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Original Articles

# Androgen receptor (AR)/miR-520f-3p/SOX9 signaling is involved in altering hepatocellular carcinoma (HCC) cell sensitivity to the Sorafenib therapy under hypoxia *via* increasing cancer stem cells phenotype

Yao Xiao[a,b,1], Yin Sun[b,1], Guodong Liu[a,b], Jie Zhao[b], Yuan Gao[b], Shuyuan Yeh[b], Liansheng Gong[a,*], Chawnshang Chang[b,c,**]

[a] Department of Hepatobiliary and Pancreatic Surgery, Xiangya Hospital, Central South University, Changsha, 410008, China
[b] George Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, The Wilmot Cancer Center, University of Rochester Medical Center, Rochester, NY, USA
[c] Sex Hormone Research Center, China Medical University/Hospital, Taichung 404, Taiwan

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ABSTRACT

Early studies indicated that the androgen receptor (AR) might play key roles to impact hepatocellular carcinoma (HCC) progression at different stages. Its linkage to hypoxia, a condition that occurs frequently during the HCC progression, however, remains unclear. Here we found that AR/miR-520f-3p/SOX9 signaling is involved in altering HCC cells sensitivity to the Sorafenib therapy under hypoxia *via* increasing the cancer stem cells (CSC) population. Mechanism dissection revealed that AR might alter the miR-520f-3p/SOX9 signaling through transcriptional regulation *via* binding to the androgen-response-elements (AREs) on the promoter region of miR-520f, which could then suppress SOX9 mRNA translation *via* targeting its 3′ untranslated region (3′UTR). The *in vivo* mouse model with orthotopic xenografts of HCC cells also validated the *in vitro* data, and a human HCC sample survey confirmed the positive linkage of AR/miR-520f-3p/SOX9 signaling to the CSC population during HCC progression. Together, these preclinical findings suggest that hypoxia may increase the HCC CSC population *via* altering the AR/miR-520f-3p/SOX9 signaling, and targeting this newly identified signaling with the small molecule, miR-520f-3p, may help in the development of the novel therapy to better suppress the HCC progression.

## 1. Introduction

Hepatocellular carcinoma (HCC) is the fifth and seventh most frequently diagnosed cancer in men and women, and the second and sixth cause of cancer death respectively across the globe [1]. In the United States, the incidence of HCC has almost tripled in the past 3 decades, rendering it the fastest rising cause of cancer-related deaths [2]. Based on understanding and characterization of novel genetic and epigenetic alterations that may help provide novel therapeutic targets for HCC treatment, the multikinase inhibitor Sorafenib is used to treat patients with advanced HCC [3,4]. According to the population-based Surveillance Epidemiology and End Results registry data, HCC age-adjusted incidence rate was at least 6 per 100,000 in 2010 [5]. In the US, 40,710 cases of liver & intra hepatic bile duct cancer were newly diagnosed in 2017 and these cancers are highly fatal with a gender ratio of incidence of 3 times higher in men than in women [6].

The obvious male dominance in HCC occurrence suggested that sex hormones and/or their receptors may play important roles for the development of HCC [7]. The role of AR signals in the HCC initiation and progression appear to be complex [8], and results from different studies indicated that AR might promote the initiation and development of HCC during the early stage [9], yet might suppress the cell invasion during the later stages of HCC [10].

Hypoxia occurs frequently during solid tumor progression due to tumor growth surpassing the supply of oxygen and nutrients by the tumor vasculature, especially in patients with portal vein tumor thrombus and treated with trans-catheter arterial chemoembolization or Sorafenib [11,12]. Hypoxia may enhance the tumor cell

* Corresponding author. Department of Hepatobiliary and Pancreatic Surgery, Xiangya Hospital, Central South University, Changsha, 410008, China.
** Corresponding author. George Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, and The Wilmot Cancer Center, University of Rochester Medical Center, Rochester, NY, 14642, USA.
*E-mail addresses:* gongliansheng8280@163.com (L. Gong), chang@urmc.rochester.edu (C. Chang).
[1] Contributed equally to this work.

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proliferation, angiogenesis, metastasis and chemo-resistance of HCC [13,14]. Mechanism dissection suggested that hypoxia might influence the tumor progression *via* altering the HIF signaling [15].

The microRNAs (miRs) are a class of noncoding RNAs, 18–25 nucleotides in length, that have been demonstrated to negatively mediate the expression of their target genes by inducing mRNA degradation or suppressing protein translation [16], primarily through binding to the 3′UTRs mRNA of protein-coding genes [17].

The SRY-box family proteins are well-established regulators of cell fate and differentiation, such as male differentiation, stemness, neurogenesis, and skeletogenesis. Dysregulation of SOX factors has been further implicated in diseases including cancer [18,19]. Aberrant SOX9 expression led to tumorigenesis in several tissue subtypes. High levels of SOX9 increased the ability of metastasis of cancer cells and led to poor prognosis in different cancers [20]. Development of HCC can be driven by a small heterogeneous population of tumor-derived cancer stem cells (CSC) or tumor-initiating cells. Studies show that SOX9 is required for CSC self-renewal and can regulate tumorigenicity by promoting symmetrical cell division of CSC in many cancers, including HCC [21,22].

Here, we found that AR/miR-520f-3p/SOX9 signaling is involved in altering HCC cells sensitivity to the Sorafenib therapy under hypoxia *via* increasing the CSC population.

## 2. Materials and methods

### 2.1. Cell culture

HCC SK-HEP-1 and Hep G2 cell lines were purchased from the American Type Culture Collection (ATCC) in 2015. Dulbecco's Modified Eagle's Medium (Invitrogen) with 10% FBS, 1% glutamine, and 1% penicillin/streptomycin was used for culture of SK-HEP-1 and Hep G2 cells. All cell lines were cultured in a 5% (v/v) $CO_2$ humidified incubator at 37 °C.

### 2.2. Hypoxia

Hypoxia (1% $O_2$, 5% $CO_2$, 94% $N_2$) was achieved by using an In Vivo2 hypoxic workstation (Ruskinn Technologies) or in a positive pressure chamber receiving gas from a custom-mixed tank (Airgas).

### 2.3. Human HCC specimens

Of the 24 eligible patients in our study from the Department of Hepatobiliary and Pancreatic Surgery, XiangYa Hospital, none had received pre-operative treatment, and all of their postoperative pathology confirmes as HCC. Two different types of tissues from each HCC patient including tumor-free tissue > 2 cm far from the tumor edge (N), and tissue from the tumor (T) were processed immediately after surgical resection. Areas of tissue necrosis and hemorrhage were excluded. The specimens from each patient were divided into two parts. One part was snap-frozen immediately after resection and was stored in liquid nitrogen until used for experiments. The other part was treated with 10% formaldehyde solution and paraffin-embedded until used for experiments.

### 2.4. Reagents and materials

GAPDH (6c5) and AR (N-20) antibodies were obtained from Santa Cruz Biotechnology. SOX9 and SOX4 antibody were purchased from One World Lab. Anti-mouse/rabbit second antibody for Western Blot was from Invitrogen. Normal rabbit IgG was also from Santa Cruz Biotechnology.

### 2.5. Lentivirus packaging

The plasmids pLVTHM-miR-520f-3p, pWPI-AR, pLKO.1-shSOX9 or pLKO.1-shAR, the psPAX2 packaging plasmid, and pMD2.G envelope plasmid, were transfected into 293T cells using the standard calcium chloride transfection method for 48 h to get the lentivirus soup. The lentivirus soups were collected and concentrated by density gradient centrifugation and used immediately or frozen at −80 °C for later use.

### 2.6. RNA extraction and qRT-PCR analysis

For RNA extraction, Trizol reagent (Invitrogen) was used to isolate total RNAs and 1 μg of total RNA was subjected to reverse transcription into cDNA using Superscript III transcriptase (Invitrogen). Determination of mRNA expression level of a gene of interest was completed using quantitative real-time PCR (qRT-PCR) conducted using a Bio-Rad CFX96 system with SYBR green. The expression of GAPDH or β-actin RNA was used to normalize the expression levels of a target gene.

### 2.7. Western blot analysis

Cells were lysed in RIPA buffer and proteins (30 μg) were separated on 8–10% SDS/PAGE gel and then transferred onto PVDF membranes (Millipore). After blocking membranes, they were incubated with appropriate dilutions of specific primary antibodies, and then blots were incubated with HRP-conjugated secondary antibodies and visualized using the ECL system (Thermo Fisher Scientific).

### 2.8. Sphere formation assay

The sphere formation assay was performed as described earlier [23,24]. Briefly, cell suspensions ($1 \times 10^3$ in 70 μl media) were mixed with 70 μl Matrigel (BD) and placed along the rim of the 24-well plates with three triplicate experiments. The culture plates were placed in 37 °C incubator for 10 min to let the mixture solidify and 500 μl media was then added into the well. Sphere numbers were counted after 7–14 days under Olympus light microscope and size differences were also examined.

