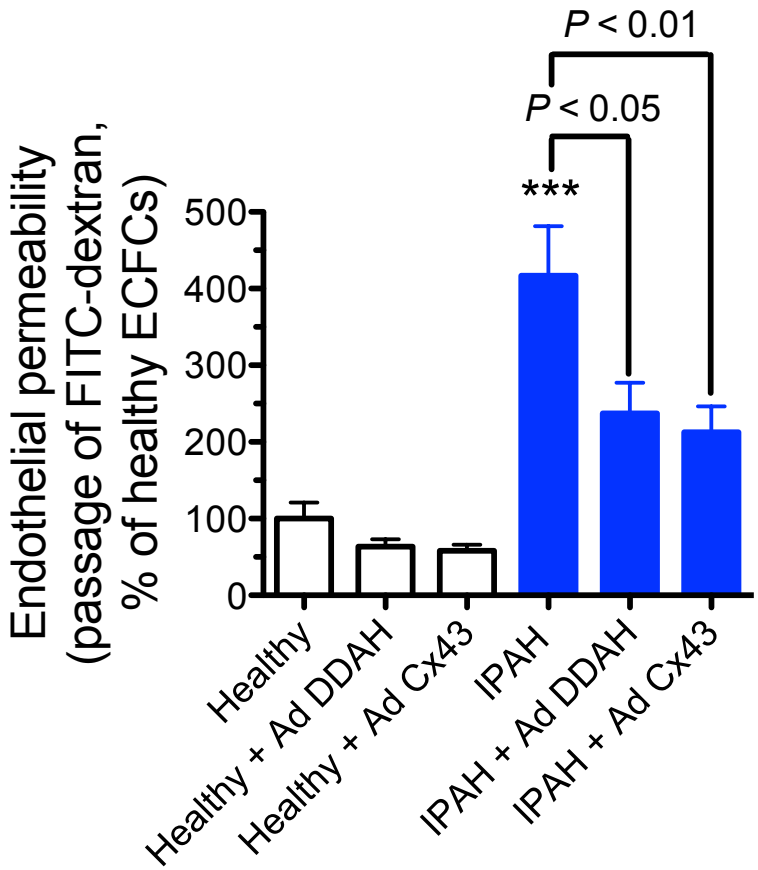


Figure 7.7. Overexpression of DDAH1 and Cx43 reduces endothelial permeability. Permeability of ECFC monolayers (assessed by the flux of FITC-labelled dextran), expressed as a proportion that observed in cells from healthy volunteers (n=6). Measurements obtained after transfection with adenoviral control or adenoviral over expression of DDAH1 (Ad DDAH) or Cx43 (Ad Cx43). Each bar represents mean±SEM. Statistics derived from two-way ANOVA, followed by Bonferroni post-hoc analysis. ***, $P < 0.001$, compared with healthy-ECFCs in control condition.

7.3.6 DDAHI and Cx43 overexpression has no effect on tube formation by ECFCs from IPAH patients

Connexins are important regulators of endothelial angiogenic responses (Gartner et al., 2012). ADMA inhibited endothelial tube formation in HPAECs and this was prevented by DDAHI over expression (Figure 7.8), consistent with previously published data (Fiedler et al., 2009). Treatment with DDAHI inhibitor L-257 or Cx43 junctional blocker Gap26 also attenuated tube formation whereas treatment with Cx43 junctional activator rotigaptide and Cx43 overexpression promoted tube formation and prevented the inhibitory influence of ADMA (Figure 7.8).

