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# MEF2A alters the proliferation, inflammation-related gene expression profiles and its silencing induces cellular senescence in human coronary endothelial cells

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## Abstract

**Background:** Myocyte enhancer factor 2A (MEF2A) plays an important role in cell proliferation, differentiation and survival. Functional deletion or mutation in MEF2A predisposes individuals to cardiovascular disease mainly caused by vascular endothelial dysfunction. However, the effect of the inhibition of MEF2A expression on human coronary artery endothelial cells (HCAECs) is unclear. In this study, expression of MEF2A was inhibited by specific small interference RNA (siRNA), and changes in mRNA profiles in response to MEF2A knockdown were analyzed using an Agilent human mRNA array.

**Results:** Silencing of MEF2A in HCAECs accelerated cell senescence and suppressed cell proliferation. Microarray analysis identified 962 differentially expressed genes (DEGs) between the MEF2A knockdown group and the negative control group. Annotation clustering analysis showed that the DEGs were preferentially enriched in gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to proliferation, development, survival, and inflammation. Furthermore, 61 of the 578 downregulated DEGs have at least one potential MEF2A binding site in the proximal promoter and were mostly enriched in the GO terms “reproduction” and “cardiovascular.” The protein–protein interaction network analyzed for the downregulated DEGs and the DEGs in the GO terms “cardiovascular” and “aging” revealed that PIK3CG, IL1B, IL8, and PRKCB were included in hot nodes, and the regulation of the longevity-associated gene PIK3CG by MEF2A has been verified at the protein level, suggesting that PIK3CG might play a key role in MEF2A knockdown induced HCAEC senescence.

**Conclusions:** MEF2A knockdown accelerates HCAEC senescence, and the underlying molecular mechanism may be involved in down-regulation of the genes related with cell proliferation, development, inflammation and survival, in which PIK3CG may play a key role.

**Keywords:** Myocyte enhancer factor 2A, Human coronary artery endothelial cells, Vascular endothelial dysfunction, Senescence, Cardiovascular disease, PIK3CG

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## Background

MEF2A is one of the members of the MEF2 family that belongs to the MADS-box superfamily and shares homology in a 58-amino acid domain that mediates DNA binding and dimerization [1]. An MEF2A-encoded protein can act as a homodimer or heterodimer and is involved in several cellular processes, including neuronal differentiation, muscle development, cell growth control, and apoptosis [2]. MEF2A has an essential DNA-binding site in the MyoDa gene control region and is a transcription factor that activates many muscle-specific, growth factor-induced, and stress-induced genes [3, 4]. The critical roles of MEF2A in myocardial development have been confirmed in many studies. Naya et al. observed that mice lacking *Mef2a* die suddenly within the first week of life and exhibit pronounced right ventricle dilation, myofibrillar fragmentation, mitochondrial disorganization, and fetal cardiac gene program activation [5]. Chen et al. found that MEF2A participates in the pathway mediating cardiomyocyte development by AKT2 [6]. The association of genetic variations in the coding sequence of MEF2A with coronary artery disease (CAD) has also been broadly investigated. The deletion of seven amino acids disrupts the nuclear localization of MEF2A and reduces the MEF2A-mediated activation of transcription, and this deletion is co-segregated with premature CAD in a family lineage [7]. Another study has reported that a novel 6 bp deletion in exon 11 of MEF2A is closely associated with premature CAD [8]. Nonsynonymous mutation or variation in the number of CAG or CCG repeats is correlated with susceptibility to CAD in some populations [9, 10]. However, the roles of MEF2A variants in CAD are controversial because not all studies can show the association between these variants and CAD [11–15]. The inflammation and dysfunction of vascular endothelial cells or smooth muscle cells are implicated in the pathogenesis of CAD. MEF2A promotes vascular smooth muscle cell (VSMC) senescence and regulates H<sub>2</sub>O<sub>2</sub>-induced VSMC senescence by interacting with miR-143, and MEF2A inhibition induces the phenotypic switching of VSMCs, leading to increased cell proliferation and migration [16, 17]. Kim et al. demonstrated that the activity of MEF2 (MEF2A and MEF2C) is remarkably impaired in pulmonary arterial endothelial cells derived from the subjects with pulmonary arterial hypertension (PAH), and the restoration of the impaired function of endothelial MEF2 rescues PAH [18]. Silencing of MEF2A in apoE<sup>-/-</sup> mice by using lentiviral shRNA considerably reduces plaque collagen content and fibrous cap thickness and increases plaque area; conversely, silencing of MEF2A has no obvious effect on plaque lipid content [19]. These studies have suggested that MEF2A plays important roles in cardiomyocytes, VSMCs, and

pulmonary arterial endothelial cells, but its functions in coronary artery endothelial cells are unclear. In this study, we found that the inhibition of MEF2A by siRNA promoted HCAEC senescence. To explore the transcriptional change in the downstream molecular network in response to MEF2A knockdown, we conducted mRNA microarray analysis and revealed that many specific pathways might be regulated by MEF2A in HCAECs. We also described a downstream molecular network controlled by MEF2A.

