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Basic Research

TUG1 Regulates Pulmonary Arterial Smooth Muscle Cell Proliferation in Pulmonary Arterial Hypertension

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See editorial by Bonnet et al., pages 1433–1434 of this issue.

ABSTRACT

Background: Pulmonary arterial hypertension (PAH) is a progressive disease, characterized by a persistent elevation of pulmonary arterial pressure and pulmonary vascular remodelling. Recent studies implicated that long noncoding RNAs (lncRNAs) play important roles in the development of various diseases. However, the underlying mechanisms of lncRNAs in PAH remain unclear. Here we show evidence for the modulation of human pulmonary smooth muscle cell (HPASMC) proliferation and vascular remodelling by lncRNA taurine upregulated gene1 (TUG1).

Methods: TUG1 expression and localization was detected by real-time polymerase chain reaction (PCR) and fluorescence *in situ* hybridization. Proliferation and apoptosis were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), western blot, bromodeoxyuridine incorporation, flow cytometry, scratch-wound assay, 4′,6-diamidino-2-phenylindole (DAPI), and caspase-3 activity. Luciferase activity and microscale thermophoresis were used to identify biomolecular interactions. The right ventricular systolic pressure

RÉSUMÉ

Contexte : L'hypertension artérielle pulmonaire est une maladie évolutive, caractérisée par une élévation persistante de la pression artérielle pulmonaire et par un remodelage vasculaire pulmonaire. Les résultats d'études récentes semblent indiquer que les ARN longs non codants (ARNlnc) jouent un rôle important dans l'apparition de diverses maladies, mais les mécanismes sous-jacents de ces ARNlnc qui interviennent dans l'hypertension artérielle pulmonaire demeurent mal compris. Nous présentons ici des données à l'appui de la modulation de la prolifération des cellules musculaires lisses pulmonaires humaines et du remodelage vasculaire par l'ARNlnc TUG1 (*taurine upregulated gene 1*).

Méthodologie : L'expression et l'emplacement de TUG1 ont été détectés par réaction de polymérisation en chaîne (PCR) en temps réel et par hybridation *in situ* en fluorescence. La prolifération et l'apoptose ont été mesurées de différentes manières : dosage par diméthylthiazolyl-diphényltétrazolium (MTT), buvardage de Western, incorporation de bromodésoxyuridine, cytométrie en flux, test de

Pulmonary arterial hypertension (PAH) is a life-threatening disease, which is defined as mean pulmonary artery pressure >20 mm Hg. The pathologic consequence of PAH is pulmonary vascular remodelling, which is related to pulmonary arterial smooth muscle cell (PASMC) proliferation and pulmonary arterial endothelial cell dysfunction. Both processes could lead to an increased pulmonary vascular resistance, right heart failure, and death.[1-4] Although numerous stimuli and pathologic conditions, including hypoxia, oxidative stress, and infection, could facilitate severe vascular remodelling in PAH,[5-7] the cellular and molecular mechanisms for pulmonary vascular remodelling are still poorly understood.

Long noncoding RNAs (lncRNAs) are a class of noncoding RNA molecules that are longer than 200 nucleotides. It is well established that lncRNAs are involved in various diseases, including PAH. For example, lncRNAs, such as PAXIP1AS1, can change multiple PAH transcriptional programs, and

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and right ventricular hypertrophy were measured to evaluate cardiopulmonary function.

Results: TUG1 was upregulated in the pulmonary arteries of mice after a hypoxic assault and showed a significant increase in patients with PAH. TUG1 knockdown significantly prevented the development of PAH *in vivo*. Moreover, TUG1 promoted the proliferative responses of HPASMCs, including cell viability, 5-bromodeoxyuridine incorporation, the expression of proliferating cell nuclear antigen, and cell-cycle progression. All these functions of TUG1 were likely to be associated with miR-328.

Conclusions: The present study indicates that TUG1, a novel potential target for the treatment of PAH, is necessary for HPASMC proliferation and pulmonary vascular remodelling.

migration cellulaire par blessure, diamidino-phénylindole (DAPI) et activité de la caspase 3. Les interactions biomoléculaires ont été déterminées par la mesure de l'activité de la luciférase et par thermophorèse à micro-échelle. La pression systolique du ventricule droit et l'hypertrophie ventriculaire droite ont été mesurées afin d'évaluer la fonction cardiopulmonaire.

Résultats : L'ARNlnc TUG1 a subi une régulation positive dans les artères pulmonaires de souris après provocation hypoxique et affichait une hausse importante chez les patients atteints d'hypertension artérielle pulmonaire. Le silençage de TUG1 a fortement inhibé l'apparition de l'hypertension artérielle pulmonaire *in vivo*. En outre, la présence de TUG1 a favorisé la réponse proliférative des cellules musculaires lisses pulmonaires humaines (y compris la viabilité des cellules), l'incorporation de 5-bromodésoxyuridine, l'expression de l'antigène nucléaire de prolifération cellulaire et la progression du cycle cellulaire. Toutes ces fonctions de TUG1 étaient vraisemblablement associées au microARN miR-328.

Conclusions : Les résultats de l'étude indiquent que l'ARNlnc TUG1, une nouvelle cible potentielle pour le traitement de l'hypertension artérielle pulmonaire, est nécessaire à la prolifération des cellules musculaires lisses pulmonaires humaines et au remodelage vasculaire pulmonaire.

therefore affects PASMC function.[8] Some lncRNAs, such as MEG3 and LnRPT, may regulate vascular smooth muscle cell (VSMC) proliferation, which plays a key role in the development of PAH.[9,10] Furthermore, a recent study has reported that lncRNA CPS1-IT suppresses the progression of PAH by regulating interleukin-1β in rat models of obstructive sleep apnea.[11] Therefore, lncRNA may act as a novel prognostic marker for PAH therapy.

Taurine upregulated gene1 (TUG1), a 7.1-kb lncRNA, involves in many critical biological processes through epigenetic mechanisms that include chromatin remodelling or acting as decoys for proteins or micro-RNAs (miRNAs). For example, TUG1 could bind to polycomb repressive complex 2 and be induced in a p53-dependent manner to repress cell-cycle progression in response to DNA damage.[12] Furthermore, TUG1 is hypoxia related.[13] However, the biological functions of TUG1 in PAH remain unknown. Here, we characterize the expression of TUG1 in PAH and define the functional role of TUG1 in regulation of human PASMC proliferation.

Materials and Methods

For detailed Material and Methods, please see the Supplemental Data.

Animals and lung tissue preparation

Adult male C57BL/6 mice (mean weight of 30 g) were purchased from the Experimental Animal Center of Harbin Medical University, which is fully accredited by the Institutional Animal Care and Use Committee. The mice were administered nontargeted control or TUG1 shRNA intranasally. Seven days later, mice were assigned to normoxia (Fi,O2 0.21) and hypoxia (Fi,O2 0.12) for 21 days as previously described.[7] At the end of the 21 days' exposure period, the mice were anaesthetized with chloral hydrate (40 mg/kg, intraperitoneally). The right ventricular (RV) systolic pressure (RVSP) was measured by right heart catheterization as previously described. Besides, the RV hypertrophy was routinely assessed using the ratio of RV weight to left ventricular (LV) weight plus septum weight (LV+S).[14] Then the organs were quickly removed for further experiments.