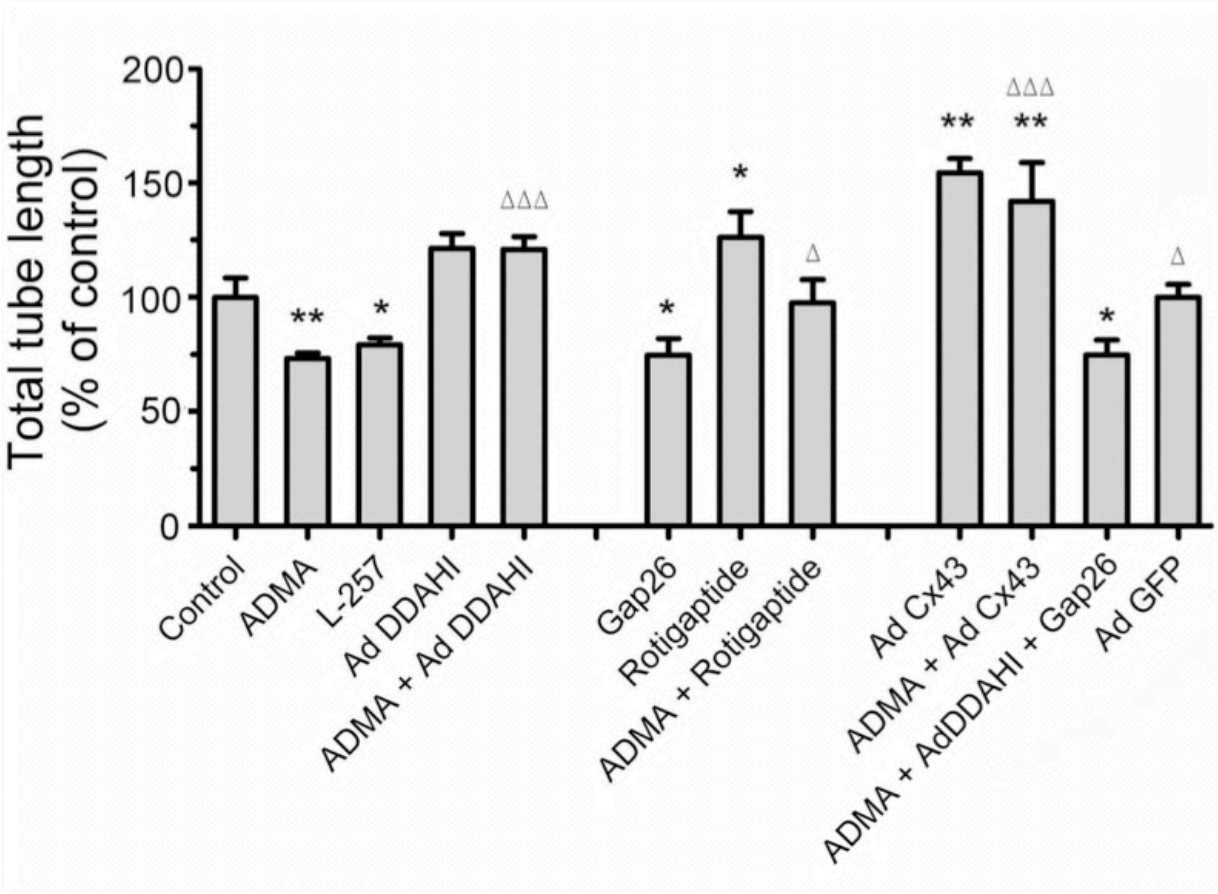


Figure 7.8. ADMA, DDAHI and Cx43 regulate Matrigel tube formation in HPAECs. (A) Tube formation in HPAECs treated with ADMA (100 µM), DDAHI inhibitor L-257 (1 µM), Cx43 gap junction blocker Gap26 (200 µM), Cx43 gap junction activator rotigaptide (100 nM) or overexpression of Cx43 or DDAHI. Each bar represents mean±SEM relative to control. Statistics derived from one-way ANOVA and Bonferroni post hoc analysis. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, comparisons with untreated controls; $^{\Delta}$, $P<0.05$; $^{\Delta\Delta\Delta}$, $P<0.001$, comparisons between ADMA + treatement and ADMA controls; N= 3.

When assessing tube formation in ECFCs, ECFCs from IPAH patients were again found to display an increased angiogenic capacity, as demonstrated by enhanced Matrigel tube formation, compared with cells from healthy volunteers (Figure 7.9). However, in contrast to normal HPAECs (Figure 7.8) and ECFCs from healthy controls, over expression of Cx43 or DDAHI did not promote tube formation by ECFCs from IPAH patients (Figure 7.9).

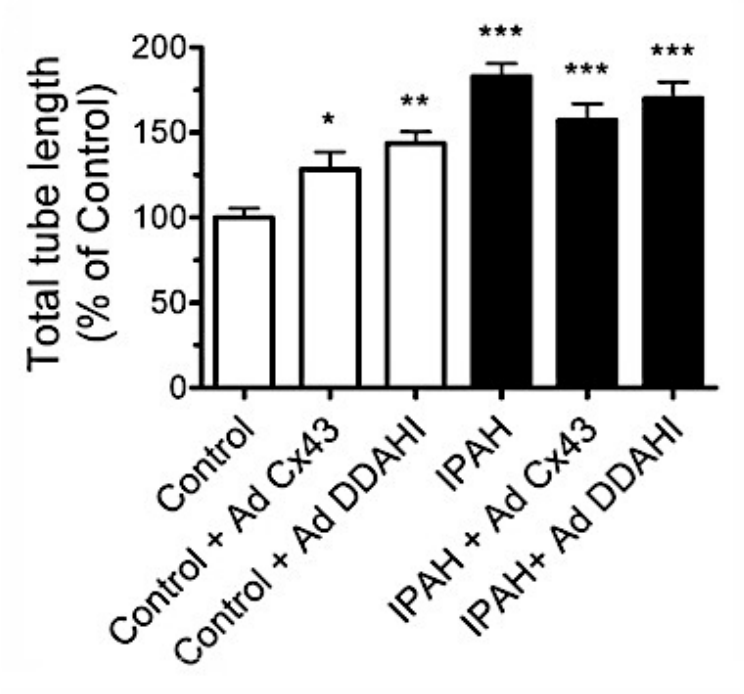


Figure 7.9. Matrigel tube formation by ECFCs and the effects of Cx43 and DDAHI over expression. Quantitative analysis showing the effects of over expression of Cx43 or DDAHI on tube formation by ECFCs from healthy volunteers (control; n=3) and IPAH patients (n=4). Each bar represents mean±SEM relative to healthy control ECFCs. Statistics derived from one-way ANOVA and Bonferroni post hoc analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with healthy controls.

7.4 Discussion

CLIC4 and endothelial dysfunction in ECFCs from IPAH patients

The main findings in the CLIC4 experiments are:

- ECFCs from IPAH patients exhibit increased CLIC4 expression and HIF-1α stabilisation.
- Increased CLIC4 expression contributes to the enhanced tube formation exhibited by ECFCs from IPAH patients.
- Inhibition of BMPR2 expression does not significantly affect CLIC4 expression in ECFCs.

We have recently described the critical role of CLIC4 in regulating several functions of HPAECs and suggested that CLIC4 could be important in the development of experimental PH (Abdul Salam et al., 2012). CLIC4 expression was found to be increased in the lungs of rats with chronic hypoxia- or MCT-induced PH, and localised predominantly to the endothelium in the remodelled pulmonary vasculature. Despite being expressed in abundance in various mammalian endothelial cells and blood vessels, including those in the kidney (Bohman et al., 2005), CLIC4 levels were reduced in renal and myocardial tissues of chronically hypoxic animals (unpublished data, Abdul Salam 2012). These observations suggest an organ-specific response to hypoxia, with CLIC4 expression having a distinct role in the development of hypoxia-induced PH.

In keeping with the observation that raised CLIC4 expression was predominantly localised to the pulmonary endothelium in IPAH lungs (Abdul-Salam et al., 2010), CLIC4 expression was also found to be raised in ECFCs derived from IPAH patients. HIF-1α expression had previously been identified within endothelial plexiform lesions (Tuder et al., 2001), in isolated PASMCs (Bonnet et al., 2006) and pulmonary ECs from IPAH patients (Fijalkowska et al., 2010), suggesting that HIF-dependent signalling may contribute to the pulmonary angioproliferative remodelling in IPAH. Recently, CLIC4 overexpression was found to promote the stabilisation and nuclear translocation of HIF-1α and increased production of VEGF in HPAECs, these effects being abrogated by inhibition of endogenous CLIC4 (Abdul Salam et al., 2012). We have now also demonstrated increased HIF-1α stabilisation in ECFCs from IPAH patients and shown that the reduction of CLIC4 expression in ECFCs from IPAH patients (to the levels found in ECFCs from healthy volunteers) abolishes their enhanced tube

forming capacity. Taken together, these observations suggest that CLIC4 is an important factor contributing to the disease-related phenotype of ECFCs from IPAH patients.