### 2.9. MTT assay

MTT assay was used to measure cell proliferation rates. Hep G2 cells were seeded in 24-well plates at 5000 per well after being transfected with plasmids for 24 h. The cell proliferation assay was performed on day 2. We added the MTT reagent to each well, then incubated the plates at 37 °C for 2 h, dissolved the precipitate in DMSO and measured the absorbance at 450 nm. Each sample was assayed in triplicate.

### 2.10. Clonogenic growth assay

To measure clonogenic survival, cells were seeded at 2000 cells per 6-well plates in growth media, and each sample was assayed in triplicate. After 7–14 days incubation, we washed the colonies with PBS, then 10% formalin was used to fix cells and 0.5% crystal violet used to stain cells. Following digital photography, the total colony area was counted under Olympus light microscope.

### 2.11. Chromatin immunoprecipitation assay (ChIP)

Normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) and protein A-agarose were used sequentially to preclear the cell lysates. We then added anti-AR antibody (2.0 μg) to the cell lysates and incubated overnight at 4 °C. IgG was used in the reaction for a negative control. Specific primer sets were designed to amplify a target sequence within the human miR-520f-3p promoter and agarose gel electrophoresis was used to identify the PCR products.

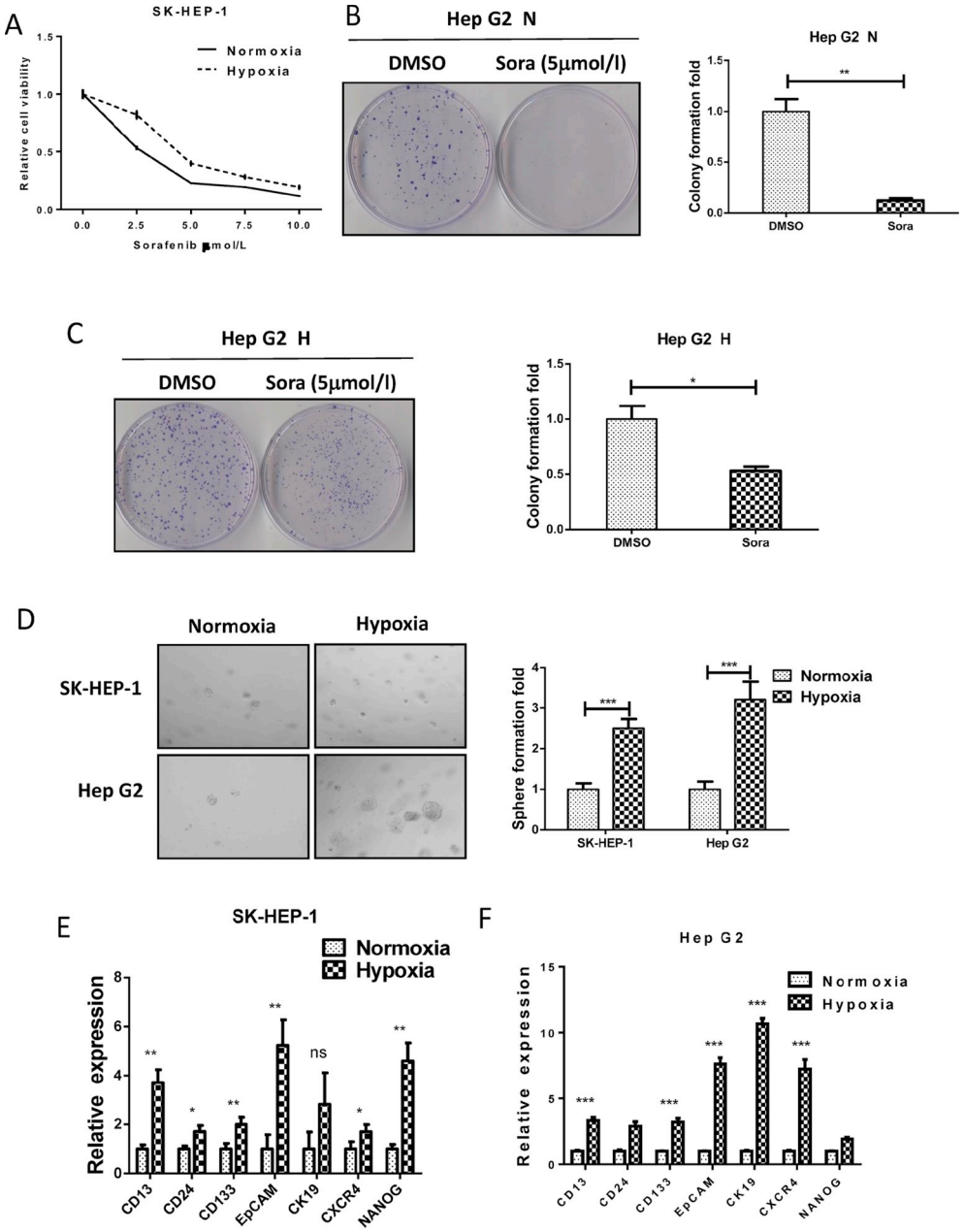


**Fig. 1. Hypoxia alters the efficacy of Chemotherapy with Sorafenib to suppress the HCC cell growth and mechanism dissection why hypoxia alters the efficacy of Sorafenib to suppress the HCC cell growth: *via* altering the CSC population.** (A) MTT proliferation change in SK-HEP-1 cells exposed to hypoxia (H) and treated with Sorafenib comparing with cells exposed to normoxia (N) with Sorafenib treatment. (B–C) Hep G2 cells were exposed to (B) normoxia or (C) hypoxia and treated with 5 μmol/l Sorafenib (Sora) or DMSO. Colony formation detects the proliferation change. (D) SK-HEP-1 and Hep G2 were exposed to hypoxia/normoxia for 48 h. Sphere formation assay were performed to evaluate the CSCs number. After 7–14 days of incubation, colonies in five random fields per each well were counted under a microscope. (E–F) Total RNA was analyzed for CSC markers in SK-HEP-1 and Hep G2 cells, including *CD13, CD24, CD133, EpCAM, CK19, CXCR4*, and *NANOG* by real-time PCR. For B-D quantitations are at the right, are mean ± SD, *p ≤ 0.05, **p < 0.01, ***p < 0.001.

### 2.12. Luciferase assay

The human 5′-promoter region of miR-520f was constructed into pGL3-basic luciferase reporter vector (Promega). Site-directed mutagenesis of the AR binding site in the miR-520f 5′ promoter was achieved with the Quick Change mutagenesis. 2033bp fragment of SOX9 3′UTR with wild type or mutant miRNA-responsive elements was cloned into the psiCHECK-2 vector (Promega) downstream of the Renilla luciferase ORF. Hep G2 and SK-HEP-1 cells were plated in 24-well plates and the cDNA were transfected with Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PRL-TK was used as an internal control that served as the baseline control response. Luciferase activity was measured 36–48 h after transfection by Dual-Luciferase Assay (Promega) according to the manufacturer's manual.

### 2.13. In vivo studies

6–8 weeks old nude mice were purchased from NCI and divided into 4 groups for injection of $1 \times 10^6$ SK-HEP-1-Luc cells transduced with Luciferase and pre-cultured as follows: vector control with normoxia (group 1, 5 mice) or hypoxia (group 2, 10 mice) for 48 h, and over-expressed miR-520f-3p (oemiR-520f-3p) group with normoxia (group 3, 5 mice) or hypoxia (group 4, 10 mice) for 48 h, Then the cells were mixed with Matrigel (1:1) and injected into the mice under the left lobes of the liver capsules. Tumor development and metastasis were monitored by non-invasive In Vivo Fluorescent Imager (IVIS Spectrum, Caliper Life Sciences) once a week. Once the tumor formation was detectable, we treated one half the mice (5 mice) from group 2 and group 4 with Sorafenib at 30 mg/kg/day and the other half of these mice were treated with control vehicle for 4 weeks. Mice were sacrificed after 8 weeks, tumors and any metastases were removed for studies. Animal experiments were approved by institutional animal care at Univeristy of Rochester Medical Center.