Human lung specimens were obtained from patients with PAH (with a mean pulmonary arterial pressure of 87 ± 15 mm Hg) undergoing lung transplantation or healthy subjects with matched PAH samples at the Lung Transplant Group, Wuxi People's Hospital Affiliated of Nanjing Medical University (Wuxi, China). This study was approved by the Ethic Committee for Use of Human Samples of the Nanjing Medical University (China), which was in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Cell culture

Human PASMCs (HPASMCs) used in the experiment were obtained from ScienCell Research Laboratories (Carlsbad, CA), and cultured in smooth muscle cell−specific medium (ScienCell Research Laboratories). The identity of HPASMCs was verified with α-actin immunocytochemistry (Sigma, St Louis, MO). Cells with typical "hill and valley" appearance at approximately 80% confluence were prepared for experiments. Before each experiment, HPASMCs were synchronized in the same cell cycle by serum withdrawal for 24 hours and then were placed in an incubator with 2% O_2 at 37°C for hypoxia.[7]

Statistical analysis

All data were expressed as means ± standard error of the mean. Data analysis was performed with the Mann-Whitney U test and the 1-way analysis of variance followed by Dunnett's and Student's t test. Differences were considered to be significant at $P \leq 0.05$.

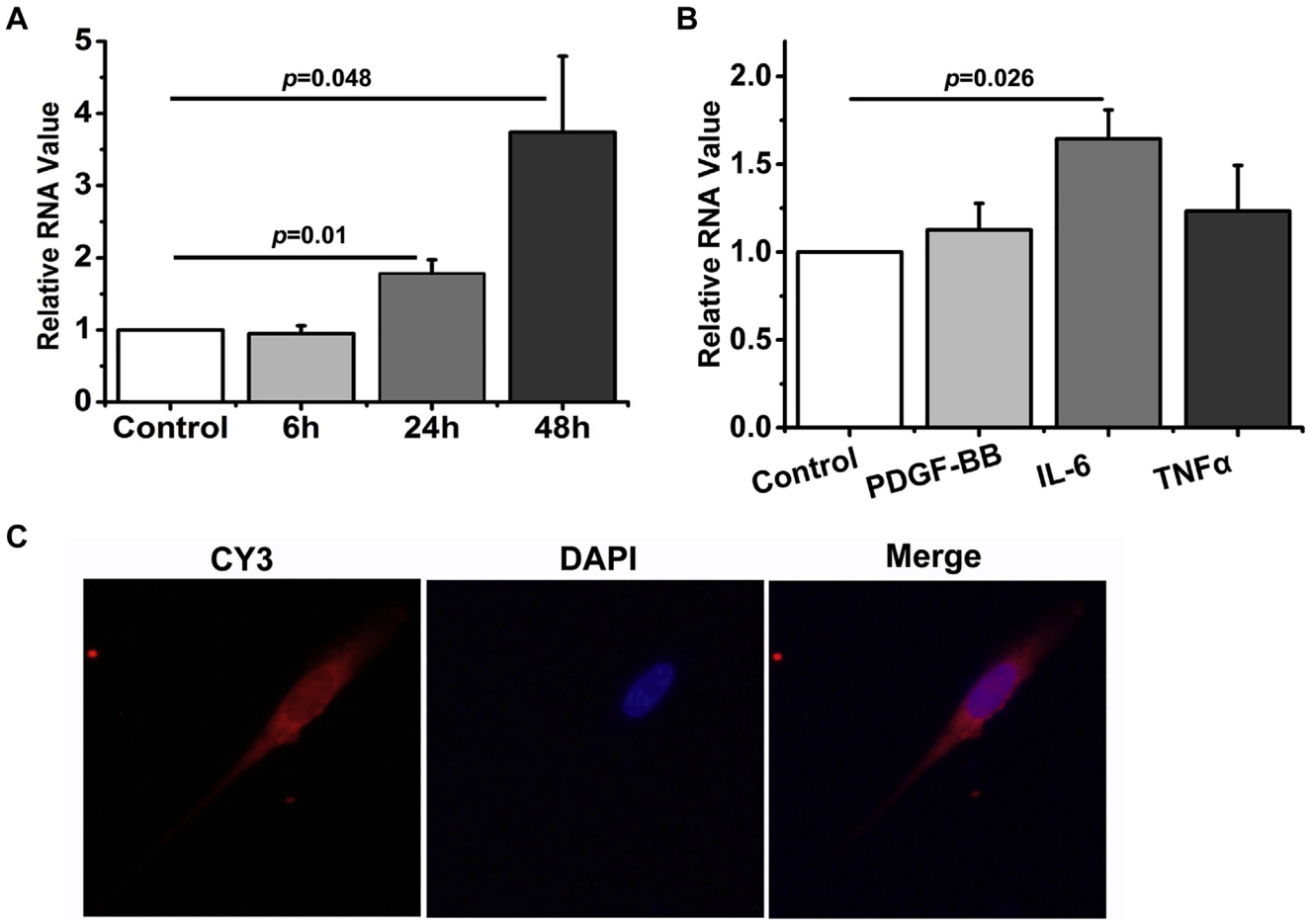

Figure 1. Effects of hypoxia on taurine upregulated gene1 (TUG1) expression. (**A**) Hypoxia increased TUG1 expression in human pulmonary artery smooth muscle cells (HPASMCs). (**B**) The expression of TUG1 after the treatment of platelet-derived growth factor-BB (PDGF-BB), interleukin-6 (IL-6), and tumour necrosis factor α (TNFα) in HPASMCs. (**C**) The subcellular localization of TUG1. HPASMCs were stained for TUG1 (**red**). The nucleus was labelled with 4′,6-diamidino-2-phenylindole (DAPI) (**blue**). All values are denoted as means ± standard error of the mean from 3 or more independent experiments.

Results

Hypoxia induces TUG1 expression in HPASMCs

Hypoxia is an important factor related to PAH. To investigate the effects of hypoxia on TUG1 in HPASMCs, we examined the expression of TUG1 by real-time polymerase chain reaction (PCR). The results showed that hypoxia induced a marked increase in the expression of TUG1 in HPASMCs (Fig. 1A). It is interesting to note that TUG1 also showed an increase after interleukin-6 (50 ng/mL) treatment, a known factor associated with PAH. However, platelet-derived growth factor-BB (PDGF-BB) (10 ng/mL) and tumour necrosis factor α (500 U/mL), other 2 PAH-related factors,[15-17] have no effects on TUG1 expression (Fig. 1B). Besides, the intracellular localization of TUG1 within the cell was assessed. We found that TUG1 was located in both cytoplasm and nucleus of HPASMCs (Fig. 1C). These findings suggest that TUG1 may be a key molecule involved in PAH.

