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TRAM-34 attenuates hypoxia induced pulmonary artery smooth muscle cell proliferation

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Abstract. – OBJECTIVE: Hypoxia is an important risk factor for pulmonary arterial remodeling in pulmonary arterial hypertension (PAH). Pulmonary artery smooth muscle cell (PASMC) proliferation is a major contributor to pulmonary vascular remodeling. The intermediate-conductance Ca^{2+}-activated K^+ channel (Kca3.1) has been implicated in disease states characterized by excessive cell proliferation, but its role in hypoxia-induced PASMC proliferation is unknown. In the present study, we sought to investigate the effect of TRAM-34 (triarylmethane-34), a selective blocker of Kca3.1, on hypoxia-induced PASMC proliferation and underlying mechanisms.

METHODS: PASMC was exposed to hypoxia (2% O_2) for 24 hours, cell proliferation and cell cycle analysis were measured by cell counting kit (CCK-8) and flow cytometry. Cell signaling were examined using Quantitative real-time PCR and Western blotting.

RESULTS: CCK8 results showed that TRAM-34 reduced PASMC proliferation under hypoxia. Flow cytometry revealed that TRAM-34 inhibited PASMC proliferation by G0/G1 arrest. Quantitative real-time PCR and western blotting results showed that Kca3.1 mRNA and protein levels were greater in PASMC after hypoxia exposure for 24 hours. Elevated BMP2 (bone morphogenetic protein 2) levels and decreased BMPR2/Smad1 signaling activation were also observed under hypoxia, which were significantly attenuated by TRAM-34 intervention.

CONCLUSIONS: These results suggest that Kca3.1 inhibition with TRAM-34 inhibited hypoxia-induced PASMC proliferation in the G0/G1 phase. The capability of TRAM-34 to increase BMPR2/p-Smad1 signaling may be part of the mechanisms for hypoxia-induced cell proliferation. Thus, our study implies that blockade of kca3.1 might provide benefits to attenuating PAH vascular remodeling.

Key Words:
TRAM-34, Kca 3.1, Hypoxia induced cell proliferation, BMPR2.Smad1.

Introduction

Pulmonary arterial hypertension (PAH) is a hemodynamic problem that can result in serious morbidity and mortality, including right-sided heart failure and sudden death[1,2]. It has been generally accepted and acknowledged that alterations in the pulmonary vasculature, commonly marked by vascular proliferation, fibrosis, remodeling, and vessel occlusion[3]. PAH results from a variety of initiating stimuli. Hypoxia is a key factor for the pathogenesis and animal models found pulmonary artery smooth muscle cell (PASMC) proliferation in small intrapulmonary arteries and induce inflammatory cell influx into the lung, releasing numerous mediators that control the remodeling of pulmonary vessels[4]. However, the precise mechanisms by which hypoxia exposure produces PASMC proliferation are still poorly understood.

Increasing evidence suggests that ion channels play an important role in cell proliferation by enhancing intracellular Ca^{2+} signaling and affecting cell cycle progression[5,6]. Cellular proliferation requires an increase in expression and function of K^+ channels[7]. Blockade of K^+ channels inhibits proliferation of many cell types, including vascular smooth muscle cells[8]. In this regard, Ca^{2+}-activated K^+ channel (Kca3.1) which regulate membrane potential and, thus, provide the driving force for Ca^{2+} entry seem to be of paramount importance. Thus, KCa3.1 could have a pivotal role in disease states characterized by excessive cell proliferation and may emerge as a promising therapeutic target for anti-proliferative treatment. TRAM-34 (triarymethane-34), a high selective blocker of Kca3.1, could suppress cell proliferation and ameliorate disease progression in models of experimental angiogenesis[9], post-interventional arterial restenosis[10], atherosclerosis[11], and asthma[12]. However, the potential involvement of

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Kca3.1 channels in hypoxia induced PASMC proliferation has so far not been investigated. In the study, we addressed the hypothesis that Kca3.1 channels may promote hypoxia-induced PASMC proliferation and TRAM-34 could ameliorate the progression.

