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Tet3 enhances IL-6 expression through up-regulation of 5-hmC in IL-6 promoter in chronic hypoxia induced atherosclerosis in offspring rats

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ABSTRACT

Aims: Tet1, Tet2, and interleukin-6 (IL-6) have been linked to atherosclerosis. Whether Tet3 has a relationship with atherosclerosis and IL-6 was unknown. This study aims to determine the link between Tet3 and IL-6, and the role of Tet3 in prenatal hypoxia-induced atherosclerosis in offspring rats.

Main methods: Pregnant rats were divided into hypoxia and control group. Their male offspring were tested at 20 months old. Hematoxylin-eosin staining and transmission electron microscopic staining were used. Gene mRNA and protein levels were measured with q-PCR or Western blotting. Cell viability and migration was tested with MTT or cell scratch assay. 5-hmC and 5-mC expression were obtained by qGlucMS-PCR; 5-hmC and 5-mC activity were obtained by dot blotting.

Key findings: Chronic prenatal hypoxia increased Tet3 and IL-6 expression, and decreased Tet3 activity in offspring rats. GlucMS-qPCR showed the percentage of 5-hmC was significantly up-regulated in the promoter of IL-6 in both the rats and cells. Moreover, 5-hmC percentage also was increased in the A7r5 cells transfected with Tet3. Furthermore, Tet3 promoted proliferation and migration of A7r5 cells. However, Tet3 was not sensitive to acute hypoxia, while influenced by HIF-1α DNA element.

Significance: Tet3 enhanced IL-6 expression though up-regulating 5-hmC percentage in the IL-6 promoter.

1. Introduction

Cardiovascular disease is a major illness that threatens human health, while the primary pathological basis of cardiovascular diseases is atherosclerosis [1,2]. Hypoxia is one of the multifactor that contributes to atherosclerosis [3,4]. The association between hypoxia and atherosclerosis has been extensively explored, but there was very limited literature about maternal hypoxia and the occurrence of offspring atherosclerosis [5–7]. Recent studies demonstrated that prenatal hypoxia contributed substantially to the incidence of ischemic myocardial injury, preeclampsia, hypertension, and insulin resistance [8–12].

Recently, inflammation was reported to be the main reason for atherosclerosis [6,13], whereas the underlying mechanisms that regulate pro-inflammatory cytokines remain largely unknown. Interleukin-6 (IL-6) was suggested to act as an upstream inflammatory cytokine that plays a central role in propagating downstream inflammatory responses, accounting for the occurrence of atherosclerosis [14]. In the past decade, ten-eleven translocation enzymes (Tet) appeared to be the most important methylcytosine dioxygenases, which could convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) [15,16]. Notably, Tet1 and Tet2 proteins have a linkage with inflammation by affecting the pro-inflammatory cytokines [17,18]. Thus, a question was raised: whether Tet3 has an association with IL-6 in prenatal hypoxia induced atherosclerosis in offspring rats. Recent reports have demonstrated that oxygen levels are correlated with Tet expression, either by up-regulating or down-regulating Tet expression in solid tumours [18,19].

The present study investigated whether Tet3 plays a role in inflammation implicated in the offspring atherosclerosis caused by chronic hypoxia during pregnancy, explored the relationship between hypoxia/HIF1-α and Tet3. In addition, further analyze the percentage of 5-mC/5-hmC of IL-6 promoter in the offspring aorta following prenatal hypoxia as well as in A7r5 cells. We proposed a potential strategy targeting Tet3 to improve atherosclerosis caused by prenatal hypoxia.

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Given that aging may affect the development of atherosclerosis, we used rats at 20 months old, equivalent to human around 55–60 years old, an atherosclerosis-prone age.

2. Material and methods

2.1. Animals

The experimental procedures were performed as previously described [6]. All procedures were approved by the Ethical Committee of Soochow University. 13 pregnant rats of hypoxia group and 8 pregnant rats of control group were enrolled in the experiment. Male offspring of two groups were chosen for the study at 20 months old. Pregnant rats were divided into control (CON, 21% oxygen) and hypoxia group (HY, 10.5% oxygen). Hypoxia was induced by a mixture of nitrogen gas and air in an individual chamber at room temperature. All animals were fed with standard rat chow ad libitum.

2.2. Histochemical analysis

At 20 month of old, the offspring were anaesthetized with sodium pentobarbital, and the thoracic aorta was collected immediately, and fixed in 2.5% glutaraldehyde solution buffer at pH 7.2 with 0.1 mol/L phosphate buffered saline or 4% paraformldehyde solution, respectively. Then tissue was kept at 4 °C for transmission electron microscopic staining and hematoxylin-eosin (HE) staining.

