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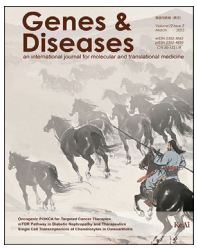

RAPID COMMUNICATION

# A novel nested gene *Aff3ir* participates in vascular remodeling by enhancing endothelial cell differentiation in mice

Gene overlap serves as a common strategy employed by bacteria and viruses to expand their genomic capacity. Over the past two decades, advances in omics studies have unveiled a prevalence of overlapped genes in eukaryotes, including mammals. However, few are reported to participate in the regulation of the cardiovascular system. In this study, we introduce a novel protein-coding nested gene, named *Aff3ir*, which contributes to endothelial maintenance by promoting the differentiation of vascular stem/progenitor cells (SPCs) into endothelial cells (ECs).

Recently, through the analysis of the microarray datasets from the laminar flow-induced mouse embryonic stem cell differentiation toward EC lineage,[1] we identified a novel gene entirely situated within intron 6 of the lymphoid nuclear protein, AF4/FMR2 member 3 (*Aff3*) gene,[2] which is henceforth designated as *Aff3* intron resident gene, *Aff3ir* (Fig. 1A). Comprising 7 exons, *Aff3ir* exhibits two transcript variants, distinguished by the inclusion/excision of exon 4 (Genbank: MH282850.1 for transcript variant 1 and MH282851.1 for transcript variant 2, originally designated as *Laf4ir*). *Aff3ir* and *Aff3* utilize opposite strands for transcription. To prevent the formation of double-stranded RNA between their precursor RNAs and the ensuing RNA decay, *Aff3ir* and *Aff3* may temporally undergo differential transcription, or there might be intron bypass during *Aff3* transcription. This distinct transcriptional pattern was evident in their response to shear stress, where *Aff3ir*, but not *Aff3*, exhibited up-regulation under laminar flow conditions, accompanied by a concurrent increase in EC markers (Fig. S1A). A more comprehensive investigation into the transcriptional regulation of *Aff3* and *Aff3ir* promises to offer new insights into the transcriptional dynamics of genes harboring large introns.

To elucidate the functional role of *Aff3ir*, we constructed a *pShuttle2-FLAG-Aff3ir-tv2* plasmid, seamlessly incorporating the entire *Aff3ir-tv2* cDNA sequence with a strategically positioned FLAG sequence downstream of the AUG codon within ORF2 (open reading frame 2) (Fig. S1B, upper segment). Immunofluorescence staining of SPCs, originating from mouse embryonic stem cells that had undergone 3-days spontaneous differentiation, transfected with *pShuttle2-FLAG-Aff3ir-tv2* revealed a robust signal for the FLAG epitope, providing compelling evidence of the translational potential of ORF2 primarily within cytosolic SPCs (Fig. S1B, lower segment). Proteomic analyses confirmed the translation of both ORF1 (open reading frame 1) and ORF2 in SPCs (Fig. S1C, with identified peptide fragment sequences underlined and a representative spectrum included). Notably, *Aff3ir* transcript variant 2 was the predominant one, generating a longer AFF3IR-ORF2 protein by removing the stop codon in exon 4. Specific antibodies against AFF3IR-ORF1 and AFF3IR-ORF2 were subsequently raised utilizing peptides QRNRRPWSVKITSDC and ADHRSTPQAGKVNRC, respectively. Western blot confirmed the translation of both in mouse aortic adventitia-derived primary SPCs,[3] with only AFF3IR-ORF2 responding to shear stress, particularly under laminar flow (Fig. 1B; Fig. S2A).

Mice tissue collection and experimental vascular remodeling models were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals under the UK Home Office Project License PPL*70/7266*. Differential expression of AFF3IR-ORF1 and AFF3IR-ORF2 was observed during embryonic development (Fig. S2B) and in adult tissues (Fig. 1C). A slight molecular weight difference in AFF3IR-ORF2 among tissues suggested varying post-translational modifications. Notably, AFF3IR-ORF2, predominantly expressed in the aorta