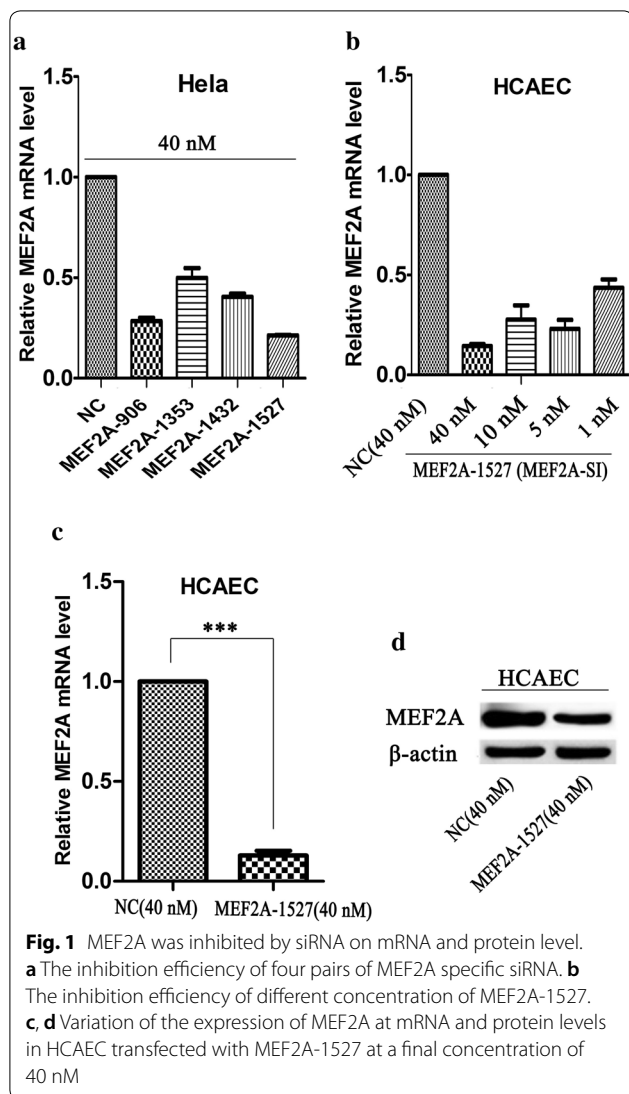
## Results

### Inhibition of MEF2A in HCAECs promoted cellular senescence and suppressed proliferation

In this study, we attempted to understand the effect of inhibiting the MEF2A expression on HCAECs. Four pairs of MEF2A-specific siRNA were designed to suppress the MEF2A expression, and MEF2A-1527 showed the highest inhibitory activity on the expression of MEF2A (Fig. 1a). MEF2A-1527 had a significant inhibitory effect on MEF2A when it was transfected at different concentrations in HCAEC (Fig. 1b), and a final concentration of 40 nM MEF2A-1527 remarkably inhibited MEF2A at both mRNA (Fig. 1c) and protein (Fig. 1d) levels in HCAEC. The results of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) staining and EdU staining showed that MEF2A knockdown accelerates HCAEC senescence (Fig. 2a) and inhibits cell proliferation (Fig. 2b).

### Identification of DEGs in response to MEF2A knockdown in HCAECs

The MEF2A-specific siRNA group and the negative control group were subjected to genome-wide expression microarray analyses to determine the underlying regulatory network in response to the inhibition of MEF2A. The raw microarray data obtained in this experiment were deposited in Gene Expression Omnibus (GEO) [20] with an accession number of GSE114114. A total of 1363 genes had more than twofold changes in the expression between the MEF2A knockdown group and the NC group, and the difference in the gene expression patterns could be seen in the heat map (Fig. 3a). The DEGs, namely, MEF2A, SERPIN1, IL1B, ENPP1, ZAK, PIK3CG, FOS, MMP1, HLA-DQB1, GUCY1A3, MYL9, GJA5, and CSF2, were selected for real-time q-PCR validation. Upregulation of MMP1 and MYL9 and downregulation of the other genes except GJA5 in the MEF2A-siRNA group were observed in qPCR and microarray. The microarray result showed that GJA5 was significantly down-regulated, but the qPCR result revealed that GJA5 was up-regulated in the MEF2A-siRNA group (Fig. 3b).



Of the down-regulated DEGs, 61 were predicted with at least one MEF2A binding site in the proximal promoter (500 bp region upstream from the transcription start site) (Additional file 1: Table S1). Database for Annotation, Visualization, and Integrated Discovery (DAVID) analysis of the 61 genes indicated that many genes were significantly enriched in the terms “reproduction” and “cardiovascular” in the GAD\_DISEASE\_CLASS category. The terms “secreted,” “disulfide bond,” “signal peptide,” and “zinc finger region:B box-type” were the most significantly enriched (Table 1). A few of these DEGs were selected for qPCR validation, and the expression of TRIM7, SGK196, BCL2A1, NR5A2, and ANK3 significantly differed between the two groups (Fig. 3c). These findings suggested for the

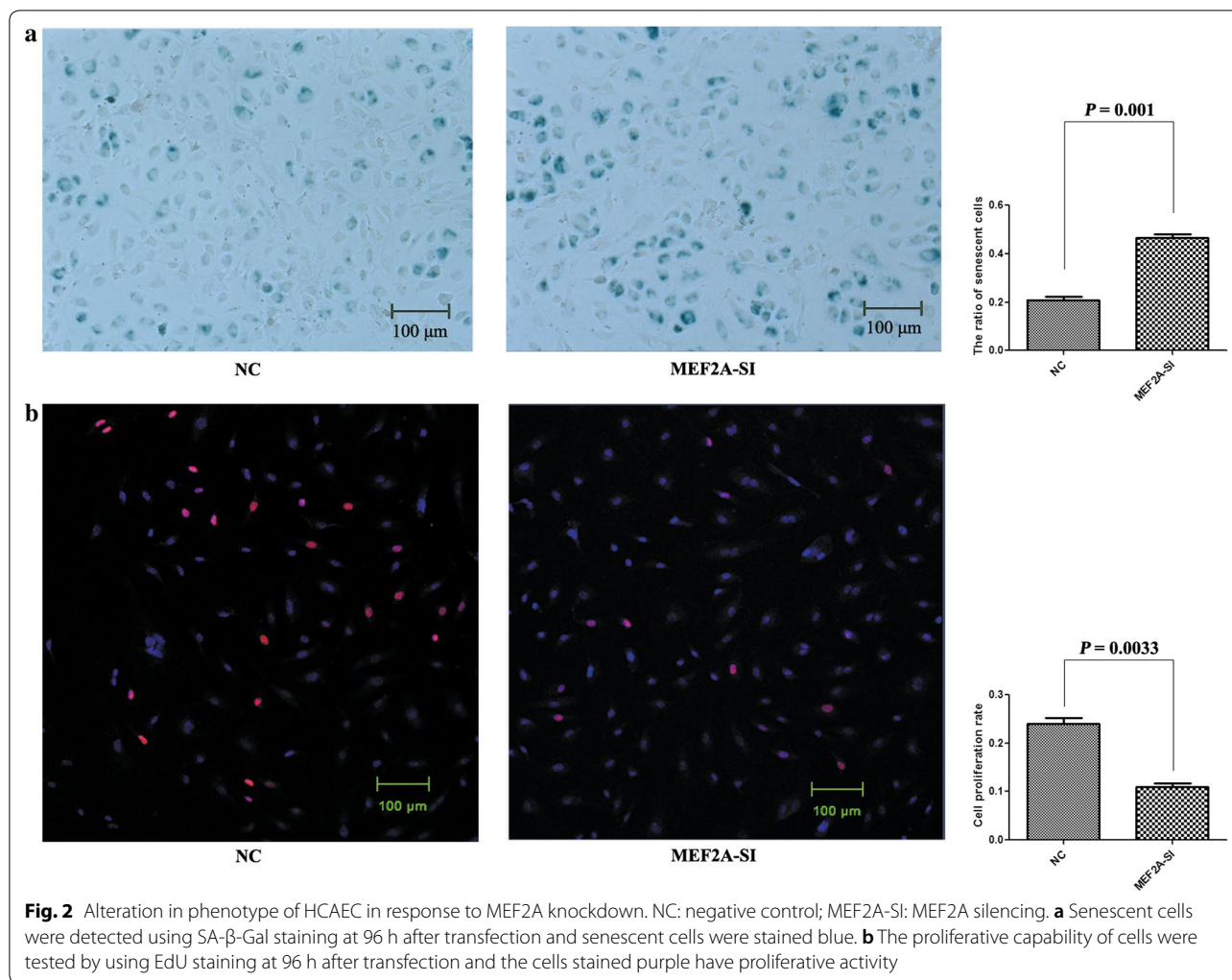
first time that the expression of these genes may be directly mediated by MEF2A.