2.3. Plasmid constructs

Over-expression plasmid pcDNA3.1-Tet3, pcDNA3.1-HIF1-α was constructed by Genewiz Biotechnology Corporation (Suzhou, China), and RNA interference (RNAi) plasmid was constructed by GenePharma Corporation (Suzhou, China). For the over-expression assay, the negative control was transfected with empty vector, pcDNA3.1 (+). For the RNAi assay, the negative control was pGPH1/GFP/Neo empty vector linked with mismatched dsRNA.

2.4. Serum values

Offspring rats were anaesthetized; blood samples were collected from the abdominal aorta with heparin sodium. Serum was used for determination of triglyceride (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL−C). TG, TC, LDL-C, and HDL-C were determined by an automatic spectrophotometer according to the manufacturer's protocols, and all testing kits were purchased from Nanjing Jiancheng Bioengineering Institute.

2.5. Cell culture and transient transfection

A7r5 cells derived from the rat aorta were purchased from Cell bank of the Chinese Academy of Sciences, then cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (HyClone, Utah State, USA). The DMEM medium containing 100 mg/ml penicillin G and 100 mg/mL streptomycin sulphate. For transient transfection, A7r5 cells were plated in 10 cm dishes over night before transfection. Then, under the condition of 1% O_2, A7r5 cells were transfected with corresponding plasmids in the presence of Lipofectamine 2000 (Invitrogen, CA, USA). Following 24–48 h post-transfection, corresponding cells collected were subjected to RNA extraction, and used for determining mRNA expression levels of corresponding genes using Quantitative Real-time PCR (q-PCR).

2.6. Quantitative Real-time PCR (q-PCR) and Western blotting

Total RNA was isolated from rat aorta tissue or A7r5 cells using RNAiso Plus reagent (Takara, Dalian, China) according to the manufacturer's protocols. All primers used were listed in Table 3. Tet3 protein abundance was assessed with Western blotting normalized to β-actin. The antibodies were purchased from Cell Signalling Corporation (CST, Boston, USA). Q-PCR and Western blot were carried out as previously reported [20].

2.7. MTT, cell scratch assays

The MTT assays, cell scratch assays of A7r5 cells were performed as recently reported [21].

2.8. Dot blot

Genomic DNA from the aorta of old offspring, as well as A7r5 cells, was extracted using the phenol-chloroform methods, and quantified with NanoDrop One (Thermo, USA). Genomic DNA was dissolved in 2 M NaOH, and boiled for 10 min at 95 °C. 300–900 ng genomic DNAs were spotted onto a charged nylon-based membrane at room temperature for 30 min, and baked at 75 °C for 1 h. The membrane was then blocked with 5% non-fat milk for 1 h. After washing in PBS three times (5 min/time), the blot was incubated with rabbit polyclonal anti-5-hmC antibody (Active Motif; 1:30,000) overnight at 4 °C. On the following day, the blot was washed in PBS three times (5 min/time) and incubated with peroxidase-conjugated anti-rabbit IgG (secondary antibody) for 1 h. The signal was visualized using ECL-Plus system (Tanon, Shanghai, China). Then, the blot was stained with methylthionine chloride for 10 min as the DNA control group.

2.9. Glucosylation-coupled methylation-sensitivity qPCR (GlucMS-qPCR)

Sequence specific detection of 5-hmC and 5-mC as a percentage of total C was performed using the EpiMark™ 5-hmC and 5-mC Analysis Kit (NEB, Beijing, China) according to published methods [22]. Briefly, Genomic DNA (5 μg) was treated with T4 Phage β-glucosyltransferase, a highly specific 5-hmC glucosyltransferase, which adds a glucose moiety to 5-hmC (generating glucosyl-5-hmC, 5-ghmC). Glucosylated genomic DNA was then digested with *Msp*I that recognizes and cleaves mC, 5-mC and 5-hmC, but not 5-ghmC. Locus specific detection of 5-hmC was determined by q-PCR using primers designed to flank at least one MspI site.

2.10. Data analysis

Statistical analyses were performed with either *t*-test or post hoc analysis of variance using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). Significance was accepted at $P < 0.05$. Data were expressed as mean ± SEM.

3. Results

3.1. Body weight

Compared with control group (CON) (607.7 ± 13.9 g), there was no significant difference in body weight in hypoxia group (HY) (614.2 ± 14.6 g) in the offspring.