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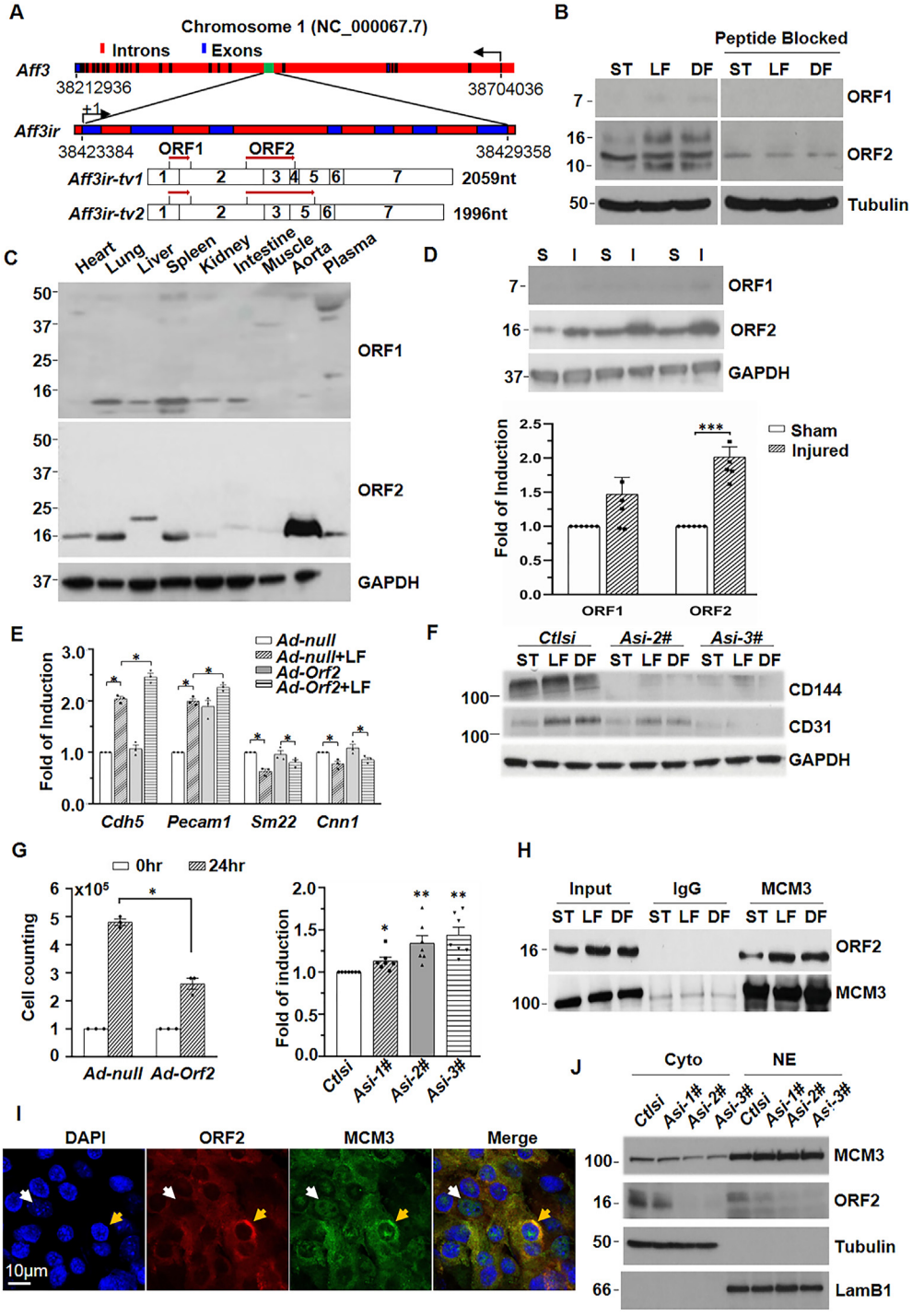


**Figure 1** The discovery of the novel gene *Aff3ir* and its involvement in vascular remodeling. **(A)** A schematic illustration of the *Aff3ir* gene structure. *Aff3ir* (green box) is in intron 6 of *Aff3*, comprising of 7 exons (blue box). The number shows the nucleotide position in chromosome 1. +1/arrow indicates the transcription initiation site and direction. Red arrows indicate the predicted short open reading frames (ORFs). **(B)** The stem/progenitor cells (SPCs) were subjected to laminar flow (LF) or disturbed flow (DF) in the dish-shaking system or kept at static condition (ST) for 24 h, followed by Western blot (WB) analysis with anti-AFF3IR-ORFs or blocking peptide. **(C)** WB analysis of AFF3IR-ORFs in tissues isolated from 10-week-old mice with GAPDH included as loading control. **(D)** The Hindlimb ischemia model was introduced into wild-type mice. The skeletal muscles were collected from the uninjured (S) or injured legs (I), followed by WB. $n = 5$. **(E)** SPCs were infected with *Ad-Orf2* at 10 multiplicity of infection (MOI) for 24 h and then subjected to LF or kept at ST for 24 h, followed by quantitative reverse transcription-PCR analysis of relative genes with *Gapdh* as internal control. The fold of induction was defined as the ratio of the target gene to *Gapdh* compared with the *Ad-null* group.