TUG1 regulates HPASMC proliferation

Proliferation plays an important role in PAH. To demonstrate the effects of TUG1 on HPASMC proliferation, we suppressed TUG1 expression, and then measured cell viability, the expression of proliferating cell nuclear antigen (PCNA) and 5-bromodeoxyuridine incorporation. The efficiency of TUG1 siRNAs is shown in Supplemental Figure S1. As shown in Figure 2A, TUG1 siRNA decreased HPASMC viability when compared with the negative control group. Consistently, PCNA expression was attenuated by adding TUG1 siRNA under normoxia (Fig. 2B). We also found that treatment with TUG1 siRNA decreased HPASMC viability, PCNA expression, and DNA synthesis under hypoxia (Fig. 2,C-E). Furthermore, we overexpressed TUG1 with a CRISPR/Cas9-based synergistic activation mediator system to identify its proliferative effects under hypoxia, and the efficiency of overexpression is shown in Supplemental Figure S2. As shown in Figure 2F, PCNA was increased by overexpressing TUG1. On the other hand, we examined the effects of TUG1 on

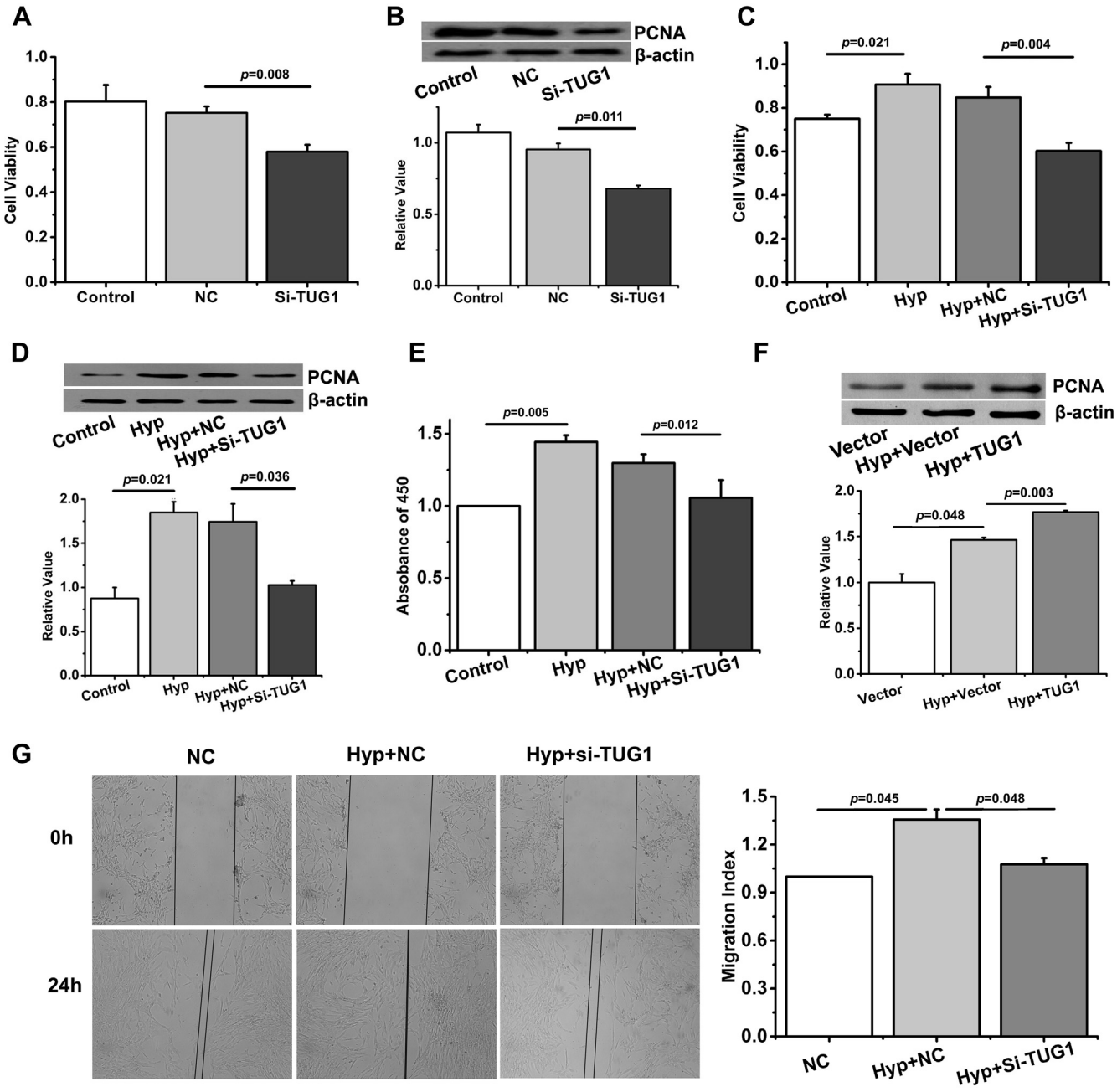

Figure 2. Effects of taurine upregulated gene1 (TUG1) on human pulmonary artery smooth muscle cell (HPASMC) proliferation. TUG1 siRNA (Si-TUG1) decreased (**A**) the HPASMC viability and (**B**) PCNA expression under normoxia. (**C**) The effects of Si-TUG1 on HPASMC viability, (**D**) proliferating cell nuclear antigen (PCNA) expression, and (**E**) DNA synthesis under hypoxia (Hyp). (**F**) TUG1 overexpression increased the expression of PCNA under HYP. (**G**) Si-TUG1 inhibited migration of HPASMCs. All values are denoted as means ± standard error of the mean from 3 or more independent experiments. NC, nontargeted control siRNA.

migration. The results showed that cell migration in response to hypoxia was significantly promoted, whereas this effect was reduced by TUG1 siRNA (Fig. 2G). All these results indicate that TUG1 regulates HPASMC proliferation.

Effects of TUG1 on cell-cycle progression

To determine whether TUG1 affects cell-cycle progression, cell-cycle distribution was detected. As shown in Figure 3A, TUG1 siRNA decreased the percentage of cells in the G_2/M+S phase under the hypoxic condition. Besides, we found that TUG1 siRNA reduced cyclin A and cyclin E expression under both normoxia and hypoxia (Fig. 3, B-E). Moreover, cyclin C and cyclin D expression was determined under hypoxia. Unlike cyclin D, cyclin C did not show a significant decrease after TUG1 siRNA treatment (Fig. 3, F and G). These results suggest that TUG1 contributes to HPASMC proliferation by regulating certain cell-cycle regulators.

