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Heat shock factor 1 in cancer-associated fibroblasts is a potential prognostic factor and drives progression of oral squamous cell carcinoma

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Heat shock factor 1 (HSF1) is highly expressed in various malignancies and is a potential modulator of tumor progression. Emerging evidence suggests that HSF1 activation in stromal cells is closely related to poor patient prognosis. However, the role of HSF1 in oral squamous cell carcinoma (OSCC) remains elusive. We aimed to investigate the function of HSF1 in cancer-associated fibroblasts (CAFs) of the tumor microenvironment (TME) and in tumor development. In the present study, we found that HSF1 was highly expressed in both CAFs and tumor cells, and was significantly correlated with poor prognosis and overall survival. Moreover, HSF1 overexpression in CAFs resulted in a fibroblast-like phenotype of Cal27 cells, induced epithelial-mesenchymal transition (EMT), and promoted proliferation, migration and invasion in Cal27 cells. HSF1 knock-down attenuated features of CAFs and reduced EMT, proliferation, migration and invasion in Cal27 cells. Furthermore, HSF1 in CAFs promoted tumor growth in nude mice. Taken together, these data suggest that HSF1 expression in CAFs drive OSCC progression, and could serve as an independent prognostic marker of patients with OSCC. Thus, HSF1 is a potent mediator of OSCC malignancy.

KEYWORDS
cancer-associated fibroblast, heat shock factor 1, invasion, migration, oral squamous cell carcinoma

1 | INTRODUCTION

Oral squamous cell carcinoma, the sixth most common cancer worldwide, is typically detected in advanced stages.[1,2] Despite advanced treatment strategies, the 5-year OS rate has remained at approximately 50%.[3] Local invasion and distant metastasis are the predominant causes of death in patients;[4] however, the underlying mechanism has not been fully explored.

Tumor microenvironment is known to have a prominent role in tumor progression.[5] As a major component of TME, CAFs participate in tumor initiation and evolution, and they promote tumor cell proliferation, angiogenesis, invasion and chemotherapy resistance by various mechanisms.[6-8] Unlike NFs, which usually form a tumor-suppressive microenvironment, CAFs are involved in ECM remodeling, tumor-enhancing inflammation, and secretion of various growth factors and cytokines that regulate EMT.[9-11] Recently, activated CAFs have been thought to be characterized by the expression of α-SMA, FSP-1, FAP, fibronectin and Vimentin.[12-14] Of note, increased infiltration of CAFs in tumor stroma is correlated with poor prognosis in many malignancies, including breast cancer, colorectal cancer and oral cancer.[15-17]

Studies investigating the activation of HSF1 in CAFs have been particularly enlightening. As the dominant regulator of the heat shock response, HSF1 is an 82-kD transcription factor that is located in the cytoplasm and responds to stress conditions.[18,19] Moreover, HSF1 is involved in the phosphorylation, nuclear accumulation and trimerization of proteins, and it can bind to HSE to induce the transcription of target genes[20-22] and participate in many biological processes, including growth, development, survival, and inflammation.[23-25] However, the activation of HSF1 has been widely correlated with tumor initiation, growth, invasion and metastasis, and HSF1 has been shown to function as a tumor promoter and potential diagnostic biomarker.[26-29] Recently, there has been growing concern regarding the role of HSF1 in the modulation of the TME. In particular, HSF1 remodels the TME by reprogramming stromal fibroblasts to promote tumor invasion and metastasis. Most α-SMA-positive CAFs have strong nuclear HSF1 staining in different tumor types including skin, lung, esophageal, gastric, colon and prostate carcinoma.[26]

Despite accumulating evidence on the effects of CAFs on TME modulation during tumor progression, little is known about HSF1 expression in CAFs that is responsible for TME reprogramming resulting in the promotion of OSCC invasion and metastasis. In the present study, we investigated the role of HSF1 in CAFs and tumor cells and then analyzed its relationship with the clinicopathological parameters and prognosis of OSCC patients. Furthermore, we characterized the functional role of HSF1 in CAFs during crosstalk with tumor cells and cancer progression and metastasis. Our results may facilitate the identification of novel therapeutic implications of HSF1 expression in CAFs in OSCC.

2 | MATERIALS AND METHODS

2.1 | Patients

One hundred and twenty-one patients (71 males and 50 females; mean age, 60.2 years; range, 34-88 years) with primary OSCC were recruited from 2008 to 2013 and treated by surgery at the Department of Oral and Maxillofacial Surgery, the Affiliated Stomatological Hospital of Nanjing Medical University. Tumor pathological grade was classified according to WHO classification criteria. Clinical stage and TNM stage were defined by the UICC and the American Joint Commission on Cancer (AJCC). All patients were followed up from 2 to 91 months, but two patients were lost during this period. This study was approved by the Nanjing Medical University Ethics Committee and was in accordance with the World Medical Association Declaration of Helsinki, and written informed consent was obtained from all patients.

2.2 | Immunohistochemistry

Paraffin sections (4 μm) of tissues were dewaxed in xylene, rehydrated in gradient ethanol, and incubated with 3% hydrogen peroxide for 10 minutes. After antigen retrieval and blocking were carried out, the sections were incubated overnight with antibodies against HSF1 (1:100; Abcam, Cambridge, MA, USA), α-SMA (1:100; Abcam), E-cadherin (1:100; Abcam) and Vimentin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then incubated with HRP-polymer anti-mouse/rabbit Kit and a DAB Detection Kit (Fuzhou Maixin Biotech, Fuzhou, China) followed by counterstaining with hematoxylin. Then, all stained sections were dehydrated in gradient alcohol, clarified in xylene and mounted with neutral gum.

2.3 | Evaluation of immunoreactivity

Histological samples were individually evaluated by two pathologists. Immunoreactivity of HSF1, E-cadherin and Vimentin in tumor cells was evaluated by IRS according to staining intensity and distribution[30] as follows: IRS = IS × PS. IS was defined as 0, negative; 1, weak; 2, moderate; or 3, strong, whereas PS was defined as 0, negative; 1, <10%; 2, 11%-50%; 3, 51%-80%; 4, >80%. The patients were divided into two groups according to the IRS: low expression, 0-4; high expression, >4. Immunoreactivity of HSF1 and α-SMA in CAFs was estimated according to the classification system described by Fujii et al:[31] 0, negative (no staining); 1, sparse (a small population of discrete stained fibroblasts); 2, focal (irregular and intermittent areas of stained fibroblasts); and 3, abundant (widespread and consecutive areas of stained fibroblasts). The highest score observed in the entire peritumoral stroma was used as the final score. Then, scores of 0-1 and 2-3 were categorized as low and high expression, respectively.