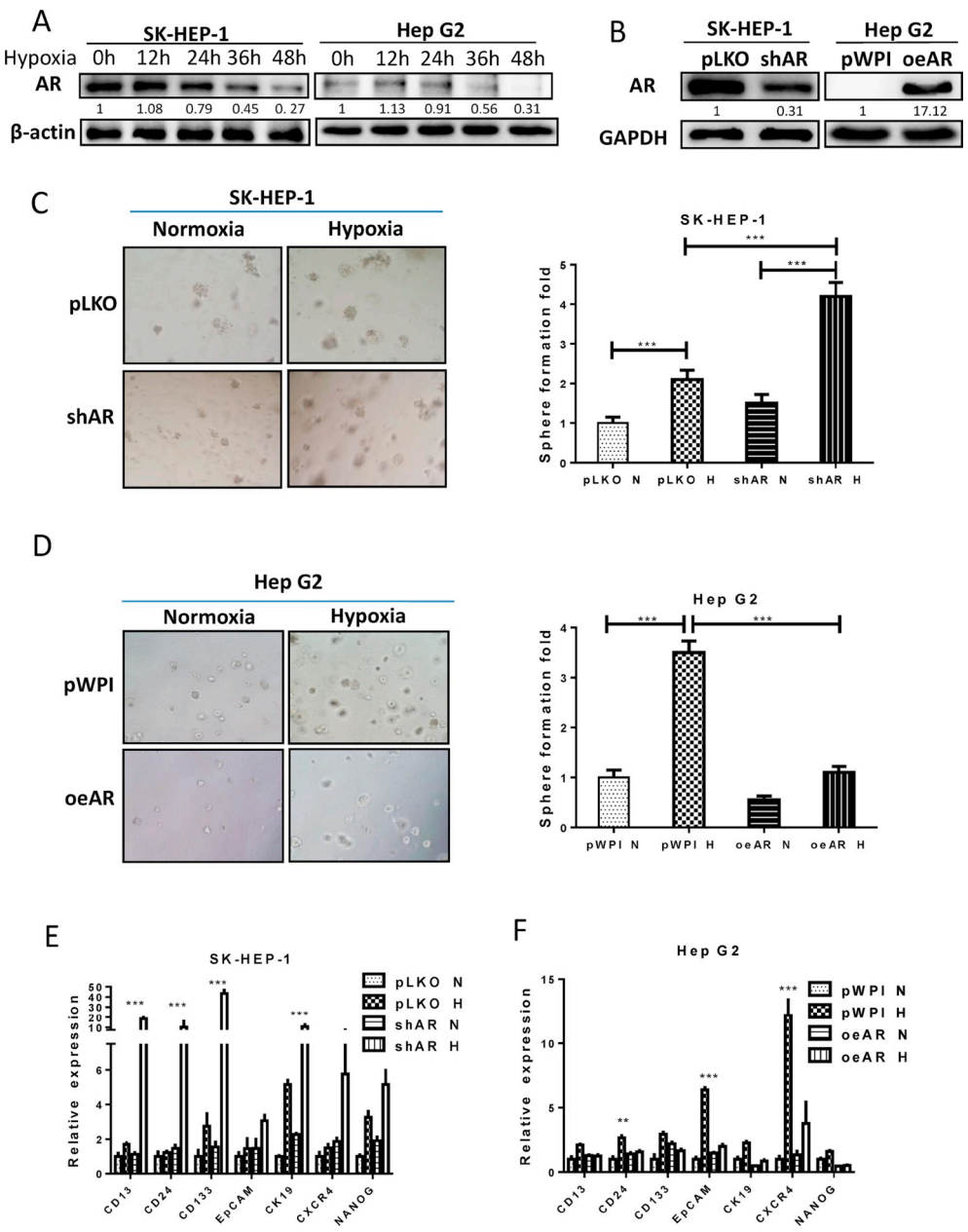


**Fig. 2. Mechanism dissection why hypoxia can alter the CSC population: *via* altering the AR expression.** (A) SK-HEP-1 and Hep G2 cells were exposed to hypoxia for 0, 12, 24, 36, or 48 h. Western blot was used to detect AR expression. (B) Western blot showed the efficiency of knocking down AR (shAR) in SK-HEP-1 cells and over-expressing AR (oeAR) in Hep G2 cells. (C-D) SK-HEP-1 (C) and Hep G2 (D) cells were virally transduced with shAR or oeAR and cells were exposed to hypoxia or normoxia for 48 h. Sphere formation assays were performed to evaluate the CSC sphere formation. After 14 days of incubation, colonies in five random fields per each well were counted under a microscope. (E–F) Total RNAs were analyzed for CSC markers including *CD13, CD24, CD133, EpCAM, CK19, CXCR4*, and *NANOG* by real-time PCR in SK-HEP-1 (E) and Hep G2 (F) cells. For (C) and (D), quantitations are at the right and are mean ± SD, ***p < 0.001.

### 2.14. Immunohistochemical (IHC) staining

Mouse tissues were fixed in 10% (v/v) formaldehyde in PBS, and fixed and embedded in paraffin. The samples were cut into 5 μm thick slices and dehydrated with xylene and used for IHC staining with specific primary antibodies against AR and SOX9. To enhance antigen exposure, the slides were treated with 1 × EDTA at 98 °C for 10 min for antigen retrieval. The slices were incubated with endogenous peroxidase blocking solution, and then were incubated with the primary antibody AR and SOX9 overnight at 4 °C. After rinsing with Tris-buffered saline, the slices were incubated with biotinylated secondary antibody for 1 h at room temperature, washed, and then incubated with enzyme conjugate horseradish peroxidase (HRP)-streptavidin. Freshly prepared DAB (Zymed, South San Francisco, CA) was used as substrate to detect HRP. Finally, slides were counter-stained with hematoxylin and mounted with aqueous mounting media. Positive cells were calculated as the number of immunopositive cells × 100% divided by total number of cells/field in 10 random fields at 400 × magnification.

### 2.15. Statistics

All statistical analyses were carried out with SPSS 19.0 (SPSS Inc, Chicago, IL). The data values were presented as the mean ± SD. Differences in mean values between two groups were analyzed by two-tailed Student's *t*-test and the mean values of more than two groups were compared with one way ANOVA. $p \leq 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Hypoxia alters the efficacy of Sorafenib to suppress the HCC cell growth

Hypoxia occurs frequently during the HCC progression [25]. Its potential impact on altering the efficacy of chemotherapy with Sorafenib to suppress the HCC cells growth, however, remains unclear. Here we first applied the MTT proliferation assay to examine the HCC