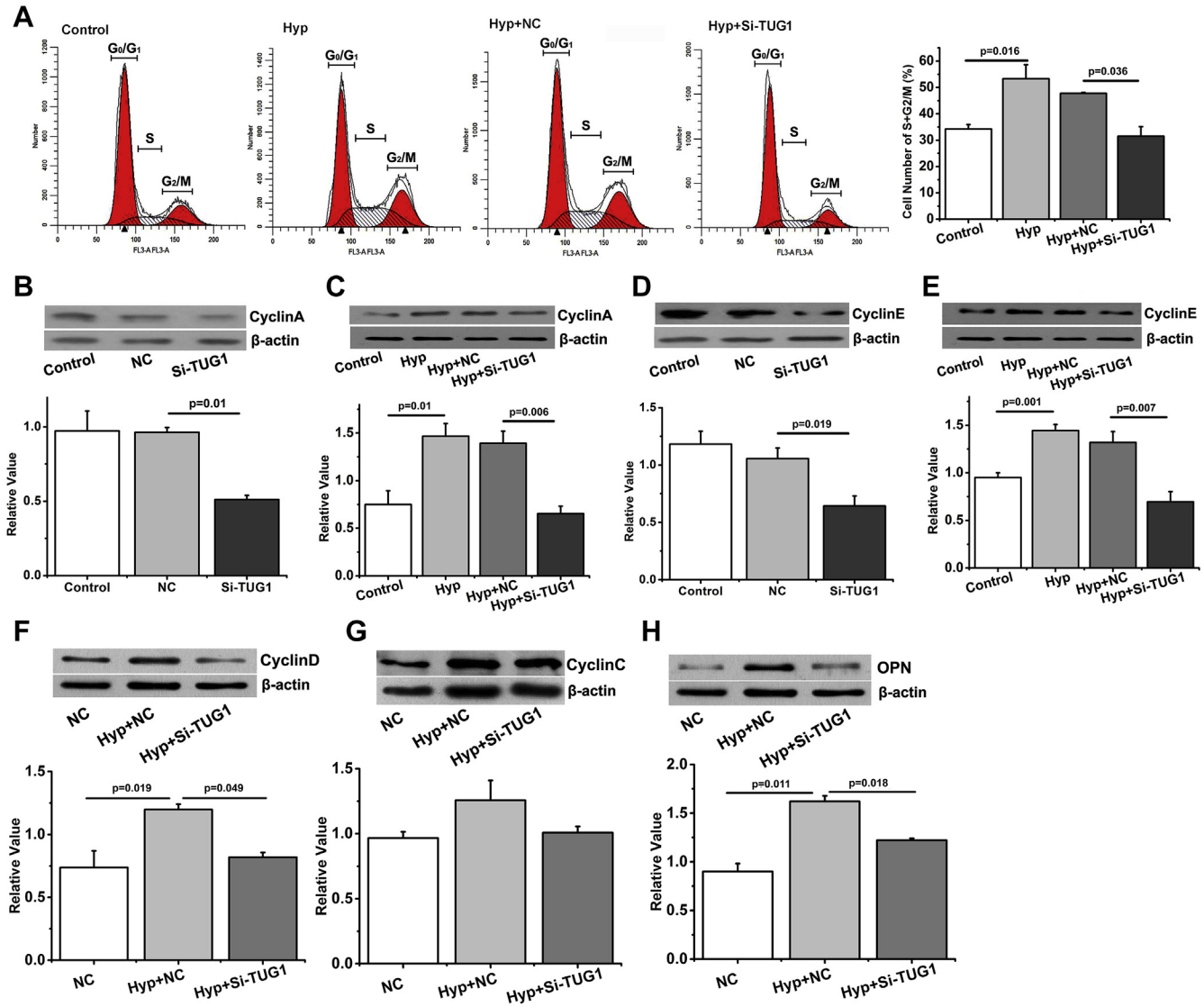

Figure 3. Taurine upregulated gene1 (TUG1) promotes cell-cycle progression. (**A**) TUG1 siRNA (Si-TUG1) decreased the percentage of human pulmonary artery smooth muscle cells in the G_2+M phase under hypoxia (Hyp). (**B**) The effect of Si-TUG1 on cyclin A under normoxia. (**C**) Si-TUG1 decreased cyclin A expression under Hyp. (**D**) Cyclin E expression was decreased by silencing TUG1 under normoxia. (**E**) Si-TUG1 decreased the expression of cyclin E under Hyp. (**F**) Si-TUG1 suppressed the expression of cyclin D under Hyp. (**G**) Cyclin C was not regulated by Si-TUG1 under Hyp. (**H**) Si-TUG1 attenuated osteopontin (OPN) expression under Hyp. All values are denoted as means ± standard error of the mean from 3 or more independent experiments. NC, nontargeted control siRNA.

Effects of TUG1 on PASMC phenotype

The hyperproliferative/secretory phenotype of PASMCs is associated with PAH. To explore the impact of TUG1 on PASMC phenotypes, osteopontin (OPN) expression, a marker of proliferative/secretory phenotype,[18,19] was examined. As shown in Figure 3H, the expression of OPN was upregulated after hypoxia treatment. However, this effect was markedly abolished in the presence of TUG1 siRNA. These results indicate that TUG1 affects HPASMC phenotype.

TUG1 regulates apoptosis

To investigate whether TUG1 was involved in the apoptotic signalling in HPASMCs, we tested caspase-3 activity and morphologic changes in nucleus. The results showed that silencing TUG1 increased the percentage of nuclear shrinkage and caspase-3 activity when compared with the negative control group (Fig. 4, A and B). To examine whether overexpression of TUG1 has an antiapoptotic effect on HPASMCs, cells were exposed to normoxia or hypoxia under serum-deprived conditions to induce apoptosis.[20] As shown in Figure 4C, TUG1 overexpression significantly promoted PASMC survival under hypoxia and serum deprivation. These findings indicate that TUG1 is a potent mediator involved in the HPASMC survival.

Apoptosis is induced by 2 main routes involving the death receptor pathway and mitochondria pathway. To identify the potential pathway related to TUG1, we examined the

