Universidade de Aveiro Departamento de Química
Ano 2013

Joana Filipa Fernandes Mendes Justino

Survivina e Smac/DIABLO: papel da apoptose na HAP experimental

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica do Professor Doutor Tiago Alexandre Henriques-Coelho, Professor Auxiliar Convidado do Departamento de Fisiologia e Cirurgia Cardiotorácica da Faculdade de Medicina da Universidade do Porto e da Professora Doutora Rita Maria Pinho Ferreira Professora Auxiliar Convidada do Departamento de Química da Universidade de Aveiro.

À minha família, à Alice

o júri

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palavras-chave

Hipertensão pulmonar, apoptose, survivina, Smac/DIABLO, remodelação vascular

Resumo

A hipertensão pulmonar (HP), é uma doença multifactorial e progressiva, caracterizada pela vasoconstrição, remodelação vascular pulmonar e trombose *in situ*, com consequente aumento da pressão arterial pulmonar e da resistência vascular pulmonar, frequentemente culminando na insuficiência cardíaca direita e morte. A remodelação vascular pulmonar, um dos principais contribuintes para o desenvolvimento e progressão da HP, é caracterizada por uma proliferação celular excessiva e uma reduzida apoptose. No entanto, pouco se sabe sobre os mecanismos moleculares subjacentes a este desequilíbrio. Assim, o presente trabalho teve como objetivo principal a avaliação da contribuição da apoptose na patogénese da HAP, através da análise da expressão cardíaca e pulmonar da survivina e do Smac/DIABLO e a sua associação às alterações hemodinâmicas e morfométricas. Para o efeito utilizou-se um modelo animal de HAP induzida por administração de monocrotalina (MCT). Os resultados demonstraram que os cardiomiócitos se encontravam hipertrofiados 7 dias após a injeção de MCT o que precedeu as alterações hemodinâmicas que se verificaram apenas ao dia 21. Sete dias após a administração de MCT observou-se sobreexpressão de survivina nos ventrículos, tendo esta aumentado progressivamente com a doença. A nível pulmonar, embora sobreexpressa ao dia 7, a expressão de survivin diminuiu com o desenvolvimento da doença. Apesar de sobreexpressa nos ventrículos e no pulmão 7 dias após injeção de MCT, a expressão do Smac/DIABLO diminuiu progressivamente com a HAP. Em conclusão, os resultados sugerem que a desregulação das vias de sinalização nas quais intervêm a survivina e o Smac/DIABLO está relacionada com a remodelação vascular pulmonar e a hipertrofia dos cardiomiócitos em resposta a estímulos apoptóticos.

Keywords Pulmonary hypertension, apoptosis, survivin, Smac/DIABLO, vascular remodelling

Abstract

Pulmonary hypertension (PH) is a multifactorial, progressive disease, characterized by vasoconstriction, pulmonary vascular remodelling and thrombosis in situ, which lead to augmentation of pulmonary arterial pressure and pulmonary vascular resistance, culminating in right heart failure and ultimately in dead. Pulmonary vascular remodelling, which is one of the major contributors to the development and progression of PH, is characterized by excessive cellular proliferation and reduced apoptosis. However, little is known about the molecular mechanisms underlying this imbalance. So, the present work aimed to study the contribution of apoptosis to the pathogenesis of PAH, through the analysis of cardiac and pulmonary expression of survivin and Smac/DIABLO along hemodynamic and morphometric alterations in an animal model of monocrotaline (MCT)-induced PAH. Results showed that cardiomyocytes were hypertrophied 7 days after MCT injection, preceeding hemodynamic alterations which were only present at day 21. Seven days after MCT administration, survivin overexpression was notorious in the ventricles and progressively increased throughout the development of MCT-induced PAH. On the other hand, although increased 7 days after MCT injection, lung survivin expression progressively decreased between the two days. The upregulation of Smac/DIABLO observed in lung, right and left ventricles 7 days after MCT administration, progressively decreased with the disease progression. In conclusion, data suggest that a deregulation in the balance between survivin and smac/DIABLO might be related with pulmonary vascular remodelling and cardiomyocytes hypertrophy in response to apoptotic stimuli.

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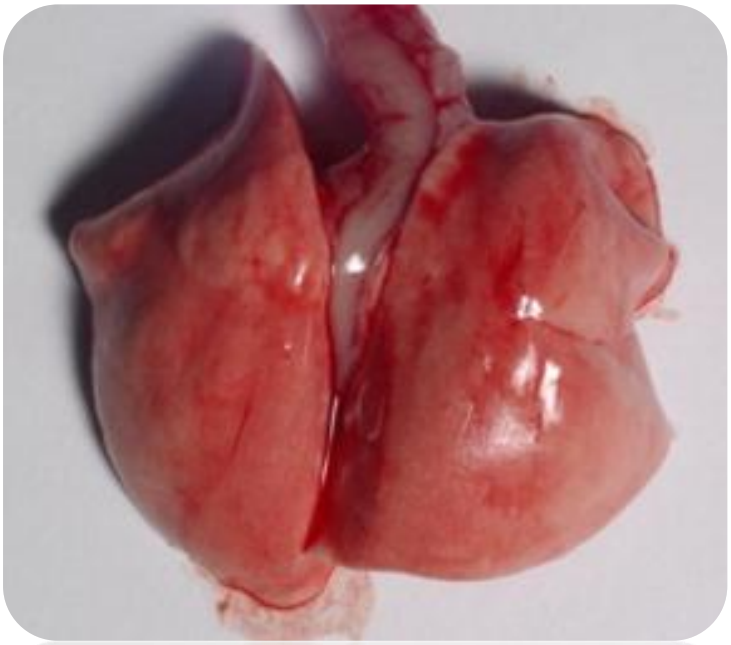
Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
5-HT	Serotonin
ADM	Adrenomedulin
AIF	Apoptosis-inducing factor
ALK	Activin receptor-like
Ang-1	Angiopoietin-1
ANP	Atrial natriuretic peptide
Apaf-1	Apoptotic protease activating factor-1
ATII	Angiotensin II
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BNP	Brain natriuretic peptide
BW	Body weight
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CCB	Calcium channel blockers
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CPI-17	C-kinase potentiated protein phosphatase-1 inhibitor
CREB	cAMP response element-binding protein
CTEPH	Chronic thromboembolic pulmonary hypertension
cyt c	Cytochrome C
EC	Endothelial cell
ECM	Extracellular matrix
E_m	membrane potential
ERK	Extracellular-signal-regulated kinase
ET-1	Endothelin-1
G	Gastrocnemius
GDF	Growth and differentiation factors
HF	Heart failure
HIV	Human immunodeficiency virus
HR	Heart rate
HW	Heart weight
IAP	Inhibitors of apoptosis proteins
IP_3	Inositol 1,4,5-triphosphate
iPAH	Idiopathic pulmonary arterial hypertension
JNK	c-Jun N-terminal kinase
K_V	Voltage-gated potassium channels
L	Lung
LV	Left ventricle
MAPK	Mitogenic-activated protein kinase

MCT	Monocrotaline
MCTP	Monocrotaline pyrrole or dehydromonocrotaline
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NF-AT	Nuclear factor of activated T cells
NF-kB	Nuclear factor kappa B
NO	Nitric oxide
NYHA	New York Heart Associaction
PA	Pulmonary artery
PAEC	Pulmonary artery endothelial cell
PAH	Pulmonary arterial hypertension
PAP	Pulmonary arterial pressure
PASMC	Pulmonary artery smooth muscle cell
PCH	Pulmonary capillary hemangiomatosis
PDGF	Platelet-derived growth factor
PGI_2	Prostacyclin
PH	Pulmonary hypertension
PK	Protein kinase
PLC	Phospholipase C
$P_{max.}$	Peak systolic pressure
PPH	Primary pulmonary hypertension
PVOD	Pulmonary veno-occlusive disease
PVR	Pulmonary vascular resistance
ROC	Receptor-operated calcium channel
ROS	Reactive oxygen species
RV	Right ventricle
RVH	Right ventricle/ventricular hypertrophy
S	Septum
SC	Subcutaneous injection
SERT	Serotonin transporter
SMC	Smooth muscle cell
SOC	Store-operated calcium channel
SPAP	Systolic pulmonary artery pressure
TGF	Transforming growth factor
Tib	Tibia
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRP	Transient receptor potential
TXA_2	Thromboxane A_2
VDCC	Voltage-dependent calcium channel
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
WHO	World Health Organization



I – Introduction

Pulmonary hypertension (PH) has been defined as a sustained elevation of the pulmonary arterial pressure (PAP) to more than 25 mmHg at rest or greater than 30 mmHg with exercise [1]. This is a multifactorial, progressive disease, with substantial mortality and morbidity. Besides elevated PAP, PH is characterized by: (i) increased pulmonary vascular resistance (PVR); (ii) alterations in the extracellular matrix (ECM); (iii) pulmonary vascular remodelling due to proliferation and migration of endothelial cells (ECs) and vascular smooth cells; (iv) progressive pulmonary vascular obliteration; (v) thrombosis and (vi) fibrosis, frequently leading to right-sided heart failure (HF) and death [2]. Untreated patients have a median survival time of 2.8 years [3]. Although it is found in both sexes at all ages, it has a higher incidence in women than men (2:1) and the average age of presentation is 36 years old [4].

Over the years the cellular and molecular mechanisms underlying the PH have been studied, not only to better understand these alterations, but also to develop therapies that may lead to a better prognosis. The therapies implemented at this moment are not entirely efficient because although they increase patients' survival, they do not lead to a cure. Therefore, the study of these mechanisms remains essential to better understand the development and progression of the disease and to plan a targeted and personalized therapy. For that reason, using a monocrotaline (MCT) animal model we studied the contribution of apoptosis to the pathogenesis of pulmonary arterial hypertension (PAH), through the analysis of the apoptotic proteins survivin and Smac/DIABLO.

II – State of Art

1. Pulmonary Hypertension: Classification, Risk Factors and Diagnosis

In 1891, during an autopsy, the German physician Ernst von Romberg observed pulmonary vascular lesions without a known cause, which he named "pulmonary vascular sclerosis", a classical description of PH (reviewed in [5]). The term primary pulmonary hypertension (PPH) was first used in 1951 after Dresdale and his associates reported data on 39 patients with unexplained PH (reviewed in [6]). Due to the rising incidence of PH in Europe, in 1973 at an international conference on PPH, World Health Organization (WHO) proposed a classification for PH that included two groups: PPH, a rare disorder that occurred without a known underlying cause, or secondary PH, a disease that developed in the presence of an underlying cause or in the presence of risk factors [6]. Later in 1998, this classification was reviewed at the Second World Symposium on PAH (Evian, France). In the "Evian Classification" PH was divided into five major groups based on pathobiologic and clinical features as well as therapeutic options [7]. During this Symposium, different risk factors for PAH: (i) drugs, toxins and chemicals (e.g. aminorex, meta-amphetamines, benfluores, cocaine, chemotherapeutic agents), (ii) demographic and medical conditions (e.g. gender, pregnancy, obesity), and (iii) diseases (HIV infection, thyroid disorders, collagen vascular disease) were categorized as definitive, very likely, possible or unlikely according to the strength of their association with PH and their probable casual role [7].

The classification was modified once more in 2003 at the Third World Symposium (Venice, Italy). The nomenclature "idiopathic pulmonary arterial hypertension" (iPAH) was adopted instead of PPH. In 2008 at the Fourth World Symposium on PH (Dana Point, California) (Table 1) the classification of PH was updated, and the term "non-PAH" was approved for other categories than PAH [8]. Additionally, left-heart disease PH was subdivided in systolic HF, diastolic HF and valvular heart disease and schistosomiasis was included as a new class of disease-associated PAH [8]. More recently, in March of 2013 the Fifth World Symposium was realized in Nice (France), however the new guidelines have not been published yet.

So, nowadays PH is classified in: (i) PAH; (ii) pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH); (iii) PH associated with left heart disease; (iv) PH associated with lung disease and/or hypoxia; (v) chronic

thromboembolic PH (CTEPH) and (vi) PH with unclear or multifactorial mechanisms (Table 1).

Table 1 | Clinical Classification of Pulmonary Hypertension, from the 4th World Symposium on Pulmonary Hypertension (Dana Point, 2008) (adapted from [8])**.**

Pulmonary Arterial Hypertension
Group 1: Pulmonary Arterial Hypertension
1.1 Idiopathic 1.2 Familial/Heritable (BMPR2, ALK1, endoglin, unknown) 1.3 Drugs and toxin-induced 1.4 Associated with: 1.4.1 Connective tissue disease 1.4.2 HIV infection 1.4.3 Portal hypertension 1.4.4 Schistosomiasis 1.4.5 Congenital heart disease 1.4.6 Chronic haemolytic anaemia 1.4.7 Others 1.5 Persistent pulmonary hypertension of the newborn
Group 1': Sub-class of Pulmonary Arterial Hypertension
1'. Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
Non-Pulmonary Arterial Hypertension
Group 2: Pulmonary Hypertension associated with left heart disease
2.1 Systolic dysfunction 2.2 Diastolic dysfunction 2.3 Valvular disease
Group 3: Pulmonary Hypertension associated with lung disease and/or hypoxia
3.1 Chronic obstructive pulmonary disease 3.2 Interstitial lung disease 3.3 Sleep-disordered breathing 3.4 Chronic exposure to high altitude 3.5 Alveolar hypoventilation disorders 3.6 Developmental abnormalities
Group 4: Chronic Thromboembolic Pulmonary Hypertension
Group 5: Pulmonary Hypertension with unclear or multifactorial mechanisms
5.1 Hematologic disorders (e.g. Myeloproliferative disorders) 5.2 Systemic disorders (e.g. vasculalitis, sarcoidosis, neurofibromatosis) 5.3 Metabolic disorders (e.g. glycogen storage disease, thyroid disorders) 5.4 Congenital heart disease (other than systemic-pulmonary shunt) 5.5 Others

Legend: BMPR2 – bone morphogenetic protein receptor-II; ALK1 – activin receptor-like kinase 1; HIV – human immunodeficiency virus

Besides the classification based on pathobiologic and clinic features, patients with PH can also be classified according to their functional capacity (Table 2). This classification has been adopted from the New York Heart Association (NYHA) for left heart disease [9]. Both clinical and functional classifications are useful for diagnosis, prognosis and therapy guidance of PH.

S

Table 2 | Functional classification of Pulmonary Arterial Hypertension (adapted from [9])**.**

Class I:	Patients with pulmonary hypertension without resulting in limitation of physical activity. Normal physical activity does not cause undue dyspnoea or fatigue, chest pain or near syncope.
Class II:	Patients with pulmonary hypertension resulting in slight limitation of physical activity. They are comfortable at rest, but normal physical activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class III:	Patients with pulmonary hypertension resulting in marked limitation of physical activity. The patient is comfortable at rest, but less than normal physical activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class IV:	Patients with pulmonary hypertension with inability to carry out any physical activity without symptoms. These patient manifest signs of right heart failure. Dyspnoea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

In advanced disease the patient may present non specific symptoms such as: chest pain, breathlessness, fatigue, weakness, palpitations, oedema, ascites and syncope [10, 11]. After careful examination, the clinician can also found: (i) hepatomegaly, (ii) right ventricular hypertrophy (RVH) and right atrial dilation on the electrocardiogram and (iii) increased systolic pulmonary artery pressure (SPAP) on the echocardiography [10-12]. For a differential diagnosis and assessment of disease severity it may be required: (i) antinuclear antibodies, (ii) autoimmune disease markers, (iii) HIV and viral hepatitis screening, (iv) coagulation disorder markers (e.g. protein S and C, von Willebrand factor) and (v) cardiac deterioration markers (e.g. cardiac troponin T, B-type natriuretic peptide (BNP)) [11, 12].

2. Pulmonary Vascular Morphology in Pulmonary Hypertension

In order to maintain its main function – gas exchange – pulmonary circulation is a low-pressure, high-flow and low-resistance system. Therefore the walls of the pulmonary arteries (PAs) are relatively thin [13]. The pulmonary vascular wall has 3 layers: (i) an outer adventitia containing fibroblasts, (ii) a media, composed of smooth muscle cells (SMCs) and an elastic lamina (the internal and external, separating the three layers), and (iii) an intima, with a single layer of ECs and a basement of connective tissue (Figure 1) [13, 14].

Pathological changes underlying PH involves vascular remodelling, which is characterized by the thickening of all three layers of pulmonary vessel wall due to the hypertrophy or hyperplasia of one or more cell types as well as increased deposition of ECM components (example: collagens) (Figure 1) [13, 15, 16]. The thickening of media (proliferation and hypertrophy of medial smooth muscle) and/or intima (EC hypertrophy and hyperplasia, oedema and thickening of the endothelial basement membrane) leads to narrowing and occlusion of pulmonary artery, increasing PVR and PAP [13, 15, 16]. In the adventia layer is also seen the proliferation of fibroblasts and the deposition of collagen [16].

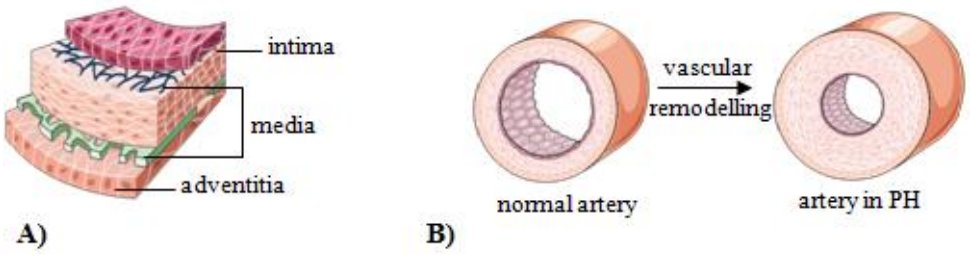


Figure 1 | Pulmonary Vascular Morphology. A) The pulmonary vascular wall has 3 layers: an outer adventitia, a media and an intima; **B)** In PH, vascular remodelling is characterized the thickening of all three layers of pulmonary vessel wall.

In severe stages of PH occurs the formation of a layer of ECM and cells (SMCs or myofibroblasts embedded in mucopolysaccharide) between the internal elastic lamina and the endothelium – neointima – which also contribute to the elevation of PVR [13, 17]. These myofibroblasts express markers such as α-smooth muscle actin and vimentin but there is no expression of smooth muscle myosin (characteristic marker of highly differentiated SMCs), cluster of differentiation 31 (CD31), CD34 or factor VIII (ECs markers) [17]. Plexiform lesions, another form of vascular remodelling, also predominates in severe stages of PH. They occur due to the disorganised proliferation of ECs supported by a myofibroblast stroma, contributing to the elevation of PVR [13, 18]. These ECs express angiogenic markers such as vascular endothelial growth factor (VEGF) and its receptors [19, 20]. In some forms of PH the release of reactive oxygen species (ROS), growth factors, cytokines and angiogenic factors from the abundant mast cells, T and B lymphocytes and macrophages that surround PAs and arterioles may play a role in vascular remodelling [18]. With time, all of these vascular remodelling events lead to the reduction of PAs' lumen due to its obliteration, resulting in PVR rise and PAP elevation. The augmentation of the PVR results in an overwork of the heart especially of the right ventricle (RV) inducing RVH [21]. Muscle mass accumulates in the RV (through the enhancement of protein synthesis and myocyte hypertrophy)

increasing wall thickness, and it assumes a more rounded shape compressing the left ventricle (LV) [21, 22]. Although in an initial phase the overwork of the RV can maintain the cardiac output, the persistent elevated resistance leads to a progressive dysfunction that frequently culminate in right heart failure [21].

3. Pathobiology of Pulmonary Hypertension

3.1. Genetic Associations

Mutations in bone morphogenetic protein (BMP) receptor 2 (BMPRII), and single nucleotide polymorphisms of K_V (voltage-gated K^+ channels) 1.5, transient receptor potential (TRP) channels and serotonin transporters (SERT) have been implicated in the development of PH.

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor (TGF) superfamily. They have multiple important roles in vascular remodelling, including inhibition of apoptosis and SMC proliferation, regulation of vasoactive peptides and growth factors, and modulation of immune cell function [23]. Signalling pathways involving BMPs involve the formation of hetero-complexes between one of type-I receptors and one of type-II receptors [24]. The TGF-β superfamily type II receptors are constitutively active serine/tyrosine kinases that initiate intracellular signalling in response to specific ligands [24]. After BMP binding, type-II receptor phosphorylates type-I receptor, activating its kinase domain [24, 25]. In turn, activated type-I receptor phosphorylates the cytoplasmic signalling proteins responsible for TGF-β superfamily signal transduction – Smad – that translocates to the nucleus where interacts with various transcription factors, regulating gene transcription (e.g. Bcl-2 and K^+) [24, 25]. There is evidence that MAPKs (mitogenic-activated protein kinase), including ERKs (extracellular-signal-regulated kinase), p38, and JNKs (c-Jun N-terminal kinases) might also be activated via TGF-β and BMPs [24-26]. Mutations in the gene coding for BMPRII (locus in chromosome 2q31-32) have been found in more than 70% of familiar PAH, and 10% to 40% in iPAH [27]. In spite of the autosomal dominant inheritance of BMPRII gene mutations, this disease has a low penetrance since only approximately 20% of individuals carrying the mutation will develop PAH [27]. One hundred and forty four mutations in BMPRII have been

reported until now, particularly missense mutations that lead to substitution of cysteine residues in the ligand-binding and/or kinase domain of BMPRII, which culminate in the traffic disruption of the mutant BMPRII to the cell surface retaining the receptor in the cytoplasm [28, 29]. On the other hand, in non-cysteine missense mutations in the kinase domain, BMPRII mutants reach the cell surface but fail to activate Smad either by disruption of its serine-threonine kinase activity or the inability to complex with type I receptors [28]. In addition, missense mutations in the cytoplasmic domain attained the cell surface and retained the ability to activate Smad signalling [28]. All these mutations will result in a common feature – gain of function ligand-independent activation of p38 pro-proliferative pathways, inhibiting Smad-dependent signalling with the consequent vascular remodelling observed in familial PAH [28, 30, 31]. Indeed, the decline of BMPRII function in the endothelium might lead to increased apoptosis favouring the development of apoptosis-resistant clones of ECs resulting in the formation of plexiform lesion in PH patients [32]. In addition, mice with BMPRII mutations showed that loss of BMPRII signalling might induce and/or predispose PH with elevation of PAP, wall thickness, pulmonary arterial musculatization and RVH [33, 34].