As heterozygous germline *BMPR2* mutations contribute to HPAH (Machado et al., 2009) and down regulation of BMP signalling has been implicated in the development of experimental PH (Morty et al., 2007; Long et al., 2009), we examined whether differences in BMPR2 expression might influence CLIC4 protein levels. Despite the marked suppression of BMPR2 mRNA and protein expression, no significant changes were found in CLIC4 protein and mRNA levels in either HPAECs or ECFCs from healthy volunteers. This is consistent with the results of an earlier study on the regulation of CLIC4 mRNA expression in isolated smooth muscle cells (Spiekerkoetter et al., 2009) and suggests that the enhanced expression of CLIC4 found in endothelial cells from IPAH patients (Abdul-Salam et al., 2010) and lungs from rats with pulmonary hypertension is not a direct manifestation of altered BMPR2 expression. It is however unclear whether TGF-β signalling affects CLIC4 expression in ECFCs, as TGF-β1 has been shown to increase CLIC4 expression in primary keratinocyte (Shukla et al., 2009).

Role of ADMA, DDAHI and Cx43 and endothelial dysfunction in ECFCs from IPAH patients

The main findings in the ADMA/DDAHI/Cx43 experiments are:

- ECFCs from IPAH patients exhibit reduced DDAHI and Cx43 expression compared with ECFCs from healthy volunteers.
- ECFCs from IPAH patients also produce more ADMA than ECFCs from healthy volunteers.
- Increasing the expression of DDAHI and Cx43 attenuates the excessive endothelial permeability and the enhanced tube forming capacity of ECFCs from IPAH patients.

ADMA was recently found to inhibit the expression and membrane localisation of Cx43 in HPAECs, while Cx37 and Cx40 were unaffected (Tsang et al., 2011). ADMA also disrupted the barrier functions of HPAECs, decreasing gap junctional communication and increasing endothelial permeability, and this was prevented by Cx43 or DDAHI overexpression. DDAHI was the predominant isoform of DDAH found in HPAECs and the manipulation of DDAHI expression did not induce a compensatory change in DDAHII expression (Tsang et al., 2011). Indeed, similar observations have recently been described in mice (Hu et al., 2011). The expression of DDAHI and Cx43 proteins was significantly reduced and

production of ADMA increased in ECFCs derived from IPAH patients. It is conceivable that the increased production of ADMA reflects decreased DDAHI expression but unclear whether circulating ECFCs and/or cells residing locally in the pulmonary vasculature (Alvarez et al., 2008; Duong et al., 2011) contribute to the circulating levels of ADMA, which are raised in IPAH patients (Pullamsetti et al., 2005). Increased ADMA release might also lead to a reduction in Cx43 expression and the disruption of barrier function observed in ECFCs from IPAH patients. Indeed, increasing the expression of DDAHI or Cx43 partially attenuated the excessive endothelial permeability displayed by these cells (Figure 7.7).

In fact, connexin expression is frequently disrupted in response to lung disease (Johnson and Koval, 2009). ADMA-induced impairment of endothelial barrier function was associated with a reduction of Cx43 expression and activation, which is reminiscent of the actions of other barrier-compromising factors such as bioactive lipids, ET-1 and thrombin (Hill et al., 1994; Spinella et al., 2003; D'Hondt et al., 2007). Intercellular junctions between endothelial cells maintain vascular permeability and integrity. Gap junctions are located in close proximity to the tight and adherens junctions that form the endothelial barrier (Derangeon et al., 2009). The interaction of Cx43 with submembrane actin cytoskeleton helps coordinate formation of protein scaffolds containing proteins such as the tight junction protein-1 (ZO-1), cadherins, and integrins and therefore is important for the formation and maintenance of intercellular contacts (Giepmans, 2004; Nagasawa et al., 2006; Kojima et al., 2007; Vitale et al., 2009). Another possibility is that Cx43 hemichannels enhance barrier function by promoting release/transfer of barrier-protective signalling molecules such as ATP (Gunduz et al., 2003), nicotinamide adenine dinucleotide (NAD+) (Umapathy et al., 2010), or prostaglandin (Birukova et al., 2007) or by helping to normalise intracellular calcium levels (Parthasarathi et al., 2006; Li et al., 2010).

In addition to effects on inter-cellular communication and barrier function, the pharmacological and genetic manipulation of DDAH and CX43 activity/expression also enhanced angiogenic responses in HPAECs. Cx43 augments several processes important in angiogenesis, such as cell-cell adhesion (Giepmans, 2004; Vitale et al., 2009), cell migration (Behrens et al., 2010) and expression of genes in VEGF- and TGF-β-signaling pathways (Walker et al., 2005). Nonetheless, in contrast to HPAECs and ECFCs from healthy volunteers, ECFCs from IPAH patients showed an elevated angiogenic capacity and apparent resistance to the effect of overexpressing DDAHI or Cx43. It is possible that other NO-independent pro-angiogenic factors might be involved. For example, basic FGF, a growth factor implicated in the pathogenesis of PAH, can act in an NO-independent manner and synergise with

VEGF in inducing endothelial permeability and angiogenesis (Arcot et al., 1995; Tille et al., 2001; Benisty et al., 2004).

