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Hypoxic Pulmonary Vascular Smooth Muscle Cell Proliferation

Shiro Mizuno, Hirohisa Toga and Takeshi Ishizaki

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<http://dx.doi.org/10.5772/45979>

1. Introduction

Pulmonary arterial smooth muscle cell (PASMC) proliferation in response to hypoxia is thought to be a key component of the vascular remodeling that occurs in chronic hypoxic pulmonary hypertension. Many pulmonary disorders, including chronic obstructive pulmonary disease, are associated with chronic hypoxia, and when pulmonary hypertension and right heart failure develop due to pulmonary vascular remodeling, patient survival is impaired. Hypoxia is one of the factors known to cause secondary pulmonary hypertension and pulmonary vascular remodeling [1]. According to a WHO statement in 1996, there were approximately 140 million people living at altitudes above 2500m and there are several areas of permanent habitation at altitudes in excess of 4000 m. After several weeks of exposure to high altitude, lowlanders develop pulmonary hypertension, which is not completely reversed by supplemental oxygen [2], suggesting development of vascular remodeling of the lung [3]. Secondary pulmonary hypertension is characterized by proliferation of vascular smooth muscle cells and pulmonary arterial fibroblasts in small pulmonary vessels [4-6]. These results suggest that hypoxic enhancement of PASMC proliferation contributes to the progression of hypoxia-associated small pulmonary arterial remodeling and secondary pulmonary hypertension. In animals, hypoxia has been shown to cause pulmonary vascular wall thickening by inducing PASMC proliferation [7-9]. Most commonly, pulmonary vascular remodeling is studied in rats or mice exposed to 10% oxygen hypoxia for 2 to 8 weeks [9,10]. In the animals exposed to chronic hypoxia, muscular arteries increase the thickness and distal extension and migration of PASMC into normally non-muscular arteries can be observed (Figure 1).

In addition, many *in vitro* studies have also addressed the proliferation of vascular smooth muscle cells upon exposure to hypoxia [11,12]. Increased levels of growth factors derived from the accumulation of hypoxia-inducible factor 1 α (HIF-1 α) are thought to regulate

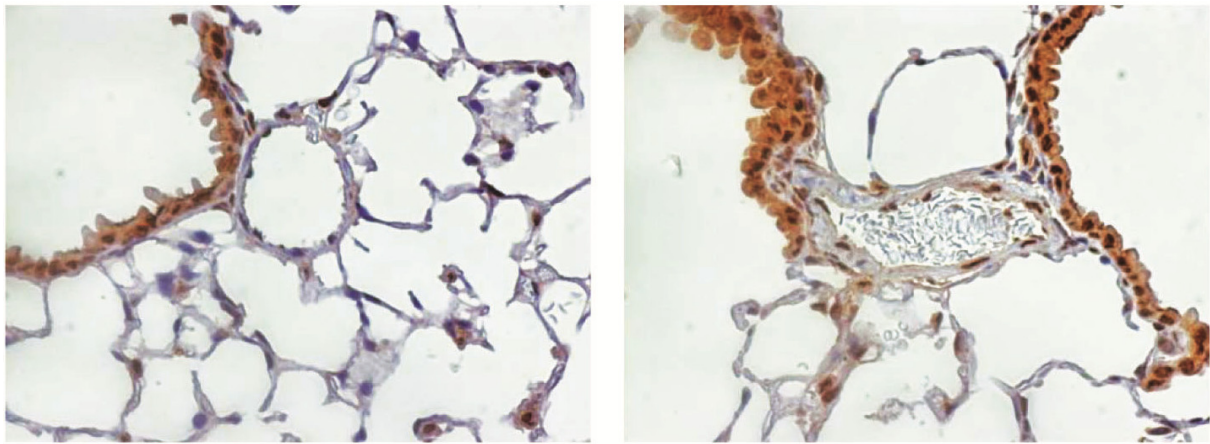


Figure 1. Proliferating cell nuclear antigen (PCNA) staining of small pulmonary arteries from mice exposed to chronic hypoxia (10% oxygen) for 8 weeks. PCNA positive cells were found in the smooth muscle layer of small pulmonary arteries (right picture) compared with normoxic control (left picture).