(Fig. 1C), was restricted to the EC layer, as confirmed by immunofluorescence staining (Fig. S2C). Intriguingly, no expression of AFF3IR-ORF2 was observed in the intact femoral artery (Fig. S2D), highlighting potential differences in flow patterns, forces, and the heterogeneous origins of ECs along the artery tree. Endothelium injury induced AFF3IR-ORF2 expression in the vessel wall (Fig. S2D) and bone marrow (Fig. S2E). Furthermore, femoral artery ligation-induced ischemia increased AFF3IR-ORF2 expression (Fig. 1D), emphasizing its role as the principal orchestrator in governing the intricate process of vascular injury repair via resident or circulating SPC activation.

As previously elucidated, *Aff3ir* mRNA and AFF3IR-ORF2 protein expression were up-regulated in response to laminar flow, concomitant with increased key EC markers. Notably, the presence of AFF3IR-ORF2-positive cells increased following vascular injury, prompting a thorough exploration of *Aff3ir*'s potential involvement in EC differentiation. As shown in Figure 1E, *Ad-Orf2* gene overexpression augmented laminar flow-induced mRNA levels of essential EC markers (*Cdh5* and *Pecam1*) and concurrently down-regulated smooth muscle cell marker genes (*Sm22* and *Cnn1*) in SPCs. This enhancement effect extended beyond laminar flow conditions, demonstrated in vascular endothelial growth factor-induced EC differentiation from SPCs (Fig. S3A). To delve deeper into the functional implications, we designed three siRNA fragments targeting distinct *Aff3ir* mRNA regions, revealing varying efficacies in knocking down AFF3IR-ORF2 protein (Fig. S3B). As expected, *Aff3ir* knockdown induced a significant reduction in baseline expression of EC marker proteins, CD31 and CD144, within SPCs. Furthermore, these siRNAs completely abrogated laminar flow-induced up-regulation of CD31 and CD144 expression (Fig. 1F), strongly implicating the active participation of AFF3IR-ORF2 in EC differentiation, particularly in response to stress-induced processes.

In the context of cell differentiation, the regulation of cell proliferation emerges as a critical imperative. As expected, overexpression of AFF3IR-ORF2 distinctly suppressed proliferation, while *Aff3ir* knockdown resulted in enhanced proliferation in differentiated SPCs (Fig. 1G). To unravel the mechanistic underpinnings of how AFF3IR-ORF2 orchestrates stem cell cycle arrest, we conducted an immunoprecipitation coupled with proteomics analysis (Spreadsheet S1). Strikingly, minichromosomal maintenance complex component 3 (MCM3), a key regulator of DNA replication initiation,[4] emerged among the uniquely identified proteins in laminar flow-treated samples. Immunoprecipitation plus Western blot analysis confirmed the physical interaction between MCM3 and AFF3IR-ORF2 in SPCs, notably potentiated under laminar flow conditions (Fig. 1H). AFF3IR-ORF2 predominantly localized within the cytosol fraction (Fig. S3C), retaining MCM3 in the cytosol, as revealed by immunofluorescence staining (Fig. 1I) upon its overexpression. In contrast, *Aff3ir* knockdown resulted in a dose-dependent reduction of MCM3 retention within the cytosol in SPCs (Fig. 1J; Fig. S3D).

Our previous studies showed that laminar flow up-regulates $p21^{waf1}$ via histone deacetylase-mediated p53 deacetylation, inducing cell cycle arrest and differentiation.[1,5] Importantly, overexpression of AFF3IR-ORF2 increased p53 and $p21^{waf1}$ expression (Fig. S3E). These collective findings underscore that AFF3IR-ORF2 exerts a negative modulatory effect on cell proliferation by retaining MCM3 within the cytosol and up-regulating the p53/$p21^{waf1}$ pathway. This regulatory milieu enhances the capacity of stem cells to embark on the journey of differentiation toward endothelial cells. In conclusion, our discoveries unveil the novel protein-coding nested gene *Aff3ir* as a participant in endothelial cell differentiation, providing fresh insights into the regulation of vascular endothelial integrity.

