

Oxygen sensing, mitochondrial biology and experimental therapeutics for pulmonary hypertension and cancer

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Abstract (300 words)

The homeostatic oxygen sensing system (HOSS) optimizes systemic oxygen delivery. Specialized tissues utilize a conserved mitochondrial sensor, often involving NDUFS2 in complex I of the mitochondrial electron transport chain, as a site of pO₂-responsive production of reactive oxygen species (ROS). These ROS are converted to a diffusible signaling molecule, hydrogen peroxide (H₂O₂), by superoxide dismutase (SOD2). H₂O₂ exits the mitochondria and regulates ion channels and enzymes, altering plasma membrane potential, intracellular Ca²⁺ and Ca²⁺-sensitization and controlling acute, adaptive, responses to hypoxia that involve changes in ventilation, vascular tone and neurotransmitter release. Subversion of this O₂-sensing pathway creates a *pseudohypoxic state* that promotes disease progression in pulmonary arterial hypertension (PAH) and cancer. Pseudohypoxia is a state in which biochemical changes, normally associated with hypoxia, occur despite normal pO₂. Epigenetic silencing of SOD2 by DNA methylation alters H₂O₂ production, activating hypoxia-inducible factor 1 α , thereby disrupting mitochondrial metabolism and dynamics, accelerating cell proliferation and inhibiting apoptosis. Other epigenetic mechanisms, including dysregulation of microRNAs (miR), increase pyruvate dehydrogenase kinase and pyruvate kinase muscle isoform 2 expression in both diseases, favoring uncoupled aerobic glycolysis. This Warburg metabolic shift also accelerates cell proliferation and impairs apoptosis. Disordered mitochondrial dynamics, increased mitotic fission and impaired fusion, promotes disease progression in PAH and cancer. Epigenetic upregulation of dynamin-related protein 1 (Drp1) and its binding partners, MiD49 and MiD51, contributes to the pathogenesis of PAH and cancer. Finally, dysregulation of intramitochondrial Ca²⁺, resulting from impaired mitochondrial calcium uniporter complex (MCUC) function, links abnormal mitochondrial metabolism and dynamics. MiR-mediated decreases in MCUC function reduce intramitochondrial Ca²⁺, promoting Warburg metabolism, whilst increasing cytosolic Ca²⁺, promoting fission. Epigenetically disordered mitochondrial O₂-sensing, metabolism, dynamics, and Ca²⁺ homeostasis offer new therapeutic targets for PAH and cancer. Promoting glucose oxidation, restoring the fission/fusion balance, and restoring mitochondrial calcium regulation are promising experimental therapeutic strategies.

Keywords

ABT-263 (Navitoclax); ABT-199 (Venetoclax); B-cell lymphoma 2 (BCL-2); DNA methylation; DNA methyltransferase (DNMT); dynamin-related protein 1 (Drp1); group 1 pulmonary hypertension, hypoxia-inducible factor 1 α (HIF-1 α); hypoxia-inducible factor 2 α (HIF-2 α); hypoxic pulmonary vasoconstriction; mammalian target of rapamycin (mTOR); microRNA (miRNA); miR-138; miR-25; mitochondrial calcium uniporter (MCU); mitochondrial dynamics protein of 49 kDa (MiD49); mitochondrial dynamics protein of 51 kDa (MiD51); mitofusin 2 (Mfn2); mitophagy; monocrotaline; oxygen sensing; peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α); pyruvate dehydrogenase (PDH); pyruvate dehydrogenase kinase (PDK); pyruvate kinase muscle isoform 2 (PKM2); reactive oxygen species (ROS); Sugen5416; survivin; von Hippel-Lindau protein (VHL)

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Abbreviations

| Abbreviation | Full name |
|-------------------------------------|--|
| 4-AP | 4-aminopyridine, a Kv channel inhibitor |
| 5-AZA | 5-aza-2'-deoxycytidine |
| 6MWD | 6-minute walk distance |
| acetyl-CoA | acetyl coenzyme A |
| AIF | apoptosis-inducing factor |
| Akt | protein kinase B |
| AMPK | AMP-activated protein kinase |
| Ang | angiopoietin |
| <i>ANGPT2</i> | gene encoding angiopoietin 2 |
| Apaf-1 | apoptosis activating factor 1 |
| AR | androgen receptor |
| ATF6 | activating transcription factor 6 |
| ATP | adenosine triphosphate |
| Bcl-2 | B-cell lymphoma 2 |
| BH3 | Bcl-2 homology 3 |
| BMPR2 | bone morphogenetic protein receptor type II, encoded by <i>BMPR2</i> |
| BNIP3 | adenovirus E1B 19-kDa-interacting protein 3 |
| BNIP3L | BNIP3-like, also known as NIX |
| BORIS | brother of regulator of imprinted sites |
| BTIC | brain tumor initiating cell |
| C76 | compound 76, a HIF-2 α inhibitor |
| [Ca ²⁺] _{cyto} | cytosolic calcium |
| [Ca ²⁺] _{mito} | mitochondrial calcium |
| Ca _L | voltage-gated calcium channel |
| CAMK | Ca ²⁺ /calmodulin-dependent protein kinase |
| CBS | cystathionine β -synthase |
| CDK | cyclin-dependent kinase |
| Cyt c | cytochrome <i>c</i> |
| DCA | dichloroacetate |
| Dnm2 | dynamamin 2 |
| DNMT | DNA methyltransferase |
| Drp1 | dynamamin-related protein 1, also called dynamamin-1-like protein |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor, encoded by <i>EGFR</i> |
| EMRE | essential MCU regulator |
| EMT | epithelial-to-mesenchymal transition |

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| ER | endoplasmic reticulum |
| ERBB2 | human epidermal growth factor receptor 2 |
| ERK2 | mitogen-activated protein kinase 1 |
| ERR | estrogen-related receptor |
| ETC | electron transport chain |
| FDG | 2- ^[18F] -Fluoro-2-deoxy-d-glucose |
| FHR | fawn hooded rats |
| FIS1 | mitochondrial fission 1 protein |
| FOXO1 | forkhead box protein O1 |
| FRAP | FKBP-rapamycin-associated protein |
| G-6-P | glucose-6-phosphate |
| GLUT | glucose transporter |
| H ₂ O ₂ | hydrogen peroxide |
| HER2 | human epidermal growth factor receptor-2 |
| HIF | hypoxia-inducible factor |
| HK | hexokinase |
| hnRNP | heterogenous nuclear ribonucleoprotein |
| HOSS | homeostatic oxygen-sensing system |
| HPV | hypoxic pulmonary vasoconstriction |
| HRE | hypoxia response element |
| HSG | hyperplasia suppressor gene |
| Hsp70 | heat shock protein 70 |
| HyPer-dCyto | compartment-targeted probe for cytosolic H ₂ O ₂ |
| HyPer-dMito | compartment-targeted probe for mitochondrial H ₂ O ₂ |
| IGF | insulin-like growth factor |
| IGFBP | IGF-binding protein |
| I _k | potassium current |
| ILK | integrin-linked kinase |
| IMM | inner mitochondrial membrane |
| IMS | intermembrane space |
| IR | ischemia-reperfusion |
| JNK | c-Jun N-terminal kinase |
| KNK437 | heat shock protein inhibitor I |
| Kv | voltage-gated potassium channel |
| LC3 | light chain 3 |
| LDH-A | lactate dehydrogenase A, encoded by <i>LDHA</i> |
| MCT | monocrotaline |
| MCU | mitochondrial Ca ²⁺ uniporter |

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|------------------------------|--|
| MCUC | mitochondrial Ca ²⁺ uniporter complex |
| mdivi-1 | mitochondrial division inhibitor |
| MET | mesenchymal-to-epithelial transition |
| Mff | mitochondrial fission factor |
| Mfn | mitofusin |
| MICU1 | mitochondrial calcium uptake 1 |
| MiD49 | mitochondrial dynamics protein of 49 kDa |
| MiD51 | mitochondrial dynamics protein 51 kDa |
| miRNA | microRNA |
| MLS | mitochondrial localization sequence |
| MMP | matrix metalloproteinase |
| MnTBAP | Mn(III)tetrakis(4-benzoic acid)porphyrin chloride, a SOD mimic |
| mPAP | mean pulmonary artery pressure |
| MPC | mitochondrial pyruvate carrier |
| MPP | mitochondrial processing peptidase |
| mtDNA | mitochondrial DNA |
| mTOR | the mammalian target of rapamycin |
| mTORC | mammalian target of rapamycin complex |
| NDP52 | nuclear dot protein 52 kDa |
| NIMA-1 | never in mitosis A-1, also known as PIN1 |
| Nrf | nuclear respiratory factor |
| NSCLC | non-small cell lung cancer |
| O ₂ ^{•-} | superoxide anion |
| OMM | outer mitochondrial membrane |
| OPA1 | optic atrophy 1 |
| OPTN | optineurin |
| OXPHOS | oxidative phosphorylation |
| p-Drp1 _{S616} | phosphorylated Drp1 at serine 616 |
| p-Drp1 _{S637} | phosphorylated Drp1 at serine 637 |
| PA | pulmonary artery |
| PAEC | pulmonary artery endothelial cell |
| PAfib | pulmonary artery fibroblast |
| PAH | pulmonary arterial hypertension |
| PAP | pulmonary artery pressure |
| PAR-2 | protease-activated receptor-2 |
| PARL | presenilin-associated rhomboid-like |
| PASMC | pulmonary artery smooth muscle cell |
| PDC | pyruvate dehydrogenase complex |

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|-----------------|--|
| PDGF | platelet-derived growth factor |
| PDH | pyruvate dehydrogenase |
| PDK | pyruvate dehydrogenase kinase |
| PET | positron emission tomography |
| PGC-1 α | peroxisome proliferator-activated receptor γ coactivator-1 α |
| PH | pulmonary hypertension |
| PHD | prolyl hydroxylase |
| PI3K | phosphoinositide 3-kinase |
| PINK1 | PTEN-induced putative kinase |
| PK | pyruvate kinase |
| PKM | pyruvate kinase muscle isoform |
| pO ₂ | partial pressure of oxygen |
| PPHN | pulmonary hypertension of the newborn |
| PTB | polypyrimidine tract-binding protein |
| PTBP1 | polypyrimidine-tract-binding protein 1 |
| PTEN | phosphatase and tensin homolog |
| PVR | pulmonary vascular resistance |
| RCC | renal cell carcinoma |
| REDOX | reduction and oxidation |
| ROS | reactive oxygen species |
| RuR | ruthenium red |
| RV | right ventricle |
| RVF | right ventricular failure |
| RVfib | right ventricular fibroblast |
| SAB | supra-aortic banding |
| siRNA | small interfering RNA |
| <i>SIRT3</i> | gene encoding sirtuin 3 |
| SMC | smooth muscle cell |
| SOD2 | superoxide dismutase |
| Su/Hx | Sugen5416/hypoxia, a preclinical model of PAH |
| <i>TEK</i> | gene encoding TEK receptor tyrosine kinase (angiopoietin-1 receptor) |
| TEMPOL | 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl, a SOD mimic |
| TFAM | mitochondrial transcription factor A |
| TGF- α | transforming growth factor- α , encoded by <i>TGFA</i> |
| Tie2 | angiopoietin-1 receptor |
| TNBC | triple negative breast cancer |
| TRPC | transient receptor potential channel |
| UCP2 | mitochondrial uncoupling protein 2, encoded by <i>UCP2</i> |
| VDAC1 | voltage-dependent-anion-selective channel 1 |

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|--------------|--|
| VEGF | vascular endothelial growth factor |
| <i>VEGFA</i> | gene encoding vascular endothelial growth factor A |
| VHL | von Hippel-Lindau protein |

1 Oxygen-sensing and experimental therapeutics in pulmonary arterial hypertension and cancer

1.1 Normal oxygen-sensing in pulmonary vs systemic arteries

Specialized tissues are specifically adapted to sense small changes in airway oxygen levels and arterial oxygen (pO_2) within the physiological range [1]. These tissues, which make up the homeostatic oxygen-sensing system (HOSS), elicit changes in respiration, vascular tone and neurosecretion to adapt to changing environmental oxygen levels, or adapt to localized changes in pO_2 within the body. Type 1 cells within the carotid body respond to hypoxia via exocytosis of neurotransmitters [2]. The smooth muscle cells (SMC) within the resistance pulmonary arteries (PA), fetoplacental arteries, and ductus arteriosus respond to changes in pO_2 through alterations in vascular tone [1, 3]. Interestingly, hypoxia elicits vasodilation of the ductus arteriosus and systemic arteries, while stimulating vasoconstriction of the fetoplacental and resistance PA. These opposing responses are coordinated to enhance oxygen uptake and systemic oxygen delivery (Figure 1).

Hypoxic pulmonary vasoconstriction (HPV) is a homeostatic mechanism that optimizes oxygen uptake by matching perfusion to ventilation in the lung. HPV is intrinsic to the pulmonary circulation, functioning most strongly in small, resistance level pulmonary arteries. In response to environmental hypoxia, HPV manifests ‘globally’ and all small pulmonary arteries constrict, resulting in a rise in pulmonary artery pressure (PAP). In contrast, in most lung diseases, such as pneumonia or atelectasis, segmental hypoxia results in localized HPV, which diverts blood flow to better-oxygenated lung segments [4]. This localized vasoconstrictor response improves oxygen uptake without elevating PAP. HPV occurs within seconds after the onset of modest levels of airway hypoxia, with constriction reaching a maximum intensity within minutes [5, 6]. Unless adverse vascular remodeling occurs, HPV is rapidly reversible upon restoration of normal airway oxygen levels [7, 8] (Figure 1).

1.1.1 Mechanisms of HPV

Although endothelial-derived vasoconstrictors and vasodilators do play an important role in modulating PA tone [9, 10], the core mechanism of HPV is intrinsic to the pulmonary artery smooth muscle cells (PASMC), and HPV persists in endothelium-denuded PA rings [11].

Oxygen sensing within the PASMC involves the coordination of an upstream sensor of alveolar oxygen and a downstream effector mechanism, comprised of several types of oxygen-sensitive ion channels. In response to changing oxygen tension, the sensor(s) within the mitochondria alters production of reactive oxygen species (ROS), notably superoxide anion. ROS are then locally and rapidly converted into H₂O₂ by mitochondrial superoxide dismutase 2 (SOD2). H₂O₂ diffuses from the mitochondria to the cell membrane where it modifies the activity of specific redox-sensitive potassium (K⁺) and calcium (Ca²⁺) channels. These channels contain redox-sensitive amino acids in key positions, such as cysteine residues, and are thereby susceptible to reduction and oxidation (REDOX) regulation of channel gating and open-state probability [12, 13]. Redox-mediated changes in the kinetics and gating of these ion channels ultimately alter membrane polarization and/or calcium influx into the oxygen-sensitive cell, leading to the physiologic response. These immediate redox changes in ion channel function are reinforced by redox regulation of enzymes, notably rho kinase, which sustains hypoxic responses.

