Department of Vascular Surgery, the Third Xiangya Hospital, Central South University, Changsha, China

Interleukin 6 induces expression of NADPH oxidase 2 in human aortic endothelial cells via long noncoding RNA MALAT1

Yan Wang*, Wanpin Nie*, Kai Yao, Zheng Wang, Hao He

Received March 25, 2016, accepted April 29, 2016

Kai Yao, MD, Department of Vascular Surgery, The Third Xiangya Hospital, Central South University, 138 Tongzipo Road, Changsha, Hunan 410013, China
xiangyayaokai2014@gmail.com
**These authors contributed equally to this work.*

Pharmazie 71: 592–597 (2016) *doi: 10.1691/ph.2016.6598*

Human abdominal aortic aneurysm (AAA) is characterized by the induction of intracellular and extracellular inflammatory cytokines and the production of reactive oxygen species (ROS) associated with localized inflammatory responses in the vascular wall. Recent studies have shown that greater circulating levels of the proinflammatory cytokine interleukin-6 (IL-6) are closely associated with AAA presence, suggesting that IL-6 plays an important role in the development of AAA. Previous in vivo studies have indicated that excess activities of NADPH oxidase (NOX), a major oxidase system for ROS production, promote AAA development. Furthermore, long noncoding RNAs (lncRNAs) are involved in the development of AAA. LncRNA MALAT1 has been found closely involved in endothelial cell functions and dysfunctions. In the present study, we explored the effects and the underlying mechanisms of IL-6 and MALAT1 on the expression/activity of NOXs in human aortic endothelial cells (HAOECs). Primary HAOECs with or without overexpression or knockdown of MALTA1 were cultured in the presence of IL-6. We found that IL-6 concentration- and time-dependently elevated the NOX activity as well as the MALAT1 level in HAOECs. Among different NOXs, only NOX2 was induced by IL-6. Overexpression and knockdown of MALAT1 respectively augmented and abolished IL6-induced expression of NOX2, NOX activity/cellular ROS production, and activation of the human NOX2 gene promoter, whereas MALAT1 alone in the absence of IL-6 treatment showed no significant effect. Knockdown of extracellular signal-regulated kinase (ERK) abolished IL6-induced expression of MALAT1. In conclusion, this study provides the first evidence that IL-6 induces expression/activity of NOX2 in HAOECs via inducing MALAT1 by an ERK-dependent mechanism. It adds new insights into the molecular mechanisms underlying AAA development.

1. Introduction

Characterized by localized structural deterioration of the aortic wall, abdominal aortic aneurysm (AAA) occurs in up to 9% of adults older than 65 years and represents the third frequent cause of cardiovascular death (Burillo et al. 2015; Franck et al. 2013). Endothelial cells, which play a major role in the maintenance of vascular integrity (Franck et al. 2013), are a critical cell type involved in AAA development (Huang et al. 2015).

Inflammation plays a key role in the pathogenesis of AAA (McCormick et al. 2007). Human AAA is characterized by induction of intracellular and extracellular inflammatory cytokines, expression of cell adhesion molecules, increase of protease expression, and release of reactive oxygen species (ROS) (Golledge et al. 2009). Interleukin-6 (IL-6) is a proinflammatory cytokine that has been implicated in various manifestations of cardiovascular disease (Takagi et al. 2014). Recent studies have shown that greater circulating IL-6 levels are closely associated with AAA presence (Takagi et al. 2014), suggesting that IL-6 plays an important role in the development of AAA.

One of the key features of AAA is significant production of ROS associated with localized inflammatory responses in the vascular wall (Emeto et al. 2016). Oxidative stress, i.e. production of ROS in excess of antioxidant protection, is said to be involved in the development of AAA (Emeto et al. 2016). Imbalance in the activity of major endogenous pro-oxidative enzymes such as NADPH oxidase (NOX) results in excessive ROS (Emeto et al. 2016). ROS are generated in the cells mainly through the NADPH oxidase (NOX) system, which is a large family of enzymes with oxidase activity as essential regulators of vascular function and dysfunction (Van Buul et al. 2005; Panieri and Santoro 2015). Since the discovery of the phagocytic NADPH oxidase (NOX2), several additional members have been identified so far (e.g. NOX1, NOX3, NOX4, NOX5) and functionally characterized in mammals. Each of them possesses specific regulatory mechanisms, downstream targets, and differential expression in tissues and cellular compartments (Panieri and Santoro 2015). Previous in vivo studies have indicated that excess NOX activity promotes AAA development (Kigawa et al. 2014). Originally found in neutrophils, the NOX system is also expressed in endothelial cells (Van Buul et al. 2005).

Studies on human genomes have revealed that transcription from <2% of the human genome yields many short or long noncoding RNAs (lncRNAs) with limited or no protein-coding capacity (Mattick and Makunin 2006). LncRNAs, defined as noncoding RNAs more than 200 nucleotides in length, play key roles in various cellular and physiological processes (Kapranov et al. 2007) and are dysregulated in a variety of human disorders (Bhan and Mandal 2015). Recent studies have suggested that lncRNAs are involved in the development of AAA (He et al. 2015; Falak et al. 2014). Metastasis associated lung adenocarcinoma transcript1 (MALAT1), a 6.5 Kb nuclear residing long noncoding RNA originally shown to control tumor metastasis and cancer cell survival (Puthanveetil et al. 2015), has been found enriched in endothelial cells and closely involved in endothelial cell functions and dysfunctions (Michalik et al. 2014; Thum and Fiedler 2014; Puthanveetil et al. 2015).

In the present study, we explored the effects and the underlying mechanisms of IL-6 and MALAT1 on the expression/activity of NOXs in human aortic endothelial cells (HAOECs).

