1 Knockdown of GAS5 restores ox-LDL-induced impaired autophagy flux
2 via upregulating miR-26a in human endothelial cells

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- 19 ox-LDL, Oxidized low-density lipoprotein
- 20 EC, endothelial cell
- 21 GAS5, growth-arrest specific transcript 5
- 22 HAECs, human aortic endothelial cells
- 23 RIP, RNA immunoprecipitation
- 24 ncRNAs, noncoding RNAs
- 25 lncRNA, long noncoding RNA
- 26 miRNA, microRNA
- 27 HFD, high-fat diet
- 28 apoE, apolipoprotein E
- 29 ceRNA, competitive endogenous RNA
- 30 miR-NC, miRNA negative control
- 31 cDNA, complementary DNA
- 32 snRNA, small nuclear RNA
- 33 SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel
- 34 TBST, Tris-based saline with Tween 20
- 35 RISC, RNA-induced silencing complex
- 36 TSLP, thymic stromal lymphopoietin
- 37 HA-VSMCs, human aorta vascular smooth muscle cells
- 38 CAD, coronary artery disease
- 39

40 **Abstract**

41 **Background:** Oxidized low-density lipoprotein (ox-LDL)-induced endothelial
42 cell (EC) injury and autophagy dysfunction play a vital role in the development
43 of atherosclerosis. LncRNAs have been identified to participate in the
44 regulation of pathogenesis of atherosclerosis. However, it remains largely
45 undefined whether growth-arrest specific transcript 5 (GAS5) could influence
46 ox-LDL-induced autophagy dysfunction in ECs.

47 **Methods:** The expressions of GAS5 and miR-26a in the plasma samples of
48 patients with atherosclerosis and ox-LDL-treated human aortic endothelial
49 cells (HAECs) were detected by qRT-PCR. Luciferase reporter assay, RNA
50 immunoprecipitation (RIP), and RNA pull down were performed to validate
51 whether GAS5 could directly interact with miR-26a. The effects of ox-LDL,
52 GAS5 or combined with miR-26a on apoptosis and autophagy were evaluated
53 by flow cytometry analysis and western blot, respectively.

54 **Results:** GAS5 expression was upregulated and miR-26a was downregulated
55 in the plasma samples of patients with atherosclerosis and ox-LDL-treated
56 HAECs. There was reciprocal inhibition between GAS5 and miR-26a
57 expressions in ox-LDL-treated HAECs. We further demonstrated that GAS5
58 directly interacted with miR-26a in ox-LDL-treated HAECs. Additionally,
59 ox-LDL administration induced apoptosis and impaired autophagy flux in
60 HAECs. Rescue experiments demonstrated that GAS5 knockdown restored
61 ox-LDL-induced impaired autophagy flux by upregulating miR-26a in HAECs.

62 **Conclusion:** Knockdown of GAS5 restores ox-LDL-induced impaired
63 autophagy flux via upregulating miR-26a in human endothelial cells, revealing

64 a novel regulatory mechanism for ox-LDL-induced impaired autophagy flux in
65 ECs through ceRNA crosstalk.

66 **Key words:** GAS5, miR-26a, ox-LDL, autophagy, HAECs, atherosclerosis

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68 **Introduction**

69
70 Atherosclerosis, a devastating and chronic multi-factorial vascular
71 cardiovascular disease, remains a leading health issue among aged people,
72 accounting for the high morbidity and morbidity of cardiovascular disease
73 worldwide [1]. It has been suggested that endothelial dysfunction is the
74 requisite for the progression of atherosclerosis [2]. Oxidized low-density
75 lipoprotein (ox-LDL) is considered as a key risk factor associated with
76 endothelial dysfunction in atherosclerosis [3]. Extensive researches within the
77 past decades have demonstrated that ox-LDL-induced autophagy dysfunction
78 in endothelial cells (ECs) plays a vital role in the development of
79 atherosclerosis [4]. Autophagy is well-known as a dynamic process of
80 recycling that plays a prominent role in degrading dysfunctional or damaged
81 proteins or intracellular organelles in eukaryotic cells [5]. Recently, increasing
82 evidence has indicated that impaired autophagy flux contributes to lipid
83 metabolism dysfunction and EC apoptosis, greatly implicated in vascular
84 endothelial dysfunction and atherosclerotic plaque development [6]. Therefore,
85 upregulation of autophagy may be a promising therapy to protect ECs from
86 ox-LDL-induced injury.

