SOX9 in prostate cancer is upregulated by cancer-associated fibroblasts to promote tumor progression through HGF/c-Met-FRA1 signaling

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Transcription factor SOX9 was a biomarker for prostate cancer (Pca) with poor prognosis. Nevertheless, the regulatory mechanism underlying SOX9 upregulation still remains unclear. Several cytokines have been reported to be involved in the regulation of SOX9, suggesting that cancer-associated fibroblasts (CAFs), one of the main sources of secreted factors in the tumor microenvironment (TME), may play a role in regulating SOX9 expression. Herein, an in vitro model of paracrine interaction between primary CAFs and Pca cells was applied to investigate the molecular mechanism of SOX9 upregulation during Pca progression. The regulatory axis was validated by in vitro experiments and The Cancer Genome Atlas data. Conditional medium of CAFs (CAF-CM) upregulated the expression of SOX9, which was mutually proved to be essential for CAF-induced tumor progression. Further analysis showed that hepatocyte growth factor (HGF) secreted by CAFs was responsible for SOX9 elevation in Pca cells, via the activation of c-Met signaling. Mechanistically, HGF/c-Met signaling specifically activated MEK1/2-ERK1/2 pathway, which induced phosphorylation and upregulation of FRA1, which then transcriptionally upregulated SOX9 by binding to the promoter of SOX9 gene. Moreover, we identified that HGF/c-Met-ERK1/2-FRA1-SOX9 axis was relatively conserved between human and mouse species by validating in mouse Pca cells. Our results reveal a novel insight into the molecular mechanism that SOX9 in Pca cells is promoted by CAFs through HGF/c-Met signaling to enroll the optimal Pca patients for HGF/c-Met inhibition treatment, since it is much more stable and easier to detect.

Abbreviations
- AR, androgen receptor
- CAF-CM, conditional medium of Pca CAFs
- CAFs, cancer-associated fibroblasts
- HGF, hepatocyte growth factor
- IHC, immunohistochemistry
- NF-CM, conditional medium of NFs
- NFs, normal fibroblasts
- Pca, prostate cancer
- SOX, Sry-related high-mobility group box
- TCGA, The Cancer Genome Atlas
- TME, tumor microenvironment
- TRE, TPA-responsive element
Introduction

SOX9 is a transcription factor belonging to the SOX (Sry-related high-mobility group box) family, which is a key regulator involved in embryonic development including sex determination [1], chondrocyte differentiation [2], neurogenesis–gliogenesis switch [3], cardiogenesis [4], and prostate development [5,6]. Previous studies showed that SOX9 played an important role in the initial stage of prostate development. Mice with conditional Sox9 knockout in prostate showed severe defect in the development of ventral prostate and abnormal differentiation of anterior prostate [5]. It is a common occurrence that genes or pathways involved in embryogenesis are reactivated in cancer initiation and development. Several studies have indicated that Sox9 was reactivated in prostate cancer (Pca) and played an essential role in the initiation and progression of Pca [6–9]. Thomsen et al. [8] observed that Sox9 elevation in mouse prostate could facilitate Pca initiation in cooperation with PTEN loss. Wang et al. [7] found that upregulation of SOX9 promoted Pca cell proliferation and invasion, as well as angiogenesis, indicating that SOX9 contributed to Pca progression. Further investigations showed that SOX9/Sox9 was detected at higher frequency in recurrent Pca [10] and strikingly upregulated in castration-resistant tumors of PTEN/Trp53 null mice [11]. When Sox9 was knocked down in androgen-independent Pca cells, it obviously weakened the clonogenicity in castrated culture medium and tumor growth in castrated mice [12].

Despite the significance of SOX9 in Pca, the key mechanism underlying SOX9 upregulation still remains unclear. A previous study revealed that SOX9 expression in Pca was regulated by Wnt/β-catenin signaling. However, the exact mechanism was not further investigated [10]. Cai et al. found that in TMPRSS2:ERG fusion-positive Pca cells, such as VCaP, SOX9 expression could be upregulated by ERG through opening an androgen receptor (AR)-regulated enhancer downstream of the SOX9 gene. However, this mechanism was strictly restricted to the ERG and AR double-positive Pca cells [9].

There has been increasing evidence implying that cytokines such as FGFs [13,14], TGF-β1 [15,16], and BMPs [17,18] are involved in the regulation of SOX9 expression. It is well known that large amounts of cytokines distributed in the tumor microenvironment (TME) play important roles in cellular communications. As one of the most abundant stromal cells in TME, cancer-associated fibroblasts (CAFs) have been demonstrated to promote tumorigenesis and progression of multiple tumors including Pca [19–24] through paracrine effects. Kinds of cytokines secreted by CAFs have been reported to contribute to cancer cell stemness through activating self-renewal pathways including Wnt, Notch, and Shh signaling [25–27]. SOX9 is also well known to be involved in maintaining stem cell populations in normal tissues [10,13,28,29] and cancer cell stemness [12,30]. In addition, SOX9 ChIP sequencing analysis and transcriptome profiling of Pca cells showed that SOX9 positively activated various Wnt pathway genes to drive invasiveness of Pca [31]. In light of these findings, we hypothesized that SOX9 elevation in Pca cells might be regulated by secreted cytokines from CAFs, and might mediate the tumor-promoting effects of CAFs.

This study was therefore designed to investigate the possible mechanism of SOX9 elevation by CAFs through an in vitro model of paracrine interaction between primary CAFs and Pca cells. To the best of our knowledge, this is the first study to illustrate the regulatory mechanism of upregulated SOX9 by CAFs during the progression of Pca.

Results

SOX9 elevation in Pca is positively correlated with cancer aggressiveness

In order to verify the clinical significance of SOX9 in human Pca, we examined SOX9 expression in specimens of radical prostatectomy from a cohort of 108 consecutive Pca patients by IHC staining. For pathological analysis, Pca with higher Gleason scores had higher expression of SOX9 (Fig. 1A,B). Particularly, specimens with Gleason scores no less than 8 showed significantly higher expression of SOX9 than 3 + 3, 3 + 4, and 4 + 3 (Fig. 1B). Moreover, Pca with advanced pathological stage (III and IV) showed higher SOX9 expression (Fig. 1C). The Spearman correlation analysis indicated that SOX9 expression was significantly associated with the percentage of Ki67-positive tumor cells (Spearman r = 0.301, P = 0.0017, n = 108; Fig. 1D). In addition, high SOX9 IHC staining in Pca patients was positively associated with Gleason scores (P = 0.004), seminal vesicle invasion (P = 0.040), and nerve invasion (P = 0.019; Table 1). These results validated previous reports [7,8,10] that SOX9 levels correlated with Pca progression.