2. Investigations and results

2.1. IL-6 induces expression of NOX2 in HAOECs

To examine the effect of IL-6 on NOX activity in aortic endothelial cells, we treated HAOECs with IL-6 (2, 4, 8, 16 and 32 ng/ml) for 5, 10, 15, 20, 25 and 35 h. As show in Fig. 1, IL-6 concentration- and time-dependently (particularly after 15 h of treatment) elevated the NOX activity in HAOECs. We used 8 ng/ml of IL-6 in subsequent experiments, because: (1) according to a meta-analysis study showing that greater circulating IL-6 levels are closely associated with AAA presence (Takagi et al. 2014), the circulating IL-6 levels in AAA patients were found within the range of 2.3-27.4 ng/ml (mostly below 10 ng/ml) in studies carried out after year 2000; (2) IL-6 at concentrations ≥ 8 ng/ml reached a plateau in elevating the NOX activity after 25 h of treatment. To examine the potential effect of IL-6 on the expression of NOXs, we next examined the mRNA levels of NOX1-5 in HAOECs treated with IL-6 (8 ng/ml). As shown in Fig. 2, IL-6 (8 ng/ml) significantly induced the expression of NOX2 after 15 h of treatment, up to 3.8 fold of that in untreated control cells at 35 h of treatment.

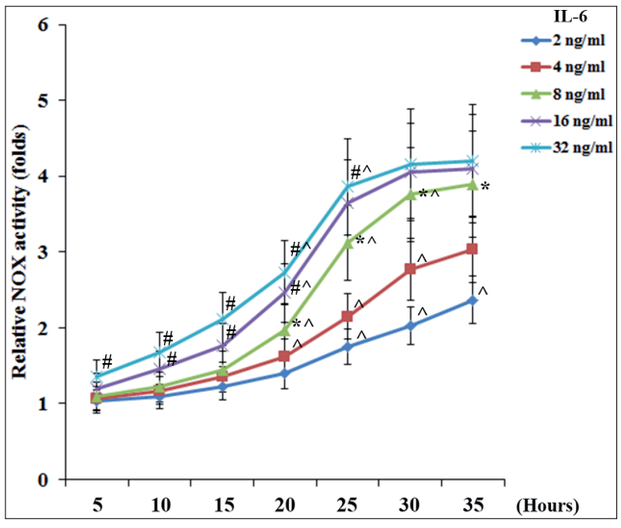

Fig. 1: NADPH oxidase (NOX) activity in human aortic endothelial cells (HAOECs) in the presence of interleukin 6 (IL-6) treatment. HAOECs were treated with IL-6 (2, 4, 8, 16 and 32 ng/ml) for 5, 10, 15, 20, 25 and 35 h. The NOX activity was measured and expressed as folds changes to that of untreated control cells (designated as 1). *p<0.05 vs. 4 ng/ml; #p<0.05 vs. 8 ng/ml; ^p<0.05 vs. immediate previous time point.

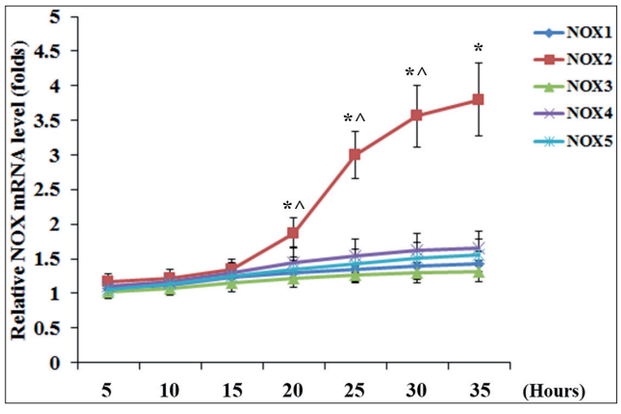

Fig. 2: NADPH oxidase (NOX) mRNA levels in human aortic endothelial cells (HAOECs) in the presence of interleukin 6 (IL-6) treatment. HAOECs were treated with IL-6 (8 ng/ml) for 5, 10, 15, 20, 25 and 35 h. The mRNA levels of NOX1-5 were measured and expressed as folds changes to those of untreated control cells (designated as 1). *p<0.05 vs. NOX1, 3, 4 and 5; ^p<0.05 vs. immediate previous time point.

2.2. IL-6 induces expression of MALAT1 in HAOECs

As shown in Fig. 3A, IL-6 concentration- and time-dependently (particularly before 25 h of treatment) elevated the MALAT1 level in HAOECs. To explore the potential effect of MALAT1 on IL6-induced expression of NOX2, we transduced HAOECs with human MALAT1 lentivirus or human MALAT1 siRNA lentivirus to overexpress or knock down MALAT1. Cells transduced with the blank control lentivirus (VC) or the scrambled siRNA lentivirus (SC) were used as controls. As shown in Fig. 3B, the VC and SC controls treated with 8 ng/ml of IL-6 showed similar data trend in the expression level of MALAT1 over time as the nontransduced cells in Fig. 3A, indicating that transduction of the blank control lentivirus or the scrambled siRNA lentivirus did not significantly affect the expression level of MALAT1. Lentiviral transduction of MALAT1 and MALAT1-siRNA resulted in significant increase and decrease of the expression of MALAT1 compared with the VC and SC controls, respectively (Fig. 3B).

2.3. MALAT1 is required for IL6-induced expression of NOX2 and NOX activity/cellular ROS production

As shown in Fig. 4, compared with the controls, MALAT1 knockdown abolished IL6-induced mRNA levels of NOX2, while MALAT1 overexpression further elevated it. Western blot analyses, NOX activity assays, and cellular ROS detection assays were subsequently performed to confirm the above findings at the NOX2 protein level. Time points 5, 20 and 35 h of IL-6 (8 ng/ml) treatment were used in these experiments because: (a) 5 h was the first detection time point in this study; (b) The NOX activity and the NOX2 expression started to rapidly increase at 20 h of IL-6 treatment, while MALAT1 expression started to reach a plateau after this time point. (c) 35 h was the last detection time point, when the NOX activity, the NOX2 expression, and the MALAT1 expression reached the highest level in this study. As shown in Fig. 5, compared with the controls, overexpression and knockdown of MALAT1 respectively augmented and abolished IL6-induced protein levels of NOX2. In parallel with these findings, the IL6-induced NOX activity (Fig. 6A) and the cellular ROS production (Fig. 6B) were augmented and largely abolished by overexpression and knockdown of MALAT1, respectively.