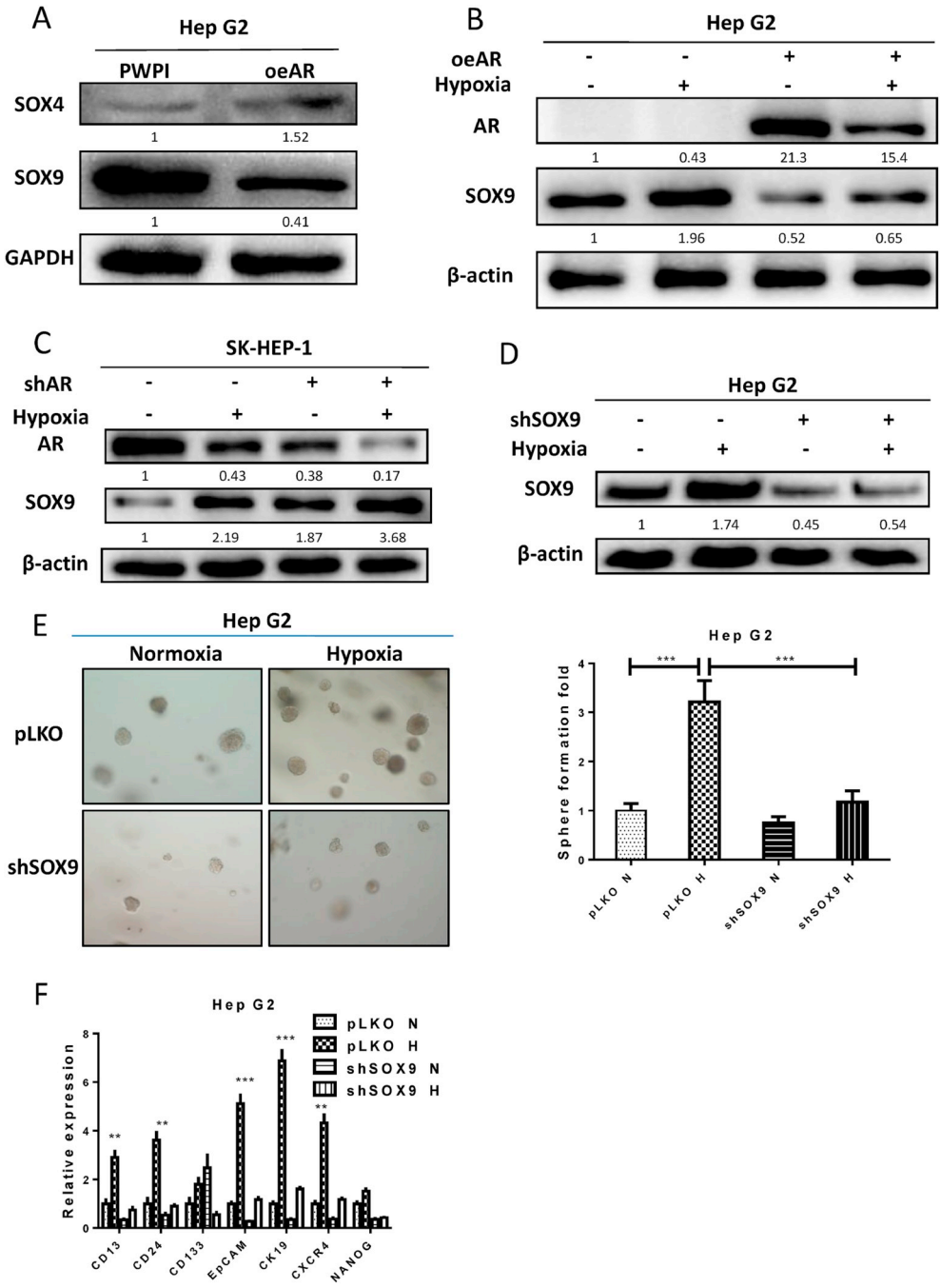


**Fig. 3. Mechanism dissection why hypoxia-suppressed AR can alter the CSC population: *via* altering SOX9 expression.** (A) Hep G2 cells were virally transduced with oeAR, and Western blot was used to detect SOX4 and SOX9 expression. (B) Western blot showed the efficiency of oeAR with/without exposure to hypoxia for 48 h in Hep G2 cells. (C) SK-HEP-1 cells were virally transduced with shAR and cells were exposed to hypoxia (+) or normoxia (−) for 48 h. Western blot was used to detect AR and SOX9 expression. (D) Western blot showed the efficiency of knocking down SOX9 (shSOX9) and exposing to hypoxia (+) or normoxia (−) for two days in Hep G2 cells. (E) Hep G2 cells were virally transduced with shSOX9 and cells were exposed to hypoxia or normoxia for 2 days. Sphere formation assays were performed to evaluate the CSC numbers. After 10 days of incubation, colonies in five random fields per each well were counted under a microscope. (F) Total RNAs in Hep G2 cells were analyzed for CSC markers including *CD13, CD24, CD133, EpCAM, CK19, CXCR4,* and *NANOG* by real-time PCR. For (E), quantitation is at the right and is mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$.

cells viability in response to the Sorafenib. The results revealed that Sorafenib treatment is less effective in suppressing HCC SK-HEP-1 cells viability in hypoxic than in normoxic conditions (Fig. 1A). Similar results were also obtained when we replaced the MTT assay with the colony formation assays in HCC Hep G2 cells (Fig. 1B–C).

Together, results from different assays in various HCC cells (Fig. 1A–C) suggest that the hypoxia can alter the efficacy of Sorafenib to suppress the HCC cell growth.

### *3.2. Mechanism dissection of how hypoxia can alter the efficacy of Sorafenib to suppress the HCC cell growth: via altering the CSC population*

To dissect the mechanism of how hypoxia alters the efficacy of Sorafenib to suppress the HCC cells growth, we focused on the CSC population, as early studies indicated that CSC might play important roles to alter drug resistance [26]. Using sphere formation assays to evaluate the CSC phenotype, we found that hypoxia could increase the CSC population in SK-HEP-1 cells and Hep G2 cells (Fig. 1D). Results from a different approach to assay the CSC population using CSC-related biomarkers assay also revealed that hypoxia could increase the expression of CSC-related biomarkers, including *CD13, CD24, CD133, EpCAM, CK19, CXCR4* and *NANOG* (Fig. 1E–F). We found that Sorafenib did not affect differential sphere formation and relative expression of CSC markers significantly under both hypoxia and normoxia in SK-HEP-1 cells (Supplementary Fig. SA-B).

Together, results (Fig. 1D–F and Supplementary Fig. SA-B) from two different assays in two HCC cell lines all suggested that hypoxia may function *via* altering the CSC population to alter the efficacy of Sorafenib to suppress the HCC cell growth.

### 3.3. Mechanism dissection of how hypoxia can alter the CSC population: via altering the AR expression

Since recent studies indicated that AR might play a key role to alter the HCC progression [8,10,27–29], and AR could also modulate the CSC population in the prostate cancer [30], we focused on the AR signaling to dissect the mechanism of how hypoxia can alter the CSC population to impact the efficacy of Sorafenib to suppress the HCC cell growth.

We first examined hypoxia's influence on the AR expression. Results from both Hep G2 and SK-HEP-1 cell lines revealed a decreased AR protein expression after being exposed to hypoxia for 36 h (Fig. 2A). We found that Sorafenib did not affect AR expression significantly under either hypoxia or normoxia in SK-HEP-1 cells (Supplementary Fig. SC). We then examined the potential impact of hypoxia-suppressed AR expression on the CSC population, and sphere formation assay results revealed that knocking-down AR with AR-shRNA increased the sphere formation more significantly under hypoxia than under normoxia (Fig. 2B–C). As expected, knocking down AR also increased the expression of CSC biomarkers including *CD13, CD24, CD133, EpCAM, CK19, CXCR4,* and *NANOG* in SK-HEP-1 cells more significantly under hypoxia than under normoxia (Fig. 2E). In contrast, adding AR led to decrease the CSC population (Fig. 2D) and expression of CSC biomarkers, including *CD13, CD24, CD133, EpCAM, CK19, CXCR4,* and *NANOG* in Hep G2 cells (Fig. 2F).

Together, results from Fig. 2A–F suggest that hypoxia may function *via* suppressing AR expression to increase the CSC population in the HCC cells.

### 3.4. Mechanism dissection of how hypoxia-suppressed AR can alter the CSC population: via altering the SOX9 expression

To further dissect the mechanism of how hypoxia-suppressed AR can alter the CSC population, we focused on the potential AR downstream genes SOX4 and SOX9, as early studies indicated they might play key roles to promote CSC formation [22,31]. Results from the western blot analysis revealed that increased AR expression *via* adding AR-cDNA led to decrease the SOX9 protein expression, yet increased the SOX4 protein expression (Fig. 3A).

Importantly, results from an interruption approach revealed that hypoxia-increased SOX9 protein expression could be altered *via* adding or knocking down AR expression in Hep G2 and SK-HEP-1 cells **(**Fig. 3B–C**,** respectively). As expected, suppressing SOX9 with SOX9-shRNA (shSOX9) led to reverse/block the hypoxia-increased SOX9 expression, CSC population and expression of CSC biomarkers (Fig. 3D–F). The 2nd AR-shRNA construct similarly increased the SOX9 protein expression and the CSC population in SK-HEP-1 cells (Supplementary Fig. SD-E) compared to pLKO control.

Together, results from Fig. 2A–F and Supplementary Fig. SC suggest that hypoxia may function *via* altering the AR/SOX9 signaling to alter the CSC population.

### 3.5. Mechanism dissection of how hypoxia-suppressed AR can alter the SOX9 expression: via de-repressing the miR-520f-3p expression

To examine the molecular mechanism underlying the regulation of SOX9 by AR, we examined the AR impact on the SOX9 mRNA expression, and results revealed that adding AR had little influence on the SOX9 mRNA expression in the HepG2 cells, and there is little decrease of SOX9 mRNA expression with knocking-down AR in the SK-Hep-1 cells (Fig. 4A), suggesting AR might function *via* altering the miRNAs to suppress SOX9 translation.

We then searched the online databases (Targetscan, miRDB, Microcosm Target, mirtarbase.mbc.nctu.edu.tw) and public literature to identify those potential miRNAs that could target SOX9, and results indicated that 11 potential miRNAs may be able to modulate the SOX9 protein expression. Importantly, results from qPCR assay also indicated that hypoxia could decrease the expression of miR-520f-3p while increasing the other 10 miRNAs (Fig. 4B). However, manipulating AR indicated that 8 miRNAs were positively regulated by AR (Fig. 4C). We therefore focused on the miR-520f-3p for the remaining studies.

We first altered the miR-520f-3p expression in HepG2 cells **(**Fig. 4D**)** and results revealed that adding miR-520f-3p could significantly suppress SOX9 protein expression (Fig. 4E) and CSC population (using HCC sphere formation assay) under the hypoxic condition **(**Fig. 4F**).** Furthermore, exogenous expression of miR-520f-3p also decreased the expression of CSC biomarkers including *CD13, CD24, CD133, EpCAM, CK19, CXCR4* in Hep G2 cells under hypoxia (Fig. 4G).

Importantly, results from the rescue assay further revealed that the effect of AR-shRNA-increased HCC sphere formation and CSC biomarker expression could be partially blocked/reversed by adding the miR-520f-3p in SK-HEP-1 cells under hypoxia (Fig. 4H–I). In a reciprocal manner, reduced expression of HCC CSC biomarkers due to AR expression could be partially blocked/reversed by miR-520f-3p inhibitor in Hep G2 cells under hypoxia (Fig. 4J). As expected, the AR-shRNA-increased SOX9 expression could be partially reversed *via* adding the miR-520f-3p in SK-HEP-1 cells under hypoxia (Fig. 4K), and AR-decreased SOX9 expression could be partially reversed *via* miR-520f-3p inhibitor in Hep G2 cells under hypoxia (Fig. 4L), suggesting that miR-520f-3p may alter the hypoxia/AR/SOX9/CSC signaling axis.

Together, results from Fig. 4**A-L** suggest that hypoxia-suppressed AR may function *via* interacting with/modulating miR-520f-3p to alter the SOX9 protein expression.