#### DEGs in response to MEF2A knockdown were mostly enriched in the DAVID GO terms related to growth, signal transduction and inflammation

Excluding the DEGs that lacked the ENTREZ GENE ID information, we found that 384 and 578 of 962 DEGs were up-regulated and down-regulated, respectively (Additional file 2: Table S2). The annotation clusters containing GO terms, including glycoprotein, glycosylation site:N-linked (GlcNAc), signal peptide, disulfide bond, secreted, and signal, were significantly enriched in the DEGs (Additional file 3: Table S3). Functional GO term enrichment analysis revealed that preferential changes in the expression of the genes in response to MEF2A knockdown were involved in diverse biological processes, including “female pregnancy,” “cell–cell signaling,” and “metabolic process.” The DEGs were likely implicated in many molecular functions, including “phosphatidylinositol-4,5-bisphosphate 3-kinase activity,” “cytokine activity,” “serine-type endopeptidase inhibitor activity,” and “growth factor activity” (Table 2). The most significantly enriched GO terms in cellular component included extracellular space, extracellular region, and plasma membrane (Table 2). Pathway enrichment analysis showed that most of the DEGs down-regulated by the MEF2A knockdown were enriched in immune-related pathways (Table 3). These GO terms are closely associated with growth, development, cell survival, and inflammation, suggesting the extensive involvement of MEF2A in mediating cell functions.

#### PIK3CG and several chemotactic factors were included in the hot nodes in protein–protein interaction (PPI) networks

The PPI network of the down-regulated DEGs contained 502 nodes and 281 edges, and the  $p$ -value of PPI enrichment was  $< 1.0 \times 10^{-16}$ . Most of the downregulated DEGs formed a complicated interaction network (Fig. 4a). GNG13, GNG2, GABBR2, CXCR7, CXCL3, IL8, CNR1, IL1B, PRKCB, PMCH, P2RY14, and PIK3CG were presented at the hot nodes and may play important roles in the biological process (Fig. 4a). The majority of the genes in the network were significantly enriched (FDR  $< 0.01$ ) in the categories of extracellular space and extracellular region in the GO term of cellular component, and many of these genes were involved in signal transduction (Table 4). The KEGG pathways “amoebiasis, *Staphylococcus aureus* infections, influenza A, rheumatoid arthritis, and olfactory transduction” were significantly enriched



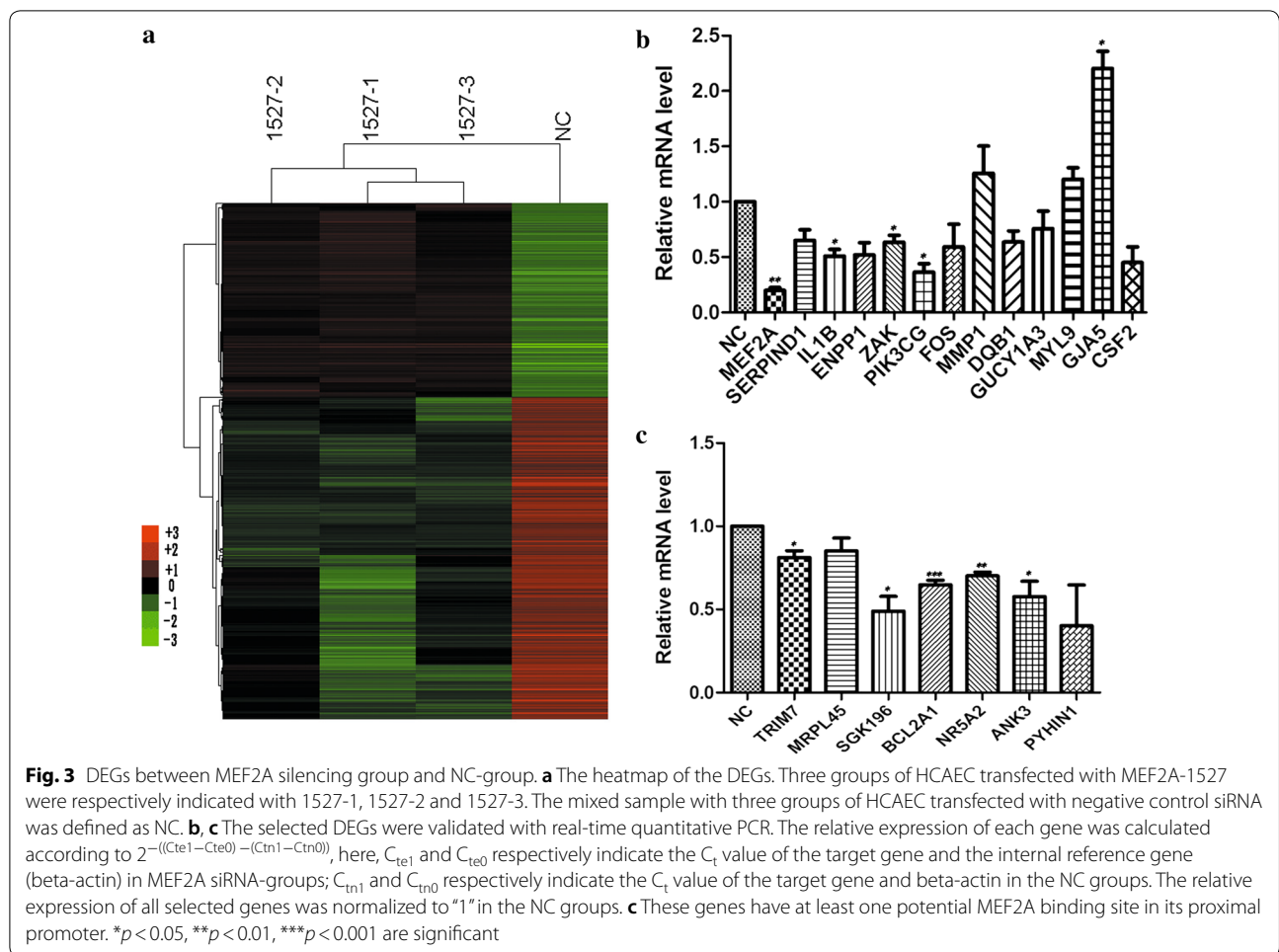
(FDR < 0.05) for the DEGs in the PPI network (Table 4). However, most of the up-regulated genes are independent of each other and only a small number of them can form an interaction network (Fig. 4b).

In order to discover the genes possibly involved in related diseases, the DEGs were annotated to the category “GAD\_DISEASE\_CLASS” and the terms “metabolic, chemdependency, cardiovascular, hematological, psych, neurological, renal, vision, pharmacogenomic, reproduction, normal variation, immune, aging, and developmental” were preferentially enriched (Fig. 5a). Among the DEGs in the GO term “aging,” 23 were up-regulated and 33 were down-regulated. The PPI network of the DEGs in the GO term “aging” showed that 22 DEGs, including PIK3CG, TXK, HDAC9, PPARG, IL1B, IL8, and PCK1, were in a correlative interaction network (Fig. 5b). A PPI network analysis performed for 244 DEGs in the GO term “cardiovascular” showed that these genes had more interactions among

themselves than the expected observation (PPI enrichment  $p < 1 \times 10^{-16}$ ), suggesting that the genes were biologically connected as a group (Fig. 5c). PIK3CG, IL8, IL1B, and CSF were included in the hot nodes with multiple interactions, implying their major role in the GO terms “aging” and “cardiovascular.” Further validation experiments showed that the mRNA and protein levels of PIK3CG were significantly down-regulated when MEF2A was inhibited (Fig. 6a), while the mRNA and protein levels of PIK3CG were significantly up-regulated when overexpressing MEF2A (Fig. 6b).