In addition, serotonin (5-HT) pathway, which has been related with PASMCs hyperplasia, also plays an important role in the development and progression of PH. Serotonin transporter is highly expressed in the PASMCs and have been implicated in vascular remodelling, since patients with iPAH have increased expression of SERT in lung tissues especially in the media of thickened PAs [35]. Furthermore, mice with SERT gene knockout developed less severe hypoxia PH than wild-type control and selective SERT inhibitors attenuate hypoxia- and MCT-induced PH [36-38]. On the other hand, elevated expression of SERT is related with increased severity of PH and transgenic mice selectively overexpressing SERT gene in SMCs developed spontaneously PH in absence of hypoxia, and overstated PH after exposure to hypoxia [39, 40]. Finally, SERT is encoded by a single gene on chromosome 17q11.2 and variants in upstream promoter region have been described. The polymorphism consists in the insertion or deletion of 44pb, designated the long (L) and the short (S) allele, respectively, affecting SERT expression and function. The L-allele induces 2- to 3-fold greater level of SERT gene transcription than S-allele [35, 41]. The L/L genotype might confer genetic susceptibility to iPAH because this homozygous form is much more frequent in these patients (65%) compared with healthy individuals (27%) [35].

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) also has been related with pulmonary vasoconstriction as well as pulmonary vascular wall thickening and remodelling underlying PH. Calcium influx through membrane is controlled by multiple ion channels including: i) voltage-dependent Ca^{2+} channel (VDCC) that is regulated through changes in membrane potential (E_m); ii) receptor-operated Ca^{2+} channels (ROC) which is activated by the interaction agonist-membrane receptor; and iii) store-operated Ca^{2+} channels (SOC) that is activated by depletion of Ca^{2+} from intracellular stores [42]. In turn, SOC and ROC function is dependent on the expression of TRP channel genes. TRP genes, such as TRPC1, TRPC4, TRPC5, TPRC6 and TPRC7 are expressed in human PASMCs, and TRPC1, TRPC3 and TRPC4 are present in PAECs [43, 44]. Additionally, TRPCs seems to play a role in PH, since PASMCs of iPAH overexpressed TRPC3 and TRPC6 mRNA and protein compared to normal individuals, enhancing cell growth and proliferation [43]. Furthermore, it has been reported that a single-nucleotide polymorphism in the TRPC6 gene promoter is associated with elevation of $[Ca^{2+}]_i$ and proliferation of PASMCs in 12% of iPAH patients', predisposing to the disease [45].

3.2. Neurohumoral Mechanisms

The primary function of the pulmonary endothelium is to maintain a low PVR through the balance between: (i) vasodilators and vasoconstrictors, (ii) mitogens (proliferation) and growth inhibitors (apoptosis) and (iii) antithrombotic and prothrombotic determinants [46, 47]. In patients with PH, pulmonary endothelial dysfunction appears to play an important role in the changes of pulmonary vasculature [48]. Besides disorganized proliferation of ECs that lead to the formation of plexiform lesions, these cells have an altered production of vasoactive mediators (such as prostacyclin (PGI_2), nitric oxide (NO), endothelin-1 (ET-1), serotonin (5-HT) and thromboxane (TXA_2)) affecting vascular tone and SMCs growth which may facilitate the pulmonary vascular hypertrophy and structural remodelling (Table 3) [47, 48]. In addition, endothelial dysfunction might affect the production of anticoagulant factors altering the balance between prothrombotic and antithrombotic determinants, impairing vascular homeostasis that can lead to thrombosis and fibrosis [48].

Table 3 | Neurohumoral alterations described in the pathobiology of PH

Mediators	Physiological Effects				Levels in PH		References
	Vascular Dilatation	Cell Proliferation	Platelet Aggregation	Apoptosis	Serum	Lungs	
PGI_2	√	✕	✕			↓	[49-51]
TXA_2	✕	√	√				[49, 50, 52]
NO	√	✕	✕	√		↓↑	[46, 53, 54]
ET-1	✕	√	√	√	↑	↑	[55-59]
5-HT	✕	√			↑	↑	[35, 39, 60-62]
VIP	√	✕	✕	✕	↓	↓	[63-65]
ADM	√	✕		✕	↑		[66, 67]
ATII	✕	√				↑	[68-70]
Ang-1				✕		↑	[71, 72]
VEGF		√		✕		↑↓	[73-77]
PDGF		√		✕		↑↓	[78, 79]
ANP, BNP						↑	[80-83]
Apelin	√	✕		√		↓	[84, 85]
Ghrelin	√						[86-88]

Legend: **5-HT**, Serotonin; **ADM**, Adrenomedulin; **Ang-1**, Angiopoiein-1; **ANP,** Atrial Natriuretic Peptide; **ATII**, Angiotensin II; **BNP,** Brain Natriuretic Peptide **ET-1**, Endothelin-1; **NO**, Nitric Oxide; **PDGF**, Platelet-Derived Growth Factor; **PGI_2**, Prostacyclin; **TXA_2**, Thromboxane A_2; **VEGF**, Vascular Endothelial Growth Factor; **VIP**, Vasoactive Intestinal Peptide; ✕, no; √, yes; ↓, decrease; ↑, increase

Contraction and relaxation as well as SMCs proliferation and apoptosis are also controlled by multiple protein kinases (PKs) (e.g. PKC, Rho kinase) and cyclic nucleotides (e.g. 3'-5'-cyclic adenosine monophosphate (cAMP) and 3'-5'-cyclic guanosine monophosphate (cGMP)). Indeed, PKA, that can be trigger by PGI_2, activates K_V channels in vascular SMCs promoting vasodilatation [89]. On the contrary, PKC, which can be trigger by ATII, ET-1 and TXA_2, is able to adjust $[Ca^{2+}]_i$ mediating contraction through activation of Ca^{2+} channels and inhibition of K^+ channels [90]. PKC is also capable to intervene in contraction through phosphorylation of CPI-17 (C-kinase potentiated Protein phosphatase-1 Inhibitor) which in turn inhibits myosin light chain (MLC) phosphatase (MLCP) increasing MLC phosphorylation [90, 91]. Inhibition of MLCP and CPI-17 phosphorylation is also achieved by Rho kinase, also promoting smooth muscle contraction [92]. Regarding cAMP role, it is hypothesize that vascular relaxation is accomplish by (i) lowering $[Ca^{2+}]$ through inhibition of inositol 1,4,5-triphosphate (IP_3) formation due to phospholipase C (PLC) inhibition, (ii) inhibition of Ca^{2+} release from sarcoplasmic reticulum, (iii) stimulation of Ca^{2+} uptake and/or extrusion, and (iv) inhibition of Ca^{2+} entry [90]. In addition, cAMP through increasing MLCP activity and reducing MLC kinase (MLCK), p42/44, MAPK and Rho kinase activities can regulate vascular tone [91].

Furthermore, $[Ca^{2+}]_i$ determines smooth muscle contraction, and its rise is the main trigger of pulmonary vasoconstriction as well as SMC migration and proliferation, leading to pulmonary vascular wall thickening and remodelling. Indeed, $[Ca^{2+}]_i$ is an important second messenger for PASMC migration and proliferation, once some signal transduction proteins involved in cellular proliferation are Ca^{2+} dependent (e.g. mitogen-activated protein II kinase) [93, 94]. The fact that resting $[Ca^{2+}]_i$ in proliferating PASMC is greater than in growth-arrested cells supports the role of enhanced Ca^{2+} influx into the cytoplasmic space is also essential for cell growth and proliferation [95]. Moreover, resting $[Ca^{2+}]_i$ is higher in PASMCs of iPAH patients than in cells of normal individuals, demonstrating once more the role of $[Ca^{2+}]_i$ in PH [96]. In addition, augmentation in $[Ca^{2+}]_i$ leads to elevation of nuclear Ca^{2+} concentration which is essential for cell cycle progression, since at least four steps are sensitive to Ca^{2+}/CaM (calmodulin) complex activation [97-99]. Additionally, Ca^{2+} influx via VDCC (voltage-dependent Ca^{2+} channels) activates several transcription factors, such as CREB (cAMP response element-binding protein), NF-AT (nuclear factor of activated T

cells) and NF-κB (nuclear factor kappa B) that are involved in cell proliferation, protein synthesis and inflammation [42].

3.3. Role of Apoptosis

Although recent therapies target specific cellular and molecular pathways involved in the pathogenesis of PAH, they are essentially vasodilators, contributing to symptoms relief and improvement but not leading to a better prognosis. So, in the last years investigations have been redirected towards a better understanding of the cellular and molecular mechanisms underlying vascular remodelling, including the role of apoptosis in PH pathogenesis. Indeed, it has been described an imbalance between mitogens and growth inhibitors in PH that along with plexiform lesions and neointima formation highlight a deregulated apoptosis and cell proliferation in the disease.

Apoptosis, or programmed cell death, is important for maintenance of tissue homeostasis through the elimination of harmful cells. In the vasculature, apoptosis modulators are multiple and complex, and might include ROS, NO and ATII systems [100-102]. Apoptosis is characterized by cell shrinkage, extensive protein cross-linking and chromatin condensation (pyknosis) and migration to nuclear membrane [103-105]. In a later stage, although cell membrane remains intact, there is DNA fragmentation (karyorrhexis) into 180 to 200 base pairs promoted by Ca^{2+} - and Mg^{2+}-dependent endonucleases and formation of apoptotic bodies that are quickly removed via phagocytosis by surrounding cells [105, 106]. Neither the process of apoptosis nor removal of apoptotic bodies is associated with an inflammatory reaction [107, 108]. Apoptosis can occur mainly via two main pathways: intrinsic (or mitochondrial) and extrinsic (or death receptor) ones (Figure 2). Both pathways converge to the same execution pathway that is initiated by the cleavage and activation of execution/effector caspases (cysteinyl aspartic acid-proteases) – caspase-3, caspase-6 and caspase-7 – resulting in the characteristic alterations previously mentioned [109].

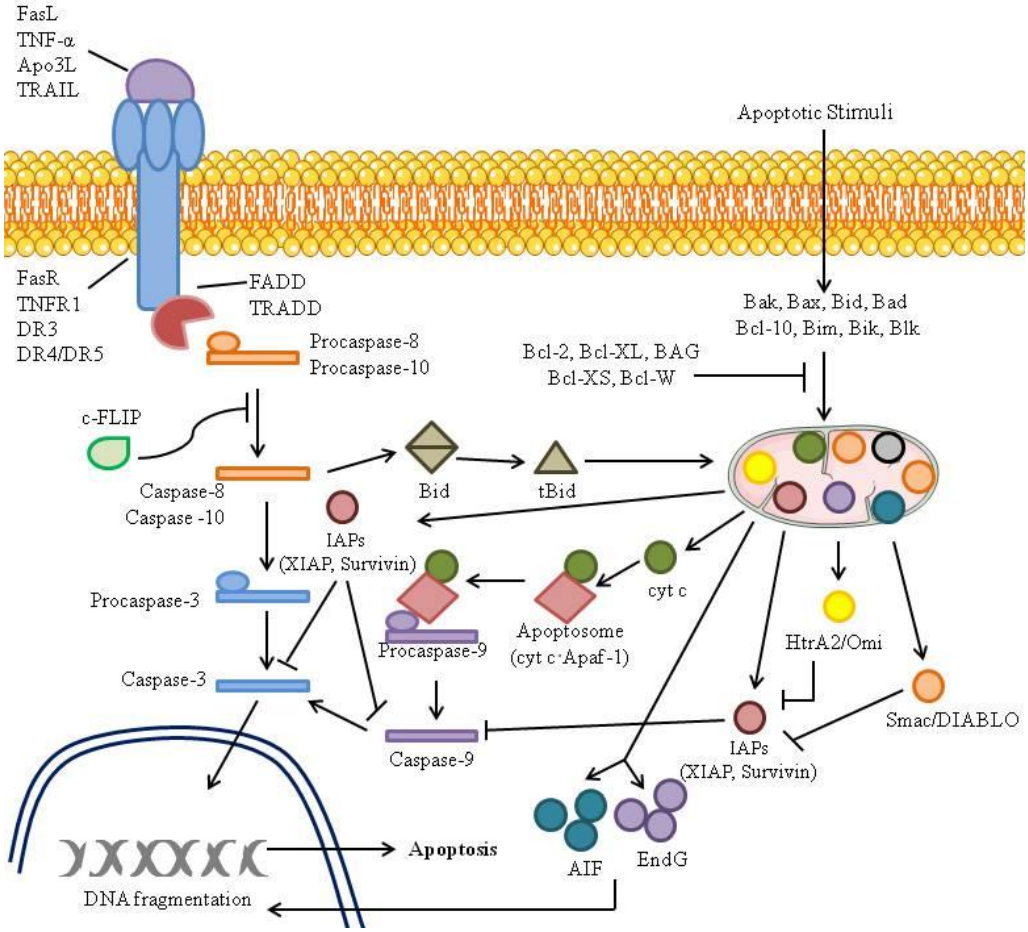


Figure 2 | Apoptosis signalling pathway. Apoptosis can occur mainly via two pathways: intrinsic and extrinsic ones. In the extrinsic pathway activation of death receptors activates initiator caspase 8 initiating the caspase cascade culminating in apoptosis. The intrinsic pathway is determined by mitochondrial permeability which is regulated through the balance between pro-apoptotic and anti-apoptotic mediators. Activation of pro-apoptotic ones lead to the release of cytochrome c (cyt c), Smac and Omi promoting apoptosis through apoptosome formation and neutralization of inhibitors of apoptosis proteins (IAPs). A crosstalk between these two pathways has been described involving, for instance, Bid cleavage by caspase 8, releasing cyt c from the mitochondria.

The extrinsic pathway involves the activation of transmembrane death receptors which belongs to the TNF (Tumor Necrosis Factor) receptor gene superfamily. These family of receptors is characterized for having a similar cysteine-rich extracellular domain and a cytoplasmic domain called "death domain" [110]. The best-characterized death receptors and corresponding ligands include: i) FasL/FasR, ii) TNF-α/TNFR1, iii) Apo3L/DR3, iv) Apo2L (or TRAIL)/DR4 and v) Apo2L (or TRAIL)/DR5 [111]. After the ligand bind the death receptor, an adaptor protein (FADD for FasL and TRADD for TNF-α) binds through its own death domain to the clustered receptor death domain

[112, 113]. This adaptor protein also has a "death effector domain" that will associate with an initiator caspase, such as caspase-8 and caspase-10, through dimerization of the death effector domain, activating it by self-cleavage [114, 115]. Then, the initiator caspase (caspase-8) trigger execution pathway through activation of downstream effector caspase, including caspase-3, culminating in apoptosis [115]. This extrinsic pathway can be inhibited trough a protein that binds both initiator caspase and/or adaptor protein, such as c-FLIP which can associate with both FADD and caspase-8 inhibiting apoptosis [116, 117].

The intrinsic pathway is determined by mitochondrial permeability which is regulated through the balance between pro-apoptotic (Bcl-10, Bak, Bax, Bid, Bad Bim, Bik and Blk) and anti-apoptotic (Bcl-2, Bcl-XL, Bcl-XS, Bcl-W and BAG) mediators [109]. When the cell is exposed to some stimuli, such as depravation of growth factors, exposition to agents that damage DNA or accumulation of unacceptable amounts of misfolded proteins, there is the activation of pro-apoptotic mediators (Bax and Bak), which translocate into the mitochondria and form channels through which cytochrome c (cyt c), Smac/DIABLO, serine protease HtrA2/Omi and other proteins escape to the cytosol [118-120]. Released cyt c activates Apaf-1 (apoptotic protease activating factor-1) and procaspase-9, forming the apoptosome (cyt c/Apaf-1) which is responsible for the activation of initiator caspase 9, culminating in the activation of execution pathway, via activation of caspase-3 [109, 115]. In addition, it was recently demonstrated that besides activating caspases, cyt c can also activate K^+ channels [121]. Smac/DIABLO and HtrA2/Omi have been described to promote apoptosis through the inhibition of IAP (inhibitors of apoptosis proteins) activity, such as XIAP [119, 120, 122]. In addition, alterations in mitochondrial permeability during apoptosis also lead to the release of AIF (apoptosis-inducing factor) and endonuclease G that induce chromatin condensation and DNA fragmentation through a caspase-independent cell death [120].

A crosstalk between these two pathways was already described involving, for instance, Bid cleavage through caspase 8 (Figure 2). Truncanted Bid translocates to the mitochondria and causes cyt c release through the homo-oligorization of Bak and/or Bax [123-125]. Recently, it was suggested that apoptosis induced by TRAIL (TNF-related apoptosis-inducing ligand) appears to require the release of mitochondrial Smac/DIABLO [126]. TRAIL induces apoptosis through activation of it death receptors, DR4 and DR5; only, TRAIL-induced apoptosis requires Bax translocation

from the cytosol to the mitochondria. In addition, this translocation of Bax depends on the caspase 8 activation and in turn activated caspase 8 cleave Bid, which translocate to mitochondria releasing Smac/DIABLO [126]. Considering recent works [127, 128] describing TRAIL as a potential therapeutic target in PH, it would be interesting to investigate TRAIL in the development of PH and if its administration can also attenuate or even reverse vascular remodelling seen in PH.

Additionally, maintenance of high concentrations of intracellular K^+ ($[K^+]_i$) is important to the regulation of normal cell volume and suppression of caspases and nucleases [129, 130]. Therefore, $[K^+]_i$ and K^+ channels seems to be important in apoptosis modulation. Indeed, elevated K^+ loss or K^+ efflux through K^+ channels (via their activation) results in reduction of $[K^+]_i$ inducing apoptotic volume decrease and activation of caspases and endonucleases that will trigger apoptosis [130, 131]. On the contrary, inhibition of K^+ channels maintain sufficient $[K^+]_i$ attenuating apoptotic volume decrease and inhibiting apoptosis [131]. Moreover, overexpression of Bcl-2 in rat PASMCs leads to reduction of K_V channels subunits inhibiting apoptosis [94]. Overexpression of Bcl-2 was also found in the lungs of iPAH patients, which might contribute to the downregulation of K_V channels in these patients [132].

In PH, the resistance to apoptosis observed in PASMCs seems to result, in part, from the diminished expression of K_V channels, since PASMCs from iPAH patients' present reduced mRNA expression of K_V channels subunits, probably due to defects in their gene transcription [133, 134]. Remillard and colleagues [135] also demonstrated that $K_V1.5$ (an α subunit of K_V channels) encoding gene of iPAH have multiple single nucleotide polymorphisms which probably contributes to altered function and/or expression of this channels in PASMCs of patients. Additionally, it is believed that certain appetite suppressants (such as aminorex, fenfluramine and dexfenfluramine) are associated with an increased risk of PAH, and they appear to contribute to K_V channels downregulation, leading to PH [136, 137]. In SMCs, BMP inhibits proliferation and induces apoptosis, on the other hand in ECs it is responsible for cell survival (proliferation) [138, 139]. However, studies had suggested that alterations in BMP axis induce PAECs apoptosis [139]. Besides the genetic susceptibility associated to PH due to mutation in BMP signalling, initial environmental injuries, such as hypoxia, increase flow, inflammation, infections, drugs or toxins, might lead to ECs apoptosis [140-142]. Apoptosis of ECs impairs endothelial function providing conditions for an increased

proliferation of the remaining endothelium and favouring the emergence of apoptosis-resistance and hyperproliferative ECs, that are characteristics of PH later stage [140, 141, 143]. Beyond the release of growth factors and other mediators, the initial apoptosis of ECs leaves SMCs more susceptible to these factors that stimulates proliferation and inhibits apoptosis of SMCs (Figure 3) [144].

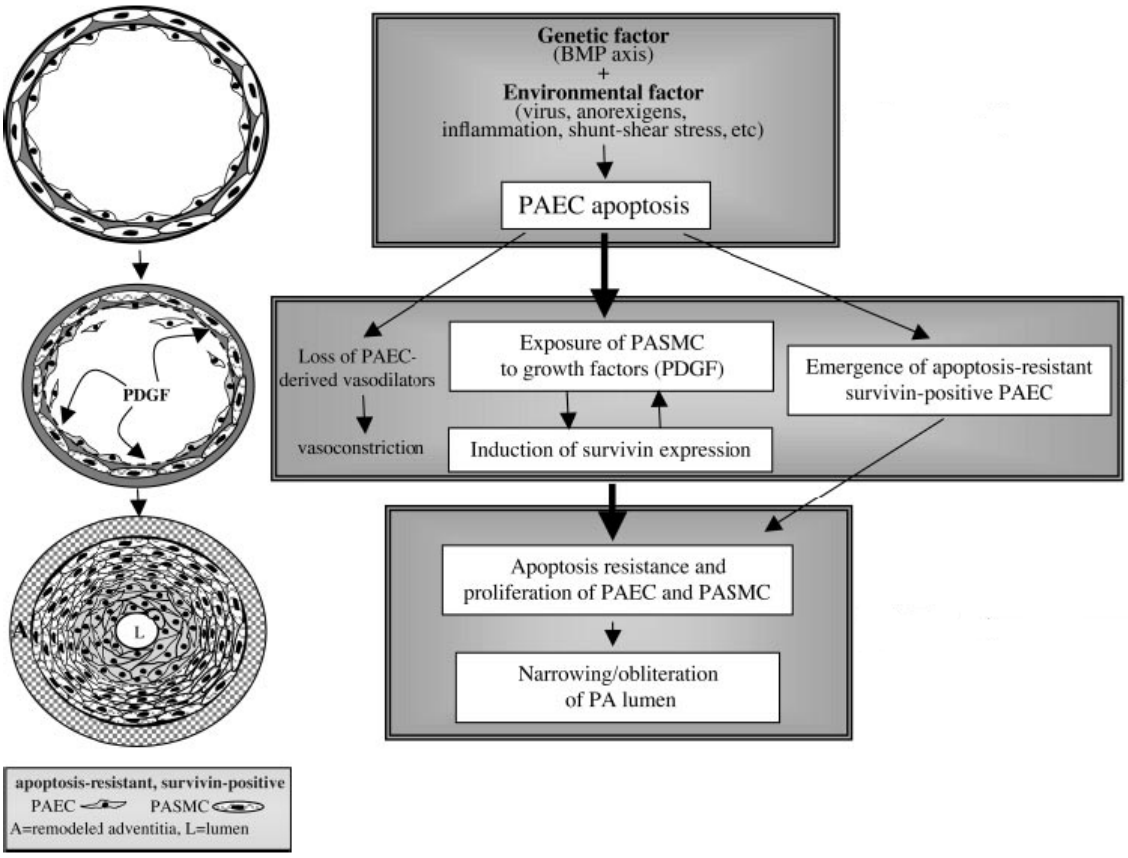


Figure 3 | Apoptotic-based theory for the development of PH. Although the mechanisms underlying PH need to be better characterized, at the moment an apoptotic-based theory for the development of PH is one of the most accepted in the scientific community. In addition to the genetic susceptibility associated to PH due to mutation in BMP signalling initial environmental factors, such as hypoxia, inflammation or drugs, might lead to PAECs apoptosis. The apoptosis of ECs leads to an endothelial dysfunction providing conditions that increase proliferation of the remaining endothelium and favouring the emergence of apoptosis-resistance, hyperproliferative ECs, expressing survivin, and vasoconstriction, that are characteristic of later stage of PH. In addition, beyond induces the release of growth factors and other mediators the initial apoptosis of ECs leaves SMCs more susceptible to those factors and all this stimulates proliferation and inhibits apoptosis in SMCS. Consequently, the apoptosis resistance and proliferation of both PAECs and PASMCs leads to narrowing and occlusion of pulmonary artery increasing PVR and PAP, frequently culminating in right-sided heart failure and death.
(adapted from [142])

3.3.1. Survivin and Smac/DIABLO

Survivin is the smallest element of the inhibitor of apoptosis protein (IAP) family [145]. Along with survivin, seven more IAP proteins were identified in humans: XIAP/hILP

(X-chromosome-linked IAP), C-IAP1/HIAP-2 (cellular inhibitor of apoptosis 1), C-IAP2/HIAP-1 (cellular inhibitor of apoptosis 2), ILP2 (IAP-like protein 2), MLIAP (livin), NAIP (neuronal apoptosis inhibitor protein) and BRUCE [146]. IAP family members are characterized by the presence of two to three baculoviral IAP repeat (BIR) domains, which can bind and inactivate caspases and prevent caspase-induced apoptosis; however, survivin only contains a BIR domain [145, 146].