Bone morphogenetic protein (BMP), a member of the Transforming Growth Factor β superfamily, could regulate embryonic tissue patterning and organogenesis, as well as the remodeling of mature tissues. PAH is characterized by pulmonary remodeling and hypoxia-induced PAH animal models indicated increased BMP2 levels compared to the control group[13]. BMPR2, a BMP ligand binding receptor, is a key factor in heritable PAH. Heterozygous BMPR2-mutant mice were more sensitive to the endothelial injury and an enhanced inflammatory response than the wild type; thus, developed persistent PAH[14]. Matthias et al[15] used antagomiR-20a to restores functional levels of BMPR2 in pulmonary arteries to prevent the development of vascular remodeling. These revealed that BMPR2 also effect in hypoxia-induced PAH. Smad, especially p-Smad was activate after the BMP ligand binding to the BMPR2 and then affect the transcriptional activities. Smad protein also contributed to the cellular response to hypoxia[16]. All of these reveals that BMP2, BMPR2, p-SMAD1 contribute to the PAH. In our study, we aimed to find if Kca3.1 regulate hypoxia-induced cell proliferation through the BMPR2/p-Smad1 pathway.

Methods

Cell Culture and Hypoxia Intervention

Pulmonary artery smooth muscle cells (PASMC) of Spragne-Dawley rats were isolated and cultured as previously described[13] with minor modifications. The arteries were excised and isolated, cut into small pieces and immersed in 10 ml phosphate-buffered saline (PBS) with 0.1 mg collagenase for 15 minutes. The endothelium and adventitia were softly removed. And the remainder of vascular tissues was placed in culture plates. Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. And cell immunostaining was performed to confirm that cells were smooth muscle cells with anti-α-smooth muscle actin (α-SMA) monoclonal antibody (NeoMarkers, Lab Vision, Fremont, CA, USA).

All cells were incubated at 5% CO_2/37°C for 48 h to 50-60% confluency prior to exposure to hypoxic conditions. Normal incubators with 21% O_2 were used for the normoxic cultures. For hypoxia (2% O_2) cultures, cells were put into gas-tight modular incubator chambers (Thermo, Carlsbad, CA, USA), which were flushed with a custom gas mixture containing 5% CO_2 and 93% N_2 for 24 hours. Hypoxia was administered simultaneously with the TRAM-34 intervention.

CCK8 and Flow Cytometry

Cell viability was assessed using the tetrazolium salt WST-8-based cell counting kit (CCK8; Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's instruction. When PASMC were cultured to reach to 90% confluence, cells were detached, seeded in 96-well plates. Subsequently, cells were incubated in low-serum culture media (DMEM, 0.1% FBS: fetal bovine serum) to be kept quiescent for 24 hours. Then, TRAM-34 (20 nM, 100 nM, 200 nM), a Kca3.1 channel inhibitor (Sigma, St Louis, MO, USA) was added to the culture media. In another 24 hours of hypoxia incubation, 10 μl of CCK8 solution was added to each well and the cells were incubated for 1 h at 37 °C. Optical density was measured on a microplate reader (SpectraMax®M7; Molecular Devices, part of MDS Analytical Technologies, Sunnyvale, CA, USA) using an absorbance wavelength of 450 nm, with a reference wavelength of 630 nm. The results were expressed as the percentage of viable TRAM-34 treated cells relative to the control cells.

The cells were lifted by incubation with 2.5 ml trypsin and neutralized by the addition of 5 ml cell culture media with FBS (Invitrogen, Carlsbad, CA, USA). 1×10^6 cells were counted, spun down at 800 g and re-suspended in 1 ml of FBS-free media. The single cell suspension was fixed in 70% ethanol for at least half an hour at 4 °C. A half an hour incubation with RNase A stock at 37°C was followed by a double phosphate-buffered saline (PBS) rinsing step. Propidium iodide (PI, Sigma) was added to samples to a final concentration of 50 mg/ mL, and nuclei were stained at 4°C for half an hour in the dark. The mean fluorescence of nuclei (usually about 5000 per sample) was quantified using a Coulter Epics Elite flow cytometry (Beckman, Brea, CA, USA) equipped with a water-cooled laser tuned at 514 nm and 500 mW. Fluorescence at 615 nm was detected by a photomultiplier screened by a long-pass filter.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (Quantitative RT-PCR) Analysis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 5 μg of total RNA for each sample using moloney murine leukemia virus (MMLV) reverse transcriptase (MBI Fermentas Inc, Ontario, Canada) and random hexamer primers, according to the manufacturer's instructions. Quantitative-PCR was performed using SYBR1 GreenER qPCR SuperMix, and samples were run on BioRad iCycler CFX (Hercules, CA, USA).