PASMC proliferation under hypoxic conditions, since a partial HIF-1 α deficiency decreases muscularization of pulmonary arterioles in animals exposed to chronic hypoxia [7]. However, it is unclear whether hypoxia is a direct mitogen or indirect mitogen induced by the mediators from endothelial cells or fibroblasts, because some investigators have shown that hypoxia is not a direct stimulus of PASMC proliferation [13,14]. This discrepancy may be explained by the severity of hypoxia. The investigators who have found PASMC proliferation by hypoxic exposure usually used the moderate hypoxia (1 – 5% oxygen) [1,11,12,15,16].

Although HIF-1 α regulates various transcriptional genes for angiogenic factors, severe hypoxia and iron depletion induce cell growth arrest. In contrast to severe hypoxia, moderate hypoxia can also enhance the proliferation of airway-smooth muscle cells, lung fibroblasts and mesangial cells [17,18]. Proliferation of PASMCs, which causes pulmonary vascular remodeling, requires re-entry of the cells into the cell cycle. We confirmed that the cultured PASMC cell cycle progresses more quickly in hypoxia and that severe hypoxia or iron depletion using an iron chelator, which mimics anoxia, caused inhibition of cell cycle progress compared to the normoxic conditions (Figure 2).

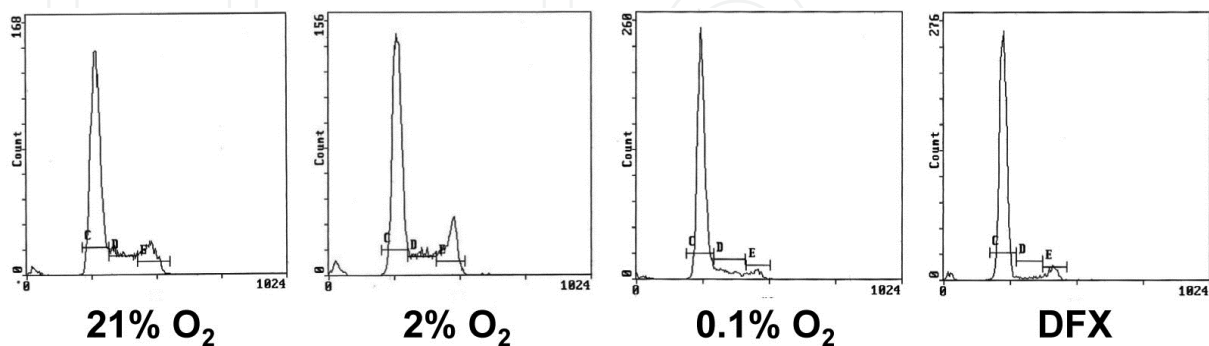


Figure 2. Cell cycle analysis of human pulmonary arterial smooth muscle cells (HPASMC) cultured in various concentration of oxygen (0.1%, 2%, 21%) or 100 μ M of iron chelator, desferroxamine (DFX) using flow cytometric analyses with propidium iodide staining. S+M phases are increased in moderate hypoxia (2% oxygen) compared to the normoxic condition. Severe hypoxia (0.1% oxygen) and iron chelator decreased the S+M phases in cultured HPASMCs.