Survivin plays an important role in apoptosis. In response to cell death stimuli, mitochondrial survivin is released to the cytosol, inhibiting apoptosis by either directly or indirectly interfering with caspase function (Figure 2) [147]. Indeed, it was reported that overexpression of survivin suppress apoptosis induced by multiple stimuli including TNF and Fas [148-150]. Some authors refer that survivin can directly bind and inhibit caspase-3, -7 and -9 activity, since survivin co-immunoprecipitated with these, and since apoptosis was suppressed with co-expression of survivin and overexpression of caspase-3, -7 and -9 [150]. On the other hand, others demonstrated that survivin inhibits apoptosis through the interaction with Smac/DIABLO, which is pro-apoptotic factor that promotes caspase activation, binding and inhibiting other IAPs activity [151]. By binding with Smac/DIABLO, survivin delays the release of Smac/DIABLO into the cytoplasm and increases its cytosolic stability, which results in prolonged cell survival [152]. Indeed, Song and colleagues [153] proposed that survivin binds Smac/DIABLO released from the mitochondria, decreasing antagonism of Smac/DIABLO to XIAP, and therefore the free XIAP directly inhibits caspase and consequently apoptosis. Survivin also blocks apoptosis downstream pro-apoptotic mediators Bax, Bik, Bak, and cyt c [148, 150]. Furthermore, it was suggested that under apoptotic stimuli involving mitochondria, survivin is also able to forms a complex with XIAP, enhancing its stability and inhibiting caspases-9 activation [154].

Apart from its role in apoptosis, survivin also regulates cell division and viability. Survivin seems to have a cell-cycle dependent expression in mitosis, being upregulated during G2/M phase of the cell cycle [155]. It was demonstrated to be essential in karyokinesis and cytokinesis through the formation of chromosomal passenger complex along with Aurora B kinase, INCENP and Borealin [156]. Indeed, survivin is necessary in multiple points of mitosis through the regulation of spindle assembly checkpoint, metaphase spindle formation and microtubule stability [155, 157, 158]. Therefore, inhibition of survivin expression/function results in multiple mitotic defects due to cell

cycle arrest, formation of aberrant multipolar spindles, absence of cytokinesis and development of multinucleated polyploidy cells [155, 157, 159].

BIRC5, the gene that encodes survivin, produces five major isoforms of the transcript through alternative splicing of survivin pre-mRNA: survivin (wild type), survivin-2B, survivin-ΔEx3, survivin-3B and survivin-2α. Survivin-2α seems to support the induction of apoptosis, while survivin (wild type), survivin-3B and survivin-ΔEx3 seem to be cytoprotective [160-163]. Some isoforms, such as survivin-3B, appears to also be implicated in cell cycle regulation [163]. It has been hypothesised that the dual role of survivin might be related with its different compartimentalization. Although nuclear survivin is associated to the regulation of cell division, mitochondrial survivin is linked to the inhibition of apoptosis, preventing the activation of effectors caspases which confers resistance to apoptosis [147, 154, 164]. On the contrary, survivin released to the cytoplasm after apoptosis initiation, loses its protective ability against apoptosis, probably due to posttranslational modifications [154, 165]. In a study [166] realized in HeLa cells it was demonstrated that differential ablation of survivin through RNA interference influences cell death. Partial reduction of the cytosolic pool of survivin through RNA interference in association to UVB irradiation resulted in the increased catalytic activity of caspases-3/-7 as well as in the proteolytic cleavage of caspase-9. On the other hand, loss of mitochondrial membrane permeability and spontaneous apoptosis were observed after complete removal of cytosolic survivin. In the same study [166] it was verified that preferential suppression of the nuclear pool of survivin culminated in cell arrest followed by re-entry into the cell cycle and polyploidy along with other mitotic defects.

In the last years, PAH has been considered a cancer like disease since almost characteristics that defines cancer, such as deregulation of growth pathways, angiogenesis, dysfunctional K_V channels, loss of expression of PGI_2 synthase gene (anti-proliferative) and augmentation of survivin expression have been associated in the disease [167-170]. Indeed, survivin is practically undetectable in differentiated adult tissues, but is highly expressed is the most common human cancers, thus making it an interesting tumor marker [171]. However, little is known about it expression and role in PAH. Until now, it was reported that in an animal model of MCT-induced PH with vascular remodelling and PAs of patients with PH survivin was found overexpressed and was related with the inhibition of apoptosis and stimulation of PASMCs

proliferation [121]. Moreover, lung transfer of survivin gene in experimental animals resulted in pulmonary vascular remodelling while adenoviral gene therapy with a mutant survivin reversed MCT-induced PH through the activation of K_V channels, induction of apoptosis and suppression of proliferation [121]. Therefore a better characterization of survivin involvement in the development and progression of PAH will potentially open new perspectives on the treatment of PAH.

4. Animal Models for the Study of PAH

Animal models have allowed the study of the pathogenesis and therapeutic strategies of multiple diseases, including PAH [172]. Although an animal model should mimic the combine characteristics of human disease, including relevant clinical, hemodymanic, biochemical and histopathological features, until now no model is able to mimic all features of the PAH [173]. Different animal models have been used to study the distinct features of PAH, namely the various underlying genetic and environmental causes.MCT and chronic hypoxia are the most frequently used animal models in the study of PH [172]. In addition, transgenic and knockout models have been used to evaluate altered expression of specific genes in the development and/or progression of PH [174, 175]. MCT model has been used for over than 50 years, however the molecular mechanisms underlying MCT-induced PH need to be better clarified [176].

4.1. Monocrotaline Animal Model of PAH

Monocrotaline (MCT) (or 12β,13β,-dehydroxy-12α,13α,14α-trmethylcrotal-1-enine) is an 11-membered macrocyclic pyrrolizidine alkaloid derived from the stems, leaves and seeds of the *Crotalaria spectabilis* that can be administrated by intraperitoneal (60 mg/kg), subcutaneous (60 mg/kg) or intravenous injection (1-5mg/kg). Although when topically or injected applied it does not cause localized toxicity, MCT is known for its hepatic and cardiopulmonary toxicity, causing lesions in multiple organs after absorption and hepatic bioactivation [15, 177]. In fact, it was described that in chronic exposure or single high doses injections of MCT causes periacinar hepatic necrosis, hepatocyte necrosis, hepatocellular megalocytosis and hepatic fibrosis [178]. However, a single lower-dose injection does not cause hepatic pathology but results in PH after

hepatic bioactivation [178, 179]. In the liver, MCT is activated and converted to reactive metabolites by cytochrome P450 3A, including dehydromonocrotaline also called monocrotaline pyrrole (MCTP) [179]. It is considered that MCTP metabolite is responsible for the toxicity and injuries associated to MCT, since intravenous administration of chemically synthesized MCTP results in lung and/or liver lesions similar to those caused by MCT [15, 179, 180].

It is believed that pulmonary vascular endothelium is the early target of MCT injury probably due to circulatory proximity to the liver and because lungs are the major vascular bed after the liver [179, 181]. In fact, within 7-14 days it was verified increased DNA synthesis and hypertrophy of ECs that occurs after an initial induction of ECs apoptosis within 4 days pos-injection of MCT [181-184]. As early as 4h after injection platelet thrombi can be detected in small arteries, and 4 days pos-injection there is an augmentation in the number of swollen mitochondria as well as ROS generation, which might be endothelial-toxic [184-186]. At day 7, it has been described an early perivascular mononuclear inflammation in MCT-treated rats, mainly due to accumulation of macrophages in the adventitia [187, 188]. MCT treatment also causes the augmentation of extracellular space in the adventitia, probably due to accumulation of oedema fluid from altered microvascular EC permeability [178]. By 8 days after MCT injection there is extension of SMCs into peripheral normally non-muscular small PAs [189]. By 12 days it was described medial hypertrophy of small arteries with a concomitant increase in PAP and resistance [185, 190]. Only after 21 days of MCT injection RVH and dysfunction has been described [190, 191]. Unfortunately, there is evidence that MCT treatment causes myocarditis affecting both right and left ventricles, which will complicate the study of RVH/failure frequently associated with severe PH [192]. In addition, MCT treated rats do not developed plexiform lesions, a characteristic of later stages of human PH [193]. However, combined treatment of MCT with pneumonectomy revealed the development of plexiform lesions [194].

Finally, MCT is also able to induce multiple cellular and molecular alterations at all layers of pulmonary vessels. Lungs of rats exposed to MCT present increased expression of multiple pro-inflammatory cytokines [195-197]. In addition, after MCT treatment it was described an increase of medial SERT and survivin expression, whereas K_V channels expression, including K_V 1.5 and K_V 2.1, were diminished [37, 121, 198]. BMPRII expression along with its signalling pathway is reduced in MCT-

treated rats, however its restoration does not improve PH [199, 200]. At intima level, MCT cause an augmentation in endothelial expression of ET-1 with a downregulation of ET_B (endothelin receptor type B) and eNOS (endothelial nitric oxide synthase) expression [201, 202]. In the adventitial layer there is an enhancement of ECM glycoproteins production, such as elastin, fibronectin, collagen and tenascin-C [203, 204].

Taken together, MCT-induced PH differs in some aspects of human PAH, but mimics several of its features, such as hemodynamic repercussions, histological alterations and high mortality. In comparison with other models of PH, MCT model offers technical simplicity, reproducibility and low cost, reasons that justify its general use in the investigation of PH [205].

5. Treatment

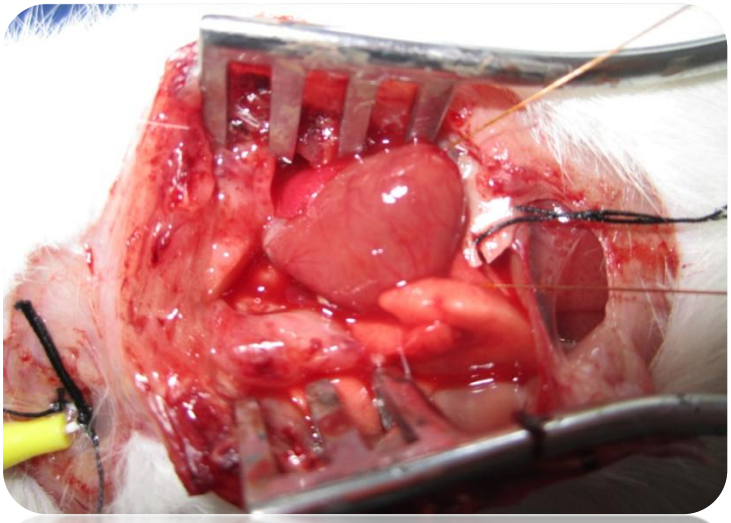
Until approximately 25 years ago, PAH was considered a fatal disease with poor survival, being its treatment only palliative [4]. The therapies were based on symptomatic relief and were adapted from other pulmonary and cardiac diseases and included oxygen supplementation, anticoagulants, diuretics, digoxin, inotropes and calcium-channel blockers (CCB) (Table 4) [206]. However, the intense research dedicated to the pathobiology of PAH has been helpful for the development of several disease-specific therapies. In fact, treatments targeting specific cellular and molecular pathways involved in the pathogenesis of PAH have being implemented and they allowed symptoms improvement and increased survival (Table 4) [207]. Despite slowing the progression of the disease, treatments do not lead to a cure but diminished the need of surgical procedures (e.g. heart/lung transplantation and atrial septostomy), performed in patients who fail to respond to the pharmacologic therapy [208-211]. As summarized in Table 4, current treatments include: i) supportive therapies, ii) calcium-channel blockers, iii) synthetic prostacyclin and prostacyclin analogues, iv) endothelin-1 receptor antagonists, v) type 5 phosphodiesterase inhibitors and vi) interventional procedures.

Table 4 | Current therapies for PAH

Drug	Route	Comments and recommendations	Adverse effects and contradictions	References
Supportive therapies				
Anticoagulants	O	Improved survival in small retrospective studies	Bleeding	[206, 207, 212]
Diuretics	O	Used to reduce fluid retention, indicated for RV volume overload	Hypotension	
Oxygen	In	Useful in patients with hypoxia		
Digoxin	O	Used for RV failure with benefit on RV function	Vomiting, arrhythmias	
Inotropes	IV	Mostly applied for end-stage RV failure	Tachycardia	
Calcium-channel blockers				
Nifedipine	O	Helpful as long term in patients with acute vasodilator responsiveness	Peripheral oedema, hypotension	[206, 213]
Diltiazem	O			
Amlodipine	O			
Synthetic prostacyclin and prostacyclin analogues				
Epoprostenol	cIV	Can have beneficial effects for years, used in combination therapy	Nausea, diarrhoea, headache, jaw pain, flushing, catheter-related sepsis/thrombosis	[207, 214-220]
Treprostinil	SC, O, IV, In	Used when oral therapy has failed; short-term benefits	Nausea, diarrhoea, rash, headache, infusion site pain	
Berapost	O	Short-term benefits; used in combination therapy	Flushing, headache	
Iloprost	In, IV	Short-term benefits; used in combination therapy	Headache, jaw pain, cough, flushing	
Endothelin-1 receptor antagonists				
Bosetan	O	Dual ET_A/ET_B antagonist, used as a fist-line drug; short-term benefits,; used in combination therapy	Headache, anaemia, oedema, hepatotoxicity	[207, 221, 222]
Sitaxsentan	O	Selective ET_A receptor antagonists	Flushing, hepatotoxicity, INR augmentation, warfarin interaction	
Ambrisentan	O		Flushing, hepatotoxicity	
Type 5 phosphodiesterase inhibitors				
Sildenafil	O	Used as an initial agent and in combination therapy	Headache, visual disturbances, nasal congestion	[207, 223, 224]
Tadafil	O		Nausea, headache, dyspnea	
Interventional procedures				
Atrial septostomy		Decompresses right heart, improves systemic output		[206, 207, 211]
Lung transplantation		Improves survival and quality of life		

Legend: cIV, continuous intravenous; In, inhaled; INR, international normalized ratio; IV, intravenous; O, oral; RV, right ventricle; SC, subcutaneous.

In addition to monotherapy, combination therapy has been an attractive option since it permits to target more than one pathophysiological mechanisms of PAH, allowing the improvement of clinical efficiency and minimizing side-effects [225, 226]. The main clinical combination therapy being considered includes: i) prostanoids and endothelin-1 receptor antagonist (e.g. bosentan + iloprost, bosentan + beraprost, bosentan + epoprostenol), ii) prostanoids and type 5 phosphodiesterase inhibitors (e.g. sildenafil + epoprostenol, sildenafil + iloprost, sildenafil + berapost, sildenafil + treprostinil) and iii) type 5 phosphodiesterase inhibitors and endothelin-1 receptors antagonists (e.g. bosentan + sildenafil, bosentan + tadafil) [227-233]. Finally, with increasing studies on the pathobiology of PAH multiple potential therapeutic targets have been suggested: i) Rho kinases inhibitors (fasudil), ii) tyrosine kinase inhibitors (imatinib), iii) stimulators and activators of soluble guanylate cyclase (riociguat), iv) VIP, v) ADM, vi) HMG-CoA reductase inhibitors (statins such as simvastatin) and vii) angiotensin II type 1 receptor antagonists (losartan)) [66, 234-243] . These potential therapeutic targets were or are being studied in patients with PAH and/or experimental models of the disease.



III – Aims

Vascular remodelling is an important event in the progression of PAH, characterized by an excessive cellular proliferation and a reduced apoptosis; however, little is known about the molecular events underlying the imbalance between cellular proliferation and apoptosis. So, the aim of the present work was to study the contribution of apoptosis to the pathogenesis of PAH, through the analysis of the apoptotic proteins survivin and Smac/DIABLO, in an animal model of MCT-induced PAH. To achieve this goal, morphometric analysis of heart and lungs of controls and animals with MCT-induced PAH was related with the pulmonary and cardiac expression of survivin and Smac/DIABLO, evaluated by immunohistochemistry and western blot.

IV – Methods and Materials

1. Experimental Design

In order to study the contribution of apoptosis to the pathogenesis of PAH, an experimental protocol was designed and is summarized in Figure 4.

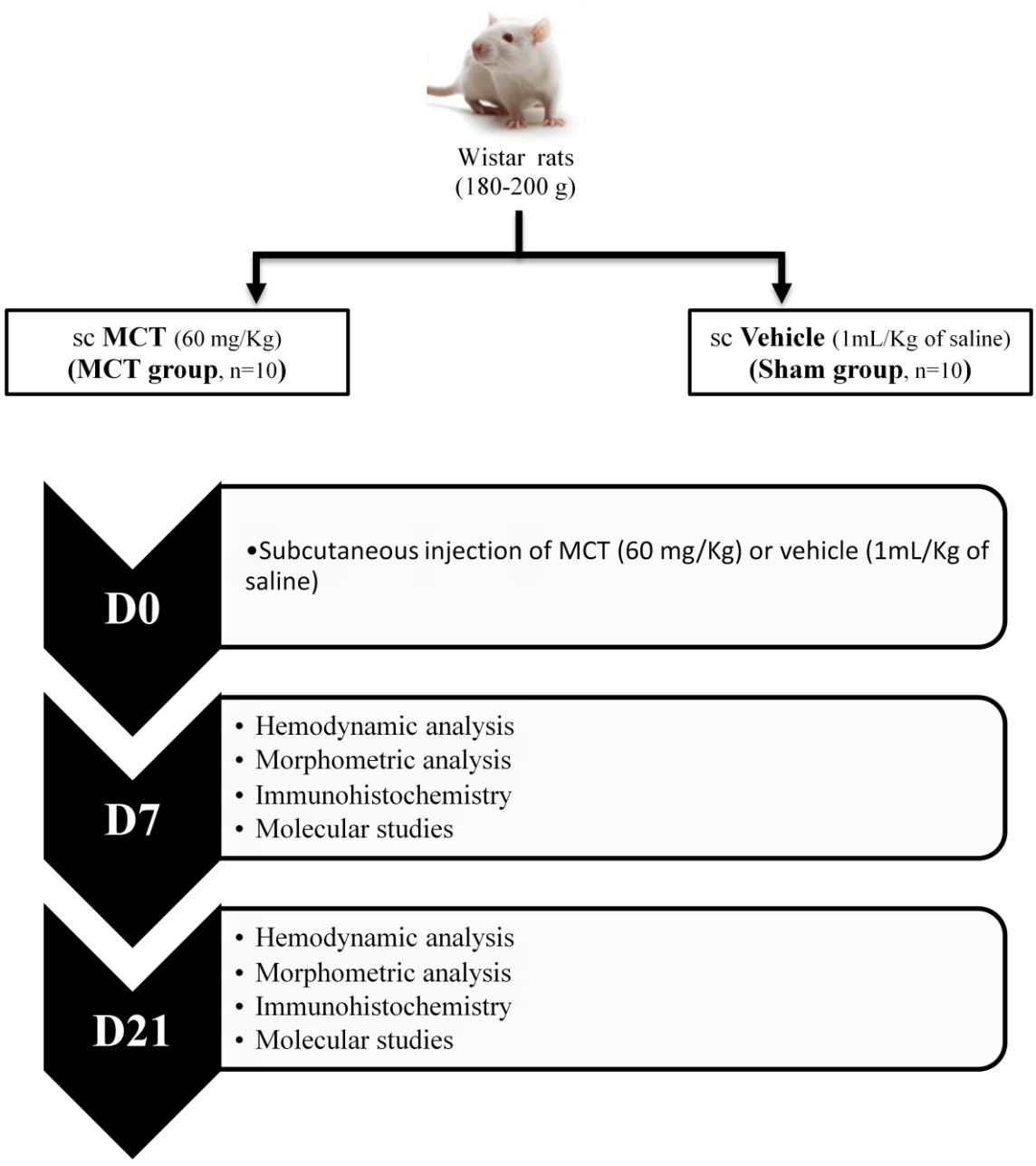


Figure 4 | Experimental protocol design (sc, subcutaneous injection)

2. Animal Protocol

Animal experiments were performed according to the Portuguese law for animal welfare (DL 129/92, DL 197/96; P 1131/97) and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Male Wistar rats (Charles River Laboratories, Barcelona, Spain) weighing 180-200g were housed in groups of 5 rats/cage, in a controlled environment under a 12:12-h light-dark cycle at room temperature of 22ºC, with free supply of food and water. Rats randomly received a subcutaneous injection of MCT (60 mg/kg, Sigma-Aldrich, Barcelona, Spain) (MCT group, n=10 per time point) or an equal volume of vehicle (1 mL/kg of saline) (Sham group, n=10 per time point).