87 Recently, increasing evidence demonstrated that noncoding RNAs
88 (ncRNAs), including the recently acknowledged long noncoding RNA (lncRNA)
89 and the well-known microRNA (miRNA), play important roles in the regulation
90 of gene expression via multiple mechanisms [7]. LncRNAs are generally
91 defined as a group of RNA transcripts longer than 200 nucleotides with limited
92 or no protein-coding potential. A wide range of documents unveil that lncRNAs
93 play important functional roles in the regulation of lipid metabolism, vascular

94 inflammation, cell proliferation, and EC apoptosis, suggesting that lncRNAs
 95 participate in the regulation of pathogenesis of atherosclerosis [8]. lncRNA
 96 growth-arrest specific transcript 5 (GAS5), located at chromosome 1q25.1,
 97 was originally isolated from mouse NIH 3T3 cells using subtraction
 98 hybridization [9]. There is striking evidence that GAS5 functions as a tumor
 99 suppressive lncRNA and is aberrantly downregulated in a variety of human
 100 cancers [10]. Notably, previous studies showed that GAS5 was increased in
 101 the plaque of atherosclerosis collected from patients and animal models and
 102 knockdown of GAS5 reduced the apoptosis of macrophages and vascular
 103 endothelial cells after ox-LDL stimulation [11, 12]. However, it remains largely
 104 undefined whether GAS5 could influence ox-LDL-induced autophagy
 105 dysfunction in ECs.

106 miRNAs are small, endogenous, single-stranded ncRNAs with 20-25
 107 nucleotides in length, which repress gene expression at the posttranscriptional
 108 level via mRNA degradation or translational inhibition. More recently,
 109 substantive studies have demonstrated that miRNAs play critical roles in the
 110 development of atherosclerosis via regulating the proliferation, migration and
 111 apoptosis of various types of cells [13]. miR-26a, a highly conserved miRNA,
 112 has been revealed to play essential roles in development, cell differentiation,
 113 apoptosis and growth. miR-26a is reported to be frequently dysregulated in
 114 cardiovascular diseases such as cardiac hypertrophy and myocardial ischemia
 115 [14, 15]. Moreover, it was previously reported that miR-26a was
 116 downregulated in high-fat diet (HFD)-fed apolipoprotein E (apoE)^{-/-} mice and
 117 ox-LDL-stimulated human aortic endothelial cells (HAECs) and miR-26a
 118 overexpression alleviated the development of atherosclerosis [16, 17].

119 Recently, a new regulatory mechanism has been proposed that lncRNAs
120 function on a competitive endogenous RNA (ceRNA) to regulate the
121 expressions and biological function of miRNAs [18]. The lncRNA-miRNA
122 interaction has been identified in various human diseases, including vascular
123 pathophysiology [19]. Since our bioinformatics analysis demonstrated that
124 GAS5 contained the complementary binding sites in miR-26a, we
125 hypothesized whether GAS5 could function as a molecular sponge of miR-26a
126 to regulate atherosclerosis progression.

127 In the present study, we investigated the effects of GAS5 on
128 ox-LDL-induced autophagy in HAECs, as well as the interaction between
129 GAS5 and miR-26a.

130 **2. Materials and methods**

131 *2.1 Clinical specimens*

132 A total of 30 atherosclerotic patients diagnosed by clinical symptoms and
133 coronary angiography at the department of cardiology in the Henan Provincial
134 People's Hospital between January 2015 and August 2016 and 30 healthy
135 subjects were enrolled in the present study. The plasma samples were
136 collected from all patients and healthy subjects and stored at -80°C for further
137 experiments. The study was performed with the approval of the Research
138 Ethics Committee of Henan Provincial People's Hospital and written informed
139 consent was obtained from each participant.