2. Cell cycle regulation of hypoxic PASMC proliferation

Under normal physiological conditions, the majority of the pulmonary vascular cells are in a quiescent state. The most important molecular event necessary for progression of the cell cycle is phosphorylation of the retinoblastoma protein by cyclin-dependent kinase (CDK)-cyclin complexes. Cell cycle progression requires the coordinated interaction of CDK and its regulatory subunits, the cyclins, to drive cells through G1 into S phase to ultimately result in cell division. Cyclin-CDK complexes activate transcription factors important in cell cycle progression. The cyclin-CDK complexes include cyclin D-CDK4/CDK6 and cyclin E-CDK2 which inactivate retinoblastoma, an antitumor and antiproliferative protein which limits E2F-mediated gene transcription [19]. CDK inhibitors are proteins that bind cyclin-CDK complexes, inhibit hyperphosphorylation of retinoblastoma, cause G1 arrest, and suppress cell proliferation [20]. CDK activity can be inhibited by CDK inhibitors, which arrest the cell cycle at each corresponding phase and inhibit cell proliferation. Two families of CDK inhibitors have been shown to regulate vascular smooth muscle cell proliferation, p21 and p27. The loss of CDK inhibitors has been implicated in tumor development [21,22], and is closely related with the state of the tumor suppressor p53 [23,24].

2.1. Role of tumor suppressor p53 and CDK inhibitor p21

The endogenous CDK inhibitor p21 plays an important role in PASMC proliferation via induction of the tumor suppressor p53 [23,25], and has been identified as a key regulator of the cell cycle in cells exposed to hypoxia and oxidative stress [26-28]. In tumors expressing wild-type p53, apoptosis occurs in hypoxic regions, whereas tumors expressing mutant p53 exhibit lower levels of apoptosis in hypoxic regions [29]. p53^{-/-} mouse embryo fibroblasts are more resistant to hypoxia-induced apoptosis, and have selective growth advantages compared to wild-type p53 cells [30]. In addition, hypoxic p53 accumulation has been linked to the hypoxia-inducible factor-1 α (HIF-1 α), which is known as a central transcriptional factor operating during hypoxia toward angiogenesis [31,32]. We recently reported that hypoxic p53 accumulation has been linked to the hypoxia inducible factor-1 α (HIF-1 α) [9]. These results support the view that the p53 protein opposes cell proliferation under hypoxia, and p53 plays a critical role as a modulator of hypoxia-induced small pulmonary arterial remodeling. Decreased expression of p21 and increased expression of HIF-1 α via suppression of p53 protein may mitigate hypoxic pulmonary arterial remodeling and PASMC proliferation. Recently, several groups identified microRNAs (miRNAs) regulated by p53 [33,34]. miRNAs are non-coding RNA molecules which modulate gene expression by binding to complementary sequences in the coding - or the 3'-untranslated region of target mRNAs. miRNAs can regulate cell proliferation, differentiation and apoptosis [35,36]. The miR34a has been shown to be the most significant miRNA induced by p53, which is closely related to induction of apoptosis and cell cycle arrest in cancer cells [37]. We have shown that this miRNA is also associated with HIF-1 α expression both in animal and human lung tissues [9,38,39].

p21 has been shown to regulate cell cycle progression through both p53-dependent and -independent pathways [24,25,40]. However, it is known that nitric oxide (NO) donors suppress proliferation of cultured PASMC via the expression of p53 and p21 [23]. NO is synthesized from L-arginine via nitric oxide synthase (NOS), and endothelial NOS plays an important regulatory role in hypertrophic and hyperplastic growth of PASMC *in vivo* and *in vitro*. Several studies have suggested that NO derived from endothelial NOS has a protective effect toward arterial smooth muscle cell proliferation [41],[42]. It is possible that the anti-proliferative effect of NO derived from pulmonary arterial endothelial cells is depend on the status of p53 in PASMC.

In our recent data from p53 knockout mice, chronic hypoxia increased p21 expression and induced medial wall thickening of small pulmonary arteries in wild type mice, and the deletion of the p53 gene prevented the hypoxic induction of p21 expression [9]. These results indicate that under hypoxic conditions, induction of the p53-p21 signaling pathway serves as a negative feed-back to prevent excessive vascular cell proliferation and vascular remodeling. Using cultured PASMCs, we confirmed that the anti-proliferative NO pathway was intact in the hypoxic condition and the protein expression of p21 was associated with HPASMC proliferation (Figure 3).