3. Hemodynamic Analysis

In order to evaluate and confirm the presence and progression of the disease, hemodynamic assessment was performed 7 and 21 days after MCT/vehicle injection. The rats were anesthetized by inhalation of a mixture of sevoflurane (4%) and oxygen, intubated for mechanical ventilation (Dual Mode, Kent Scientific, Connecticut, USA) and placed over a heating pad (body temperature is maintained at 37°C). Under binocular surgical microscopy (Wild M651.MS-D, Leica; Herbrugg, Switzerlad), the right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution) to compensate for perioperative losses. The heart was exposed by a median sternotomy and the pericardium was widely opened. Right ventricular hemodynamic function was measured with pressure-volume (PV) catheter (PVR-1045, Millar instruments, Houston, USA). Data was continually acquired (MPVS 300, Millar Instruments, Houston, USA) and digitally recorded at 1000Hz (ML880 PowerLab 16/30, Millar TM instruments, Houston, USA). After complete instrumentation, the animal preparation was allowed to stabilize for 15 minutes. Hemodynamic recording were made under basal with respiration suspended at end-expiration. Heart rate (HR) and RV peak systolic pressure (P_{max}) were obtained and analysed using PVAN 3.5 (Millar Instruments, Houston, USA).

4. Tissue Preparation for Morphometric, Immunohistochemistry and Molecular Analysis

The heart, lungs and right *gastrocnemius* muscle were excised and weighted. The right tibia was also excised and its length was measured with a millimetric ruler. Under binocular magnification (×3.5), the RV free wall was dissected from the left ventricle (LV) + septum (S) and weighted separately. Heart, lungs, RV and LV + S weights were normalized to body weight (BW) and *gastrocnemius* weight was normalized to tibia length. Samples from RV, LV and lung were fixed and included in paraffin for light microscopy, or frozen with liquid nitrogen for molecular studies.

5. Morphometric Analysis

Samples of RV, LV (midway between the apex and base) and lung were fixed in 4% (v/v) buffered paraformaldehyde followed by dehydration with graded ethanol, diaphanization with xylene and included in paraffin blocks. Serial sections (4 µm of thickness) of paraffin blocks were cut by a microtome (RM2125RTS, Leica, Nussloch, Germany) and mounted on silane-coated slides. The slides were dewaxed in xylene and hydrated through graded alcohols. Sections were stained for haematoxylin-eosin by immersing slides in Mayer's haematoxylin solution for 5 minutes followed by immersion in aqueous eosin solution for 5 minutes. Slides were still submitted to graded alcohols and xylene and mounted with Entellan. Studied samples were observed at light microscopy (Dialux 20, Leitz, Wetzlar, Germany), photographed with a digital camera (XC30, Olympus, California, USA) and measured with a digital image analyzer (cell^B life science basic imaging software, Olympus, California, USA). Five images with random microscopic fields (magnification of ×400) were obtained from each section to compensate for variations within sections. Only round to ovoid muscle fibbers with a nuclear profile were counted to measure the cardiomyocytes surface area (CSA). Around 500 cardiomyocytes per group per time point were analyzed. On pulmonary specimens, external diameter and medial wall thickness in muscular arteries (20-25 arteries/lung) were analyzed. Orthogonal intercepts were used to generate eight random measurements of external diameter (distance between the external lamina) and sixteen random measurements of medial thickness (distance between the internal and external lamina). For each artery medial hypertrophy was expressed as follows: % wall thickness = [(medial thickness × 2) / (external diameter)] × 100.

6. Immunohistochemistry

Immunohistochemistry was performed to determine survivin and Smac/DIABLO expression in the RV. Sections (4 µm) were placed on SuperFrost®Plus slides (Thermo Scientific, Massachusetts, USA). After deparaffinization and rehydration, slides were subjected to heat induced antigen retrieval by immersion in 10 mM sodium citrate buffer (pH 6.0) in the microwave for 30 minutes. Peroxidase activity was blocked by 3% hydrogen peroxide for 10 minutes. Blockage of non-specific binding was performed with 5% normal goat serum (G9023-10mL, Sigma-Aldrich, Barcelona, Spain) in TBS-T (100 mM Tris, 1.5 mM NaCl, pH 8.0; 0.1% Tween-20) for 1 hour at room temperature flowed by 15 minutes of washes in TBS-T. Sections were encircled with a pap pen (Vector Laboratories, California, USA) to prevent splitting leakage of the flowing incubation solutions. Endogenous avidin-biotin expression was blocked using an endogenous avidin + biotin blocking system (ab3387, abcam, Cambridge, UK) according to manufacturer's instructions, being followed by incubation with the primary antibodies (1:500 dilution; rabbit anti-Survivin (ab469, abcam, Cambridge, UK) or dilution 1:250; rabbit anti-Smac/DIABLO (ab8115, abcam, Cambridge, UK)) overnight at 4°C. After incubation with primary antibodies, slides were washed 3 times, 5 minutes each with TBS-T and incubated with goat anti-rabbit IgG secondary antibody (1:250 dilution; ab6720, abcam, Cambridge, UK) for 2 hours at room temperature. Slides were submitted to another 3 washes in TBS-T prior being incubated with Streptavidin protein, HRP (1:1000 dilution; ab7403, abcam, Cambridge, UK). To visualize the peroxidase activities in sections 3,3-diaminobenzidine (DAB, ab94665, abcam, Cambridge, UK) was used. Finally, slides were counterstained with Mayer's haematoxylin, submitted to graded alcohols and xylene and mounted with Entellan. Negative control reactions included omission of the primary antibody. The slides were observed and photographed with a microscope (Dialux 20, Leitz, Wetzlar, Germany) under ×400 magnification. Survivin and Smac/DIABLO expression was qualitatively determined as positive (cytoplasmatic staining) or negative in around 500 cardiomyocyte per group per time point.

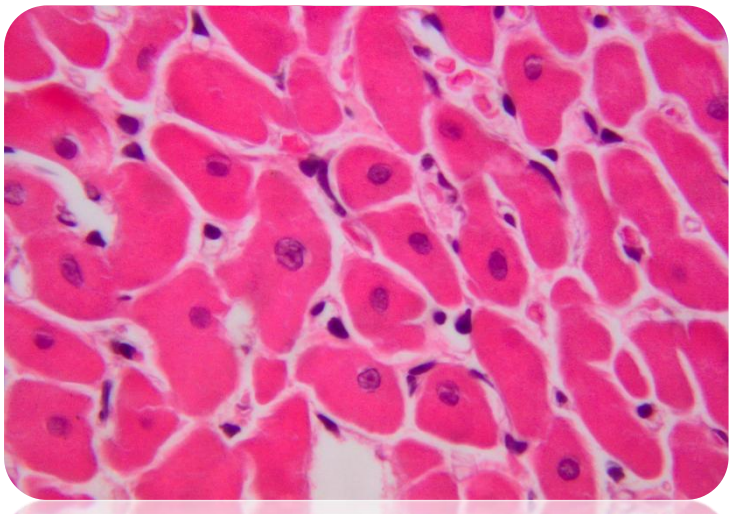
7. Western Blot

RV, LV and lung samples (n=6 per group per time point) previously frozen with liquid nitrogen were homogenised in phosphate buffer (13 mM KH_2PO_4, 54mM $NaHPO_4$, pH 7.4) (in the proportion of 1:20) with a Bio-Gen PRO200 homogeniser (Pro 200, Pro Scientific, Connecticut, USA). Total protein concentration was spectrophotometrically determined with the colorimetric method RC-DC protein assay (Bio-Rad, California, USA). The optic density was determined at 750nm in a microplate reader (UVM340, Asys, Cambridge, UK). Simultaneously, a calibration curve was performed using different concentrations of bovine serum albumin (BSA).

Equivalent amounts of total protein from the homogenised RV, LV and lung of each group were electrophoresed on a 12.5 % SDS-PAGE at 200 V at room temperature as described by Laemmli [244]. Proteins were electrotransfered to a nitrocellulose membrane (0,2 µm, Bio-Rad, California, USA) in 25 mM Tris, 192 mM and 20 % methanol at 200 mA. Successful transfer was confirmed by staining the membranes with Ponceau S. Nonspecific binding sites were blocked with 5 % (w/v) dry non-fat milk in TBS-T (100 mM Tris, 1.5 mM NaCl, pH 8.0 (TBS) and 0.5 % Tween 20). Membranes were incubated with primary antibody (rabbit anti-Survivin (ab469, abcam, Cambridge, UK), 1:1000 dilution or rabbit anti-Smac/DIABLO (ab8115, abcam, Cambridge, UK), 1:500 dilution) overnight at 4°C with agitation. Afterwards, blots were washed in TBS-T, incubated with a secondary antibody (LI-COR IRDye® 800CW, Nebraska, USA) and washed in TBS-T. Immunoreactive bands were observed under fluorescence using an Odyssey system (LI-COR Odyssey, Nebraska, USA) and the results were analysed with Quantity One software v. 4.6.3 (Bio-Rad, California, USA)

8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software v. 5.0 (GraphPad Software, California, USA). All data are presented as mean ± SEM and were compared using Two Way ANOVA. When treatments were significantly different, Students-Newman Keuls post-hoc test was selected to perform pairwise multiple comparisons. Results were considered significantly different when $p<0.05$.



V – Results

1. Hemodynamic Evaluation and Morphometric Analysis

In order to confirm and evaluate RV dysfunction and the development of PAH through MCT injection, hemodynamic evaluation and morphometric analysis were performed 7 and 21 days after MCT injection. As represented in Table 5, at D7 no significant hemodynamic alterations were noted. However, at D21, RV peak systolic pressure in the MCT treated group was significantly enhanced when compared with Sham group.

Table 5 | Hemodynamic evaluation of MCT-induced PAH.

	D7		D21	
	Sham	**MCT**	**Sham**	**MCT**
Heart rate (bpm)	349 ± 22	357 ± 10	340 ± 14	376 ± 23
RV P_{max} (mmHg)	27.3 ± 2.2	31.5 ± 1.5	26.3 ± 1.2	38.9 ± 2.7 *

Data are present as mean ± SEM. Sham, sham group; MCT, monocrotaline group; P_{max}, peak systolic pressure; RV, right ventricle. *$p < 0.05$ vs Sham of the same day

Table 6 | Morphometric alterations in MCT-induced PAH

	D7		D21	
	Sham	**MCT**	**Sham**	**MCT**
Body weight (g)	230.4 ± 8.2	214.6 ± 3.2	290.6 ± 4.5 [a]	260 ± 4.9 *, [a]
HW/BW (g/Kg)	3.031 ± 0.079	3.129 ± 0.061	2.794 ± 0.069	3.282 ± 0.113 *
RV/(LV+S) (g/g)	0.282 ± 0.017	0.332 ± 0.021	0.302 ± 0.012	0.467 ± 0.049 *, [a]
RV/BW (g/Kg)	0.583 ± 0.038	0.332 ± 0.021	0.584 ± 0.013	0.911 ± 0.095 *, [a]
(LV+S)/BW (g/Kg)	2.070 ± 0.058	2.011 ± 0.047	1.957 ± 0.060	1.956 ± 0.042
L/BW (g/Kg)	5.436 ± 0.307	5.896 ± 0.400	4.782 ± 0.323	7.241 ± 0.464 *, [a]
G/tib (g/cm)	0.382 ± 0.012	0.374 ± 0.004	0.440 ± 0.016	0.430 ± 0.008

Data are present as mean ± SEM. Sham, Sham group; MCT, monocrotaline group; HW, heart weight; BW, body weight; RV, right ventricle; LV+S, left ventricle plus septum; L, lungs; G, gastrocnemius; tib, tibia. *$p < 0.05$ vs Sham of the same day; [a] $p < 0.05$ vs D7 of the same treatment group.

The morphometric alterations registered in MCT-induced PAH are summarized in Table 6. At day 21, animals from the MCT group evidenced a significant body weight loss in comparison to the Sham group. There was a significant augmentation of body weight in both groups between days 7 and 21. In addition, there were no significant alterations in the ratio G/tib.

In MCT treated animals, lung weight increased between time-points, and was significantly higher in MCT group than in Sham group. As expressed in Figure 5, at D21 media of pulmonary artery is significantly hypertrophied in MCT-treated animals comparing with Sham group.

The ratio HW/BW was significantly higher in MCT-treated animals compared to Sham on day 21 after injection. Moreover, RV/(LV+S) and RV/BW indexes were both significantly increased on day 21 in MCT-treated animals, indicating that these ones developed RV hypertrophy. Also, as expressed in Figure 5, MCT-treated animals developed cardiomyocytes hypertrophy since D7. The RV hypertrophy increased with the progression of the disease since RV cardiomyocytes cross sectional area was significantly higher on day 21 than on day 7. There were no differences in the (LV+S)/BW parameter in both MCT and Sham groups.

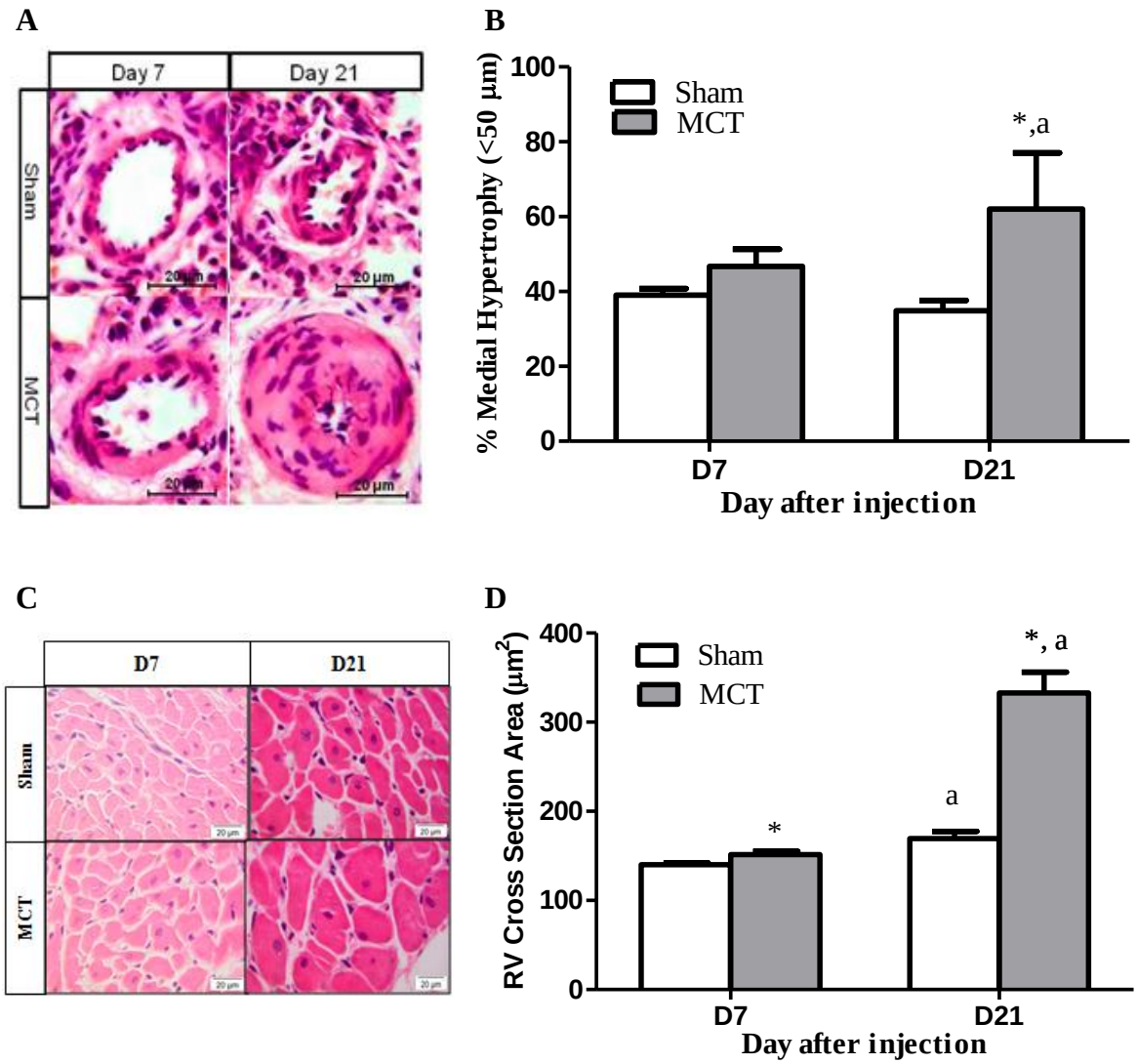


Figure 5 | Pulmonary arterial and right ventricular cardiomyocyte hypertrophy. A and **C**: Histological appearance of small pulmonary arteries and right ventricular cardiomyocytes, respectively, stained with hematoxylin and eosin of Sham and MCT groups 7 and 21 days after injection. **B:** Medial layer thickness expressed as percentage of all thickness. **D:** Right ventricular cardiomyocytes hypertrophy expressed as cardiomyocytes cross-sectional area (μm^2). Data are present as mean ± SEM. Sham, Sham group; MCT, monocrotaline group. $^{*}p < 0.05$ vs Sham of the same day; $^{a}p < 0.05$ vs D7 of the same treatment group.

2. Survivin and Smac/DIABLO Expression

With the aim to study the role of apoptosis in PAH, the expression of survivin and Smac/DIABLO were analysed in RV, LV and lungs.

Survivin and Smac/DIABLO expression in RV were analysed through immunohistochemistry and western blot, and the results are shown in Figure 6 and Figure 7, respectively. MCT treatment significantly raised survivin expression in both day 7 and 21 after injection as compared with Sham group. Sham groups did not present

any alterations in survivin expression. Contrarily, MCT treatment significantly decreased Smac/DIABLO expression in comparison with Sham groups. As present in Figure 8, MCT treatment has induced an increase in survivin levels ans a decresed in Smac/DIABLO levels in LV, in a similar way of those noted in RV.

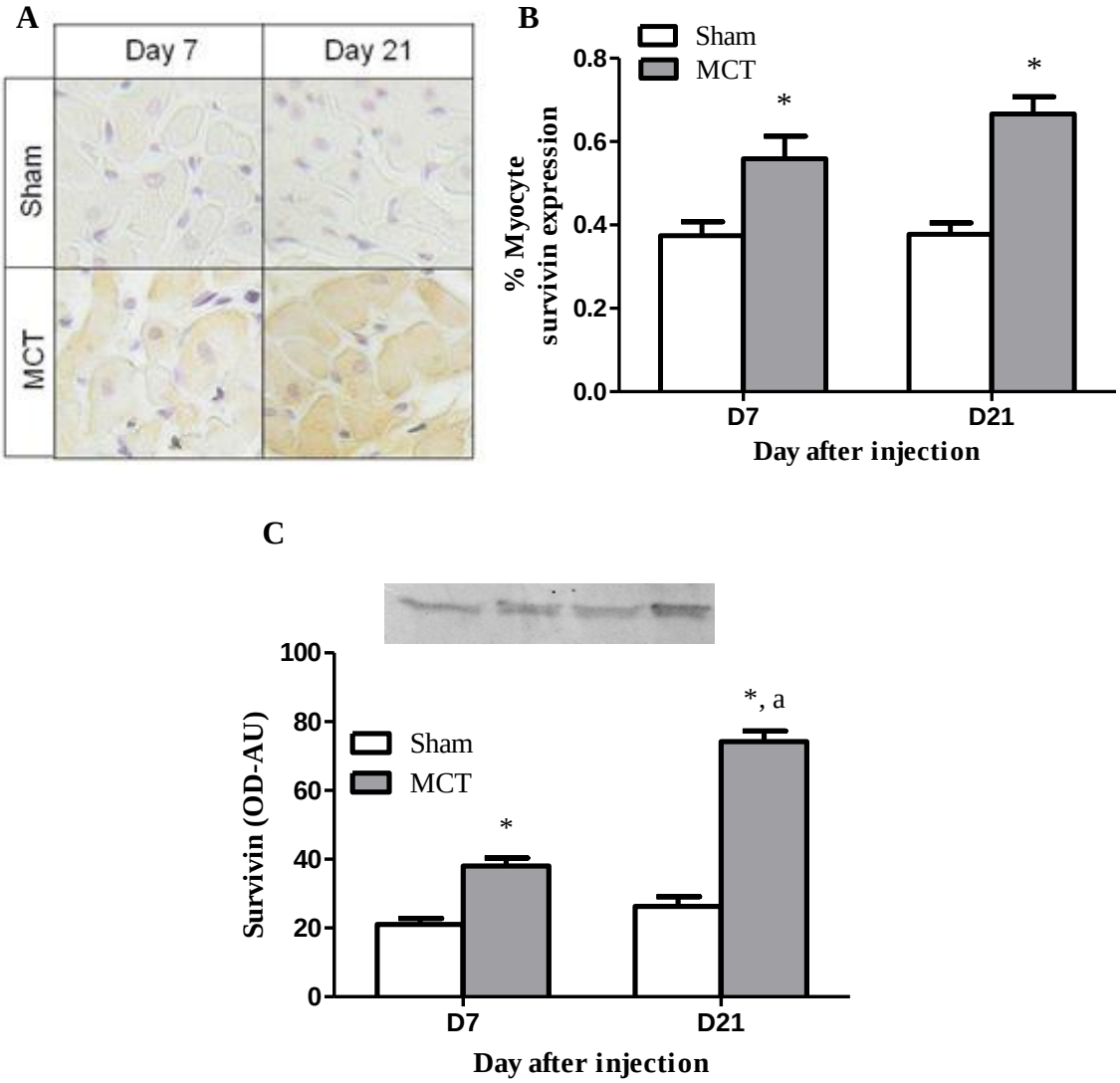


Figure 6 | Right Ventricle survivin expression, evaluated through immunohistochemistry and western blot. **A:** Representative image of immunohistochemistry for survivin expression in the right ventricle of Sham and MCT groups 7 and 21 days after injection. **B:** Survivin expression in right ventricle expressed as percentage of stained cardiomyocytes in immunohistochemistry. **C:** Right ventricle survivin expression evaluated through western blot with a representative image of the results. Data are present as mean ± SEM. Sham, Sham group; MCT, monocrotaline group; RV, right ventricle. $^{*}p < 0.05$ vs Sham of the same day; $^{a}p < 0.05$ vs D7 of the same treatment group.