140 *2.2. Cell culture and transfection*

141 HAECs were obtained from American Type Culture Collection (ATCC,
142 Manassas, VA, USA) and were cultured in endothelial cell medium containing
143 endothelial cell growth factors, 10% heat-inactivated fetal bovine serum (FBS,

144 Hyclone, Logan, UT, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin
 145 (Sangon, Shanghai, China) at 37°C in a humidified atmosphere of 5% CO₂.
 146 Cells in logarithmic phase were collected for further experiments. When grown
 147 to 70-80% confluence, HAECs were transiently transfected with miR-26a
 148 mimic (miR-26a), miRNA negative control (miR-NC), miR-26a inhibitor
 149 (anti-miR-26a), inhibitor negative control (anti-miR-NC), pcDNA-GAS5 (GAS5),
 150 pcDNA empty control (pcDNA), siRNA against GAS5 (si-GAS5), siRNA
 151 negative control (si-NC) (all from RiboBio Co., Ltd., Guangzhou, China) using
 152 Lipofectamine 2000 reagent (Invitrogen, CA, Carlsbad, USA). Following 48 h
 153 of transfection, HAECs were treated with different concentrations of ox-LDL
 154 (0.25, 0.5, and 1 mg/L) (UnionBiol, Beijing, China) for 24 h.

155 2.3. Quantitative real-time PCR

156 Total RNA was extracted from clinical samples or treated HAECs using TRIzol
 157 reagent (Invitrogen) and complementary DNA (cDNA) was synthesized from 1
 158 µg of total RNA using a High-Capacity cDNA Reverse Transcription kit
 159 (Applied Biosystems, Foster City, CA, USA). The expressions of GAS5 and
 160 miR-26a were examined using a SYBR Premix Ex Taq II (Takara, Dalian,
 161 China) and TaqMan MicroRNA Assay Kit (Applied Biosystems) on a CFX96
 162 real-time PCR System (Bio-Rad, Hercules, CA, USA), respectively. The
 163 expressions of GAS5 and miR-26a were normalized to GAPDH and U6 small
 164 nuclear RNA (snRNA), respectively. The relative gene expression was
 165 calculated using the $2^{-\Delta\Delta C_t}$ method.

166 2.4. Western blot

167 Total proteins were extracted from the treated HAECs using RIPA buffer
 168 containing protease inhibitors (Roche, Nutley, NJ, USA). Equal quantities of

169 protein were fractionated by a 10% sodium dodecyl sulfate-polyacrylamide gel
 170 (SDS-PAGE) and transferred to a nitrocellulose membranes (Millipore,
 171 Billerica, MA, USA). After being blocked in 5% skimmed milk in Tris-based
 172 saline with Tween 20 (TBST) for 1 h at room temperature, the membranes
 173 were incubated with primary antibodies against LC3-II (1:1000, Abcam,
 174 Cambridge, UK), LC3-I (1:1000, Abcam), p62 (1:2000, Cell Signaling
 175 Technology, Danvers, MA, USA). After washing with TBST, the membranes
 176 were incubated with HRP-conjugated secondary antibody for 2 h at room
 177 temperature. The protein bands were visualized using a chemiluminescence
 178 kit (GE Healthcare, Buckinghamshire, UK) and protein intensity was quantified
 179 with Image-Pro Plus 6.0 software (GE Healthcare).

180 *2.5. Flow cytometry analysis*

181 Cell apoptosis was assessed using a FITC-Annexin V Apoptosis Detection Kit
 182 (BD Bioscience, San Jose, CA, USA). HAECs were seeded into 24-well plates
 183 and exposed to ox-LDL at a series of concentration (0.25, 0.5, and 1 mg/L) for
 184 24 h, or transfected with si-NC, si-GAS5, si-GAS5 + anti-miR-NC, si-GAS5 +
 185 anti-miR-26a, followed by stimulation with 1 mg/L ox-LDL. Then cells were
 186 collected and digested using 0.25% trypsin. After washed twice with ice-cold
 187 PBS, cells were resuspended with 500 μ l of 1 $\times$ binding buffer solution and
 188 incubated with 5 μ l of Annexin-V FITC and 5 μ l propidium iodide (PI) for 15 min
 189 in the dark. Apoptotic cells were measured by a BD FACSCalibur flow
 190 cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed with FlowJo
 191 software (TreeStar, Ashland, OR, USA).

192 *2.6. Luciferase reporter assay*

193 The wild-type fragment of GAS5 including the miR-26a binding sites and its
194 mutant sequence were subcloned into psiCHECK-2 luciferase reporter vector
195 (Promega, Madison, WI, USA) and named as GAS5-WT and GAS-MUT. For
196 luciferase reporter assay, HAECs were cotransfected with 100 ng constructed
197 luciferase reporter vectors, together with 20 ng Renilla luciferase vector, and
198 100 nM miR-26a or miR-NC using Lipofectamine 2000 (Invitrogen), followed
199 by treatment with 1 mg/L ox-LDL. At a point of 48 h transfection, cells were
200 harvested and the luciferase activity was detected using the dual-Luciferase
201 Reporter Assay System (Promega). The relative luciferase activity was
202 normalized to the Renilla luciferase activity.