3.2. Morphological changes in the aorta of the old offspring

Using transmission electron microscopy and HE staining, the aorta of the CON offspring exhibited flattened endothelial cells on the tunica intima (Fig. 1, black arrow), regular parallel-arranged smooth muscle cells (Fig. 1B, green box), and complete elastic membranes with no

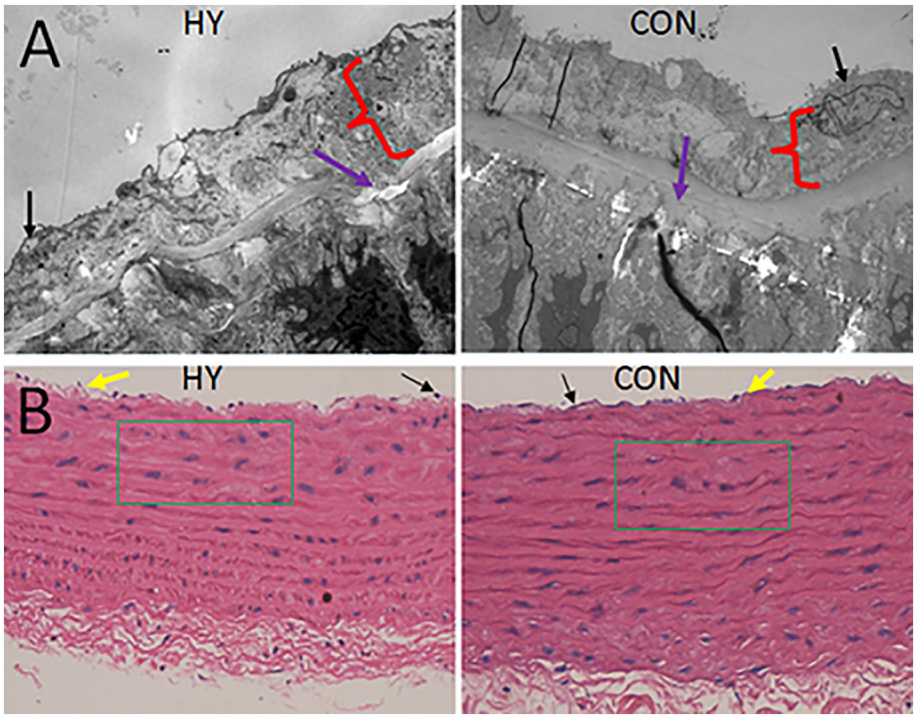

Fig. 1. Representative transmission electron microscope and HE staining of aorta in offspring rats. A (transmission electron microscopy): black arrow indicates endothelial cells, there are intact endothelial cells in CON, whereas the endothelial cells are naked, even desquamated in HY. Purple arrow indicates internal elastic lamina (IEL), it was broken through by smooth muscle cells in the HY group (magnification, ×0.8 K). Red brace indicates intima thickness, it is significantly thicker in HY than that of CON. B (HE staining): black arrow represents endothelial cells, yellow arrow represents the thickness of intima, and green box indicates the interaction between smooth muscle cells and IEL (magnification, ×100). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

across of smooth muscle cells. However, there were naked endothelial cells on the tunica intima with desquamated (Fig. 1, black arrow) in HY group, and the vascular intima seemed thicker, compared to CON group (Fig. 1A, red brace). Disarranged elastic membranes with smooth muscle cells migrating into the intima were also seen in the HY group (Fig. 1A, purple arrow). Compared with the young adult offspring as previously reported [6], there were no significant differences except the thicker tunica intima.

3.3. Biochemical values of rat offspring serum

In all the biochemical values tested, no difference was observed between the two groups (Table 1). Compared to the young adult offspring (data were shown in Table 2), TG, TC, and LDL-C of the older offspring were significantly higher; while HDL-C was lower [6]. The reason may be that physiological functions of old rats declined with age, leading to decrease of hormone levels and metabolism. The detailed mechanism of the increase of TG/TC/LDL-C in old offspring rats needs to be investigated in future.

3.4. Tet3 expression and activity in the offspring and A7r5 cells

Q-PCR and Western blot were used to evaluate Tet3 gene expression in the aorta of the offspring. Tet3 showed higher expression of mRNA and protein in HY at 20 month of offspring rats (Fig. 2A, B). Then, we tested whether activities of Tet3 were consistent with the expression of mRNA and protein. As shown in Fig. 2C, 5-hmC level was much lower in HY group at 20 month using a series of genomic DNA dilution.

3.5. Tet3 potentiates IL-6 expression in A7r5 cells

Under hypoxic condition (1% O_2), A7r5 cells transfected with over-expression plasmid pcDNA3.1-Tet3 showed an increased Tet3 expression, accompanied with higher value of IL-6 expression. In contrast, A7r5 cells transfected with RNA interference plasmid pGPH1/GFP/Neo-Tet3, Tet3 expression was decreased significantly, associated with lower value of IL-6 expression. The efficacy of RNAi sounded more pronounced relative to over-expression (Fig. 3).