2.4. IL-6/MALAT1 signaling induces NOX2 at the gene promoter/transcription level in HAOECs

To explore how IL-6/MALAT1 signaling could induce the expression of NOX2 in HAOECs, we transfected HAOECs with a human NOX2 gene promoter/luciferase reporter. As shown in Fig. 7A, luciferase reporter assays revealed that IL-6 time-dependently increased the human NOX2 gene promoter/luciferase reporter activities, which was abolished by knocking down MALAT1 and augmented by overexpressing MALAT1, respectively. As shown in Fig. 7B, overexpression and knockdown of MALAT1 in the absence of IL-6 treatment showed no significant effect on the human NOX2 gene promoter activity, the NOX2 mRNA level, the NOX activity, and the cellular ROS content, indicating that MALAT1 alone had no significant effect on the expression/activity of NOX2 in HAOECs.

2.5. IL-6 induces MALAT1 in HAOECs by an ERK-dependent mechanism

It has been shown that ERK is a major downstream effector of IL6 (Reeves and Compton 2011; Wei et al. 2013). As shown in Fig. 8, although IL-6 did not increase the total ERK1/2 amount, it markedly increased the activation phosphorylation of ERK1/2 by 3.7-4.5 folds compared with that in untreated control cells, indicating that IL-6 activated ERKs in HAOECs. ERK1/2 siRNA significantly decreased the expression of ERK1/2, resulting in decreased ERK activation as manifested by markedly decreased activation phosphorylation of ERK1/2 (Fig. 8). As shown in Fig. 9, knockdown of ERK abolished IL6-induced expression of MALAT1, suggesting that IL-6 induced MALAT1 in HAOECs mainly by an ERK-dependent mechanism.

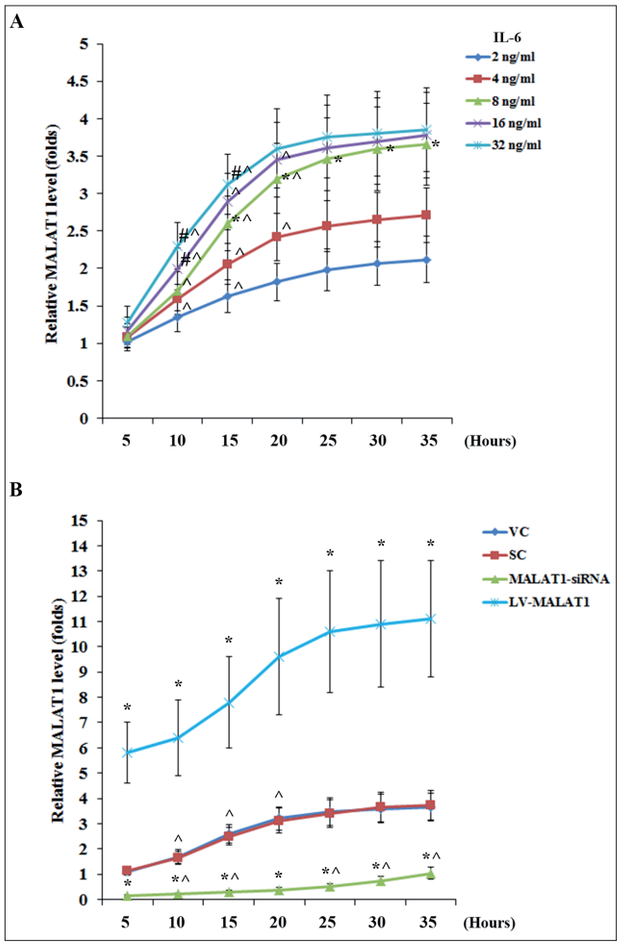

Fig. 3: Overexpression and knockdown of MALAT1 in human aortic endothelial cells (HAOECs) in the presence of interleukin 6 (IL-6) treatment. *(A)* HAOECs were treated with IL-6 (2, 4, 8, 16 and 32 ng/ml) for 5, 10, 15, 20, 25 and 35 h. The MALAT1 levels were measured and expressed as folds changes to those of untreated control cells (designated as 1). *p<0.05 vs. 4 ng/ml; #p<0.05 vs. 8 ng/ml; ^p<0.05 vs. immediate previous time point. *(B)* HAOECs were transduced with human MALAT1 lentivirus (LV-MALAT1) or human MALAT1 siRNA lentivirus (MALAT1-siRNA) to overexpress or knock down MALAT1. Cells transduced with the blank control lentivirus (VC) or the scrambled siRNA lentivirus (SC) were used as controls. Ten hours after transduction, the cells were were treated with IL-6 (8 ng/ml) for 5, 10, 15, 20, 25 and 35 hours. The MALAT1 levels were measured and expressed as folds changes to those of untreated control cells (designated as 1). *p<0.05 vs. VC and SC; ^p<0.05 vs. immediate previous time point.

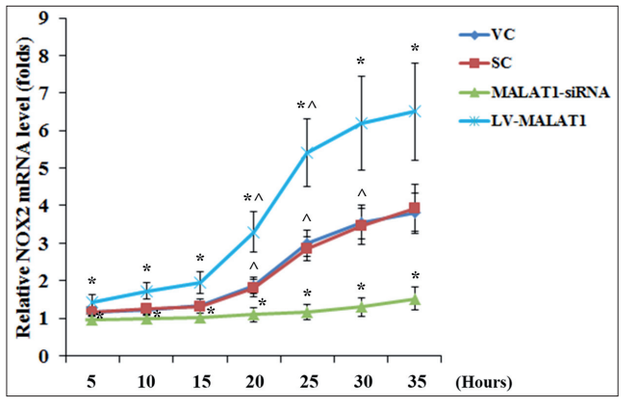

Fig. 4: NADPH oxidase 2 (NOX2) mRNA levels in human aortic endothelial cells (HAOECs) with overexpression and knockdown of MALAT1 in the presence of interleukin 6 (IL-6) treatment. HAOECs were transduced with human MALAT1 lentivirus (LV-MALAT1) or human MALAT1 siRNA lentivirus (MALAT1-siRNA) to overexpress or knock down MALAT1. Cells transduced with the blank control lentivirus (VC) or the scrambled siRNA lentivirus (SC) were used as controls. Ten hours after transduction, the cells were were treated with IL-6 (8 ng/ml) for 5, 10, 15, 20, 25 and 35 h. The mRNA levels of NOX2 were measured and expressed as folds changes to those of untreated control cells (designated as 1). *p<0.05 vs. VC and SC; ^p<0.05 vs. immediate previous time point.

