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RAPID COMMUNICATION

The AKT1-mTOR signaling cascade is crucial for SOX3 to promote hepatocarcinogenesis

Sex-determining region Y-box protein 3 (SOX3) is a member of the SoxB1 transcription factors subfamily including SOX1 and SOX2, first identified based on homology to the high mobility box.[1] The oncogenic potential of the high mobility group box protein SOX3 has been widely validated. However, there have been inconsistent reports of SOX3 expression in hepatocellular carcinoma (HCC). For example, Zhang et al showed that SOX3 mRNA levels were not significantly different between the tumor and its corresponding adjacent normal tissues.[2] Contrarily, a noteworthy contrast was revealed by Li and colleagues,[3] who reported an evident up-regulation of SOX3 expression in 188 HCC samples from the TCGA database, compared with 50 normal liver tissues.[3] In contrast to these findings, Yang et al offered compelling evidence indicating the association of SOX3 with tumor progression and unfavorable prognosis in HCC.[4] However, the specific role and mechanism of SOX3 in these reports had not been investigated.

To determine whether SOX3 expression was up- or down-regulated in HCC, we analyzed the expression of SOX3 using TCGA database and found that SOX3 expression for 371 HCC and 50 normal liver samples, in most cases, was very low. However, SOX3 expression was significantly elevated in some HCC samples (Fig. S1A), suggesting that SOX3 may be up-regulated in partial but not all HCC patients. Next, analysis of patient samples showed that SOX3 mRNA and protein levels were significantly up-regulated in some HCC tissues compared with paracancerous tissues (Fig. S1B–D). Furthermore, immunohistochemistry analyses of 90 patients in tissue microarrays showed that SOX3 protein level was not obviously different between most HCC tissues and paracancerous tissues, while 30% of patients had higher SOX3 protein levels in HCC tissues compared with the normal tissues, like Figure S1E. Thus, SOX3 is up-regulated in some but not all HCC tissues, which explains the variable reports on SOX3 expression in HCC.

To determine whether SOX3 was functionally involved in HCC, we first checked SOX3 expression in various HCC cells. The results showed the expression of SOX3 was low in HepG2, but higher in Huh7 (Fig. S2A, B). Thus, we used HepG2 for overexpression and Huh7 for knockdown studies, respectively (Fig. S2C). CCK8, colony formation, and EdU proliferation assays confirmed that SOX3 could promote the proliferation of HCC cells (Fig. 1A–C). Transwell assays showed that SOX3 could promote HCC cell migration and invasion (Fig. 1D). Next, *in vivo* experiment identified that SOX3-overexpressed cells formed significantly larger tumors compared with the control (Fig. 1E). In addition, tumors in the overexpressing SOX3 group possessed higher levels of the proliferation marker Ki67 compared with the control group (Fig. 1F). In contrast, SOX3 knockdown tumor cells formed smaller tumors compared with the control group (Fig. S3). Furthermore, hematoxylin and eosin staining showed that SOX3 enhanced lung metastasis of tumor cells (Fig. 1G).

We next investigated the molecular mechanism by which SOX3 promoted HCC cell proliferation and metastasis. RNA sequencing analysis identified 1342 differentially expressed genes (DEGs) ($p < 0.05$), with 549 DEGs up-regulated and 793 down-regulated. Among them, 262 genes were changed by over two folds (47 up-regulated and 215 down-regulated genes) (Fig. S4A, C). Venn diagram analyses confirmed reproducibility among the biological triplicates (Fig. S4B). KEGG pathway analysis revealed a number of biological pathways, including phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, enriched among the DEGs due to overexpression of SOX3 (Fig. S4D). The heat map analysis results showed that among the DEGs with increased expression due to SOX3 expression, many, including AKT1, MAP2K3 (mitogen-activated protein kinase kinase 3), and STAT2 (signal transducer and activator of transcription 2), were associated with the AKT signaling pathway (Fig. 1H). Furthermore, independent quantitative PCR analysis of a number of genes in the ATK pathway, such as AKT1, were indeed expressed at significantly higher levels in SOX3 transfected-HepG2 cells than in vector transfected-HepG2 cells (Fig. S4E).