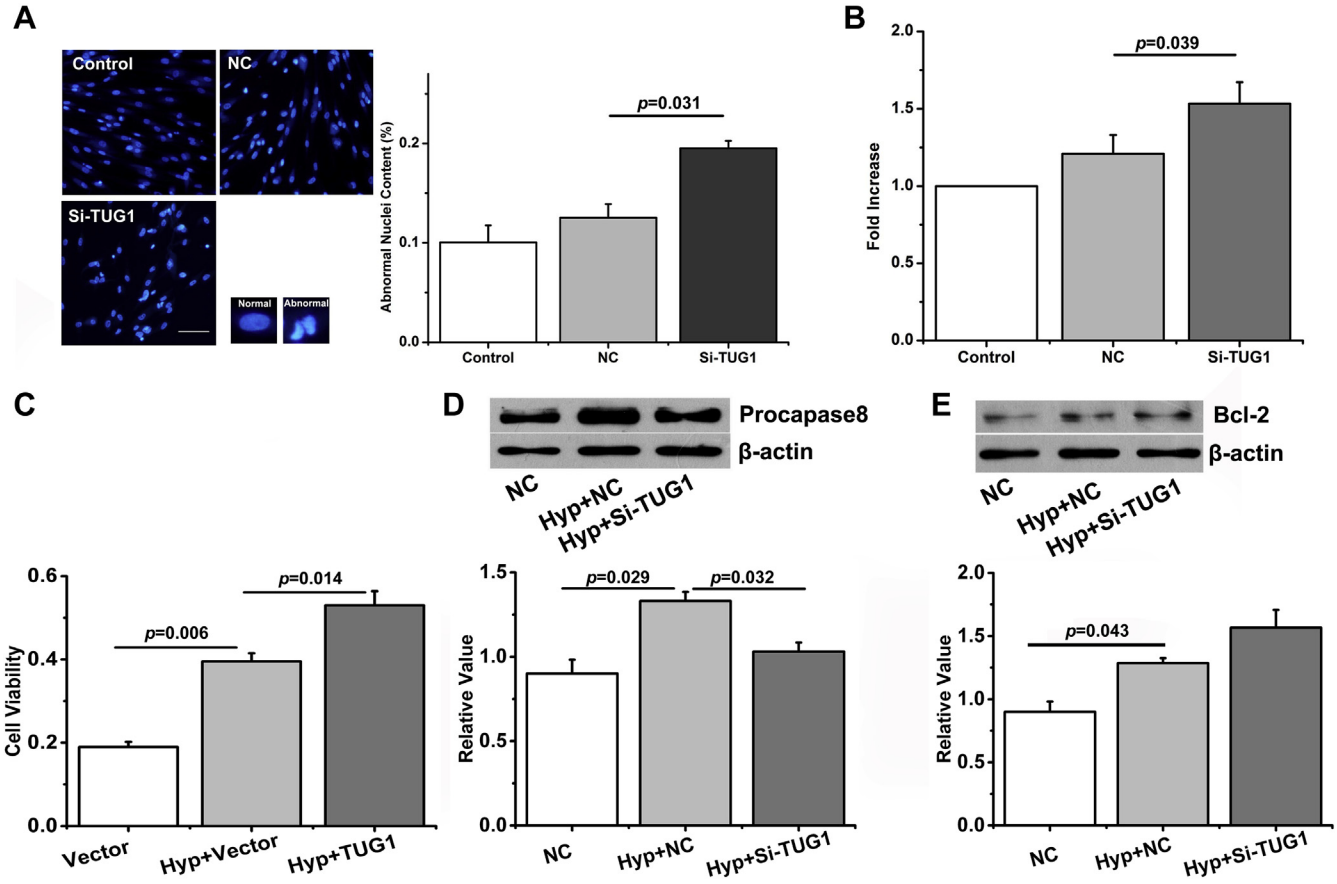

Figure 4. The effects of taurine upregulated gene1 (TUG1) on apoptosis. (**A**) TUG1 siRNA (Si-TUG1) treatment induced morphologic changes in nucleus. (**B**) Si-TUG1 increased caspase-3 activity. (**C**) Effect of TUG1 overexpression on the pulmonary artery smooth muscle cell survival under hypoxia (Hyp). (**D**) The expression of procaspse-8 was decreased by Si-TUG1. (**E**) Si-TUG1had no effects on Bcl-2 expression. All values are denoted as means ± standard error of the mean from 3 or more independent experiments. NC, nontargeted control siRNA.

expression of procaspase-8 and Bcl-2.[21,22] The results showed that hypoxia increased procaspase-8 expression, which was blocked by TUG1 siRNA (Fig. 4D). However, Bcl-2 was not regulated by si-TUG1 (Fig. 4E). Therefore, it is likely that TUG1 inhibits apoptosis through the death receptor apoptotic pathway in HPASMCs.

Verification of interaction between TUG1 and miR-328-3p

We previously demonstrated that miR-328 plays a dominant role in pulmonary vascular remodelling.[23] To explore the association of TUG1 with miRNA-328, we performed a bioinformatics analysis and predicted that TUG1 contains a potential binding site for miR-328-3p (Fig. 5A). To confirm this prediction, we constructed luciferase reporter assays, which contain miR-328-3p binding sites (5493-5512 nt). We found that miR-328-3p decreased luciferase levels, whereas a treatment with miR-328-3p inhibitor, AMO-328-3p, reversed this effect. On the other hand, either miR-328-3p or AMO-328-3p had no noticeable effect, when putative miR-328-3p binding site was mutated (Fig. 5B). Furthermore, microscale thermophoresis results further validated the binding of TUG1with miR-328-3p. The results showed that the microscale thermophoresis signal was decreased for binding of miR-328-3p to TUG1 in a dose-dependent manner (Fig. 5C). Moreover, we examined the influence of TUG1 on miR-328-3p expression in HPASMCs. As shown in Figure 5D, miR-328-3p was upregulated by TUG1 siRNA under normoxia. Similar results were observed under the hypoxic condition (Fig. 5E).

Because IGF-1R, CaV1.2, and PIM-1 are known as direct targets of miR-328 in PAH, the effects of TUG1 on these proteins were then examined. We found that hypoxia increased the expression of IGF-1R, CaV1.2, and PIM, and these effects were reversed by TUG1 siRNA (Fig. 5, F to H).[23-25] Together, these results imply that TUG1 inhibits miR-328 through a competitive endogenous RNA function in HPASMCs.

MiR-328-3p reverses the effect of TUG1 on cell proliferation

To elucidate the interplay of TUG1 and miR-328 on HPASMC proliferation, miR-328 mimics or AMO-328 was used. We found that miR-328 mimics had a similar effect as TUG1 siRNA, which caused a marked decrease in BrdU incorporation. The effect of TUG1 siRNA was