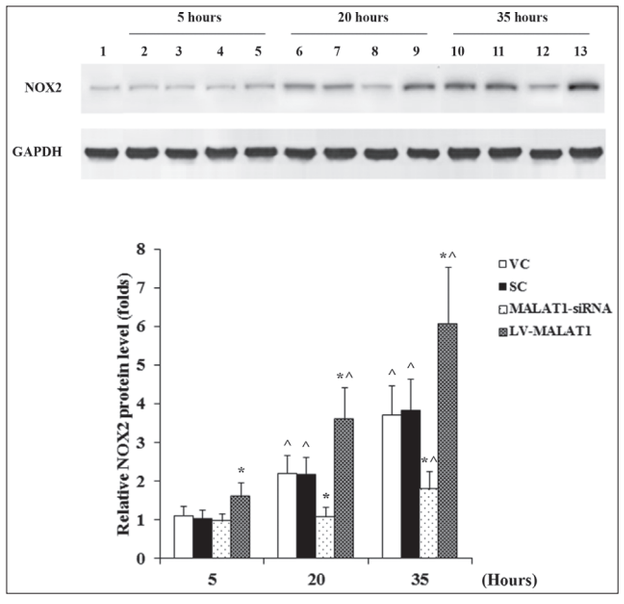

Fig. 5: NADPH oxidase 2 (NOX2) protein levels in human aortic endothelial cells (HAOECs) with overexpression and knockdown of MALAT1 in the presence of interleukin 6 (IL-6) treatment. HAOECs were transduced with human MALAT1 lentivirus (LV-MALAT1) or human MALAT1 siRNA lentivirus (MALAT1-siRNA) to overexpress or knock down MALAT1. Cells transduced with the blank control lentivirus (VC) or the scrambled siRNA lentivirus (SC) were used as controls. Ten hours after transduction, the cells were were treated with IL-6 (8 ng/ml) for 5, 20 and 35 h. The protein levels of NOX2 were determined with Western blot analyses. *Lane 1*, untreated control cells; *lanes 2, 6 and 10,* VC; *lanes 3, 7 and 11,* SC; *lanes 4, 8 and 12,* MALAT1-siRNA; *lanes 5, 9 and 13,* LV-MALAT1. GAPDH was used as a loading control. Density of the NOX2 blot was normalized against that of the GAPDH blot to obtain a relative blot density, which was expressed as fold changes to that of untreated control cells (designated as 1). *p<0.05 vs. VC and SC; ^p<0.05 vs. immediate previous time point.

3. Discussion

Human AAA is characterized by induction of intracellular and extracellular inflammatory cytokines and production of reactive oxygen species (ROS) associated with localized inflammatory responses in the vascular wall (Golledge et al. 2009). Recent studies have shown that greater circulating levels of the proinflammatory cytokine IL-6 are closely associated with AAA presence (Takagi et al. 2014), suggesting that IL-6 plays an important role in the development of AAA. Previous in vivo studies have indicated that excess activities of NOX, a major oxidase system for ROS production (Van Buul et al. 2005; Panieri and Santoro 2015), promote AAA development (Kigawa et al. 2014). Recent studies have suggested that lncRNAs are involved in the development of AAA (He et al. 2015; Falak et al. 2014). LncRNA MALAT1 has been found closely involved in endothelial cell functions and dysfunctions (Michalik et al. 2014; Thum and Fiedler 2014; Puthanveetil et al. 2015). Using primary HAOECs as a cell model, we have found in this study that MALAT1 mediates IL6-induced expression/activity of NOX2 in aortic endothelial cells, a critical cell type involved in AAA development (Huang et al. 2015).

We used a concentration range of 2-32 ng/ml for IL-6 treatment, based on a recent meta-analysis study showing that the circulating IL-6 levels in AAA patients were within the range of 2.3-27.4 ng/ml (mostly below 10 ng/ml) in studies carried out after year 2000 (Takagi et al. 2014). Our results showed that IL-6 within the range of 2-32 ng/ml concentration- and time-dependently elevated the