Primers for Kca3,1 PCR (194 bp) were (forward) 5'-TCAATGCCACGGGACACCT-3' and (reverse) 5'- TTCTCCGCCTTGTTGAACTCC-3'. Primers for β-actin PCR (194 bp) were (forward) 5'-CCT GTA TGC CTC TGG TCG TAC C-3' and (reverse) 5'-TCT CGG CTG TGG TGG TGA AG-3'. BMP2 PCR (191 bp) were (forward) 5'- ACGGACTGCGGTCTCCTAAA-3' and (reverse) 5'- AGCAGCCTCAACTCAAACTCG -3'. BMPR2 PCR (290 bp) were (forward) 5'- TGGCATCGGTTTCTGTATTAGC-3' and (reverse) 5'- ATGTTCCATCAAAGGCACTCTG-3'. The PCR program was initiated by a 2 min denaturation step at 95°C, followed by 35 cycles of 95°C for 5 s, 60°C for 15 s and 95°C for 10 s, and melt curve at 65°C to 95°C for 5 min. PCR results were analyzed by BioRad CFX software.

Western Blotting Analysis

Cells were prepared in lysis buffer, containing 50 mM Tris-HCl, 150 mM NaCl, 1% nonyl phenoxypolyeth xylethanol (NP)-40, 0.5% sodium deoxycholate, 2 mM NaF, 2 mM EDTA, 0.1% SDS, and a protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN, USA). Equivalent amounts of protein (10 μg) from each sample were separated on 10% SDS-polyacrylamide gels, and then transferred onto 0.45 μM polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Primary antibodies used were kca3.1 antibody (1:500; Sigma, St Louis, MO, USA), BMP2 antibody (1:400;Boster, Wuhan, China), BMPR2 antibody (1:500, Boaosen, Beijing, China), p-Smad1 antibody (1:400; Boster Biotechnology, China); GAPDH antibody (1:2000, Epitomics, Burlingame, CA, USA). The signals were developed using Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

Statistical Analysis

Values were expressed as mean ± SD. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's HSD test for post hoc multiple comparisons (SPSS for Windows version 13.0, Chicago, IL, USA). A value of $p < 0.05$ was considered to indicate significance.

Results

Up-regulation of Kca3.1 Expression in Hypoxia Treated PASMC

To compare the expression levels of Kca3.1 in rat primary PASMCs from the hypoxia group and the control group, cell samples were used for Quantitative RT-PCR and Western blotting analysis. The results show that Kca3.1 RNA and protein levels were significantly increased in the hypoxia group (Figure 1, $p < 0.05$ vs Con).