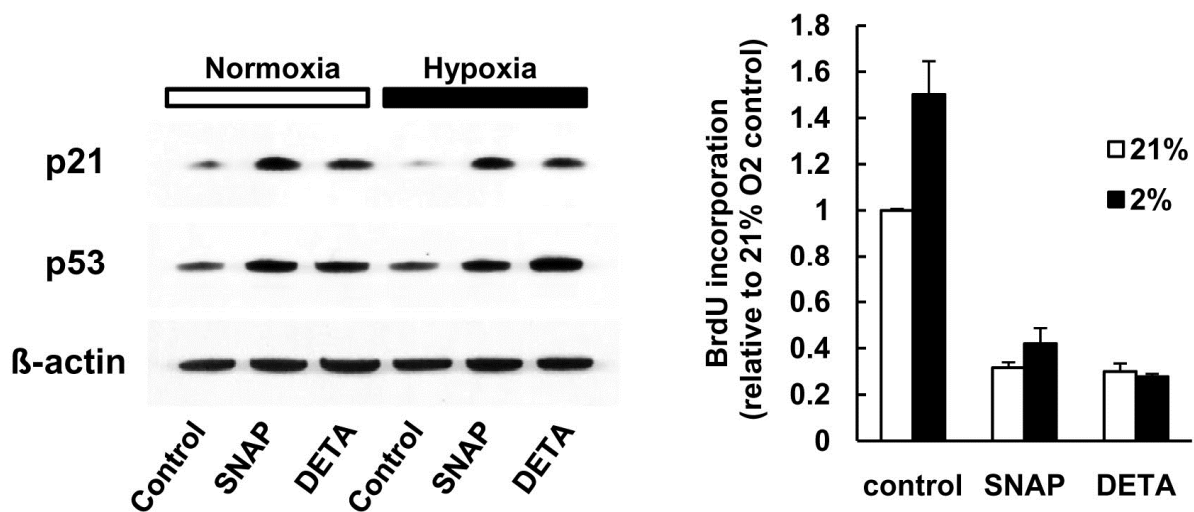


Figure 3. Western blot analysis of p21 and p53 (left photographs) and BrdU incorporations in cultured PASMC exposed hypoxia (2% oxygen) and NO donors (SNAP and DETANO) (right graph). Moderate hypoxia decreased p21 and the NO donors increased the both p21 and p53 protein expressions. The NO donors suppressed DNA amplification in cultured PASMCs during hypoxia.

2.2. CDK inhibitor p27

The suppressive effect of hypoxia on p27 expression has been demonstrated in mice with pulmonary hypertension induced by hypoxia [8]. However, the expression of p27, which blocks the cell cycle at the G0/1 phase, is regulated by several mechanisms including transcription, protein degradation and translation [43-45]. We reported that hypoxia-induced down-regulation of p27 was not mediated by hypoxia *per se*, but rather by mitogenic factors

including PDGF and hypoxia enhanced p27 protein degradation. The moderate hypoxia enhanced the proliferation of serum-stimulated PASMCM in accordance with promoted p27 protein degradation, probably via the induction of growth factors [12]. The prostacyclin analogue suppressed PASMCM proliferation under both hypoxic and normoxic conditions by blocking p27 mRNA degradation through an increase in intracellular cAMP. Hypoxia may activate HIF-1 α regulated growth factors and cell growth signaling such as mitogen activated protein kinase (MAPK). We also found that hypoxic exposure and p53 regulate MAPK activation in cultured PASMCMs (Figure 4). To clarify the effect of p53 on the activation of MAPK in hypoxic PASMCM proliferation, we performed gene silencing of p53 to the cultured PASMCM. The gene silencing of p53 suppressed MAPK activation, which indicates hypoxic activation of MAPK and p53 are also associated with the degradation of p27 and hypoxic PASMCM proliferation.