### 3.6. Mechanism dissection of how hypoxia-suppressed AR can alter the miR-520f-3p expression: via transcriptional regulation

To further dissect the molecular mechanism(s) how AR can regulate the miR-520f-3p expression at the transcriptional level, we searched for the potential androgen-response-elements (AREs) on the 2 Kb region of the miR-520f promoter using JASPAR database, and results revealed that 6 putative AREs were located on the regions (−1944∼-1930; −1888∼-1874; −1268∼-1254; −668∼-654; −666∼-652; and −449∼-435) (Fig. 5A). We then applied the chromatin immunoprecipitation (ChIP) assay to verify their binding to AR *in vivo*, and results revealed that AR could bind to the ARE-2/3 region (Fig. 5B).

We then constructed the miR-520f promoter luciferase reporter construct by inserting a 1 kb 5′ promoter region of miR-520f into the pGL3 luciferase backbone as well as a version with the mutated ARE (Fig. 5C). As expected, the luciferase assay results revealed that adding AR significantly increased luciferase activity in HepG2 cells transfected with wild type ARE, but not in the cells with mutant ARE (Fig. 5D). When we replaced the HepG2 cells with the SK-HEP-1 cells (Fig. 5E), knocking down AR significantly decreased the luciferase activity in the SK-HEP-1 cells transfected with wild type ARE, but not in the cells with mutant ARE.

Together, results from Fig. 5A–E suggested that AR could increase miR-520f-3p expression at a transcriptional level *via* binding to the ARE located in its 5′ promoter region of the miRNA precursor.

### 3.7. Mechanism dissection of how hypoxia/AR/miR-520f-3p axis can alter the SOX9 expression: via binding to the 3'UTR of SOX9 mRNA

To dissect the mechanism of how hypoxia/AR/miR-520f-3p axis can alter the SOX9 expression at the molecular level, we first searched the potential miRNAs targeting sites located on the 3′UTR of SOX9 mRNA (http://www.targetscan.org/vert_71/). We then applied the reporter assay with the psiCHECK-2 vector carrying the wild type 3′UTR and a deletion mutant without the miR-520f-3p target site (Fig. 5F). Results from the luciferase assay revealed that adding miR-520f-3p markedly

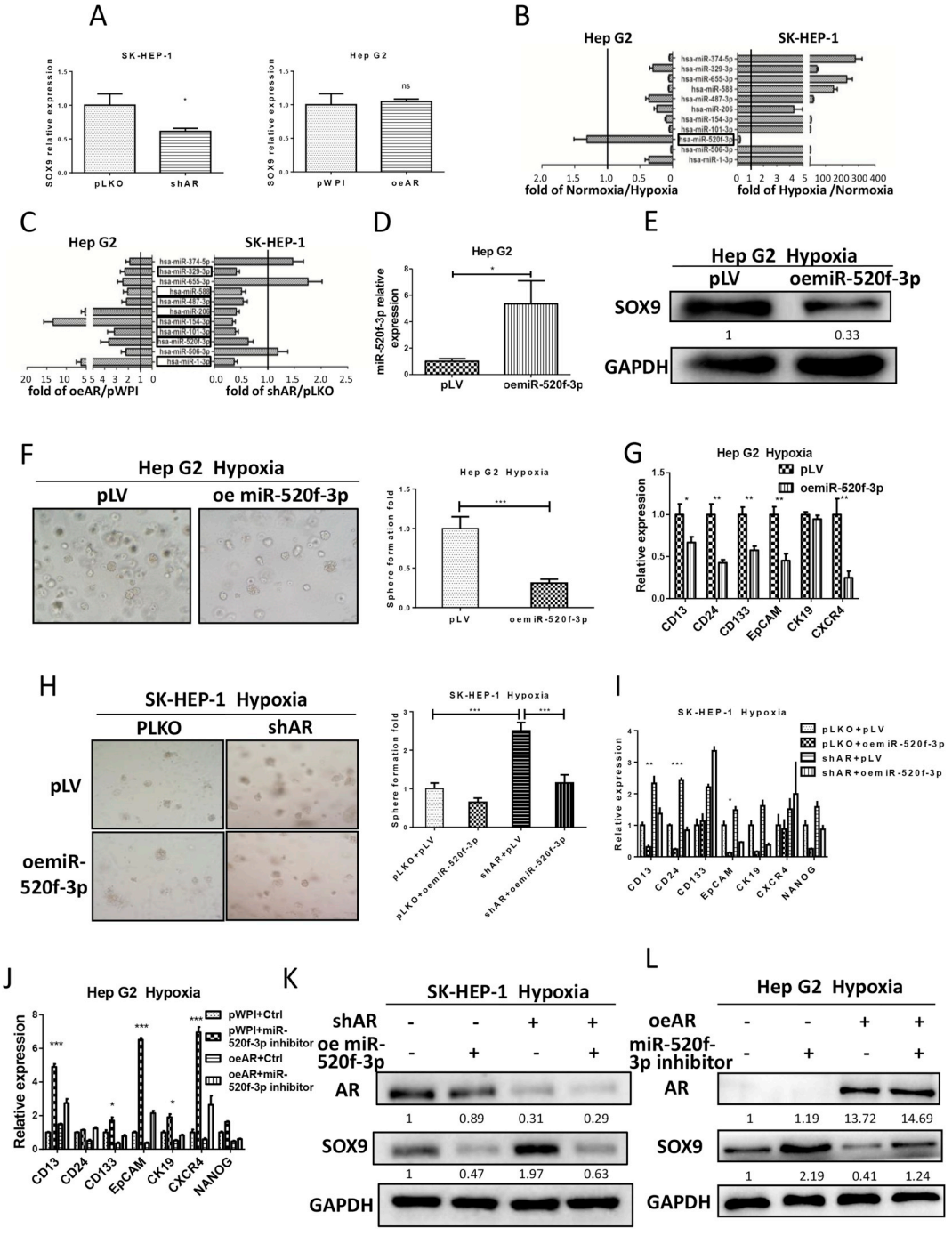


**Fig. 4. Mechanism dissection how hypoxia-suppressed AR can alter the SOX9 expression: *via* de-repressing the miR-520f-3p expression.** (A) Real-time PCR showed SOX9 expression after knocking down AR (shAR) in SK-HEP-1 and overexpressing AR (oeAR) in Hep G2 cells. (B–C) Real-time PCR assay for screening a set of miRNAs that can regulate SOX9 in (B) Hep G2 and SK-HEP-1 cells treated with hypoxia compared with normoxia for 48 h and in (C) Hep G2 cells with oeAR compared with pWPI (left) and in SK-HEP-1 cells shAR compared with pLKO vector control (right). (D) Real-time PCR assay to test expression (quantification) of miR-520f-3p relative to U6 in Hep G2 cells with stable overexpression of miR-520f-3p (oemiR-520f-3p). (E) Hep G2 cells were virally transduced with oemiR-520f-3p. Western blot was used to detect SOX9 expression. (F) Hep G2 cells were virally transduced with oemiR-520f-3p and cells exposed to hypoxia (H) for 48 h. Sphere formation assays were performed to evaluate the CSC numbers. After 10 days of incubation, colonies in five random fields per each well were counted under a microscope. (G) Total RNAs were analyzed for CSC markers, including *CD13, CD24, CD133, EpCAM, CK19,* and *CXCR4* by real-time PCR. (H) SK-HEP-1 cells were virally transduced with shAR and oemiR-520f-3p, and cells were exposed to hypoxia for 48 h. Sphere formation assays were performed to evaluate the CSC numbers. After 14 days of incubation, colonies in five random fields per each well were counted under a microscope. (I–J) Total RNA was analyzed for CSC markers including *CD13, CD24, CD133, EpCAM, CK19, CXCR4,* and *NANOG* by real-time PCR. (K) Western blot in SK-HEP-1 cells showed the efficiency of ± shAR and ± oemiR-520f-3p after exposing to hypoxia for 48 h. (L) Western blot in Hep G2 cells (showed the efficiency of ± oeAR and treating with/without miR-520f-3p inhibitor and exposing to hypoxia for 48 h. For (D), (F), and (H), quantitations are at the right and are mean ± SD, * < 0.05, ***p < 0.001.

decreased luciferase activity in HepG2 cells while miR-520f-3p inhibitor treatment significantly increased luciferase activity in SK-HEP-1 cells while both cell lines were transfected with wild type SOX9 3′UTR, but not the mutant SOX9 3′UTR (Fig. 5G–H), suggesting that miR-520f-3p can directly bind to the 3′UTR of SOX9 mRNA to suppress its protein expression.