Pharmacologic and electrophysiological studies using the patch clamp technique in isolated PASMC have identified voltage-gated potassium channels (K_v), as well as large-conductance, voltage-gated calcium channels (Ca_L), as major effectors of HPV. In the resistance pulmonary vessels, K_v channels are the predominant regulators of resting membrane potential and are major effectors of HPV [14]. The whole cell-patch clamp method has shown that a tonic, normoxic outward potassium current (I_K) within the PASMC prevents Ca_L channels from opening during normoxia. Pharmacological profiling of the ion channel responsible for HPV points to K_v channels. The K_v channel inhibitor 4-aminopyridine (4-AP), mimics the pulmonary vasoconstrictor effects of hypoxia and like hypoxia inhibits I_K and elicits membrane depolarization [15] (Figure 2). The vasoconstrictor effects of 4-AP and hypoxia are not additive, suggesting they act through a shared mechanism. In contrast, inhibitors of other channel types, such as TASK, BK_{Ca} and K_{ATP} channels do not cause normoxic pulmonary vasoconstriction and hypoxia causes constriction when superimposed on lungs treated with effective doses of these channel blockers.

In PASMC, but not in systemic arterial SMC, hypoxia inhibits Kv as well as other K⁺ channel types. The tonic egress of intracellular K⁺ down its gradient (140 mM intracellular to 5 mM extracellular) establishes a resting membrane potential of ~-60mV in normoxic PASMC. Kv channel inhibition, whether initiated by hypoxia or 4-AP, leads to accumulation of positive charges within the cytoplasm and plasma membrane depolarization (from ~-60mV to ~-20mV). This degree of membrane depolarization activates Ca_L channels, which have a voltage sensor [16]. The resulting influx of calcium down its colossal concentration gradient (2 mM extracellular to 100 nM intracellular) initiates SMC contraction. The source of increasing cytosolic [Ca²⁺] that follows SMC membrane depolarization is mainly derived from the extracellular Ca²⁺ pool, as indicated by studies showing that inhibition of Ca_L channels by verapamil completely inhibits the hypoxic pressor response in the isolated rat lung as well as HPV in isolated PA rings [17, 18]. These electrical and ionic responses begin within seconds and account for the initiation of HPV. However, there is also an important role in the release of intracellular calcium stores and calcium sensitization in HPV, particularly with more prolonged exposure to hypoxia [19, 20].

Although several families of Kv channels exist, the Shaker family (Kv) is the most widely distributed and is implicated in HPV [21, 22]. Within this family of ion channels, Kv1.5 and Kv2.1 are central to the process of HPV. In Kv1.5 knockout mice, a significant reduction in the 4-AP- and oxygen-sensitive portions of I_K and an impairment in HPV are observed [23]. Kv channels are tetramers and the heterotetrameric composition of a Kv channel can impact its oxygen sensitivity. For example, PASMC expressing Kv1.5/Kv1.2 heterotetrameric channels have increased sensitivity to hypoxia in comparison to cells expressing homomeric channels [24]. In addition, the heterotetrameric Kv2.1/Kv9.3 channel is also oxygen-sensitive, suggesting it has a role in HPV [25] (Figure 3).

1.1.2 ROS as Mediators of Oxygen-Sensing

Weir and Archer *et al.* first demonstrated that changes in the redox status of the PASMC regulate PASMC membrane polarization and contraction [11]. Specifically, antioxidants cause membrane depolarization of PASMC and elicit vasoconstriction of PA rings, whereas oxidants cause membrane hyperpolarization and relaxation of constricted PA rings. These findings

suggest that in the PA, reducing agents mimic hypoxia whilst oxidants mimic normoxia. Additionally, these findings demonstrated that changing the redox status of the SMC directly affects I_K . Reducing or oxidizing key cysteine residues within Kv channels alters their open state probability and can thereby regulate membrane potential, Ca_L opening and vascular tone [26]. Beyond the indirect effects of redox state on Ca^{2+} flux mediated by changes in membrane potential, Ca_L channels are directly responsive to changes in pO_2 [27].

The mechanism responsible for altering redox status during SMC oxygen-sensing is thought to reside within the electron transport chain (ETC) of the mitochondria. As electrons flow through the inner mitochondrial membrane, leakage of electrons occurs at several distinct sites [28]. Leaked electrons from complexes I and III combine with molecular oxygen to produce superoxide anion ($O_2^{\cdot-}$), a toxic form of ROS [29-31]. Superoxide is quickly converted into hydrogen peroxide (H_2O_2) by the actions of SOD2, a less toxic ROS with a larger diffusion radius [3, 32]. Both superoxide anion and H_2O_2 are capable of oxidizing different classes of cellular targets; H_2O_2 has an important role as a signaling molecule due to its ability to oxidize thiol moieties on cysteine or methionine residues, causing structural and functional changes in target proteins through the formation of disulfide bridges [33]. This redox signaling mechanism is one of the means by which mitochondria to regulate ion channels and enzymes.

There is teleological logic to the ETC serving as a sensor since the ETC relies on oxygen as its terminal electron acceptor, and this upstream redox sensor function leads to changes in protein function that optimize oxygen delivery to mitochondria. During HPV, a decrease in the electron flux through the ETC leads to decreased ROS production, shifting the cell to a more reduced state and promoting reductive modification of K^+ channels. These modifications reduce the open state probability of Kv channels causing membrane depolarization, leading to Ca^{2+} influx and subsequent vasoconstriction. Hypoxia does not have this effect on renal [34] or mesenteric arterial SMC [35], a reminder that there is mitochondrial diversity within vascular SMC, with PASMC having mitochondria that are functionally different than those in systemic arterial SMC [34]. While both pulmonary and systemic arteries constrict in response to 4-AP, the PAs uniquely constrict to hypoxia whilst systemic arteries relax. Moreover, the ETC inhibitors, rotenone and antimycin A cause pulmonary vasoconstriction but elicit

systemic arterial vasodilatation. Thus it is mitochondrial heterogeneity (rather than ion channel heterogeneity) that primarily determines the differential response of pulmonary versus systemic vascular beds to hypoxia. Mitochondrial heterogeneity includes increased expression of key enzymes in in PASMCMitochondria, including SOD2, and their unique ability to vary ROS production in response to physiologic hypoxia, which is absent in systemic arterial SMC. Differential production of mitochondria-derived, normoxic vasodilating factors, including H₂O₂, underlies the opposing effects of hypoxia on the PAs versus systemic arteries.

Although the role of the mitochondria in producing ROS is well established, the vector of change in ROS production in response to physiological hypoxia (decreased vs increased) remains controversial. While several investigators, including our group, report that ROS decrease in direct proportion to falling pO₂ with hypoxia [36-41], other groups found that ROS production increases in response to hypoxia [42-48]. These contrasting findings have been attributed to the degree of hypoxia used to assess HPV (hypoxia vs anoxia), the use of freshly isolated PASMCMitochondria versus passaged cell lines, as well as the complexity of measuring ROS in subcellular compartments. An interesting finding by Lopez-Barneo *et al.* suggests that ROS production during hypoxia differs depending on the mitochondrial compartment measured [49]. In the carotid body type 1 cell, hypoxia increases ROS production in the mitochondrial intermembrane space (IMS) while decreasing ROS production in the mitochondrial matrix. Similarly, Waypa *et al.* demonstrated that hypoxia induces compartmental effects on ROS production within vascular SMC, with the mitochondrial matrix becoming more reduced and the cytosol and mitochondrial IMS becoming more oxidized in response to hypoxia [50]. However, our group has consistently reported that in freshly isolated, resistance level PASMCMitochondria or resistance level PA rings, under physiologic hypoxia (pO₂ 40-60mmHg) and physiologic pH (7.35-7.45), ROS decrease, both in aggregate, in the cytosol and in the mitochondria. Both Lopez-Barneo and Waypa's findings, in terms of effects of hypoxia on mitochondrial ROS production in the mitochondrial matrix, are thus consistent with our findings. The relative role of ROS compartments within the mitochondria on oxygen sensing requires further exploration.

It is important to note that despite the controversy pertaining to the direction of ROS changes in response to hypoxia, and whether or not these changes depend on the cellular compartment

measured, there is a consensus that ROS are mitochondrially-derived and act as the diffusible signaling molecules in HPV. Therefore, the signalling ROS must be able to travel out from the mitochondria towards the cytosol and cell membrane, to modulate the activity of redox-sensitive ion channels and elicit changes in vascular tone. In our recent publication, using dynamic, compartment-targeted probes (HyPer-dMito for mitochondrial H₂O₂ and HyPer-dCyto for cytosolic H₂O₂ respectively), we demonstrated decreased levels of H₂O₂ in both the mitochondria and cytosol of PASMC following exposure to acute, physiologic, hypoxia [51] (Figure 3).

1.2 HPV and Pulmonary Arterial Hypertension

Pulmonary hypertension (PH) is defined by a resting mean PAP (mPAP) \geq 20 mmHg but is further subdivided based on underlying aetiology and pathogenesis [52]. According to the Fifth World Symposium on Pulmonary Hypertension, PH is divided into 5 groups: PH due to pulmonary vascular disease (i.e., pulmonary arterial hypertension, PAH, and congenital heart disease Group 1), PH due to left heart disease (Group 2), PH due to lung disease or hypoxia (Group 3), PH due to chronic thromboembolic disease (CTEPH, Group 4), or PH with unclear and/or multifactorial mechanisms (Group 5) [53]. PH of any type increases mortality and can cause right ventricular failure (RVF) and death. Though most forms of PH are not driven by environmental hypoxia (except for Group 3 PH), epigenetic changes in the oxygen sensing pathway (which mimic key biochemical aspects of hypoxia) are involved in many forms of PH.

A hallmark of PAH is adverse pulmonary vascular remodeling, which contributes to increased pulmonary vascular resistance (PVR). The pulmonary arterial wall is divided into three layers: the intima, media and adventitia. Pulmonary artery endothelial cells (PAEC) and PASMC are the principal components of the intimal and medial layers, respectively. The adventitial layer, whose principal resident cells are fibroblasts (PAfib), is less studied. All three layers are involved in the pulmonary remodeling in PH and this is particularly evident in Group 1 PH, where PAEC dysfunction, increased PASMC proliferation and concomitant impaired apoptosis lead to intimal hyperplasia and medial hypertrophy. Within the adventitia, fibrosis

is also observed due to fibroblast proliferation and increased production of collagen, accompanied by a variable degree of perivascular inflammation [10, 54].

1.3 Abnormal oxygen-sensing in PAH – normoxic activation of Hypoxia-Inducible Factor (HIF)

In response to tissue hypoxia, the HIF family of transcription factors is activated and initiates the transcription of hundreds of genes used to combat hypoxia-induced stress [55, 56]. The HIF protein complex is comprised of two subunits, an alpha subunit (HIF- α), which exists in the cytoplasm, in three distinct isoforms (HIF-1 α , HIF-2 α and HIF-3 α) and a beta subunit (HIF- β), which is stably expressed in the nucleus, independent of pO₂. Although both HIF subunits are persistently synthesized within the cell, the presence of HIF- α subunits is inversely proportional to the concentration of oxygen within the cell [57], reflecting a tonic process of oxygen-dependent HIF- α degradation.

Under normoxic conditions, oxygen-sensitive prolyl hydroxylases (PHD) modify proline residues within the HIF- α subunit. Proline hydroxylation allows the von Hippel-Lindau protein (VHL) to ubiquitinate HIF- α , targeting it for proteasomal degradation [58]. Under hypoxic conditions, PHD becomes inactive. Consequently, HIF- α is not targeted for degradation by VHL, allowing HIF- α to accumulate within the cytoplasm and translocate into the cell nucleus where it dimerizes with HIF- β to form the active HIF complex [58]. HIF complexes in the nucleus are capable of binding to hypoxia response elements (HRE) located within regulatory elements of genes that control processes related to oxygen delivery and oxygen deprivation, including pathways involved in cell metabolism, angiogenesis, erythropoiesis, cell proliferation, and apoptosis [59-61].

While activation of the HIF pathway by environmental hypoxia is often adaptive for the organism, normoxic activation of the HIF- α pathway is a hallmark of numerous pathologies. A rare but highly instructive example is Chuvash disease, which results from a homozygous missense mutation in the VHL gene. Patients with Chuvash disease manifest sustained pulmonary vasoconstriction, polycythemia and spontaneous PH, all findings that would be

expected in the presence of environmental hypoxia, but which occur in the absence of tissue hypoxia [62-64]. In the absence of a functional VHL protein, HIF-1 α subunits become transcriptionally active. Thus, despite normoxic conditions, HIF target genes, such as erythropoietin and vascular endothelial growth factor (VEGF) are upregulated [65-67], causing these patients to display phenotypes similar to those observed during chronic hypoxia. Collectively, the role of pathologic VHL signaling and HIF-1 α activation highlights one example of how subversion of oxygen-sensing pathways contributes to the pathological processes which promote disease pathogenesis in both PAH and cancer (Figure 4).

Chuvash disease demonstrates that a genetic mutation that causes HIF-1 α activation results in a *pseudohypoxic* environment that drives a hyperproliferative, apoptosis-resistant PASMC phenotype. However, disordered oxygen sensing can also occur by epigenetic mechanisms. Fawn hooded rats (FHR), a strain of rat that like Chuvash patients develops PH and polycythemia spontaneously, have normoxic activation of HIF-1 α signaling and spontaneously develop PAH, characterized by excessive proliferation and impaired apoptosis of PASMC [68]. In contrast to Chuvash patients, normoxic activation of HIF-1 α in PAH is largely a result of an epigenetic reduction in expression of mitochondrial SOD2, the main source of mitochondrial-derived H₂O₂. HIF-1 α activation is largely regulated by the cell's redox status. Hypoxic stabilization of HIF-1 α is prevented by H₂O₂ and sulfhydryl oxidants where it is enhanced by reducing agents [69]. This redox regulation of HIF-1 α parallels the redox regulation of ion channels that mediate HPV. In both cases the data support the conclusion that physiologic levels of hypoxia (>40mmHg) is a state of reduction whilst normoxia is a state of oxidation, as originally proposed by Archer and Weir [70].

In both the FHR-PAH model and patients with PAH, SOD2 expression is decreased within the lungs and PA [71]. SOD2 deficiency in the FHR is the result of covalent cytosine methylation of two key CpG islands within the SOD2 gene promoter and enhancer regions [68]. These methylation events, controlled by DNA methyltransferases (DNMT), interfere with the binding of transcription factors. In FHR, upregulation of DNMT1 within the lungs, as well as DNMT3B within PASMC may explain the observed hypermethylation of the SOD2 gene.