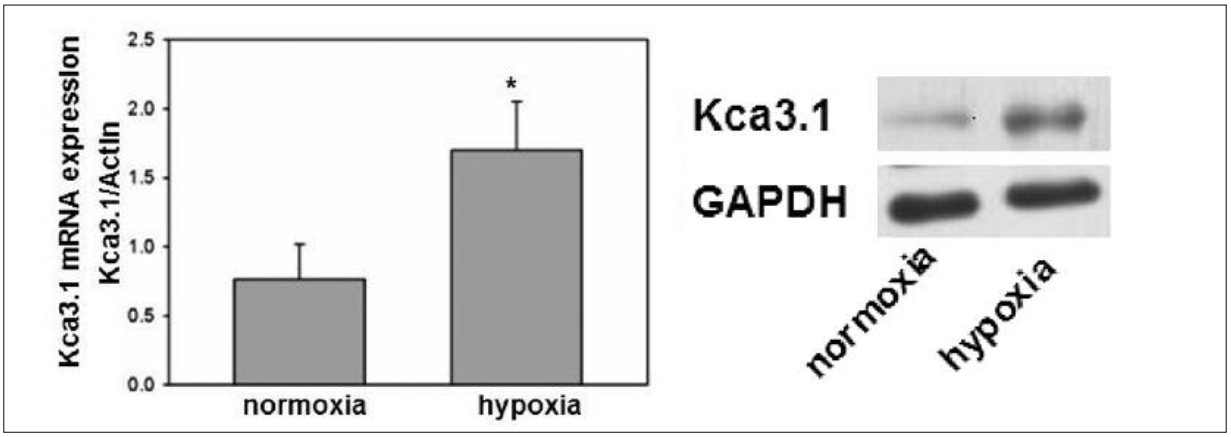

Figure 1. Kca3.1 mRNA and protein expression of PASMC. Hypoxia induced Kca3.1 mRNA and protein expression increase. Results are expressed as mean ± SD. Statistical differences were assessed by the Tukey's HSD test. *$p < 0.05$ versus normoxia conditions.

Pharmacological Blockage of Kca3.1 Ameliorate PASMC Proliferation

To determine whether hypoxia could induce PASMC proliferation and whether Kca3.1 is involved in the proliferation, CCK8 of different groups were compared. Optical density analysis revealed notable increase of PASMC in the hypoxia group, and pharmacological blockade of Kca3.1 with TRAM-34 dose-dependently decreased hypoxia-induced cell proliferation incorporation of CCK8 (Figure 2A) with maximum effects observed at 200 nM (0.77 ± 0.07 versus 1.13 ± 0.13 in hypoxia-treated cells, n = 6, $p <$ 0.05). TRAM-34 inhibited cell proliferation in the 100 nM and 200 nM.

To elucidate further of the mechanism by which inhibiting Kca3.1 channels suppresses proliferation of PASMC, flow cytometry was used to detect TRAM-34 on cell cycle progression. To ensure G0/G1 phase synchronization, cells were switched to quiescent medium 24h before intervention. Stimulation with hypoxia resulted in a distinct cell shift from phase G0/G1 87.7% to phases S 8.7% and G2/M 3.6% as compared with unstimulated cells (G0 /G1: 77.9%; S: 14.2%; G2/M: 7.9%). In the presence of TRAM-34, this hypoxia-induced shift was prevented (G0/G1: 80.5%; S: 10%; G 2/M: 9.5% for 100 nM; G0/G1: 83.1%; S: 7%; G 2/M: 9.9% for 200 nM), indicating that KCa3.1 inhibition caused a cell cycle arrest in phase G0/G1 (Figure 2B).

Kca3.1 Inhibition Increased Hypoxia Induced BMPR2/Smad Signaling Activation

BMP2 levels was increased in the hypoxia group, TRAM-34 down-regulated the expression of BMP2. To determine the involvement of Kca3.1 pathway in hypoxia-induced PASMC proliferation, we assessed BMPR2/Smad signaling activation with cell samples. In the hypoxia group, BMPR2 levels were significantly lower than in the control group. Both 100 nM and 200 nM TRAM-34 treatment increased the BMPR2 concentration as compared with the hypoxia-induced group by Quantitative RT-PCR (Figure 3).

BMPR2 and p-Smad1 were detected by Western blotting analysis. Results showed that both protein levels were markedly decreased in the hypoxia-induced group compared to the control group (Figure 4). Blockade of kca3.1 with TRAM-34 resulted in increase of BMPR2 and p-Smad1 as compared with the hypoxia-induced group.

Discussion

Excessive proliferation of PASMC is a hallmark of pulmonary artery remodeling in PAH. Hypoxia is a key stimuli for PAH. In the preliminary experiment, cell biochemical changes provided that the Kca3.1 mRNA and protein increased in the hypoxia group. In this study, we investigated the role of calcium-activated potassium channel Kca3.1 in hypoxia induced PASMC proliferation. The main findings of the present study indicated: (1) the high selective blocker of Kca3.1 channel could ameliorate hypoxia induced cell proliferation and (2) the inhibitory effect of TRAM-34 may be through the BMPR2/p-Smad1 pathway.