203 *2.7. RNA immunoprecipitation (RIP) assay*

204 Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford,
205 MA, USA) was used to perform RIP assay was conducted using the. Briefly,
206 ox-LDL-treated HAECs at 80-90% confluence were collected and lysed in
207 complete RIP lysis buffer. Next, the supernatant from cell lysates was
208 harvested by centrifugation and then 100 µl of cell extracts were incubated
209 with RIP buffer containing A + G magnetic beads conjugated with human
210 anti-Argonaute2 (Ago2) antibody (Abcam) or corresponding negative control
211 IgG (Abcam). To remove the non-specific binding, the Sepharose beads were
212 incubated with Proteinase K to digest the protein and subsequently the
213 precipitated RNA was isolated by TRIzol reagent (Invitrogen). The purified
214 RNA was subjected to qRT-PCR analysis.

215 *2.8. RNA pull-down assay with biotinylated GAS5*

216 Briefly, biotinylated DNA probe complementary to GAS5 was amplified by PCR
217 with a T7-containing primer and cloned into the plasmid vector GV394

(Genechem, Shanghai, China). The resultant plasmids were linearized with *Xho*I. Biotin-labeled RNAs were then reversely transcribed with the Biotin RNA Labeling Mix (Roche, Indianapolis, IN, USA) and T7 RNA polymerase (Roche), treated with RNase-free DNase I (Roche) and purified with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA). The bound RNAs were extracted for further evaluation by qRT-PCR analysis.

2.9. RNA pull-down assay with biotinylated miR-26a

Biotinylated miR-26a (bio-miR-26a-WT), biotinylated mutant (bio-miR-26a-MUT), and biotinylated NC (bio-NC) were synthesized by GenePharma (Shanghai, China). HAECs were transfected with biotinylated miRNA using Lipofectamine 2000 (Invitrogen) and collected at 48 post-transfection. Cell lysates were incubated with M-280 streptavidin magnetic beads (Invitrogen). The bound RNAs were purified using TRIzol reagent (Invitrogen) for further qRT-PCR analysis.

2.10. Statistical analysis

The data are presented as the mean $\pm$ standard deviation (SD). All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). The significance of differences was estimated by Student's *t* test or one-way analysis of variance (ANOVA). Differences were considered to be statistically significant when *P* value < 0.05.

3. Results

3.1. GAS5 expression was upregulated and miR-26a was downregulated in the plasma samples of patients with atherosclerosis and ox-LDL-treated HAECs

242 The expressions of GAS5 and miR-26a in the plasma samples from
243 atherosclerotic patients and healthy controls were firstly detected by qRT-PCR.
244 The results indicated that GAS5 expression was abnormally higher while
245 miR-26a expression was aberrantly lower in the plasma samples from patients
246 with atherosclerosis compared with that from healthy controls (Fig. 1A and 1B).
247 Next, we further analyzed the expressions of GAS5 and miR-26a in HAECs
248 treated with different concentrations of ox-LDL (0.25, 0.5, and 1 mg/L).
249 qRT-PCR analysis demonstrated that ox-LDL stimulation dose-dependently
250 increased GAS5 expression and decreased miR-26a expression in HAECs
251 (Fig. 1C and 1D). Collectively, these data suggested that abnormally
252 expressed GAS5 and miR-26a may be associated with the development of
253 atherosclerosis.

254 *3.2. The relationship between GAS5 and miR-26a expression in* 255 *ox-LDL-treated HAECs*

256 To address the interaction between GAS5 and miR-26a, HAECs were
257 transfected with GAS5, si-GAS5, miR-26a, anti-miR-26a, or respective
258 controls, followed by ox-LDL stimulation. We found that GAS5 expression was
259 strikingly elevated by GAS5 transfection and greatly reduced by si-GAS5
260 introduction in ox-LDL-treated HAECs (Fig. 2A). However, ox-LDL-treated
261 HAECs transfected with GAS5 showed a remarkable decline of miR-26a
262 expression and ox-LDL-treated HAECs introduced with si-GAS5 exhibited a
263 substantial enhancement of miR-26a expression (Fig. 2B). Besides, miR-26a
264 expression was considerably enhanced following transfection of miR-26a but
265 dramatically reduced after treatment with anti-miR-26a in ox-LDL-treated
266 HAECs (Fig. 2C). On the contrary, GAS5 expression was obviously lowered in

HAECs treated with miR-26a and ox-LDL but evidently augmented in HAECs treated with anti-miR-26a and ox-LDL (Fig. 2D). Together, these findings uncovered that there was reciprocal inhibition between GAS5 and miR-26a expressions in ox-LDL-treated HAECs.