To explore the possible roles of SOX9 in Pca tumorigenesis and progression, we assessed the effects of SOX9 on proliferation, migration, invasion, and stemness in Pca cells. DU145 cells transiently transfected
with pcDNA3.1(+)-SOX9 (Fig. 2A,B) showed a remarkably enhanced growth rate compared with the negative control group (Fig. 2C). Transwell assay revealed that forced expression of SOX9 notably accelerated cell migration and invasion of DU145 cells (Fig. 2D,E). Spheroid formation assay indicated that overexpression of SOX9 significantly increased both the size and the number of cell spheres (Fig. 2F,G). Consistently, immunoblotting analysis showed that SOX9 overexpression led to the increase in MMP1 (metastasis-associated protein) and CD44, CD133, c-Myc, Oct4, and ALDH1A1 (tumor stemness-associated markers; Fig. 2H,I). Then, we knocked down SOX9 by siRNA (siSOX9-1 and siSOX9-2) in another Pca cell line PC3, which had a higher endogenous expression of SOX9 (Fig. 2J,K). Compared with negative control siRNA (siNC), in vitro assays revealed that SOX9 knockdown markedly inhibited cell growth (Fig. 2L), as well as cell migration, invasion (Fig. 2M,N), and cancer stemness (Fig. 2O,P). In addition, decreased protein levels of CD44, CD133, and c-Myc were also detected in the SOX9 knockdown cells (Fig. 2Q).

To investigate whether forced expression of SOX9 promotes tumor growth of Pca cells in vivo, DU145 cells stably overexpressing SOX9 or vector control (Fig. 2R) were injected subcutaneously in nude mice. Compared with the control group, xenografts of SOX9-overexpressing cells had greater tumor volumes and growth rates (Fig. 2S,T). Moreover, IHC staining showed that overexpression of SOX9 promoted the expression of Ki67 and CD44 and CD133 in DU145 xenograft tumors (Fig. 2U,V).

SOX9 in Pca is upregulated by CAFs and essential for CAF-induced tumor-promoting effects

To assess whether SOX9 in Pca cells could be regulated by paracrine factors from CAFs, we isolated CAFs and corresponding normal fibroblasts (NFs) from fresh prostate biopsy specimens (Fig. 3A). Immunofluorescence staining showed that in the biopsy tissues of both normal prostate cancer and prostate cancer, epithelial cells expressed E-cadherin prominently, while stromal cells expressed fibronectin markedly (Fig. 3B). As for CAF-specific biomarker, α-SMA was barely detected in the stromal area of normal tissues, but strongly detected in the prostate cancer stromal and nearly colocalized with SOX9.
with fibronectin (Fig. 3B). Cell immunofluorescence (Fig. 3C), western blotting (Fig. 3D), and qRT-PCR (Fig. 3E) were also performed to verify the purity of cultured fibroblasts. Altogether, the fibroblast identity of NFs and CAFs has been confirmed and found to be sustaining after several passages (data not shown). Then, we verified the tumor-promoting effects of CAFs by wound-healing assay, transwell assay, and spheroid formation assay, and it revealed that treatment with CAF-CM remarkably increased the abilities of cell migration, invasion (Fig. 4A–D), and cancer stemness (Fig. 4E,F), in comparison with both control medium and conditional medium from NFs (NF-CM). Consistently, western blotting showed that MMP1 and MMP3, and markers of cancer stemness (CD44, CD133) were elevated in the CAF-CM-treated cells (Fig. 4G).

To investigate the paracrine effects of CAFs on SOX9 expression in Pca cells, CAF-CM and NF-CM were added to DU145 and 22Rv1 cells for 12 and 24 h. Western blotting and qRT-PCR revealed that both CAF-CM and NF-CM increased SOX9 protein expression and mRNA level, but the effect of CAF-CM was more apparent (Fig. 5A,B). These results suggested that paracrine factors from stromal fibroblasts upregulated SOX9 in Pca cells. Since CAFs promoted both the aggressiveness of Pca and the expression of SOX9, we wondered whether SOX9 played the role as a bridge in the process of CAF-mediated tumor promotion. RNA interference involving three siRNA targeting different regions was employed to knock down SOX9 expression, and then, transwell invasion assay and spheroid formation assay showed that knockdown of SOX9 significantly reduced the capabilities of cell invasion and sphere formation potentiated by CAF-CM (Fig. 5C–F). Concomitantly, western blotting analysis further showed that protein expressions of CD44 and CD133 were reduced in SOX9 knockdown cells (Fig. 5G). These data implied that SOX9 played an essential role in the CAF-mediated Pca progression.

HGF is a key paracrine factor secreted by CAFs to upregulate SOX9 in Pca cells

In order to identify the key paracrine factors secreted by CAFs involved in the activation of SOX9, a homemade qRT-PCR array consisting of 52 selected TME-related secretory factors was applied to screen in DU145 cells and three pairs of NFs and CAFs for the possibly responsible factors. Hierarchical clustering and heatmap indicated that in the genes that barely expressed in DU145 cells but highly expressed in fibroblasts, HGF was the most upregulated one in CAFs compared with NFs (Fig. 6A). Considering HGF and its tyrosine kinase receptor c-Met are known to be involved in promoting Pca progression [32,33], we focused our attention on the role of HGF in CAF-mediated SOX9 upregulation and tumor-promoting effects. Firstly, qRT-PCR results verified that mRNA level of HGF in CAFs was about 13-fold higher than in NFs, but barely detected in DU145 cells (Fig. 6B). ELISA showed that no or negligible levels of HGF were produced by Pca cells (22Rv1, PC3, DU145), while all CAFs secreted more abundant HGF compared with their paired NFs (Fig. 6C), suggesting that HGF was preferentially secreted by CAFs in TME. Consistently, western blotting analysis confirmed a

### Table 1. Clinicopathologic characteristics of Pca cohort (n = 108) with SOX9 expression.

| Clinicopathologic features | SOX9 IHC staining | P value |
|---------------------------|-------------------|---------|
| Age (year)                |                   |         |
| ≤ 70                      | 28 (46.7%)        | 0.897   |
| > 70                      | 32 (53.3%)        |         |
| PSA (ng·mL⁻¹)             |                   |         |
| ≤ 10                      | 16 (26.7%)        | 0.072   |
| > 10                      | 25 (41.7%)        |         |
| ≥ 20                      | 19 (31.7%)        |         |
| Gleason scores            |                   | 0.004   |
| 3 + 3                     | 13 (21.7%)        |         |
| 3 + 4                     | 27 (45.0%)        |         |
| 4 + 3                     | 15 (25.0%)        |         |
| ≥ 8                       | 5 (8.3%)          |         |
| Pathological stage        |                   | 0.063   |
| I + II                    | 44 (73.3%)        |         |
| III + IV                  | 16 (26.7%)        |         |
| T stage                   |                   | 0.161   |
| pT1 + T2                  | 45 (75.0%)        |         |
| pT3 + T4                  | 15 (25.0%)        |         |
| Bilaterally involved      |                   | 0.778   |
| No                        | 10 (16.7%)        |         |
| Yes                       | 50 (83.3%)        |         |
| Seminal vesicle invasion  |                   | 0.040   |
| No                        | 53 (88.3%)        |         |
| Yes                       | 7 (11.7%)         |         |
| Spermaduct invasion       |                   | 0.698   |
| No                        | 57 (95.0%)        |         |
| Yes                       | 3 (5.0%)          |         |
| Nerve invasion            |                   | 0.019   |
| No                        | 14 (23.3%)        |         |
| Yes                       | 46 (76.7%)        |         |
| Vessel carcinoma embolus  |                   | 0.898   |
| No                        | 52 (86.7%)        |         |
| Yes                       | 8 (13.3%)         |         |
| AR IHC staining           |                   | 0.224   |
| Negative of Low           | 37 (61.7%)        |         |
| High                      | 23 (38.3%)        |         |