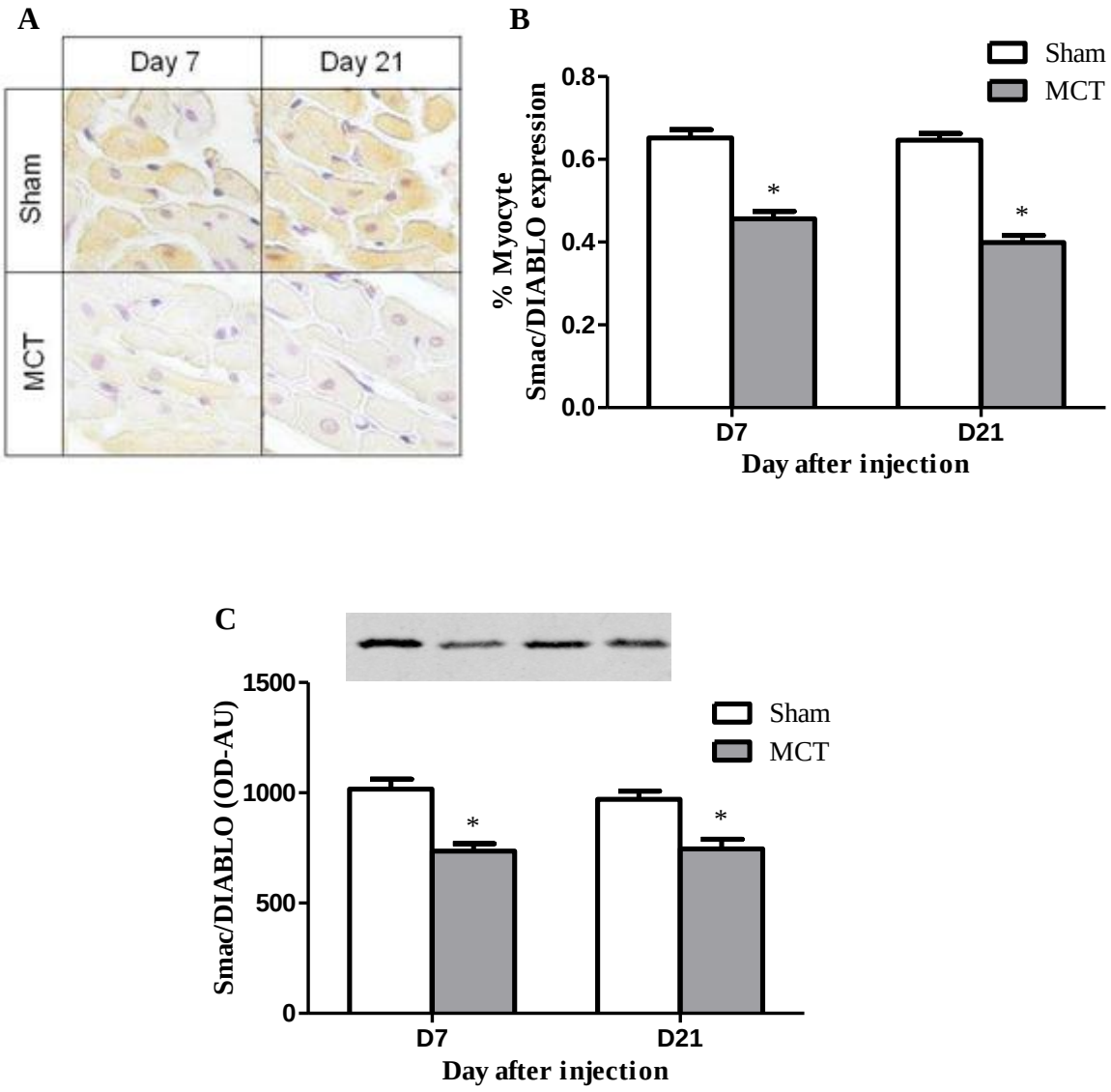


Figure 7 | Smac/DIABLO expression in right ventricle, evaluated through immunohistochemistry and western blot. **A:** Representative image of immunohistochemistry for Smac/DIABLO expression in the right ventricle of Sham and MCT groups 7 and 21 days after injection. **B:** Smac/DIABLO expression in right ventricle expressed as percentage of stained cardiomyocytes in immunohistochemistry. **C:** Right ventricle Smac/DIABLO expression evaluated through western blot with a representative image of the results. Data are present as mean ± SEM. Sham, Sham group; MCT, monocrotaline group; RV, right ventricule. $^{*}p < 0.05$ vs Sham of the same day; $^{a}p < 0.05$ vs D7 of the same treatment group.

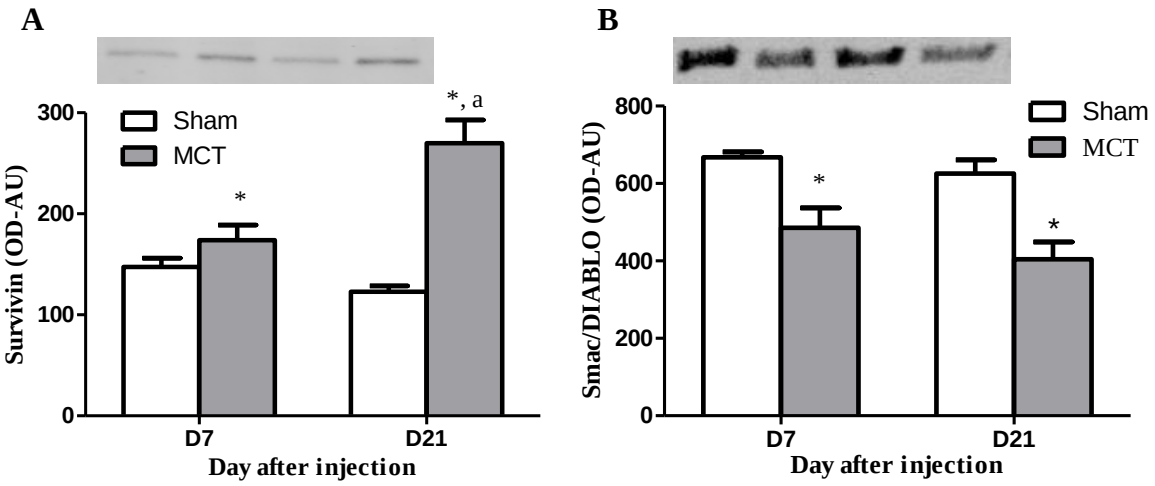


Figure 8 | Survivin (A) and Smac/DIABLO (B) expression in left ventricle, evaluated through western blot of Sham and MCT groups 7 and 21 days after injection, with respective representative images. Data are present as mean ± SEM. Sham, Sham group; MCT, monocrotaline group. $^{*}p < 0.05$ vs Sham of the same day; $^{a}p < 0.05$ vs D7 of the same treatment group.

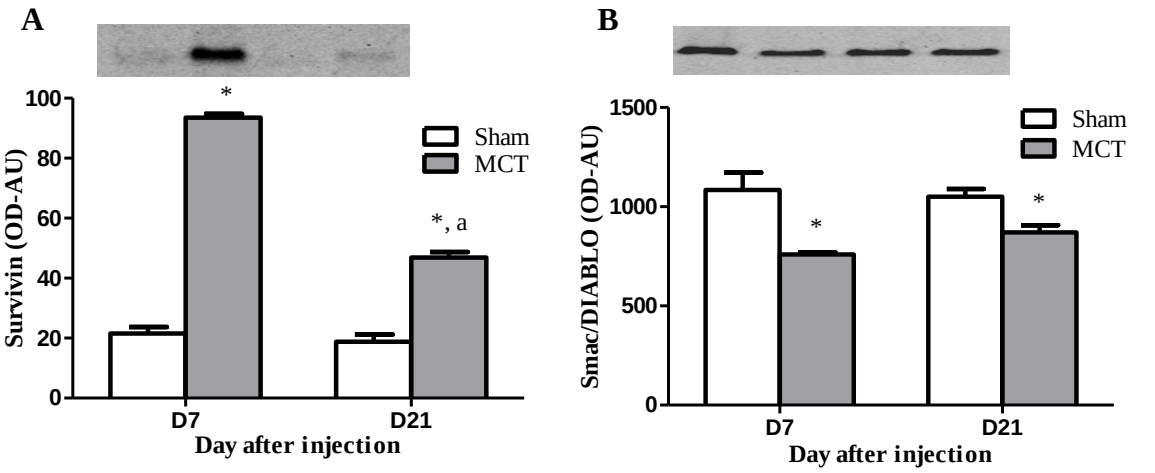
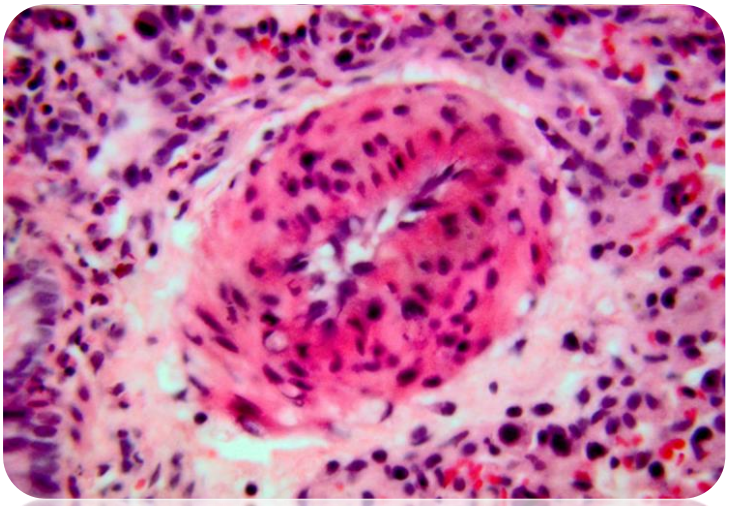


Figure 9 | Lung survivin (A) and Smac/DIABLO (B) expression, evaluated through western blot of Sham and MCT groups, 7 and 21 days after injection, with respective representative images. Data are present as mean ± SEM. Sham, Sham group; MCT, monocrotaline group. $^{*}p < 0.05$ vs Sham of the same day; $^{a}p < 0.05$ vs D7 of the same treatment group.

In respect to lung (Figure 9), survivin expression was significantly augmented in MCT groups. However, in MCT-treated animals, between day 7 and 21 after injection, there was a significant reduction of survivin expression, while no differences were noted between Sham groups. In contrast, pulmonary Smac/DIABLO expression significantly lowered with MCT treatment in both time-points in comparison with Sham groups.



VI – Discussion

PAH is a multifactorial, progressive disease with substantial mortality and morbidity. Over the years, cellular and molecular mechanisms underlying PAH have been studied in order to better understand PAH pathogenesis as well as to develop therapies that might lead to a better prognosis. Thus, studies focusing the development, progression and treatment of PAH are still essential. Most of the research performed with animal models of this disease has been focused on advanced stages of the disease (25-35 days after MCT injection), being scarce the ones conducted in the early phases, probably reflecting the fact that most patients with PAH are usually diagnosed at advance stages. We believe that the study of PAH in early stages will allow a better understanding of pathophysiologic pathways that might be therapeutically modulated aiming to cure PAH. For that reason, the present study analysed the role of apoptosis in two time-points of disease progression in a well characterized animal model of MCT-induced PAH.

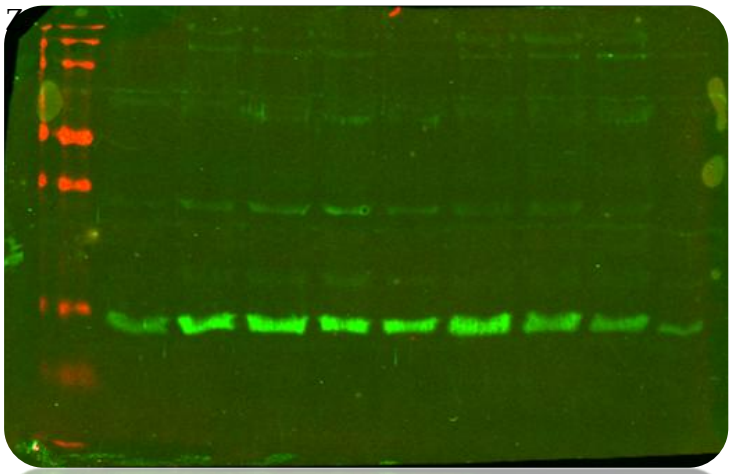
MCT administration affects mainly hepatic and cardiopulmonary systems, promoting vasoconstriction, ECs hypertrophy, SMCs hypertrophy and hyperplasia and inflammation at lung level and also RV hypertrophy [15, 182, 193]. Several studies reported that, in rats, the severity of these lesions depends on MCT dose, administration route and the animal age at the time of treatment [180, 245]. Although, not all the typical findings of human PAH are mimicked by this animal model (such as plexiform lesions present in human PAH and absent in the experimental model), it has identical hemodynamic and some morphological features, like pulmonary artery medial layer remodelling and RV hypertrophy, which are the focus of our study [15, 193]. Our results, showed that MCT treatment, after 21 days, induced an augmentation of RV peak systolic pressure (Table 5) in association with increased RV mass (Table 6) and cardiomyocyte hypertrophy (Figure 5), confirming the previous studies. Also, as described before, our results demonstrated that MCT injection promoted pulmonary arterial wall thickness (Figure 5), which is the primary cause of pressure overload, leading to the increase of PVR and PAP and consequently RV hypertrophy. Therefore, RV hypertrophy seems to reflect a compensatory response in which RV adapts to the sustained afterload elevation [21, 246]. Interestingly, although there were no hemodynamic neither morphological alterations (Table 5 and Table 6, respectively) at RV, our results demonstrated for the first time that cardiomyocytes were hypertrophied at day 7 after MCT injection (Figure 5). These results suggest that besides heart

overwork due to pulmonary hypertrophy and PVR, other mechanisms, such as neurohumoral ones, might lead to RV hypertrophy.

In the last years, angiogenesis, dysfunctional K_V channels, loss expression of prostacyclin synthase gene (anti-proliferative), deregulation of growth signalling pathways and augmentation of survivin expression (anti-apoptotic) have been observed in the development and/or progression of PAH [167-170]. In order to evaluate the contribution of survivin pathway to PAH pathogenesis, we evaluated the cardiac and pulmonary expression of this anti-apoptotic protein as well as of its antagonist, Smac/DIABLO. In 2008, a study carried out by Levkau and colleagues [159] revealed several key features of survivin in the heart. They demonstrated that in the neonatal period, mice deficient for cardiac survivin presented a decrease number of total cardiomyocytes and a marked cardiomyocytes polyploidy due to multiple rounds of DNA replication without cytokinesis. This altered phenotype led to progressive heart failure and ultimately to death. In the same study survivin overexpression was associated with the induction of cell division and the protection of cardiomyocytes from doxorubicin-induced apoptosis. Moreover, they found only residual levels of survivin in normal human heart, but these were extraordinarily increased in the heart of patients with end-stage of heart failure. Interestingly, hemodynamic support with LVAD (left ventricular assist device) resulted in a marked reduction of expression of cardiomyocyte survivin, raising the hypothesis that cardiac survivin expression is load-dependent [159]. Wohlschlaeger and colleagues [247] also described that survivin was overexpressed in congestive heart failure but decreased after unloading. They also demonstrated a positive correlation between cardiomyocytes survivin expression and cardiac hypertrophy and DNA content. In the present study, survivin was significantly enhanced at day 7 after MCT injection and progressively increased throughout the development of MCT-induced PAH (Figure 6). Moreover, cardiomyocyte hypertrophy (Figure 5) was accompanied by increased expression of survivin (Figure 6) both of which progressively increased until day 21. LV expression of survivin had a similar pattern to RV (Figure 8). These results suggest that survivin might be involved in the cardiac remodelling process underlying PAH. Previously, McMurtry and colleagues [121] also demonstrated that lung survivin overexpression preceded hemodynamic alterations verified in the disease. Our results (Table 5 and Figure 9) corroborate McMurtry findings. Therefore, taking into consideration: i) the overexpression of RV,

LV and lung survivin at day 7 after MCT injection, ii) the similar survivin expression profile between LV and RV, and iii) the occurrence of survivin overexpression before hemodynamic alterations, makes us believe that besides pulmonary arterial hypertrophy other mechanisms, perhaps neurohumoral ones, are involved in RV hypertrophy. In particularly, we hypothesised that pulmonary vascular remodelling phenomena may be signalized to RV, possibly through neurohumoral mediators, such as ET-1, ATII, PDGF and catecholamines, inducing survivin overexpression and cardiomyocytes hypertrophy, a favourable cardiac remodelling response to the disease, as previously suggested [21, 248].

The expression of Smac/DIABLO was the reverse of survivin expression with decreasing levels noticed from day 7 to day 21 after MCT administration, in both RV and LV as well as in lung (Figure 6, Figure 8 and Figure 9, respectively). Data suggest that expression of Smac/DIABLO decreases with the progression of PAH. Smac/DIABLO is known for its pro-apoptotic properties through its interaction with IAP proteins, including survivin, eliminating their inhibitory effect on caspases [119, 151, 249]. On the other hand, survivin expression is also able to delay the release of smac/DIABLO from the mitochondria, preventing its pro-apoptotic function [152]. Several studies also revealed a reciprocal expression of survivin and Smac/DIABLO in different types of cancers [250-253]. So, we believe that deregulation in the balance between survivin and Smac/DIABLO might be related with pulmonary vascular remodelling and cardiomyocytes hypertrophy in response to apoptotic stimuli.



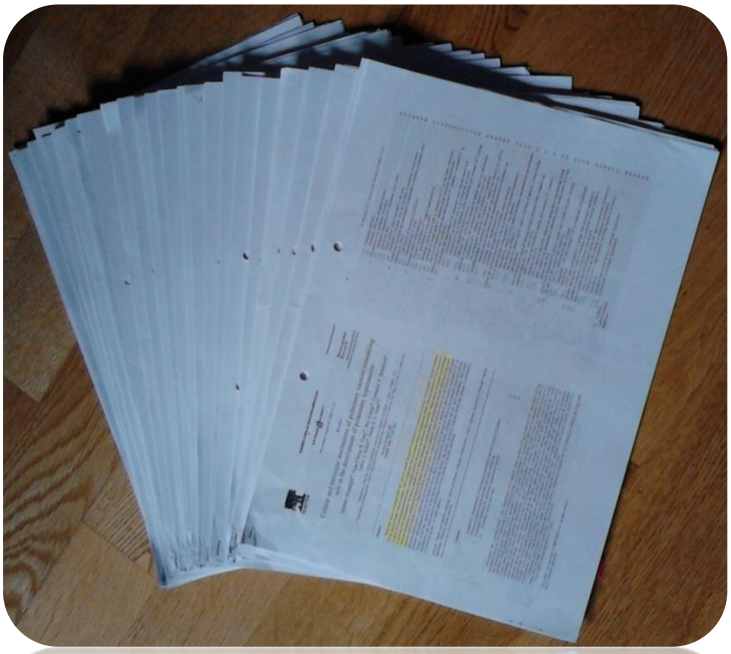
VII – Conclusions

Aiming to evaluate the contribution of apoptosis to the pathogenesis of PAH, MCT animal model was used to analyse pulmonary and cardiac expression of survivin and Smac/DIABLO. Data allowed us to conclude that:

i) cardiomyocytes hypertrophy was present 7 days after MCT injection, proceeding hemodynamic alterations;
ii) RV and LV survivin overexpression was demonstrated 7 days after MCT injection and progressively increased throughout the development of MCT-induced PAH;
iii)although increased 7 days after MCT injection, lung survivin expression progressively decreased between time-points;
iv)RV, LV and lung Smac/DIABLO expression was upregulated 7 days after MCT injection but progressively decreased throughout the development of MCT-induced PAH.

Taken together, data suggest that a deregulation in the balance between survivin and Smac/DIABLO might be related with pulmonary vascular remodelling and cardiomyocytes hypertrophy in response to apoptotic stimuli. Therefore, we consider that a therapy targeting survivin and/or Smac/DIABLO might be a future option for the treatment of PAH.

In the future, we intend to study survivin expression in cellular fractions (nuclear, cytoplasmic and mitochondrial) as well as the existence of possible posttranslational modifications that regulate cellular location of this protein and, consequently, its activity. Data obtained in the present study also leads us to therapeutically target the balance between survivin and Smac/DIABLO using terameprocol which is a drug widely used in cancers therapeutics.