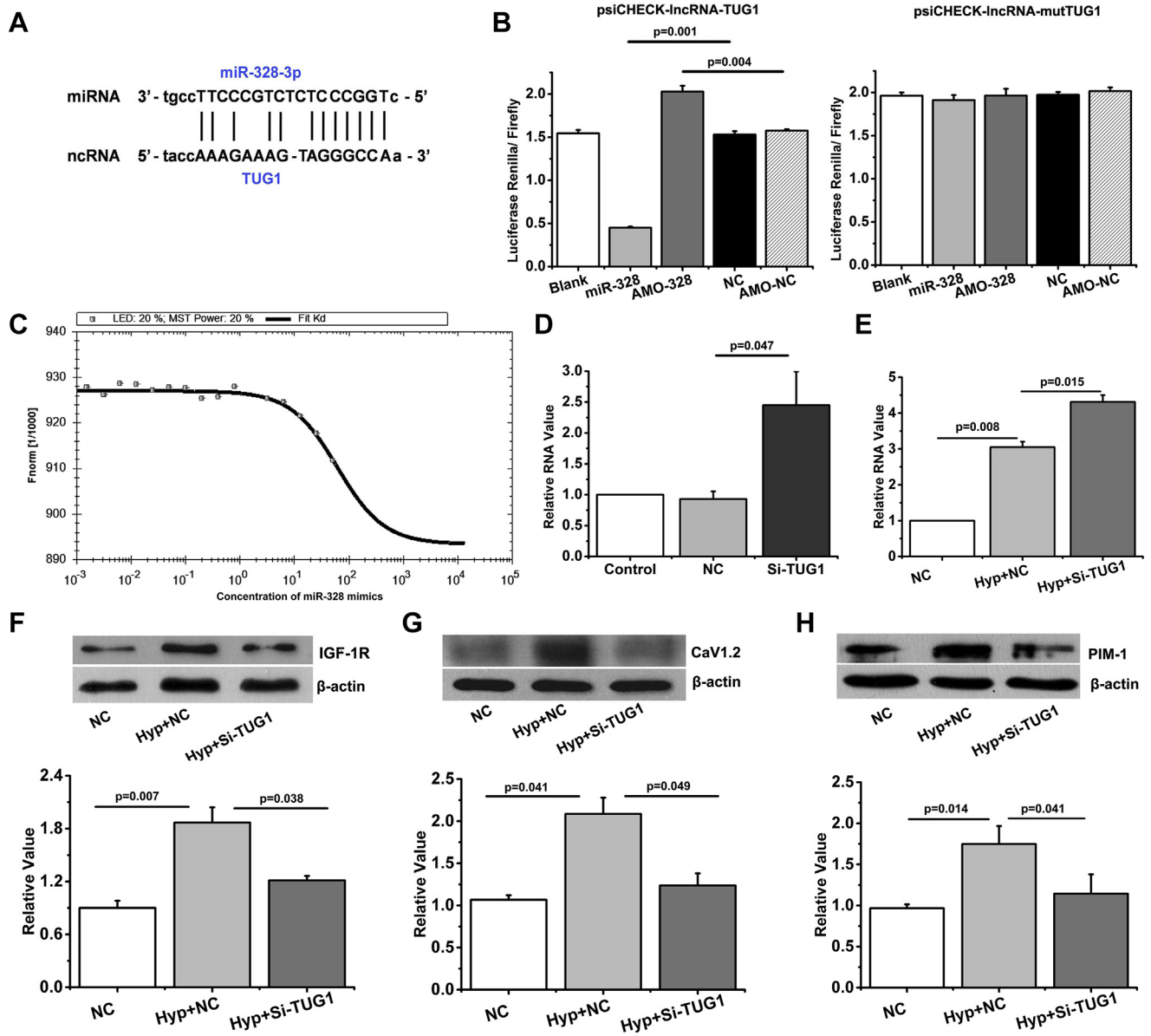

Figure 5. Taurine upregulated gene1 (TUG1) directly binds to miR-328-3p. (**A**) MiR-328-3p binding sequence in TUG1. (**B**) Luciferase reporter activities showed the interaction between miR-328 and TUG1. (**C**) MiR-328 interaction with TUG1 was tested by microscale thermophoresis analysis. (**D**) The effect of TUG1 on miR-328-3p expression under normoxia. (**E**) Silencing TUG1 increased miR-328-3p expression under hypoxia (Hyp). TUG1 siRNA (Si-TUG1) attenuated (**F**) insulin-like growth factor 1 receptor (IGF-1R) expression, (**G**) CaV1.2 expression, and (**H**) PIM-1 expression under Hyp. All values are denoted as means ± standard error of the mean from 3 or more independent experiments. AMO-NC, antisense oligonucleotides against nontargeted control; NC, nontargeted control siRNA.

reversed by AMO-328 (Fig. 6A). Consistently, TUG1 siRNA suppressed the expression of PCNA, cyclin A and cyclin E, which were all blocked by AMO-328 treatment (Fig. 6, B-D). Moreover, we investigated the interactions between TUG1 and miR-328 on proliferation under hypoxia. The results showed that TUG1 siRNA suppressed BrdU incorporation and PCNA expression in HPASMCs exposed to hypoxia, which were attenuated by AMO-328 (Fig. 6, E and F). These results indicate that the effects of TUG1 on PASMC proliferation appear to be mediated by miR-328-3p.

The expression of TUG1 in PAH

To determine the expression pattern of lncRNA TUG1 in the development of PAH, TUG1 expression in patients with PAH was determined. We observed that the walls of pulmonary vessels in patients with PAH were thicker than those from normal subjects, and the expression of TUG1 in the lung tissues of patients with PAH was significantly increased compared with control (Fig. 7, A and B). Moreover, the expression and cellular localization of TUG1 in pulmonary arteries of hypoxic mice were identified (Fig. 7, C and D).

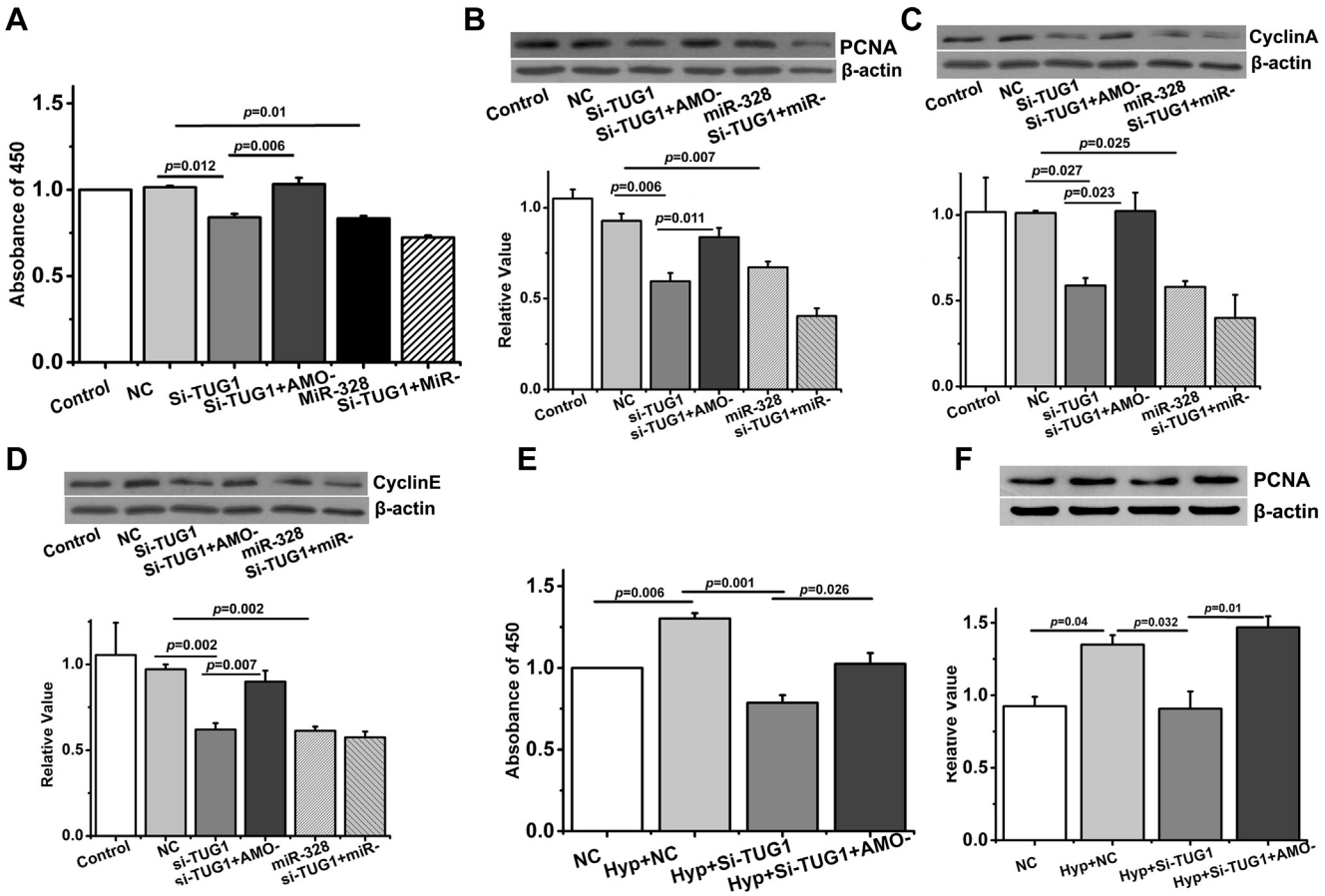

Figure 6. MiR-328 reverses the effects of taurine upregulated gene1 (TUG1) on human pulmonary artery smooth muscle cell proliferation. (**A**) MiR-328 reversed the effect of TUG1 on DNA synthesis. (**B**) TUG1 siRNA (Si-TUG1) decreased the expression of PCNA via miR-328. Antisense oligonucleotides against miR-328 (AMO-miR-328) reversed the effects of Si-TUG1 on (**C**) cyclin A expression and (**D**) cyclin E expression. AMO-miR-328 reversed the impacts of Si-TUG1 on (**E**) BrdU incorporation and (**F**) proliferating cell nuclear antigen (PCNA) expression under hypoxia (Hyp). All values are denoted as means ± standard error of the mean from 3 or more independent experiments. miR-, miR-328; NC, nontargeted control siRNA.