3.3. *GAS5 directly interacted with miR-26a in ox-LDL-treated HAECs*

Based on the reciprocal repression between GAS5 and miR-26a expression, we guessed whether the crosstalk between GAS5 and miR-26a is through direct interaction. Accordingly, bioinformatics analyses were performed to predict the potential miRNAs for GAS5. As a result, the prediction showed the potential binding sites for miR-26a on GAS5, as displayed in Fig. 3A. To verify whether GAS5 could directly interact with miR-26a, we cloned the wild-type or mutated fragment of GAS5 into psiCHECK-2 luciferase reporter vector and performed luciferase reporter assay. The results manifested that cotransfection with GAS5-WT and miR-26a significantly decreased the luciferase activity, but cotransfection with GAS5-MUT and miR-26a did not affect the luciferase activity in ox-LDL-stimulated HAECs (Fig. 3B). It is reported that miRNAs exerted their gene silencing functions via binding to Ago2, a core component of the RNA-induced silencing complex (RISC) [20]. Then RIP assay was performed in cell extracts from HAECs treated with utilizing the antibody against Ago2 and the results presented that GAS5 and miR-26a were both preferentially enriched in Ago2 pellets compared with control IgG immunoprecipitates (Fig. 3C). To confirm whether miR-26a could directly interact with GAS5, we applied a biotin-labeled miRNA pull down to capture GAS5 using M-280 streptavidin magnetic beads from HAECs transfected with biotinylated miR-26a and the results suggested that GAS5

292 was pulled down as analyzed by qRT-PCR, while miR-26a-MUT with mutant
 293 binding sites of GAS5 led to the inability of miR-26a to pull down GAS5 (Fig.
 294 3D). Also, we used the opposite pull down system to confirm whether GAS5
 295 could pull down miR-26a using a biotin-labeled specific GAS5 probe. We
 296 observed a significant amount of miR-26a in the GAS5 pulled down pellet
 297 compared with control group as analyzed by qRT-PCR (Fig. 3D). These results
 298 demonstrated that GAS5 could directly bind to miR-26a in ox-LDL-treated
 299 HAECs.

300 *3.4. ox-LDL administration induced apoptosis and impaired autophagy flux in* 301 *HAECs*

302 Next, we analyzed the effects of ox-LDL on the apoptosis of HAECs. HAECs
 303 were treated with different concentrations of ox-LDL (0.25, 0.5, and 1 mg/L)
 304 and then flow cytometry analysis was conducted. As shown in Fig. 4A, the
 305 percentage of apoptotic rate was specifically increased following ox-LDL
 306 treatment in a dose-dependent manner. Moreover, we further evaluated the
 307 influence of ox-LDL on the autophagy flux in HAECs by detecting the protein
 308 levels of autophagy markers LC3 and p62. The western blot analysis indicated
 309 that the LC3-II/LC3-I ratio was distinctly decreased while p62 expression was
 310 notably increased in ox-LDL-treated HAECs dose-dependently, suggesting
 311 that ox-LDL induced impaired autophagy flux in HAECs (Fig 4B). Taken
 312 together, these results demonstrated that ox-LDL administration induced
 313 apoptosis and weakened autophagy in HAECs.

314 *3.5. GAS5 knockdown restored ox-LDL-induced impaired autophagy flux by* 315 *upregulating miR-26a in HAECs*

To figure out the effects of GAS5 or combined with miR-26a on ox-LDL-induced impaired autophagy flux, HAECs were transfected with si-GAS5, si-NC, si-GAS5 + anti-miR-26a, si-GAS5 + anti-miR-NC, and then exposed to 1 mg/L ox-LDL. Flow cytometry analysis proved that silencing of GAS5 effectively suppressed ox-LDL-induced apoptosis in HAECs, which was partially recuperated by inhibition of miR-26a (Fig. 5A). The subsequent western blot analysis displayed that transfection with si-GAS5 obviously boosted the LC3-II/LC3-I ratio and reduced p62 level in ox-LDL-treated HAECs (Fig. 5B). However, these effects were significantly reversed by anti-miR-26a treatment (Fig. 5B), suggesting that miR-26a suppression attenuated GAS5 knockdown-mediated restoration of ox-LDL-induced impaired autophagy flux in HAECs. These results manifested that GAS5 knockdown restored ox-LDL-induced impaired autophagy flux by upregulating miR-26a in HAECs.