2.4 | Fibroblast isolation, cell culture and preparation of conditioned media

Tumor tissues and matched proximal nonmalignant tissues were obtained during surgery or pathological examination. The fibroblast isolation protocol was approved by the Nanjing Medical University Ethics Committee. Fresh tissues were washed with PBS, cut into approximately 1 mm^3 pieces, distributed on plates and placed in a 5% CO_2 incubator at 37°C for 4 hours to adhere. Then, the tissue pieces were maintained in DMEM (Gibco, Grand Island, NY, USA) with 15%

FBS (Gibco) for 1-2 weeks. Purified fibroblasts were maintained in DMEM with 10% FBS. The cell line Cal27 was purchased from ATCC (Manassas, VA, USA) and maintained in DMEM (high glucose) containing 10% FBS. Cells were seeded into T25 culture flasks and grown in 5 mL serum-free media for 48 hours until the cells were approximately 80% confluent. CM was collected and centrifuged at 188 g for 30 minutes to remove cellular debris.

2.5 | Immunofluorescence

Cells were fixed with 4% PFA for 20 minutes, permeabilized with 1% Triton X-100 for 15 minutes, and then incubated with goat serum for 1 hour. Subsequently, the cells were incubated with antibodies against cytokeratin (CK, 1:200; Abcam), Vimentin (1:200; Santa Cruz Biotechnology), α-SMA (1:200; Abcam), FSP-1 (1:250; Abcam) and FAP (1:250; Abcam) at 4°C overnight. After washing with PBS, cells were incubated with secondary antibodies (1:50) in the dark for 1 hour at 37°C. Then, cell nuclei were stained with DAPI (1:1000; Beyotime, Shanghai, China) for 1 minute. Immunofluorescence was visualized using a Zeiss LSM-710 laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.6 | Real-time RT-PCR and western blotting

Real-time RT-PCR and western blotting were carried out as previously described in our study.[32] Primer sequences for real-time RT-PCR are listed in Table S1. Primary antibodies for western blotting were as follows: β-actin as a control (1:500; Proteintech, Rosemont, IL, USA), HSF1 (1:1000; Abcam), α-SMA (1:400; Abcam), FSP-1 (1:1000; Abcam), FAP (1:800; Abcam), E-cadherin (1:1000; Abcam), Vimentin (1:500; Santa Cruz Biotechnology) and Snail (1:500; Abcam).

2.7 | Cell proliferation assay

Cells were plated in 96-well plates (3000 cells/well) for 24 hours incubation. CCK-8 (10 μL; Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well and incubated for 4 hours. Absorbance was determined at 0, 2, 4, and 6 days at 450 nm.

2.8 | Wound-healing and invasion assays

Cells were plated in six-well plates and grown to 90% confluence. A pipette tip was used to scratch wounds, and then cells were incubated with CM. Migrating cells at the wound front were photographed at 0, 12, and 24 hours. Cell invasion assays were carried out by using 8-μm pore Transwell filters (Costar, Lowell, MA, USA) that were precoated with Matrigel (Corning, Bedford, MA, USA). Cells (1.0×10^5) were resuspended in 200 μL serum-free medium and added to the upper chamber, while the lower chamber was filled with CM as the chemoattractant. After incubation for 24 hours, the upper chambers were fixed with 4% PFA and stained with crystal violet (Sigma-Aldrich, St Louis, MO USA). Migratory cells on the lower surface of the chamber were counted and photographed (Olympus, Tokyo, Japan).

2.9 | Three-dimensional coculture system

Fibroblasts were resuspended in FBS, and then type IA collagen, 5 × DMEM and reconstitution buffer (50 mmol/L NaOH,
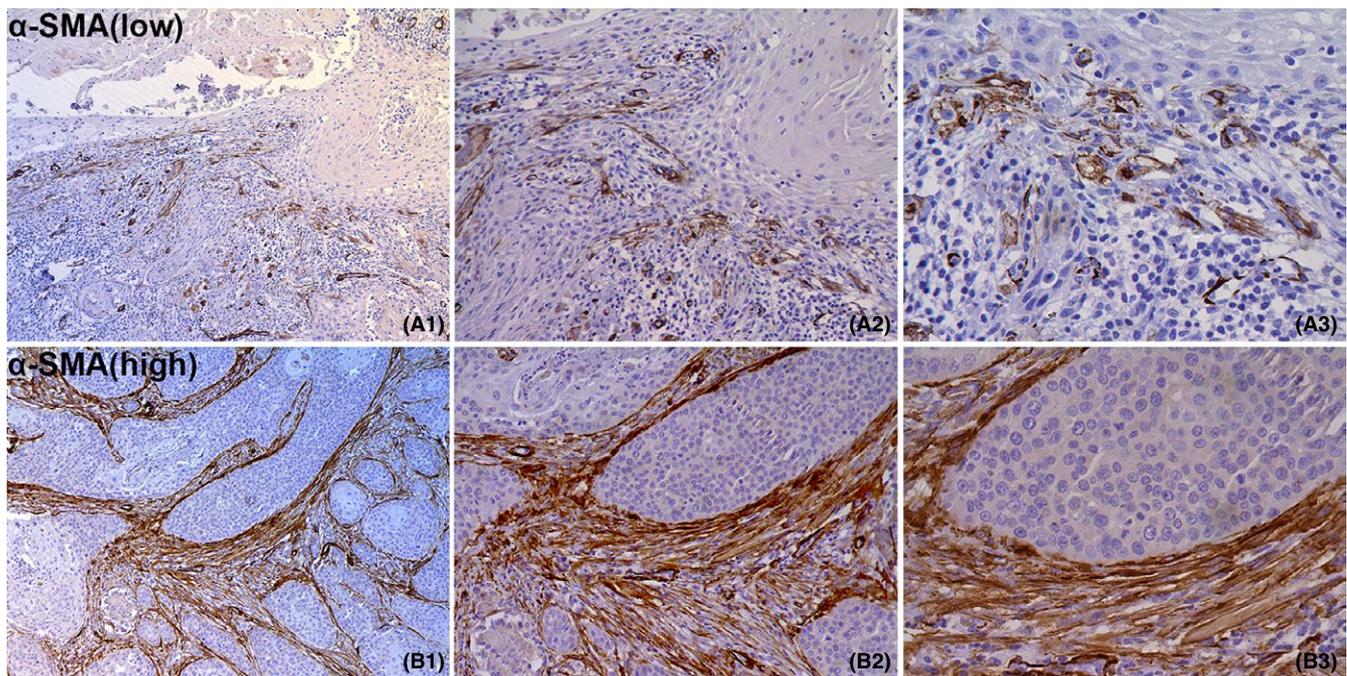