The results showed that TUG1 was potently upregulated in pulmonary arteries from hypoxic mice, including in PASMCs, revealed by α-smooth muscle actin (α-SMA) staining. We also examined TUG1 expression in other tissues, whereas no significant differences were observed between normoxic and hypoxic mice (Supplemental Fig. S3). All these data from PAH animal models and human patients reveal that TUG1 may play a key role in the development of PAH.

The impacts of TUG1 on cardiopulmonary function of mice

To investigate the role of TUG1 in PAH, we knocked down TUG1 in mice. The efficiency of TUG1 silencing was confirmed by real-time PCR (Supplemental Fig. S4). We then measured the RVSP and RV hypertrophy. Compared with normoxic mice, hypoxic mice developed PAH with a significant increase in RVSP and RV hypertrophy. Surprisingly, these effects were reversed by TUG1 shRNA (Fig. 8, A and B). Moreover, we examined the morphologic changes of pulmonary vessels. The results showed that hypoxia increased the thickness of pulmonary vascular walls, which was reversed by TUG1 knockdown (Fig. 8C). These results indicate that inhibiting TUG1 improves cardiopulmonary function and alleviates pulmonary vascular remodelling in PAH mice.

Discussion

PAH is a life-threatening disease characterized by progressive pulmonary vascular remodelling and increased pulmonary vascular resistance. Hallmarks of pulmonary vascular remodelling include aberrant PASMC proliferation and apoptosis, which eventually lead to the narrowing of the pulmonary artery lumen. In this study, we highlighted 3 novel concepts. First, lncRNA TUG1 is upregulated in PAH and TUG1 knockdown significantly prevents the development of PAH *in vivo*. Second, TUG1 regulates PASMC proliferation and apoptosis, leading to pulmonary vascular remodelling. Third, the effects of TUG1 on proliferation are related to miR-328.

LncRNAs have been shown to link with the development of many diseases. However, their functions in PAH remain enigmatic. In the present study, we focused on a hypoxia-related lncRNA, TUG1, which was upregulated in the lung tissues of patients with PAH. To the best of our knowledge,

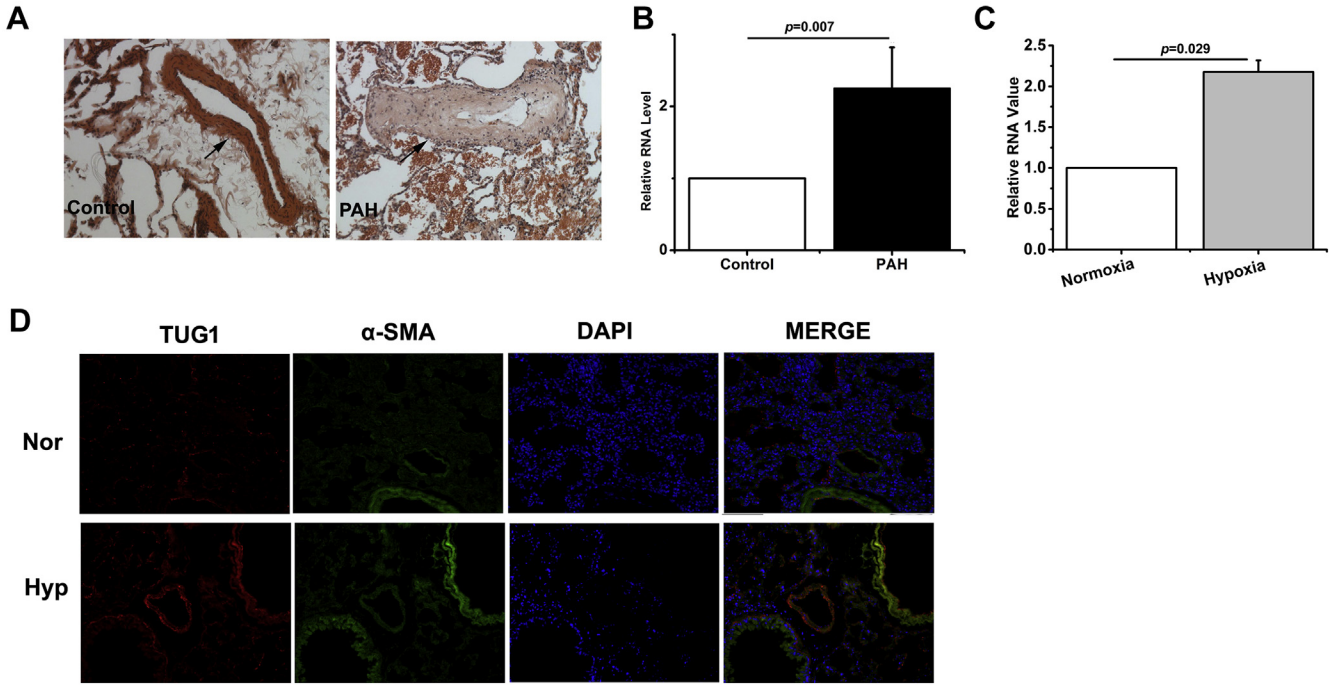

Figure 7. The expression of taurine upregulated gene1 (TUG1) in the pulmonary arterial hypertension (PAH) mice and patients with PAH. (**A**) The morphology of pulmonary arteries in patients with PAH. (**B**) The expression of TUG1 in the lung tissues of patients with PAH. (**C**) Hypoxia (Hyp) increased TUG1 expression in pulmonary arteries of mice. (**D**) The cellular localization and expression of TUG1 in pulmonary arteries of mice. Lung sections were stained for TUG1 (**red**) and α-smooth muscle actin (α-SMA) (**green**). The nucleus was labelled with 4′,6-diamidino-2-phenylindole (DAPI) (**blue**). All values are denoted as means ± standard error of the mean from 4 or more independent experiments. Nor, normoxia.

this is the first report showing that TUG1 is involved in the development of PAH. It is known that TUG1 is a highly conserved lncRNA in mammals. In the present study, we found that TUG1 was also increased in mouse models of PAH. More importantly, lower expression levels of TUG1 were correlated with the improvement of cardiopulmonary function. Although whether the functions of TUG1 are associated with sequence conservation of interspecies is still unknown, evidence from the field of PAH is so far encouraging.

Hyperproliferation of PASMCs, which is precisely regulated by cyclins, contributes significantly to vascular remodelling and PAH.[26] It is known that the level of cyclins could be precisely controlled by post-translational or translational mechanisms. For example, small noncoding RNAs, such as miR-15 family, participate in the cell-cycle progression through translational regulation.[27,28] Thus, understanding how PASMC proliferation is initiated and cell-cycle control mechanisms is critical to investigate the mechanisms of PAH. Recently, TUG1 has been identified as an essential mediator of proliferation in cancer cells.[29] In the present study, we found that TUG1 knockdown reduced PASMC proliferation, downregulated the proportion of cells in the S and G_2/M phase, and decreased the expression of cyclin A, cyclin D, and cyclin E. The information thus provided direct evidence that TUG1 regulates PASMC proliferation by coordinated control of cyclins leading to vascular remodelling.