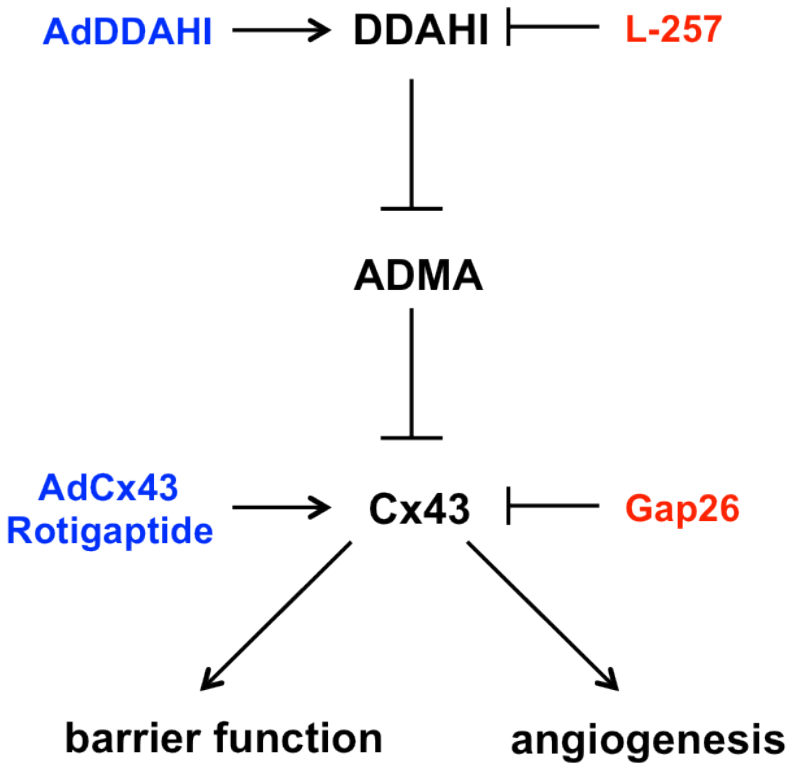


Figure 7.10. Schematic diagram of the proposed role of ADMA/DDAH in the regulation of HPAECs and ECFCs functions. ADMA inhibits Cx43 expression and gap junctional communication, which destabilises inter-cellular junctions, contributing to the breakdown of endothelial barrier function and inhibition of angiogenesis in HPAECs and ECFCs from healthy volunteers. The DDAHI inhibitor, L-257 and Cx43 inhibitor, Gap26 mimic the effects of ADMA, while over expression of DDAHI, Cx43 or treatment with rotigaptide, have a protective effect. In ECFCs from IPAH patients, decreased DDAHI expression might lead to reduced ADMA metabolism, which leads to increased ADMA release and inhibition of Cx43 expression. Loss of Cx43 could lead to the impaired barrier function (increased permeability) in the IPAH cells.

Conclusion and future perspective

The findings in this chapter indicate that increased tube forming capacity exhibited by ECFCs from IPAH patients is mediated by excessive CLIC4 expression. Decreased DDAHI and Cx43 expression may also contribute to the disrupted barrier functions of ECFCs from IPAH patients. It will be of interest to see whether these cells produce more VEGF than ECFCs from healthy controls and if this is mediated through CLIC4 and the HIF-1α stabilisation. This is of particular relevance to PAH as circulating VEGF levels are raised in IPAH patients (Selimovic et al., 2009; Kumpers et al., 2010). It would also be interesting to explore whether the altered expression of these proteins affects the other dysfunctional phenotypic characteristics of ECFCs from IPAH patients. In this regard, we recently reported that CLIC4 over expression increases the endothelial permeability of HPAECs (Abdul Salam et al., 2012). In fact, VEGF has also been shown to increase endothelial permeability by inducing VE-cadherin destabilisation (Gavard, 2009), providing a hypothetical mechanism for CLIC4-induced changes in the barrier functions of ECFCs. Manipulation of CLIC4 expression has however been found to have no effect on the wound recovery rate of endothelial cells (Tung et al., 2009), whereas Cx43 has been shown to mediate migration of HeLa cells and mouse embryonic EPCs (Behrens et al., 2010). Therefore, it will be worthwhile to investigate the role of DDAHI/ADMA/Cx43 pathway in the impaired migration of ECFCs from IPAH patients.

In summary, this chapter has demonstrated that blood-derived ECFCs can be used to investigate novel aspects of signalling dysregulation that might contribute to the pathogenic dysfunction of endothelial cells in IPAH patients. The data also provides a proof of concept indicating that ECFCs represent an accessible surrogate cell type to study endothelial dysfunction in IPAH.

Chapter 8:

Conclusions and

Future Work

Chapter 8 – Conclusions and future work

The studies in this thesis aimed to establish if ECFCs are a credible endothelial cell model for IPAH. The investigations commenced with the finding of an altered colony-forming capacity in IPAH patients. Specifically, the cells lacked the age-dependent decline in colony formation that was observed in healthy individuals. The frequency of colonies was also associated with clinical features and disease severity in IPAH patients. *In vitro* expansion of the colonies gave rise to cells with typical endothelial progenitor cell characteristics (Yoder et al., 2007); namely cobblestone-like morphology, expression of endothelial markers, lack of hematopoetic/monocytic markers, *in vitro* Matrigel tube formation and clonogenic growth. Importantly, ECFCs derived from IPAH patients exhibited apoptosis-resistance, enhanced angiogenic capacity, impaired migration and disrupted barrier function. BMPR2 expression has been found to be impaired in PAH patients and animal models, but did not appear to differ between ECFCs from patients and healthy subjects. Among the other TGF-β receptors analysed, expression of ALK5 was reduced in IPAH cells and may be associated with impaired TGF-β1/ALK5/Smad2/3 signalling. The ALK1 and ALK5 signalling pathways had opposing effects on ECFC survival, tube formation and migration and lower ALK5 expression might contribute to distinct effects on apoptosis and migration in ECFCs from IPAH patients. Differences in CLIC4, HIF-1α, DDAHI and Cx43 expression and ADMA production were also found that mediated, at least in part, the dysregulation of tube formation and endothelial permeability in cells derived from IPAH patients. Collectively, these findings provide support for the contention that blood-derived ECFCs represent a suitable surrogate endothelial cell model to investigate the pathogenic mechanisms underlying endothelial dysfunction in IPAH.