FIGURE 1 Expression of α-smooth muscle actin (α-SMA) is observed in cancer-associated fibroblasts (CAFs). Absent or reduced α-SMA-positive staining in CAFs (A1-A3, but most CAFs in the tumor stroma showed strong cytoplasmic expression of α-SMA (B1-B3). (A1, B1, 100×; A2, B2, 200×; A3, B3, 400×)

TABLE 1 Associations between clinical variables and expression levels of α-SMA, HSF1 in stroma, and HSF1 in tumor cells

Variable	No. patients	α-SMA			HSF1 in tumor stroma			HSF1 in tumor cells		
		Low	High	P-value	Low	High	P-value	Low	High	P-value
Gender										
Male	71	25	46	0.713	47	24	0.836	34	37	0.280
Female	50	16	34		34	16		19	31	
Age (years)										
≤50	24	11	13	0.167	19	5	0.155	14	10	0.109
>50	97	30	67		62	35		39	58	
Tumor location										
Tongue	49	17	32	0.189	34	15	0.462	20	29	0.226
Gingiva	25	11	14		16	9		9	16	
Buccal mucosa	31	9	22		19	12		14	17	
Palate	4	0	4		2	2		2	2	
Lower lip	4	3	1		4	0		4	0	
Jaw	4	0	4		2	2		1	3	
Other	4	1	3		4	0		3	1	
Tumor size										
T1	19	9	10	**0.018**	11	8	0.575	7	12	0.822
T2	67	25	42		49	18		32	35	
T3	26	7	19		18	8		12	14	
T4	9	0	9		3	6		2	7	
Lymph node metastasis										
N0	68	33	35	**<0.001**	47	21	0.565	33	35	0.235
N	53	8	45		34	19		20	33	
Distant metastasis										
M0	119	41	78	0.548	79	40	1.000	51	68	0.190
M	2	0	2		2	0		2	0	
Clinical stage										
I	11	6	5	**<0.001**	6	5	0.938	5	6	0.137
II	41	22	19		29	12		20	21	
III	34	8	26		23	11		18	16	
IV	35	5	30		23	12		10	25	
Pathological grade										
I	71	33	38	**<0.001**	49	22	0.290	36	35	**0.041**
II	42	8	34		30	12		16	26	
III	8	0	8		2	6		1	7	
Recurrence										
No	80	36	44	**<0.001**	74	6	**<0.001**	52	28	**<0.001**
Yes	38	3	35		5	33		0	38	
Lost	3	2	1		2	1		1	2	
Follow up										
Alive	87	36	51	**0.001**	74	13	**<0.001**	52	35	**<0.001**
Dead	32	3	29		5	27		0	32	
Lost	2	2	0		2	0		1	1	

H, high expression; HIF1, heat shock factor 1; L, low expression; M0, no metastasis; M, metastasis; N0, no nodal metastasis; N, nodal metastasis; α-SMA, alpha smooth muscle actin.
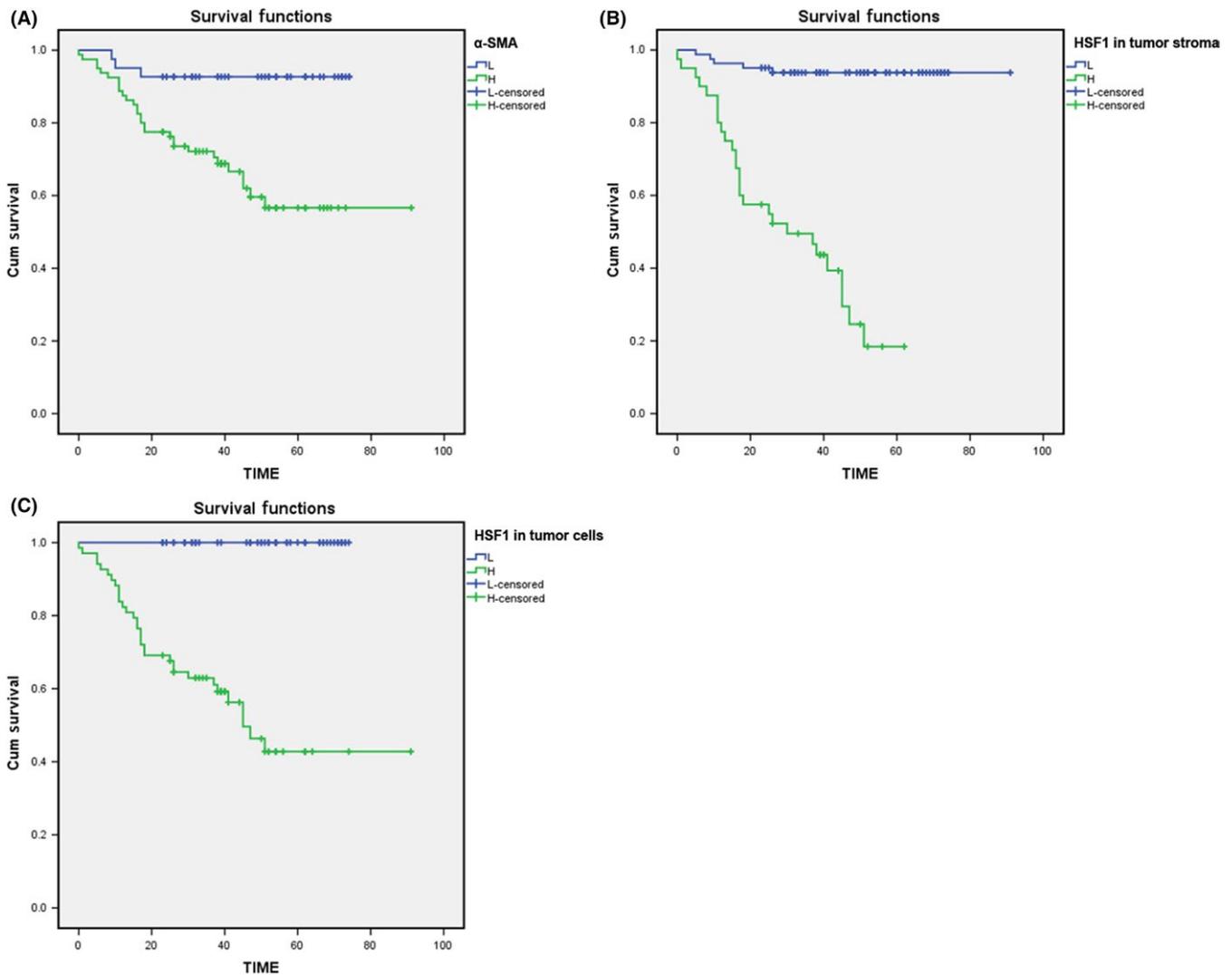Bold values signify P < 0.05.