## Discussion

In the present study, inhibition of MEF2A by siRNA promoted HCAEC senescence, suggesting that it has an anti-aging functional role. This protective role appears to be different from its role in VSMC, which exhibits a detrimental aging-promoting role [16, 17]. However, an in vivo research has shown that MEF2A may have an



**Table 1** GO annotation for the down-regulated DEGs with potential MEF2A binding sites in the proximal promoter

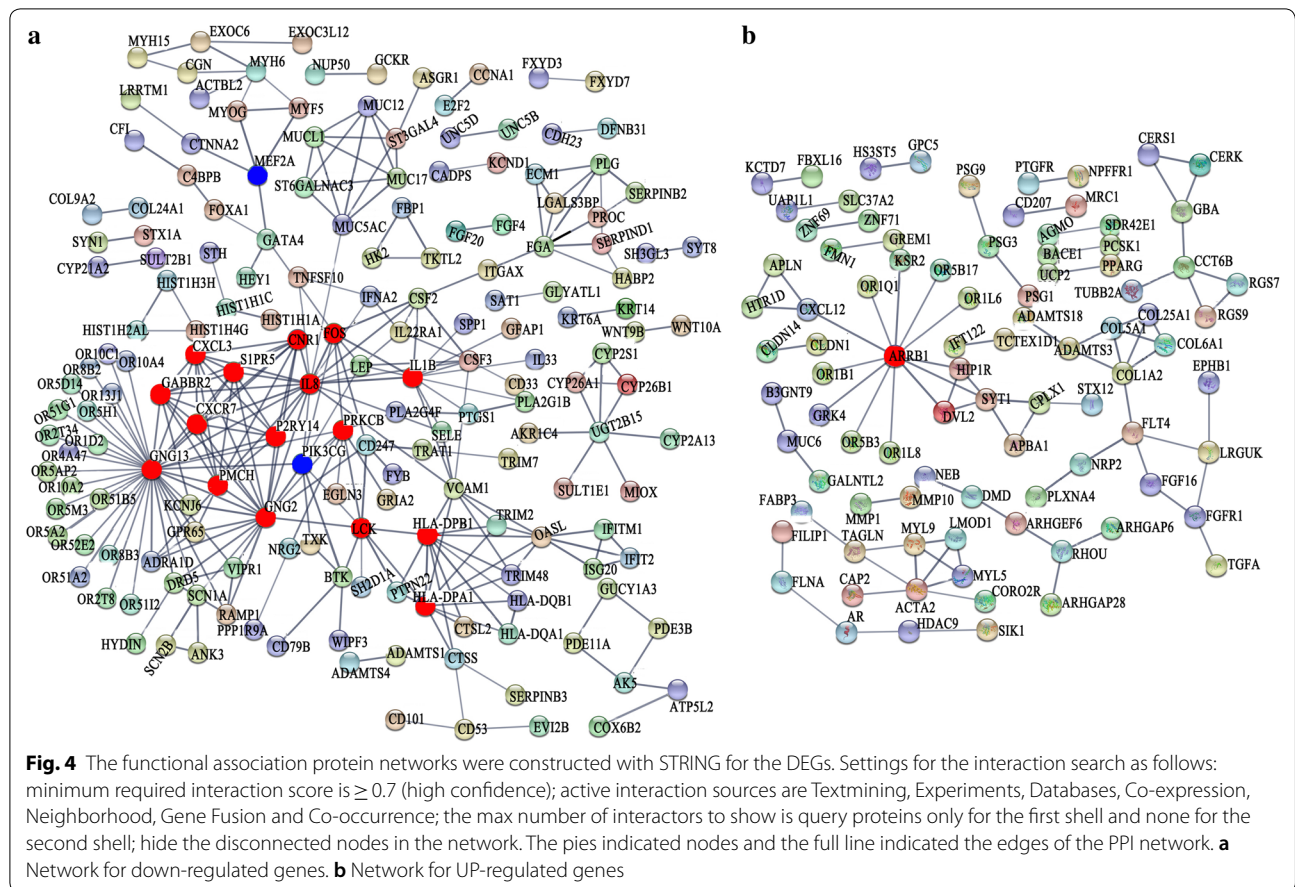
| Category          | Term                                 | Count (%)  | p-value | Fold enrichment |
|-------------------|--------------------------------------|------------|---------|-----------------|
| GAD_DISEASE_CLASS | Reproduction                         | 8 (13.33)  | 0.03294 | 2.51            |
| GAD_DISEASE_CLASS | Cardiovascular                       | 21 (35)    | 0.03316 | 1.48            |
| UP_KEYWORDS       | Secreted                             | 18 (30)    | 0.00002 | 3.14            |
| UP_KEYWORDS       | Disulfide bond                       | 21 (35)    | 0.00097 | 2.10            |
| UP_KEYWORDS       | Signal                               | 23 (38.33) | 0.00181 | 1.90            |
| UP_KEYWORDS       | Glycoprotein                         | 22 (36.67) | 0.01248 | 1.66            |
| UP_KEYWORDS       | Cell junction                        | 7 (11.67)  | 0.01257 | 3.56            |
| UP_KEYWORDS       | Cell membrane                        | 16 (26.67) | 0.03143 | 1.73            |
| UP_KEYWORDS       | Citrullination                       | 3 (5)      | 0.03161 | 10.61           |
| UP_KEYWORDS       | Actin-binding                        | 4 (6.67)   | 0.04397 | 5.01            |
| UP_KEYWORDS       | Thick filament                       | 2 (3.33)   | 0.04765 | 40.35           |
| UP_SEQ_FEATURE    | Signal peptide                       | 23 (38.33) | 0.00012 | 2.30            |
| UP_SEQ_FEATURE    | Disulfide bond                       | 19 (31.67) | 0.00132 | 2.18            |
| UP_SEQ_FEATURE    | Zinc finger region:B box-type        | 3 (5)      | 0.01710 | 14.75           |
| UP_SEQ_FEATURE    | Glycosylation site:N-linked (GlcNAc) | 20 (33.33) | 0.03095 | 1.58            |
| UP_SEQ_FEATURE    | Domain:B30.2/SPRY                    | 3 (5)      | 0.03312 | 10.34           |