Treatment of FHR with the DNMT inhibitor, 5-aza-2'-deoxycytidine (5-AZA), restores SOD2 expression and corrects the rates of PASMC proliferation and apoptosis [68]. The therapeutic potential of 5-AZA is particularly noteworthy as it is approved in the treatment of patients with myeloproliferative disorders [72]. Therapy with the SOD mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) also regresses PAH in the FHR, evident by its ability to reduce right ventricular (RV) hypertrophy, improve functional capacity, and decrease muscularization of pulmonary precapillary resistance vessels [68]. These results are consistent with the effects of 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), a SOD mimetic shown to decrease HPV in rats, as well as recombinant SOD1, which reduces PVR in persistent pulmonary hypertension in newborn lambs [73, 74]. Epigenetic silencing of SOD2 by hypermethylation also enhances cell proliferation in multiple cancer types, including pancreatic cancer and myeloma [75-77]. As in PAH, overexpression of SOD2 increases H₂O₂ levels in cancer cells, which decreases rates of cell proliferation and tumor growth [75, 77, 78]. DNMT3B is also involved in epigenetic silencing of SOD2 within cancer cells. In human breast cancer and A549 cells (a non-small cell lung cancer cell line), depletion of DNMT3B reactivates methylation-silenced gene expression, and induces apoptosis [79].

The HIF-1 α and HIF-2 α subunits have similar DNA binding and dimerization domains but have distinct transactivation domains and effect unique gene targets. For example, HIF-1 α regulates glycolytic genes whilst HIF-2 α does not [80]. However, a number of hypoxia-regulated genes, such as *VEGFA* (encoding VEGF-A), are regulated by both HIFs. Activation of HIF-2 \square occurs in the PAEC of patients with idiopathic PAH and in rodent PAH models [81]. HIF-2 \square activation, secondary to PHD deficiency, contributes to obliterative pulmonary vascular remodeling, leading to RVF and death [82, 83]. HIF-2 \square ^{+/-} mice exposed to chronic hypoxia exhibit diminished increases in RV systolic pressure and RV hypertrophy compared to wild type mice [84]. In PAEC, HIF-2 \square activation induces the production of the vasoconstrictor endothelin-1 and alters nitric oxide homeostasis by increasing the expression of arginase, both effects which contribute to the development of HPV. Pharmacological inhibition of HIF-2 \square translation by the inhibitor compound 76 (C76) [85] can improve survival in rodent models of PAH by inhibiting obliterative pulmonary vascular remodelling, reducing RV hypertrophy and cardiac fibrosis and inhibiting RVF [81]. C76 treatment is found to be selective for HIF-2 \square , without affecting HIF-

1 α in human lung microvascular ECs. Selective HIF-2 α inhibitors, such as PT2385 and its more potent analog, PT2977, have already been tested in clinical trials in the treatment of patients with solid tumors, including glioblastoma and renal cell carcinoma (NCT03216499, NCT02293980, NCT03401788, NCT03634540, NCT02974738). The relative importance of HIF-1 α versus HIF-2 α remains unclear in PAH and cancer.

1.4 HIF-activated metabolic changes in PAH and cancer

PAH is marked by metabolic abnormalities, most notably a shift from oxidative metabolism to aerobic glycolysis, in which glycolysis rates are disproportionately increased in relation to mitochondrial pyruvate utilization and thus glycolysis is considered ‘uncoupled’ [86]. This uncoupling effect is mediated in part, by the actions of HIF-1 α . The two major targets of HIF-1 α responsible for this effect are lactate dehydrogenase A (LDH-A) and pyruvate dehydrogenase kinase (PDK) 1 [87, 88]. LDH-A converts pyruvate into lactate, using NADH as a co-factor. This provides a means of replenishing the pool of NAD⁺ which is required for glycolysis, which would normally be replenished through mitochondrial metabolism. PDK1 phosphorylates and inactivates PDH, preventing the production of acetyl-CoA from pyruvate, and decreasing the net oxidative metabolic rate. In monocrotaline (MCT)-PAH rats, both PDK1 and PDK3 are upregulated and the resulting PDH inhibition contributes to the fibrogenic, hyperproliferative Warburg phenotype of RV fibroblasts (RVfib) [89]. Inhibition of either PDK1 or PDK3 reduces proliferation, mitochondrial fission, and collagen production within the RVfib, suggesting these PDK isoforms are possible therapeutic targets for treating PAH.

The HIF-1 α -PDK-PDH pathway is not the only way that PAH and cancer cells develop Warburg metabolism. Pyruvate kinase (PK) is responsible for the final step of glycolysis, converting phosphoenolpyruvate into pyruvate. PK muscle isoform (PKM) 1 and 2 are isoforms of PK, formed by alternative splicing of the PKM2 RNA transcript. In cancer, a switch from PKM1 to PKM2 expression is partially responsible for increased rates of aerobic glycolysis [90]. Increased expression of PKM2, but not PKM1, increases tumor growth. Within cancer cells, PKM2 is not only a target of HIF-1 α , but also acts as a transcriptional co-activator of HIF-1 α , increasing HIF-1 α binding to HRE and inducing expression of known HIF targets [91]. In this way, PKM2 participates in a positive feedback loop which serves to promote Warburg

metabolism. PKM2 has also been observed to associate with HIF-2 α , increasing its transcriptional activity [91]. In HeLa (human cervical adenocarcinoma) and Hep3B (hepatocellular carcinoma) cells, PKM2 and HIF-1 α expression are associated with increased expression of metabolic genes, including LDH-A and PDK1. Although no association has been found between PKM2-HIF-2 activation and metabolic enzymes, both PKM2-induced HIF-1 and HIF-2 activation induce the expression of VEGF [91].

HIF-1 α also promotes aerobic glycolysis through the regulation of a number of other proteins involved in glucose metabolism. HIF-1 α activation increases the expression of glucose transporters (GLUT) 1 and 3, as well as glycolytic enzymes hexokinase (HK) 1 and 2, aldolase A, enolase, 6-phosphofructo-1-kinase, and phosphoglycerate kinase 1 [92-95]. In both cancer cells and vascular cells from PAH patients, the upregulation of glucose transporters appears to be compensatory, allowing massive increases in glucose flux to maintain ATP homeostasis. It is the reliance of these diseased cells on glucose uptake which accounts for the clinical utility of 2-[¹⁸F]-Fluoro-2-deoxy-d-glucose (FDG)-positron emission tomography (PET) in the detection of cancers and PAH [96, 97]. Using FDG as a radiotracer, PET can visualize the increase in glucose flux that are required to maintain energy homeostasis *in vivo*.

While originally believed to not play a role in the regulation of the glycolytic pathway, HIF-2 α is capable of regulating some of the same gene targets as HIF-1 α , although its role in HIF-activated Warburg metabolism is not well defined [80, 98-101] (Figure 5).

1.5 Abnormal oxygen sensing in cancer

Overexpression/activation of HIF-1 α is common in cancer and can be both oxygen-dependent (due to inadequate blood supply and tumor hypoxia) and oxygen-independent (due to genetic alteration of upstream regulators).

1.5.1 HIF-activated cell proliferation in cancer

One of the major phenotypes of cancer is cell hyperproliferation and apoptosis resistance. It is common for cancer cells to undergo autocrine stimulation by the growth/survival factors they secrete. HIFs regulate a wide variety of growth factors, including transforming growth factor-

□ (TGF-□, encoded by *TGFA*), insulin-like growth factor-2 (IGF-2, encoded by *IGF2*), VEGF, endothelin 1, adrenomedullin and erythropoietin.

TGF-□ is a mitogenic polypeptide belonging to the epidermal growth factor (EGF) family. In clear cell renal cell carcinoma (RCC), the tumor suppressor gene *VHL* is often mutated bi-allelically (*VHL*^{-/-}). As a result, HIF-1 α is activated regardless of the oxygen level in the tissue. Gunaratnam *et al.* found that in *VHL*^{-/-} RCC cells, HIF-1 α promotes cell proliferation by activating the TGFA/EGF receptor (EGFR) pathway [102]. In pancreatic cancer, activation of protease-activated receptor-2 (PAR-2) upregulates both HIF-1 α and HIF-2 α by the integrin-linked kinase (ILK) signaling pathway, which in turn increases the transcription level of *TGFA* [103]. This leads to the progression of human pancreatic cancer. HIF-1 α -dependent *TGFA* activation is also observed in human non-small cell lung cancer (NSCLC) [104].

EGFR (encoded by *EGFR*) is a member of the ErbB family of receptors which includes four receptor tyrosine kinases. Gain-of-function mutations of *EGFR* are found in many types of cancer. The Semenza group first found that activation of phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/protein kinase B (Akt)/FKBP-rapamycin-associated protein (FRAP) pathway contribute to the normoxic activation of HIF-1 α in prostate cancer [105]. Peng *et al.* later reported a crosstalk between the EGFR pathway and the HIF-1 α pathway contributing to the apoptosis-resistant phenotype of cancer cells [106]. In breast cancer cells, activation of EGFR by EGF stimulation increases survivin-mediated apoptosis resistance under normoxic condition. However, EGF does not increase survivin expression in normal mammary epithelial cells. EGFR upregulates normoxic HIF-1 α expression through the PI3K/Akt pathway.

Human epidermal growth factor receptor-2 (HER2), a ErbB family member, is overexpressed in 20-25% of breast cancers and is correlated with both HIF-1 α and HIF-2 α expression [107]. Independent of oxygen availability, HER2 activation increases the levels of HIF-2 α signaling in breast cancers, suggesting that HER2 activation may exacerbate tumor pathology [108]. HIF-2 α -specific inhibition, through the use of siRNA or C76, decreases HER2-positive breast

cancer cell growth rates, suggesting that targeting HIF-2 α may be a therapeutic strategy for treatment of HER2 positive breast cancers.

IGF-2 is a protein growth factor with insulin-like structure. It stimulates mitosis and is increased in many cancers. The Semenza group first demonstrated the reciprocal positive regulation between HIF-1 α and IGF-2 [109]. Both insulin and IGFs (IGF-1 and IGF-2) can induce HIF-1 α activation under normoxic condition. Activated HIF-1 α initiates the expression of its downstream genes, including IGF-2, IGF-binding protein (IGFBP)-2 and IGFBP-3. This suggests a role for HIF in a positive feedback loop in the autocrine regulation of tumor growth factors. Furthermore, Mohlin *et al.* showed that in neuroblastoma, the expression levels of HIF-1 α and IGF-2 are correlated, and this correlation is strongest in high-stage tumors [110]. The expression of both IGF-2 and HIF-1 α in neuroblastoma is hypoxia-dependent.

1.5.2 HIF-activated angiogenesis in cancer

In cancer cells, localized hypoxia can occur and activation of angiogenesis counteracts this hypoxia by increasing the delivery of oxygen and nutrients to the tumor. HIF is a master regulator of angiogenesis, promoting vessel growth by activating multiple pro-angiogenic pathways. Pro-angiogenic genes, including *VEGFA*, *ANGPT2* (encoding angiopoietin 2) and *TEK* (encoding TEK receptor tyrosine kinase), are often used as biomarkers for tumor hypoxia, and are implicated in HIF-mediated angiogenesis.

Central to the process of angiogenesis, HIF-1 α and HIF-2 α regulate the expression of VEGF [56, 111-113]. VEGF increases vessel permeability and is implicated in the proliferation and migration of endothelial cells and pericytes during neovascularization. Activation of HIF-1 α and HIF-2 α , due to loss of VHL function, occurs in highly vascularized tumors, although the relative roles of these HIFs in angiogenesis varies depending on the cell type [113]. For example, in RCC, VHL-associated hemangioblastoma, and liver hemangioma, HIF-2 α is the dominant HIF isoform controlling VEGF gene expression and tumor vasculogenesis [98, 114]. In contrast, HIF-1 α plays a major role in oral cancer and breast cancer [115, 116].

HIF is also known to play a role in the activation of angiopoietin (Ang) 1 and Ang2, as well as the angiopoietin 1 receptor, Tie2. During angiogenesis, Ang1 supports the action of VEGF, acting as an agonist for the Tie2 receptor. Through Ang1/Tie2 signaling, Ang1 plays a role in vascular remodeling and stabilization of newly formed vessels by supporting interactions between endothelial cells and their surrounding environment. In contrast, Ang2 antagonizes the Tie2 receptor, destabilizing existing vessels and shifting them to a more plastic state. Ang2 upregulation is also involved in the pathogenesis of multiple cancer types, including colorectal cancer [117-121]. Ang2 is a direct target of HIF-2 α and may also be indirectly targeted by HIF-1 α , since VEGF activation can also stimulate Ang2 expression [122, 123]. Ang2 repression regresses tumor vascularization and tumor metastasis in mammary carcinoma as well as pancreatic insulinoma [124]. It has also been reported that Tie2 expression is partially controlled by HIF-2 α in endothelial cells. Within tumor-infiltrating myeloid cells, Tie2 knockdown decreases tumor angiogenesis [124, 125].

1.5.3 HIF-activated epithelial-to-mesenchymal transition (EMT) in cancer

EMT is critical during embryonic development, tissue regeneration, organ fibrosis and wound healing [126]. This process describes the transition of a cell from an epithelial state, where cells exhibit epithelial cell-to-cell junctions and apical-basal polarity, towards a mesenchymal state, in which cells lack polarity and have heightened motility [127]. Although often viewed as a binary switch, EMT often involves only the acquisition of certain mesenchymal traits, while retaining some epithelial traits, which leads to mixed epithelial/mesenchymal phenotypes [128]. In cancer, by increasing cell motility and the ability to degrade components of the extracellular matrix, EMT activation allows cancer cells to invade the bloodstream, settle in the microvasculature, and extravasate from the vasculature to begin proliferating in secondary sites.

Adherence of epithelial cells to each other and to the basement membrane is maintained by cell adhesion molecules, most notably E-cadherin, and by tight junction proteins. The transition of epithelial cells from the expression of E-cadherin to N-cadherin, as well as the increased expression of the mesenchymal markers fibronectin and vimentin, indicate EMT transition [129]. EMT is controlled largely by EMT transcription factors which bind to the promoter

regions and repressing the expression of various cell adhesion genes. These transcription factors, including TWIST, Snail, SIP1, Zeb1 and Slug, have also been implicated in the progression of different cancer cell types and are controlled in part by HIF-1 α [130-139]. TWIST is a master regulator of mesoderm development and embryonic morphogenesis that is also implicated in cancer metastasis [140]. HIF-1 α regulates TWIST expression, promoting EMT and cancer metastasis [133]. The co-expression of HIF-1 α with TWIST and Snail is associated with worsened prognosis in patients with head or neck cancers [133]. Furthermore, HIF-1 α -mediated TWIST activation plays an important role in the progression of ovarian epithelial cancers and is associated with decreased survival [134]. Similar to TWIST, upregulation of Snail induces invasion and metastasis in a variety of cancer types and promotes mammary tumor recurrence [130, 141, 142].