The P values were analyzed by the chi-square test or Fisher’s exact test, and bold values mean P < 0.05.
SOX9 in Pca is regulated by CAFs via HGF-FRA1 axis

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SOX9 overexpression promotes Pca progression in vitro and in vivo. (A, B) Efficiency of transient overexpression of SOX9 in DU145 cells was validated by qRT-PCR (A) and western blotting (B). (C) MTT assay showed that forced expression of SOX9 obviously accelerated the proliferation of DU145 cells. (D, E) SOX9 overexpression significantly promoted migration and invasion of DU145 cells by transwell assay. (F, G) Spheroid formation assay demonstrated that SOX9 augmentation increased both the size and the number of cell spheres. (H, I) Levels of proteins related to metastasis (H) and cancer stemness (I) were detected by western blotting in DU145 cells after overexpressing SOX9. (J, K) SOX9 mRNA and protein levels in PC3 cells transiently transfected with siNC, siSOX9-1, and siSOX9-2 were analyzed by qRT-PCR (J) and western blotting (K). (L) MTT assay indicated that SOX9 knockdown significantly suppressed cell proliferation. (M–P) The effects of SOX9 knockdown in PC3 cells on migration, invasion, and sphere formation were measured by transwell assay (M, N) and spheroid formation assay (O, P). (Q) Western blotting analysis showed that cancer stemness markers in PC3 cells with SOX9 knocked down were obvious decreased. (R) The efficiency of SOX9 overexpression in lentiviral infected DU145 cells was validated by western blotting. (S) The growth rates of subcutaneous tumors formed of control (n = 6) or SOX9-overexpressing (n = 6) DU145 cells. (T) Photograph of dissected tumors in control or SOX9-overexpressing groups. (U) Representative pictures of HE staining and IHC staining of SOX9, Ki67, CD44, and CD133 in the xenografts. Scale bar represents 50 μm. (V) Percentage of Ki67-positive cells and semi-quantitation of CD44 and CD133 IHC staining in the transplanted tumors. Each in vitro experiment was repeated for three times independently, and a representative result was presented in the figure. Values were expressed as mean ± SD and analyzed by the unpaired t-test between two groups. *P < 0.05; **P < 0.01; ***P < 0.001.

strong increase in c-Met phosphorylation (Tyr1234/1235) in DU145 and 22Rv1 cells following the treatment with CAF-CM compared with NF-CM, while c-Met remained nearly unphosphorylated in control medium (Fig. 6D), which suggested that tyrosine residues 1234 and 1235 of c-Met in Pca cells could not autophosphorylate without stimulation by stromal fibroblasts. The correlation between the expression of HGF and ACTA2 (which encodes α-SMA) was evaluated by Pearson’s correlation analysis using mRNA expression profiles of Pca from TCGA, and it turned out that HGF expression was significantly correlated with ACTA2 (Pearson’s r = 0.406, P < 0.0001, n = 499; Fig. 6E).}

Previous studies have demonstrated that HGF/c-Met played an important role in tumorigenesis and tumor progression of Pca [32,33]. Herein, we treated DU145 and 22Rv1 cells with recombinant human HGF (PeproTech, 100-39H, Rocky Hill, NJ, USA) in vitro, and the abilities of cell migration, invasion, and sphere formation were detected. We found that 10 ng·mL⁻¹ HGF was sufficient to significantly increase the number of migrated cells or invaded or to the down surface of transwell membrane (Fig. 7A–D), and enhance the number and the size of formed spheroids (Fig. 7E,F). The key role of HGF in the paracrine effects of CAFs was further validated by an ATP-competitive c-Met inhibitor, capmatinib (INCB28060; Sellick Chemicals, S2788, Boston, MA, USA). In vitro assays showed that the inhibition of HGF/c-Met pathway in DU145 and 22Rv1 cells with pretreatment of 10 nM capmatinib remarkably abrogated the effects of CAF-CM on cell migration, invasion (Fig. 7G–J), and tumor-initiating abilities (Fig. 7K,L).

Since HGF has been identified as a key secretory factor in CAF-mediated tumor promotion, and SOX9 was also verified essential for Pca progression induced by CAFs, we wonder whether there was a connection between HGF and SOX9 expression. Western blotting analysis demonstrated that treatment with 30 ng·mL⁻¹ recombinant human HGF obviously upregulated SOX9 expression since 1 h upon stimulation in both DU145 and 22Rv1 cells (Fig. 8A). 10 ng·mL⁻¹ HGF was already sufficient to induce the increase in SOX9 expression, as well as 30 ng·mL⁻¹ HGF (Fig. 8B). When capmatinib was combined in the HGF or CAF-CM treatment, the upregulation of SOX9 caused by HGF or CAF-CM was markedly abrogated (Fig. 8B, C). In addition, in vivo experiments were performed to further investigate the comprehensive role of HGF/c-Met signaling in the regulation of tumor growth and SOX9 expression. DU145 cells alone or accompanied by equivalent CAFs were injected subcutaneously into nude mice, and 3 weeks later, vehicle or capmatinib was given to the tumor-bearing mice twice a day. Compared with the DU145-alone group, tumors of DU145 with CAFs had more rapid growth rates and greater tumor sizes, while capmatinib treatment significantly abrogated the CAF-mediated acceleration of tumor growth (Fig. 8D,E). IHC staining demonstrated that SOX9 expression in Pca cells was upregulated by CAFs in vivo and could be abolished by capmatinib, which strongly inhibited the phosphorylation of c-Met in Pca cells (Fig. 8F). Correspondingly, Ki67 and CD44 showed a similar pattern to SOX9 (Fig. 8F). Taken together, HGF plays a central role in upregulating SOX9 in Pca cells and mediating the tumor-promoting effects of CAFs.
SOX9 in Pca is regulated by CAFs via HGF-FRA1 axis