3.6. The effect of hypoxia on Tet3

Fig. 4 showed that Tet3 mRNA levels were not altered between 1% O_2 and 21% O_2. With transfection of HIF-1α over-expression plasmid (pcDNA3.1- HIF-1α), Tet3 showed a higher level compared to transfection with pcDNA3.1 in A7r5 cells (Fig. 4).

3.7. Hypoxia up-regulated 5-hmC and down-regulated 5-mC percentage of IL-6 promoter

In order to further analyze the mechanism of Tet3 induced IL-6 expression by chronic hypoxia leading to atherosclerosis, GlucMS-qPCR was used to determine the percentage of 5-hmC and 5-mC in IL-6 promoter. The results showed that 5-hmC percentage of IL-6 promoter was up-regulated from (2.39%) in CON to (5.58%) in HY old offspring, 5-mC percentage was down-regulated from (85.36%) to (72.28%) in CON and HY group, respectively (Fig. 5A). In the case of A7r5 cells, 5-hmC percentage of IL-6 promoter was up-regulated in HY group [CON (1.84%) vs. HY (4.72%)], and 5-mC percentage was down-regulated [CON (94%) vs. HY (68.47%)] (Fig. 5B). Furthermore, in the case of A7r5 cells transfected with over-expression or RNA interference

Table 1
Biochemical values of rat offspring.

Offspring	TC (m mol/L)	TG (m mol/L)	HDL-C (m mol/L)	LDL-C (m mol/L)	LDL-C/HDL-C
HY	1.50 ± 0.28	1.23 ± 0.18	0.48 ± 0.08	0.68 ± 0.12	1.42 ± 0.11
CON	1.78 ± 0.23	1.36 ± 0.25	0.40 ± 0.10	0.58 ± 0.09	1.45 ± 0.09

Table 2
Biochemical values of adult rat offspring (Zhang, 2016).

Offspring	TC (m mol/L)	TG (m mol/L)	HDL-C (m mol/L)	LDL-C (m mol/L)	LDL-C/HDL-C
HY	1.37 ± 0.12*	0.64 ± 0.07	0.90 ± 0.13**	0.27 ± 0.05*	0.3 ± 0.07*
CON	0.90 ± 0.09	0.72 ± 0.10	1.55 ± 0.19	0.12 ± 0.03	0.08 ± 0.02

* $p > 0.05$
** $p > 0.01$

Table 3
Gene primers.

Gene	Forward primer	Reverse primer
Tet1	ATGCCACCAACTGTGGAGAG	TTGAGCACCTCGCATTTCCT
Tet2	CTACAGCCCAGGAAAGCACA	TGTCATTTCCCCCTTCCTGC
Tet3	GTAGACCAGAAGCCCGACTG	TAAGAGGACACAGCTTCGGC
IL-6 hmC	GGGATCTGGACAAGTCATCCC	GGGTATCTATCTGCTGCCACT
β-actin	CCTAAGGCCAACCGTGAAAAG	GTACGACCAGAGGCATACAGG

plasmid of Tet3, 5-hmC showed higher value (16.18%) in over-expression group, no difference was observed between NC (8.10%) and RNA interference group (6.33%); 5-mC showed great value in RNA interference group (86.44%), no difference was observed between NC (45.90%) and over-expression group (29.03%)(Fig. 5C).

3.8. Tet3 promoted proliferation and migration of A7r5 cells

Proliferation and migration of smooth muscle cells are important characteristics of atherosclerosis [23]. As shown in Fig. 6A, over-expression of Tet3 significantly promoted proliferation of A7r5 cells at 48 and 72 h, while knockdown of Tet3 blocked the proliferation. In the case of migration of A7r5 cells, Fig. 6B showed that over-expression of Tet3 increased the migration significantly at 72 h, while knockdown of Tet3 blocked the migration at 72 h.

3.9. Hypoxia affected gene expression of Dnmt1, Dnmt3A and Dnmt3B

Compared with CON group, chronic hypoxia down-regulated mRNA of Dnmt1, whereas mRNA levels of Dnmt3B were up-regulated in HY group (Fig. 7A, B, C). However, Dnmt3A mRNA levels were unchanged. In the case of acute hypoxia, all mRNA levels of DNMTs remained unchanged between two groups (1% O_2 and 21% O_2), although there was a tendency of down-regulation of Dnmt1 and an up-regulation of Dnmt3B in HY group (Fig. 7D, E, F). In addition, RNA interference of Tet3 significantly decreased mRNA of DNMT3A and DNMT3B (Fig. 7G).