Although less studied than HIF-1 α , HIF-2 α is also implicated in EMT and cancer pathogenesis. In pancreatic cancer tissue, HIF-2 α activation is associated with more aggressive cancer phenotypes and lymph node metastasis [143]. HIF-2 α also increases the expression of matrix metalloproteinase (MMP) 2 and MMP9, enzymes which implicated in tumor metastasis and EMT [144]. Additionally, in gastric cancer cells, both HIF-2 α and HIF-1 α expression is correlated with more advanced clinical stage and are upregulated in metastasizing cancers. Small interfering RNA (siRNA) against both HIF-1 subtypes reduces the migration and invasion of both gastric and lung cancer cells, suggesting a possible role for both HIF-1 subtypes in promoting cancer metastasis [145, 146].

2 Mitochondrial biology and experimental therapeutics in PAH and cancer

2.1 Aerobic glycolysis (Warburg effect) and their experimental therapeutics

2.1.1 Glycolysis and glucose oxidation

Changes in mitochondrial metabolism in disease include alterations in aerobic glycolysis, fatty acid oxidation, as well as the induction of glutaminolysis [147]. These metabolic changes are also associated with altered mitochondrial ROS production, bioenergetics and disruptions in both mitochondrial membrane potential and mitochondrial morphology [148].

Glucose is transported into the cytosol by GLUTs in the cell membrane. In the cytosol, glucose is first converted to glucose-6-phosphate (G-6-P) with the enzyme, HK. After a sequence of catalytic reactions, G-6-P is converted to pyruvate by PK. Pyruvate is the final product of glycolysis and when oxygen is present, it enters the mitochondria via the mitochondrial pyruvate carrier (MPC) [149]. Within the mitochondrial matrix, pyruvate is oxidized by PDH to form acetyl-CoA, which feeds Krebs' cycle. Krebs' cycle generates the electron donors (NADP and FADH) that enter the ETC at Complex I and II respectively, leading to oxidative phosphorylation (OXPHOS) and ATP generation. Glycolysis is a cytoplasmic pathway that converts glucose into lactate and generates adenosine triphosphate (ATP). Glucose oxidation in the mitochondria generates 32 ATP molecules per molecule of glucose, while glycolysis in the cytosol only generates 2 moles of ATP per mole of glucose.

Normally, glycolysis and glucose oxidation are coupled to each other. In certain diseases, notably those characterized by excess rates of cell proliferation and impaired apoptosis, pyruvate is primarily converted to lactate by the enzyme LDH and glucose oxidation is depressed, even in the presence of oxygen. Such uncoupled aerobic glycolysis, was first discovered in cancer cells by Warburg about one century ago [150]. Warburg metabolism confers a competitive advantage to affected cells since it largely eliminates cell death by mitochondria-mediated apoptosis.

Uncoupled aerobic glycolysis contributes to the hyperproliferative and apoptosis-resistant phenotype in many cancer cell types, promoting tumor growth [151]. In PAH, uncoupled aerobic glycolysis also promotes a cancer-like phenotype in PA vascular cells [68, 152, 153] and RVfib [89]. These acquired metabolic abnormalities are largely epigenetically initiated and contribute to adverse PA modeling and other key pathologic features of PAH, including increased PVR. For RV myocytes, which are not proliferative, uncoupled aerobic glycolysis results in mitochondrial metabolic dysfunction, which leads to RV myocyte hypertrophy, reduced contractility and even apoptosis [154, 155]. The elevated glucose flux required to support energy homeostasis in a state of uncoupled glycolysis is also pathologic. Increased glucose levels in the PAH RV contribute to RV dysfunction by a form of posttranslational modification of proteins called O-GlcNAcylation [156]. Increased O-GlcNAcylation of

mitochondrial proteins in MCT RVs leads to mitochondrial dysfunction and RVF in MCT-PAH and this glucose-related dysfunction can be reversed by colchicine [156].

Abnormalities in the many upstream regulators that affect the mitochondrial OXPHOS pathway can promote or facilitate the Warburg effect (See recent review in [148]). However, since PKM2 and PDH are rate-limiting enzymes for pyruvate production and the conversion of pyruvate to acetyl-CoA, respectively (which are essential steps for glucose oxidation), we focus on the canonical regulation, and nuclear translocation of PKM2 and PDH as key regulators of Warburg metabolism (Figure 5).

2.1.2 PKM2 In the Nucleus

2.1.2.1 Nuclear Translocation of PKM2 and its Regulation

PKM2 is predominately found in the cytosol. However, under various conditions, PKM2 can translocate into the nucleus. In the nucleus, PKM2 functions as a coactivator for several transcriptional factors to promote the Warburg effect. Factors that promote PKM2 nuclear translocation include inflammatory and proapoptotic factors like interleukin-3, UV irradiation, H₂O₂, and somatostatin analog TT-232 [157, 158]. Additionally, phosphorylation of PKM2 at serine 202 by Akt, under IGF-1 stimulation, induces PKM2 nuclear translocation [159].

PKM2 nuclear translocation involves the EGF-mitogen-activated protein kinase 1 (ERK2)-PKM2 axis. ERK2 activation by mitogenic growth factors, such as platelet-derived growth factor (PDGF) and EGF, exposes its docking groove to PKM2. ERK2 phosphorylates PKM2 at serine 37 (S37), after which never in mitosis A-1 (NIMA-1, also known as PIN1)-dependent cis–trans isomerization and conversion of PKM2 S37 from a tetramer to a monomer occurs. Monomeric PKM2 then binds to importin α 5 and is translocated into the nucleus [160]. This is most likely only one of several mechanisms for nuclear translocation by phosphorylation of PKM2 because phosphorylation of PKM2 at serine 202 by Akt (under IGF-1 stimulation) is not dependent on S37 phosphorylation [159]. Jumonji C domain containing dioxygenase 5 can also directly interact with PKM2 and promote PKM2 translocation into the nucleus [161]. Conversely, sirtuin 6 reduces PKM2 translocation into the nucleus by deacetylating PKM2 at lysine 433, resulting in reduced cell proliferation [162].

2.1.2.2 Nuclear PKM2 as a Coactivator

In cancer cells and under hypoxic conditions, nuclear PKM2 functions as a coactivator of HIF-1 α . PKM2 interacts directly with HIF-1 α and recruits the coactivator p300-acetyltransferase to enhance HIF-1 α binding to the HRE of HIF-1 α target genes [91]. PKM2 is also a target gene of HIF-1 α , creating a positive feedback loop that maintains and continuously promotes the proglycolytic shift seen in cancer cells [91].

Nuclear PKM2 also directly regulates the Warburg effect through transcriptional regulation of c-Myc. Once bound to β -catenin, which is phosphorylated at tyrosine 333 (Y333), the PKM2- β -catenin complex phosphorylates histone H3 at threonine 11, causing the dissociation of histone deacetylase 3 from the c-Myc promoter region [163]. PKM2-induced c-Myc expression creates a positive feedback loop since c-Myc increases the expression of heterogeneous nuclear ribonucleoproteins (hnRNP) A1, hnRNPA2, and polypyrimidine tract-binding protein (PTB), which promotes the alternative splicing of PKM2 over PKM1 [164]. Furthermore, c-Myc promotes the Warburg effect by increasing the transcription of HK2, LDHA, and PDK1 [165]. Thus, PKM2 promotes Warburg metabolism through diverse and mutually reinforcing mechanisms.

2.1.3 PDH in the Nucleus

The pyruvate dehydrogenase complex (PDC) consists of three enzymes: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) [166]. Together, PDC irreversibly catalyzes the conversion of pyruvate to acetyl-CoA, thus linking glycolysis to Krebs' cycle. Sutendra *et al.* demonstrated the presence of PDC in the nucleus of primary fibroblasts from human lungs, normal small airway epithelial cells, and A549 cells [167]. Nuclear PDC-E1 levels increase in parallel to decreasing mitochondrial PDC-E1 levels under serum stimulation without change in total PDC. The N-terminus of newly translated PDC contains a mitochondrial localization sequence (MLS), which is cleaved by mitochondrial-processing peptidase when entering the mitochondrial matrix [168]. Nuclear PDC lacks the mitochondrial localization sequence, demonstrating that newly translated PDC is first processed in the mitochondria prior to translocating to the nucleus.

PDC-E1 translocation from the mitochondria to the nucleus occurs during the S phase and is undetected within the nucleus of quiescent cells. Nuclear PDC-E1 is most likely constitutively active, given the absence of nuclear PDK. This also suggests that nuclear PDC-E1 is regulated differently than mitochondrial PDC-E1. Nuclear PDC-E1 can generate acetyl-CoA from pyruvate. Cells treated with siPDC-E1 produce significantly less acetyl-CoA, while increasing levels of pyruvate restore levels of acetyl-CoA, indicating the presence of nonlimiting amounts of functional nuclear PDC-E1. Acetyl-CoA, produced by nuclear PDH-E1, is utilized for histone acetylation of histones H2B, H3, and H4, which are involved in S phase entry. However, nuclear PDC inhibition does not alter acetylation of its other targets, such as p53 and FOXO1, indicating that nuclear PDC's acetylation activity is tightly regulated [167]. Interestingly, nuclear PDC cannot be subject to phosphorylation regulation because PDK is absent from the nucleus. Instead, nuclear PDC may be regulated by the availability or proximity of acetyltransferases or deacetylases [167].

The mechanism by which PDC translocation occurs remains unelucidated. Although the nuclear pore complex can allow the translocation of large protein complexes, it remains unclear how a large complex like PDC is exported across the mitochondrial membrane. The authors indicate that the process may be dependent on heat shock protein 70 (Hsp70). Hsp70 is involved in the nuclear translocation of several proteins [169]. Nuclear Hsp70 levels increase with serum stimulation in parallel with nuclear PDC-E1 levels. PDC's PDK-binding site contains a putative Hsp70-binding motif. Hsp70 may allow PDC to remain active after translocation to the nucleus by competing with PDK for PDC's PDK-binding site. Decreasing Hsp70 levels, using a heat shock protein inhibitor I (KNK437), or siRNA against Hsp70, decreases levels of nuclear PDC-E1.

2.1.4 Epigenetic Regulation of PKM2

PKM2 expression can be regulated at multiple levels. One important mechanism involves the epigenetic creation of splice variants by hnRNPs. hnRNPs are a group of c-Myc regulated splicing factors (hnRNPA1, hnRNPA2, and PTBP1) that regulate PKM2 expression by altering

PKM pre-mRNA. By binding to splicing signals that flank PKM exon 9, hnRNPs promote PKM2 expression [164].

MiRNAs are another epigenetic means by which PKM2 expression is regulated. MiRNAs are a class of small, endogenous, non-coding RNAs that serve to regulate post-transcriptional gene expression by targeting specific mRNAs for degradation or inhibiting their translation by binding to the 3'-untranslated region [170]. miRNAs regulate PKM2 activity in a tissue-specific manner either directly by targeting the PKM2 mRNA or indirectly through PTBP1. A summary of studies investigating miRNAs that target PKM2 in pulmonary hypertension and cancer cells along with their associated mechanism of action is provided in *Table 1*.

DNA methylation is another method through which PKM2 expression is regulated. Desai *et al.* discovered that intron 1 of the *PKM* gene is hypermethylated, resulting in reduced *PKM* expression. Conversely, hypomethylation of intron 1 of the *PKM* gene is associated with elevated PKM2 expression in various cancer types. This suggests that epigenetic regulation through DNA methylation of intron 1 may be a key mechanism through which PKM2 expression is increased in cancer [171]. Interestingly, Singh *et al.* observed higher DNA methylation at *PKM* exon 10 in breast cancer cells compared to normal human mammary epithelial cells, which correlates with the inclusion of exon 10 of the *PKM* gene and increased PKM2 expression. DNA methylation recruits Brother of Regulator of Imprinted Sites (BORIS), a CCCTC-binding factor paralog, to bind to exon 10. BORIS increases RNA polymerase II activity at exon 10, which favours increased inclusion of exon 10 and thus increases PKM2 expression. Depletion of DNMT3B, but not DNMT1 or 3A, decreases exon 10 methylation, which correlates with decreased RNA polymerase II occupancy, and reduced exon 10 inclusion and PKM2 expression [172]. This DNA methylation-mediated recruitment of BORIS at exon 10 of *PKM* gene is also observed in head and neck cancer, which leads to the alternative splicing and generation of the PKM2 splice isoform [173]. These results suggest that DNA methylation is critical in regulating PKM2 expression in various types of cancers (Figure 6), albeit there is tumor heterogeneity in DNMT's *PKM* gene target sites.

2.1.5 Epigenetic Regulation of PDH

PDH is also epigenetically regulated. In PAH PSMCs, hypermethylation of the CpG islands in the SOD2 promoter, mediated by upregulation in DNMT1 and DNMT3B, reduce SOD2 gene expression [174]. The resulting decrease in H₂O₂ activates HIF-1 α , which upregulates the transcription of PDK (among other key enzymes that inhibit oxidative metabolism) [68]. Inhibition of PDH through PDK-mediated phosphorylation inhibits acetyl-CoA production and promotes the Warburg effect. This pathway has been termed the DNMT-SOD2-HIF-1 α /PDK/PDH pathway [149].

2.1.6 Therapeutics targeting of PDH and PKM2 in PAH

Many *in vitro*, preclinical and clinical therapeutic studies have been performed to restore glucose oxidation to normalize mitochondrial metabolic function and thus cellular function in PAH. Here we focus on these two targets, i.e., PDH and PKM2, since their activity is directly involved in the glucose oxidation pathway. Additional information on clinical trials on mitochondrial metabolism in PAH can be found in previous reviews [148, 175]. A summary of therapeutics targeting PDK/PDH and PKM2 in PAH along with their associated mechanism of action is provided in *Table 2*.

2.1.6.1 Inhibition of PDK

Dichloroacetate (DCA) is a metabolic modulator that inhibits all four PDK isoforms. Inhibiting PDK enhances PDH activity and OXPHOS. *In vitro*, DCA has been shown to restore glucose oxidation and inhibit cell proliferation and apoptosis resistance in PSMC [176, 177], PAfib [178, 179] and RVfib [89]. DCA also restores glucose oxidation in PAH RV myocytes *in vitro* [180] and enhances RV contractility *ex vivo* [181] in animal models of PAH. *In vivo*, DCA has been shown to improve the hemodynamic function of lungs and RV in animal models of PH. DCA reverses PA remodeling in MCT-PAH rats [97, 176], reduces RV fibrosis and improves RV systolic and diastolic function in MCT rats and FHR [89, 154, 180, 181].

Though many studies have demonstrated the benefits of DCA in animal models of PAH, clinical trials in patients are limited. We searched on Clinicaltrials.gov using “pulmonary hypertension” in “Condition or disease” and “DCA or glycolysis” in “Other terms” and only found 1 clinical study using DCA to treat patients with idiopathic PAH (NCT01083524; Phase

I, completed). In this small clinical trial, 16 patients out of 20 completed the trial. The data from this study suggest that DCA is safe to use in patients with PAH and demonstrate that DCA leads to a reduction in mPAP and PVR and improvement in functional capacity [182]. However, DCA is not effective if patients have loss-of-function SNPs in *UCP2* (encoding mitochondrial uncoupling protein 2, UCP2) and *SIRT3* (encoding sirtuin 3) [182]. Further clinical studies on larger populations of PAH patients (both Phases II and III) are required to assess the efficacy and safety of DCA and a personalized approach to this metabolic therapy is likely required.