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Fig. 3. Characterization of targeted Pca biopsy tissues and isolated fibroblasts. (A) Representative images of mpMRI (a–c) and HE staining of pathological tissue section of prostate (d). ADC, apparent diffusion coefficient; DWI, diffusion-weighted image; T2WI, T2-weighted image. The arrows indicated the main tumor focus. (B) Immunofluorescence staining showed the cellular location and expression level of E-cadherin, fibronectin, and α-SMA in normal prostate biopsy tissues and prostate cancer biopsy tissues. Scale bar represents 50 μm. (C) Cell immunofluorescence staining demonstrated that the established CAFs showed high expression of α-SMA compared with corresponding NFs, and DU145 cells were used as an epithelial cell control. Scale bar represents 50 μm. (D) The protein levels of E-cadherin, vimentin, fibronectin, and α-SMA in DU145 cells and four pairs of established primary NFs and CAFs were measured by western blotting. (E) The relative mRNA levels of CAF-specific genes including ACTA2, FAP, S100A4, and TNC in DU145 cells, NFs, and CAFs were detected by qRT-PCR. ACTB was set as the normalization control. Immunofluorescence, western blotting, and qRT-PCR experiments were repeated for three times independently, and a representative result was presented in the figure. Values were expressed as mean ± SD and analyzed by the unpaired t-test between two groups. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 4. CAF-CM enhances the abilities of migration, invasion, and cancer stemness of Pca cells. (A, B) Cell migration affected by NF-CM and CAF-CM was evaluated by wound-healing assay. (C, D) The influence of NF-CM and CAF-CM on cell migration and invasion was measured by transwell assay. (E, F) Spheroid formation assay showed that Pca cells treated with CAF-CM formed significantly more and larger spheroids compared with control media and NF-CM. (G) Markers of cancer metastasis and stemness in Pca cells treated with indicated media were assessed by western blotting. Each in vitro assay involving CAF-CM and NF-CM treatment was conducted by using at least three pairs of CAFs and NFs, and repeated for three times independently, with a representative result presented in the figure. Values were expressed as mean ± SD and analyzed by the unpaired t-test between two groups. *P < 0.05; **P < 0.01; ***P < 0.001.
SOX9 in Pca is upregulated by paracrine factors from CAFs and essential for CAF-mediated tumor progression. (A, B) The protein level (A) and mRNA expression (B) of SOX9 in DU145 and 22Rv1 cells after treatment of control medium or NF-CM and CAF-CM for 12 and 24 h. (C, D) SOX9 knockdown abolished CAF-induced cell invasion of DU145 and 22Rv1 cells. (E, F) Knockdown of SOX9 obviously decreased the sphere size and increased the number by CAF-CM. (G) Western blotting showed that SOX9 knockdown abrogated the enhanced expression of stemness markers (CD44, CD133) induced by CAF-CM. Each in vitro assay involving CAF-CM and NF-CM treatment was conducted by using at least three pairs of CAFs and NFs, and repeated for three times independently, with a representative result presented in the figure. Values were expressed as mean ± SD and analyzed by the unpaired t-test between two groups. *P < 0.05; **P < 0.01; ***P < 0.001.

HGF upregulates SOX9 in Pca via MEK1/2-ERK1/2 pathway

Previous studies have reported that HGF induced dimerization and phosphorylation of c-Met, resulting in the activation of several downstream oncogenic signaling pathways including RAS/MAPK, PI3K/AKT, and STAT3 [34]. To specify which pathway was involved in the regulation of SOX9 expression by HGF, we tested the total expression and phosphorylation level of representative proteins of RAS/MAPK, PI3K/AKT, and STAT3 pathways in DU145 cells treated with 30 ng·mL⁻¹ HGF by western blotting. It turned out that the phosphorylation of ERK1/2 (Thr202/Tyr204) was most enhanced in cells treated with HGF, while phosphorylation of JNK (Thr183/Tyr185) was increased slightly and others including p38, Akt, Stat3, and NF-κB remained unaffected (Fig. 9A). In a time course of HGF treatment, phosphorylation of ERK1/2 in both DU145 and...
22Rv1 cells started rising as early as 10 min upon stimulation and lasted for at least 2 h (Fig. 9B). Further analysis revealed that phosphorylation of ERK1/2 by HGF was dose-dependent, and 30 ng·mL⁻¹ HGF induced a strikingly increased phosphorylation of ERK1/2, which could be abolished by capmatinib pretreatment (Fig. 9C). As expected, CAF-CM also caused a remarkable enhancement of ERK1/2 phosphorylation compared with the control medium and NF-CM, which could also be abolished by capmatinib (Fig. 9D,E). Then, MEK1/2 inhibitor U0126 (Selleck Chemicals, S1102) and ERK1/2 inhibitor SCH772984 (Selleck Chemicals, S7101) were adopted to block MEK1/2-ERK1/2 pathway, and we found that the
Fig. 7. CAFs promotes migration, invasion, and sphere formation of Pca cells through HGF/c-Met signaling. (A–D) The abilities of migration and invasion of Pca cells treated with recombinant human HGF and capmatinib were measured by transwell migration (A, B) and invasion (C, D) assay. (E, F) Sphere formation ability of Pca cells treated with recombinant human HGF and capmatinib was evaluated by spheroid formation assay. (G, H) Wound-healing assay showed that the enhanced migration of Pca cells by CAFs was abolished by capmatinib. (I, J) Transwell assay also indicated that capmatinib could abrogate CAF-induced migration and invasion of Pca cells. (K, L) Spheroid formation assay demonstrated that blocking HGF/c-Met signaling could reduce the size and the number of formed spheres of Pca cells. Each in vitro assay was repeated for three times independently, and experiments involving CAF-CM treatment were conducted by using at least three batches of CAFs derived from different patients. A representative result was presented in the figure. Values were expressed as mean ± SD and analyzed by the unpaired t-test between two groups. *P < 0.05; **P < 0.01; ***P < 0.001.
upregulation of SOX9 induced by HGF or CAF-CM was obviously abrogated (Fig. 9F,G). In addition, when MEK1/2-ERK1/2 pathway was inhibited, it repressed the reinforced abilities of cell invasion and sphere formation induced by HGF (Fig. 9H–K). These results implied that MEK1/2-ERK1/2 pathway was responsible for the SOX9 upregulation and the tumor-promoting effects induced by HGF secreted from CAFs.

**FRA1 is the downstream effector of MEK1/2-ERK1/2 signaling and transcriptionally upregulates SOX9**

Previous reports showed that HGF treatment in hepatocellular carcinoma cells enhanced the activity of AP-1 proteins, which then mediated HGF-induced effects [35–37]. Dimers of the FOS and JUN family proteins bind to the TPA-responsive element (TRE, 5'-TGAG/CGT-3').