FIGURE 2 Survival analysis of 121 patients with oral squamous cell carcinoma (OSCC). A, Survival curves of 121 OSCC patients with low or high expression of α-smooth muscle actin (α-SMA) (log-rank test, $P < 0.001$). B, Survival curves of 121 OSCC patients with low or high expression of heat shock factor 1 (HSF1) in the tumor stroma (log-rank test, $P < 0.001$). C, Survival curves of 121 OSCC patients with low or high expression of HSF1 in tumor cells (log-rank test, $P = 0.001$). L, low expression; H, high expression

260 mmol/L $NaHCO_3$, and 200 mmol/L HEPES) were sequentially added to the fibroblasts and uniformly mixed. The mixture was added to 12-well plates and allowed to solidify in an incubator at 37°C for 30-60 minutes. Cal27 cells were resuspended in the coculture medium and then transferred onto the surface of the gelatinized fibroblast layer. The coculture medium was refreshed every day. After 3 days, the gels were transferred onto a supporter in six-well plates and were cultured at the air-liquid interface. Then, the gels were fixed with 4% PFA, embedded in paraffin and cut into 4-μm sections for H&E staining.

2.10 | Cell transfection

Human HSF1-encoding lentiviral vectors were constructed by GeneChem Co., Ltd (Shanghai, China). The sequence for HSF1-targeting shRNA is CCAAGTACTTCAAGCACAA, and the scrambled

sequence is TTCTCCGAACGTGTCACGT. CAFs were seeded in six-well plates and cultured to 40% confluence, and lentiviruses were used to infect CAFs according to the manufacturer's instructions. Cells in the control group (CAFs-G) and in the experimental group (CAFs-H) were cultured at 37°C in a 5% CO_2 incubator for 8-12 hours, and then the medium was refreshed. Fluorescence microscopy was used to observe transfection efficiency, and real-time RT-PCR and western blotting were used to detect shRNA interference efficiency 72 hours later.

2.11 | Tumor xenografts

BALB/c nude mice (4-6 weeks old, female) were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and raised under specific pathogen-free conditions in the Animal Core Facility of Nanjing Medical University. All experimental procedures were

TABLE 2 Univariate Cox regression analysis of overall survival

Covariate	P-value	Risk ratio	(95% CI)
Gender (male, female)	0.369	1.375	(0.686, 2.755)
Age, y (≤50, >50)	0.120	2.569	(0.783, 8.436)
Tumor location (tongue, gingiva, buccal mucosa, palate, lower lip, jaw, other)	0.976	0.997	(0.792, 1.254)
Tumor size (T1-T4)	0.110	1.421	(0.923, 2.217)
Lymph node metastasis	0.660	1.169	(0.584, 2.340)
Clinical stage (I, II, III, IV)	0.573	1.109	(0.773, 1.591)
Pathological grade	**0.024**	1.822	(1.082, 3.068)
α-SMA	**0.003**	6.002	(1.824, 19.744)
HSF1 in tumor stroma (low, high)	**<0.001**	17.163	(6.530, 45.112)
HSF1 in tumor cells (low, high)	**0.003**	75.409	(4.309, 1319.683)