2.1.6.2 Inhibition of PKM2

Decreasing the PKM2/PKM1 ratio reduces glycolysis and restores glucose oxidation, resulting in reduced cell proliferation in *in vitro* studies of PAEC and PAfib from PAH patients. This antiproliferative effect can be achieved regardless whether PKM2 inhibition is achieved by PTBP1 inhibition, administration of a miR-124 mimic, or administration of shikonin, a Chinese herbal remedy [152, 153]. In a model of group 2 PH, shikonin (2 mg/kg/d for 2 weeks) normalized PKM2/PKM1 expression and improved RV systolic pressure while regressing RV hypertrophy [183]. Stabilizing the tetramer formation, using a small molecule TEPP-46 (a member of the thieno[3,2-b]pyrrole[3,2-d]pyridazinones class) is another strategy to inhibit PKM2. TEPP-46 decreases lactate production and normalize glucose metabolism in human PAH PAfib [153]. However, further preclinical studies safety/efficacy studies will be required to better evaluate optimal dosing and toxicities of these PKM2-targeted drugs before preceding to clinical trials in PAH patients.

2.1.7 Therapeutics targeting of PDH and PKM2 in cancer

The concept of aerobic glycolysis was established much earlier in cancer than in PAH, and many experimental therapeutics have focused on targeting glycolysis (see reviews [151, 184-186]). Here we focus on the therapeutics that target PDK (or PDH) and PKM2. A summary of therapeutics targeting PDK/PDH and PKM2 in cancer along with their associated mechanism of action is provided in *Table 2*.

2.1.7.1 Inhibition of PDK

Archer and Michelakis hold a patent for the use of PDK inhibitors to treat cancer (US11/911,299). *In vitro*, DCA has no effect on non-cancerous 293T cells [187], but reduces cell proliferation and/or increases apoptosis in many cancer cell lines including *In vitro*, DCA has no effect on non-cancerous 293T cells [187], but reduces cell proliferation and/or increases apoptosis in many cancer cell lines including A549 (NSCLC), M059K (glioblastoma), MCF-7 (breast cancer), human prostate cancer cells, pancreatic cancer cell lines (PANC-1, BXPC-3), and endometrial cancer cell lines (Ishikawa, RL95-2, KLE, AN3CA, and SKUT1B) [187-190].

The HIF inhibitor echinomycin, reduces PDK1 expression and restores glucose oxidation in RKO (colon cancer) and Su.86 (pancreatic cancer) cancer cells *in vitro* [191]. *In vivo*, DCA decreases xenograft tumor growth of A549 tumors in rats [188] and is therapeutic in a xenograft pancreatic cancer mouse model [190]. Echinomycin also delays tumor growth in a murine RKO xenograft model [191].

There are a few clinical trials using DCA to treat cancer. The first clinical report by Michelakis *et al.* found that 4 out of 5 patients with glioblastoma demonstrated radiologic regression by magnetic resonance imaging and were clinically stable after 15 months of oral DCA administration [192]. In a single case report, a middle-aged man with non-Hodgkin's lymphoma who had relapsed after standard chemotherapy showed complete remission after over 4 years' treatment with oral DCA (1 g/day as monotherapy) [193]. However, ~1 week of oral DCA treatment (6.25 mg/kg/12h) did not show any benefits for 6 patients with stage IV metastatic breast cancer or advanced stage (IIIB/IV) NSCLC in an open-label Phase II trial [194]. Regarding the optimal dose of DCA, oral DCA (~8 mg/kg/12h) treatment for 4 weeks in a Phase I trial was well-tolerated in 15 adult patients with recurrent malignant gliomas or other tumors metastatic to the brain [195]. However, 6.25 mg/kg/12h is the dose recommended for Phase II trials, based on a Phase I trial [196]. The major toxicity of DCA is a reversible peripheral neuropathy in genetically susceptible patients [197]. The benefits of DCA may depend on the type and stage of tumor and the degree to which the PDK is activated. It is unknown whether there is individual variation in DCA efficacy in cancer therapy depending on a

patient's genetic commodifier profile (i.e. the presence of SNPs in UCP2 and *SIRT3*), as is the case in PAH.

2.1.7.2 Inhibition of PKM2

In vitro, drugs derived from plants such as resveratrol, apigenin, shikonin (and shikonin's enantiomeric isomer, alkannin) can reduce PKM2 expression and/or activity, resulting in inhibition of proliferation and glycolysis and induction of apoptosis in several cancer cell lines including DLD1, HeLa, MCF-7, HCT116, HT29, MCF-7, MCF-7/Adr, MCF-7/Bcl-2, MCF-7/Bcl-xL, A549, and HCC [198-201]. The non-specific or off-target effect of these drugs is unknown. PKM2 synthetic peptide aptamers and small molecules targeting PKM2 have also been shown to reduce the growth of tumor cells [174, 202], indicating the pathologic role of the PKM2 isoform in cancer cells. Overexpression of miR-1294, which inhibits PKM2 expression, inhibits cell proliferation, migration, and invasion, and induces apoptosis in osteosarcoma cells [203]; however, miRs have many targets and this miR intervention would not selectively target PKM2. Increased expression of the PKM2 tetramer, which is the active form of PKM2, inhibits glycolysis and promotes glucose oxidation [204]. PKM2 activators, such as 3-(trifluoromethyl)-1H-pyrazole-5-carboxamide and TEPP-46, promote PKM2 tetramerization and inhibit cell proliferation in lung cancer cell lines including NCI-H1975, A549 and NCI-H1299 [204, 205]. Currently, no clinical trial has been conducted to use PKM2-targeted drugs to treat cancer.

2.2 Mitochondrial dynamics as a target for experimental therapeutics in PAH and cancer

While the primary role of mitochondria is to generate ATP through OXPHOS, mitochondria also play a number of noncanonical roles which are important for cell survival and death. These include mitochondrial fission and fusion [206], mitochondrial quality control (via mitophagy) [207], mitochondrial biogenesis [208], calcium regulation [209], and mitochondria-mediated apoptosis [210].

Mitochondria are highly dynamic organelles which continuously undergo division (fission) and joining (fusion) [206]. In normal physiological conditions, mitochondrial dynamics are tightly regulated by two groups of proteins (fission mediators and fusion mediators), most of

which are large GTPases. Dysregulation of mitochondrial fission and fusion has been identified in PAH and cancers and the most common imbalance is fission exceeding fusion, resulting in excessive mitochondrial fragmentation. This fission/fusion imbalance contributes to the excess rate of cell proliferation, apoptosis resistance and increased migration seen in both syndromes [209, 211-214]. In cancer, a fragmented mitochondrial network is also associated with an alteration of bioenergetic and biosynthetic functions that support tumor initiation [215].

2.2.1 Mitochondrial fission and its experimental therapeutics

Increased mitochondrial fission is mainly due to increased expression level and/or activity of Drp1 and/or its binding partners [206]. Epigenetic regulation [212], transcriptional regulation [216] and post-translational modifications [211] are each involved in the dysregulation of fission and fusion mediators in PAH and cancer.

Fission proteins include Drp1 and its four binding partners: Mff (mitochondrial fission factor), FIS1 (mitochondrial fission 1 protein), MiD49 (mitochondrial dynamics protein of 49 kDa) and MiD51 (mitochondrial dynamics protein 51 kDa) [217-221]. Since Drp1 does not have an outer mitochondrial membrane (OMM) binding domain, it requires its binding partners/membrane receptors to facilitate OMM attachment [217].

During mitochondrial fission, endoplasmic reticulum (ER) first wraps around the mitochondria and marks the fission site [222]. Next, cytosolic Drp1 is activated by post-translational modifications, and recruited to the OMM where it interacts with one or more binding partners [217]. On the OMM, Drp1 polymerizes into a ring-like structure, at a site demarcated by the ER and binding partners. This ring constricts the mitochondrial membrane, utilizing the energy generated from GTP hydrolysis [223]. There are two major phosphorylation sites in human Drp1: serine 616 (p-Drp1_{S616}) and serine 637 (p-Drp1_{S637}). Phosphorylation at S616 promotes mitochondrial fission [224-226] while phosphorylation at S637 decreases mitochondrial fragmentation [227, 228]. Drp1's binding partners also likely focus the constriction apparatus, guiding division of the mitochondrial membranes.

Drp1 can only constrict mitochondria down to a diameter of ~30nm [229], a model of sequential constriction by multiple fission mediators was recently proposed. Lee *et al.* demonstrated that dynamin 2 (Dnm2), a mediator in endocytosis [230] and vesicular trafficking [231], completes the final step of mitochondrial fission [232]. However, whether Dnm2 is essential for mitochondrial fission is controversial. Fonseca *et al.* found that dynamin triple-knock out does not defect mitochondrial fission in mouse fibroblasts [233]. Clarification of the roles of Drp1 and Dnm2 requires additional research.

2.2.1.1 Increased mitochondrial fission in PAH

Hyperfragmentation of the mitochondrial network, whether due to increased mitochondrial fission [214] or decreased fusion [216], is observed in both PAH patients and experimental models of PAH. This pathological mitochondrial phenotype contributes not only to hyperproliferation and aerobic glycolysis of PASMCM [212, 214] and RVfib [89, 234], but also to RV dysfunction in PAH [235]. The consequences of a fragmented mitochondrial network are highly contextual, ranging from metabolic inefficiency with ROS production, to impending apoptosis or increasing cell division.

Mitochondrial fission is coordinated with mitosis ensuring equitable distribution of mitochondria to daughter cells. In rapidly dividing cells, fragmented mitochondria often signify increased rates of mitotic fission, as is the case for PAH PASMCM. The Archer lab first identified increased expression level of both total and activated form of Drp1 (p-Drp1_{S616}) in PASMCM from patients and rodent preclinical models of PAH [214]. In both animals and patients, HIF- and cyclin B1/CDK1-dependent activation of Drp1 occurs. The activation of Drp1 (increased p-Drp1_{S616}) in PAH PASMCM is pathologically relevant as inhibiting Drp1 reduces rates of cell proliferation and causes cell cycle arrest [214]. Activation of Drp1 in PAH PASMCM is also caused by decreased level of the inactivated form of Drp1 (p-Drp1_{S637}), which can be reversed by treprostinil, a synthetic analog of prostacyclin [236].

Elevation of the expression of several Drp1 binding partners is also observed in PAH, notably including FIS1 [214], MiD49 and MiD51 [212]. Different from Drp1, MiD49 and MiD51 are epigenetically upregulated by decreased miR-34a-3p in PAH PASMCM. This increase in MiD

expression ultimately leads to accelerated mitotic fission and cell cycle progression via an ERK1/2 and CDK4-dependent mechanism [212] (Figure 7). Interestingly, a SNP (exm1300952) in the MiD49 gene (*SMCR7*) is associated with a higher risk of PAH [237]. Elevated MiD51 expression is also reported in the left ventricle (LV) and RV of a rodent SAB model of group 2 PH [238].

The heart is intriguing because it has both dividing cells (RVfib) and vascular cells and nondividing cells (cardiomyocytes). While fission is seen in both cell types, the consequences are quite different. In RVfib, increased Drp1-mediated mitochondrial fission is associated with hyperproliferation and increased collagen production, which ultimately results in RV stiffness and RV failure [234]. However, increased mitochondrial fission in RV myocytes, likely also due to increased Drp1 activity and Drp1-FIS1 interaction, is associated with an increase in mitochondrial ROS production and a spontaneous ischemia-reperfusion (IR) injury, which can be attenuated by Drp1 inhibitors [235].

2.2.1.2 Increased mitochondrial fission in cancer

Excessive mitochondrial fission has been confirmed in various cancer tissues/cells, including lung cancer [211], breast cancer [239], brain cancer [240], pancreatic cancer [241], melanoma [242] and this increase in fission is associated with accelerated cell cycle progression, hyperproliferation, apoptosis resistance, invasion, and migration [243]. This makes mitochondrial fission an appealing therapeutic target and a potential biomarker in cancer.

Excessive fission in cancer cells is due to increased expression levels/post translational activation of the fission protein, Drp1 and dysregulation of Drp1 binding partners MiD49 and MiD51. Our group demonstrated that in lung cancer, increased p-Drp1_{S616} and decreased p-Drp1_{S637}, along with decreased mitofusin (Mfn) 2, contribute to the net increase in mitochondrial fragmentation in NSCLC cell lines (A549 and H1993), as well as in tumors from patients [211]. Upregulation of Drp1 also occurs in invasive breast carcinoma, in which case it is accompanied by downregulation of Mfn1 [239]. Normalization of Drp1 or Mfn1 expression in this study inhibited lamellipodia formation by breast cancer cells, a key step for cancer metastasis [239]. Brain tumor initiating cells (BTICs) are stem-cell like cells present in

brain tumors. The mitochondria in BTICs are more fragmented compared to those in non-BTICs [240]. Cyclin-dependent kinase (CDK) 5 activates Drp1 in BTICs by phosphorylation at S616 while Ca²⁺/calmodulin-dependent protein kinase (CAMK) inhibits Drp1 in non-BTICs by phosphorylation at S637. P-Drp1_{S616} inhibits the downstream target, AMP-activated protein kinase (AMPK), a central cellular sensor of energy stress. Increased activity of the CDK5/CAMK-Drp1-AMPK pathway has prognostic value in patients with primary glioblastomas [240]. Drp1 is also upregulated in pancreatic cancer [244] and thyroid tumors [245].

Although there is much evidence supporting the pro-tumorigenesis function of Drp1, the role of Drp1 binding partners in cancer, especially MiD49 and MiD51, is less clearly understood. We recently discovered a parallel upregulation of MiD49 and MiD51 in NSCLC and invasive breast carcinoma [213]. Similar to our observation in PAH cells, this pathway contributes to increased mitotic fission, apoptosis resistance and accelerated cell cycle progression in cancer cells [213]. In many cancers and PAH, the MiDs are increased because of an upstream epigenetic mechanism, namely downregulation of miR-34a-3p (Figure 7). However, in pancreatic cancer, MiD49 is downregulated and is thought to play a role of tumor suppressor [246]. Downregulation of MiD49 in pancreatic cancer is associated with tumor growth and metastasis [246]. The reason for these opposing observations on the role of MiDs is unclear.