Phenotypic differences between ECFCs from IPAH patients and healthy volunteers

The discovery of an age-dependent decline of ECFC colony formation in healthy individuals is supported by a recent report describing similar findings found in the rhesus monkey (Shelley et al., 2012). It is interesting that the older IPAH patients (over 62 years) did not follow this trend and the underlying mechanism needs to be clarified. Further investigation would be to compare the telomerase activity of these cells as this is thought to correlate with the greater number/colony forming capacity of ECFCs in cord blood compared with adult peripheral blood (Ingram et al., 2004). Determining the origin of circulating ECFCs may also help to understand their potential contribution

to endothelial repair and pulmonary vascular remodelling in IPAH. Endothelial cells isolated from both human pulmonary arteries (Duong et al., 2011) and the rat pulmonary microvasculature (Alvarez et al., 2008) have been shown to contain ECFCs, indicating that these cells reside locally in walls of pulmonary vessels as well as in bone marrow. Studies exploring the microvascular (*Griffonia simplicifolia* lectin binding and CD36 positive) and macrovascular (*Helix Pomatia* lectin binding) endothelial phenotype (Swerlick et al., 1992; Comhair et al., 2012) of ECFCs may provide additional insight into their vascular origin and potential contribution to plexiform lesions, the latter representing a monoclonal proliferation (Lee et al., 1998) of macro- rather than micro-vascular endothelial cells (Comhair et al., 2012). Further experiments with ECFCs derived from both explanted lung tissues and contemporary peripheral blood samples, involving the rigorous use of the single cell clonogenic assay (replating cells from the primary colony to produce secondary and tertiary colonies), may help to determine whether the source of ECFCs influences observed differences in the frequency and proliferation of ECFCs from healthy volunteers and IPAH patients.

To establish ECFCs as an effective endothelial cell model for IPAH, a clear understanding of their functional phenotype is required. I have shown that ECFCs from IPAH patients exhibit several characteristics (most notably resistance to apoptosis) that are reminiscent of ECs in the diseased pulmonary vasculature of IPAH patients, studied both *in situ* and as cultured cells *in vitro* (Masri et al., 2007; Tu et al., 2011). I also observed that ECFCs from IPAH patients were more angiogenic *in vitro* but it will be important to determine whether this can be replicated *in vivo*, using for example cellularised gel implants or Matrigel plugs in nude mice (Melero-Martin et al., 2007; Yoder et al., 2007). It is unclear how the results of the cell migration (wound healing) and proliferation assays relate to the potential contribution of ECFCs in the pathogenesis of IPAH, but impaired migration or proliferation could be deleterious to endothelial repair and vascular homeostasis. It also is interesting to note that patients with HHT, who harbour mutations of endoglin or ALK1, have also been found to give rise to ECFCs that display impaired migratory and proliferative capacity *in vitro* (Fernandez et al., 2007).

Dysregulated TGF-β signalling contributes to the functional phenotype of ECFCs from IPAH patients

ECFCs from IPAH patients displayed differences in ALK5 expression and the canonical TGF-β1/Smad2 signalling pathway, when compared with cells isolated from healthy volunteers. Studies into the regulation of endothelial cell functions by TGF-β signalling have been complicated by the opposing responses this can induce, due to biphasic concentration-dependent effects and the distinct effects

of activating ALK1/TGF-β and ALK5/TGF-β receptor pathways. Indeed, differing results have also been found using the same model/cell type, as shown for example by studies into the mechanism by which endoglin regulates endothelial cell growth. Earlier studies reported pro-proliferative and pro-migratory responses elicited by endoglin in mouse embryonic ECs, whereas others found that endoglin inhibited proliferation and migration; these disparate findings probably reflect signalling via Smad-independent as well as Smad-dependent pathways (Lebrin et al., 2004; Pece-Barbara et al., 2005; Ray et al., 2010; Pan et al., 2012). In the present study, one of the most consistent findings was the resistance to apoptosis in serum-deprived ECFCs from IPAH patients and the opposing effects of ALK1 and ALK5 signalling, apoptosis (caspase-3/7 activity) being attenuated by ALK1 activation and ALK5 inhibition. Nevertheless, the mechanisms controlling these effects and the significance of the depletion in ALK5, endoglin and ActRIIA expression in IPAH-derived cells have not been fully explored. Increasing evidence demonstrates that stimulation of TGF-β and BMP signalling can activate intracellular non-Smad pathways that regulate a range of cellular functions (Zhang, 2009). These include the MAP kinase pathways (p38, ERK, JNK), Rho GTPase and phosphatidylinositol-3-kinase/AKT pathways. In endothelial cells, the activation of p38 MAPK by BMP9 and TGF-β1 has been shown to mediate ET-1 synthesis, tube formation, migration and apoptosis (Ferrari et al., 2009; Park et al., 2012), whereas ALK1/endoglin-mediated ERK and JNK signalling promoted proliferation and migration (David et al., 2007b; Pan et al., 2012). In fact, interactions between these signalling pathways can have unexpected effects on endothelial cell apoptosis and involve different isoforms of p38 MAPK (Ferrari et al., 2012). I only considered Smad signalling in this study and the examination of non-Smad pathways may provide further insight into the dysfunctional phenotype of ECFCs from IPAH patients.

Disparate findings in functional phenotypes and canonical BMP9 signalling activation between ECFCs from IPAH patients in this study and HPAH patients with *BMPR2* mutations by others suggests possible variation in the molecular mechanisms of ECFC dysfunction between HPAH patients with *BMPR2* mutations and IPAH patients (Toshner et al., 2009; Toshner et al., 2011). Furthermore, Yeager and co-workers identified TGF-βRII and Bax microsatellite instabilities in the endothelial cells of plexiform lesions in IPAH lungs, while the same group and others failed to identify these mutations in the plexiform lesions of APAH and HPAH lungs (Yeager et al., 2001; Yeager et al., 2002; Machado et al., 2005). Identifying these mutations in ECFCs from IPAH patients would provide a more direct link between the monoclonal proliferation *in vitro* (ECFC colony formation) and *in vivo* (plexiform lesions) and add weight to the suggestion that ECFCs have a role in the pathogenesis of IPAH. Thus, ECFCs might provide an accessible cell source to investigate the formation of plexiform lesions in the lungs of IPAH patients. Unfortunately, only two ECFC samples were derived from HPAH patients in this

study and the *BMPR2* mutation status of the IPAH patients was not available. Given the necessary time and facilities I would have liked to investigate the receptor signalling and functional properties of ECFCs from well phenotyped and genotyped patients, with and without known *BMPR2*, *ALK1* and *SMAD* mutations associated with PAH.