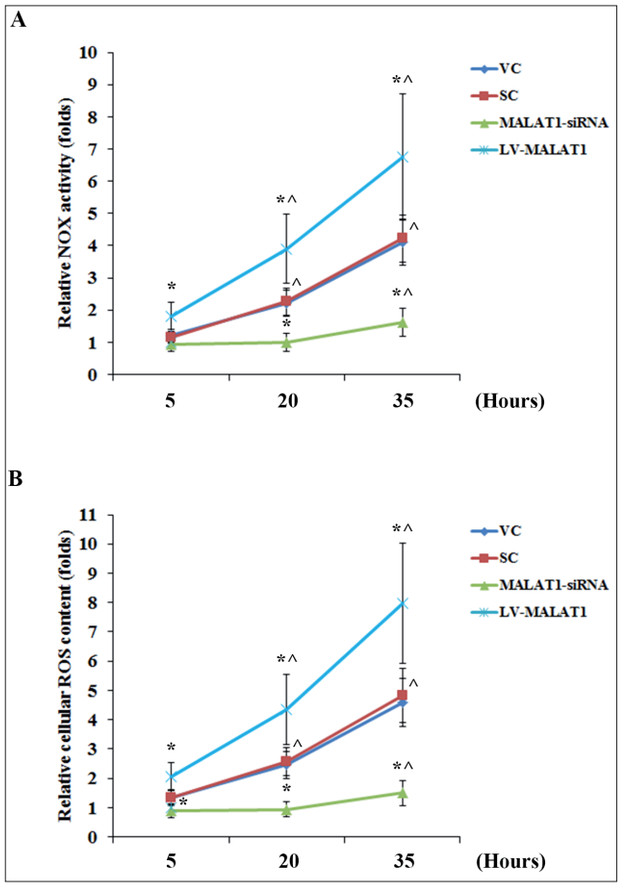

Fig. 6: NADPH oxidase (NOX) activity and reactive oxygen species (ROS) content in human aortic endothelial cells (HAOECs) with overexpression and knockdown of MALAT1 in the presence of interleukin 6 (IL-6) treatment. HAOECs were transduced with human MALAT1 lentivirus (LV-MALAT1) or human MALAT1 siRNA lentivirus (MALAT1-siRNA) to overexpress or knock down MALAT1. Cells transduced with the blank control lentivirus (VC) or the scrambled siRNA lentivirus (SC) were used as controls. Ten hours after transduction, the cells were were treated with IL-6 (8 ng/ml) for 5, 20 and 35 h. (A) The NOX activity and (B) cellular ROS content were measured and expressed as folds changes to those of untreated control cells (designated as 1). *p<0.05 vs. VC and SC; ^p<0.05 vs. immediate previous time point.

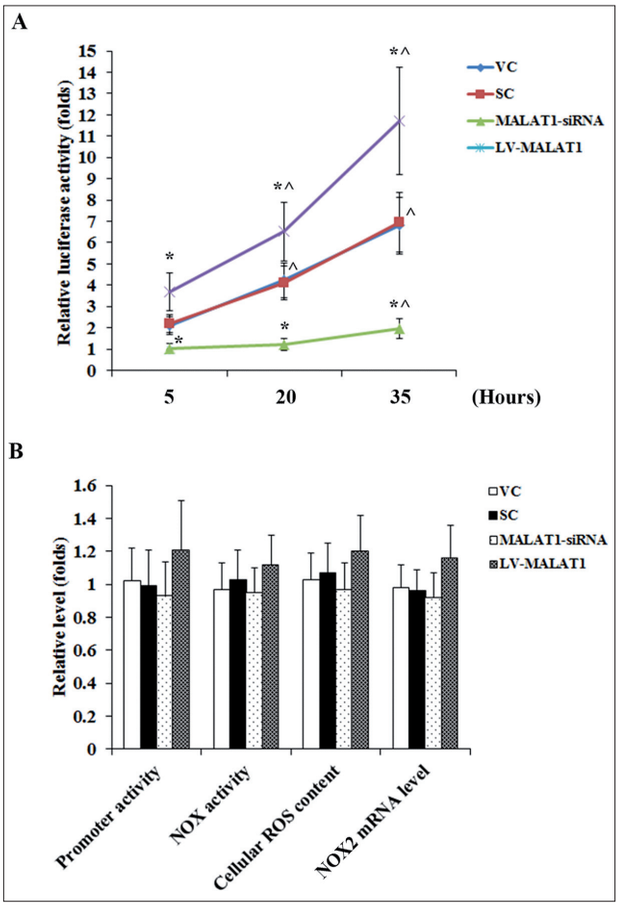

Fig. 7: NADPH oxidase 2 (NOX2) gene promoter activity in human aortic endothelial cells (HAOECs) with overexpression and knockdown of MALAT1 in the presence or absence of interleukin 6 (IL-6) treatment. HAOECs were transduced with human MALAT1 lentivirus (LV-MALAT1) or human MALAT1 siRNA lentivirus (MALAT1-siRNA) to overexpress or knock down MALAT1. Cells transduced with the blank control lentivirus (VC) or the scrambled siRNA lentivirus (SC) were used as controls. Ten hours after transduction, the cells were transfected with human NOX2 gene promoter/luciferase reporter plasmids and then cultured in the presence of IL-6 (8 ng/ml) for 5, 20 and 35 h. The promoter/luciferase activities were measured and expressed as fold changes to those of untreated control cells (designated as 1). *p<0.05 vs. VC and SC; ^p<0.05 vs. immediate previous time point. (B) The NOX2 promoter/luciferase activity, the NOX activity, cellular ROS content, and the NOX2 mRNA levels were also measured in the absence of IL-6 treatment.

NOX activity as well as the MALAT1 level in HAOECs. 8 ng/ml of IL-6 treatment was used for subsequent experiments, because IL-6 at concentrations ≥8 ng/ml reached a plateau in elevating the NOX activity and the MALAT1 level after 25 h of treatment. Overexpression and knockdown of MALAT1 respectively augmented and abolished IL6-induced expression of NOX2, suggesting that MALAT1 is a critical mediator for IL6-induced expression of NOX2 in HAOECs. In parallel with this, overexpression and knockdown of MALAT1 respectively augmented and largely abolished IL6-induced NOX activity and cellular ROS production, suggesting that IL6-induced expression of NOX2 could be a major contributor to IL6-induced NOX activity/ROS production in HAOECs. Nevertheless, we cannot exclude the possibility that MALAT1 can also mediate IL6-induced activation of NOXs in HAOECs, which will be explored in our future studies.

We used a range of 5-35 h for IL-6 treatment, because the NOX activity and the NOX2 expression only started to rapidly increase after 15 h of IL-6 treatment and reached a plateau at 25-30 h. In contrast, the MALAT1 level rapidly increased within 20 h of IL-6 treatment and then reached a plateau. Of timing, the IL6-induced expression of MALAT1 is a premise for the following augmented expression of NOX2, as indicated in the MALAT1 overexpression and knockdown experiments. This is in agreement with our finding that MALAT1 is a critical mediator for IL6-induced expression of NOX2 in HAOECs.