![Fig. 8. HGF upregulates SOX9 in Pca cells and promotes tumor growth in vivo.](image)

(A, B) Western blotting showed that recombinant human HGF induced continuous SOX9 elevation in Pca cells (A) and upregulation of SOX9 by HGF was in a dose-dependent fashion (B). (C) Western blotting and qRT-PCR showed that capmatinib abrogated SOX9 upregulation, which was induced by CAF-CM. (D) Dissected xenografts collected at the end point formed of DU145 cells with vehicle (n = 5) treatment, and DU145 cells were accompanied by equivalent CAFs with vehicle (n = 5) or capmatinib (n = 5) treatment. (E) The growth rates of subcutaneous xenografts in the three groups. (F) Representative images of IHC staining of phos-c-Met (Tyr1234/1235), SOX9, Ki67, and CD44 in the xenografts. Scale bar represents 50 μm. Western blotting and qRT-PCR were repeated for three times independently, and experiments involving CAF-CM treatment were conducted by using at least three batches of CAFs derived from different patients. A representative result is presented in the figure. Values were expressed as mean ± SD and analyzed by the unpaired t-test between two groups. *P < 0.05; **P < 0.01; ***P < 0.001.
CTCA-3') sequence and transcriptionally upregulate target genes. Since previous research has demonstrated that putative AP-1 binding sites were found in the promoter region of mouse Sox9 gene [38], we deduced that AP-1 transcription factors may be the downstream mediators of activated MEK1/2-ERK1/2.
signaling to upregulate SOX9. In order to identify which API proteins were specifically activated by HGF, we analyzed the protein expression of all the members of AP-1 family, including c-Jun, JunB, JunD, c-Fos, FosB, Delta FosB, FRA1, and FRA2, in DU145 cells treated with 30 ng-mL\(^{-1}\) HGF. The results revealed an obvious increase of FRA1 protein level, while the other members remained nearly unaffected (Fig. 10A). Further investigation showed that HGF treatment induced remarkable phosphorylation (Ser265) and increase in total expression of FRA1, as early as 10 min in DU145 cells and 30 min in 22Rv1 cells after HGF exposure, indicating that the induction of phosphorylation and upregulation of FRA1 protein was quite rapid (Fig. 10B). Similar to the patterns of phosphorylation and upregulation of FRA1 cells after HGF exposure, indicating that the induction of phosphorylation and upregulation of FRA1 by HGF were also in a dose-dependent fashion, and the markedly phosphorylation and upregulated expression of FRA1 induced by 30 ng-mL\(^{-1}\) HGF could be restored by capmatinib similar to that observed in the control treatment (Fig. 10C). Correspondingly, CAF-CM induced higher upregulation of FRA1 protein expression and its phosphorylation level compared with NF-CM (Fig. 10D). When capmatinib was preadded along with CAF-CM, the levels of phos-FRA1 and total FRA1 expression were decreased to levels in the control treatment (Fig. 10E). U0126 and SCH772984 were used to inhibit MEK1/2-ERK1/2 pathway; then, both the phosphorylation and upregulation of FRA1 stimulated by HGF treatment induced remarkable phosphorylation level compared with NF-CM (Fig. 10D). U0126 and SCH772984 were used to inhibit MEK1/2-ERK1/2 pathway; then, both the phosphorylation and upregulation of FRA1 stimulated by HGF were abolished obviously (Fig. 10F,G). These data suggested that FRA1 was a downstream factor activated by CAF-secreted HGF through MEK1/2-ERK1/2 pathway.

To evaluate the hypothesis that FRA1 upregulates SOX9, we transiently overexpressed FRA1, and FOS and JUN in DU145 cells (AR-negative). Forced expression of FRA1, FOS, and JUN all increased the protein level of SOX9, but FRA1 had the most obvious upregulating effect (Fig. 10H, left). qRT-PCR also showed a similar outcome that FRA1 increased SOX9 mRNA level most efficiently (Fig. 10H, right). In another two AR-positive Pca cell lines, 22Rv1 and LNCaP, SOX9 expression also increased when FRA1 was overexpressed (Fig. 10I), indicating that upregulation of SOX9 by FRA1 was AR-independent. When FRA1 was depleted by specific siRNA, the upregulation of SOX9 induced by HGF or CAF-CM was obviously abrogated (Fig. 10J,K). Interestingly, FRA1 knockdown also visibly decreased the phosphorylation level of c-Met (Tyr1234/1235), suggesting that a positive feedback loop may exist between HGF/c-Met pathway and FRA1 expression (Fig. 10J,K). To investigate the transcriptional regulation of SOX9 by FRA1, we analyzed the sequence of human SOX9 promoter, and one TRE sequence (−551 ~ −545) was found (Fig. 11A). ChIP assay was performed using specific FRA1 and phos-FRA1 (Ser265) antibodies in DU145 cells stably overexpressing FRA1, and the precipitated DNA was amplified by two sets of primers across TRE sequence. The results showed both FRA1 and phos-FRA1 (Ser265) could bind to the target segment more than normal rabbit IgG (Fig. 11B,C), which indicated that FRA1 could transcriptionally upregulate SOX9. Further investigation showed that in DU145 cells treated with HGF (Fig. 11D) or CAF-CM (Fig. 11E), colocalization of FRA1 and SOX9 within the nuclei was quite apparent, and since 3 h after CAF-CM stimulation, FRA1 and SOX9 expression started increasing (Fig. 11E). In addition, Pca cells with FRA1 overexpressed formed larger xenograft tumors in mice compared with the control group (Fig. 11F-J). These data collectively indicated that FRA1 was the downstream effector of MEK1/2-ERK1/2 activated by HGF and transcriptionally mediated SOX9 upregulation.

**HGF/c-Met-FRA1-SOX9 axis is validated in mouse Pca cells and TCGA database**

On the one hand, mouse Hgf shares remarkable homology with human HGF at both mRNA (percent identity: about 88%) and protein (percent identity: higher than 90%) levels, and human and murine HGF/Hgf are cross-reactive. On the other hand, TRE sequences were found in the promoter region of both human and mouse SOX9/Sox9 gene (Figs 11A and 12E). In addition, binding site B (~600 bp ~ −593 bp) in the mouse Sox9 promoter was found conserved with that found in the human SOX9 promoter (~551 ~ −545). Therefore, we assumed that Hgf/c-Met-Erk1/2-Fra1 signaling was also responsible for the upregulation of Sox9 in mouse. Western blotting was carried out in a mouse Pca cell line, RM-1, and the results showed that recombinant murine Hgf (PeproTech, 315-23) also promoted Sox9 expression through c-Met-Erk1/2-Fra1 axis (Fig. 12A–D). ChIP assay with antibody against phos-Fra1 (Ser265) was performed in RM-1 cells treated with 30 ng-mL\(^{-1}\) Hgf for 6 h, and it turned out that the activated Fra1 could bind to all the three TRE sequence located in the promoter of Sox9 gene (Fig. 12E–G). We also exploited TCGA database and found that Pearson’s correlation analysis showed moderate positive correlation between MET and SOX9 (Pearson’s \(r = 0.268\), \(P < 0.0001\), \(n = 499\);
SOX9 in Pca is regulated by CAFs via HGF-FRA1 axis

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A
HGF (30 ng·mL⁻¹)
  p-c-Met (Tyr1234/1235)
  c-Met
  c-Jun
  JunB
  JunD
  p-FRA1 (Ser265)
  FRA1
  β-actin