Novel therapeutic perspectives

It has been suggested that the inhibition of the ALK5 receptor could have therapeutic benefit as the receptor is thought to mediate abnormal proliferation of VSMCs from patients with HPAH, and increased ALK5/TGF-β signalling is considered to promote the proliferation and migration of PASMCs in experimental models of PH (Zaiman et al., 2008; Long et al., 2009; Thomas et al., 2009). Nonetheless, we need to more fully understand the potential significance of impaired ALK5 expression and regulation of apoptosis in the pulmonary endothelium before undertaking more general targeting of ALK5 expression in the pulmonary vascular system. In fact, other groups have observed that ALK1 (not ALK5) expression is affected in the lungs and PASMCs of MCT-treated rats (Zakrzewicz et al., 2007) and selective down regulation of ALK5 may offer growth advantages to abnormal pulmonary endothelial cells (Richter et al., 2004). The coordinated activity of both type 1 receptors is necessary for the normal regulation of VEGF in endothelial cells (Shao et al., 2009) and the inhibition of ALK5 and Smad2 activation has been found to enhance the angiogenic function and survival of endothelial cells (Froese et al., 2011). Further studies are also warranted in view of the intricate interaction between ALK1 and ALK5 signalling in regulating endothelial functions and our current understanding of the role of endothelial cell apoptosis in the initiation versus progression of pulmonary vascular remodelling and formation of plexiform lesions (Sakao et al., 2005). The present results indicate that ALK5 inhibition protects ECFCs and PAECs from apoptosis whereas ALK1 inhibition had the opposite effect and reversed the apoptosis-resistant property of ECFCs from IPAH patients. It would be interesting to see what effect selective ALK1 and ALK5 inhibition might have on the early versus late stages of pulmonary vascular remodelling in animal model where angiogenic (plexiform-like) lesions are also manifested (Abe et al., 2010).

Excessive CLIC4 and impaired DDAHI and Cx43 expression were identified in ECFCs from IPAH patients, suggesting novel therapeutic strategies that target these proteins. The general CLIC inhibitor NPPB was found to attenuate hypoxia-induced angiogenesis (tube formation *in vitro*) and endothelial permeability in HPAECs (Abdul Salam et al., 2012). To date there is no selective CLIC4 inhibitor available to explore the functional significance of reducing CLIC4 expression in PH, but

preliminary results with CLIC4 knockout mice suggest that they may be protected from the effects of hypoxia on cardiovascular remodelling. In contrast, rotigaptide is a Cx43-specific activator that has been found to reverse the detrimental effects of ADMA on HPAEC functions (Tsang et al., 2011). Rotigaptide is a stable anti-arrhythmic peptide that increases phosphorylation of Ser-368 in the C-terminus of Cx43, rapidly improves Cx43 membrane targeting and enhances intercellular gap-junctional conduction (Dhein et al., 2010). Studies over-expressing different connexins in HeLa cells have shown that rotigaptide specifically affects Cx43 and Cx45 but not other connexins present in the vascular system (Dhein et al., 2010). The effect of rotigaptide on hypoxia-induced pulmonary vasoconstriction remains to be established but it is reported to have no effect on vascular tone in the forearm of healthy subjects, either in the presence or absence of the nitric oxide synthase inhibitor L-NMMA (Lang et al., 2008), and to have a protective effect against endothelial ischaemia reperfusion injury (unpublished data, Dr Ninian Lang, University of Edinburgh, 2012). This suggests that impaired Cx43 expression/function may contribute to endothelial dysfunction in conditions of oxidative stress (Pullamsetti et al., 2011) and testing drugs such as rotigaptide on ECFCs might reduce the need to use animal models of PH. Indeed, potential therapeutic targeting of the DDAH/Cx43 pathway in patients with PAH will require careful consideration as increased Cx43 expression may affect the phenotype and proliferation of smooth muscle cells and enhance neointimal formation in a systemic model of arterial injury (Song et al., 2009). Furthermore, DDAH/NO signalling has been implicated in pulmonary fibrosis and conditions associated with inflammation and increased inducible NOS activity (Pullamsetti et al., 2011). ECFCs from IPAH patients and healthy controls may represent a useful means of testing drugs such as rotigaptide and could reduce the need for using animal models of PH.

Limitations

There are a number of limitations to these studies. Firstly, the sample size was relatively small and there was a lack of usable ECFC populations (one out of ten) from patients in WHO functional class I and II. The results of the functional studies may therefore be more applicable to patients with severe functional impairment and the latter stages of the disease than the mechanisms initiating endothelial dysfunction in IPAH. Microarray analysis of gene and microRNA expression in ECFCs from all four WHO functional classes may provide more information on the factors implicated in disease initiation and progression.

Second, the ECFCs were studied in isolation and reproducing the *in vivo* environment requires a multi-cellular system. Blood-derived smooth muscle progenitor cells (SMPCs) could provide an

analogous non-invasive approach to obtain patient-specific smooth muscle cells (Simper et al., 2002). The initial steps of culturing SMPCs are very similar to those required for ECFCs, indicating that both cell types could be derived from the same blood sample. SMPCs are also reported to express smooth muscle cell markers and display a typical "hill-and-valley" smooth muscle cell morphology that is readily distinguished from the cobblestone appearance of ECFCs (Wang et al., 2012). Co-culturing both cell types from the same patient could recreate a more credible *in vitro* model and enhance our understanding of the paracrine relationship between them. Furthermore, no reprogramming is required. Epigenetic changes are thought to have a role in the development of PAH (Zhao et al., 2012) and the reprogramming involved in the production of induced pluripotent stem cells (IPScs) might affect their cellular functions through methylation and acetylation events (Saha and Jaenisch, 2009). Furthermore, if induced vascular progenitor cells (iVPCs) are required, which can differentiate into vascular smooth muscle and endothelial cells, then ECFCs may represent a useful starting cell type. Unlike fibroblasts, they may require only partial reprogramming and retain the 'epigenetic memory' of their endothelial lineage (Yin et al., 2012).