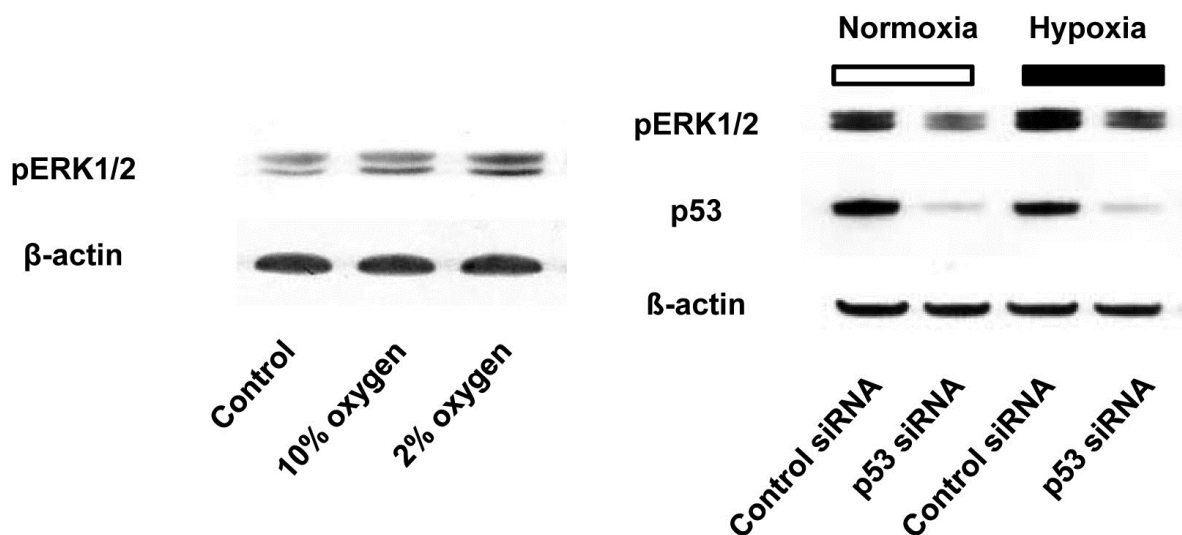


Figure 4. Left figure shows protein expressions of phosphorylated ERK1/2 in cultured PASMCM exposed hypoxia (2 - 10% oxygen). Right figure shows effect of p53 gene silencing on ERK1/2 phosphorylation in cultured PASMCM exposed to hypoxia (2% oxygen). Hypoxic exposure induced MAPK activation, and the p53 protein expression are suppressing the MAPK activation.

Previous findings have suggested that p27 mRNA stability is controlled by interactions between MAPK [46] and Rho-dependent translation [47]. Further, cAMP induces cell relaxation through Rho GTPase activation [48,49], which might be an important target of hypoxic pulmonary vascular remodeling [50,51]. These reports imply that the Rho and MAPK interaction contributes to p27 mRNA stability during exposure to agents that elevate cAMP and hypoxia. These interactions may be also possible in smooth muscle cells. The Rho inhibitor Y-27632 inhibited human aortic smooth muscle proliferation in response to platelet derived growth factor and markedly suppressed neointima formation associated with decreased expression of p27 in rat carotid artery [52]. Regarding the effect of Rho on the expression of p27 in PASMCM, we simply measure the p27 protein expression using Rho inhibitor in cultured PASMCM and found that the p27 protein expression was increased by Y-27632 (Figure 5).