2.2.1.3 Therapeutic targeting of fission mediators to reduce mitochondrial hyperfragmentation

Due to the pivotal role of Drp1 in mitochondrial fission and its related pathological conditions, the GTPase domain of Drp1 has become a target domain for drug development. In 2008, the Nunnari lab discovered the first Drp1 inhibitor, mitochondrial division inhibitor (mdivi-1) [247]. They first screened ~23,000 chemical compounds in yeast using a growth assay. Candidate compounds which inhibited yeast mitochondrial fission suppressed the growth defect of mitochondrial fusion mutants (*fzo1-1* and *mgm1-5*). This was followed by a screen based on steady-state mitochondrial morphology. Only 3 potential mitochondrial division inhibitors were identified after two rounds of screening. Among them, mdivi-1 was the most efficacious. Mdivi-1 selectively inhibited the GTPase activity of Dnm1 (the yeast dynamin-

related GTPase) and interfered with its self-assembly. Mdivi-1 also inhibited mitochondrial division [247]. At their study demonstrated that mdivi-1 inhibited mitochondrial apoptosis in mammalian cells by blockage of Bid-activated Bax/Bak-dependent cytochrome *c* (Cyt *c*) release. However, in the hyperproliferative human cells in PAH and cancer, mdivi-1 is both antiproliferative and pro-apoptotic agent. These therapeutic effects reflect mdivi-1's inhibitory effect on mitotic fission and cell cycle progression. In human PAH PASMC, inhibition of Drp1 by mdivi-1 prevents mitotic fission and arrests the cell cycle at the G2/M interphase [214]. In vivo, mdivi-1, administered to a rat model of pulmonary hypertension induced by administration of the HIF-activator cobalt, improves pulmonary vascular hemodynamics, RV function and exercise capacity, while simultaneously reducing mitochondrial fragmentation in PASMC in the vascular media. Rehman in our group first employed mdivi-1 as a treatment in a preclinical model of cancer [211]. Mdivi-1 significantly regressed tumor growth in a mouse xenograft model of human NSCLC. Subsequently, the efficacy of mdivi-1 has been validated by many other groups in various solid tumors, including brain tumors [240], breast cancer [248, 249] and melanoma [250].

In 2013, Qi *et al.* took a different approach to inhibiting Drp1-mediated fission. Rather than target the GTPase domain, they created a selective peptide P110, which inhibits Drp1 activity by blocking the interaction of Drp1 and FIS1 [251]. P110 has been successfully used to protect the RV in an *ex vivo*, preclinical treatment of RV-ischemia-reperfusion (IR) injury [235]. Since Drp1-mediated mitochondrial hyperfragmentation is also associated with increased apoptosis in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and Huntington's disease [252-257], the therapeutic efficacies of mdivi-1 and P110 have also been tested and shown to be beneficial in these non-hyperproliferative, neurological conditions [258-260]. However, P110 would only be expected to be therapeutic in conditions in which pathologic fission requires FIS1 binding, which does not appear to be a major mechanism of fission in PAH or most cancers.

Although the therapeutic efficacy of mdivi-1 on PAH and cancer is supported by an increasing number of literatures, a putative lack of specificity for Drp1 [261] is a concerning fact that may lead to off-target effects. Recently, our lab discovered a novel Drp1 inhibitor, Drpitor1a [262]

(patent pending), which is more specific and 50X more potent than mdivi-1 (Figure 8). Like mdivi-1, Drpitor1a inhibits lung cancer tumor growth and RV-IR injury in rodent disease models [262]. It is noteworthy that in our study neither mdivi-1 nor Drpitor1a inhibited complex 1 activity (i.e. we did not detect off target effects on Complex 1 or other dynamins). A summary of therapeutics targeting mitochondrial fusion/fission mediators in PAH and cancer is provided in *Table 3*.

2.2.2 Therapeutic targeting of fusion mediators to reduce mitochondrial hyperfragmentation

In mammalian cells, mitochondrial fusion is primarily mediated by three large GTPases: Mfn1, Mfn2 on the OMM, and OPA1 (optic atrophy 1) on the inner mitochondrial membrane (IMM) [263, 264]. OPA1 functionally requires Mfn1, but not Mfn2, to regulate mitochondrial fusion, indicating functional differences between Mfn1 and Mfn2 [265]. In addition, there is a Ras-binding domain at the N-terminus of Mfn2 which is absent in Mfn1, suggesting specific roles of Mfn2. Previous studies have indicated that overexpression of Mfn2 (initially called the hyperplasia suppressor gene, HSG) can suppress proliferation and induce apoptosis of vascular SMCs [266, 267].

2.2.2.1 Regulation of Mfn2

Expression of Mfn2 is regulated both at transcriptional and post-translational levels. The transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α), transcriptional regulator of Mfn2, is downregulated in human and rodent PAH PSMCs [216]. Mfn2 expression is also regulated at the posttranslational level by the ubiquitin-proteasome system [268]. In response to cellular stress, Mfn2 is phosphorylated at serine 27 by c-Jun N-terminal kinase (JNK), leading to the recruitment of the ubiquitin ligase (E3) Huwe1 and resulting in ubiquitin-mediated proteasomal degradation of Mfn2. This degradation of Mfn2 can result in mitochondrial fragmentation and apoptosis [269]. Mfn2 is also phosphorylated by PTEN-induced putative kinase (PINK1) at serine 442, leading to Parkin-mediated ubiquitination and proteasomal degradation [270]. In addition, Mfn2 expression is

controlled by the PI3K-Akt-mTOR pathway, which plays an important role in activation-induced, proteasomal downregulation of Mfn2 in human peripheral blood T cells [271].

2.2.2.2 Dysregulation of Mfn2 in PAH and its experimental therapeutics

Mfn2 expression is downregulated in the medial layer (PASMC) of pulmonary vasculature in PAH patients and in the PAs of experimental rodent models of PAH (MCT-PAH and Su/Hx-PAH) [216]. Augmenting Mfn2 expression by adenovirus-mediated gene transfer increases mitochondrial fusion, inhibits cell proliferation and induces apoptosis in PAH PASMC, suggesting potential therapeutic benefit of Mfn2 therapy in human and experimental PAH [216]. Furthermore, augmenting Mfn2 by airway nebulization of adenovirus containing the Mfn2 gene increases lung vascularity and decreases PVR and PA medial thickness in the Su/Hx-PAH model [216] (Figure 9).

2.2.2.3 Role of Mfn2 in the pathogenesis and therapy of cancer

Downregulation of Mfn2 in cancer was first identified in urinary bladder cancers [272] and lung cancer [211], and later, this phenomenon is confirmed in various types of cancer, including breast cancer [273], hepatocellular carcinoma [274], colorectal cancer [275] and gastric cancer [276]. Augmenting Mfn2 level in cancer cells elongates the mitochondrial network [211], arrests cell cycle transition from G1 to S phase [272], inhibits cell proliferation and colony formation, decreases mitochondrial membrane potential, triggers mitochondria-associated apoptosis, and impairs the invasion and migration abilities of cancer cells [275-278].

Consistent with these findings, Xu *et al.* reported that overexpression of Mfn2 suppresses cancer progression through the inhibition of the mammalian target of rapamycin complex (mTORC) 2/Akt signaling pathway. Conversely, silencing of Mfn2 in MCF7 breast cancer cells and A549 NSCLC cells promotes cancer cell viability, colony formation, and cancer cell invasion *in vitro* and this is replicated in a xenotransplant murine model. In the Mfn2-silenced cancer cells, the mTORC2/Akt signaling pathway, which is activated in most cancers, becomes further activated leading to the growth and metastasis of cancer cells. Mfn2 directly interacts with mTORC2 and suppresses its kinase activity, which inactivates Akt, thereby inhibiting tumor growth [273]. Xue *et al.* reported that overexpression of Mfn2 by adenovirus-mediated

gene transfer in a pancreatic cancer cell line (AsPC-1) induces autophagy by inhibiting the PI3K/Akt/mTOR signaling pathway with the net effect of inhibiting cancer cell proliferation and ROS production [277].

Mfn2 expression can also be increased pharmacologically. A recent study demonstrates that oral leflunomide, an FDA-approved disease modifying drug for rheumatoid arthritis, increases Mfn2 expression 2-fold in tumors and improves the median survival of mice with spontaneous tumors by 50% compared with the control group. The mechanism of tumor suppression by leflunomide reflects reduced mitochondrial mass and ATP production [279].

There are some data that show opposing effects of Mfn2 in lung adenocarcinoma [280]. In this study, Mfn2 knockdown inhibited cell proliferation and invasion and caused cell cycle arrest [280]. However, the authors did not investigate the effect of silencing Mfn2 on mitochondrial morphology, nor did they report the effects on the expression level of other fission/fusion mediators, including Drp1 and MiDs, leaving questions about the mechanism of action of their Mfn2 knockdown. In ovarian cancer, increased Mfn2 expression is associated with its upstream regulator, cystathionine β -synthase (CBS). Although molecular inhibition of Mfn2 decreased mitochondrial fusion in ovarian cancer cells, the inhibition of cell growth might be due to a compensatory decrease in Drp1, which was not investigated in that study [281].

Mfn2 expression is also epigenetically regulated. Pan *et al.* reported that the expression of Mfn2 is negatively regulated by miR-125a, which is downregulated in several types of human cancer, including breast cancer [282], lung cancer [283], ovarian cancer [284], medulloblastoma [285] and gastric cancer [286]. Augmenting miR-125a in PANC-1 pancreatic cancer cells downregulates Mfn2 expression, resulting in mitochondrial fission, activation of mitochondria-dependent apoptosis and suppression of cancer cell migration. However, Mfn2 may be not the only target of miR-125a since miR-125a mimic also inhibits the expression level of two fission mediators, Drp1 and Fis1. Therefore, the mechanism by which miR-125a mediates mitochondria-dependent apoptosis and suppression of cancer cell migration requires further investigation [287].

Thus, Mfn2 has been reported to have both tumor-suppressing and tumor-promoting functions, depending on the cancer type suggesting its role in cancer is more complicated than expected. The validity and therapeutic implications of the apparent differences in the roles of Mfn2 in various tumor types require further experimental study. Our own research favours the interpretation that augmenting Mfn2 in general has an antiproliferative effects (in rapidly proliferating cells).

2.3 Mitophagy in PAH and cancer

Mitophagy is a specialized form of autophagy that serves as a quality control mechanism. Mitophagy selectively removes dysfunctional mitochondria without killing the cell. This is achieved by targeting damaged mitochondria for proteolytic degradation [288]. The proteolytic system of mitochondria is comprised of two AAA protease complexes in the inner membrane which degrade unfolded membrane proteins [289]. The IMM and OMM proteins are also targeted for degradation by cytosolic proteasomes [290]. Mitophagy is mediated by the coordinated activation of PINK1 and Parkin. In a damaged or dysfunctional mitochondrion, the mitochondrial membrane potential depolarizes. This damage may result from oxidative stress, with the mitochondria itself generating ROS or reflect damage caused by external stimuli, like irradiation or chemotherapeutic agents. With mitochondrial damage, inhibition of mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like (PARL) proteases stabilize PINK1 in the OMM [291, 292]. PINK1 in turn recruits Parkin, which polyubiquitinates several OMM proteins such as voltage-dependent-anion-selective channel 1 (VDAC1), Mfn1 and Mfn2. These polyubiquitinated proteins are then marked by adaptor proteins, such as p62, optineurin (OPTN) and nuclear dot protein 52 kDa (NDP52), causing them to be recognized by light chain 3 (LC3), leading to the formation of autophagosome [291]. There is limited evidence of increased mitophagy in experimental PH. Mice with PH due to endothelial-specific knockout of *UCP2* exposed to intermittent hypoxia have an increase in mitophagy which in turn inhibits mitochondrial biogenesis and induces apoptosis in PAECs [293].

Mitophagy is also dysregulated in many cancers [294]. Loss of function mutation of critical genes inhibits mitophagy resulting in the accumulation of dysfunctional mitochondria, which contributes to tumorigenesis. Loss of Parkin has been reported to suppress mitophagy and contribute to the development of various cancers [295]. In a murine model, loss of Parkin results in hyperproliferative hepatocytes, leading to microscopic hepatocellular cancer [296]. Furthermore, depletion of either PINK1 or Parkin stimulates K-Ras-driven pancreatic adenocarcinoma [297]. PINK1 and Parkin also play an important role in the stability of HIF-1 α in that parkin ubiquitinates HIF-1 α at lysine 477 (K477), leading to its degradation [298]. In breast cancer, high HIF-1 α expression correlates with low Parkin expression and increased susceptibility to metastasis [299]. PINK1 and Parkin are also critical in maintaining metabolic homeostasis and regulation of the cell cycle. In pancreatic adenocarcinoma, loss of PINK1 or Parkin function increases ROS production, HIF-1 α expression and Warburg metabolism and these pathologic changes are reversed by silencing HIF-1 α [297]. Thus, PINK1 or Parkin inactivation promotes Warburg metabolism in cancers through HIF-1 α stabilization [297]. Parkin has also been shown to regulate the stability of protein complexes such as FBX4 Cullin-RING ligase complex and to control cell cycle progression by regulating cyclin levels and by interacting with Cdc20/Cdh1 to enforce mitotic checkpoint control and genetic stability [300].

Unlike PINK1/Parkin-mediated mitophagy, which removes damaged or dysfunctional mitochondria, healthy mitochondria can be eliminated in response to nutritional stress to allow the cells to utilize the energy stored within [301]. Stress-induced mitophagy receptors, B-cell lymphoma 2 (Bcl-2) and adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L, also known as NIX) are present in the OMM and interact directly with LC3 or LC3 homologs to promote mitophagy of healthy mitochondria [302]. Chromosome 10q26.3, which contains the BNIP3 locus, is often deleted in metastatic triple-negative breast cancer and is associated with poor metastasis-free survival [303, 304]. In addition, epigenetic silencing of BNIP3 has been reported during progression of tumors' invasiveness to metastasis in several cancers, including lung, gastric, pancreatic, liver, and in hematological malignancies [305-308]. Chemoresistance and poor prognosis in pancreatic cancer is associated with inactivation of BNIP3 [305, 309]. In an experimental murine model of breast cancer, loss of

BNIP3 resulted in higher tumor growth, faster progression to invasive carcinoma, decreased latency of lung metastasis and decreased overall survival [303]. Furthermore, silencing of BNIP3 increased tumor growth in an orthotropic model of breast cancer [310]. Thus, loss of BNIP3 decreases mitophagy and leads to increased invasiveness, ROS and HIF-1 α expression [303]. Taken together, these observations indicate that BNIP3 negatively regulates the expression of HIF-1 α and conversely, BNIP3 inactivation promotes tumorigenesis and invasiveness. In contrast to BNIP3, BNIP3L promotes tumorigenesis. In *KRAS*-mutated pancreatic ductal adenocarcinoma, loss of BNIP3L is associated with decreased tumorigenesis, reduced mitophagy, increased oxidative metabolism and better prognosis, suggesting a divergent role of these mediators of mitophagy with one acting as a tumor suppressor and the other as a promoter of tumor growth [311].