4. Discussion

In the present study, we demonstrated that GAS5 was upregulated and miR-26a was downregulated in the plasma samples of patients with atherosclerosis and ox-LDL-treated HAECs. Our study demonstrated that GAS5 directly interacted with miR-26a in ox-LDL-treated HAECs. Mechanistically, GAS5 knockdown restored ox-LDL-induced impaired autophagy flux by upregulating miR-26a in HAECs. Thus, GAS5 intervention may be a promising strategy to prevent atherosclerosis.

Dysregulation of autophagy has been documented to be closely associated with many diseases, including cardiovascular disease [21]. Autophagy is well-known to become dysfunctional in atherosclerosis, suggesting its protective role. It has been shown that the protective effect on

autophagy might be beneficial to the therapy of atherosclerosis [22]. Recent studies showed that ox-LDL could induce vascular EC autophagy dysfunction and apoptosis in apoE^{-/-} mice [23]. Accordingly, we used ox-LDL to treat ECs and VSMCs to stimulate the pathological changes that occur during the early stage of atherosclerosis. During autophagy process, a cytosolic form of LC3 (LC3-I) is conjugated to form LC3-phosphatidylethanolamine conjugate (LC3-II) to promote autophagosome formation [24]. Thus, the ratio of LC3-II to LC3-I is considered as a marker for monitoring autophagy activity [25]. Additionally, p62, known as an autophagic substrate, is used as another widely marker of autophagy flux and can be selectively degraded by autophagy [26]. In our study, we found that ox-LDL stimulation induced apoptosis and impaired autophagy flux in HAECs, as demonstrated by the reduced ratio of LC3-II/LC3-I and increased expression of p62, which was consistent with the previous studies [27-29].

Recently, substantive studies have shown that ncRNAs including lncRNAs or miRNAs are identified as vital regulator of the physiological process of atherosclerosis [30]. For example, it was revealed that thymic stromal lymphopoietin (TSLP)-induced activation of lncRNA HOTAIR played a protective role in ox-LDL-induced EC injury by facilitating cell proliferation and migration and suppressing apoptosis in ECs [31]. In addition, miR-126 was reported to alleviate EC injury in atherosclerosis by restoring autophagic flux via inhibiting of PI3K/Akt/mTOR [32]. Recently, ample evidence has suggested that lncRNAs suppress the expressions and biological functions of miRNAs by acting as a ceRNA [33]. For example, it was demonstrated that silencing of H19 inhibited lipid accumulation and inflammation response in ox-LDL-treated

Raw264.7 cells by upregulating miR-130b [34]. LncRNA H19 expression was found to be increased in atherosclerotic patient serum and ox-LDL-stimulated human aorta vascular smooth muscle cells (HA-VSMCs), and knockdown of H19 suppressed proliferation and induced apoptosis in ox-LDL-induced HA-VSMCs through modulating WNT/ β -catenin signaling [35]. LncRNA MEG3 expression was reported to be downregulated in coronary artery disease (CAD) tissues than in normal arterial tissues, and ectopic expression of MEG3 increased EC proliferation and migration through inhibiting miR-21 expression [36].

In our study, we demonstrated that GAS5 was significantly upregulated and miR-26a was remarkably downregulated in the plasma samples of patients with atherosclerosis and ox-LDL-treated HAECs, which was in accordance with the previous studies [11, 12, 16, 17]. Given the inverse expression changes between GAS5 and miR-26a in atherosclerosis, we focused on the interaction between GAS5 and miR-26a. Luciferase reporter assay, RIP and RNA pull down manifested that GAS5 could directly interact with miR-26a by functioning as a ceRNA in ox-LDL-treated HAECs. Furthermore, rescue experiments demonstrated that we found that GAS5 knockdown alleviated ox-LDL-induced apoptosis and restored ox-LDL-induced impaired autophagy flux in HAECs by upregulating miR-26a.