4. Discussion

Hypoxia during pregnancy could lead to early morphological changes in atherosclerosis in the rat offspring, the underlying mechanisms still remain elusive [5–7]. Recently, Tet family members have caught attention in the progression of atherosclerosis in murine, although the role of Tet3 has not been reported previously, and need to be explored [24–26]. In the present study, the mechanism of Tet3 was investigated during the development of atherosclerosis. To the best of our knowledge, this is the first report about the relationship between hypoxia and Tet3 in processing of atherosclerosis in old offspring rats.

In the present study, the biochemical values in the circulation seemed comparable between CON and HY groups. Compared to the young adult offspring, TC and TG were higher. This probably could be attributed to the physiological function of old rats declines with age, which leads to the decrease of hormone levels and metabolism, leading to weaker ability in clearing TC and TG-rich lipoprotein in the old offspring during aging.

Hypoxia is associated with altered Tet expression in the development of atherosclerosis. For example, Tet1 was increased in atherosclerotic plaques, while loss of Tet2 in human somatic cells accelerated the progression of atherosclerosis [24,27]. In addition, Tet2 was reported to repress IL-6 levels and promote THP-1 derived macrophage autophagy in mice [26,28]. Moreover, research on cancer indicated that hypoxia increased expression of Tet1 and Tet3, leading to breast tumor malignancy through a TET-TNFα-p38-MAPK signaling axis [29]. So far, no connection between hypoxia and Tet3 was established

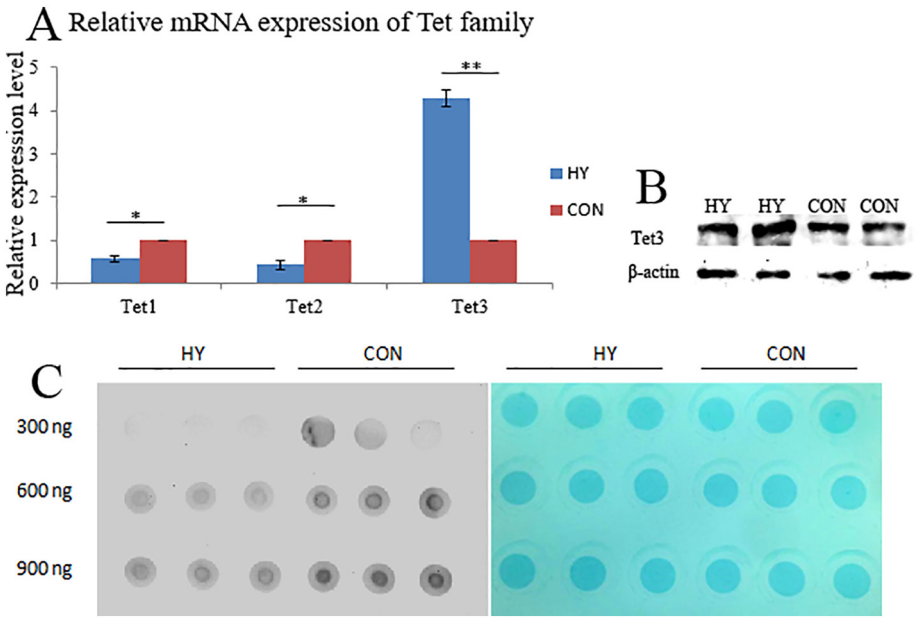

Fig. 2. Expression and activities of Tet3. A: Tet mRNA abundance in 24 month of hypoxia induced offspring rats. B: Tet protein expression in 24 month of offspring rats between HY and CON groups. C: 5-hmC activities was detected in HY and CON groups using a series of 3 fold dilution. HY: prenatal hypoxia (10.5%O_2), CON: prenatal control (21%O_2), *: $p < 0.05$; **: $p < 0.01$.