2.4 Mitochondrial Biogenesis in PAH and cancer

Mitochondrial biogenesis is characterized by the division of pre-existing mitochondria to produce new mitochondria to meet energy requirements caused by the changes in environmental and physiological conditions [208]. This process is regulated by the transcription factor, PGC-1 α which promotes mitochondrial biogenesis by activating nuclear respiratory factor (Nrf) 1 and mitochondrial transcription factor A (TFAM), which drives transcription and replication of mitochondrial DNA (mtDNA) [312-315].

In human and experimental models of PAH, mitochondrial biogenesis is inhibited due to the reduced expression of its mediators [316, 317]. In a bovine model of persistent pulmonary hypertension of the newborn (PPHN), expression of PGC-1 α , various ETC subunits, and mtDNA copy number are decreased [318]. In addition, mice lacking bone morphogenetic protein receptor type II (*BMPR2*) in the endothelium show impaired mitochondrial biogenesis along with increased mitochondrial ROS production, decreased mitochondrial membrane potential and a predilection to the development of PH [319]. Furthermore, in a time-course study conducted with RV, gastrocnemius, and LV in MCT-PAH rats, an early decrease in the expression of genes promoting mitochondrial biogenesis (sirtuin 1, PGC-1 α and TFAM) is observed in the skeletal muscles followed by a similar decrease in RV myocytes and gastrocnemius muscle; conversely, their expression in the LV remained unchanged [320].

Unlike PAH, mitochondrial biogenesis is *increased* in cancers. An early study by Lee *et al.* demonstrated that in arsenic-induced Bowen's Disease (a form of skin cancer), tumor growth is promoted by upregulation of TFAM. In addition, the transcription factors of TFAM, Nrf1 and Nrf2, are also upregulated by arsenic [321]. Consistent with these findings, several other studies demonstrated a link between increased mitochondrial biogenesis and tumor growth. Inhibition of tumorigenesis and impaired OXPHOS are associated with the loss of mtDNA [322, 323]. Furthermore, a link between increased expression of TFAM and cancer progression is reported [324, 325] which aligns with the findings that loss of TFAM suppresses K-Ras-induced tumor formation in lungs [326].

As a transcription coactivator, PGC-1 α interacts with various transcription factors. It is not only the master regulator of mitochondrial biogenesis, but also a key regulator of OXPHOS. PGC-1 α enhances OXPHOS, mitochondrial biogenesis and oxygen consumption rates in invasive breast cancer cells. In human epidermal growth factor receptor 2 (ERBB2)⁺ breast cancer, PGC-1 α and estrogen-related receptor (ERR) α positively regulate the expression of genes for glutamine metabolism [327]. The PGC-1 α /ERR α axis also supports *de novo* lipogenesis[327]. Furthermore, there is a strong correlation between PGC-1 α expression and the formation of distant metastases in invasive breast cancer cells; conversely, silencing PGC-1 α suppresses invasiveness and inhibits metastasis [328].

In melanoma, there are two subpopulations of cells expressing different levels of PGC-1 α which have distinct metabolic phenotypes [329]. The PGC-1 α -upregulated melanoma cells are OXPHOS-dependent and have increased ROS detoxification capacities, allowing them to survive under oxidative stress conditions [329]. A high PGC-1 α level is also inversely correlated with vertical growth of human melanoma and its metastasis [330]. On the contrary, low PGC-1 α expressing melanoma cells are more glycolytic and sensitive to killing by ROS-inducing drugs [329].

In prostate cancer, the role of PGC-1 α is controversial. Androgen receptor (AR) is a transcription factor directly regulated by PGC-1 α [331] and PGC-1 α is upregulated in various

prostate cancer cell lines. Activation of PGC-1 α /AR promotes cancer cell growth in AR-expressing prostate cancer cells [331]. Tennakoon *et al.* further demonstrated that the AR-dependent cell growth in prostate cancer is due to the activation of a metabolic sensor AMPK/PGC-1 α pathway [332]. In contrast, Torrano *et al.* reported PGC-1 α to be a tumor suppressor in prostate cancer [333]. They found that PGC-1 α is downregulated in prostate cancer and noted that patients with lower PGC-1 α in the prostate cancer tissue have worse disease-free survival [333]. Mechanistically, PGC-1 α might suppress tumor progression and metastasis by activation of an ERR α -dependent transcriptional program [333]. These opposing results are a reminder of the complexity of cancer and suggest that other factors, such as AR or AMPK, may modulate the impact of PGC-1 α on tumorigenesis.

In pancreatic cancer, the oncogene c-Myc binds to the promoter region of PGC-1 α and inhibits its transcription [334]. Pancreatic cancer stem cells have a low c-Myc/PGC-1 α ratio, a high level of OXPHOS and are more sensitive to metformin. In contrast, differentiated pancreatic tumor cells have a high c-Myc/PGC-1 α ratio. When pancreatic cancer stem cells develop metformin resistance during treatment, they show an intermediate metabolic phenotype, with reduced OXPHOS but increased glycolysis, due to an increase in the c-Myc/PGC-1 α ratio [334]. Therefore, pharmacological inhibition of c-Myc may be a potential therapeutic intervention for pancreatic cancer by preventing/reversing their resistance to metformin

2.5 Mitochondrial calcium homeostasis in PAH and cancer

The hallmarks of hyperproliferative diseases include uncontrolled cell division and evasion of apoptosis. Dysregulation of intracellular Ca²⁺ in various compartments can modulate signaling pathways relevant to these events.

In PAH, increases in cytosolic calcium ([Ca²⁺]_{cyto}) promote pulmonary vasoconstriction and proliferation of PASMC [335-337]. A multifactorial mechanism is involved in the elevation of [Ca²⁺]_{cyto}, which includes influx through the L-type calcium channels, activation of transient receptor potential channels (TRPC) and activation of store-operated calcium channels [338-340]. Mitochondrial calcium ([Ca²⁺]_{mito}) homeostasis plays a critical role in the regulation of intracellular calcium and metabolism. Normal physiologic levels of [Ca²⁺]_{mito} are also required

to maintain the activity of the 3 mitochondrial dehydrogenases, including PDH. $[Ca^{2+}]_{mito}$ is suppressed in PAH PASMC [209]. Increased $[Ca^{2+}]_{mito}$ causes suppression of cell proliferation and increase in spontaneous apoptosis both in PAH PASMC and in cancer cells [209, 341].

Although there are complementary routes of calcium entry into mitochondria, the primary mechanism of calcium influx occurs through a multichannel complex, mitochondrial calcium uniporter complex (MCUC), located in the inner mitochondrial membrane [342]. The MCUC plays an important role in maintaining the cytosolic and mitochondrial calcium balance which is critical for activation of mitochondrial calcium-dependent dehydrogenases [343]. The MCUC is a multi-protein complex with a pore-forming subunit comprised of the mitochondrial calcium uniporter (MCU) and the essential MCU regulator (EMRE). The MCUC also includes 3 regulatory subunits including the negative regulator, mitochondrial calcium uptake 1, (MICU1). Decreases in MCU expression occur in both cancer and PAH. In PAH, MICU1 expression is also increased [209, 344]. This decrease in MCU expression and MCUC function contributes to decreased $[Ca^{2+}]_{mito}$ and increased $[Ca^{2+}]_{cyto}$, resulting in PDH inhibition and mitochondrial fission respectively [345]. These changes, in turn, promote cell proliferation and resistance to apoptosis in human and experimental PAH [209]. The expression of MCU is epigenetically regulated [209, 346]. Marchi *et al.* first reported that miR-25 is an upstream regulator of MCU. In human colon cancers and cancer-derived cells, upregulation of miR-25 silences MCU, which increases the resistance to apoptotic stimuli. Conversely, anti-miR-25 inhibits cell proliferation and increases cell apoptosis [344]. In PAH PASMC, the decreased expression of MCU is due to the increase of two regulatory miRNAs: miR-25 and miR-138. Augmenting anti-miR-25 or miR-138 restore MCU expression and regresses experimental PAH [209] (Figure 10).

The role of MCU in cancer has been most investigated in breast cancer. Unlike the case in PAH and colon cancer cells [209, 344], MCU expression is increased in various types of breast cancer tissues/cells, including estrogen receptor negative breast cancers [341] and triple negative breast cancer (TNBC) [347]. Tosatto *et al.* found that in TNBC, the expression of MCU is positively correlated with tumor size and lymph node infiltration [347]. Increased MCU and decreased MICU1 levels were correlated with worse patient outcomes [348].

Likewise, silencing MCU blunts cancer cell invasion, inhibits tumor growth, lymph node infiltration, and lung metastasis in a xenograft model of TNBC. In TNBC, HIF-1 α is the downstream target of MCU [347]. However, MCU is not always crucial for cell biology in breast cancer cell lines. In MDA-MB-468 breast cancer cells, stimulation of EGF does not alter the expression level of MCU [349]. In MDA-MB-231 cells, manipulation (overexpression or silencing) of MCU does not change cell survival, indicating that in this breast cancer cell line, MCU activity is not a major pathogenetic factor [348]. However, even this finding is open to debate as inhibition of MCU by ruthenium red (RuR) or siRNA abolishes serum-induced migration in the same cell line [350].

Another mechanism for regulation of mitochondrial calcium stores under physiological conditions involves UCP2 [351]. Heterologous overexpression of UCP2 in cardiomyocytes inhibits intramitochondrial calcium influx and decreases ROS production [352]. Consistent with these findings, SMCs lacking UCP2 switch to glycolysis due to dysregulation of the calcium-sensitive enzyme, PDH. Cells lacking UCP2 also exhibit impaired calcium flux from the ER to mitochondria [353]. In PASMC exposed to hypoxia, Nogo-B, a regulator of ER structure is upregulated by activating transcription factor 6 (ATF6), an ER stress-sensitive transcription factor. Induction of Nogo-B increases the distance between ER and mitochondria, resulting in the decrease of phospholipid and calcium transfer between ER and mitochondria. These changes finally result in increased cell proliferation by inhibiting mitochondria-dependent apoptosis, as seen in PAH [354].

These data suggest that calcium regulation in cancer is complex and not always MCU-dependent. The role of MCU in cellular pathology is disease- and tissue-specific. Normalizing MCU level might be a novel therapeutic target in PAH and in some forms of cancer. However, attention to the basal level of pathway derangement (up or down regulated) will likely be important. As with Mfn2, one cannot make a generalization regarding the role of MCU in all tumors as there appears to be significant heterogeneity in the effects of the MCUC on tumor progression.

2.6 Apoptosis in PAH and cancer

Apoptosis is an essential physiological process by which multicellular organisms maintain quality control and eliminate abnormal cells to maintain homeostasis. At the molecular and cellular level, a series of morphological and biochemical changes are involved in apoptosis, including the formation of apoptotic bodies, shrinkage of the cells, caspase activation and DNA fragmentation [355].

In PAH, resistance to apoptosis displayed by abnormal PASMCs result in the proliferation/apoptosis imbalance, which contributes to vascular obstruction and elevation of the PVR. In many cases, PAH patients receive a diagnosis at an advanced stage of the disease when many changes in the vessel wall layers have already taken place; therefore, cell growth inhibition may have limited value in reducing PVR at this stage. Thus, much current research is directed toward targeting these unwanted cells by induction of apoptosis [356].

Likewise, in cancer, the imbalance of proliferation and apoptosis results in abnormal accumulation of cells. Apoptosis-resistance contributes to cellular multiplication and tumorigenesis; therefore, substantial current cancer research focuses on advancing novel therapeutic inducers to execute apoptosis [357].

Two crucial signalling pathways regulate apoptosis: the extrinsic (cell receptor) and the intrinsic (mitochondrial) pathways. Cell viability is governed at the molecular level by a balance between pro-apoptotic and anti-apoptotic signals. Within the mitochondrial pathway, anti-apoptotic signalling is mediated by several gene families that promote the integrity of the outer mitochondrial membrane, the most prominent being the Bcl-2 family (BCL-2, BCL-XL and Mcl-1) [358]. Increased expression of anti-apoptotic proteins is involved in the development and progression of PAH as well as cancer [359]. Conversely, Bax, Bak and Bcl-2 homology 3 (BH3) proteins are pro-apoptotic. Therefore, stimulating the pro-apoptotic signal or blocking anti-apoptotic function could offer a potential therapeutic benefit in reversing pulmonary vascular remodelling in PAH and in regressing cancer.

The PI3K/AKT/mTOR signalling pathway represents a key regulatory mechanism to control pro- and anti-apoptotic Bcl-2 family proteins' activity. Small-molecule inhibitors of this signalling pathway offer new means to regulate mitochondrial apoptosis [360]. PI3K inhibitors prime neuroblastoma cells for chemotherapy by increasing apoptosis through an increased expression of pro-apoptotic Bcl-2 family proteins [361]. In this study, the use of dual-class I PI3K/mTOR inhibitor (PI103) synergistically enhanced apoptosis induced by doxorubicin in several neuroblastoma cell lines. The FDA-approved mTOR inhibitors (e.g., Rapamycin) are used systemically as immunosuppressive but when administered locally (via the coating on coronary artery stents) are antiproliferative agent dramatically reduce local arterial restenosis by reducing intimal proliferation[362]. Rapamycin can trigger apoptosis in primary human ECs, human umbilical vein ECs, and aortic ECs by inhibiting mTORC2 [363, 364]. Rapamycin is a candidate pro-apoptotic drug for human pulmonary vascular cells in PAH as well. It reduces the proliferation of PASMCs from MCT rats and patients with idiopathic PAH [365, 366]. In hypoxia-induced PH mice, systemically administered rapamycin decreased the thickening and proliferation of the pulmonary vasculature as well as RV hypertrophy [367]. However, McMurtry *et al.* reported that neither rapamycin nor rapamycin + atorvastatin has therapeutic benefits for the MCT-PAH model [368]. These discordant findings may reflect differences between the pathogenesis of preclinical models of PH (hypoxia vs MCT) as well as the methodology used (*in vitro* study vs *in vivo* study). In a clinical study, everolimus, another mTOR inhibitor, improved PVR and 6-minute walk distance (6MWD) in 8 of the 10 patients with PAH or CTEPH [369]. A Phase 1 clinical trial of albumin-bound mTOR inhibitor (ABI-009) is also now being evaluated in patients with severe PAH; the study is expected to end by December 2020 (NCT02587325).

Molecules that directly target pro- or anti-apoptotic regulator proteins, such as ABT-737 and ABT-263 (Navitoclax), potentially have greater specificity than rapamycin. These BH3-mimetic small molecules activate BAX and BAK by displacing activator BH3 from BCL-2 and BCL-XL [370, 371], or selectively inhibit BCL-2, like ABT-199 (Venetoclax). In April 2016, the first inhibitor of BCL-2 (Venetoclax, Venclexta[®]) was approved by the FDA for the treatment of patients with chronic lymphocytic leukemia who have a 17p deletion [372]. This drug has a more specific, pure, apoptotic activity compared to other cancer medications that

affect both apoptosis and autophagy, such as anthracyclines, proteasome inhibitors, or taxanes [373-375].