**Table 2 The top 10 enriched biological process, cell component and molecular function GO terms for DEGs, which were sorted respectively by *p*-value in ascending**

| Category         | Term  | Count | <i>p</i> -value | Fold enrichment |
|------------------|---|-------|-----------------|-----------------|
| GOTERM_BP_DIRECT | GO:0007565 ~ female pregnancy   | 13    | 2.86E-04        | 3.534242        |
| GOTERM_BP_DIRECT | GO:0007267 ~ cell-cell signaling  | 24    | 3.61E-04        | 2.286233        |
| GOTERM_BP_DIRECT | GO:0008152 ~ metabolic process  | 18    | 5.93E-04        | 2.592425        |
| GOTERM_BP_DIRECT | GO:0007165 ~ signal transduction  | 69    | 0.002168        | 1.438003        |
| GOTERM_BP_DIRECT | GO:0001525 ~ angiogenesis   | 20    | 0.002302        | 2.170042        |
| GOTERM_BP_DIRECT | GO:0008277 ~ regulation of G-protein coupled receptor protein signaling pathway | 7     | 0.00493         | 4.342866        |
| GOTERM_BP_DIRECT | GO:0030168 ~ platelet activation  | 12    | 0.007956        | 2.524796        |
| GOTERM_BP_DIRECT | GO:0006687 ~ glycosphingolipid metabolic process                                | 7     | 0.009997        | 3.763817        |
| GOTERM_BP_DIRECT | GO:0071526 ~ semaphorin-plexin signaling pathway                                | 6     | 0.010737        | 4.399266        |
| GOTERM_BP_DIRECT | GO:0016266 ~ O-glycan processing  | 8     | 0.011451        | 3.226129        |
| GOTERM_CC_DIRECT | GO:0005615 ~ extracellular space  | 109   | 1.54E-11        | 1.95324         |
| GOTERM_CC_DIRECT | GO:0005576 ~ extracellular region   | 123   | 2.57E-11        | 1.844064        |
| GOTERM_CC_DIRECT | GO:0005886 ~ plasma membrane  | 214   | 1.33E-04        | 1.253453        |
| GOTERM_CC_DIRECT | GO:0005578 ~ proteinaceous extracellular matrix                                 | 25    | 3.33E-04        | 2.251656        |
| GOTERM_CC_DIRECT | GO:0016021 ~ integral component of membrane                                     | 251   | 0.001739        | 1.17346         |
| GOTERM_CC_DIRECT | GO:0005581 ~ collagen trimer  | 11    | 0.004686        | 2.886035        |
| GOTERM_CC_DIRECT | GO:0009986 ~ cell surface   | 36    | 0.00631         | 1.603245        |
| GOTERM_CC_DIRECT | GO:0005887 ~ integral component of plasma membrane                              | 76    | 0.015603        | 1.296444        |
| GOTERM_CC_DIRECT | GO:0002177 ~ manchette  | 3     | 0.015728        | 14.48265        |
| GOTERM_CC_DIRECT | GO:0005884 ~ actin filament   | 8     | 0.017555        | 2.9708          |
| GOTERM_MF_DIRECT | GO:0046934 ~ phosphatidylinositol-4,5-bisphosphate 3-kinase activity            | 10    | 8.46E-04        | 3.963234        |
| GOTERM_MF_DIRECT | GO:0005125 ~ cytokine activity  | 18    | 8.47E-04        | 2.513051        |
| GOTERM_MF_DIRECT | GO:0004867 ~ serine-type endopeptidase inhibitor activity                       | 12    | 0.001915        | 3.039842        |
| GOTERM_MF_DIRECT | GO:0008083 ~ growth factor activity   | 16    | 0.002531        | 2.426869        |
| GOTERM_MF_DIRECT | GO:0008201 ~ heparin binding  | 15    | 0.005735        | 2.30363         |
| GOTERM_MF_DIRECT | GO:0008401 ~ retinoic acid 4-hydroxylase activity                               | 3     | 0.009368        | 18.42904        |
| GOTERM_MF_DIRECT | GO:0005102 ~ receptor binding   | 25    | 0.009977        | 1.74023         |
| GOTERM_MF_DIRECT | GO:0005509 ~ calcium ion binding  | 42    | 0.017368        | 1.439367        |
| GOTERM_MF_DIRECT | GO:0032395 ~ MHC class II receptor activity                                     | 4     | 0.021097        | 6.552547        |
| GOTERM_MF_DIRECT | GO:0001665 ~ alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase activity | 3     | 0.022182        | 12.28603        |

**Table 3 The top 10 significant KEGG pathway enriched in the down-regulated DEGs**

| Category     | Term   | Count | <i>p</i> -value | Fold enrichment |
|--------------|--|-------|-----------------|-----------------|
| KEGG_PATHWAY | hsa05146:amoebiasis                                | 11    | 9.00E-04        | 3.585377        |
| KEGG_PATHWAY | hsa05164:influenza A                               | 14    | 0.001468        | 2.779885        |
| KEGG_PATHWAY | hsa04650:natural killer cell mediated cytotoxicity | 11    | 0.002616        | 3.115164        |
| KEGG_PATHWAY | hsa05323:rheumatoid arthritis                      | 9     | 0.003677        | 3.533523        |
| KEGG_PATHWAY | hsa05150:Staphylococcus aureus infection           | 7     | 0.004323        | 4.478704        |
| KEGG_PATHWAY | hsa04612:antigen processing and presentation       | 8     | 0.006025        | 3.636842        |
| KEGG_PATHWAY | hsa04610:complement and coagulation cascades       | 7     | 0.014078        | 3.505072        |
| KEGG_PATHWAY | hsa05143:African trypanosomiasis                   | 5     | 0.014207        | 5.234848        |
| KEGG_PATHWAY | hsa05332:graft-versus-host disease                 | 5     | 0.014207        | 5.234848        |
| KEGG_PATHWAY | hsa04740:olfactory transduction                    | 20    | 0.020697        | 1.73183         |