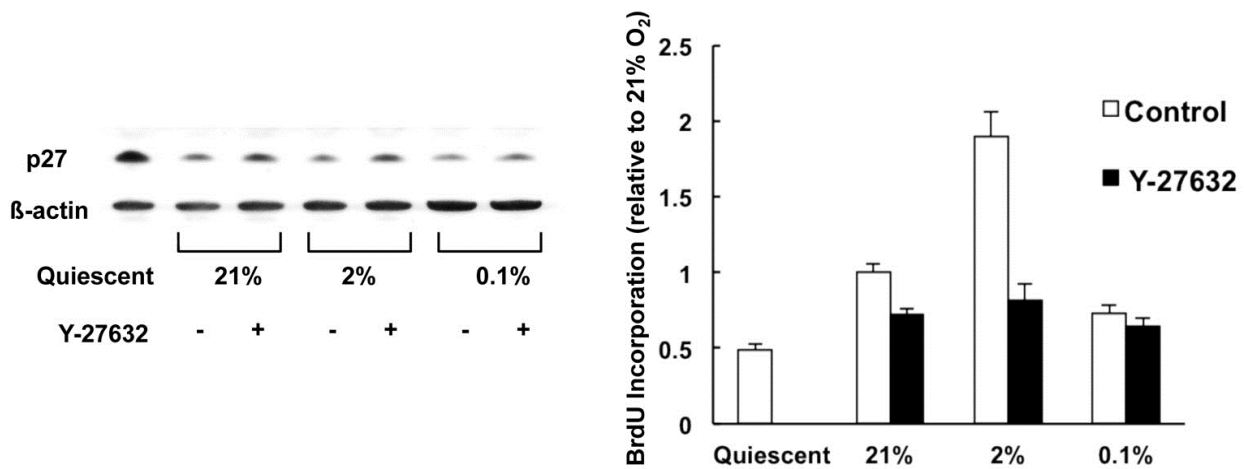


Figure 5. p27 protein expression in cultured PASMC exposed to hypoxia with 10 μ M of Rho inhibitor Y-27632 (left figure) and BrdU incorporations in cultured PASMC exposed to hypoxia with or without Y-27632 (right graph). The p27 protein expression was increased by the treatment of Rho inhibitor both in the normoxic and hypoxic conditions with association of the cell proliferation.

3. Conclusion

It is well accepted that hypoxia is a cause of pulmonary vascular remodeling and PASMC proliferation. In the aggregate, research conducted by us and others suggests that decreased oxygen levels affect PASMC proliferation. Decreased expression of p27 and signal transduction via p53 and p21 play critical roles in the fine-tuning of hypoxic PASMC proliferation. *In vitro* studies have demonstrated that hypoxia has direct mitogenic effect on cultured PASMC by increased production of growth factors and decreased expression of CDK inhibitors. The cell cycle regulation by p53 may be closely related with the severity of hypoxia and HIF-1 α status (Figure 6).

However, the hypoxia induced remodeling of the pulmonary circulation including PASMC proliferation is a highly complex process, which may have numerous interactions between the vascular cells, especially between endothelial cells and lung fibroblasts. Because of that, it is difficult to explain the *in vivo* pulmonary vascular remodeling using single cell culture experiments. For example, hypoxic endothelial cells and adventitial fibroblasts around PASMC may also be able to release mitogenic factors for PASMC proliferation, and damage of vascular endothelial cells by hypoxia can lead the decrease of anti-proliferative mediator production resulting PASMC proliferation. In addition, hypoxic proliferation of lung fibroblasts can secrete matrix proteins, which may play an important role for the proliferation of PASMC [53]. Further studies are necessary to characterize the role of cell cycle regulations on the hypoxic vascular cell proliferation, and to clarify the interactions between PASMC, pulmonary vascular endothelial cells and fibroblasts. We believe that a better understanding of the genetic and cellular mechanisms of hypoxic pulmonary remodeling will lead to improved modes of therapy for hypoxia-associated changes in lung tissue structure and aid in the remodeling of pulmonary hypertension.