CI, confidence interval; HIF1, heat shock factor 1; α-SMA, alpha smooth muscle actin.
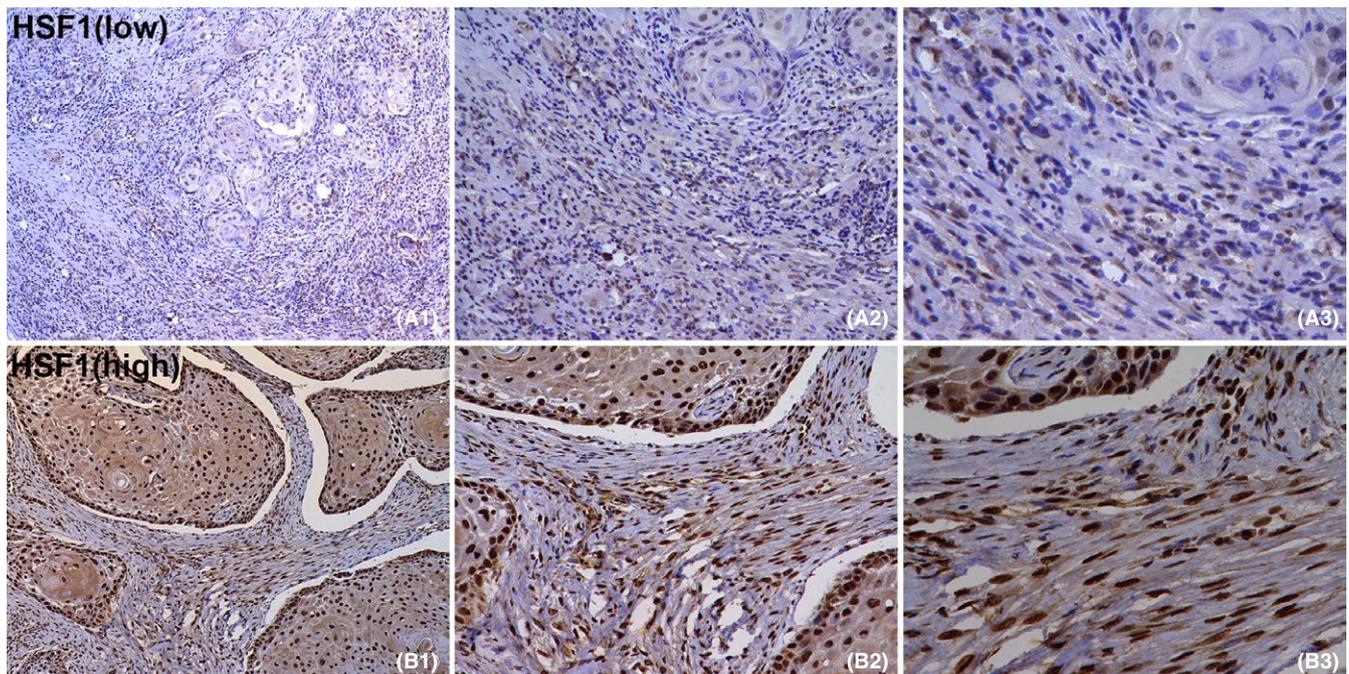Bold values signify $P < 0.05$.

FIGURE 3 Heat shock factor 1 (HSF1) expression in tumor stroma of human oral squamous cell carcinoma (OSCC) specimens. Low expression level (A1-A3) and high expression level (B1-B3) of HSF1 in tumor stroma of OSCC specimens (A1, B1, 100×; A2, B2, 200×; A3, B3, 400×)

approved by the Animal Ethics and Welfare Committee of Nanjing Medical University. Cal27 cells (1×10^6) were s.c. injected with 1×10^6 CAFs-G or CAFs-H in the right axilla of mice. Tumor sizes were measured and tumor volumes were calculated using the equation: volume (mm^3) = (length × width^2)/2. At approximately 24 days, the mice were killed, and xenograft tumors were dissected for subsequent analyses.

2.12 | Statistical analysis

Student's t test, chi-squared test and Fisher's exact test were carried out to evaluate the relationship of α-SMA, HSF1 in stroma, and HSF1 in tumor cells with clinicopathological parameters. Pearson's correlation analysis was used to estimate the correlation among HSF1 in tumor cells,

α-SMA and HSF1 in stroma. Cox regression models were used to assess the prognosis of patients. OS was evaluated by Kaplan-Meier analysis and the log-rank test. Statistical significance in in vitro and in vivo experiments was assessed using one-way ANOVA and independent-samples t test. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Infiltration of CAFs is associated with OSCC progression

In the present study, we stained OSCC specimens with PBS instead of antibodies against α-SMA and HSF1 as negative controls
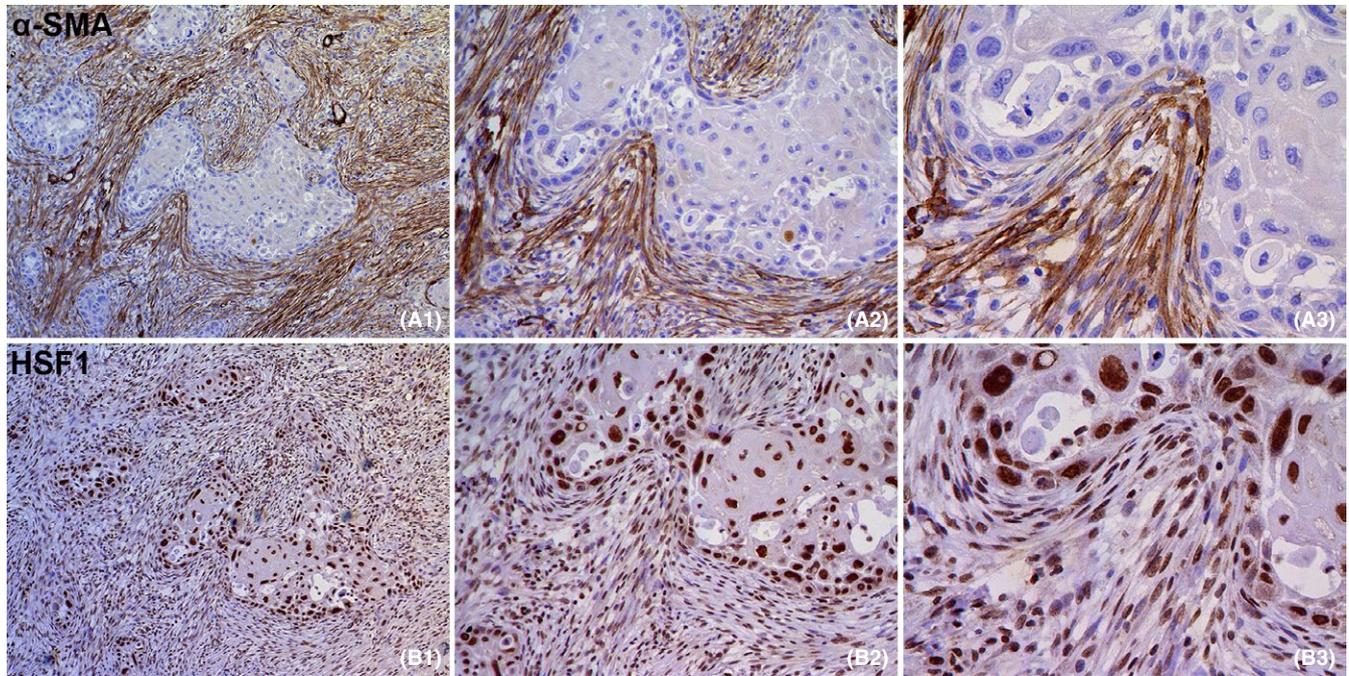
FIGURE 4 Heat shock factor 1 (HSF1) expression in the tumor stroma was consistent with α-smooth muscle actin (α-SMA) staining. α-SMA-positive cancer-associated fibroblasts (A1-A3) were abundant in the stroma of oral squamous cell carcinoma; in the same region, HSF1 expression (B1-B3) was strongly positive. (A1, B1, 100×; A2, B2, 200×; A3, B3, 400×)