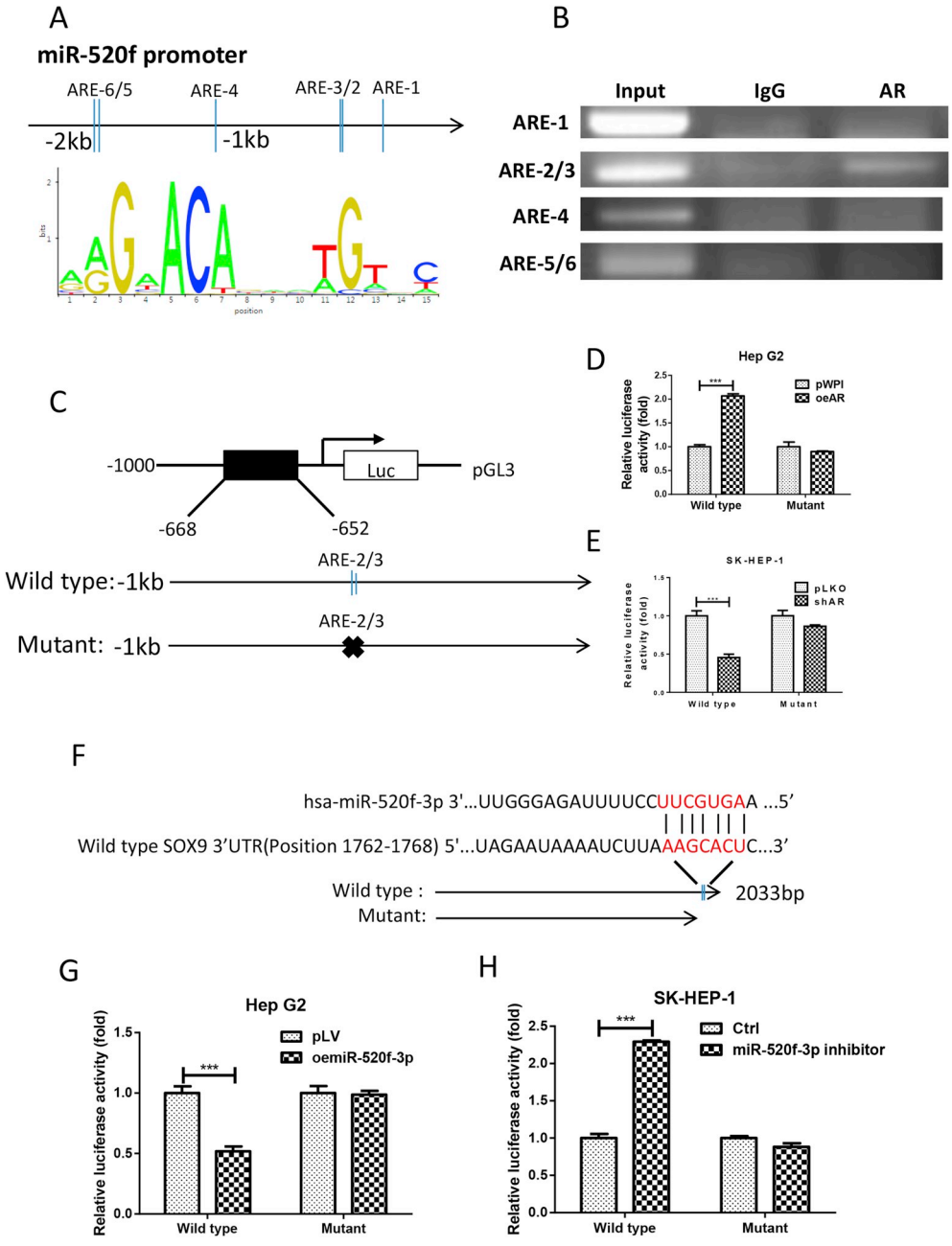


**Fig. 5. Mechanism dissection how hypoxia-suppressed AR can alter the miR-520f-3p expression: *via* transcriptional regulation and mechanism dissection how hypoxia/AR/miR-520f-3p axis can alter the SOX9 expression: *via* binding to the 3'UTR of SOX9-mRNA.** (A) 6 putative AREs predicted by JASPAR from the miR-520f promoter and ARE motif sequences. (B) ChIP assay results of 6 AREs of the miR-520f promoter in SK-HEP-1 cells. (C) The wild type and mutant pGL3-miR-520f promoter reporter constructs. (D–E) Luciferase activity after transfection of wild type or mutant miR-520f promoter reporter construct in Hep G2 cells with oeAR (D) and SK-HEP-1 cells with shAR (E) compared to the control cells. (F) Sequence alignment of the SOX9 3'UTR with wild type versus mutant potential miR-520f-3p targeting sites. (G-H) Luciferase reporter activity after transfection of wild type or mutant SOX9 3'UTR reporter construct in Hep G2 cells with/without oemiR-520f-3p (G) and SK-HEP-1 cells treated with/without miR-520f-3p inhibitor (H) compared to the control cells. For (D), (E), (G) and (H), quantitations are at the right and are mean ± SD, ***p < 0.001.

### *3.8. AR/miR-520f-3p/SOX9-modulated CSC population led to alter the Sorafenib sensitivity in HCC cells under the hypoxic condition*

As the CSC phenotype has been frequently linked to the drug resistance, we then tested the potential impact of the newly identified AR/miR-520f-3p/SOX9/CSC signaling axis, under the hypoxia condition, on the efficacy of Sorafenib, which is used clinically to treat HCC patients.

Using the interruption approaches, comparing hypoxia to normoxia, we found that targeting this newly identified AR/miR-520f-3p/SOX9/CSC signaling with targeting the AR or miR-520f-3p or SOX9 could all led to alter the efficacy of Sorafenib to suppress the HCC cell growth (Fig. 6A–D).

### *3.9. Human clinical study to link the AR/miR-520f-3p/SOX9 signaling to the HCC progression*

To link the above *in vitro* results with human HCC progression, we used immunohistochemistry (IHC) staining to detect AR and SOX9 protein level in HCC samples, and the results indicated that AR expression was higher in normal (N) tissues compared to tumor (T) tissues with a reverse relationship of SOX9 expression (Fig. 7A). Results from the TCGA data analysis also indicated that AR expression is higher in normal tissues compared to tumor tissue, and adjacent tumor tissue, while SOX9 expression is higher in tumor tissue compared to normal tissue, consistent with a negative correlation between AR and SOX9 (Fig. 7B). With the increase of HCC grades and stages, there is a corresponding decrease of AR level and HCC patients who had a higher AR expression had a higher survival rate (Fig. 7C). In contrast, with increase of HCC grade and stage, there is a corresponding increase of SOX9 level and HCC patients who had a higher SOX9 expression had a lower survival rate (Fig. 7D). Importantly, these data also indicated that AR had a negative correlation with CSC biomarkers such as *CD24, CD133, EpCAM, CK19* and *CXCR4* (Fig. 7E).

Together, results from a human clinical survey (Fig. 7A–E) are consistent with the above preclinical studies using multiple HCC cell lines showing AR/miR-520f-3p/SOX9/CSC signaling may play important roles to alter the HCC progression.