To determine whether SOX3 induced tumorigenesis via AKT1, we first analyzed the mRNA and protein levels of AKT1 in cells with varying levels of SOX3 expression. The results showed that SOX3 could indeed promote the expression of AKT1 (Fig. S5A, B). SOX family proteins are known to be a

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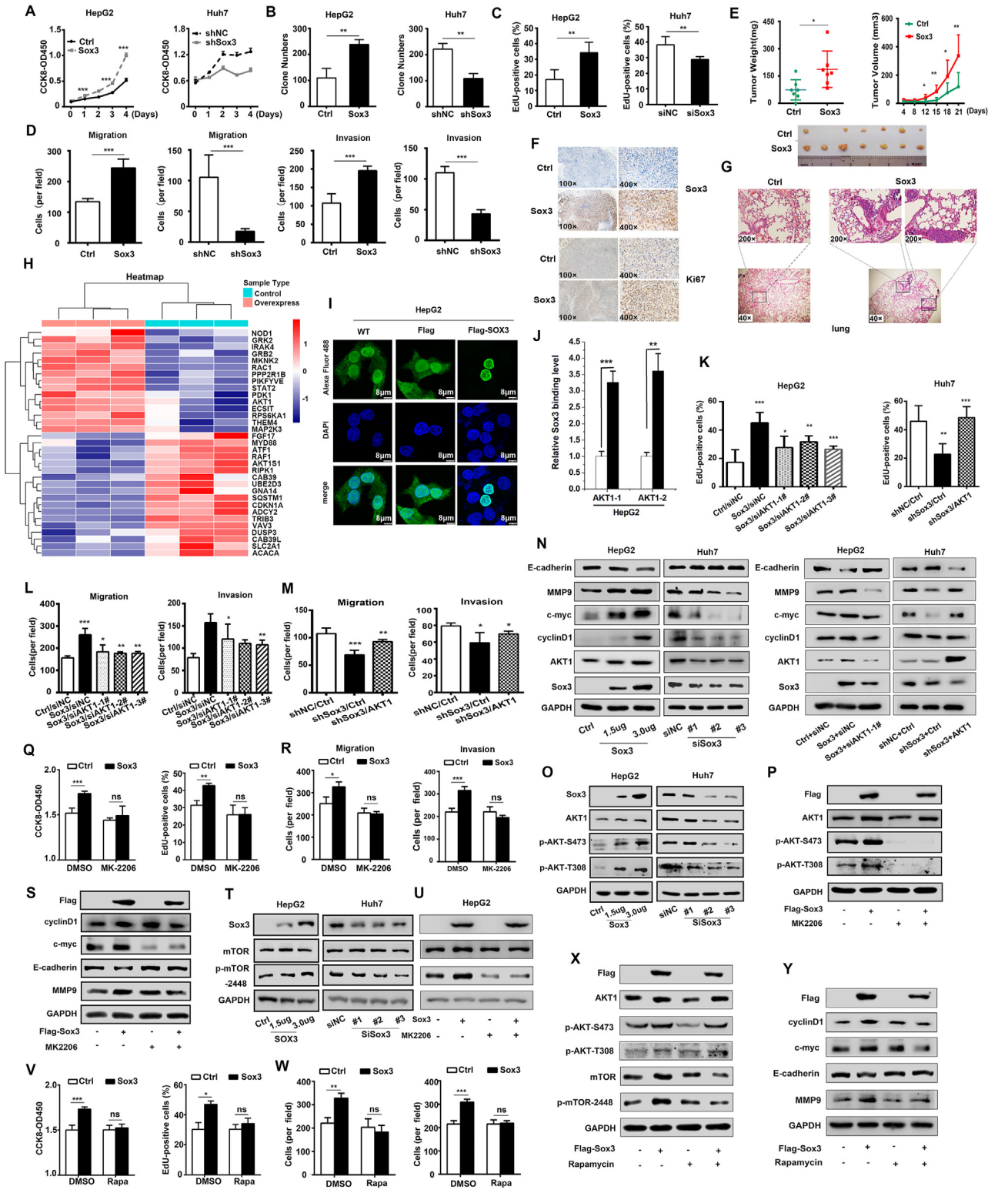

Figure 1 SOX3 activate AKT1-mTOR signaling cascade to promote hepatocarcinogenesis. **(A)** Growth rates of HepG2 and Huh7 cells were determined with CCK8 assay at 0, 24, 48, 72 h post-transfection with Sox3-overexpressing or control vector. **(B)** Number of colonies formed by HepG2 cells transfected with control or SOX3-overexpressing vector (left panel) or by Huh7 cells transfected with control or SOX3-knockdown shRNA (right panel). **(C)** EdU staining (green) was used to evaluate cell proliferation in different transfected cells, HepG2-ctrl, HepG2-SOX3, Huh7-siNC, and Huh7-siSOX3. **(D)** Transwell assay was performed to compare cell migration and invasion between HepG2-ctrl and HepG2- SOX3, or between Huh7-shNC and Huh7-shSOX3 cells. 50 μg matrigel was used to cover the bottom of transwell insert before seeding cells for invasion assay compared with migration assay. **(E)** Representative images of xenograft tumors 22 days after HepG2-Ctrl/SOX3-GFP cells was subcutaneously injected into six-week-old male