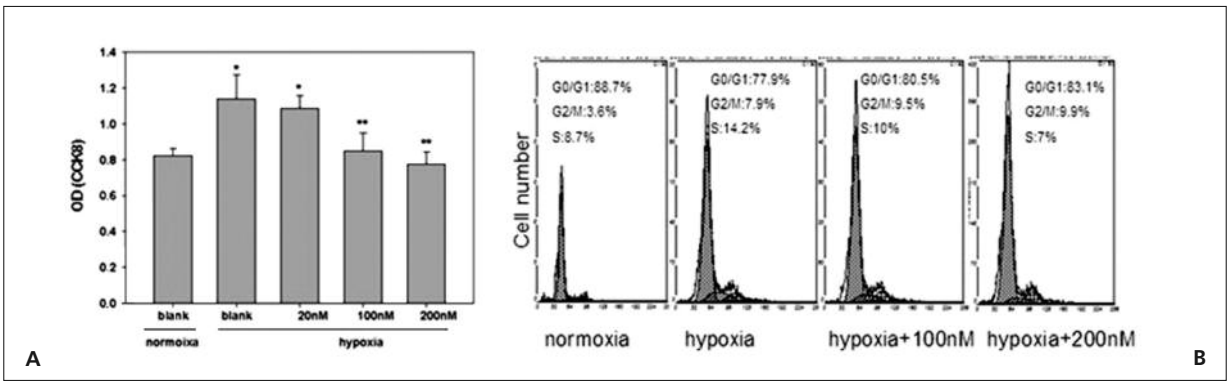

Figure 2. CCK-8 and flow cytometry results of hypoxia and TRAM-34 treated PASMC. ***A,*** OD value is positively related with cell number. Hypoxia stimulated PASMC proliferation while TRAM-34 intervention could ameliorate the stimuli. Results are expressed as mean ± SD. Statistical differences were assessed by the Tukey's HSD test. *$p < 0.05$ versus normoxia conditions. **$p <$ 0.05 versus hypoxia group without TRAM-34 intervention. B, TRAM-34 inhibited PASMC proliferation by G0/G1 arrest.

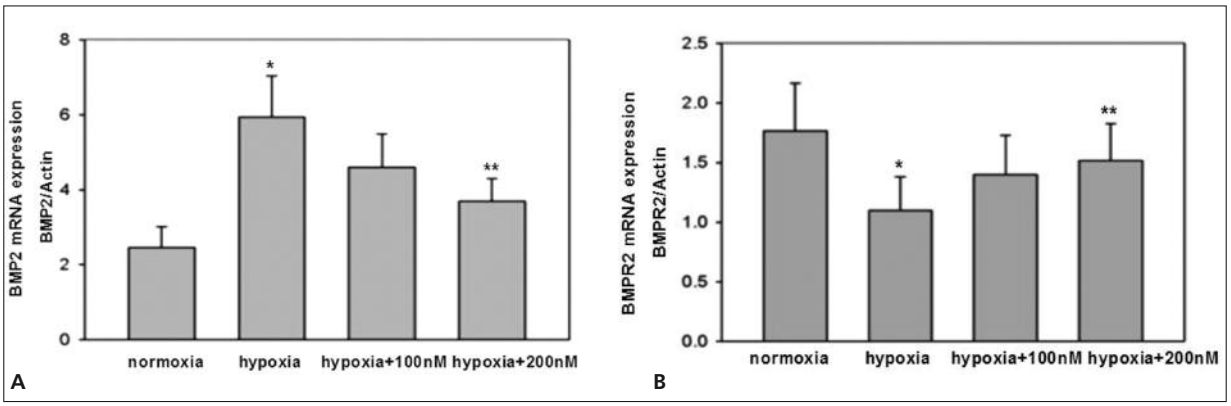

Figure 3. BMP2 and BMPR2 mRNA expression measured by Quantitative RT-PCR. Results are expressed as mean ± SD. Statistical differences were assessed by the Tukey's HSD test. *$p < 0.05$ versus normoxia conditions.**$p < 0.05$ versus hypoxia group without TRAM-34 intervention.