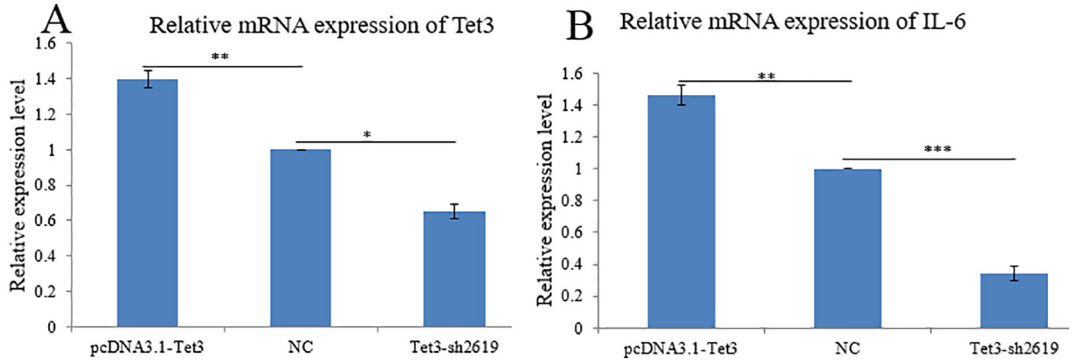

Fig. 3. Tet3 enhances IL-6 expression. A: Tet3 mRNA was detected when A7r5 cells transfected with Tet3 overexpression plasmid (pcDNA3.1-Tet3) or RNA interference plasmid (Tet3-sh2619). B: IL-6 mRNA was evaluated under the same condition of A. For the over-expression assay, the negative control was transfected with empty vector, pcDNA3.1 (+). For the RNAi assay, the negative control was pGPH1/GFP/Neo empty vector linked with mismatched dsRNA. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

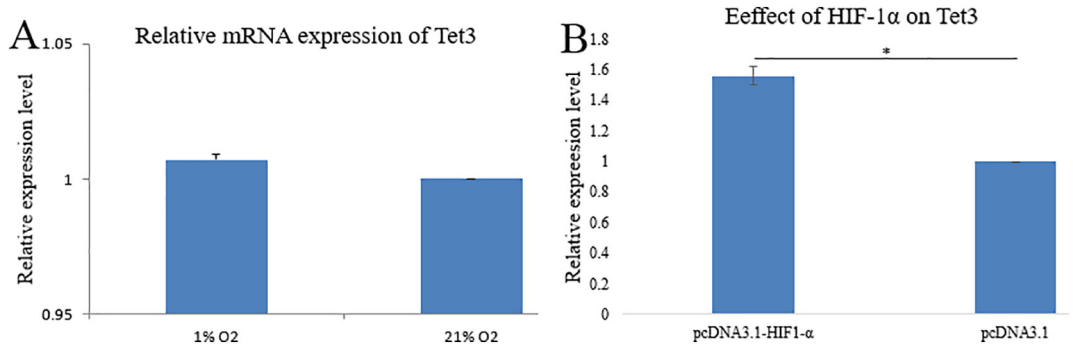

Fig. 4. The effect of acute hypoxia on Tet3 expression. A: mRNA level of Tet3 exposed to 1% O_2 and 21% O_2. B: under the condition of 1% O_2, the effect of HIF-1α DNA element on Tet3 mRNA expression was evaluated. *: $p < 0.05$.

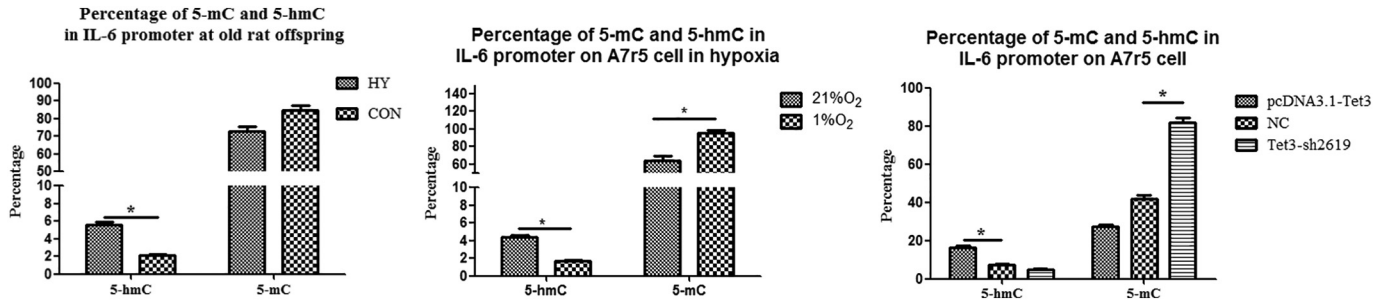

Fig. 5. The percentage of 5-mC and 5-hmC in IL-6 promoter. Left, the percentage on offspring rats of HY and CON groups; middle, the corresponding percentage on A7r5 cells exposed to 1% O_2 and 21% O_2; right, the corresponding percentage on A7r5 cells transfected with pcDNA3.1-Tet3, NC, or Tet3-sh2619, respectively. HY: prenatal hypoxia (10.5%O_2), CON: prenatal control (21%O_2), *: $p < 0.05$.

regarding the development of atherosclerosis. The present study uncovered that chronic hypoxia increased Tet3 levels at 20 month old offspring. However, the activity of Tet3 was reduced in HY group compared to CON, the reason may be that Tet1 and Tet2 had more powerful ability to transform 5-mC to 5-hmC. Tet3 was increased by transfection of HIF-1α, with no change when exposed to acute hypoxia, suggesting that Tet3 was not sensitive to hypoxia, rather influenced by HIF-1α DNA element under the condition of acute hypoxia.