DU145
  +
  +
  +
  +
  +
  +
  +

DU145
  +
  +
  +
  +
  +
  +
  +

B
HGF (30 ng·mL⁻¹)
p-FRA1 (Ser265)
FRA1
β-actin

DU145
  0 m
  10 m
  30 m
  1 h
  6 h
  12 h

DU145
  0 m
  10 m
  30 m
  1 h
  6 h
  12 h

E
CAF-CM
Capma
p-FRA1 (Ser265)
FRA1
β-actin

DU145
  +
  +
  +
  +

D
NF-CM
CAF-CM
p-FRA1 (Ser265)
FRA1
β-actin

DU145
  +
  +
  +
  +

G
CAFCM
U0126 (10 μM)
SCH772984 (1 μM)
p-FRA1 (Ser265)
FRA1
β-actin

DU145
  +
  +
  +
  +

H
HGF (30 ng·mL⁻¹)
U0126 (10 μM)
SCH772984 (1 μM)
p-FRA1 (Ser265)
FRA1
β-actin

DU145
  +
  +
  +
  +

C
HGF (ng·mL⁻¹)
  Capma
  p-FRA1 (Ser265)
  FRA1
  β-actin

DU145
  0
  10
  30
  30

DU145
  0
  10
  30
  30

F
HGF (30 ng·mL⁻¹)
U0126 (10 μM)
SCH772984 (1 μM)
p-FRA1 (Ser265)
FRA1
β-actin

DU145
  +
  +
  +
  +

DU145
  +
  +
  +
  +

J
siRNA
HGF (30 ng·mL⁻¹)
p-c-Met (Tyr1234/1235)
c-Met
p-FRA1 (Ser265)
FRA1
SOX9
β-actin

DU145
  siNC
  siNC
  siFRA1-1
  siFRA1-2
  siFRA1-3

K
siRNA
CAFCM
p-c-Met (Tyr1234/1235)
c-Met
p-FRA1 (Ser265)
FRA1
SOX9
β-actin

DU145
  siNC
  siNC
  siFRA1-1
  siFRA1-2
  siFRA1-3

L
22Rv1
Vector
FRA1
SOX9
β-actin

LNCaP
Vector
FRA1
SOX9
β-actin

Vector
3xFGF: c-Fos
3xFGF:FRA1
3xFGF:c-Jun
SOX9
β-actin

Relative mRNA expression

SOX9

1.0
1.5
2.0
2.5

ns
**

5420

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and it promotes Pca growth, migration, invasion, and study, we found that SOX9 is positively correlated regulated by HGF/c-Met-ERK1/2-FRA1 signaling was involved in prostate development and reactivated in Transcription factor SOX9 is a critical regulator (Pearson’s r

Fig. 12H). MET and FRA1 (Pearson’s r = 0.268, P < 0.0001, n = 499; Fig. 12I), and FRA1 and SOX9 (Pearson’s r = 0.312, P < 0.0001, n = 499; Fig. 12J). These results strongly suggested that SOX9 expression regulated by HGF/c-Met-ERK1/2-FRA1 signaling was a relatively common mechanism in Pca cells.

Discussion

Transcription factor SOX9 is a critical regulator involved in prostate development and reactivated in Pca initiation and progression [5–9]. In the current study, we found that SOX9 is positively correlated with the Gleason score and pathologic staging of Pca, and it promotes Pca growth, migration, invasion, and cancer stemness both in vitro and in vivo. Since Pca with higher SOX9 is more aggressive with worse prognosis, it is urgently needed to uncover the key mechanism underlying SOX9 upregulation. Although previous study has revealed that in AR-positive Pca cells with TMPRSS2:ERG gene fusions, SOX9 expression could be upregulated by cooperation of ERG and AR [9], how SOX9 is elevated in AR-negative or TMPRSS2:ERG fusion-negative Pca cells still remains elusive.

Cancer-associated fibroblasts is the main type of stromal cell in TME, providing a supportive microenvironment to induce and maintain more aggressive biological behaviors of cancer cells [39]. Increasing evidences support that CAFs promote tumorigenesis and progression of various cancers including Pca via secreting multiple paracrine factors including TGF-β1, IL-6, HGF, EGF, VEGF, and FGF families [39,40]. Meanwhile, previous studies showed that several kinds of cytokines such as FGFs, TGF-β1, and BMPs induced the upregulation of SOX9 [13–18]. On the basis of these results, we hypothesized that the paracrine effects of CAFs might be involved in upregulating SOX9 in Pca cells. In fact, in vitro model of paracrine interaction between CAFs and Pca cells and in vivo experiments in our study demonstrated that SOX9 expression in Pca cells could indeed be upregulated by the paracrine effects of CAFs, both in AR-positive and in AR-negative Pca cells (22Rv1 and DU145, respectively), suggesting an undiscovered mechanism underlying SOX9 elevation in Pca cells regardless of AR status.

Emerging evidences have demonstrated that HGF/c-Met plays a crucial role in tumorigenesis and cancer progression [41]. In our study, we identified that HGF was the most highly secreted factors by CAFs in TME compared with NFs and Pca cells. Furthermore, HGF was proved to be the key contributor for the upregulation of SOX9 in Pca cells and the tumor progression promoted by CAFs. Since the clinical significance of HGF/c-Met in Pca has been recognized, several agents targeting HGF/c-Met signaling including cabozaantinib, foretinib, crizotinib, and rilotumumab underwent clinical trials [42]. In spite of the strong antitumor effects in preclinical studies, therapeutic agents targeting HGF/c-Met did not provide significant survival benefits for Pca patients in clinical trials [43]. One possible explanation for this seemingly inconsistent result is that enrolled patients in most clinical trials were not highly selected. Patients without activated HGF/c-Met may get very limited benefits from HGF/c-Met inhibition agents. Rhee et al. [37] found that the half-life of phosphorylated c-Met (Tyr1234/1235) is so short as < 10 min that no reliable detection of the phosphorylation status of c-Met could be performed in human specimens. Since the main challenge facing the efficacy of HGF/c-Met inhibition for Pca treatment is optimal patients’ enrollment, while capturing the activation status of HGF/c-Met signaling via detecting phos-c-Met by IHC staining in Pca tissues is quite unpractical, effective markers substituted for activation of HGF/c-Met signaling are urgently needed. Our work revealed that the growth of xenograft tumor with high SOX9 expression upregulated by CAFs could be effectively inhibited by capmatinib (Fig. 8D–F), and in vitro
experiments indicated that HGF induced relatively continuous upregulation of SOX9 in Pca cells (Fig. 8A). Overall, SOX9 may serve as an alternative marker for the activated HGF/c-Met signaling to enroll optimal Pca patients for HGF/c-Met inhibition treatment. Most importantly, SOX9 is much easier to detect, making it an ideal biomarker to predict treatment response of HGF/c-Met inhibition.

The binding of HGF to its receptor c-Met triggers the activation of multiple downstream pathways...
**Materials and methods**

**Cell lines and cell culture**

Four human Pca cell lines, LNCaP, 22Rv1, DU145, and PC3, were obtained from the Cell Bank of Type Culture Collection, Chinese Academy of Science (Shanghai, China). RM-1, a mouse prostate cancer cell line, was purchased from the American Type Culture Collect. All human cell lines have been authenticated using STR profiling within the last 3 years. All experiments were performed with mycoplasma-free cells.