VIII – Bibliography

1. Kovacs, G., Berghold, A., Scheidl, S., and Olschewski, H.; *Pulmonary arterial pressure during rest and exercise in healthy subjects: a systematic review*. European Respiratory Journal. 2009. **34**(4): p. 888-894.
2. McLaughlin, V.V., Archer, S.L., Badesch, D.B., Barst, R.J., Farber, H.W., Lindner, J.R., Mathier, M.A., McGoon, M.D., Park, M.H., Rosenson, R.S., Rubin, L.J., Tapson, V.F., and Varga, J.; *ACCF/AHA 2009 expert consensus document on pulmonary hypertension a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society, Inc.; and the Pulmonary Hypertension Association*. Journal of the American College of Cardiology. 2009. **53**(17): p. 1573-1619.
3. D'Alonzo, G.E., Barst, R.J., Ayres, S.M., Bergofsky, E.H., Brundage, B.H., Detre, K.M., Fishman, A.P., Goldring, R.M., Groves, B.M., Kernis, J.T., Levy, P.S., Pietra, G.G., Reid, L.M., Reeves, J.T., Rich, S., Vreim, C.E., Williams, G.W., and Wu, M.; *Survival in patients with primary pulmonary hypertension. Results from a national prospective registry*. Annals of Internal Medicine. 1991. **115**(5): p. 343-349.
4. Rich, S., Dantzker, D.R., Ayres, S.M., Bergofsky, E.H., Brundage, B.H., Detre, K.M., Fishman, A.P., Goldring, R.M., Groves, B.M., Koerner, S.K., Levy, P.C., Reid, L.M., Vreim, C.E., and Williams, G.W.; *Primary pulmonary hypertension. A national prospective study*. Annals of Internal Medicine. 1987. **107**(2): p. 216-223.
5. Fishman, A.P.; *Primary pulmonary arterial hypertension: a look back*. Journal of the American College of Cardiology. 2004. **43**(12 Suppl S): p. 2S-4S.
6. McLaughlin, V.V., Davis, M., and Cornwell, W.; *Pulmonary arterial hypertension*. Current Problems in Cardiology. 2011. **36**(12): p. 461-517.
7. Simonneau, G., Galie, N., Rubin, L.J., Langleben, D., Seeger, W., Domenighetti, G., Gibbs, S., Lebrec, D., Speich, R., Beghetti, M., Rich, S., and Fishman, A.; *Clinical classification of pulmonary hypertension*. Journal of the American College of Cardiology. 2004. **43**(12 Suppl S): p. 5S-12S.
8. Simonneau, G., Robbins, I.M., Beghetti, M., Channick, R.N., Delcroix, M., Denton, C.P., Elliott, C.G., Gaine, S.P., Gladwin, M.T., Jing, Z.C., Krowka, M.J., Langleben, D., Nakanishi, N., and Souza, R.; *Updated clinical classification of pulmonary hypertension*. Journal of the American College of Cardiology. 2009. **54**(1 Suppl): p. S43-54.
9. Ito, T., Ozawa, K., and Shimada, K.; *Current drug targets and future therapy of pulmonary arterial hypertension*. Current Medicinal Chemistry. 2007. **14**(6): p. 719-733.
10. Barst, R.J., McGoon, M., Torbicki, A., Sitbon, O., Krowka, M.J., Olschewski, H., and Gaine, S.; *Diagnosis and differential assessment of pulmonary arterial hypertension*. Journal of the American College of Cardiology. 2004. **43**(12 Suppl S): p. 40S-47S.
11. Galie, N., Hoeper, M.M., Humbert, M., Torbicki, A., Vachiery, J.L., Barbera, J.A., Beghetti, M., Corris, P., Gaine, S., Gibbs, J.S., Gomez-Sanchez, M.A., Jondeau, G., Klepetko, W., Opitz, C., Peacock, A., Rubin, L., Zellweger, M., and Simonneau, G.; *Guidelines for the diagnosis and treatment of pulmonary hypertension: the Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT)*. European Heart Journal. 2009. **30**(20): p. 2493-2537.
12. Rosenkranz, S.; *Pulmonary hypertension: current diagnosis and treatment*. Clin Res Cardiol. 2007. **96**(8): p. 527-541.
13. Jeffery, T.K., and Morrell, N.W.; *Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension*. Progress in Cardiovascular Diseases. 2002. **45**(3): p. 173-202.
14. Seeley, R.R., Stephens, T.D., and Tate, P.; *Anatomia&Fisiologia*. 6th ed. 2003: McGraw (Lusociência - Edições Técnicas e Científicas, Lda.).
15. Schultze, A.E., and Roth, R.A.; *Chronic pulmonary hypertension--the monocrotaline model and involvement of the hemostatic system*. Journal of Toxicology and Environmental Health Part B, Critical Reviews. 1998. **1**(4): p. 271-346.
16. Meyrick, B., and Reid, L.; *Hypoxia-Induced Structural-Changes in the Media and Adventitia of the Rat Hilar Pulmonary-Artery and Their Regression*. American Journal of Pathology. 1980. **100**(1): p. 151-178.
17. Yi, E.S., Kim, H., Ahn, H., Strother, J., Morris, T., Masliah, E., Hansen, L.A., and Park, K.; *Distribution of obstructive intimal lesions and their cellular phenotypes in chronic pulmonary hypertension - A morphometric and immunohistochemical study*. American Journal of Respiratory and Critical Care Medicine. 2000. **162**(4): p. 1577-1586.
18. Tuder, R.M., Groves, B., Badesch, D.B., and Voelkel, N.F.; *Exuberant Endothelial-Cell Growth and Elements of Inflammation Are Present in Plexiform Lesions of Pulmonary-Hypertension*. American Journal of Pathology. 1994. **144**(2): p. 275-285.

19. Tuder, R.M., Chacon, M., Alger, L., Wang, J., Taraseviciene-Stewart, L., Kasahara, Y., Cool, C.D., Bishop, A.E., Geraci, M., Semenza, G.L., Yacoub, M., Polak, J.M., and Voelkel, N.F.; *Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis*. Journal of Pathology. 2001. **195**(3): p. 367-374.
20. Hirose, S., Hosoda, Y., Furuya, S., Otsuki, T., and Ikeda, E.; *Expression of vascular endothelial growth factor and its receptors correlates closely with formation of the plexiform lesion in human pulmonary hypertension*. Pathology International. 2000. **50**(6): p. 472-479.
21. Bogaard, H.J., Abe, K., Vonk Noordegraaf, A., and Voelkel, N.F.; *The right ventricle under pressure: cellular and molecular mechanisms of right-heart failure in pulmonary hypertension*. Chest. 2009. **135**(3): p. 794-804.
22. Haworth, S.G.; *The cell and molecular biology of right ventricular dysfunction in pulmonary hypertension*. European Heart Journal Supplements. 2007. **9**(H): p. H10-H16.
23. Humbert, M., Morrell, N.W., Archer, S.L., Stenmark, K.R., MacLean, M.R., Lang, I.M., Christman, B.W., Weir, E.K., Eickelberg, O., Voelkel, N.F., and Rabinovitch, M.; *Cellular and molecular pathobiology of pulmonary arterial hypertension*. Journal of the American College of Cardiology. 2004. **43**(12 Suppl S): p. 13S-24S.
24. Miyazono, K., Maeda, S., and Imamura, T.; *BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk*. Cytokine and Growth Factor Reviews. 2005. **16**(3): p. 251-263.
25. Nohe, A., Keating, E., Knaus, P., and Petersen, N.O.; *Signal transduction of bone morphogenetic protein receptors*. Cellular Signalling. 2004. **16**(3): p. 291-299.
26. Massague, J., and Chen, Y.G.; *Controlling TGF-beta signaling*. Genes and Development. 2000. **14**(6): p. 627-644.
27. Austin, E.D., and Loyd, J.E.; *Genetics and mediators in pulmonary arterial hypertension*. Clinics in Chest Medicine. 2007. **28**(1): p. 43-57, vii-viii.
28. Rudarakanchana, N., Flanagan, J.A., Chen, H., Upton, P.D., Machado, R., Patel, D., Trembath, R.C., and Morrell, N.W.; *Functional analysis of bone morphogenetic protein type II receptor mutations underlying primary pulmonary hypertension*. Human Molecular Genetics. 2002. **11**(13): p. 1517-1525.
29. Machado, R.D., Aldred, M.A., James, V., Harrison, R.E., Patel, B., Schwalbe, E.C., Gruenig, E., Janssen, B., Koehler, R., Seeger, W., Eickelberg, O., Olschewski, H., Elliott, C.G., Glissmeyer, E., Carlquist, J., Kim, M., Torbicki, A., Fijalkowska, A., Szewczyk, G., Parma, J., Abramowicz, M.J., Galie, N., Morisaki, H., Kyotani, S., Nakanishi, N., Morisaki, T., Humbert, M., Simonneau, G., Sitbon, O., Soubrier, F., Coulet, F., Morrell, N.W., and Trembath, R.C.; *Mutations of the TGF-beta type II receptor BMPR2 in pulmonary arterial hypertension*. Human Mutation. 2006. **27**(2): p. 121-132.
30. Yang, X., Long, L., Southwood, M., Rudarakanchana, N., Upton, P.D., Jeffery, T.K., Atkinson, C., Chen, H., Trembath, R.C., and Morrell, N.W.; *Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension*. Circulation Research. 2005. **96**(10): p. 1053-1063.
31. Nohe, A., Hassel, S., Ehrlich, M., Neubauer, F., Sebald, W., Henis, Y.I., and Knaus, P.; *The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways*. Journal of Biological Chemistry. 2002. **277**(7): p. 5330-5338.
32. Morrell, N.W.; *Pulmonary hypertension due to BMPR2 mutation: a new paradigm for tissue remodeling?* Proc Am Thorac Soc. 2006. **3**(8): p. 680-686.
33. West, J., Fagan, K., Steudel, W., Fouty, B., Lane, K., Harral, J., Hoedt-Miller, M., Tada, Y., Ozimek, J., Tuder, R., and Rodman, D.M.; *Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle*. Circulation Research. 2004. **94**(8): p. 1109-1114.
34. Hong, K.H., Lee, Y.J., Lee, E., Park, S.O., Han, C., Beppu, H., Li, E., Raizada, M.K., Bloch, K.D., and Oh, S.P.; *Genetic ablation of the BMPR2 gene in pulmonary endothelium is sufficient to predispose to pulmonary arterial hypertension*. Circulation. 2008. **118**(7): p. 722-730.
35. Eddahibi, S., Humbert, M., Fadel, E., Raffestin, B., Darmon, M., Capron, F., Simonneau, G., Dartevelle, P., Hamon, M., and Adnot, S.; *Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension*. Journal of Clinical Investigation. 2001. **108**(8): p. 1141-1150.
36. Eddahibi, S., Hanoun, N., Lanfumey, L., Lesch, K.P., Raffestin, B., Hamon, M., and Adnot, S.; *Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene*. Journal of Clinical Investigation. 2000. **105**(11): p. 1555-1562.
37. Guignabert, C., Raffestin, B., Benferhat, R., Raoul, W., Zadigue, P., Rideau, D., Hamon, M., Adnot, S., and Eddahibi, S.; *Serotonin transporter inhibition prevents and reverses monocrotaline-induced pulmonary hypertension in rats*. Circulation. 2005. **111**(21): p. 2812-2819.

38. Marcos, E., Adnot, S., Pham, M.H., Nosjean, A., Raffestin, B., Hamon, M., and Eddahibi, S.; *Serotonin transporter inhibitors protect against hypoxic pulmonary hypertension*. American Journal of Respiratory and Critical Care Medicine. 2003. **168**(4): p. 487-493.
39. MacLean, M.R., Deuchar, G.A., Hicks, M.N., Morecroft, I., Shen, S., Sheward, J., Colston, J., Loughlin, L., Nilsen, M., Dempsie, Y., and Harmar, A.; *Overexpression of the 5-hydroxytryptamine transporter gene: effect on pulmonary hemodynamics and hypoxia-induced pulmonary hypertension*. Circulation. 2004. **109**(17): p. 2150-2155.
40. Guignabert, C., Izikki, M., Tu, L.I., Li, Z., Zadigue, P., Barlier-Mur, A.M., Hanoun, N., Rodman, D., Hamon, M., Adnot, S., and Eddahibi, S.; *Transgenic mice overexpressing the 5-hydroxytryptamine transporter gene in smooth muscle develop pulmonary hypertension*. Circulation Research. 2006. **98**(10): p. 1323-1330.
41. Eddahibi, S., Chaouat, A., Morrell, N., Fadel, E., Fuhrman, C., Bugnet, A.S., Dartevelle, P., Housset, B., Hamon, M., Weitzenblum, E., and Adnot, S.; *Polymorphism of the serotonin transporter gene and pulmonary hypertension in chronic obstructive pulmonary disease*. Circulation. 2003. **108**(15): p. 1839-1844.
42. Fernandez, R.A., Sundivakkam, P., Smith, K.A., Zeifman, A.S., Drennan, A.R., and Yuan, J.X.; *Pathogenic role of store-operated and receptor-operated ca(2+) channels in pulmonary arterial hypertension*. J Signal Transduct. 2012. **2012**: p. 951497.
43. Yu, Y., Fantozzi, I., Remillard, C.V., Landsberg, J.W., Kunichika, N., Platoshyn, O., Tigno, D.D., Thistlethwaite, P.A., Rubin, L.J., and Yuan, J.X.; *Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension*. Proceedings of the National Academy of Sciences of the United States of America. 2004. **101**(38): p. 13861-13866.
44. Firth, A.L., Remillard, C.V., and Yuan, J.X.; *TRP channels in hypertension*. Biochimica et Biophysica Acta. 2007. **1772**(8): p. 895-906.
45. Yu, Y., Keller, S.H., Remillard, C.V., Safrina, O., Nicholson, A., Zhang, S.L., Jiang, W., Vangala, N., Landsberg, J.W., Wang, J.Y., Thistlethwaite, P.A., Channick, R.N., Robbins, I.M., Loyd, J.E., Ghofrani, H.A., Grimminger, F., Schermuly, R.T., Cahalan, M.D., Rubin, L.J., and Yuan, J.X.; *A functional single-nucleotide polymorphism in the TRPC6 gene promoter associated with idiopathic pulmonary arterial hypertension*. Circulation. 2009. **119**(17): p. 2313-2322.
46. Yildiz, P.; *Molecular mechanisms of pulmonary hypertension*. Clinica Chimica Acta. 2009. **403**(1-2): p. 9-16.
47. Stenmark, K.R., and Mecham, R.P.; *Cellular and molecular mechanisms of pulmonary vascular remodeling*. Annual Review of Physiology. 1997. **59**: p. 89-144.
48. Budhiraja, R., Tuder, R.M., and Hassoun, P.M.; *Endothelial dysfunction in pulmonary hypertension*. Circulation. 2004. **109**(2): p. 159-165.
49. Christman, B.W., Mcpherson, C.D., Newman, J.H., King, G.A., Bernard, G.R., Groves, B.M., and Loyd, J.E.; *An Imbalance between the Excretion of Thromboxane and Prostacyclin Metabolites in Pulmonary-Hypertension*. New England Journal of Medicine. 1992. **327**(2): p. 70-75.
50. Narumiya, S., Sugimoto, Y., and Ushikubi, F.; *Prostanoid receptors: structures, properties, and functions*. Physiological Reviews. 1999. **79**(4): p. 1193-1226.
51. Tuder, R.M., Cool, C.D., Geraci, M.W., Wang, J., Abman, S.H., Wright, L., Badesch, D., and Voelkel, N.F.; *Prostacyclin synthase expression is decreased in lungs front patients with severe pulmonary hypertension*. American Journal of Respiratory and Critical Care Medicine. 1999. **159**(6): p. 1925-1932.
52. Mandegar, M., Fung, Y.C.B., Huang, W., Remillard, C.V., Rubin, L.J., and Yuan, J.X.J.; *Cellular and molecular mechanisms of pulmonary vascular remodeling: role in the development of pulmonary hypertension*. Microvascular Research. 2004. **68**(2): p. 75-103.
53. Birschmann, I., and Walter, U.; *Physiology and pathophysiology of vascular signaling controlled by guanosine 3',5'-cyclic monophosphate-dependent protein kinase*. Acta Biochimica Polonica. 2004. **51**(2): p. 397-404.
54. Coggins, M.P., and Bloch, K.D.; *Nitric oxide in the pulmonary vasculature*. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007. **27**(9): p. 1877-1885.
55. Galie, N., Manes, A., and Branzi, A.; *The endothelin system in pulmonary arterial hypertension*. Cardiovascular Research. 2004. **61**(2): p. 227-237.
56. Giaid, A.; *Nitric oxide and endothelin-1 in pulmonary hypertension*. Chest. 1998. **114**(3 Suppl): p. 208S-212S.
57. Giaid, A., Yanagisawa, M., Langleben, D., Michel, R.P., Levy, R., Shennib, H., Kimura, S., Masaki, T., Duguid, W.P., and Stewart, D.J.; *Expression of endothelin-1 in the lungs of patients with pulmonary hypertension*. New England Journal of Medicine. 1993. **328**(24): p. 1732-1739.

58. Luscher, T.F., and Barton, M.; *Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs*. Circulation. 2000. **102**(19): p. 2434-2440.
59. Stewart, D.J., Levy, R.D., Cernacek, P., and Langleben, D.; *Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease?* Annals of Internal Medicine. 1991. **114**(6): p. 464-469.
60. MacLean, M.R., Herve, P., Eddahibi, S., and Adnot, S.; *5-hydroxytryptamine and the pulmonary circulation: receptors, transporters and relevance to pulmonary arterial hypertension*. British Journal of Pharmacology. 2000. **131**(2): p. 161-168.
61. MacLean, M.R.; *Pulmonary hypertension and the serotonin hypothesis: where are we now?* International Journal of Clinical Practice Supplement. 2007. (156): p. 27-31.
62. Herve, P., Launay, J.M., Scrobohaci, M.L., Brenot, F., Simonneau, G., Petitpretz, P., Poubeau, P., Cerrina, J., Duroux, P., and Drouet, L.; *Increased plasma serotonin in primary pulmonary hypertension*. American Journal of Medicine. 1995. **99**(3): p. 249-254.
63. Haberl, I., Frei, K., Ramsebner, R., Doberer, D., Petkov, V., Albinni, S., Lang, I., Lucas, T., and Mosgoeller, W.; *Vasoactive intestinal peptide gene alterations in patients with idiopathic pulmonary arterial hypertension*. European Journal of Human Genetics. 2007. **15**(1): p. 18-22.
64. Said, S.I.; *The Viktor Mutt Memorial Lecture. Protection by VIP and related peptides against cell death and tissue injury*. Annals of the New York Academy of Sciences. 2000. **921**: p. 264-274.
65. Said, S.I.; *Mediators and modulators of pulmonary arterial hypertension*. Am J Physiol Lung Cell Mol Physiol. 2006. **291**(4): p. L547-558.
66. Nagaya, N., and Kangawa, K.; *Adrenomedullin in the treatment of pulmonary hypertension*. Peptides. 2004. **25**(11): p. 2013-2018.
67. Kakishita, M., Nishikimi, T., Okano, Y., Satoh, T., Kyotani, S., Nagaya, N., Fukushima, K., Nakanishi, N., Takishita, S., Miyata, A., Kangawa, K., Matsuo, H., and Kunieda, T.; *Increased plasma levels of adrenomedullin in patients with pulmonary hypertension*. Clin Sci (Lond). 1999. **96**(1): p. 33-39.
68. Morrell, N.W., Atochina, E.N., Morris, K.G., Danilov, S.M., and Stenmark, K.R.; *Angiotensin converting enzyme expression is increased in small pulmonary arteries of rats with hypoxia-induced pulmonary hypertension*. Journal of Clinical Investigation. 1995. **96**(4): p. 1823-1833.
69. Morrell, N.W., Upton, P.D., Kotecha, S., Huntley, A., Yacoub, M.H., Polak, J.M., and Wharton, J.; *Angiotensin II activates MAPK and stimulates growth of human pulmonary artery smooth muscle via AT1 receptors*. American Journal of Physiology. 1999. **277**(3 Pt 1): p. L440-448.
70. Marshall, R.P., McAnulty, R.J., and Laurent, G.J.; *Angiotensin II is mitogenic for human lung fibroblasts via activation of the type 1 receptor*. American Journal of Respiratory and Critical Care Medicine. 2000. **161**(6): p. 1999-2004.
71. Brindle, N.P., Saharinen, P., and Alitalo, K.; *Signaling and functions of angiopoietin-1 in vascular protection*. Circulation Research. 2006. **98**(8): p. 1014-1023.
72. Du, L., Sullivan, C.C., Chu, D., Cho, A.J., Kido, M., Wolf, P.L., Yuan, J.X., Deutsch, R., Jamieson, S.W., and Thistlethwaite, P.A.; *Signaling molecules in nonfamilial pulmonary hypertension*. New England Journal of Medicine. 2003. **348**(6): p. 500-509.
73. Cross, M.J., Dixelius, J., Matsumoto, T., and Claesson-Welsh, L.; *VEGF-receptor signal transduction*. Trends in Biochemical Sciences. 2003. **28**(9): p. 488-494.
74. He, H., Venema, V.J., Guo, X.L., Venema, R.C., Marrero, M.B., and Caldwell, R.B.; *Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through Flk-1/KDR activation of c-Src*. Journal of Biological Chemistry. 1999. **274**(35): p. 25130-25135.
75. Gerber, H.P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B.A., Dixit, V., and Ferrara, N.; *Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation*. Journal of Biological Chemistry. 1998. **273**(46): p. 30336-30343.
76. Voelkel, N.F., Vandivier, R.W., and Tuder, R.M.; *Vascular endothelial growth factor in the lung*. Am J Physiol Lung Cell Mol Physiol. 2006. **290**(2): p. L209-221.
77. Eliceiri, B.P., Paul, R., Schwartzberg, P.L., Hood, J.D., Leng, J., and Cheresh, D.A.; *Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability*. Molecular Cell. 1999. **4**(6): p. 915-924.
78. Heldin, C.H., and Westermark, B.; *Mechanism of action and in vivo role of platelet-derived growth factor*. Physiological Reviews. 1999. **79**(4): p. 1283-1316.
79. Perros, F., Montani, D., Dorfmuller, P., Durand-Gasselin, I., Tcherakian, C., Le Pavec, J., Mazmanian, M., Fadel, E., Mussot, S., Mercier, O., Herve, P., Emilie, D., Eddahibi, S., Simonneau, G., Souza, R., and Humbert, M.; *Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension*. American Journal of Respiratory and Critical Care Medicine. 2008. **178**(1): p. 81-88.

