Dear Editor,

Hypoxic microenvironment is clinically associated with metastasis and poor prognosis of numerous cancers. Previous studies have shown that many protein-coding genes and microRNAs are regulated upon hypoxia and involved in the progression of cancer. However, the roles of lncRNAs in the hypoxia-responsive gene networks and how lncRNA-related signaling network regulates hypoxia induced tumor metastasis is still not clear.

To identify potential key lncRNAs associated with HCC, lncRNA sequencing analysis was performed in 61 paired HCC tissues and corresponding para-tumor tissues. Among the differentially expressed lncRNAs (Supplementary Fig. S1), FAM99A was found to be downregulated by more than sevenfolds (Supplementary Fig. S2). RT-qPCR validated the downregulation of FAM99A in another cohort of 103 HCC tissues (Fig. 1a). In addition, the downregulation of FAM99A was also observed in the TCGA dataset (Supplementary Fig. S3). Further clinical pathology data analysis shows that the decreased expression of FAM99A significantly correlated with tumor capsule, differentiation, and recurrence (Supplementary Table S1) and poor prognosis (Fig. 1b). Univariate and Multivariate Cox regression analysis demonstrates that FAM99A low expression served as an independent prognostic factor for overall survival of HCC patients (Supplementary Table S2).

The next cellular phenotype studies show that FAM99A overexpression inhibited migration and invasion of SK-Hep-1 cells (Fig. 1c and Supplementary Fig. S6), while FAM99A knockdown promoted migration and invasion of Hep3B cells (Supplementary Figs. S5, S6), both in transwell and wound-healing assay. Whether FAM99A can regulate EMT in HCC cells was further investigated. As shown in Fig. 1d, FAM99A increased the expression of epithelial markers E-cadherin and β-catenin, but decreased the E-cadherin repressor snail and slug as well as mesenchymal markers N-cadherin and Vimentin in SK-Hep-1 cells. Conversely, FAM99A knockdown in Hep3B cells induced a reverse trend (Fig. 1d).

The in vivo tail vein injection mouse model shows that FAM99A dramatically inhibited HCC lung metastasis of both SK-Hep-1 and SMMC-7721 cells in mouse model (Fig. 1e and Supplementary Fig. S7). Furthermore, FAM99A over-expression significantly prolonged the survival of tumor bearing mice (Supplementary Fig. S8).

To explore the molecular mechanisms by which FAM99A exerted its functions in HCC, bioinformatics analysis was carried out by using TargetScan, miRanda and RNAhybrid, we found that miR-92a which may promote tumor metastasis via E-cadherin1 was a potential target of FAM99A (Fig. 1f). Luciferase reporter assay shows that transfection with miR-92a significantly inhibited the luciferase activity in 293T cells, while miR-92a mimic failed to regulate the luciferase activity when the binding site was mutated (Fig. 1f), which indicated that miR-92a directly bond to FAM99A. Moreover, FAM99A over-expression significantly decreased the expression of miR-92a (Supplementary Fig. S9). Furthermore, we also found the significantly increased miR-92a level in HCC tissues comparing to adjacent para-tumor tissues (Supplementary Fig. S10). Besides, qRT-PCR assay shows that there was a negative correlation between the expression of FAM99A and miR-92a in HCC tissues (n = 53) (Supplementary Fig. S11).

Moreover, the effect of FAM99A on HCC cells migration and invasion was rescued by co-transfection of miR-92a (Supplementary Figs. S12 and S13). Meanwhile, the change of EMT-related protein expression induced by FAM99A was also abrogated by miR-92a (Fig. 1g).

Recent studies have shown that aberrant expression of some lncRNAs was attributed to hypoxic microenvironment of cancers,2,3 we wonder if the decreased expression of FAM99A in HCC was regulated by hypoxia. As shown in Figs. S14 and S15, both hypoxia and CoCl2 treatment decreased the expression of FAM99A, and the hypoxia induced FAM99A downregulation could be inhibited by HIF-1 inhibitor 2-ME2 (Fig. 1h), indicating that hypoxia reduced FAM99A expression was in a HIF-1α dependent manner. To understand the underlying mechanism of FAM99A down-regulation under hypoxia, we surveyed the promoter region of the FAM99A and identified 3 hypoxia response elements (HREs) (Fig. 1i). The CHIP assay only shows enrichment of the fragment containing HRE3 (−2712 to −2436 bp) in HIF-1α immunoprecipitated chromatin (Fig. 1i). More importantly, hypoxia could dramatically increase the binding of HIF-1α to FAM99A promoter (Supplementary Fig. S16). The luciferase analysis confirms that hypoxia significantly inhibited full-length and HRE3 of FAM99A promoter activity while without affect the HRE1 or HRE2 activity of FAM99A promoter in 293T cells (Fig. 1j). The TCGA data analysis also shows that there was a significant negative correlation between FAM99A and HIF-1α expression in HCC (Supplementary Fig. S17). Taken together, these data suggest that FAM99A is transcriptionally inhibited by HIF-1α during hypoxia.