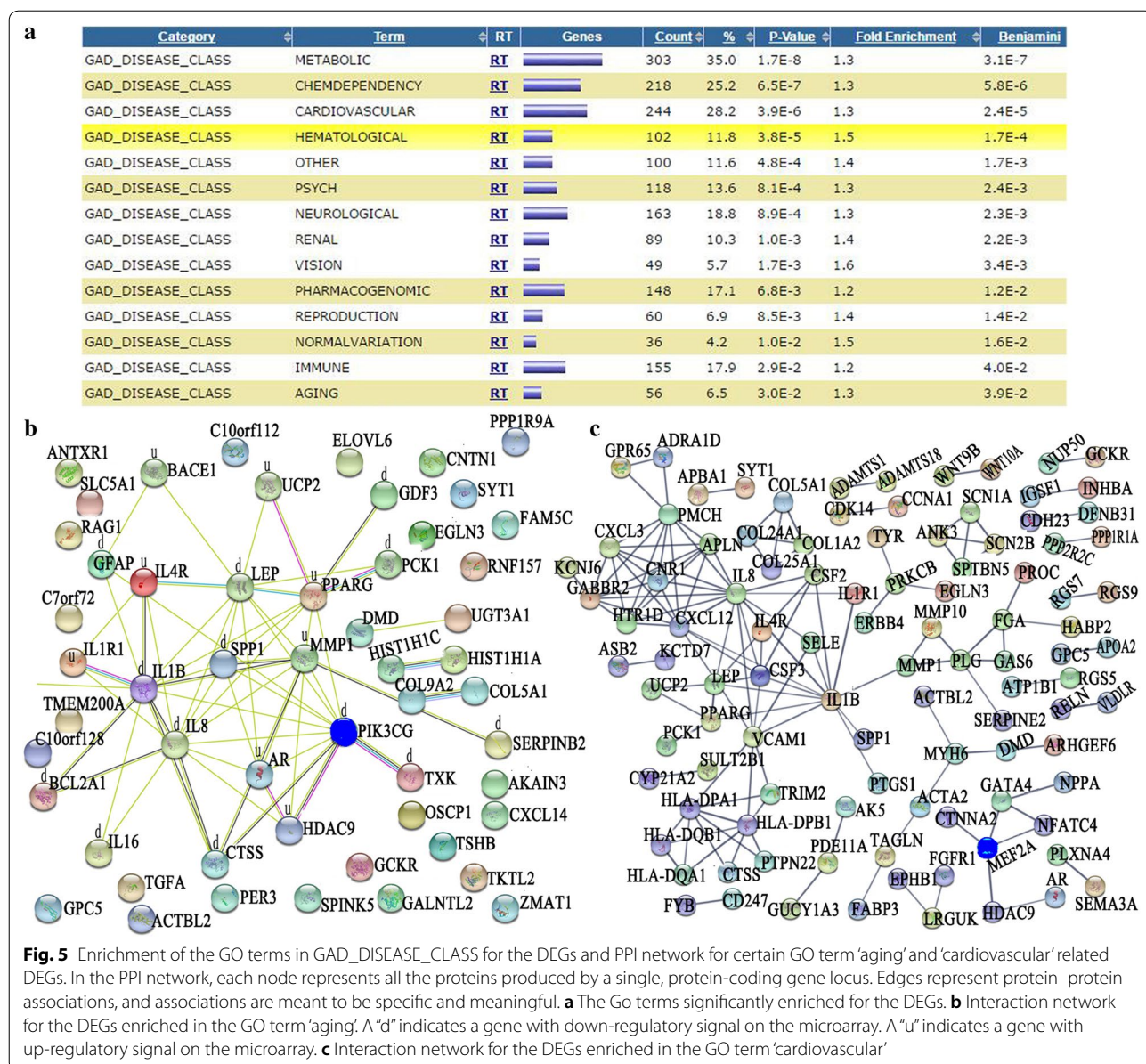
**Table 4** GO annotation for the down-regulated DEGs in the PPI network

| Pathway ID              | Pathway description   | Count in gene set | False discovery rate (FDR) |
|-------------------------|---|-------------------|----------------------------|
| Biological process (GO) |   |                   |                            |
| GO:0050793              | Regulation of developmental process                             | 84                | 0.000902                   |
| GO:0048387              | Negative regulation of retinoic acid receptor signaling pathway | 8                 | 0.00358                    |
| GO:0051239              | Regulation of multicellular organismal process                  | 88                | 0.00358                    |
| GO:0045595              | Regulation of cell differentiation                              | 60                | 0.00957                    |
| GO:0050995              | Negative regulation of lipid catabolic process                  | 6                 | 0.00957                    |
| Cellular component (GO) |   |                   |                            |
| GO:0005615              | Extracellular space   | 66                | 8.23e-07                   |
| GO:0005576              | Extracellular region  | 145               | 0.000952                   |
| GO:0071944              | Cell periphery  | 143               | 0.0129                     |
| GO:0005886              | Plasma membrane   | 139               | 0.0259                     |
| KEGG pathways           |   |                   |                            |
| 05146                   | Amoebiasis  | 10                | 0.0173                     |
| 05150                   | <i>Staphylococcus aureus</i> infection                          | 7                 | 0.0173                     |
| 05164                   | Influenza A   | 14                | 0.0173                     |
| 05323                   | Rheumatoid arthritis  | 9                 | 0.0173                     |
| 04740                   | Olfactory transduction  | 21                | 0.0413                     |

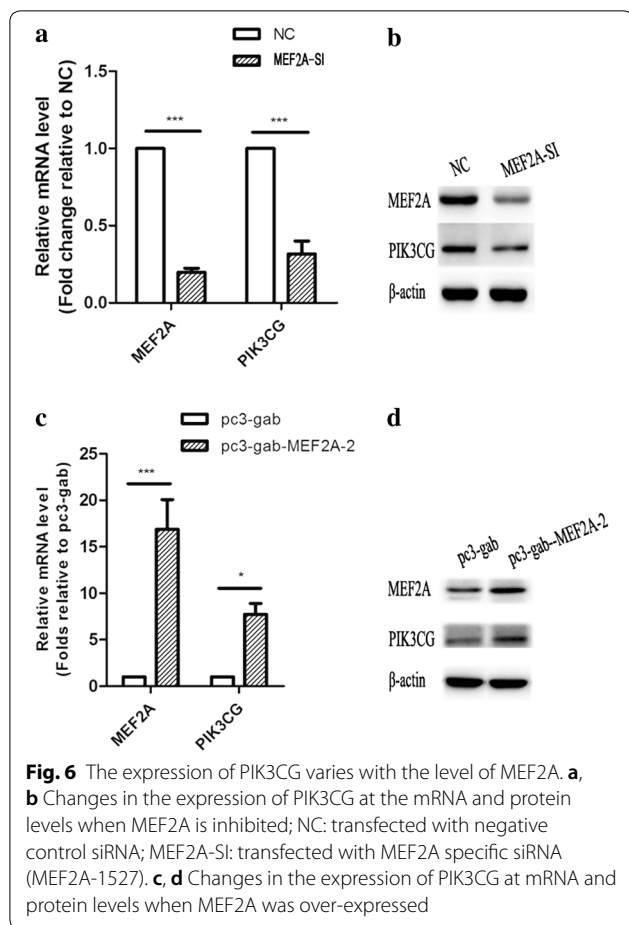
anti-atherosclerotic function in ApoE<sup>-/-</sup> mice [19], and functional deletion in MEF2A gene is co-segregated with premature CAD in several family lineages [7, 8], suggesting the protective role of MEF2A in vessels. The above findings imply that a decrease in MEF2A expression in vascular endothelial cells may result in vessel dysfunction. Therefore, it is important to understand the profile and the interaction network of the DEGs affected by knockdown of MEF2A in HCAEC.