class of transcription factors that regulate intracellular gene expression. Our results also showed that SOX3 was more distributed in the nucleus (Fig. 1I; Fig. S5C—E). Further, Chromatin immunoprecipitation experiment confirmed that SOX3 could directly bind to the promoter of AKT1 (Fig. 1J) to regulate AKT1 expression. Subsequently, we found that the changes due to overexpression of SOX3, such as increased cell proliferation, DNA synthesis, and migration and invasion abilities in HCC cells, were all reversed following AKT1 knockdown (Fig. 1K, L; Fig. S5F left panel). On the other hand, the effect of SOX3 knockdown could be prevented by overexpression of AKT1 (Fig. 1K, L; Fig. S5F right panel). Moreover, the levels of proteins involved in cell proliferation and metastasis such as c-myc, cyclin D1, MMP9 (matrix metallopeptidase 9), and E-cadherin were regulated by SOX3 in an AKT-dependent manner in HCC cells (Fig. 1N). Taken together, these findings suggest that AKT1 acted downstream of SOX3 and that SOX3 activated AKT signaling in part by increasing AKT1 expression to enhance HCC cell proliferation and metastasis.

Considering the pivotal role of AKT1 in the signaling cascade involving PI3K/AKT/mTOR, we next investigated whether SOX3 regulated the PI3K/AKT/mTOR signaling cascade. Western blot analysis showed that SOX3 overexpression significantly enhanced not only the expression of AKT1 but also induced phosphorylation of AKT1-Ser473 (S473) and AKT1-Thr308 (T308) in a concentration-dependent manner in HepG2 cells, while in Huh7 cells, SOX3 knockdown had the opposite effect (Fig. 1O). To investigate AKT signaling's role in SOX3-induced carcinogenesis, we treated SOX3-transfected HepG2 cells with MK2206, an inhibitor of AKT phosphorylation at serine 473 and threonine 308 (Fig. 1P). We found that inhibiting AKT signaling with MK2206 significantly reduced SOX3-induced cell proliferation, migration, and invasion (Fig. 1Q and R). Additionally, SOX3's regulation of the levels of proteins involved in cell proliferation and metastasis, including c-myc, cyclin D1, MMP9, and E-cadherin, was also abolished by MK2206 treatment (Fig. 1S). Similar results were obtained with SC-66, another specific inhibitor of the PI3K/AKT pathway (Fig. S6A—E).

Since AKT activation can lead to induction of mTOR signaling,[5] we next analyzed if mTOR signaling was regulated by SOX3. First, we found that SOX3 induced mTOR-S2448 phosphorylation, which is involved in the activation of mTOR pathway (Fig. 1T). In addition, when HCC cell lines were treated with two different AKT1 inhibitors (MK2206 and SC-66), the SOX3-induced mTOR-S2448 phosphorylation was prevented (Fig. 1U; Fig. S6E). Furthermore, rapamycin (an mTORC1 inhibitor) treatment blocked the effects of SOX3 on HCC cells, including cell proliferation, migration, and invasion (Fig. 1V, W) as well as on the levels of cyclin D1, c-myc, MMP-9, and E-cadherin (Fig. 1X, Y). Additionally, similar results were obtained with another mTOR inhibitor, LY303511 (Fig. S6F—J). Thus, we conclude that SOX3 enhances HCC cell proliferation, migration, and invasion by activating the AKT1/mTOR signaling cascade (Fig. S7).

In this research, we discovered that SOX3 was expressed at high levels in HCC tissues compared with the associated noncancerous tissues. We also show that SOX3 is critical for promoting HCC cell proliferation, migration, invasion, and metastasis. Mechanistically, we found that SOX3 induced the mTOR (target of rapamycin) signaling pathway by