HDAC1 interacted with HIF-1α to downregulate the expression of HIF-1α downstream target genes.4 Therefore, we hypothesized that hypoxia induced FAM99A downregulation was depending on HDAC1 and its mediated histone deacetylation. First, we found that HDAC1 but not HDAC3 negatively regulated FAM99A expression (Supplementary Figs. S18 and S19). Moreover, the HDAC inhibitor TSA increased the expression of FAM99A in a dose dependent manner (Supplementary Fig. S20), and the suppression of FAM99A by hypoxia was impaired at the presence of TSA (Fig. 1k).

Received: 8 April 2020 Revised: 8 May 2020 Accepted: 13 June 2020 Published online: 07 July 2020
The data from TCGA further confirmed a significant negative correlation between HDAC1 and FAM99A expression while no correlation between HDAC3 and FAM99A in HCC tissues (Supplementary Fig. S22). ChIP assay also showed a significant interaction between HDAC1 and FAM99A promoter (Supplementary Fig. S23); more importantly, hypoxia promoted the HDAC1 binding to FAM99A promoter region (Supplementary Fig. S23).

Next, we further investigate whether HDAC1 mediated histone deacetylation is involved in the negative regulation of FAM99A by...
Hep3B cells with FAM99A knockdown, respectively. e Representative images and quantification data of metastatic nodules in the lung tissues of mice. Stable cell lines of FAM99A overexpression or control were injected by tail vein in B-NDG mice. Upper left: macroscopic and fluorescent images. Upper right: quantification of lung metastatic nodules. Lower: representative images of lung metastatic nodules stained with H&E (magnification, ×200; scale bar, 20 μm). f Up: prediction of miR-92a binding sites in FAM99A (FAM99A-WT) and the design of FAM99A mutation sequence (FAM99A-Mut). down: luciferase reporter assays were performed in 293T cells co-transfected with the miR-92a mimic and FAM99A-WT or FAM99A-Mut reporter plasmid. g Western blotting analysis of EMT-associated markers in SK-Hep-1 cells stably transfected with FAM99A or co-transfected with miR-92a and FAM99A. h qRT-PCR analyzing the expression of FAM99A under treatment with or without hypoxia inhibitor (2-ME) under hypoxic conditions. i Up: The schematic illustration of HRE regions in FAM99A promoter and the primers of ChIP quantitative PCR (ChIP-qPCR). Down: ChIP-qPCR assay was performed to identify the binding region between HREs of FAM99A promoter and HIF-1α. j Up: The schematic illustration of luciferase reporter gene plasmid. Down: Luciferase reporter gene assays were performed to compare the luciferase activity of HREs or full length of FAM99A promoter under normal oxygen or hypoxic conditions; PC, positive control. k qRT-PCR analysis of FAM99A expression in SK-Hep-1 cells cultured with or without different concentrations of TSA for 24 h under normoxic or hypoxic conditions. I ChIP-qPCR analysis was conducted on the HRE3 using anti-acetyl-histone H3 and H4 under normoxic or hypoxic conditions. m Representative images of IHC staining in human HCC tissues (magnification, ×200; scale bar, 20 μm). n Schematic overview of HIF-1α and HDAC1 mediated regulation of FAM99A-miR92a signaling contributes to hypoxia induced hepatocellular carcinoma metastasis. Error bars symbolized standard deviation acquired from three independent experiments and all the data were shown as mean ± SD, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

ACKNOWLEDGEMENTS
This work was supported by the National Natural Science Foundation of China (81672376, 81972714, and 81602102), the State Key Project on Infectious Diseases of China (2018ZX1073204), Joint Funds of Fujian Provincial Health and Education Research (2019-WJ-19), the Startup Fund for scientific research, Fujian Medical University (2018QH1201, 2018QH1199), the Scientific Foundation of Fujian Province (2019D003).

ADDITIONAL INFORMATION
The online version of this article (https://doi.org/10.1038/s41392-020-00223-6) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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