$n = 3$. **(F)** SPCs were transfected with siRNAs indicated for 72 h and then subjected to LF, DF, or kept at ST for 24 h, followed by WB analysis. **(G)** SPCs were infected with *Ad-Orf2* at 10 MOI for 24 h and then sub-cultured for another 24 h, followed by cell number counting (left, $n = 3$) or transfected with *Aff3ir* siRNA indicated for 48 h and then sub-cultured for another 48 h, followed by proliferation assessment with MTT assay (right, fold induction was defined as the ratio of A570 nm of *Asi* to that of *Ctlsi* with that of *Ctlsi* set as 1.0; $n = 7$). **(H)** SPCs were subjected to LF or DF or kept at ST for 24 h, followed by immunoprecipitation plus WB analysis. **(I)** Mouse C166 endothelial cells that did not express endogenous AFF3IR-ORF2 were infected with *Ad-Orf2* at 10 MOI for 24 h, followed by immunofluorescence staining. White or orange arrows indicated ORF2-negative or representative positive cells, respectively. **(J)** SPCs were transfected with *Aff3ir* siRNAs followed by cellular fractionation plus WB. Tubulin and Lamin B1 were included as loading controls for cytosolic and nuclear fractions, respectively. Cyto, cytosol; NE, nuclear extract. Data presented were representative images or mean ± standard error of the mean using one-way or two-way ANOVA with GraphPad Prism 8 multiple comparison tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

## Conflict of interests

The authors declared no conflict of interests.

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## Author contributions

Y.Z., M.E., and Y.L. contributed to experimental design, performance, data analysis, and manuscript writing. L.H., A.A., X.Y., S.L., X.B., and Y.T. contributed to experimental performance. Y.G. and A.M. contributed to data analysis; T.C. and A.M.S. contributed to manuscript writing. T.L., M.Z., and L.Z. contributed to experimental design, data analysis, and manuscript writing. All authors were involved in critical evaluation and intellectual contribution to the manuscript.

## Data availability

All data are included in the Supplementary Information or available from the corresponding authors upon reasonable request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2024.101339.

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Yue Zhao [a,b,c,1], Mazdak Ehteramyan [b,1], Yi Li [b,1], Xuefeng Bai [b], Lei Huang [a,b], Yingtang Gao [d,e], Angshumonik Angbohang [b], Xiaoping Yang [b], Steven Lynham [b], Andriana Margariti [f], Ajay M. Shah [b], Yaling Tao [g], Ting Cai [g], Tong Li [a,c,d,e,***], Min Zhang [b,**], Lingfang Zeng [b,*]

[a] *Department of Heart Center, Nankai University Affiliated Third Center Hospital, Tianjin 300170, China*
[b] *School of Cardiovascular and Metabolic Medicine and Sciences, King's College London British Heart Foundation Centre of Excellence, Faculty of Life Science and Medicine, King's College London, London SE5 9NU, United Kingdom*
[c] *School of Medicine, Nankai University, Tianjin 300071, China*
[d] *Tianjin Key Laboratory of Extracorporeal Life Support for Critical Diseases, Nankai University Affiliated Third Center Hospital, Tianjin 300170, China*
[e] *Tianjin Institute of Hepatobiliary Disease, Nankai University Affiliated Third Centre Hospital, Tianjin 300170, China*
[f] *Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast BT9 7BL, United Kingdom*
[g] *Ningbo Institute of Life and Health Industry, University of Chinese Academy of Sciences, Ningbo, Zhejiang 315010, China*

*Corresponding author.

**Corresponding author.

***Corresponding author. Tianjin Key Laboratory of Extracorporeal Life Support for Critical Diseases, Nankai University Affiliated Third Center Hospital, Tianjin 300170, China.

*E-mail addresses:* litong3zx@sina.com (T. Li), min.zhang@kcl.ac.uk (M. Zhang), lingfang.zeng@kcl.ac.uk (L. Zeng)

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[1] These authors contributed equally to this work.