TABLE 3 Multivariate Cox regression analysis of overall survival

Covariate	P-value	Risk ratio	(95% CI)
Gender (male, female)	0.112	1.895	(0.861, 4.173)
Age, y (≤50, >50)	0.443	1.873	(0.377, 9.302)
Tumor location (tongue, gingiva, buccal mucosa, palate, lower lip, jaw, other)	0.164	1.219	(0.922, 1.611)
Tumor size (T1-T4)	0.521	1.217	(0.668, 2.217)
Lymph node metastasis	0.516	0.676	(0.207, 2.203)
Clinical stage (I, II, III, IV)	0.503	1.300	(0.603, 2.805)
Pathological grade	0.444	1.239	(0.716, 2.145)
α-SMA	0.316	0.451	(0.095, 2.137)
HSF1 in tumor stroma (low, high)	**0.001**	8.185	(2.320, 28.872)
HSF1 in tumor cells (low, high)	0.914	176138.264	(0.000, 6.148*10^100)

CI, confidence interval; HIF1, heat shock factor 1; α-SMA, alpha smooth muscle actin.
Bold values signify $P < 0.05$.

and observed no staining for α-SMA or HSF-1 (Figure S1). Then, we stained for α-SMA, a specific marker for CAFs, and evaluated its expression in 121 primary OSCC samples and in normal oral mucosa samples. Expression of α-SMA was sparse or absent in the stroma of normal oral mucosa (Figure S2a1, a2, a3), but high expression of α-SMA was observed in the tumor stroma (66.12%, 80/121) (Figure 1). Additionally, α-SMA expression was significantly correlated with tumor size ($P = 0.018$), lymph node metastasis ($P < 0.001$), clinical stage ($P < 0.001$), pathological grade ($P < 0.001$), recurrence ($P < 0.001$) and death ($P = 0.001$).

However, there was no significant relationship between α-SMA expression and gender, age, tumor location or distant metastasis (Table 1). Nevertheless, the expression of α-SMA in stroma was associated with poor OS (Figure 2A, $P < 0.001$) and exerted a poor prognostic impact on the survival rate according to univariate Cox analyses ($P = 0.003$, Table 2). Based on these results, we concluded that the stromal infiltration of CAFs is significantly associated with OSCC invasion, metastasis and poor prognosis and that α-SMA-positive CAFs have the potential to promote OSCC progression.
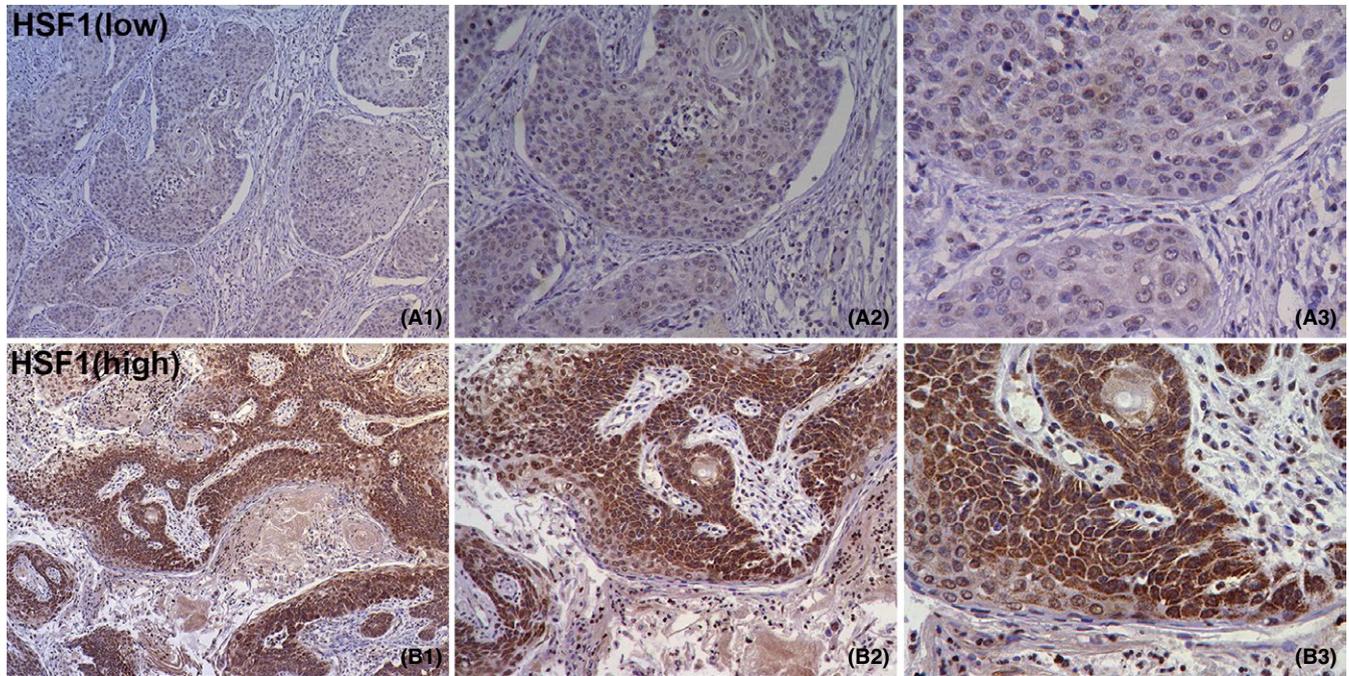
FIGURE 5 Heat shock factor 1 (HSF1) expression in tumor cells of human oral squamous cell carcinoma (OSCC) specimens. Low expression level (A1-A3) and high expression level (B1-B3) of HSF1 in the tumor cells of OSCC specimens (A1, B1, 100×; A2, B2, 200×; A3, B3, 400×)