5. Conclusions

In summary, our study provided the evidence that GAS5 knockdown restored ox-LDL-induced impaired autophagy flux by upregulating miR-26a in HAECs, revealing a novel regulatory mechanism for ox-LDL-induced impaired autophagy flux in ECs through ceRNA crosstalk. GAS5/miR-26a axis may

391 extend our knowledge of the pathological mechanism of atherosclerosis and
392 provided the potential therapeutic target for the treatment of atherosclerosis.

393

394 **Author Contributions**

395 This work was designed and conceived by Weijie Liang. The experiment
396 procedures and data analysis were carried out by Taibing Fan and Lin Liu. The
397 manuscript was prepared by Weijie Liang and Lianzhong Zhang.

398

399 **Conflicts of interest**

400 The authors have no conflict of interest to declare.

401

402 **Acknowledgements**

403 Not applicable

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509

510 **Figure legends**

511

512 **Figure 1.** GAS5 expression was upregulated and miR-26a was downregulated

513 in the plasma samples of patients with atherosclerosis and ox-LDL-treated

514 HAECs. qRT-PCR analysis of GAS5 (A) and miR-26a (B) in the plasma

515 samples from patients with atherosclerosis and healthy subjects. qRT-PCR

516 analysis of GAS5 (C) and miR-26a (D) in the HAECs treated with different

517 doses of ox-LDL (0.25, 0.5, and 1 mg/L) for 24 h. * $P < 0.05$.

518 **Figure 2.** The relationship between GAS5 and miR-26a expression in

519 ox-LDL-treated HAECs. (A and B) The expressions of GAS5 and miR-26a

520 were examined by qRT-PCR in HAECs after transfection with GAS5, si-GAS5,

521 or corresponding controls, followed by administration with 1 mg/L ox-LDL for

522 24 h. (C and D) The expressions of GAS5 and miR-26a were examined by

523 qRT-PCR in HAECs after introduction with miR-26a, anti-miR-26a, or matched

524 controls, followed by administration with 1 mg/L ox-LDL for 24 h. * $P < 0.05$.

525 **Figure 3.** GAS5 directly interacted with miR-26a in ox-LDL-treated HAECs. (A)

526 Predict binding sites between GAS5 and miR-26a. (B) Luciferase reporter

527 assay was performed to detect the luciferase activity in HAECs after

528 cotransfection with GAS5-WT or GAS5-MUT and miR-26a or miR-NC,

529 followed by treatment with ox-LDL. (C) Anti-Ago2 RIP assay was performed in

530 cellular lysates from HAECs treated with ox-LDL to explore the association

531 between GAS5 and miR-26a.

532 (D) HAECs were transfected with biotinylated miR-26a (bio-miR-26a-WT),

533 biotinylated mutant (bio-miR-26a-MUT), and biotinylated NC (bio-NC),

534 followed by treatment with ox-LDL, and collected at 48 post-transfection for

535 pull-down assay with biotinylated miR-26a. Detection of miR-26a using

536 qRT-PCR in the samples pulled down by biotinylated GAS5 and NC probe. **P*
537 < 0.05.

538 **Figure 4.** ox-LDL administration induced apoptosis and weakened autophagy
539 flux in HAECs. (A) Flow cytometry analysis was performed to assess the
540 apoptosis of HAECs treated with different concentrations of ox-LDL (0.25, 0.5,
541 and 1 mg/L). (B) Western blot was conducted to determine the protein levels of
542 LC3-II, LC3-I and p62 in HAECs treated with different doses of ox-LDL (0.25,
543 0.5, and 1 mg/L). **P* < 0.05.

544 **Figure 5.** GAS5 knockdown restored ox-LDL-induced impaired autophagy flux
545 by upregulating miR-26a in HAECs. HAECs were transfected with si-GAS5,
546 si-NC, si-GAS5 + anti-miR-26a, si-GAS5 + anti-miR-NC, and then exposed to
547 1 mg/L ox-LDL for 24 h. (A) Flow cytometry analysis was performed to detect
548 apoptosis of the treated HAECs. (B) Western blot was used to analyze the
549 protein levels of LC3-II, LC3-I and p62 in the treated HEACs. **P* < 0.05.

A **B** **C** **D**

A

Position: chr1:173833309-173833315

GAS5 5'... GAUGAUCUCAAUCCAACUUGAA...3'

miR-26a 3' UCGGAUAGGACCUAUUGAACUU 5'

B

C

D

A **B**

A **B**