Third, ECFCs were cultured and analysed under static conditions that were devoid of the *in vivo* milieu. In particular, they were not subjected to the shear stress present within the pulmonary circulation of PAH patients (Voelkel and Tuder, 1995), yet endothelial cell functions are known to be influenced by shear stress (Ando and Yamamoto, 2011). The CELLMAX artificial capillary system could be used to recreate a pulsatile flow environment and could be used to observe the differences in functional responses between ECFCs from healthy volunteers and IPAH patients (Sakao et al., 2005).

Fourth, the concentration-dependent effects of agonists such as BMP4 and BMP9 were not fully explored in this study. The concentrations used were based on published reports investigating effects on endothelial cells. But BMP9 can activate both pSmad1/5/8 and pSmad2 in HPAECs, depending on the concentration used (Upton et al., 2009), and might have biphasic effects on ECFCs that serve to further distinguish cells derived from IPAH patients and healthy volunteers.

Novelty and value

The value of this study is that it represents the first concerted attempt to demonstrate that blood-derived ECFCs are an effective endothelial cell model in IPAH. These cells exhibited significant differences in several pathways that are considered to be fundamental to the initiation and progression of the disease. As such, they provide a valuable surrogate source of patient specific endothelial-like cells that may be used to test potential drugs, identify novel therapeutic strategies and increase our understanding of mechanisms that underlie IPAH. This approach could be particularly powerful when combined with the genotyping and deep phenotyping of patients, contributing to the sub-classification of IPAH as a disease category and the development of personalised medicine. As ECFCs have an established endothelial lineage they may also represent a useful cell type for the development of IPS cells. On the other hand, the demonstration that ECFCs have a disease-related phenotype raises doubts about their suitability for cell-based therapies.

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Appendices

APPENDICES

Appendix 1 – Healthy volunteers demographics

Sample Code	Sex	Age	ECFC colony No.	ECFC colony frequency	Highest passage No.
Healthy 1	F	27	1	0.100	9
Healthy 2	F	38	1	0.063	0
Healthy 3	M	27	2	0.171	≥9
Healthy 4	M	26	1	0.056	7
Healthy 5	F	29	1	0.062	≥8
Healthy 6	F	27	4	0.340	≥10
Healthy 7	M	23	3	0.286	4
Healthy 8	M	24	5	0.362	4
Healthy 9	M	23	7	0.574	4
Healthy 10	M	25	3	0.288	0
Healthy 11	F	27	0	0.000	-
Healthy 12	F	33	1	0.059	≥11
Healthy 13	F	28	1	0.053	10
Healthy 14	F	28	3	0.300	7
Healthy 15	F	25	2	0.185	6
Healthy 16	F	24	6	0.405	9
Healthy 17	F	46	1	0.087	0
Healthy 18	F	57	0	0.000	-
Healthy 19	F	43	2	0.215	6
Healthy 20	F	23	6	0.383	≥10
Healthy 21	F	24	2	0.157	9
Healthy 22	M	30	1	0.104	2
Healthy 23	F	23	2	0.200	4
Healthy 24	F	46	2	0.228	3
Healthy 25	F	54	0	0.000	-

Table A1.1. Demographics and colony formation of 25 healthy volunteers.

Appendix 2 – PAH patients demographics

Sample Code	Sex	Age	Diagnosis	Time from diagnosis	WHO class	ECFC colony No.	ECFC colony frequency	Highest passage No.
IPAH 1	F	62	1.1 IPAH	61	3	1	0.090	9
IPAH 2	F	56	1.1 IPAH	21	3	2	0.200	≥17
IPAH 3	M	65	1.1 IPAH	17	3	1	0.104	10
IPAH 4	M	28	1.1 IPAH	80	1	2	0.176	7
IPAH 5	M	35	1.1 IPAH	0.5	2	0	0.000	-
IPAH 6	M	75	1.1 IPAH	55	3	0	0.000	-
IPAH 7	F	67	1.1 IPAH	0.1	3	7	0.330	≥14
IPAH 8	F	27	1.1 IPAH	34	4	2	0.170	0
IPAH 9	M	37	1.1 IPAH	48	2	0	0.000	-
IPAH 10	F	54	1.1 IPAH	58	1	6	0.450	0
IPAH 11	M	49	1.1 IPAH	51	3	12	1.160	2
IPAH 12	F	46	1.1 IPAH	79	3	8	0.480	6
IPAH 13	M	82	1.1 IPAH	16	3	2	0.240	0
IPAH 14	M	38	1.1 IPAH	53	3	9	0.450	3
IPAH 15	M	72	1.1 IPAH	25	3	6	0.940	4
IPAH 16	F	49	1.1 IPAH	73	2	2	0.250	3
IPAH 17	M	47	1.1 IPAH	21	1	1	0.110	0
IPAH 18	F	44	1.1 IPAH	13	3	3	0.325	≥10
IPAH 19	F	24	1.1 IPAH	9	4	4	0.270	9
IPAH 20	M	51	1.1 IPAH	16	4	0	0.000	-
IPAH 21	F	42	1.1 IPAH	12	2	3	0.260	11
IPAH 22	F	37	1.1 IPAH	46	3	7	0.610	≥10
IPAH 23	F	35	1.1 IPAH	90	3	2	0.170	1
IPAH 24	F	37	1.1 IPAH	122	2	0	0.000	-
IPAH 25	F	47	1.1 IPAH	91	2	0	0.000	-
IPAH 26	M	44	1.1 IPAH	19	3	1	0.115	0
IPAH 27	M	44	1.1 IPAH	17	2	1	0.130	2
IPAH 28	F	27	1.1 IPAH	18	2	2	0.230	1
IPAH 29	F	39	1.1 IPAH	48	3	6	0.600	≥10
IPAH 30	M	46	1.1 IPAH	14	3	9	0.500	6
IPAH 31	F	27	1.1 IPAH	60	4	1	0.130	≥10
IPAH 32	F	33	1.1 IPAH	25	3	2	0.168	0
IPAH 33	F	22	1.1 IPAH	57	2	0	0.000	-
IPAH 34	F	28	1.1 IPAH	48	3	0	0.000	-
IPAH 35	M	29	1.1 IPAH	88	2	2	0.225	MSC-like
IPAH 36	M	45	1.1 IPAH	79	3	4	0.296	7
HPAH 1	F	37	1.2.1 HPAH	0.2	4	8	0.696	≥8
HPAH 2	F	35	1.2.1 HPAH	73	2	10	1.000	≥9

Table A2.1. Demographics and colony formation of PAH patients.