In our study, the DEGs in response to MEF2A knockdown were significantly enriched in the GO terms associated with growth, proliferation, development,

survival, and inflammation. The down-regulated genes in MEF2A-knocked down HCAECs were involved in a complicated regulatory network. Among the hot nodes, PIK3CG, PRKCB, and IL8 have been extensively investigated. PIK3CG plays important roles in immunity, hypertension, longevity, and circulation [21, 22]. p110 $\gamma$ , the encoding protein of PIK3CG, is principally involved in the control of leukocyte chemotaxis and inflammatory reactions in the immune system, affects multiple aspects of adrenergic receptor signaling and cardiac function in cardiomyocytes [21], and participates in angiotensin II induced calcium influx and governing the vascular contractile response [22]. Thus, p110 $\gamma$  has been considered







as a promising target for drug design in the treatment of heart failure and hypertension, whereas the function of p110 $\gamma$  in vascular endothelial cells is unclear. In our study, the GO term analysis based on the GAD\_disease indicated that PIK3CG participated in the aging pathway and interacted with many inflammatory factors in the PPI network. In practice, mass evidences have indicated that PI3K pathway is involved in cellular senescence and proliferation [23–26]. In the present study, multiple experiments showed that the expression of PIK3CG varied with the expression level of MEF2A, suggesting that the expression of PIK3CG is regulated by MEF2A. The expression of PIK3CG was significantly down-regulated in MEF2A-knocked down HCAECs, suggesting that PIK3CG may be involved in the molecular mechanism of MEF2A silencing induced senescence. In addition, many inflammatory factors were overtly down-regulated simultaneously with PIK3CG, indicating that the down-regulation of inflammatory factors in response to MEF2A silencing might be mediated by PIK3CG.

PRKCB might promote the removal of LDLs from the blood by increasing the expression of LDLR and SR-A

and reducing foam cell formation, which might mitigate atherosclerosis. However, the characteristics of PRKCB that plays a pro-inflammatory role in endothelial cells and promotes VSMC proliferation and migration are associated with pro-atherosclerosis as observed in PRKCB knockout mice [27].

IL8 is another extensively studied gene that serves as a risk factor of cardiovascular disease [28]. The PPI network shows a large number of interactions between IL8 and other genes, suggesting that MEF2A-mediated inflammatory pathway might greatly depend on the participation of IL-8. Suzuki et al. [29] revealed that MEF2A may mediate vascular inflammation by stimulating MCP-1 expression in VSMCs and macrophage infiltration. MEF2A and MEF2D may function as activators or repressors of gene transcription in human macrophages [30]. In our study, many genes involved in macrophage infiltration were downregulated in MEF2A-knocked down HCAECs, suggesting the role of MEF2A in regulating inflammation. Furthermore, 8 of the top 10 KEGG pathways enriched by the down-regulated DEGs were involved in immune-related diseases, indicating that MEF2A participated in mediating the immune system.

Guanine nucleotide binding protein subunit gamma 2 and 13 (GNG2 and GNG13) play key roles in signal transduction pathways. In our study, they were significantly down-regulated in MEF2A-knocked down HCAECs. GNG13 is colocalized with alpha-gustducin in taste receptor cells, and antibodies against Ggamma13 block the denatonium-induced increase in inositol triphosphate (IP3) in taste tissue [31]. In our study, most olfactory receptors were distinctively down-regulated in MEF2A-knocked down HCAECs, and almost all of them interacted with GNG13 in the PPI network. These findings suggested that the expression of olfactory receptors might be regulated by MEF2A through GNG13 and PI3K.

DAVID GO functional annotation enrichment based on GAD revealed that the cardiovascular system was one of the most prominently enriched terms of the DEGs in response to MEF2A knockdown. More than 200 DEGs were related to the cardiovascular system and exhibited complicated interactions with one another. Many hot nodes in the PPI network are inflammatory factors, including IL8, IL1B, CXCL3, CXCL12, and HLA genes, which are associated with cardiovascular diseases [28, 32–34]. MEF2A functional deletion carriers were subjected to premature CAD [7, 8], strongly supporting the important roles of MEF2A in providing protection against cardiovascular disease. Our findings show that numerous genes in inflammatory pathways were down-regulated as MEF2A was silenced, indicating that

MEF2A may be an important regulator to mediate the expression of inflammatory factors, and that this role of MEF2A appears to be inconsistent with its protective effect against CAD. Alternative splicing of the pre-mRNA of MEF2A resulted in different isoforms which have distinct activity and are differentially distributed in various tissues [35]. Aberrant alternative splicing isoforms were observed to be associated with myotonic dystrophy and neuromuscular disorders [36]. More than 20 MEF2A isoforms have been submitted to NCBI database so far. Yet the functional diversity of the isoforms is unclear. Silencing of MEF2A by siRNA may alter the proportion of different splicing isoforms compared to in the normal physiological status of the cells. Therefore, when we attempt to explore the underlying molecular mechanism that MEF2A participate in a certain biological or pathological process, which MEF2A alternative splicing isoforms exert major effect on the process should be taken into account. We found that the functional role of MEF2A in vascular endothelial cells is inconsistent with its roles in VSMC reported by other studies, possibly due to differential splicing of MEF2A in the two kinds of cells.

Among the down-regulated DEGs, 61 genes were found with at least one potential MEF2A binding site in the proximal promoter. If extended the search region in the promoters of the DEGs, there would be more genes found with MEF2A binding sites. Therefore, many DEGs may be directly regulated by MEF2A via binding to their cis-elements in the promoters. TRIM7, SGK196, BCL2A1, NR5A2, ANK3 and PYHIM9 have MEF2A binding site in their proximal promoter, which were down-regulated in MEF2A-knocked down HCAEC via verifying by qPCR. These genes are respectively involved in metabolism, development, differentiation, apoptosis and survival [37, 38].

## Conclusion

MEF2A knockdown in HCAECs promotes cell senescence and remarkably alters mRNA profile, primarily related to signal transduction, proliferation, development, inflammation, and other processes, and may lead to cell dysfunction by promoting cellular senescence and inflammation or by inhibiting cell proliferation. PIK3CG has been shown to be associated with longevity, and its expression varies with the expression level of MEF2A in HCAEC, suggesting that PIK3CG may play an important role in cell senescence induced by MEF2A silencing.

## Materials and methods

### Cell culture

The primary HCAECs were purchased from Cell Applications and cultured in EGM-2 medium (Lonza, USA) supplemented with growth factors. The cells were further

grown at 37 °C in a humidified atmosphere with 5% (v/v) CO<sub>2</sub> and passaged at 1:3 when they reached approximately 85–95% confluency.

### Construction of MEF2A overexpression plasmid

The complete MEF2A coding region fragment was amplified using high fidelity PCR polymerase and cDNA template derived from HCAEC, and the amplified fragment was ligated into the mammalian expression vector pc3-gab at the sites *EcoRI* and *BglII*. Then, transformation and plasmid extraction were performed, and the inserted encoding sequence was verified by Sanger sequencing to be same to the MEF2A alternative splice variant XM\_005254915.2\_V\_X15.

### Transfection

siRNA was diluted to a 20 μM solution with RNase-free and sterile deionized water and stored below – 20 °C. HCAECs were seeded in a plate at a density of 7500 cells/cm<sup>2</sup> 18–24 h before transfection and cultured at 37 °C in a humidified atmosphere with 5% (v/v) CO<sub>2</sub>. Lipofectamine<sup>®</sup> RNAiMAX and Lipofectamine<sup>®</sup> 3000 reagent were respectively used to guide siRNA and plasmids into the cells, and the procedure was performed in accordance with the manufacturer's instructions. The next treatments were performed at 72 h post-transfection.