**Clinical Pca tissues and immunohistochemistry (IHC) analyses**

This study was conducted in accordance with the Declaration of Helsinki principles. It was approved by the Ethics Committee of Drum Tower Hospital, Medical School of Nanjing University (Nanjing, China), and was undertaken with the understanding and written consent of each subject. Human Pca specimens along with corresponding clinicopathologic features were collected from January 2011 to January 2014 (n = 108). The IHC staining was performed as described previously [36], and the scores of SOX9 IHC staining were performed independently by two well-trained pathologists. The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), and the staining range of tumor cells was scored as 0 (0%), 1 (1–10%), 2 (11–50%), and 3 (51–100%). The final score was obtained as the multiplication of the intensity score by the staining proportion, and a score ≤ 3 was defined as low expression or negative, while > 3 as high expression.

**Isolation and culture of primary prostate stromal fibroblasts**

Human prostate biopsy specimens were obtained by multiparametric magnetic resonance imaging (mpMRI)-targeted...
Fig. 12. Validation of HGF/c-Met-FRA1-SOX9 axis in mouse Pca cells and Pca mRNA profiles from TCGA. (A) Western blotting showed that recombinant murine Hgf induced upregulation of Sox9 in a time-dependent fashion, as well as phos-Erk1/2 (Thr202/Tyr204), phos-Fra1 (Ser265), and Fra1, in the mouse Pca cell line, RM-1. (B) Increased expression of Sox9 following the treatment of 30 ng·mL⁻¹ Hgf could be abrogated by capmatinib. Expression of phos-Erk1/2 (Thr202/Tyr204), phos-Fra1 (Ser265), and Fra1 presented a similar pattern to Sox9. (C) Inhibition of Mek1/2-Erk1/2 signaling by U0126 and SCH772984 decreased the Hgf-induced upregulation of Sox9, phos-Fra1 (Ser265), and Fra1. (D) Protein expression of Sox9 in RM-1 cells transiently transfected with siRNA targeting Fra1. (E) Three TRE sequences were found in the promoter of Sox9 gene, and binding site B (−600 bp − −593 bp) was conserved among human being and mouse. (F, G) ChIP assay indicated that phos-FRA1 (Ser265) could bind to all the three segments around TRE sequence. Results were normalized to the input. (H–J) Pearson’s correlation analysis between MET and SOX9 (H), MET and FRA1 (I), and FRA1 and SOX9 (J) in Pca data from TCGA. (K) qRT-PCR analysis demonstrated that SOX9 knockdown in PC3 cells significantly decreased the relative mRNA expression of several AP-1 family members, including FRA1, FRA2, and JUN. Western blotting, ChIP assay, and qRT-PCR were repeated for three times independently, and a representative result is presented in the figure. Values were expressed as mean ± SD and analyzed by the unpaired t-test between two groups. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
biopsy technique (Fig. 3A), which provided a powerful guarantee for the accurate acquisition of normal tissues and cancer specimens. All human sample collection protocols were approved by the Ethics Committee of Drum Tower Hospital, Medical School of Nanjing University, and prior written informed consent was obtained from all individuals. All the patients had a higher level of PSA than 10 ng·mL⁻¹ and prominent lesions in prostate, which were found in mpMRI.

Collection of conditioned medium

Pca cells, NFs, or CAFs were seeded into 10-cm dishes at the density of 2 × 10⁶ cells per dish. About 12 h after cell seeding, the culture medium was removed and the cells were washed twice gently with PBS, and then, 8 mL serum-free medium was added. The cells were cultured for another 36 h at 37 °C; then, the medium was collected and centrifuged at about 1000 g for 10 min, and strained through a 0.2-μm sterile filter (Merck Millipore, SLGP033RB, Carrigtwohill, Co. Cork, Ireland) to remove any cells and cell debris, and then stored at −20 °C until ready for use.

MTT assay

(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent (Beyotime, C0009, Shanghai, China) was applied to detect cell viability. The absorbance was detected at 570 nm with a reference wavelength at 630 nm by using microplate reader (TECAN Group Ltd., Infinite M200pro, Männedorf, CH/CHE, Switzerland).

Wound-healing assay

1 × 10⁶ cancer cells were seeded into 6-well plates per well, and when adherent cells grew to about 90% confluency, an artificial wound was scratched with a 200 μL pipette tip. Different conditioned media or chemicals were added after the cells were gently washed twice with PBS; then, images were taken at this time and appropriate end time to estimate the area covered by migrated cells.

Transwell migration and invasion assay

Transwell® inserts with 8.0 μm pore size (Costar, 3422, Kennebunk, ME, USA) were used to evaluate the migration and invasion ability of Pca cells. For migration assay, 5 × 10⁵ cells suspended in 150 μL serum-free medium were seeded in the upper transwell chamber, while for invasion assay, 1 × 10⁵ cells in 150 μL serum-free medium were inoculated in the upper chamber, which was precoated with diluted Matrigel® matrix (Corning, 356234, Bedford, MA, USA). The lower chamber was added with 500 μL complete medium containing 10% FBS. After incubation for appropriate time, cells that migrated or invaded to the lower surface of the polycarbonate membrane were fixed with paraformaldehyde and stained with 0.5% crystal violet staining solution (Beyotime, C0121).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol® reagent (Invitrogen, 1596018, Carlsbad, CA, USA) according to the manufacturers’ protocol. Reverse transcription was performed using PrimeScript® RT Reagent Kit with gDNA eraser (TaKaRa Biotechnology (Dalian), DRR047A, Dalian, China). qRT-PCR was prepared using SYBR® Premix Ex Taq (TaKaRa Biotechnology (Dalian), RR420A) and performed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, 4376600, Foster City, CA, USA). Relative mRNA expression levels were normalized to ACTB by the 2⁻ΔΔC₅T method. All the primer sequences used in the study are listed in Table S1.

qRT-PCR array analysis

Home-made qRT-PCR array was used to evaluate the relative mRNA expression of 52 selected TME-related secretory factors in DU145 cells and three pairs of NFs and CAFs. These secretory factors were chosen by studying the lists of Human Chemokine Array Kit (R&D Systems, ARY017, Minneapolis, MN, USA), Human Cytokine Array Kit.
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(R&D Systems, ARY005B), and Human XL Cytokine Array Kit (R&D Systems, ARY022B) for reference, and further screening through surveying extensive literature focusing on TME research. Total RNA was isolated and reverse-transcribed to cDNA, which then was used for qRT-PCR with specific primers. ACTB was set as an internal control, and adjusted $-$ΔΔCt values were adopted to draw the hierarchical clustering and heatmap by R language.

Protein extraction and western blotting

Cells were lysed in RIPA buffer (Beyotime, P0013) containing protease inhibitor cocktail (Thermo Fisher Scientific, A32955, Waltham, MA, USA) and phosphatase inhibitor cocktail (Thermo Fisher Scientific, A32957). Proteins in the lysates were separated by SDS/PAGE and then transferred onto PVDF membranes. After blocked with 5% nonfat milk, the membranes were probed with diluted primary antibodies overnight at 4 ºC, then washed with PBST three times and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using an electrochemiluminescence system. The information about primary antibodies is listed in Table S2.