We found that IL-6/MALAT1 signaling activated the human NOX2 gene promoter, which provides a mechanistic explanation for IL-6/MALAT1 signaling-induced expression of NOX2 in HAOECs. Interestingly, MALAT1 alone in the absence of IL-6 treatment showed no significant effect on the NOX2 gene promoter, suggesting that other IL6-induced factor(s) is required for MALAT1 to activate the NOX2 gene promoter in HAOECs. LncRNAs reportedly regulate gene expression through epigenetic mechanisms including chromatin remodeling, regulation of splicing, and by acting as sponges for microRNAs (Hu et al. 2012; Mercer and Mattick 2013). Our future studies will reveal how IL-6/MALAT1 signaling activates the human NOX2 gene promoter in HAOECs.

In agreement with previous studies showing ERK as a major downstream effector of IL6 (Reeves and Compton 2011; Wei et al. 2013), we found that IL-6 significantly activated ERK in HAOECs. Knockdown of ERK abolished IL6-induced expression of MALAT1, indicating that ERK is required for IL6-induced expression of MALAT1 in HAOECs. Nevertheless, we cannot exclude the possibility that other signaling pathways downstream of IL-6 may also induce MALAT1 in HAOECs.

In conclusion, this study provides the first evidence that IL-6 induces expression/activity of NOX2 in HAOECs via inducing

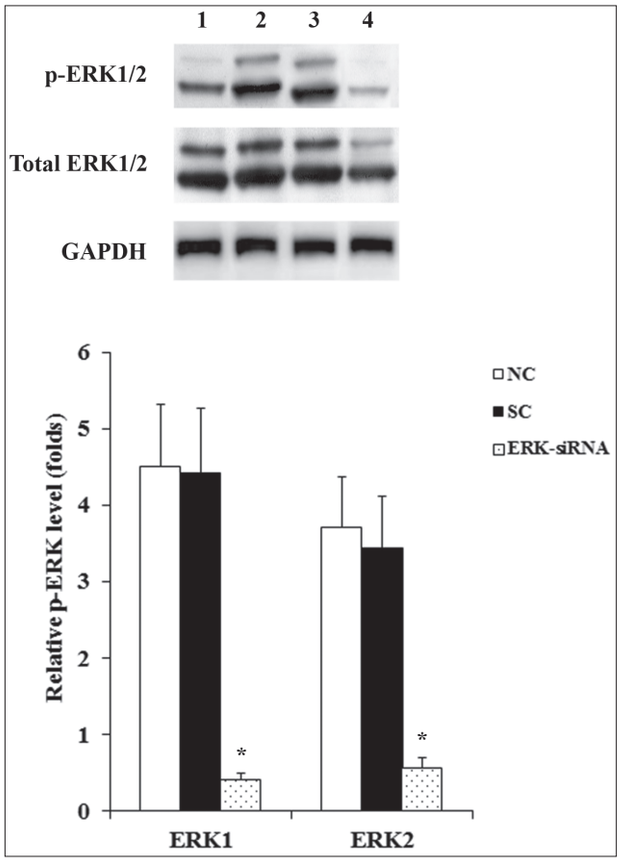

Fig. 8: Knockdown of extracellular signal-regulated kinase (ERK) in human aortic endothelial cells (HAOECs) in the presence of interleukin 6 (IL-6) treatment. HAOECs transfected with human ERK1/2 siRNA or scramble control siRNA (SC) were treated with IL-6 (8 ng/ml) for 20 h. Then the expression levels of total or phosphorylated ERK1/2 levels were determined with Western blot analyses. *Lane 1*, untreated control cells. *Lanes 2-4,* cells treated with IL-6: *lane 2*, normal control cells (NC); *lane 3*, cells transfected with SC; *lane 4*, cells transfected with ERK1/2 siRNA. GAPDH was used as a loading control. Density of the phosphorylated (p)-ERK1/2 blots was respectively normalized against that of the GAPDH blot to obtain a relative blot density, which was expressed as fold changes to that of untreated control cells (designated as 1). *p<0.05 vs. NC and SC.

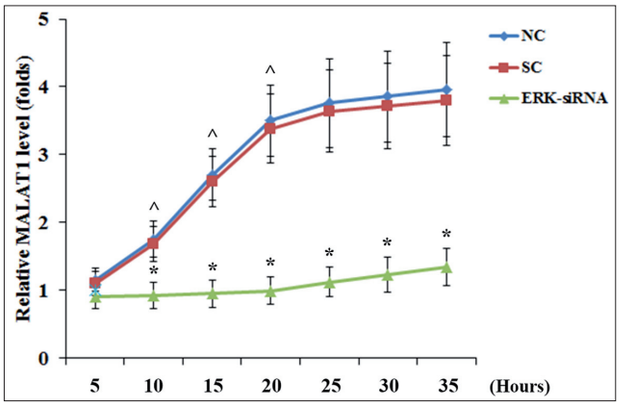

Fig. 9: Effect of extracellular signal-regulated kinase (ERK) knockdown on MALAT1 levels in human aortic endothelial cells (HAOECs) in the presence of interleukin 6 (IL-6) treatment. HAOECs transfected with human ERK1/2 siRNA or scramble control siRNA (SC) were treated with IL-6 (8 ng/ml) for 5, 10, 15, 20, 25 and 35 h. Nontransfected cells were used as a normal control (NC). The MALAT1 levels were measured and expressed as folds changes to those of untreated control cells (designated as 1). *p<0.05 vs. NC and SC; ^p<0.05 vs. immediate previous time point.

MALAT1 by an ERK-dependent mechanism. It adds new insights into the molecular mechanisms underlying AAA development.

4. Experimental

4.1. Cell culture and treatments

Primary HAOECs (Cat. No. 304-05a) and endothelial cell growth medium (Cat. No. 211F-500) were purchased from Cell Applications Inc. (San Diego, CA, USA). HAOECs were cultured in endothelial cell growth medium supplemented with 5% fetal bovine serum (Thermo Fisher Scientific, Beijing, China) and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, Beijing, China) in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were cultured in the presence or absence of 2, 4, 8, 16 or 32 ng/ml of recombinant human IL-6 (Cat. No. I1395; Sigma-Aldrich) for 5, 10, 15, 20, 25, 30 and 35 h. For extracellular signal-regulated kinase (ERK) knockdown, SignalSilence p44/42 MAPK (ERK1/2) siRNA (Cat. No. 6560; Cell Signaling Technology, Danvers, MA, USA) was transfected into HAOECs using Lipofectamine 3000 transfection reagent (Cat. No. L3000008; Thermo Fisher Scientific) by the manufacturer's protocol. Ten hours after transfection, the cells were subject to subsequent experiments. All the experiment results were normalized against the number of viable cells (per 10^4 viable cells) to exclude the potential effects of treatments on HAOECs proliferation and survival.