### Detection of cell senescence and proliferation

The cells were subjected to SA-β-Gal staining by using a β-galactosidase staining kit (Cell Signaling Technology, USA) in accordance with the manufacturer's instructions. The stained cells were examined with a high-resolution microscope at 400× magnification, and blue stain corresponded to senescent cells. Cell proliferation was detected with an EdU staining kit (Ribobio, China) in accordance with the manufacturer's instructions. The stained cells were imaged with a fluorescence inversion microscope system at 400× magnification, and purple stain indicated proliferating cells. The senescence rate and the proliferation rate were respectively determined by calculating the proportion of the senescent cells and the proliferating cells in the total number of cells in 15 visions per well. The results were expressed as the mean of triplicates ± standard error of the mean (SEM).

### RNA isolation

Total RNA of cells was isolated with Trizol reagent (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's protocol. RNA concentration was determined by measuring the absorbance at 260 nm with a NanoVue spectrophotometer (GE Healthcare, UK). RNA integrity was assessed through electrophoresis on agarose gel.

**Table 5** The primer sequences for real time quantitative PCR

| Serial no. | Genes    | Forward primer (5' to 3') | Reverse primer (5' to 3') | Tm (°C) |
|------------|----------|---------------------------|---------------------------|---------|
| 1          | MEF2A    | AGCAGCCCTCAGCTCTCTTG      | GGTAAAATCGGTTCGGACTTG     | 60      |
| 2          | PIK3CG   | TCCTCTTTGTGATGGAACTT      | TGTGTGATGACGAAGGGCT       | 60      |
| 3          | SERPIND1 | TGATTCTCAACTGCATCTACTTC   | CCTCTCTCATTAGCCG          | 60      |
| 4          | IL1B     | ACAGTGGCAATGAGGATG        | TGTAGTGGTGGTCGGAGA        | 60      |
| 5          | ENPP1    | ACTGCGAAAGTATGCTGA        | TTCTTGTTACGGATGAC         | 60      |
| 6          | ZAK      | GCGGTGGAGGAAGATTTG        | TGACTGAGGACCCTGAGTATTT    | 60      |
| 7          | FOS      | TACTACCACTCACCCGAAGA      | GAATGAAGTTGGCACTGGA       | 58      |
| 8          | MMP1     | AAACCCCAAAGCGTGTG         | GGGTAGAAGGGATTGTGC        | 60      |
| 9          | DQB1     | CTGGTGATGATGAAATGAC       | TTGCTCTGGCAGATTCA         | 60      |
| 10         | GUCY1A3  | GGAGCCGAGTCTATCTCA        | TGTGCTGTGCCAATGTTT        | 60      |
| 11         | MYL9     | AACGCCTTTGCCTGCTTC        | CATCTCGTCCACTTCTCATC      | 60      |
| 12         | GJA5     | GGCAAGGAAATAGTAGGG        | TTGGCAGTCTCATTAGGG        | 60      |
| 13         | CSF2     | CCTGTGCAACCCAGATTA        | TGGTACCAGCAGTCAAA         | 60      |
| 14         | TRIM     | GGGTGTCACATAATTGTTGT      | GCCCTAACTGGTATGTTACG      | 60      |
| 15         | MRPL45   | GAAATGTTGTCAGGGTGTG       | CCTTAAAGTCACATCGCTCA      | 60      |
| 16         | SGK196   | ACCTGGAAGAAACACTAAACC     | GGTCGTTGGAGTCGCACA        | 60      |
| 17         | BCL2A1   | CATCATAAAGTGGGAAGAA       | ATTATGAACTCCGCAACAAA      | 60      |
| 18         | NR5A2    | CGTGGAGGAAGGAATAAG        | TCAGGTCAGAGGGCATAG        | 60      |
| 19         | ANK3     | CCTATTATGTTGATTGTTATGG    | CTTGCTGGTGTCTCTAATCT      | 60      |
| 20         | PYHIN1   | AAGGAGAATGCCACAATATC      | TGTCTCTGGATGAAACTATGC     | 60      |

### Microarray analysis

Three independent groups of HCAECs transfected with MEF2A-specific siRNA (MEF2A-Homo-1527) were separately analyzed with an Agilent human mRNA array (AHMA), and three independent groups of HCAECs transfected with negative control siRNA were collected together into a tube. RNA was extracted for AHMA analysis. Three MEF2A-specific siRNA groups were indicated as 1527-1, 1527-2, and 1527-3, and the mixed control group was designated as NC. The AHMA was designed with eight identical arrays/slide (8 × 60 K format), and each array contained probes examining about 27,958 Entrez gene RNAs. The whole microarray analyses, including sample preparation, labeling, hybridization, scanning, and treatment of original data, were processed by CapitalBio Corporation (Beijing, China). DEGs were analyzed by using the DAVID v6.8, and a protein–protein interaction (PPI) network was searched using the STRING tool [39]. The DEGs containing a potential MEF2A binding site in their promoter were predicted with LASAGNA-Search 2.0 [40].

### Real-time fluorescence quantitative PCR (q-PCR)

Total RNA was reverse transcribed by using a ReverTra Ace qPCR RT kit (Toyobo, Japan), and q-PCR was performed with SYBR<sup>®</sup> Green mix (Takara, China) in accordance with the manufacturer's instructions.

Alteration in the gene expression relative to the control group was analyzed with a comparative CT method by using beta-actin for endogenous control and NC cell for calibration. The primers used in this study are shown in Table 5.

### Statistical analysis

Data were expressed as mean ± SEM with more than three independent experiments. The statistical significance of the comparison between groups was examined with Student's t test, and  $p < 0.05$  indicates significant differences.

### Additional files

**Additional file 1: Table S1.** The information of the predicted MEF2A binding sites on the proximal promoter of the DEGs.

**Additional file 2: Table S2.** The genes with more than twofold change in expression in the siRNA group compared to the negative control. Up, up-regulated in MEF2A specific siRNA group; down, down-regulated in MEF2A specific siRNA group.

**Additional file 3: Table S3.** Annotation for the differentially expressed genes.

## Abbreviations

MEF2A: myocyte enhancer factor 2A; HCAEC: human coronary artery endothelial cells; DEG: differentially expressed genes; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; CAD: coronary artery disease; SA- $\beta$ -Gal: senescence-associated  $\beta$ -galactosidase; VSMC: vascular smooth muscle cell; PAH: pulmonary arterial hypertension; LDL: low density lipoprotein cholesterol; LDLR: low density lipoprotein cholesterol receptor; PPI: protein–protein interaction; siRNA: small interference RNA; AHMA: Agilent human mRNA array; DAVID: Database for Annotation, Visualization, and Integrated Discovery.

## Authors' contributions

YX and BL conceptualized the study. LW, WJ and LP performed the experiment. YX, WL, AL and YZ helped analyze and interpreted the data. WO, BL and SL prepared the manuscript. All authors read and approved the final manuscript.

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## Competing interests

The authors declare that they have no competing interests.

## Availability of data and materials

The datasets generated and analysed during the current study are available in the Gene Expression Omnibus (GEO) repository with an accession number of GSE114114 [20].

## Consent for publication

Not applicable.

## Ethics approval and consent to participate

Not applicable.

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