Immunofluorescence

For cell immunofluorescence, cells seeded on the culture slides were fixed with 4% paraformaldehyde for 20 min, then washed three times with PBS, and permeabilized with 0.3% Triton X-100 for 10 min. For tissue staining, prostate samples were cut into frozen sections and fixed with acetone for 10 min, then rehydrated in distilled water, and permeabilized in 0.3% Triton X-100 for 20 min. Cells or tissues were incubated with primary antibodies overnight at 4 ºC, then stained with fluorochrome-conjugated secondary antibodies at room temperature for 1 h and counterstained with DAPI for 5 min. Photographs were captured by fluorescence microscope (Olympus, DP72, Tokyo, Japan) or laser confocal microscope (Leica, TCS SP5, Wetzlar, Germany). The antibodies used in the immunofluorescence staining are listed in Table S2.

siRNA oligos, plasmid constructs, and transfection

siRNA against SOX9 and FRA1/Fra1 were designed and synthesized by GenePharma (Shanghai, China). The full length of SOX9 and FRA1, FOS, and JUN coding DNA sequence (CDS) were cloned into pcDNA3.1(+)-vector and pcDNA3.1(+)-3×Flag vector, respectively, to construct the overexpression plasmids pcDNA3.1(+)-SOX9 and pcDNA3.1(+)-3×Flag-FRA1, pcDNA3.1(+)-3×Flag-JUN, pcDNA3.1(+)-3×Flag-FOS, and pcDNA3.1(+)-3×Flag-JUN. In order to establish SOX9- or FRA1-stably overexpressing Pca cells, the SOX9 CDS and 3×Flag-FRA1 DNA, which were subcloned from pcDNA3.1(+)-3×Flag-FRA1 construct, were inserted into the lentiviral vector pCDH-CMV-MCS-EF1-Puro, and DU145 cells were transduced with lentiviral particles, which were produced in HEK293FT cells cotransfected with overexpression lentiviral plasmids and ViraPowerTM Packaging Mix (Invitrogen, K4975-00). All the transient transfection of siRNA oligos and plasmids was performed with the Lipofectamine™ 3000 Reagent (Invitrogen, L3000015) following the manufacturer’s instructions. The sequences of siRNA are listed in Table S3.

Enzyme-linked immunosorbent assay (ELISA)

In order to determine HGF concentrations in the conditioned medium of Pca cells, NFs, and CAFs, human HGF ELISA kit (MULTISCIENTES (Lianke), 70-EK1H01, Hangzhou, China) was applied according to the manufacturer’s instructions. Briefly, 100 µL diluted conditioned medium and standard samples were added to the 96-well plates; subsequently, 50 µL biotin labeling anti-HGF antibody was also added per well. After incubation on a microplate shaker for 2 h at room temperature, the plates were washed by wash buffer six times and tapped dry; then, 100 µL diluted HRP-labeled streptavidin was added to each well. Following incubation for 45 min at room temperature, the plates were washed and tapped dry; then, 100 µL tetramethylbenzidine solution was added to each well. After incubating for 20 min at 37 ºC in the dark, 100 µL stop buffer was added to terminate the reaction. Then, the optical density was determined using microplate reader (TECAN, Infinite M200pro) at 450 nm with a reference wavelength at 630 nm.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted with SimpleChIP Plus Sonication Chromatin IP Kit (Cell Signaling Technology, #56383, Danvers, MA, USA) following the manufacturers’ instruction. The sonicated cell lysates containing protein–DNA complex were immunoprecipitated with anti-FRA1 or anti-phos-FRA1 antibody, and anti-normal rabbit IgG antibody was used as negative control and anti-H3 antibody as positive control. The ChIP-enriched DNA was analyzed by qRT-PCR and PCR assay with specific primers located at the promoter region of SOX9/Sox9 gene (Table S1).

In vivo xenograft tumor model

Animal experiments were approved and performed in accordance with the Institutional Animal Care and Use Committee of Drum Tower Hospital, Medical School of Nanjing University. Six-week-old male BALB/c nude mice, which were purchased from the Animal Core Facility of Nanjing Medical University (Nanjing, China), were housed in the...
specific pathogen-free environment at the Animal Laboratory Center of Nanjing Drum Tower Hospital. In order to determine whether CAFs and HGF play an important role in regulating tumorigenesis and tumor development, $2 \times 10^6$ DU145 cells alone or mixed with equivalent CAFs were injected subcutaneously into the right flank of nude mice. 21 days postinoculation, vehicle or capmatinib (10 mg·kg$^{-1}$ body weight) was given to the tumor-bearing mice via oro-gastric gavage twice a day. Tumor diameters were measured every 3 days, and tumor volumes were calculated as length $\times$ width$^2$ $\times$ π/6. The tumors were harvested 4 h after the last dosing and fixed in paraformaldehyde. For the purpose of assessing the effects of SOX9 and FRA1 on the proliferation of prostate cancer, $4 \times 10^6$ DU145 derived cells were injected into the right flank of nude mice subcutaneously; then, tumors were measured every 3 days and collected at the end point for IHC staining. The scores of CD44 and CD133 IHC staining were determined by multiplying the staining intensity (0: none, 1: weak, 2: moderate, 3: strong) and the staining area (0: 0%, 1: 1−50%, 2: 50−80%, 3: 80–100%). The IHC results were independently judged by two well-trained pathologists.

**Statistical analysis**

Continuous normally distributed variables were presented as mean ± standard deviation (SD), and unpaired Student’s t-test was used to evaluate the statistical significance between two groups. Categorical data were analyzed by the chi-square test or Fisher’s exact test. Pearson’s correlation test was applied to analyze the correlation between two continuous normally distributed factors, while Spearman’s correlation was used to analyze the relationship between categorical variables. All data analysis was performed by using IBM SPSS STATISTICS 21.0 (IBM, Armonk, NY, USA), and $P < 0.05$ was considered statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

HG and XQ designed the study. HQ and BJ isolated the primary prostate stromal fibroblasts and performed all the cell assays. HQ, CP, and WC performed IHC and IF assays on human and mouse tissues. HQ, YY, and BJ performed the PCR, WB, and ELISA experiments. HQ, ZZ, and MC performed the in vivo experiments. YY, JG, and XZ analyzed the data and performed the statistics. WD and MD analyzed and interpreted the data. HQ, WC, XQ, and HG wrote the manuscript. HG supervised the study.

**Peer Review**

The peer review history for this article is available at https://publons.com/publon/10.1111/febs.15816.

**Data accessibility**

No data were deposited in public database or repository. The data and materials involved in the current study are available from the corresponding author on reasonable request.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primer sequences used in qRT-PCR and ChIP assay.

Table S2. List of antibodies used in this study.

Table S3. siRNA sequence for gene knockdown.