Inflammation was considered as a contributing factor to atherosclerosis, and IL-6 is an upstream inflammatory cytokine that plays a central role in affecting the downstream inflammatory responses in atherosclerosis [28,30,31]. The present study showed that over-expression of Tet3 in A7r5 cells enhanced IL-6 expression, while enhanced expression of IL-6 was attributed to increased percentage of 5-hmC and decreased percentage of 5-mC in IL-6 promoter, which was also repeated and proved in the aorta of the old offspring rats. DMNT1 was reported to propagate DNA methylation patterns during replication, whereas DNMT3A and DNMT3B were shown to install DNA methylation patterns [32]. However, recent publications demonstrated that DNMT1 was also responsible for installing DNA methylation patterns during oocyte methylome [33]. The present study showed Tet3 RNA interference had no effects on DNMT1, while reduced the expression of DNMT3A and DNMT3B in A7r5 cells. These results indicated Tet3 might be associated with installing DNA methylation rather than propagating DNA methylation in our experiments.

In conclusion, the present study shed light on that chronic hypoxia could enhance Tet3 expression, while decrease Tet3 activities in the offspring rats. The up-regulation of IL-6 expression in the aorta of the offspring could be ascribed to up-regulation of 5-hmC content in the promoter, leading to acceleration of atherosclerotic progression. As significance in clinical implications, Tet3 could be taken as a potential candidate for therapeutic target in early treatment and prevention of

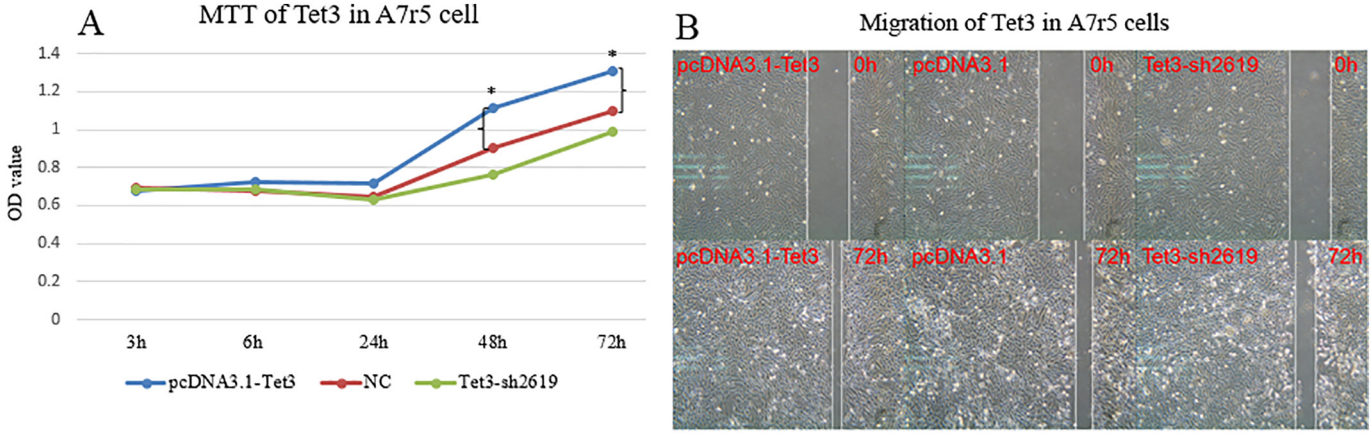

Fig. 6. Proliferation and migration of Tet3. A, MTT assay of different plasmid construction of Tet3 on A7r5 cells during different time points. B, migration assay was performed by cell scratch using corresponding plasmid construction, and evaluated at 72 h after transfection. Tet3 overexpression plasmid (pcDNA3.1-Tet3), RNA interference plasmid (Tet3-sh2619). For the over-expression assay, the negative control was transfected with empty vector, pcDNA3.1 (+). For the RNAi assay, the negative control was pGPH1/GFP/Neo empty vector linked with mismatched dsRNA. *: $p < 0.05$.