BALB/C nude mice (7 mice per group) (down panel). Tumor weight at day 22 and tumor growth curves by volume were calculated (upper panel). **(F)** Representative images of immunohistochemical analysis of SOX3 expression and Ki67 (for cell proliferation) in the xenografts from mice injected with HepG2-Ctrl or SOX3-GFP cells. **(G)** Representative hematoxylin-eosin staining of lung tissues from the different mouse groups showing larger metastatic tumor regions in mice injected with SOX3-GFP cells. **(H)** Clustering analysis of differentially expressed genes which might involve in PI3K-AKT signaling pathway using normalized counts in a heat map ($|\log_2$ fold change$| > 1$, adjusted p-value <0.05). **(I)** Immunofluorescence staining was used to detect the expression and distribution of SOX3 in wild-type HepG2 cells transfected with pEF-flag or pEF-flag-SOX3 plasmids. **(J)** Chromatin immunoprecipitation analysis of SOX3 occupancy on AKT1 promoter in HepG2 cells transfected with pEF-flag-SOX3 plasmids. AKT1-1 and AKT1-2 represent two different primers used to detect the AKT1 promoter sequence. **(K)** The proliferating cells was detected by EdU in HepG2 cells transfected with SOX3, followed by transfection with siAKT1-1#, siAKT1-2#, siAKT1-3#, or the control (siNC), and in Huh7 cells transfected with siSOX3, followed by transfection with plasmid expressing FLAG-AKT1 or the control (Ctrl). **(L, M)** Cell migration and invasion assays for the indicated cell groups. **(N)** Western blot analyses for cell proliferation- and EMT-related proteins (c-myc, cyclin D1, MMP9, and E-cadherin) in HepG2 and Huh7 cells at 48 h post-transfection. **(O)** Western blot analyses for the levels of total AKT1 and phosphorylated forms AKT1-S473 and AKT1-T308 in HepG2 cells transfected with the lentivirus expressing SOX3 and in Huh7 cells transfected with siNC, siSOX3-1#, siSOX3-2#, or siSOX3-3#. **(P)** Western blot analyses for the levels of total AKT1 and phosphorylated forms AKT1-S473 and AKT1-T308 in HepG2-Ctrl (−) or -SOX3 (Flag-SOX3) cells after treatment with MK-2206 (0.5 μmol). **(Q)** Cell proliferation determined by CCK8 or EdU staining assays for HepG2 cells transfected with Ctrl or SOX3-expressing vector, followed by treatment with AKT1 inhibitor MK-2206 (1 μmol). **(R)** Cell migration and invasion assays for HepG2 cells transfected with Ctrl or SOX3-expressing vector, followed by treatment with AKT1 inhibitor MK-2206 (0.5 μmol). **(S)** Western blot analyses for the levels of proteins related to EMT and cell proliferation in HepG2-Ctrl/SOX3 cells after MK-2206 (0.5 μmol) treatment. **(T)** Western blot analyses for the levels of total mTOR and phosphorylated mTOR-S2448 in HepG2 cells transfected with the lentivirus expressing SOX3 or control, and in Huh7 cells transfected with siNC, siSOX3-1#, siSOX3-2#, or siSOX3-3#. **(U)** Western blot analyses for the levels of total mTOR and phosphorylated mTOR-S2448 in HepG2 cells transfected with the lentivirus expressing SOX3 or control, followed by treatment with AKT1 inhibitor MK-2206 (0.5 μmol). **(V)** Cell proliferation determined by CCK8 or EdU staining assays for HepG2 cells transfected with the lentivirus expressing SOX3 or control, followed by treatment with mTOR inhibitor rapamycin (2 μmol). **(W)** Cell migration and invasion analyses for HepG2 cells transfected with the lentivirus expressing SOX3 or control, followed by treatment with mTOR inhibitor rapamycin (1 μmol). **(X)** Western blot analyses for the levels of total AKT1, phosphorylated forms AKT1-S473 and AKT1-T308, total mTOR, and phosphorylated form mTOR-S2448 after treatment with rapamycin (1 μmol). **(Y)** Western blot analyses for the levels of proteins related to EMT and cell proliferation after rapamycin (1 μmol) treatment. EMT, epithelial—mesenchymal transition. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

binding to the AKT1 promoter to promote its expression and thereby activate AKT1 signaling.

Ethics declaration

All human samples were collected at Hubei Cancer Hospital with written informed consent from all human participants. The research was approved by the Ethics Committee of Hubei Cancer Hospital (approval number: LLHBCH2021YN-027). All animal experiments were approved by the Ethics Committee of Wuhan University (150013, Wuhan, China) and performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, China, 1998).

Author contributions

Yang Liu: methodology, investigation, formal analysis, and writing - review & editing; **Feifei Song:** methodology, investigation, and formal analysis; **Xianhuang Zeng:** methodology and investigation; **Siqi Yang:** data analysis and software; **Ze Wang:** investigation and methodology; **Wajeeha Naz and Tanzeel Yousaf:** methodology and writing - review & editing; **Junwei Sun:** project administration and resources; **Yangjun Zhang:** funding acquisition and manuscript review; **Ying Zhou:** investigation and methodology; **Mingxiong Guo:** conceptualization, funding acquisition, supervision, and writing - review & editing; **Yun-Bo Shi:** conceptualization and critical revision of the manuscript; **Geng Tian:** conceptualization, funding acquisition, and project administration; **Guihong Sun:** conceptualization, funding acquisition, data curation, resources, supervision, and writing - review & editing.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2024.101316.

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