Rybka *et al.* assessed the pro-apoptotic effects of BH3 mimetic drugs ABT-263 (Navitoclax), ABT-199 (Venetoclax), ABT-737, and Obatoclax in experimental PAH [376]. Each of these agents promotes the death of human PSMCs *in vitro*. Moreover, in the rat Su/Hx-PAH model, ABT-263 promotes a beneficial death of PSMCs and reverses pulmonary vascular remodelling. This demonstration that induction of apoptosis is beneficial in PAH fits well with the benefit reported with metabolic modulators, like DCA, which inhibits cell proliferation, promotes apoptosis and also regresses experimental PAH in most preclinical models [176, 377], and is also effective in selected patients [182].

Another mitochondrial protein identified as a key signaling molecule for apoptosis is Cyt c, which is located on the IMM. During cellular stress, Cyt c is released from the mitochondria and acts as a signaling molecule. In the cytosol, Cyt c interacts with apoptosis activating factor 1 (Apaf-1), leading to apoptosome formation and caspase activation, initiating the cell death pathway. An increase in cytosolic Cyt c, in association with Apaf-1, is a trigger for the activation of caspases (e.g., caspases-3, -5, and -7), which activates the cysteine proteases that serve as the central executioners of the apoptotic pathway [378].

Inhibiting survivin, an inhibitor of apoptosis that is upregulated in cancer and PAH, has potential therapeutic benefit in decreasing adverse vascular remodeling in experimental PAH [379]. McMurtry *et al.* showed that the delivery of phosphorylation-deficient survivin to PSMCs from MCT-PAH rats reduces proliferation and increases apoptosis. The survivin mutant depolarizes PSMC mitochondria and initiates a leak of Cyt c and apoptosis-inducing factor (AIF) in the cytoplasm, thereby mediating mitochondria-dependent apoptosis. Gene therapy with a survivin mutant improves hemodynamics, reduces remodeling of the resistance PAs, and prolongs survival in MCT-PAH rats. Another study has shown that treating human PSMC with Puerarin, the primary active ingredient extracted from the root of a Chinese medicinal herb, kudzu, induces Cyt c release and caspase-9 activation in hypoxic but not

normoxic human PASMCs *in vitro*. Puerarin decreases Bcl-2 and increases Bax expression [380].

Once established, PAH is characterized by apoptosis resistance, reminiscent of the apoptosis-resistant state of cancer. Induction of mitochondria-dependent apoptosis is a potential therapeutic strategy to regress PAH and cancer [379].

Conclusion

Oxygen sensing is mediated by mitochondria and their ability to transduce changes in pO_2 into redox signals that regulate ion channels and enzymes. In both PAH and cancer, the oxygen sensing pathway is abnormal, and cells behave as if they were hypoxic, often despite abundant ambient O_2 . Dysregulation of the oxygen sensing mechanisms at many levels contributes to shared features of PAH and cancer, notably increased rates of cell proliferation and impaired apoptosis. In both PAH and cancer, changes in mitochondrial metabolism, calcium handling and dynamics create a “pseudohypoxic” environment in which the normal mitochondrial mechanisms of oxygen sensing are subverted. Aberrant mitochondrial functions shared by PAH and cancer include a shift to aerobic glycolysis (the Warburg phenomenon), abnormal mitochondrial dynamics (notably increased fragmentation due to a fission/fusion imbalance), abnormal mitophagy and impaired mitochondrial biogenesis and impaired mitochondrial calcium homeostasis (due to impaired function of the MCUC). Many of these abnormalities are epigenetically regulated and/or reflect post-translational modification of mitochondrial pathways. Many of epigenetically triggered pathways are therapeutically tractable. Recent preclinical studies demonstrate the therapeutic potential of targeting mitochondrial metabolic targets, such as PDK and PKM2, mitochondrial dynamic targets (Drp1, MiD49, MiD51 and Mfn2), regulators of mitochondrial mediated apoptosis (BCL-2), and pathways that regulate intramitochondrial calcium (such as the MCUC). Mitochondrial targets are readily regulated using miRNA, siRNA and small molecule therapeutics.

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Table 1. Summary of studies on miRNAs that target PKM2 in pulmonary hypertension and cancer cells and associated mechanistic pathway.

| MiRNA | Species | Cell Type | Mechanism |
|---|------------------------|---|--|
| miR-1, miR-133b [381], miR-124, miR-137 & miR-340 [382] | Human | Colorectal cancer | PTBP1/PKM2 pathway |
| miR-124 [152, 153, 383] | Human Rat Bovine | PAH-blood outgrowth endothelial cells PAH-fibroblasts Colorectal cancer | miR-124/PTBP1/PKM2 pathway |
| miR-133a & -133b [384] | Human | Tongue squamous cell carcinoma | Directly targets PKM mRNA |
| miR-122 [385] | Human | Hepatocellular carcinoma | Directly targets PKM2 mRNA |
| miR-148a & miR-326 [386] | Human | Thyroid cancer | Directly targets PKM2 mRNA |
| miR-148a & miR-152 [387] | Human | Breast cancer | Directly targets PKM2 mRNA |
| miR-152 [388] | Human | Breast cancer | miR-152/ β -catenin/PKM2 pathway |
| miR-372 [389] | Human | Liver cancer | miR-372/YB-1/ β -catenin/LEF/TCF4-PKM2 pathway |
| miR-491-5p [390], miR-1294 [203] | Human | Osteosarcoma | Directly targets PKM2 mRNA |
| miR-361-5p [391] | Human | Prostate cancer | miR-361-5p/Sp1/PKM2 pathway |

| | | | |
|------------------|-------|-----------------|----------------------------|
| miR-let-7a [392] | Human | Cervical cancer | Directly targets PKM2 mRNA |
|------------------|-------|-----------------|----------------------------|

Table 2. Therapeutics targeting aerobic glycolysis in PAH and cancer

| Therapy | Category | Mechanism | Tested disease(s) |
|-------------|----------------|---|--|
| DCA | Small compound | PDK inhibitor | PAH [97, 176] [89, 154, 180, 181] [182] Lung cancer, glioblastoma, breast cancer, prostate cancer [187-190], glioblastoma [192], non-Hodgkin's lymphoma [193] |
| siPTBP1 | siRNA | Decrease PKM2/PKM1 ratio by inhibition of PTBP1 | PAH [152, 153] |
| miR-124 | miRNA | Decrease PKM2/PKM1 ratio by inhibition of PTBP1 | PAH [152, 153] |
| shikonin | Small compound | Decrease PKM2/PKM1 ratio | PAH [152, 153], Group 2 PH [183] |
| TEPP-46 | Small compound | Stabilize PKM2 tetramer form | PAH [153] Lung cancer [204, 205] |
| echinomycin | Small compound | Inhibits PDK1 by inhibiting HIF | Colon cancer, pancreatic cancer [191], lung cancer [188], pancreatic cancer [190] |
| miR-1294 | miRNA | Inhibits PKM2 expression | Osteosarcoma [203] |

Table 3. Therapeutic agents targeting mitochondrial fission/fusion mediators in PAH and cancer

| Therapy | Category | Target | Tested disease(s) |
|------------|----------------|-----------------------|--|
| siDrp1 | siRNA | Drp1 | PAH [214], lung cancer |
| mdivi-1 | Small compound | Drp1 GTPase domain | PAH [214], lung cancer [211], brain tumors [240], breast cancer [248, 249], melanoma [250] |
| P110 | Peptide | Drp1-FIS1 interaction | RV-IR injury [235] |
| Drpitor1a | Small compound | Drp1 GTPase domain | Lung cancer [262], RV-IR injury [262] |
| siMiD49/51 | siRNA | MiD49, MiD51 | PAH [212], lung cancer [213], breast cancer [213] |
| miR-34a-3p | miRNA | MiD49, MiD51 | PAH [212], lung cancer [213], breast cancer [213] |

| | | | |
|-------------|----------------|------|-------------------------------------|
| Adv-Mfn2 | Adenovirus | Mfn2 | PAH [216], lung cancer [211] |
| leflunomide | Small compound | Mfn2 | Mouse spontaneous tumors [279] |
| miR-125a | miRNA | Mfn2 | PANC-1 pancreatic cancer cell [287] |

Figure legends

Figure 1. System for Homeostatic Oxygen Sensing.

The homeostatic oxygen-sensing system (HOSS) is made up of specialized tissues specifically adapted to sense small changes in oxygen levels. These include the type 1 cells within the carotid body, smooth muscle cells (SMC) within the resistance pulmonary arteries (PA), fetoplacental arteries, and ductus arteriosus. Hypoxia increases the afferent carotid-sinus nerve fiber action potential frequency, which stimulates respiration. Hypoxia also elicits vasodilation of the ductus arteriosus and systemic arteries, while stimulating vasoconstriction of the fetoplacental and resistance PA.

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Figure 2. The mechanism of acute hypoxic pulmonary vasoconstriction.

Middle: Pulmonary artery (PA) pressure is increased while renal artery pressure is decreased during hypoxia.

- A. Potassium channel current is reduced in PA smooth muscle cells (SMC) during hypoxia.
- B. Percentage decrease in SMC length with hypoxia is only observed in PAs with diameters of less than 600 μ m.
- C. PA pressure response to hypoxia is largely abolished by treatment with verapamil, a calcium channel blocker.
- D. PA pressure response to hypoxia is increased by BAY K8644, a calcium channel agonist.
- E. PA pressure increases in response to decreased pO₂.

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Figure 3. The Redox Theory of HPV.

- A. Under normoxic conditions, ETC produces higher level of ROS and the elevated NAD(P)/NAD(P)H ratio oxidizes the sulfhydryl groups on K⁺ channels, keeping them open, while the Ca_L channel remains closed.
- B. Under hypoxic conditions, decreased ROS production and reduced NAD(P)/NAD(P)H ratio inhibit the opening of K⁺ channels and leads to the opening of Ca_L channels. Influx of Ca²⁺ further triggers Ca²⁺ release from the sarcoplasmic reticulum (SR), stimulating actin and myosin and triggering vasoconstriction.

Figure 4. Role of HIF-1 under normoxia, hypoxia and pseudohypoxia.

In normoxic conditions, prolyl hydroxylases (PHDs) modify proline residues within the HIF-1 α subunit. This allows the von Hippel-Lindau protein (VHL) to ubiquitinate HIF-1 α , targeting it for proteasomal degradation. Under hypoxic conditions, PHD is inactivate. As a consequence, HIF-1 α is not targeted for degradation by VHL. This allows HIF-1 α to accumulate within the cytoplasm and translocate into the cell nucleus where it dimerizes with HIF-1 β to form the active HIF complex. HIF complexes in the nucleus are capable of binding to hypoxia response elements (HRE) located within regulatory elements of genes known to control processes related to oxygen delivery and oxygen deprivation, including pathways involved in cell metabolism, angiogenesis, erythropoiesis, cell proliferation, and apoptosis. Normoxic activation of HIF-1 α (pseudohypoxia, as seen in PAH and cancer) contributes to the metabolic shift, cell proliferation and angiogenesis.

Figure 5. Mediators of Warburg metabolism in PAH and cancer.

miR: microRNA; BMPR2: bone morphogenetic protein receptor type 2; PTBP1: polypyrimidine tract-binding protein; PKM: pyruvate kinase muscle isozyme; HIF-1 α : hypoxia-inducible factor-1 α ; ERK: extracellular signal related kinase; DNMT: DNA methyltransferase; SOD2: superoxide dismutase 2; ETC: electron transport chain; PDH: pyruvate dehydrogenase; PDK: pyruvate dehydrogenase kinase; MCUC: mitochondrial calcium uniporter complex; NFAT: nuclear factor of activated T cells; EGFR: endothelial growth factor receptor; LDHA: lactate dehydrogenase A; MPT: mitochondrial pyruvate transporter; PDGF-R: platelet-derived growth factor receptor; STAT3: signal transducer and activator of transcription 3.

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Figure 6. Mechanisms involved in imbalanced PKM2/PKM1 and therapeutic targets.

PKM: pyruvate kinase muscle isozyme isoform; PTBP1: polypyrimidine tract binding protein 1; hnRNP: heterogeneous nuclear ribonucleoprotein; TCA: tricarboxylic acid; ROS: reactive oxygen species; HDAC: histone deacetylase; SAHA: suberoylanilide hydroxamic acid.

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Figure 7. Pathways involved in increased MiD49/51 induced mitochondrial fission, cell proliferation and apoptosis resistance in A) PAH and B) cancer.

A) The expression of MiD49 and MiD51, is increased in PAH due to the downregulation of their regulatory microRNA, miR-34a-3p. This increase in MiD expression drives pathological mitochondrial fission by sequestering activated Drp1 to the outer mitochondrial membrane (OMM), cell proliferation and apoptosis resistance via PDGFR- α / β -Ras-Raf-Erk pathway. B) In cancer, downregulation of miR-34a-3p leads to MiD upregulation which in turn drives mitochondrial fission by sequestering activated Drp1 to the outer mitochondrial membrane (OMM), cell proliferation and apoptosis resistance via Akt-mTOR-S6K axis.

Figure 8. Drpitor1a, a novel Drp1 GTPase inhibitor.

A: Synthesis of Drpitor1a.

B: Predicted interaction between Drpitor1a and the GTPase domain of Drp1.

C: The binding locations of Drpitor1 (red), Drpitor1a (blue), and guanylyl-imidodiphosphate (GMP-PNP) (orange and green) in the GTPase domain of Drp1.

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Figure 9. Therapeutic effects of Mfn2 in PAH.

A. Overexpression of Mfn2 by adenoviral mediated gene transfer (Adv-Mfn2) induces mitochondrial fusion in control PASMCM and PAH PASMCM. Cells were loaded with the potentiometric dye TMRM (red) and imaged with confocal microscope to assess the mitochondrial network structure. Scale bar: 50 μ m.

B. Representative CT angiogram showing pruning of small pulmonary arteries in Su/Hx rats, which can be reversed by augmenting Mfn2. Su/Hx rats were treated either with Ad-GFP or Ad-Mfn2. Compared to the control, Su/Hx rats exhibit decreased percentage of small pulmonary arteries whilst Ad-Mfn2 treatment increased the percentage of small pulmonary arteries.

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Figure 10: MCU dysregulation in PAH PASMCM

Representative images of the photoactivation experiments showing mitochondrial network in A) Normal PASM C B) PAH PASM C. The cells were co-transfected with specified siRNA or specified plasmids, then photoactivatable mitochondrial matrix targeted green fluorescent protein (mito-PA-GFP) plasmid and then loaded with potentiometric dye TMRM (red).

- A. siMCU treatment increased mitochondrial fission and restricted the diffusion of the green signal outside the white box in normal PASM C.
- B. MCU augmentation inhibited mitochondrial fission as shown by the increased diffusion of the green signal outside the white box. Scale bar: 5 μ m.
- C. Mechanism of downregulation of MCU in PAH PASM C.

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