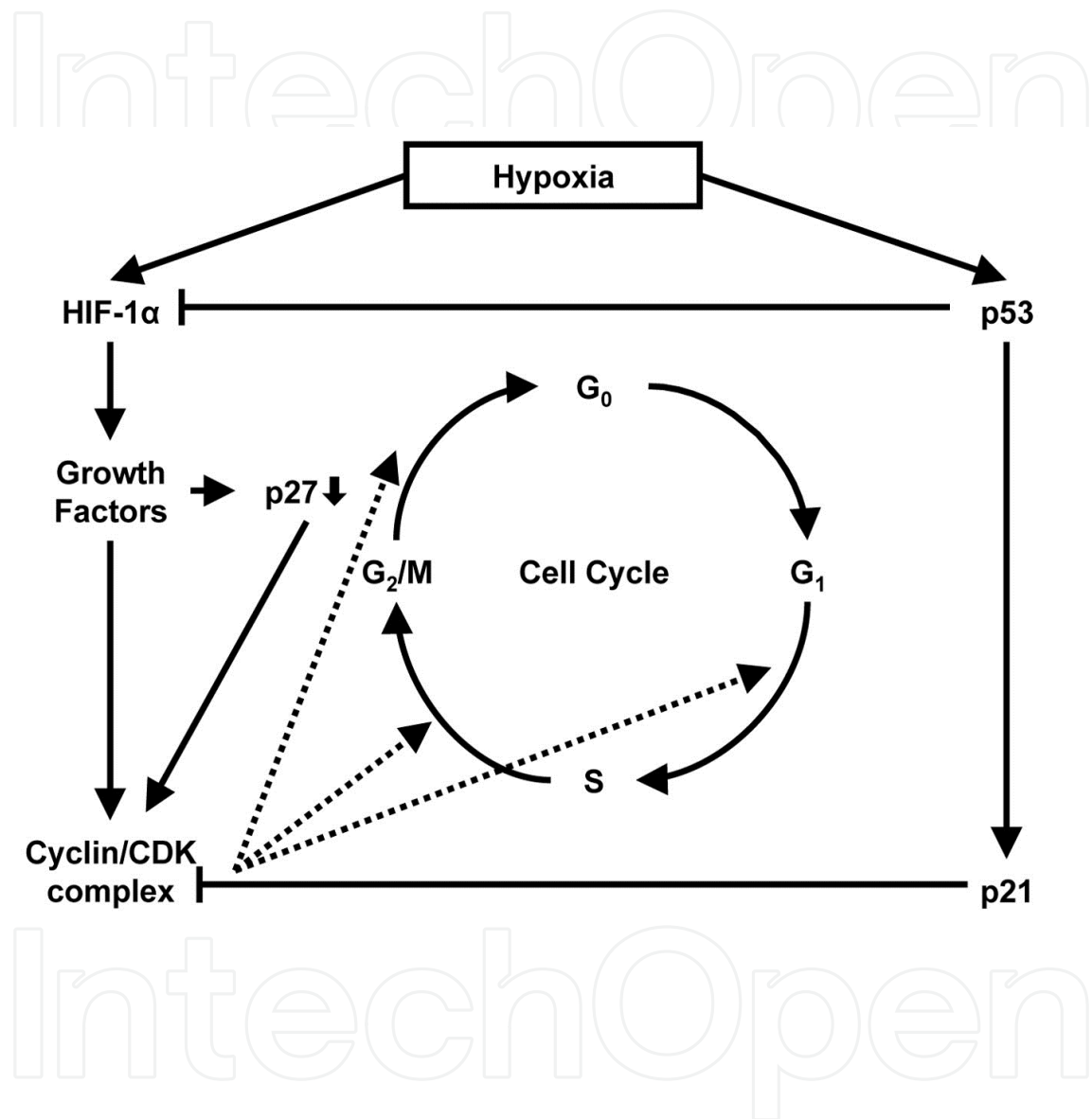


Figure 6. Schematic depicting molecular interactions in the hypoxic PASMC proliferation: Hypoxic exposure increases HIF-1 α and p53 protein expression. Up-regulated p53 induces p21 expression and the p21 inhibit G₁/0 transition via inhibition of cyclin/CDK complex. In contrast, HIF-1 α activation causes up-regulation of growth factors that reduce the expression of p27. Reduced expression of p27 and increased HIF-1 α transactivation induces cyclin/CDK complex, which causes progress of cell cycle of pulmonary smooth muscle cell.

Author details

Shiro Mizuno^{1*}, Hirohisa Toga¹ and Takeshi Ishizaki²

¹*Division of Respiratory Disease, Kanazawa Medical University, Ishikawa, Japan*

²*Department of Fundamental Nursing, University of Fukui, Fukui, Japan*

Acknowledgement

We wish to thank Prof. Norbert F Voelkel, Virginia Commonwealth University, Richmond, VA, USA and Dr. Herman J. Bogaard, VU University Medical Center, Amsterdam, the Netherlands, for their critical reading of this manuscript.

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