80. Anand-Srivastava, M.B.; *Natriuretic peptide receptor-C signaling and regulation*. Peptides. 2005. **26**(6): p. 1044-1059.
81. Levin, E.R., Gardner, D.G., and Samson, W.K.; *Natriuretic peptides*. New England Journal of Medicine. 1998. **339**(5): p. 321-328.
82. Pedram, A., Razandi, M., and Levin, E.R.; *Natriuretic peptides suppress vascular endothelial cell growth factor signaling to angiogenesis*. Endocrinology. 2001. **142**(4): p. 1578-1586.
83. Nagaya, N., Nishikimi, T., Uematsu, M., Satoh, T., Kyotani, S., Sakamaki, F., Kakishita, M., Fukushima, K., Okano, Y., Nakanishi, N., Miyatake, K., and Kangawa, K.; *Plasma brain natriuretic peptide as a prognostic indicator in patients with primary pulmonary hypertension*. Circulation. 2000. **102**(8): p. 865-870.
84. Andersen, C.U., Hilberg, O., Mellemkjaer, S., Nielsen-Kudsk, J.E., and Simonsen, U.; *Apelin and pulmonary hypertension*. Pulm Circ. 2011. **1**(3): p. 334-346.
85. Kawamata, Y., Habata, Y., Fukusumi, S., Hosoya, M., Fujii, R., Hinuma, S., Nishizawa, N., Kitada, C., Onda, H., Nishimura, O., and Fujino, M.; *Molecular properties of apelin: tissue distribution and receptor binding*. Biochimica et Biophysica Acta. 2001. **1538**(2-3): p. 162-171.
86. Kishimoto, I., Tokudome, T., Hosoda, H., Miyazato, M., and Kangawa, K.; *Ghrelin and cardiovascular diseases*. Journal of Cardiology. 2012. **59**(1): p. 8-13.
87. Kojima, M., and Kangawa, K.; *Ghrelin: structure and function*. Physiological Reviews. 2005. **85**(2): p. 495-522.
88. Tesauro, M., Schinzari, F., Caramanti, M., Lauro, R., and Cardillo, C.; *Metabolic and cardiovascular effects of ghrelin*. Int J Pept. 2010. **2010**: p.
89. Beech, D.J.; *Actions of neurotransmitters and other messengers on Ca2+ channels and K+ channels in smooth muscle cells*. Pharmacology and Therapeutics. 1997. **73**(2): p. 91-119.
90. Cogolludo, A., Moreno, L., and Villamor, E.; *Mechanisms controlling vascular tone in pulmonary arterial hypertension: implications for vasodilator therapy*. Pharmacology. 2007. **79**(2): p. 65-75.
91. Somlyo, A.P., and Somlyo, A.V.; *Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase*. Physiological Reviews. 2003. **83**(4): p. 1325-1358.
92. Loirand, G., Guerin, P., and Pacaud, P.; *Rho kinases in cardiovascular physiology and pathophysiology*. Circulation Research. 2006. **98**(3): p. 322-334.
93. Chao, T.S., Byron, K.L., Lee, K.M., Villereal, M., and Rosner, M.R.; *Activation of MAP kinases by calcium-dependent and calcium-independent pathways. Stimulation by thapsigargin and epidermal growth factor*. Journal of Biological Chemistry. 1992. **267**(28): p. 19876-19883.
94. Mandegar, M., Remillard, C.V., and Yuan, J.X.; *Ion channels in pulmonary arterial hypertension*. Progress in Cardiovascular Diseases. 2002. **45**(2): p. 81-114.
95. Golovina, V.A., Platoshyn, O., Bailey, C.L., Wang, J., Limsuwan, A., Sweeney, M., Rubin, L.J., and Yuan, J.X.; *Upregulated TRP and enhanced capacitative Ca(2+) entry in human pulmonary artery myocytes during proliferation*. Am J Physiol Heart Circ Physiol. 2001. **280**(2): p. H746-755.
96. Kuhr, F.K., Smith, K.A., Song, M.Y., Levitan, I., and Yuan, J.X.; *New mechanisms of pulmonary arterial hypertension: role of Ca(2)(+) signaling*. Am J Physiol Heart Circ Physiol. 2012. **302**(8): p. H1546-1562.
97. Allbritton, N.L., Oancea, E., Kuhn, M.A., and Meyer, T.; *Source of nuclear calcium signals*. Proceedings of the National Academy of Sciences of the United States of America. 1994. **91**(26): p. 12458-12462.
98. Means, A.R.; *Calcium, calmodulin and cell cycle regulation*. FEBS Letters. 1994. **347**(1): p. 1-4.
99. Kahl, C.R., and Means, A.R.; *Regulation of cell cycle progression by calcium/calmodulin-dependent pathways*. Endocrine Reviews. 2003. **24**(6): p. 719-736.
100. Irani, K.; *Oxidant signaling in vascular cell growth, death, and survival : a review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling*. Circulation Research. 2000. **87**(3): p. 179-183.
101. Pollman, M.J., Yamada, T., Horiuchi, M., and Gibbons, G.H.; *Vasoactive substances regulate vascular smooth muscle cell apoptosis. Countervailing influences of nitric oxide and angiotensin II*. Circulation Research. 1996. **79**(4): p. 748-756.
102. Yamada, T., Horiuchi, M., and Dzau, V.J.; *Angiotensin II type 2 receptor mediates programmed cell death*. Proceedings of the National Academy of Sciences of the United States of America. 1996. **93**(1): p. 156-160.
103. Bortner, C.D., and Cidlowski, J.A.; *A necessary role for cell shrinkage in apoptosis*. Biochemical Pharmacology. 1998. **56**(12): p. 1549-1559.

104. Nemes, Z., Jr., Friis, R.R., Aeschlimann, D., Saurer, S., Paulsson, M., and Fesus, L.; *Expression and activation of tissue transglutaminase in apoptotic cells of involuting rodent mammary tissue*. European Journal of Cell Biology. 1996. **70**(2): p. 125-133.
105. Kerr, J.F., Wyllie, A.H., and Currie, A.R.; *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics*. British Journal of Cancer. 1972. **26**(4): p. 239-257.
106. Bortner, C.D., Oldenburg, N.B., and Cidlowski, J.A.; *The role of DNA fragmentation in apoptosis*. Trends in Cell Biology. 1995. **5**(1): p. 21-26.
107. Kurosaka, K., Takahashi, M., Watanabe, N., and Kobayashi, Y.; *Silent cleanup of very early apoptotic cells by macrophages*. Journal of Immunology. 2003. **171**(9): p. 4672-4679.
108. Savill, J., and Fadok, V.; *Corpse clearance defines the meaning of cell death*. Nature. 2000. **407**(6805): p. 784-788.
109. Elmore, S.; *Apoptosis: a review of programmed cell death*. Toxicologic Pathology. 2007. **35**(4): p. 495-516.
110. Locksley, R.M., Killeen, N., and Lenardo, M.J.; *The TNF and TNF receptor superfamilies: integrating mammalian biology*. Cell. 2001. **104**(4): p. 487-501.
111. Ashkenazi, A., and Dixit, V.M.; *Death receptors: signaling and modulation*. Science. 1998. **281**(5381): p. 1305-1308.
112. Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M.; *FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis*. Cell. 1995. **81**(4): p. 505-512.
113. Hsu, H., Xiong, J., and Goeddel, D.V.; *The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation*. Cell. 1995. **81**(4): p. 495-504.
114. Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S., and Dixit, V.M.; *An induced proximity model for caspase-8 activation*. Journal of Biological Chemistry. 1998. **273**(5): p. 2926-2930.
115. Hengartner, M.O.; *The biochemistry of apoptosis*. Nature. 2000. **407**(6805): p. 770-776.
116. Kataoka, T., Schroter, M., Hahne, M., Schneider, P., Irmler, M., Thome, M., Froelich, C.J., and Tschopp, J.; *FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation*. Journal of Immunology. 1998. **161**(8): p. 3936-3942.
117. Scaffidi, C., Schmitz, I., Krammer, P.H., and Peter, M.E.; *The role of c-FLIP in modulation of CD95-induced apoptosis*. Journal of Biological Chemistry. 1999. **274**(3): p. 1541-1548.
118. Hsu, Y.T., Wolter, K.G., and Youle, R.J.; *Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis*. Proceedings of the National Academy of Sciences of the United States of America. 1997. **94**(8): p. 3668-3672.
119. Du, C., Fang, M., Li, Y., Li, L., and Wang, X.; *Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition*. Cell. 2000. **102**(1): p. 33-42.
120. van Loo, G., Saelens, X., van Gurp, M., MacFarlane, M., Martin, S.J., and Vandenabeele, P.; *The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet*. Cell Death and Differentiation. 2002. **9**(10): p. 1031-1042.
121. McMurtry, M.S., Archer, S.L., Altieri, D.C., Bonnet, S., Haromy, A., Harry, G., Puttagunta, L., and Michelakis, E.D.; *Gene therapy targeting survivin selectively induces pulmonary vascular apoptosis and reverses pulmonary arterial hypertension*. Journal of Clinical Investigation. 2005. **115**(6): p. 1479-1491.
122. van Loo, G., van Gurp, M., Depuydt, B., Srinivasula, S.M., Rodriguez, I., Alnemri, E.S., Gevaert, K., Vandekerckhove, J., Declercq, W., and Vandenabeele, P.; *The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity*. Cell Death and Differentiation. 2002. **9**(1): p. 20-26.
123. Wei, M.C., Lindsten, T., Mootha, V.K., Weiler, S., Gross, A., Ashiya, M., Thompson, C.B., and Korsmeyer, S.J.; *tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c*. Genes and Development. 2000. **14**(16): p. 2060-2071.
124. Li, H., Zhu, H., Xu, C.J., and Yuan, J.; *Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis*. Cell. 1998. **94**(4): p. 491-501.
125. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X.; *Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors*. Cell. 1998. **94**(4): p. 481-490.
126. Deng, Y., Lin, Y., and Wu, X.; *TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO*. Genes and Development. 2002. **16**(1): p. 33-45.
127. Hameed, A.G., Arnold, N.D., Chamberlain, J., Pickworth, J.A., Paiva, C., Dawson, S., Cross, S., Long, L., Zhao, L., Morrell, N.W., Crossman, D.C., Newman, C.M., Kiely, D.G., Francis, S.E., and Lawrie, A.; *Inhibition of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) reverses experimental pulmonary hypertension*. Journal of Experimental Medicine. 2012. **209**(11): p. 1919-1935.

128. Hameed, A.G., Arnold, N.D., Pickworth, J., Chamberlain, J.C., Newman, C.M.H., Crossman, D.C., Francis, S.E., and Lawrie, A.; *Trail Is a Potential Novel Therapeutic Target in Pulmonary Arterial Hypertension*. Thorax. 2011. **66**: p. A3-A3.
129. Remillard, C.V., and Yuan, J.X.; *Activation of K+ channels: an essential pathway in programmed cell death*. Am J Physiol Lung Cell Mol Physiol. 2004. **286**(1): p. L49-67.
130. Dallaporta, B., Hirsch, T., Susin, S.A., Zamzami, N., Larochette, N., Brenner, C., Marzo, I., and Kroemer, G.; *Potassium leakage during the apoptotic degradation phase*. Journal of Immunology. 1998. **160**(11): p. 5605-5615.
131. Bortner, C.D., Hughes, F.M., Jr., and Cidlowski, J.A.; *A primary role for K+ and Na+ efflux in the activation of apoptosis*. Journal of Biological Chemistry. 1997. **272**(51): p. 32436-32442.
132. Geraci, M.W., Moore, M., Gesell, T., Yeager, M.E., Alger, L., Golpon, H., Gao, B., Loyd, J.E., Tuder, R.M., and Voelkel, N.F.; *Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis*. Circulation Research. 2001. **88**(6): p. 555-562.
133. Yuan, J.X., Aldinger, A.M., Juhaszova, M., Wang, J., Conte, J.V., Jr., Gaine, S.P., Orens, J.B., and Rubin, L.J.; *Dysfunctional voltage-gated K+ channels in pulmonary artery smooth muscle cells of patients with primary pulmonary hypertension*. Circulation. 1998. **98**(14): p. 1400-1406.
134. Yuan, X.J., Wang, J., Juhaszova, M., Gaine, S.P., and Rubin, L.J.; *Attenuated K+ channel gene transcription in primary pulmonary hypertension*. Lancet. 1998. **351**(9104): p. 726-727.
135. Remillard, C.V., Tigno, D.D., Platoshyn, O., Burg, E.D., Brevnova, E.E., Conger, D., Nicholson, A., Rana, B.K., Channick, R.N., Rubin, L.J., O'Connor D, T., and Yuan, J.X.; *Function of Kv1.5 channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension*. Am J Physiol Cell Physiol. 2007. **292**(5): p. C1837-1853.
136. Weir, E.K., Reeve, H.L., Huang, J.M., Michelakis, E., Nelson, D.P., Hampl, V., and Archer, S.L.; *Anorexic agents aminorex, fenfluramine, and dexfenfluramine inhibit potassium current in rat pulmonary vascular smooth muscle and cause pulmonary vasoconstriction*. Circulation. 1996. **94**(9): p. 2216-2220.
137. Wang, J., Juhaszova, M., Conte, J.V., Jr., Gaine, S.P., Rubin, L.J., and Yuan, J.X.; *Action of fenfluramine on voltage-gated K+ channels in human pulmonary-artery smooth-muscle cells*. Lancet. 1998. **352**(9124): p. 290.
138. Zhang, S., Fantozzi, I., Tigno, D.D., Yi, E.S., Platoshyn, O., Thistlethwaite, P.A., Kriett, J.M., Yung, G., Rubin, L.J., and Yuan, J.X.; *Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells*. Am J Physiol Lung Cell Mol Physiol. 2003. **285**(3): p. L740-754.
139. Teichert-Kuliszewska, K., Kutryk, M.J., Kuliszewski, M.A., Karoubi, G., Courtman, D.W., Zucco, L., Granton, J., and Stewart, D.J.; *Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension*. Circulation Research. 2006. **98**(2): p. 209-217.
140. Sakao, S., Taraseviciene-Stewart, L., Lee, J.D., Wood, K., Cool, C.D., and Voelkel, N.F.; *Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells*. FASEB Journal. 2005. **19**(7): p. 1178-+.
141. Sakao, S., Tatsumi, K., and Voelkel, N.F.; *Endothelial cells and pulmonary arterial hypertension: apoptosis, proliferation, interaction and transdifferentiation*. Respir Res. 2009. **10**: p. 95.
142. Michelakis, E.D.; *Spatio-temporal diversity of apoptosis within the vascular wall in pulmonary arterial hypertension - Heterogeneous BMP signaling may have therapeutic implications*. Circulation Research. 2006. **98**(2): p. 172-175.
143. Taraseviciene-Stewart, L., Kasahara, Y., Alger, L., Hirth, P., Mc Mahon, G., Waltenberger, J., Voelkel, N.F., and Tuder, R.M.; *Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension*. FASEB Journal. 2001. **15**(2): p. 427-438.
144. Sakao, S., Taraseviciene-Stewart, L., Wood, K., Cool, C.D., and Voelkel, N.F.; *Apoptosis of pulmonary microvascular endothelial cells stimulates vascular smooth muscle cell growth*. Am J Physiol Lung Cell Mol Physiol. 2006. **291**(3): p. L362-368.
145. Srinivasula, S.M., and Ashwell, J.D.; *IAPs: what's in a name?* Molecular Cell. 2008. **30**(2): p. 123-135.
146. Schimmer, A.D.; *Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice*. Cancer Research. 2004. **64**(20): p. 7183-7190.
147. Dohi, T., Beltrami, E., Wall, N.R., Plescia, J., and Altieri, D.C.; *Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis*. Journal of Clinical Investigation. 2004. **114**(8): p. 1117-1127.
148. Deveraux, Q.L., and Reed, J.C.; *IAP family proteins--suppressors of apoptosis*. Genes and Development. 1999. **13**(3): p. 239-252.

149. O'Connor, D.S., Schechner, J.S., Adida, C., Mesri, M., Rothermel, A.L., Li, F., Nath, A.K., Pober, J.S., and Altieri, D.C.; *Control of apoptosis during angiogenesis by survivin expression in endothelial cells*. American Journal of Pathology. 2000. **156**(2): p. 393-398.
150. Tamm, I., Wang, Y., Sausville, E., Scudiero, D.A., Vigna, N., Oltersdorf, T., and Reed, J.C.; *IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs*. Cancer Research. 1998. **58**(23): p. 5315-5320.
151. Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J., and Vaux, D.L.; *Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins*. Cell. 2000. **102**(1): p. 43-53.
152. Ceballos-Cancino, G., Espinosa, M., Maldonado, V., and Melendez-Zajgla, J.; *Regulation of mitochondrial Smac/DIABLO-selective release by survivin*. Oncogene. 2007. **26**(54): p. 7569-7575.
153. Song, Z., Yao, X., and Wu, M.; *Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis*. Journal of Biological Chemistry. 2003. **278**(25): p. 23130-23140.
154. Dohi, T., Okada, K., Xia, F., Wilford, C.E., Samuel, T., Welsh, K., Marusawa, H., Zou, H., Armstrong, R., Matsuzawa, S., Salvesen, G.S., Reed, J.C., and Altieri, D.C.; *An IAP-IAP complex inhibits apoptosis*. Journal of Biological Chemistry. 2004. **279**(33): p. 34087-34090.
155. Li, F., Ambrosini, G., Chu, E.Y., Plescia, J., Tognin, S., Marchisio, P.C., and Altieri, D.C.; *Control of apoptosis and mitotic spindle checkpoint by survivin*. Nature. 1998. **396**(6711): p. 580-584.
156. van der Waal, M.S., Hengeveld, R.C., van der Horst, A., and Lens, S.M.; *Cell division control by the Chromosomal Passenger Complex*. Experimental Cell Research. 2012. **318**(12): p. 1407-1420.
157. Altieri, D.C.; *Molecular circuits of apoptosis regulation and cell division control: the survivin paradigm*. Journal of Cellular Biochemistry. 2004. **92**(4): p. 656-663.
158. Giodini, A., Kallio, M.J., Wall, N.R., Gorbsky, G.J., Tognin, S., Marchisio, P.C., Symons, M., and Altieri, D.C.; *Regulation of microtubule stability and mitotic progression by survivin*. Cancer Research. 2002. **62**(9): p. 2462-2467.
159. Levkau, B., Schafers, M., Wohlschlaeger, J., von Wnuck Lipinski, K., Keul, P., Hermann, S., Kawaguchi, N., Kirchhof, P., Fabritz, L., Stypmann, J., Stegger, L., Flogel, U., Schrader, J., Fischer, J.W., Hsieh, P., Ou, Y.L., Mehrhof, F., Tiemann, K., Ghanem, A., Matus, M., Neumann, J., Heusch, G., Schmid, K.W., Conway, E.M., and Baba, H.A.; *Survivin determines cardiac function by controlling total cardiomyocyte number*. Circulation. 2008. **117**(12): p. 1583-1593.
160. Badran, A., Yoshida, A., Ishikawa, K., Goi, T., Yamaguchi, A., Ueda, T., and Inuzuka, M.; *Identification of a novel splice variant of the human anti-apoptopsis gene survivin*. Biochemical and Biophysical Research Communications. 2004. **314**(3): p. 902-907.
161. Mahotka, C., Wenzel, M., Springer, E., Gabbert, H.E., and Gerharz, C.D.; *Survivin-deltaEx3 and survivin-2B: two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties*. Cancer Research. 1999. **59**(24): p. 6097-6102.
162. Caldas, H., Honsey, L.E., and Altura, R.A.; *Survivin 2alpha: a novel Survivin splice variant expressed in human malignancies*. Mol Cancer. 2005. **4**(1): p. 11.
163. Knauer, S.K., Bier, C., Schlag, P., Fritzmann, J., Dietmaier, W., Rodel, F., Klein-Hitpass, L., Kovacs, A.F., Doring, C., Hansmann, M.L., Hofmann, W.K., Kunkel, M., Brochhausen, C., Engels, K., Lippert, B.M., Mann, W., and Stauber, R.H.; *The survivin isoform survivin-3B is cytoprotective and can function as a chromosomal passenger complex protein*. Cell Cycle. 2007. **6**(12): p. 1502-1509.
164. Fortugno, P., Wall, N.R., Giodini, A., O'Connor, D.S., Plescia, J., Padgett, K.M., Tognin, S., Marchisio, P.C., and Altieri, D.C.; *Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function*. Journal of Cell Science. 2002. **115**(Pt 3): p. 575-585.
165. Dallaglio, K., Marconi, A., and Pincelli, C.; *Survivin: a dual player in healthy and diseased skin*. Journal of Investigative Dermatology. 2012. **132**(1): p. 18-27.
166. Beltrami, E., Plescia, J., Wilkinson, J.C., Duckett, C.S., and Altieri, D.C.; *Acute ablation of survivin uncovers p53-dependent mitotic checkpoint functions and control of mitochondrial apoptosis*. Journal of Biological Chemistry. 2004. **279**(3): p. 2077-2084.
167. Adnot, S.; *Lessons learned from cancer may help in the treatment of pulmonary hypertension*. Journal of Clinical Investigation. 2005. **115**(6): p. 1461-1463.
168. Adnot, S., and Eddahibi, S.; *Lessons from oncology to understand and treat pulmonary hypertension*. International Journal of Clinical Practice Supplement. 2007. (158): p. 19-25.
169. Hanahan, D., and Weinberg, R.A.; *The hallmarks of cancer*. Cell. 2000. **100**(1): p. 57-70.
170. Rai, P.R., Cool, C.D., King, J.A., Stevens, T., Burns, N., Winn, R.A., Kasper, M., and Voelkel, N.F.; *The cancer paradigm of severe pulmonary arterial hypertension*. American Journal of Respiratory and Critical Care Medicine. 2008. **178**(6): p. 558-564.