3.2 | Heat shock factor 1 expression in CAFs promotes OSCC progression

To further validate whether HSF1 expression in CAFs contributes to OSCC progression, we evaluated the level of stromal HSF1 in 121 primary OSCC samples. No staining or extremely low staining of HSF1 was observed in normal oral mucosa (Figure S2b1, b2, b3, red arrow). Of the 121 tumors, 40 (33.06%) showed strong immunoreactivity for HSF1 in tumor stroma (Figure 3). Surprisingly, HSF1 expression in the tumor stroma (Figure 4B1, B2, B3) was unexpectedly consistent with α-SMA staining (Figure 4A1, A2, A3). High expression of HSF1 was mostly observed in the same regions with high expression of α-SMA, which suggested HSF1 activation in CAFs. Furthermore, HSF1 expression in CAFs was associated with OSCC recurrence ($P < 0.001$) and death ($P < 0.001$), respectively. Almost 86.8% (33/38) of samples from patients with recurrence and 84.4% (27/32) of samples from patients who died of recurrence showed a prominent increase in the expression of HSF1 in the stroma, whereas only 7.5% (6/80) of samples from patients without recurrence expressed a high level of HSF1 in the stroma. Therefore, patients who experienced recurrence or died of recurrence expressed a distinctly higher level of HSF1 in the stroma than patients without recurrence. Significant associations were not observed between stromal HSF1 expression and gender, age, tumor location, tumor size, lymph node metastasis, distant metastasis, clinical stage, or pathological grade (Table 1). However, high expression of HSF1 in the stroma was significantly correlated with OS (Figure 2B, $P < 0.001$). Univariate and multivariate Cox regression analyses indicated that HSF1 in the stroma was an independent

prognostic factor ($P < 0.001$, $P = 0.001$, Tables 2 and 3, respectively). Thus, HSF1 expression in CAFs is closely related to poor prognosis and severely affects the survival rate of OSCC patients.