4.2. NOX activity assay

NOX activity was measured as previously described (Valencia et al. 2013). The assay uses exogenous NADPH+ and FAD+ to transfer an electron to the catalytic subunit gp91-phox, which will form a superoxide anion. This free radical reduces cytochrome c and is detected by absorbance at 550 nm over time. Superoxide dismutase 300 U/ml ((Sigma-Aldrich) was used to determine the superoxide anion produced by the NOX assay.

4.3. Real-time quantitative RT-PCR

RNA was prepared from cells using TRIzol reagent (Thermo Fisher Scientific) followed by purification with TURBO DNA-free System (Ambion, Austin, TX, USA). cDNA was synthesized using SuperScript II reverse transcriptase (Thermo Fisher Scientific) and random hexamer primers (Thermo Fisher Scientific). RT-qPCR was performed using an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific) and the fluorescent dye SYBR Green Master Mix (Thermo Fisher Scientific) as described by the manufacturer. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used for normalization. The primers used were as follows: for NOX1, 5'-GACAATGGGAAACTGGGTGGTTAAC-3' (forward) and 5'-GGGAAGTTTGAATCCCGCGGATGTCA-3' (reverse); for NOX2, 5'-CTGGACAGGAATCTCACCTTTCAT-3' (forward) and 5'-AATTTATCTACACGTTACCACACTTAG-3' (reverse); for NOX3, 5'-TATCATGATGGGGTGCTGGATTTTG-3' (forward) and 5'-ACAAGGAGAATATTCTCAATTCTTCA-3' (reverse); for NOX4, 5'-GCCAACGAAGGGGTTAAACA-3' and 5'-CGGGAACCAATATGTTCGTTCTTC-3' (reverse); For NOX5, 5′-ATGAGTGGCACCCCTTCACCATCAG-3′ (forward) and 5′-TTCGAGTGGTTTGTGAGCCTGCTGAC-3′ (reverse); for MALAT1, 5'-GTGATGCGAGTTGTTCTCCG-3' (forward) and 5'-CTGGCTGCCTCAATGCCTAC-3' (reverse); for GAPDH, 5'-GACTCATGACCACAGTCCATGC-3' (forward) and 5'-AGAGGCAGGGATGATGTTCTG-3' (reverse). The PCR amplification condition was: 20 s at 95 °C followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Relative quantification of the NOX mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and normalized against that of GAPDH in the same sample. Each experiment was repeated for three independent times in duplicates.

4.4. Lentiviral transduction

Human MALAT1 lentiviral vector (Cat. No. LV212703) and blank control lentivector (Cat. No. LV587) were purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). The MALAT1 lentiviral vector or blank control lentivector was transfected with the packaging vectors psPAX2 and pMD2.G into 293T cells by calcium chloride to produce the lentivirus. HAOECs were transduced with the blank control or MALAT1-expressing lentivirus. Human MALAT1 siRNA lentivirus (Cat. No. iV012699) and scrambled siRNA lentivirus (Cat. No. LVP015-G) were purchased from Applied Biological Materials Inc. and directly used to transduce HAOECs. Ten hours after transduction, the cells were subject to subsequent experiments.

4.5. Western blot analysis

HAOECs were lysed with a hypotonic buffer containing 2% Nonidet-P and a protease inhibitor cocktail (Sigma-Aldrich) by sonication three times for 3 s on ice and supernatant was obtained after centrifugation at 2000 for 15 min at 4 °C. Equal amount of proteins for each sample were separated by 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk powder in TBS-T for 2 h and incubated for 1 h with a 1:1000 dilution of mouse anti-human NOX2 monoclonal antibody (Cat. No. 611415; BD Biosciences, Shanghai, China), mouse anti-human ERK monoclonal antibody (Cat. No. M3807; Sigma-Aldrich), mouse anti-human activated (diphosphorylated) ERK1/2 antibody (Cat. No. M8159; Sigma-Aldrich), or mouse anti-human GAPDH monoclonal antibody (Cat. No. sc-32233; Santa Cruz Biotechnology, Dallas, TX, USA), and then washed and revealed using bovine anti-mouse secondary antibody (1:5000, 1 hour) (Cat. No. sc-2371; Santa Cruz Biotechnology). Peroxidase was revealed with a GE Healthcare ECL kit (Shanghai, China) using x-ray films (Thermo Fisher Scientific, Shanghai, China). Three independent experiments were performed.

4.6. Cellular ROS detection assay

ROS were measured with a DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (Cat. No. ab113851; Abcam, Cambridge, UK) according to the manufacturer's protocol. In brief, cells were plated at 7×10^4 cells per well in black 96-well plates and incubated with 25 μM DCFDA for 45 min. Fluorescence was detected using a Victor3 1420 Multilabel Counter (PerkinElmer, Shanghai, China).

4.7. Luciferase assay

The human NOX2 (also named CYBB) gene promoter/luciferase reporter (Cat. No. S700494) and LightSwitch Luciferase Assay Kit (Cat. No. LS010) were purchased from SwitchGear Genomics (Shanghai, China). HAOECs were transfected with the human NOX2 promoter/luciferase reporter plasmids (SwitchGear Genomics) using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific). The luciferase assays were performed using the LightSwitch Luciferase Assay Kit (SwitchGear Genomics) following the manufacturer's protocol. Plasmid PRL-CMV (Promega; Madison, WI, USA) encoding *Renilla reniformis* luciferase (at one fifth molar ratio to test plasmids) was co-transfected with test plasmids in each transfection as an internal control for data normalization. Each experiment was repeated for three independent times in duplicates.