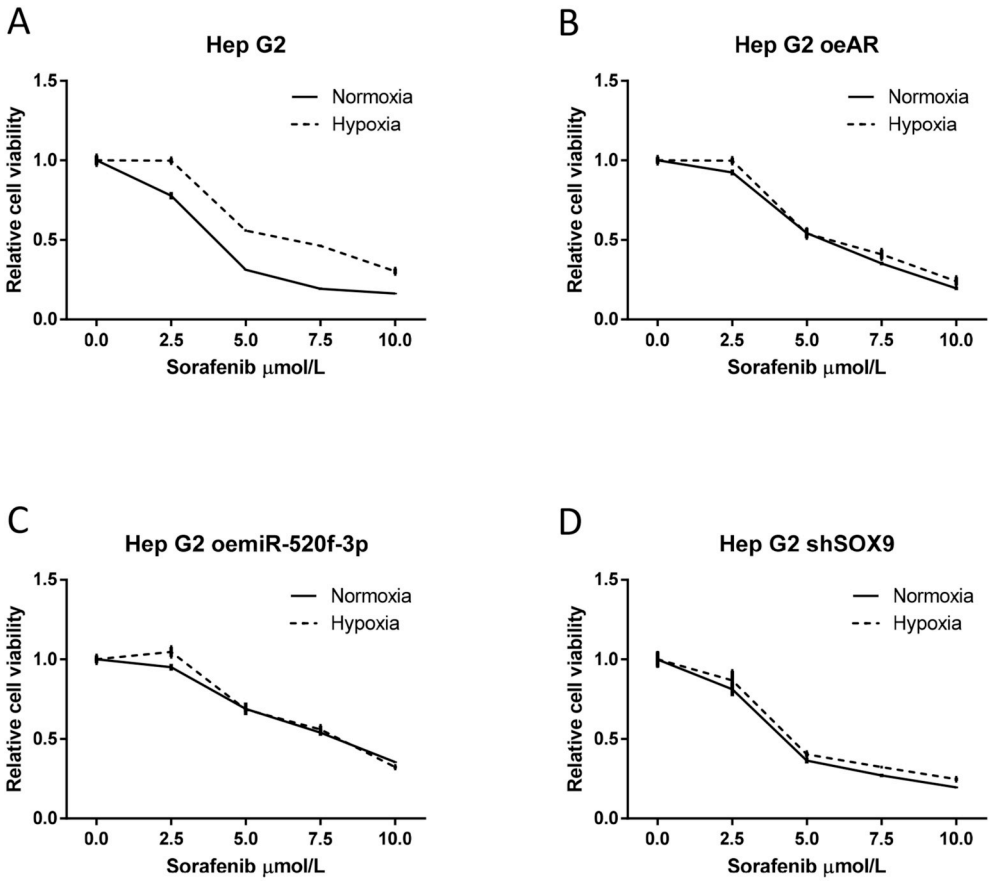


**Fig. 6. AR/miR-520f-3p/SOX9-modulated CSC population altered the chemotherapy with Sorafenib sensitivity in HCC cells under the hypoxia condition.** (A) MTT proliferation change in Hep G2 cells with/without Sorafenib treatment and exposed to hypoxia compared with cells exposed to normoxia. (B) Hep G2 cells were virally transduced with oeAR, then cells exposed to hypoxia and normoxia and treated with/without Sorafenib. MTT detected the proliferation change. (C) Hep G2 cells were virally transduced with oemiR-520f-3p, then cells exposed to hypoxia and normoxia and treated with/without Sorafenib. MTT detected the proliferation change. (D) Hep G2 cells were virally transduced with shSOX9, then cells exposed to hypoxia and normoxia and treated with/without Sorafenib. MTT detected the proliferation change.

### *3.10. Preclinical study using the in vivo mouse model to demonstrate the role of AR/miR-520f-3p/SOX9/CSC signaling in the HCC progression*

Finally, to confirm the above *in vitro* data in the *in vivo* animal model, we applied the preclinical study using the orthotopic xenografts of the SK-HEP-1 cells expressing firefly luciferase and tranfected with vector control or oe-miR-520f-3p. These HCC cells were first divided into 4 treatment groups, vector control with (Group 2) or without (Group 1) hypoxia for 48 h, and oe-miR-520f-3p group with (Group 4) and without (Group 3) hypoxia for 48 h and then cells were inoculated into the left lobes of liver capsules of nude mice and tumor sizes and metastases were monitored weekly *via* In Vivo Imaging Systems (IVIS) analysis. Once the tumor formation was detectable, we also compared the efficacy of Sorafenib in the groups 2 *vs* 4 (hypoxia with and without miR-520f-3p). After 8 weeks, we sacrificed the mice and examined the tumor weights and numbers of metastases.

The results revealed that xenografts of HCC cells with oe-miR-520f-3p (pre-cultured under hypoxia or normoxia) had significantly slower tumor growth and metastasis than the control group. After treating with Sorafenib 30 mg/kg/day for 4 weeks, the tumor size reduction was more significant in cells with oe-miR-520f-3p group than the control group (Fig. 8A). After 8 weeks, we sacrificed the mice and both the tumor volume and weight measurement also confirmed the conclusion that oe-miR-520f-3p suppressed tumor growth under both normoxia and hypoxia pre-culture conditions. In addition, with Sorafenib treatment of 30 mg/kg for 4 weeks, the tumor size reduction was more significant in groups with oe-miR-520f-3p xenografts than in the control group (Fig. 8B–D). The IHC staining also indicated that AR expression decreased dramatically in tissues of mice xenografts with cells pre-cultured under hypoxia condition, while SOX9 expression increased in this hypoxia group and decreased dramatically in oe-miR-520f-3p group consistent with the *in vitro* findings (Fig. 8E).

Together, these *in vivo* animal results indicated that AR/miR-520f-3p/SOX9 signaling played a critical role in altering the CSC population *in vivo*, and expression of miR-520f-3p could enhance the Sorafenib efficacy to better suppress the HCC progression.

## 4. Discussion

Hypoxia is a frequent occurrence during HCC progression, or may be a direct consequence of therapeutic procedures like TACE (12). Hypoxia has been linked with a variety of cellular processes such as CSC phenotypes and changes of cellular metabolism, including changes resulting in the Warburg effect [32,33]. Consistent with these findings, we also found that hypoxia alters the efficacy of Sorafenib to suppress the HCC cell growth *via* promoting CSC population evidenced by the sphere formation assays as well as CSC marker genes expression.

The role of androgen/AR signaling in HCC initiation and progression appears to be contradictory. Studies with genetically modified animals with altered AR expression indicated that AR promoted HBV-, HCV-, and carcinogen-induced HCC initiation [34–36]. However, AR has been shown to suppress HCC cell invasion and metastasis *in vivo* at a late stage [10,29]. In our study, we found that hypoxia could decrease AR protein expression to increase the CSC phenotype/population in HCC cells, consistent with AR being a tumor suppressor for late stage HCC. At the same time, our studies can not exclude a possibility that hypoxia might mediate Sorafinib resistance *via* AR/SOX9 signaling independently of the latter's ability to increase CSC.

It is well known that miRNAs play crucial regulatory roles affecting HCC development and progression [37] by directly interacting with specific mRNAs through base pairing, then inhibiting the expression of targets [38]. The miRNAs can be regulated during carcinogenesis, and miRNAs can act as oncogenes or tumor suppressor genes due to the nature of their target genes. As a consequence, abnormal miRNAs