The pathogenesis of PAH is a complicated and multifactorial process. Although PASMC proliferation is more classically associated with vascular remodelling, apoptosis also influence the degree of remodelling.[1,30,31] In this study, we have shown that TUG1 siRNA induced apoptosis of HPASMCs, and such an effect was mediated by regulation of procaspase-8, indicating that the death receptor pathway is likely to be required for the TUG1 antiapoptotic process in human PASMCs. Therefore, it is reasonable to speculate that the antiapoptotic effect of TUG1 may be another essential mechanism during vascular remodelling.

It has been reported that PAH-PASMCs exhibit a proliferative/secretory phenotype, which is a key process for VSMCs to regain their proliferative and migratory capacities.[26,32] Here, we identified that silencing TUG1 attenuated OPN expression, a marker of proliferative/secretory phenotype of PASMC.[19,33] New information obtained from our study indicates that TUG1 might be an important contributor of hypoxic PASMC phenotypical changes in the progression of PAH.

Subcellular localizations of lncRNAs, like proteins, are related to their function. For example, some lncRNAs may exert their effects in the nucleus by interacting with proteins and DNAs, or by forming subnuclear bodies to maintain their integrity, whereas others are exported to the cytoplasm to carry out their regulatory roles.[34-36] Our results identified that TUG1 localized at both cytoplasm and nucleus in PASMC, which is consistent with previous reports.[37,38] It has been reported that the interaction of TUG1 with polycomb repressive complex 2 in the nucleus induces locus-specific methylation of histone.[37] Moreover, the subnuclear structure-specific localization of TUG1 may serve as the actual docking sites responsible for relocation of polycomb 2 protein-bound growth control gene promoters, which associates with cell proliferation.[39] Here, we have focused on the role of cytoplasmic TUG1, which could act as a competitive

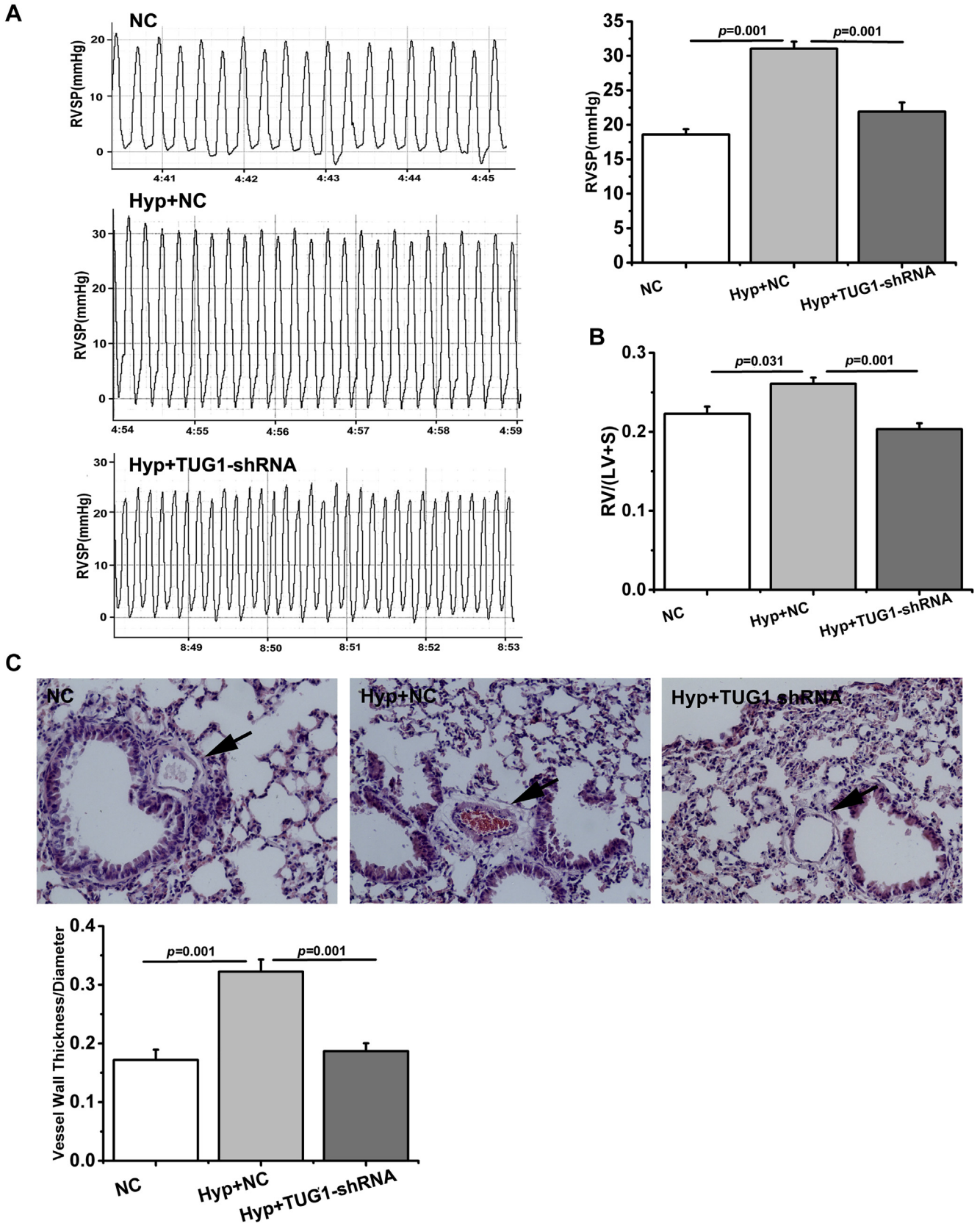

Figure 8. Taurine upregulated gene1 (TUG1) knockdown prevents hypoxia (Hyp)-induced pulmonary arterial hypertension. (**A**) The right ventricular (RV) systolic pressure (RVSP), (**B**) RV hypertrophy, and (**C**) pulmonary vascular wall thickness of hypoxic mice were attenuated by TUG1 shRNA. The **black arrows** show the pulmonary arteries. All values are denoted as means ± standard error of the mean from 4 or more independent experiments. LV, left ventricular; NC, nontargeted control shRNA; S, septum.

endogenous RNA for miR-328. It is known that lncRNAs can compete with miRNAs to alter cellular and important physiological functions. A recent study has demonstrated that lncRNA TUG1 sponges miR-204-5p, a PAH-related miRNA, to positively regulate Runx2 expression and promote osteoblast differentiation in calcific aortic valve disease progression.[40,41] In this study, we found that TUG1 could directly bind to miR-328. Importantly, this observed interaction leads to noticeable changes in proliferation and cell-cycle progression of HAPSMCs. Taken together, TUG1 acts as a "sponge" to bind miR-328 and regulates its function in cytoplasm. However, further studies are needed to determine the function of nuclear TUG1.

In conclusion, our results have shown that lncRNA-TUG1 is involved in the development of PAH and promotes PASMC proliferation, and such effects are likely to be mediated through miR-328. These findings provide a novel insight on the molecular mechanisms of pulmonary vascular remodelling, which may have major implications for PAH therapy.

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Disclosures

The authors have no conflict of interest to disclose.

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Supplementary Material

To access the supplementary material accompanying this article, visit the online version of the *Canadian Journal of Cardiology* at www.onlinecjc.ca and at https://doi.org/10.1016/j.cjca.2019.07.630.