4.8. Statistical analysis

Statistical analyses were performed with SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). All values were expressed as Mean±SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by *post hoc* pairwise comparisons using Tukey's tests. $p<0.05$ was considered statistically significant in this study.

Acknowledgements: This work was supported by Hunan Provincial Bureau of Science and Technology (grant #20156015), Changsha, China.

Conflict of interest: None declared.

References

Bhan A, Mandal SS (2-15) LncRNA HOTAIR: A master regulator of chromatin dynamics and cancer. Biochim Biophys Acta 1856: 151-164.

Burillo E, Lindholt JS, Molina-Sánchez P, Jorge I, Martinez-Pinna R, Blanco-Colio LM, Tarin C, Torres-Fonseca MM, Esteban M, Laustsen J, Ramos-Mozo P, Calvo E, Lopez JA, Vega de Ceniga M, Michel JB, Egido J, Andrés V, Vazquéz J, Meilhac O, Martin-Ventura JL (2015) ApoA-I/HDL-C levels are inversely associated with abdominal aortic aneurysm progression. Thromb Haemost 113: 1335-1346.

Emeto TI, Moxon JV, Au M, Golledge J (2016) Oxidative stress and abdominal aortic aneurysm: potential treatment targets. Clin Sci (Lond) 130: 301-315.

Falak S, Schafer S, Baud A, Hummel O, Schulz H, Gauguier D, Hubner N, Osborne-Pellegrin M (2014) Protease inhibitor 15, a candidate gene for abdominal aortic internal elastic lamina ruptures in the rat. Physiol Genomics 46: 418-428.

Franck G, Dai J, Fifre A, Ngo S, Justine C, Michineau S, Allaire E, Gervais M (2013) Reestablishment of the Endothelial Lining by Endothelial Cell Therapy Stabilizes Experimental Abdominal Aortic Aneurysms. Circulation 127: 1877-1887.

Golledge AL, Walker P, Norman PE, Golledge J (2009) A systematic review of studies examining inflammation associated cytokines in human abdominal aortic aneurysm samples. Dis Markers 26: 181-188.

He Q, Tan J, Yu B, Shi W, Liang K (2015) Long noncoding RNA HIF1A-AS1A reduces apoptosis of vascular smooth muscle cells: implications for the pathogenesis of thoracoabdominal aorta aneurysm. Pharmazie 70: 310-315.

Hu W, Alvarez-Dominguez JR, Lodish HF (2012) Regulation of mammalian cell differentiation by long non-coding RNAs. EMBO Rep 13: 971-983.

Huang CK, Luo J, Lai KP, Wang R, Pang H, Chang E, Yan C, Sparks J, Lee SO, Cho J, Chang C (2015) Androgen receptor promotes abdominal aortic aneurysm development via modulating inflammatory interleukin-1α and transforming growth factor-β1 expression. Hypertension 66: 881-891.

Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, Stadler PF, Hertel J, Hackermüller J, Hofacker IL (2007) RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science 316: 1484-1488.

Kigawa Y, Miyazaki T, Lei XF, Nakamachi T, Oguchi T, Kim-Kaneyama JR, Taniyama M, Tsunawaki S, Shioda S, Miyazaki A (2014) NADPH oxidase deficiency exacerbates angiotensin II–induced abdominal aortic aneurysms in mice. Arterioscler Thromb Vasc Biol 34:2413-2420.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods 25: 402-408.

Mattick JS, Makunin IV (2006) Non-coding RNA. Hum Mol Genet 15: R17-R29.

McCormick ML, Gavrila D, Weintraub NL (2007) Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 27: 461-469.

Mercer TR, Mattick JS (2013) Structure and function of long noncoding RNAs in epigenetic regulation. Nat Struct Mol Biol 20: 300-307.

Michalik KM, You X, Manavski Y, Doddaballapur A, Zörnig M, Braun T, John D, Ponomareva Y, Chen W, Uchida S, Boon RA, Dimmeler S (2014) Long noncoding RNA MALAT1 regulates endothelial cell function and vessel growth. Circ Res 114: 1389-1397.

Panieri E, Santoro MM (2015) ROS signaling and redox biology in endothelial cells. Cell Mol Life Sci 72: 3281-3303.

Puthanveetil P, Chen S, Feng B, Gautam A, Chakrabarti S (2015) Long non-coding RNA MALAT1 regulates hyperglycaemia induced inflammatory process in the endothelial cells. J Cell Mol Med 19: 1418-1425.

Reeves MB, Compton T (2011) Inhibition of inflammatory Interleuk 1 in-6 activity via ERK-MAPK signaling antagonizes human cytomegalovirus reactivation from dendritic cells from Latency. J Virol 85: 12750-12758.

Takagi H, Watanabe T, Mizuno Y, Kawai N, Umemoto T; All-Literature Investigation of Cardiovascular Evidence Grou (2014) Circulating interleukin-6 levels are associated with abdominal aortic aneurysm presence a meta-analysis and meta-regression of case control studies. Ann Vasc Surg 28: 1913-1922.

Thum T, Fiedler J (2014) LINCing MALAT1 and angiogenesis. Circ Res 114: 1366-1368.

Valencia A, Sapp E, Kimm JS, McClory H, Reeves PB, Alexander J, Ansong KA, Masso N, Frosch MP, Kegel KB, Li X, DiFiglia M (2013) Elevated NADPH oxidase activity contributes to oxidative stress and cell death in Huntington's disease. Hum Mol Genet 22: 1112-1131.

Van Buul JD, Fernandez-Borja M, Anthony EC, Hordijk PL (2005) Expression and Localization of NOX2 and NOX4 in Primary Human Endothelial Cells. Antioxid Redox Signal 7: 308-317.

Wei LH, Chou CH, Chen MW, Rose-John S, Kuo ML, Chen SU, Yang YS (2013) The role of IL-6 trans-signaling in vascular leakage: implications for ovarian hyperstimulation syndrome in a murine model. J Clin Endocrinol Metab 98: E472-E484.