Table A2.2

Sample Code	Treatment – target vasodilators	Treatment - others	mPAP (mmHg)	PVR (dynes. s.cm^5)	CI (l/min/ m^2)	6MWD (m)
IPAH 1	Bosentan (125 mg bd), Sildenafil (50 mg tds),	Warfarin	39	NA	NA	210
IPAH 2	Bosentan (125 mg bd), Sildenafil (25 mg tds)	Warfarin	70	1545	NA	418
IPAH 3	Bosentan (125 mg bd)	Warfarin	66	800	2.25	285
IPAH 4	Bosentan (125 mg bd), Sildenafil (25 mg tds), Treprostinil (75 ng/kg/min)	Warfarin	46	764	1.58	570
IPAH 5	Nifedipine (10 mg bd)	Atorvastatin (10 mg qd)	50	364	3.7	567
IPAH 6	Sitaxsentan (100 mg qd)	Warfarin, Atorvastatin (8 mg qd)	36	270	3.86	420
IPAH 7	Naïve		68	905	1.96	396
IPAH 8	Sildenafil (50 mg tds), Ambrisentan (5 mg qd)	Warfarin	72	1448	1.9	471
IPAH 9	Bosentan (125 mg bd), Sildenafil (25 mg tds)	Warfarin, Simvastatin (80 mg qd)	60	644	2.67	555
IPAH 10	Bosentan (125 mg bd), Sildenafil (50 mg tds), Treprostinil (3 ng/kg/min)	Warfarin	72	1730	1.72	457
IPAH 11	Bosentan (125 mg bd), Sildenafil (50 mg tds)	Warfarin, Simvastatin (80 mg qd)	42	865	1.25	339
IPAH 12	Bosentan (125 mg bd), Sildenafil (50 mg tds)	Warfarin, Simvastatin (40 mg qd)	57	696	2.34	336
IPAH 13	Sildenafil (12.5 mg tds)	Warfarin	50	675	2.82	120
IPAH 14	Sildenafil (25 mg tds)		49	427	3.5	525
IPAH 15	Bosentan (125 mg bd)	Warfarin	49	NA	NA	264
IPAH 16	Sildenafil (25 mg tds)	Warfarin	NA	NA	NA	468
IPAH 17	Nifedipine (80 mg bd)		50	516	2.95	540
IPAH 18	Bosentan (125 mg bd), Sildenafil (25 mg tds)	Warfarin	67	1169	2.17	330
IPAH 19	Treprostinil (25 ng/kg/min), Sildenafil (20mg tds)	Warfarin	56	872	2.26	120
IPAH 20	Bosentan (125 mg tds), Sildenafil (50 mg tds)	Warfarin	73	1352	1.5	NA
IPAH 21	Nifedipine (40 mg bd), Sildenafil (20 mg tds)		50	802	2.66	438
IPAH 22	Bosentan (125mg bd), Sildenafil (75mg tds)	Warfarin	55	1672	1.28	NA

Table A2.2 (continued)

Sample Code	Treatment – target vasodilators	Treatment - others	mPAP (mmHg)	PVR (dynes. s.cm^5)	CI (l/min/ m^2)	6MWD (m)
IPAH 23	Sildenafil (25 mg tds), Treprostinil (42.5 ng/kg/min)	Warfarin	51	573	2.3	NA
IPAH 24	Treprostinil (0.046 mL per hr), Sildenafil (25 mg tds)	Warfarin	53	NA	NA	396
IPAH 25	Bosentan (125 mg bd)	Warfarin	43	NA	NA	450
IPAH 26	Sildenafil (50 mg tds), Ferrous Sulphate (200mg bd)	Warfarin	79	1248	1.98	258
IPAH 27	Sildenafil (50 mg tds)	Warfarin	59	682	2.47	495
IPAH 28	Treprostinil (41 ng/kg/min)	Warfarin	56	906	2.13	456
IPAH 29	Sildenafil (25 mg tds) + Treprostinil (45 ng/kg/min)	Warfarin	71	NA	NA	368
IPAH 30	Sildenafil (25 mg tds)	Warfarin	51	1252	2.12	480
IPAH 31	Bosentan (125 mg bd, Treprostinil (110 ng/kg/min)	Warfarin	66	1472	1.59	300
IPAH 32	Bosentan (125 mg bd), Sildenafil (25 mg tds)	Warfarin	52	472	4.1	325
IPAH 33	Ambrisentan (5 mg qd), Sildenafil (25 mg tds)	Warfarin	NA	NA	NA	345
IPAH 34	Bosentan (125 mg bd), Sildenafil (50 mg tds)	Warfarin	57	664	3.15	330
IPAH 35	Sildenafil (50 mg tds), Treprostinil (42.5ng/kg/min)	Warfarin	39	NA	NA	322
IPAH 36	Bosentan (125 mg bd), Sildenafil (75mg tds), Nebulised Iloprost (5 µg x5/day)	Warfarin	NA	NA	NA	120
HPAH 1	Naïve		79	2510	1.15	NA
HPAH 2	Bosentan (125 mg bd), Sildenafil (20mg tds)		NA	NA	NA	NA

Table A2.2. PAH patients treatments and clinical characteristicss of 36 IPAH patients and 2 HPAH patients. 6MWD, 6 minutes walk distance; CI, cardiac index; mPAP, mean pulmonary arterial pressure; PVR, pulmonary vascular resistance.