The Kca3.1 channel has been proposed to contribute to disease states characterized by excessive proliferation[10-13]. The results of CCK8 (cell counting kit-8) showed that hypoxia increases the PASMC proliferation while TRAM-34 administration ameliorates the hypoxia effect. In support of these observations, we show that selective pharmacological inhibition of Kca3.1 channel is capable of suppressing hypoxia-induced proliferation of PASMC via cell cycle arrest in G0/G1 phase. The importance of Kca3.1 in excessive proliferation may be explained by its ability to enhance the driving force for Ca^{2+} influx via membrane hyperpolarization and, thus, sustain a high intracellular Ca^{2+} concentration needed for gene transcription[17,18]. Membrane hyperpolarization mediated by potassium channels is known to promote Ca^{2+} influx in the G1 of the cell cycle. Blockade of these channels diminished Ca^{2+} concentration, halted cell cycle in G0/G1 and suppressed cancer cell proliferation[19,20]. Here, we demonstrate that selective Kca3.1 inhibition with TRAM-34 is able to suppress the proliferation of PASMC.

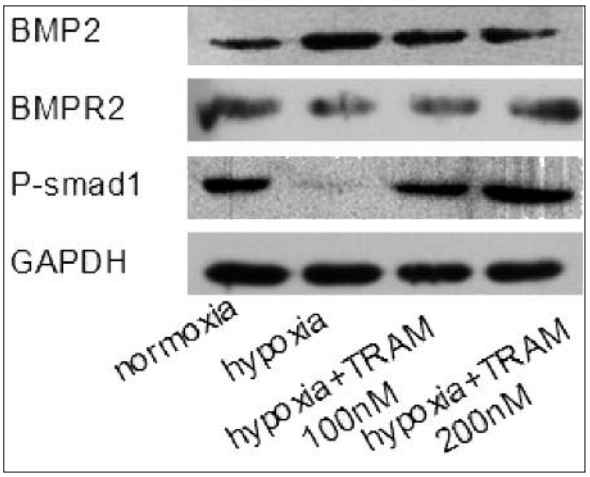

Figure 4. BMP2, BMPR2 and p-Smad1 protein expression was measured by western blot. GAPDH was used as control. Hypoxia stimuli could induce BMP2 increasing while TRAM-34 intervention decreased the expression. BMPR2 and P-smad1 protein expression increased after interfering with TRAM-34 in the hypoxia groups.

The type 2 receptor for bone morphogenetic protein (BMPR-2, a member of the TGF-β receptor family) is required for recognition of all bone morphogenetic protein (BMP) and might regulate vascular cell survival and proliferation, but the underlying mechanisms are complicated[21]. BMP ligands signal by binding the BMPR-2 in conjunction with type I receptors to activate SMADs[22]. TGF-β superfamily ligands signal through both SMAD-dependent and SMAD-independent pathways. SMAD signaling occurs via either SMAD2/3 (TGF-β) or SMAD1/5/8 (BMP), which translocate to the nucleus with the common SMAD (SMAD4) to directly regulate target gene transcription[23]. Whether high selective blockage of Kca3.1 could affect the BMP2 expression, and whether TRAM-34 suppress PASMC proliferation through the BMPR2/Smad1 signaling is still unknown. For the phosphorylated Smads affect the transcription factor, we just test the P-Smad1 expression. In our study, Kca3.1 mRNA and protein expression were significantly decreased in the hypoxia cells, indicating that hypoxia could promote the Kca3.1 expression. We also found the

BMP2 levels increased and BMPR2, p-Smad1 decreased, TRAM-34 administration ameliorate their expression, indicating a possible involvement of BMP2 in the hypoxia-induced growth of PASMC. In our study, the increased Kca3.1 may be mediated by a mechanism dependent on BMPR2/P-Smad1 signaling in the hypoxia stimuli. The detailed mechanism itself warrants further study.

Conclusions

Our findings demonstrate that Kca3.1 channels play an important role in the hypoxia induced proliferation of PASMC. TRAM-34, a selective blockage of Kca3.1, may through the BMPR2/P-Smad1 signaling to ameliorate the proliferation stimulated by hypoxia.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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