171. Ambrosini, G., Adida, C., and Altieri, D.C.; *A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma*. Nature Medicine. 1997. **3**(8): p. 917-921.
172. Campian, M.E., Hardziyenka, M., Michel, M.C., and Tan, H.L.; *How valid are animal models to evaluate treatments for pulmonary hypertension?* Naunyn-Schmiedebergs Archives of Pharmacology. 2006. **373**(6): p. 391-400.
173. Robbins, I.M.; *Advancing therapy for pulmonary arterial hypertension: can animal models help?* American Journal of Respiratory and Critical Care Medicine. 2004. **169**(1): p. 5-6.
174. Said, S.I., Hamidi, S.A., Dickman, K.G., Szema, A.M., Lyubsky, S., Lin, R.Z., Jiang, Y.P., Chen, J.J., Waschek, J.A., and Kort, S.; *Moderate pulmonary arterial hypertension in male mice lacking the vasoactive intestinal peptide gene*. Circulation. 2007. **115**(10): p. 1260-1268.
175. Rothman, R.B., Cadet, J.L., Dersch, C.M., McCoy, M.T., Lehrmann, E., Becker, K.G., Bader, M., Alenina, N., and Baumann, M.H.; *Altered gene expression in pulmonary tissue of tryptophan hydroxylase-1 knockout mice: implications for pulmonary arterial hypertension*. PLoS One. 2011. **6**(3): p. e17735.
176. Lalich, J.J., and Merkow, L.; *Pulmonary arteritis produced in rat by feeding Crotalaria spectabilis*. Laboratory Investigation. 1961. **10**: p. 744-750.
177. Kurozumi, T., Tanaka, K., Kido, M., and Shoyama, Y.; *Monocrotaline-induced renal lesions*. Experimental and Molecular Pathology. 1983. **39**(3): p. 377-386.
178. Wilson, D.W., Segall, H.J., Pan, L.C., Lame, M.W., Estep, J.E., and Morin, D.; *Mechanisms and pathology of monocrotaline pulmonary toxicity*. Critical Reviews in Toxicology. 1992. **22**(5-6): p. 307-325.
179. Kasahara, Y., Kiyatake, K., Tatsumi, K., Sugito, K., Kakusaka, I., Yamagata, S., Ohmori, S., Kitada, M., and Kuriyama, T.; *Bioactivation of monocrotaline by P-450 3A in rat liver*. Journal of Cardiovascular Pharmacology. 1997. **30**(1): p. 124-129.
180. Dumitrascu, R., Koebrich, S., Dony, E., Weissmann, N., Savai, R., Pullamsetti, S.S., Ghofrani, H.A., Samidurai, A., Traupe, H., Seeger, W., Grimminger, F., and Schermuly, R.T.; *Characterization of a murine model of monocrotaline pyrrole-induced acute lung injury*. BMC Pulm Med. 2008. **8**: p. 25.
181. Thomas, H.C., Lame, M.W., Dunston, S.K., Segall, H.J., and Wilson, D.W.; *Monocrotaline pyrrole induces apoptosis in pulmonary artery endothelial cells*. Toxicology and Applied Pharmacology. 1998. **151**(2): p. 236-244.
182. Lappin, P.B., Ross, K.L., King, L.E., Fraker, P.J., and Roth, R.A.; *The response of pulmonary vascular endothelial cells to monocrotaline pyrrole: cell proliferation and DNA synthesis in vitro and in vivo*. Toxicology and Applied Pharmacology. 1998. **150**(1): p. 37-48.
183. Meyrick, B.O., and Reid, L.M.; *Crotalaria-induced pulmonary hypertension. Uptake of 3H-thymidine by the cells of the pulmonary circulation and alveolar walls*. American Journal of Pathology. 1982. **106**(1): p. 84-94.
184. Ryan, J., Bloch, K., and Archer, S.L.; *Rodent models of pulmonary hypertension: harmonisation with the world health organisation's categorisation of human PH*. International Journal of Clinical Practice Supplement. 2011. (172): p. 15-34.
185. Rosenberg, H.C., and Rabinovitch, M.; *Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension*. American Journal of Physiology. 1988. **255**(6 Pt 2): p. H1484-1491.
186. Lalich, J.L., Johnson, W.D., Raczniak, T.J., and Shumaker, R.C.; *Fibrin thrombosis in monocrotaline pyrrole-induced cor pulmonale in rats*. Archives of Pathology and Laboratory Medicine. 1977. **101**(2): p. 69-73.
187. Wilson, D.W., Segall, H.J., Pan, L.C., and Dunston, S.K.; *Progressive inflammatory and structural changes in the pulmonary vasculature of monocrotaline-treated rats*. Microvascular Research. 1989. **38**(1): p. 57-80.
188. Meyrick, B., Perkett, E.A., and Brigham, K.L.; *Inflammation and models of chronic pulmonary hypertension*. American Review of Respiratory Disease. 1987. **136**(3): p. 765-767.
189. Rabinovitch, M.; *Investigational approaches to pulmonary hypertension*. Toxicologic Pathology. 1991. **19**(4 Pt 1): p. 458-469.
190. Meyrick, B., Gamble, W., and Reid, L.; *Development of Crotalaria pulmonary hypertension: hemodynamic and structural study*. American Journal of Physiology. 1980. **239**(5): p. H692-702.
191. Lourenco, A.P., Roncon-Albuquerque, R., Jr., Bras-Silva, C., Faria, B., Wieland, J., Henriques-Coelho, T., Correia-Pinto, J., and Leite-Moreira, A.F.; *Myocardial dysfunction and neurohumoral activation without remodeling in left ventricle of monocrotaline-induced pulmonary hypertensive rats*. Am J Physiol Heart Circ Physiol. 2006. **291**(4): p. H1587-1594.
192. Akhavein, F., St-Michel, E.J., Seifert, E., and Rohlicek, C.V.; *Decreased left ventricular function, myocarditis, and coronary arteriolar medial thickening following monocrotaline administration in adult rats*. Journal of Applied Physiology. 2007. **103**(1): p. 287-295.

193. Stenmark, K.R., Meyrick, B., Galie, N., Mooi, W.J., and McMurtry, I.F.; *Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure*. Am J Physiol Lung Cell Mol Physiol. 2009. **297**(6): p. L1013-1032.
194. White, R.J., Meoli, D.F., Swarthout, R.F., Kallop, D.Y., Galaria, II, Harvey, J.L., Miller, C.M., Blaxall, B.C., Hall, C.M., Pierce, R.A., Cool, C.D., and Taubman, M.B.; *Plexiform-like lesions and increased tissue factor expression in a rat model of severe pulmonary arterial hypertension*. Am J Physiol Lung Cell Mol Physiol. 2007. **293**(3): p. L583-590.
195. Bhargava, A., Kumar, A., Yuan, N., Gewitz, M.H., and Mathew, R.; *Monocrotaline induces interleukin-6 mRNA expression in rat lungs*. Heart Dis. 1999. **1**(3): p. 126-132.
196. Suzuki, A., Kakusaka, I., Kiyatake, K., Nakano, K., Kaneko, N., and Kuriyama, T.; *[Interleukin-2 activity in monocrotaline-treated rat lungs]*. Nihon Kyobu Shikkan Gakkai Zasshi. 1995. **33**(6): p. 625-629.
197. Gillespie, M.N., Goldblum, S.E., Cohen, D.A., and McClain, C.J.; *Interleukin 1 bioactivity in the lungs of rats with monocrotaline-induced pulmonary hypertension*. Proceedings of the Society for Experimental Biology and Medicine. 1988. **187**(1): p. 26-32.
198. McMurtry, M.S., Bonnet, S., Wu, X., Dyck, J.R., Haromy, A., Hashimoto, K., and Michelakis, E.D.; *Dichloroacetate prevents and reverses pulmonary hypertension by inducing pulmonary artery smooth muscle cell apoptosis*. Circulation Research. 2004. **95**(8): p. 830-840.
199. Morty, R.E., Nejman, B., Kwapiszewska, G., Hecker, M., Zakrzewicz, A., Kouri, F.M., Peters, D.M., Dumitrascu, R., Seeger, W., Knaus, P., Schermuly, R.T., and Eickelberg, O.; *Dysregulated bone morphogenetic protein signaling in monocrotaline-induced pulmonary arterial hypertension*. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007. **27**(5): p. 1072-1078.
200. McMurtry, M.S., Moudgil, R., Hashimoto, K., Bonnet, S., Michelakis, E.D., and Archer, S.L.; *Overexpression of human bone morphogenetic protein receptor 2 does not ameliorate monocrotaline pulmonary arterial hypertension*. Am J Physiol Lung Cell Mol Physiol. 2007. **292**(4): p. L872-878.
201. Frasch, H.F., Marshall, C., and Marshall, B.E.; *Endothelin-1 is elevated in monocrotaline pulmonary hypertension*. American Journal of Physiology. 1999. **276**(2 Pt 1): p. L304-310.
202. Yorikane, R., Miyauchi, T., Sakai, S., Sakurai, T., Yamaguchi, I., Sugishita, Y., and Goto, K.; *Altered expression of ETB-receptor mRNA in the lung of rats with pulmonary hypertension*. Journal of Cardiovascular Pharmacology. 1993. **22 Suppl 8**: p. S336-338.
203. Naeije, R., and Dewachter, L.; *[Animal models of pulmonary arterial hypertension]*. Revue des Maladies Respiratoires. 2007. **24**(4 Pt 1): p. 481-496.
204. Jones, P.L., and Rabinovitch, M.; *Tenascin-C is induced with progressive pulmonary vascular disease in rats and is functionally related to increased smooth muscle cell proliferation*. Circulation Research. 1996. **79**(6): p. 1131-1142.
205. Gomez-Arroyo, J.G., Farkas, L., Alhussaini, A.A., Farkas, D., Kraskauskas, D., Voelkel, N.F., and Bogaard, H.J.; *The monocrotaline model of pulmonary hypertension in perspective*. Am J Physiol Lung Cell Mol Physiol. 2012. **302**(4): p. L363-369.
206. Puri, A., McGoon, M.D., and Kushwaha, S.S.; *Pulmonary arterial hypertension: current therapeutic strategies*. Nat Clin Pract Cardiovasc Med. 2007. **4**(6): p. 319-329.
207. Chopra, S., Badyal, D.K., Baby, P.C., and Cherian, D.; *Pulmonary arterial hypertension: advances in pathophysiology and management*. Indian J Pharmacol. 2012. **44**(1): p. 4-11.
208. Badesch, D.B., Abman, S.H., Simonneau, G., Rubin, L.J., and McLaughlin, V.V.; *Medical therapy for pulmonary arterial hypertension: updated ACCP evidence-based clinical practice guidelines*. Chest. 2007. **131**(6): p. 1917-1928.
209. Ghofrani, H.A., Rose, F., Schermuly, R.T., Olschewski, H., Wiedemann, R., Kreckel, A., Weissmann, N., Ghofrani, S., Enke, B., Seeger, W., and Grimminger, F.; *Oral sildenafil as long-term adjunct therapy to inhaled iloprost in severe pulmonary arterial hypertension*. Journal of the American College of Cardiology. 2003. **42**(1): p. 158-164.
210. Ikeda, D., Tsujino, I., Ohira, H., Itoh, N., Kamigaki, M., Ishimaru, S., Sakaue, S., and Nishimura, M.; *Addition of oral sildenafil to beraprost is a safe and effective therapeutic option for patients with pulmonary hypertension*. Journal of Cardiovascular Pharmacology. 2005. **45**(4): p. 286-289.
211. Keogh, A.M., Mayer, E., Benza, R.L., Corris, P., Dartevelle, P.G., Frost, A.E., Kim, N.H., Lang, I.M., Pepke-Zaba, J., and Sandoval, J.; *Interventional and surgical modalities of treatment in pulmonary hypertension*. Journal of the American College of Cardiology. 2009. **54**(1 Suppl): p. S67-77.
212. Fuso, L., Baldi, F., and Di Perna, A.; *Therapeutic strategies in pulmonary hypertension*. Front Pharmacol. 2011. **2**: p. 21.
213. Rich, S., Kaufmann, E., and Levy, P.S.; *The effect of high doses of calcium-channel blockers on survival in primary pulmonary hypertension*. New England Journal of Medicine. 1992. **327**(2): p. 76-81.

214. Badesch, D.B., McLaughlin, V.V., Delcroix, M., Vizza, C.D., Olschewski, H., Sitbon, O., and Barst, R.J.; *Prostanoid therapy for pulmonary arterial hypertension*. Journal of the American College of Cardiology. 2004. **43**(12): p. 56s-61s.
215. Galli, G.L., Skovgaard, N., Abe, A.S., Taylor, E.W., and Wang, T.; *The role of nitric oxide in the regulation of the systemic and pulmonary vasculature of the rattlesnake, Crotalus durissus terrificus*. J Comp Physiol B. 2005. **175**(3): p. 201-208.
216. Waxman, A.B., and Zamanian, R.T.; *Pulmonary arterial hypertension: new insights into the optimal role of current and emerging prostacyclin therapies*. American Journal of Cardiology. 2013. **111**(5 Suppl): p. 1A-16A; quiz 17A-19A.
217. Barst, R.J., Rubin, L.J., Long, W.A., McGoon, M.D., Rich, S., Badesch, D.B., Groves, B.M., Tapson, V.F., Bourge, R.C., Brundage, B.H., Koerner, S.K., Langleben, D., Keller, C.A., Murali, S., Uretsky, B.F., Clayton, L.M., Jobsis, M.M., Blackburn, S.D., Shortino, D., and Crow, J.W.; *A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension*. New England Journal of Medicine. 1996. **334**(5): p. 296-301.
218. Sitbon, O., Humbert, M., Nunes, H., Parent, F., Garcia, G., Herve, P., Rainisio, M., and Simonneau, G.; *Long-term intravenous epoprostenol infusion in primary pulmonary hypertension: prognostic factors and survival*. Journal of the American College of Cardiology. 2002. **40**(4): p. 780-788.
219. Barst, R.J., McGoon, M., McLaughlin, V., Tapson, V., Rich, S., Rubin, L., Wasserman, K., Oudiz, R., Shapiro, S., Robbins, I.M., Channick, R., Badesch, D., Rayburn, B.K., Flinchbaugh, R., Sigman, J., Arneson, C., and Jeffs, R.; *Beraprost therapy for pulmonary arterial hypertension*. Journal of the American College of Cardiology. 2003. **41**(12): p. 2119-2125.
220. Hoeper, M.M., Schwarze, M., Ehlerding, S., Adler-Schuermeyer, A., Spiekerkoetter, E., Niedermeyer, J., Hamm, M., and Fabel, H.; *Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue*. New England Journal of Medicine. 2000. **342**(25): p. 1866-1870.
221. Rubin, L.J., Badesch, D.B., Barst, R.J., Galie, N., Black, C.M., Keogh, A., Pulido, T., Frost, A., Roux, S., Leconte, I., Landzberg, M., and Simonneau, G.; *Bosentan therapy for pulmonary arterial hypertension*. New England Journal of Medicine. 2002. **346**(12): p. 896-903.
222. Elshaboury, S.M., and Anderson, J.R.; *Ambrisentan for the treatment of pulmonary arterial hypertension: improving outcomes*. Patient Prefer Adherence. 2013. **7**: p. 401-409.
223. Garg, N., Sharma, M.K., and Sinha, N.; *Role of oral sildenafil in severe pulmonary arterial hypertension: clinical efficacy and dose response relationship*. International Journal of Cardiology. 2007. **120**(3): p. 306-313.
224. Galie, N., Brundage, B.H., Ghofrani, H.A., Oudiz, R.J., Simonneau, G., Safdar, Z., Shapiro, S., White, R.J., Chan, M., Beardsworth, A., Frumkin, L., and Barst, R.J.; *Tadalafil therapy for pulmonary arterial hypertension*. Circulation. 2009. **119**(22): p. 2894-2903.
225. Channick, R.N.; *Combination therapy in pulmonary arterial hypertension*. American Journal of Cardiology. 2013. **111**(8 Suppl): p. 16C-20C.
226. O'Callaghan, D.S., Savale, L., Jais, X., Natali, D., Montani, D., Humbert, M., Simonneau, G., and Sitbon, O.; *Evidence for the use of combination targeted therapeutic approaches for the management of pulmonary arterial hypertension*. Respiratory Medicine. 2010. **104 Suppl 1**: p. S74-80.
227. Hoeper, M.M., Taha, N., Bekjarova, A., Gatzke, R., and Spiekerkoetter, E.; *Bosentan treatment in patients with primary pulmonary hypertension receiving nonparenteral prostanoids*. European Respiratory Journal. 2003. **22**(2): p. 330-334.
228. McLaughlin, V.V., Oudiz, R.J., Frost, A., Tapson, V.F., Murali, S., Channick, R.N., Badesch, D.B., Barst, R.J., Hsu, H.H., and Rubin, L.J.; *Randomized study of adding inhaled iloprost to existing bosentan in pulmonary arterial hypertension*. American Journal of Respiratory and Critical Care Medicine. 2006. **174**(11): p. 1257-1263.
229. Humbert, M., Barst, R.J., Robbins, I.M., Channick, R.N., Galie, N., Boonstra, A., Rubin, L.J., Horn, E.M., Manes, A., and Simonneau, G.; *Combination of bosentan with epoprostenol in pulmonary arterial hypertension: BREATHE-2*. European Respiratory Journal. 2004. **24**(3): p. 353-359.
230. Stiebellehner, L., Petkov, V., Vonbank, K., Funk, G., Schenk, P., Ziesche, R., and Block, L.H.; *Long-term treatment with oral sildenafil in addition to continuous IV epoprostenol in patients with pulmonary arterial hypertension*. Chest. 2003. **123**(4): p. 1293-1295.
231. Wilkens, H., Guth, A., Konig, J., Forestier, N., Cremers, B., Hennen, B., Bohm, M., and Sybrecht, G.W.; *Effect of inhaled iloprost plus oral sildenafil in patients with primary pulmonary hypertension*. Circulation. 2001. **104**(11): p. 1218-1222.
232. Gomberg-Maitland, M., McLaughlin, V., Gulati, M., and Rich, S.; *Efficacy and safety of sildenafil added to treprostinil in pulmonary hypertension*. American Journal of Cardiology. 2005. **96**(9): p. 1334-1336.

233. Morice, A.H., Mulrennan, S., and Clark, A.; *Combination therapy with bosentan and phosphodiesterase-5 inhibitor in pulmonary arterial hypertension*. European Respiratory Journal. 2005. **26**(1): p. 180; author reply 180-181.
234. Baliga, R.S., MacAllister, R.J., and Hobbs, A.J.; *New perspectives for the treatment of pulmonary hypertension*. British Journal of Pharmacology. 2011. **163**(1): p. 125-140.
235. Ishikura, K., Yamada, N., Ito, M., Ota, S., Nakamura, M., Isaka, N., and Nakano, T.; *Beneficial acute effects of rho-kinase inhibitor in patients with pulmonary arterial hypertension*. Circ J. 2006. **70**(2): p. 174-178.
236. Souza, R., Sitbon, O., Parent, F., Simonneau, G., and Humbert, M.; *Long term imatinib treatment in pulmonary arterial hypertension*. Thorax. 2006. **61**(8): p. 736.
237. Ghofrani, H.A., Morrell, N.W., Hoeper, M.M., Olschewski, H., Peacock, A.J., Barst, R.J., Shapiro, S., Golpon, H., Toshner, M., Grimminger, F., and Pascoe, S.; *Imatinib in pulmonary arterial hypertension patients with inadequate response to established therapy*. American Journal of Respiratory and Critical Care Medicine. 2010. **182**(9): p. 1171-1177.
238. Ghofrani, H.A., and Grimminger, F.; *Soluble guanylate cyclase stimulation: an emerging option in pulmonary hypertension therapy*. Eur Respir Rev. 2009. **18**(111): p. 35-41.
239. Grimminger, F., Weimann, G., Frey, R., Voswinckel, R., Thamm, M., Bolkow, D., Weissmann, N., Muck, W., Unger, S., Wensing, G., Schermuly, R.T., and Ghofrani, H.A.; *First acute haemodynamic study of soluble guanylate cyclase stimulator riociguat in pulmonary hypertension*. European Respiratory Journal. 2009. **33**(4): p. 785-792.
240. Petkov, V., Mosgoeller, W., Ziesche, R., Raderer, M., Stiebellehner, L., Vonbank, K., Funk, G.C., Hamilton, G., Novotny, C., Burian, B., and Block, L.H.; *Vasoactive intestinal peptide as a new drug for treatment of primary pulmonary hypertension*. Journal of Clinical Investigation. 2003. **111**(9): p. 1339-1346.
241. Nagaya, N., Okumura, H., Uematsu, M., Shimizu, W., Ono, F., Shirai, M., Mori, H., Miyatake, K., and Kangawa, K.; *Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats*. Am J Physiol Heart Circ Physiol. 2003. **285**(5): p. H2125-2131.
242. Kao, P.N.; *Simvastatin treatment of pulmonary hypertension: an observational case series*. Chest. 2005. **127**(4): p. 1446-1452.
243. Morrell, N.W., Higham, M.A., Phillips, P.G., Shakur, B.H., Robinson, P.J., and Beddoes, R.J.; *Pilot study of losartan for pulmonary hypertension in chronic obstructive pulmonary disease*. Respir Res. 2005. **6**: p. 88.
244. Laemmli, U.K.; *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature. 1970. **227**(5259): p. 680-685.
245. Todd, L., Mullen, M., Olley, P.M., and Rabinovitch, M.; *Pulmonary toxicity of monocrotaline differs at critical periods of lung development*. Pediatric Research. 1985. **19**(7): p. 731-737.
246. Rhodes, C.J., Davidson, A., Gibbs, J.S., Wharton, J., and Wilkins, M.R.; *Therapeutic targets in pulmonary arterial hypertension*. Pharmacology and Therapeutics. 2009. **121**(1): p. 69-88.
247. Wohlschlaeger, J., Meier, B., Schmitz, K.J., Takeda, A., Takeda, N., Vahlhaus, C., Levkau, B., Stypmann, J., Schmid, C., Schmid, K.W., and Baba, H.A.; *Cardiomyocyte survivin protein expression is associated with cell size and DNA content in the failing human heart and is reversibly regulated after ventricular unloading*. J Heart Lung Transplant. 2010. **29**(11): p. 1286-1292.
248. Abbate, A., Scarpa, S., Santini, D., Palleiro, J., Vasaturo, F., Miller, J., Morales, C., Vetrovec, G.W., and Baldi, A.; *Myocardial expression of survivin, an apoptosis inhibitor, in aging and heart failure. An experimental study in the spontaneously hypertensive rat*. International Journal of Cardiology. 2006. **111**(3): p. 371-376.
249. Sun, C., Nettesheim, D., Liu, Z., and Olejniczak, E.T.; *Solution structure of human survivin and its binding interface with Smac/Diablo*. Biochemistry. 2005. **44**(1): p. 11-17.
250. Mansour, A., Nabil, M., Ali-Labib, R., Said, H., and Annos, F.; *Reciprocal expression of survivin and SMAC/DIABLO in primary breast cancer*. Med Oncol. 2012. **29**(4): p. 2535-2542.
251. McNeish, I.A., Lopes, R., Bell, S.J., McKay, T.R., Fernandez, M., Lockley, M., Wheatley, S.P., and Lemoine, N.R.; *Survivin interacts with Smac/DIABLO in ovarian carcinoma cells but is redundant in Smac-mediated apoptosis*. Experimental Cell Research. 2005. **302**(1): p. 69-82.
252. Ogura, A., Watanabe, Y., Iizuka, D., Yasui, H., Amitani, M., Kobayashi, S., Kuwabara, M., and Inanami, O.; *Radiation-induced apoptosis of tumor cells is facilitated by inhibition of the interaction between Survivin and Smac/DIABLO*. Cancer Letters. 2008. **259**(1): p. 71-81.
253. Bao, S.T., Gui, S.Q., and Lin, M.S.; *Relationship between expression of Smac and Survivin and apoptosis of primary hepatocellular carcinoma*. Hepatobiliary Pancreat Dis Int. 2006. **5**(4): p. 580-583.