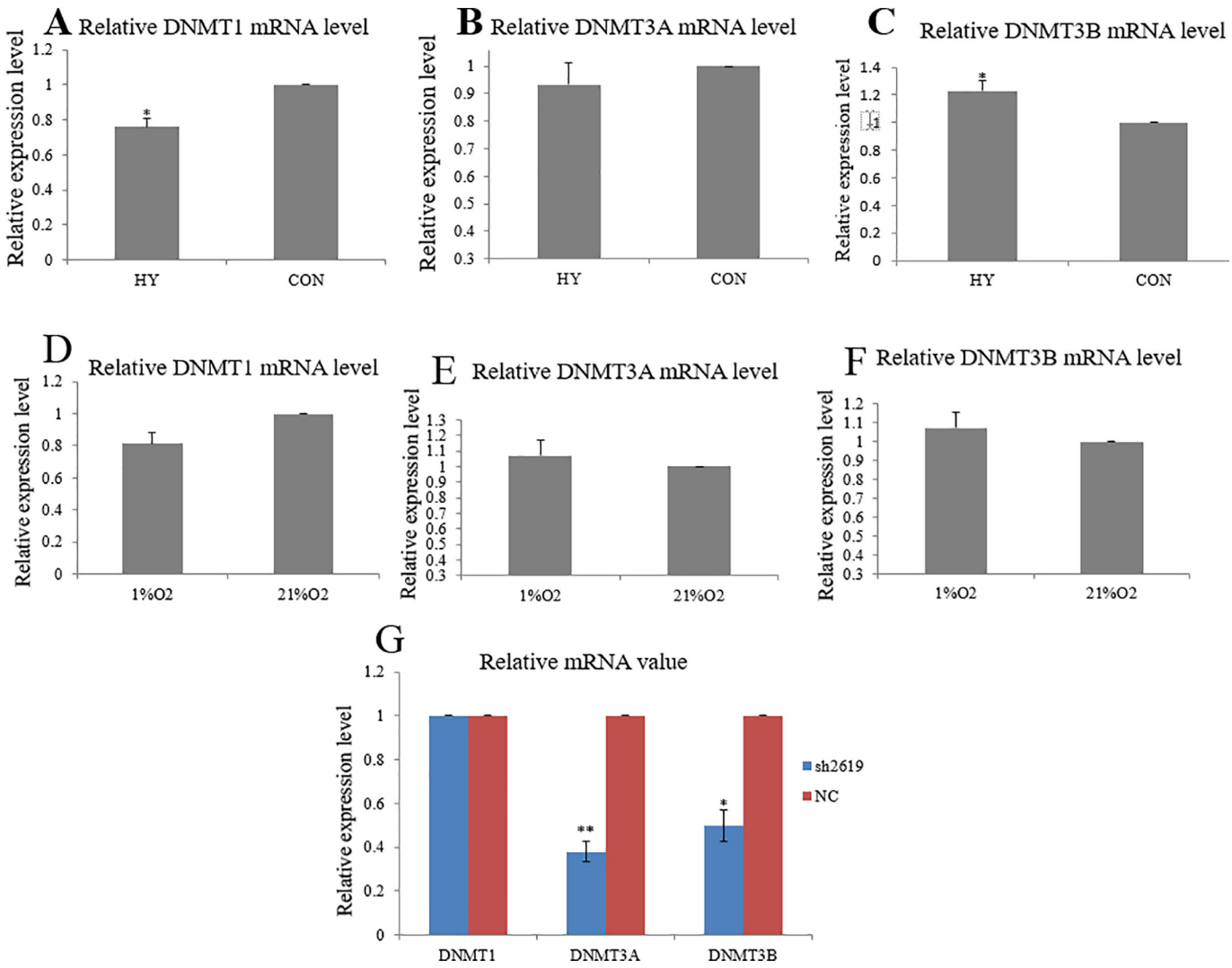

Fig. 7. DNMTs expression.
A, B, C indicates the mRNA expression of DNMT1, DNMT3A, and DNMT3B between HY and CON groups of offspring rats. D, E, F indicates the he mRNA expression of DNMT1, DNMT3A, and DNMT3B in A7r5 cells exposed to 1% O_2 and 21% O_2. G indicates DNMT1, DNMT3A, and DNMT3B evaluated when A7r5 cells transfected with Tet3-sh2619 under the condition of 1% O_2. *: $p < 0.05$.

atherosclerosis. Further analysis of Tet3 should be taken into research in future.

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Author contribution

Study & experimental design, Pengjie Zhang, Miao Sun, Zhice Xu; Experimental operation & and data analysis, Pengjie Zhang, Xionghui Cheng, Yueming Zhang, Hongyu Su, Yumeng Zhang, Lingjun Li, Xiuwen Zhou; Manuscript preparation, Pengjie Zhang, Xionghui Chen; Manuscript review & editing, Zhice Xu; Funding acquisition, Pengjie Zhang, Xionghui Chen, Zhice Xu.

Conflicts of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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