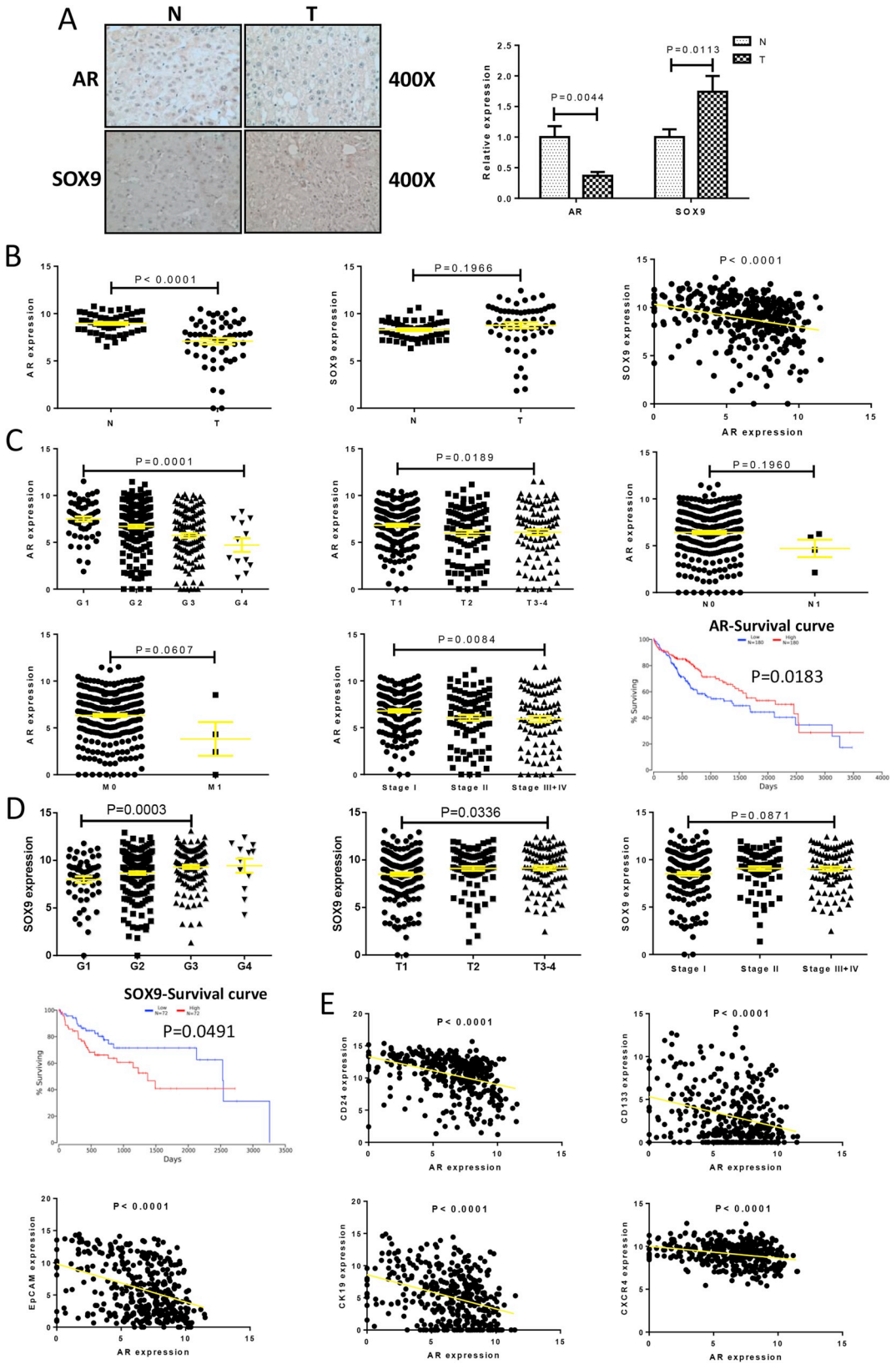


**Fig. 7. Human clinical study linking the AR/miR-520f-3p/SOX9 axis to the HCC progression.** (A) IHC for AR and SOX9 expression in normal (N) tissues and tumor (T) tissues in HCC patients' samples, quantitation at the right. (B) HCC patients' data from TCGA show different expressions of AR (left), SOX9 (middle) in normal (N) tissues and tumor (T) tissues, and the correlation analysis (right) of AR and SOX9. (C) AR expression in different grades G1-4 (upper left), grades T1-4 (upper middle), grades N0-N1 (upper right) and grades M0-M1 (lower left); different stages Stage I-IV (lower middle) of HCC, and survival curve (lower right) showed that patients who have a higher AR expression have a higher survival rate. (D) SOX9 expression in different grades G1-4 (upper left), Grade T1-T4 (upper middle); different stages Stage I-IV (upper right; and survival curve (lower left) showed that patients who have a higher SOX9 expression have a lower survival rate. (E) The correlation between AR and CSC biomarkers, such as *CD24, CD133, EpCAM, CK19* and *CXCR4*. All quantitations are mean ± SD, *p < 0.5, **p < 0.01, ***p < 0.001.

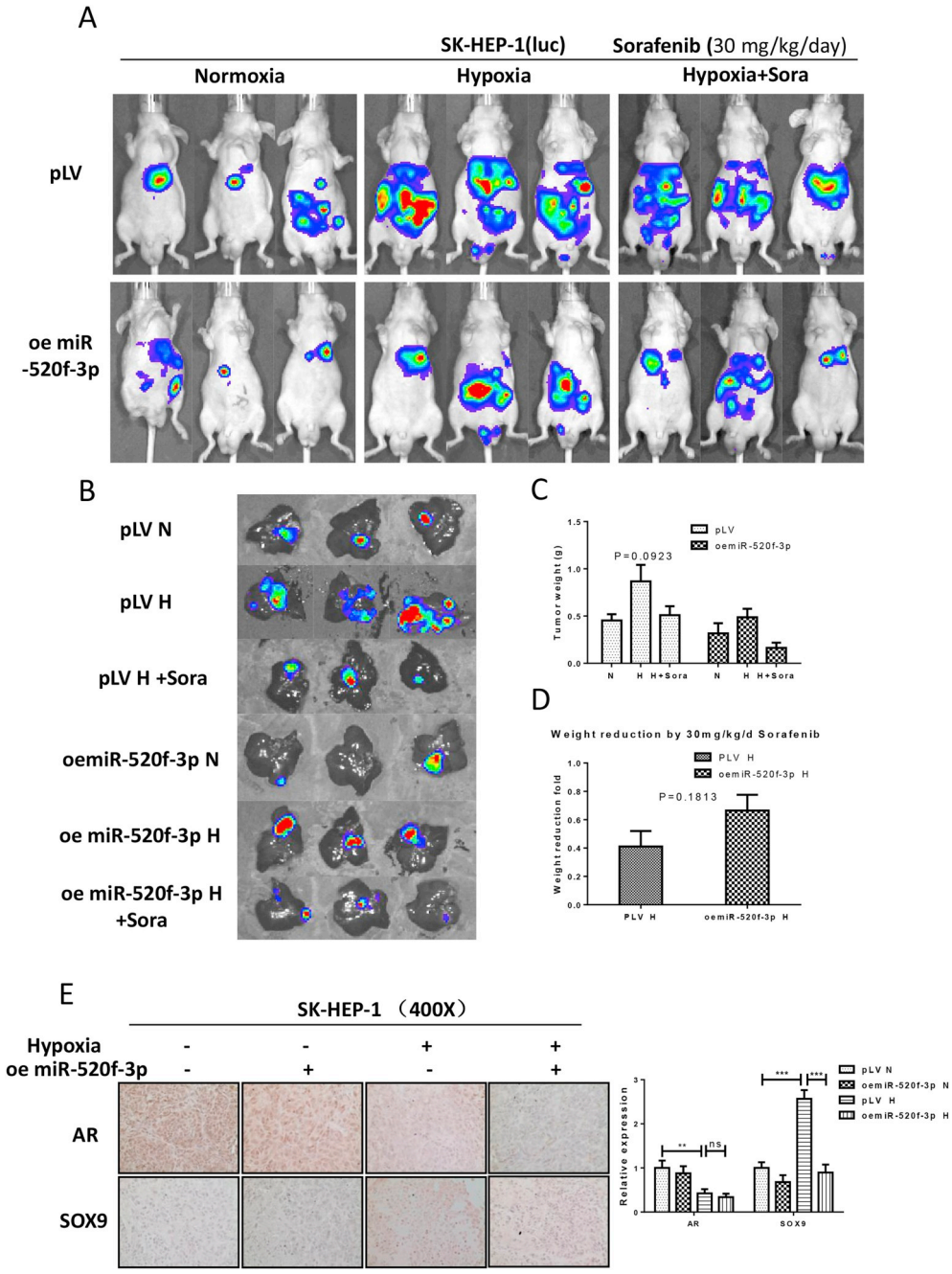


**Fig. 8. Preclinical study using *in vivo* mouse model to demonstrate the role of AR/miR-520f-3p/SOX9/CSC signaling axis in HCC progression.** SK-HEP-1-cells were transduced with Luciferase and with either pLV or oemiR-520f-32 and then grown under normoxia (N) or hypoxia (H) conditions for 48 h. Then mice were injected with the cells for xenografts. After ~2 weeks the mice were treated with/without 30 mg/kg/day Sorafenib (Sora) for 4 weeks. (A) IVIS imaging was used to determine the tumor size and metastasis in mice. (B) After sacrificing the mice the tumor tissues were obtained for imaging. (C) Weights of the xenografts were shown in 6 groups. (D) Weight reductions of the xenografts were shown in two groups after Sorafenib treatment. (E) The IHC for AR, SOX9 of xenografts in four groups. Quantitation is at the right and is mean ± SD, **p < 0.01, ***p < 0.001.

expression is a ubiquitous feature of solid tumors, including HCC [39]. The miR423-5p can be used as a useful tool to predict response to Sorafenib in HCC patients [40]. Dysregulation of miR-520f expression has been investigated in several human cancers and was verified to be involved in the pathological process of tumor development [41,42]. In our study, AR could increase miR-520f-3p expression at a transcriptional level *via* binding to the ARE located in the 5′ promoter region of its precursor, and miR-520f-3p can directly bind to the 3′UTR of SOX9 mRNA to suppress its protein expression. Whether hypoxia in HCC mainly employs this signaling pathway to regulate the CSC phenotype to confer Sorafenib resistance remains to be examined although our findings supported the notion that miR-520f-3p could suppress HCC CSC formation likely increasing HCC cell sensitivity to the Sorafenib therapy.

During tumorigenesis, SOX9 is up-regulated in a number of tumors and plays an essential role for tumor progression as an oncogene [43]. Connected to this role, it could regulate cellular proliferation, senescence, and self-renewal and it was found that SOX9 is highly expressed in liver CSC and its higher expression is positively correlated with a decreased survival in HCC patients [44]. In addition, high levels of SOX9 expression enhanced the tumorigenic and metastasis-seeding abilities of human breast cancer [45] as well as epithelial-mesenchymal transition (EMT) that is positively associated with CSC [45,46]. SOX9 could affect cervical cancer chemoresistance to Cisplatin [47] while in glioma, SOX9 could promote metastasis, which could be attributed to the ability of SOX9 to increase cell migration, invasion, and stimulate the EMT process [48]. Our data showed that AR could alter hypoxia-induced liver CSC formation *via* regulating SOX9 expression *via* altering the miRNA-520f-3p expression.

Drug resistance can be induced by many factors, and accumulating evidence has established that the CSC may contribute to the drug resistance and recurrence in cancer [49,50]. Indeed, the CSC population

is more resistant to conventional cancer therapeutics than the non-CSC population [51], partly attributable to a lower reactive oxygen species (ROS) level attained in the CSC population. This lowered ROS level protects cells from therapeutics-induced DNA damage, therefore they are more resistant to conventional chemotherapies. Clinically, we can increase the sensitivity of chemotherapy drugs by reducing the production of stem cells.

In conclusion, hypoxia may increase HCC CSC population *via* altering the AR/miR-520f-3p/SOX9 signaling, and AR may alter the miR-520f-3p/SOX9 signaling through transcriptional regulation *via* binding to the AREs on the promoter region of miR-520f, which may then suppress SOX9 mRNA translation through targeting its 3′ UTR. A potential therapy to target this newly identified signaling may help improve the targeted therapy with Sorafenib to better suppress the HCC progression.

## Conflicts of interest

The authors declare no potential conflicts of interest.

## Acknowledgement

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.004.

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