3.3 | Heat shock factor 1 expression in tumor cells promotes OSCC progression

Higher HSF1 expression was also observed in tumor cells of OSCC patients. HSF1-positive cancer cells showed strong, dense, brownish staining, located mostly in the nucleus and partly in the cytoplasm (Figure S3b1, b2, b3). However, no staining for α-SMA was observed in the cancer cells (Figure S3a1, a2, a3). In addition, 56.20% (68/121) of primary OSCC samples showed high expression of HSF1 in tumor cells (Figure 5B1, B2, B3) and 43.80% (53/121) showed low expression (Figure 5A1, A2, A3). Significant associations were observed between HSF1 expression in tumor cells and pathological grade ($P = 0.041$), recurrence ($P < 0.001$) and death ($P < 0.001$). Furthermore, 49.3% (35/71) of grade I tumors had high expression of HSF1, and the proportion was 61.9% (26/42) and 87.5% (7/8) for grade II and III tumors, respectively. In addition, patients who experienced recurrence or died of recurrence had an increased expression of HSF1, whereas 35.0% (28/80) of patients without recurrence expressed a high level of HSF1. A significantly higher expression of HSF1 was observed in tumor samples from patients who experienced recurrence ($P < 0.001$) or died of recurrence ($P < 0.001$) than in tumor samples from patients without recurrence, respectively (Table 1). Moreover, the high expression of HSF1 in tumor cells exerted a poor prognostic impact on the survival rate (Figure 2C, $P = 0.003$, Table 2). Remarkably, the expression of HSF1 in tumor
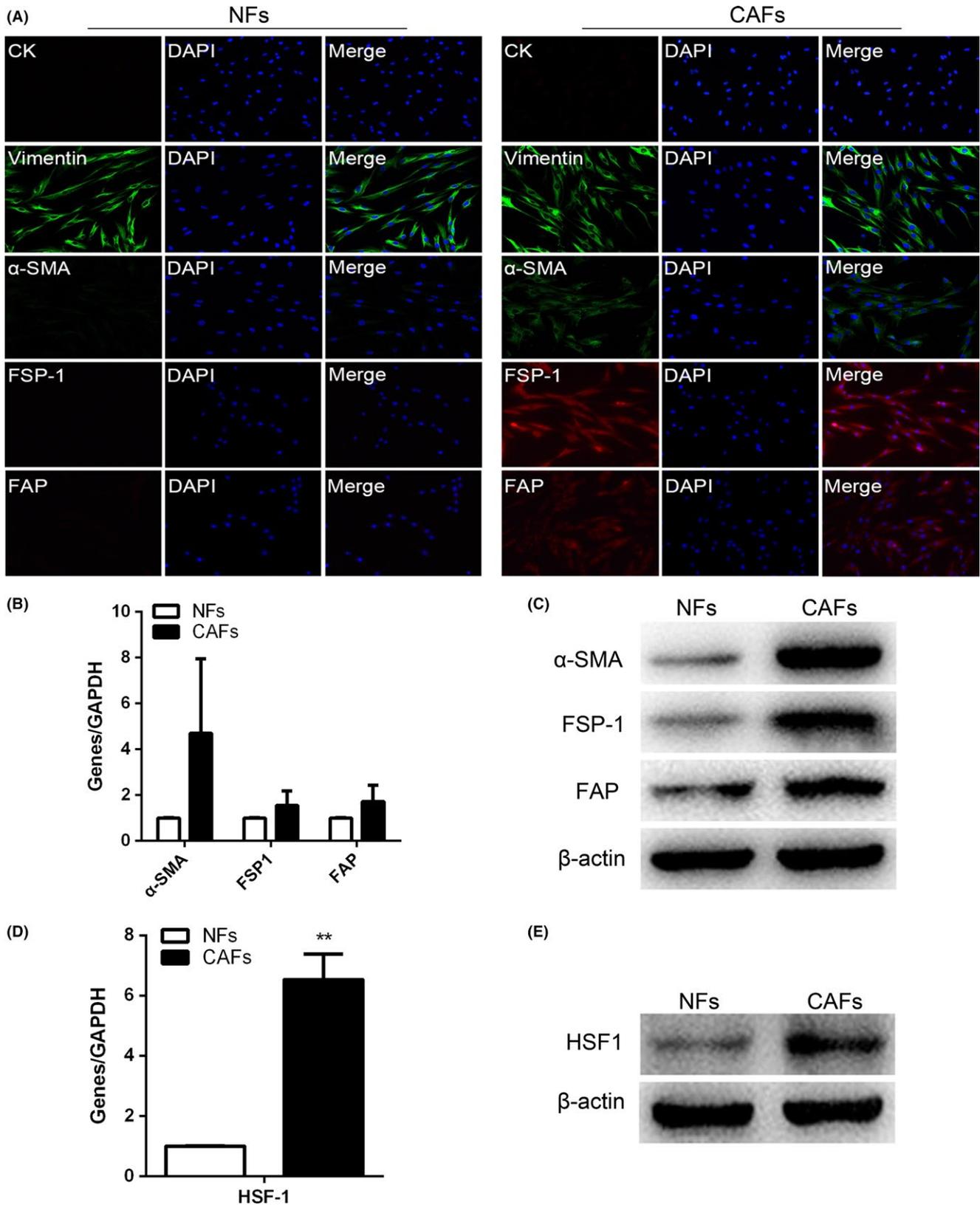
FIGURE 6 Characteristics of cancer-associated fibroblasts (CAFs) derived from oral squamous cell carcinoma patients, and the overexpression of heat shock factor 1 (HSF1) in primary CAFs. A, Immunofluorescence analysis of the epithelial marker cytokeratin (CK), mesenchymal marker Vimentin and CAFs markers α-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP-1) and fibroblast activation protein (FAP) in CAFs and normal fibroblasts (NFs). Green: CK, Vimentin and α-SMA; Red: FSP-1 and FAP; Blue: DAPI (100×). B, Gene expression of α-SMA, FSP-1 and FAP was measured by real-time RT-PCR. C, Protein levels of α-SMA, FSP-1 and FAP were determined by western blotting; β-actin served as loading control. D,E, HSF1 mRNA and protein expression in CAFs. Data represent the mean ± SD in B and D; **$P < 0.01$
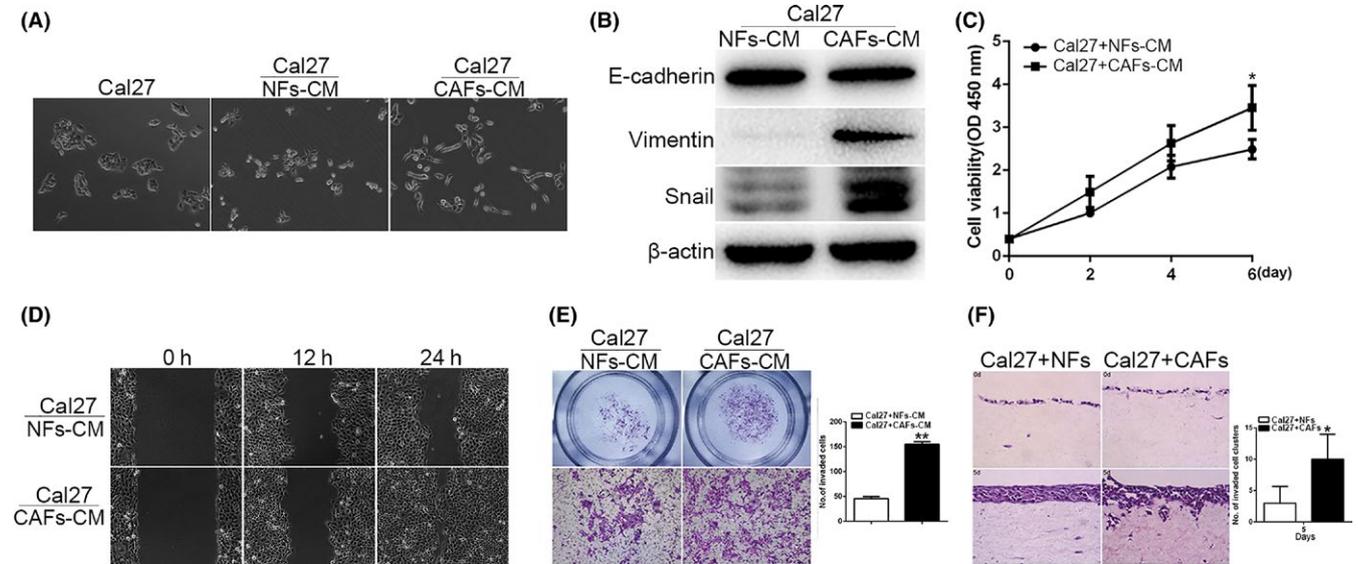
FIGURE 7 Heat shock factor 1 (HSF1)-positive cancer-associated fibroblasts (CAFs) promote epithelial-mesenchymal transition (EMT), proliferation, migration and invasion in Cal27 cells. A, Morphological changes of Cal27 cells induced by HSF1-positive CAFs (100×). B, Expression of EMT markers E-cadherin, Vimentin and Snail was examined by western blotting; β-actin served as loading control. C, CCK-8 assay was used to evaluate the proliferation of Cal27 cells treated with conditioned medium (CM) derived from CAFs or normal fibroblasts (NFs). D, Wound-healing assay for migration in Cal27 cells treated with CM derived from CAFs and NFs. E, Cells invading the filters coated with Matrigel after 24 h (upper panel); photographs taken at 100× magnification (bottom panel), and quantification of cell invasion in five different random fields (right panel). F, Representative images of Cal27 cells cocultured with primary CAFs and NFs in a 3-D coculture system, and quantification of invaded cell clusters (400×). Data represent the mean ± SD in C, E and F; *P < 0.05, **P < 0.01

cells was strongly correlated with the expression of α-SMA and HSF1 expression in CAFs ($r = 0.353$, $r = 0.620$, $P < 0.001$, Table S2).

3.4 | Heat shock factor 1 is upregulated in primary CAFs

In vitro, CAFs and NFs were established to further explore the role of HSF1 in CAFs. First, primary matched pairs of CAFs and NFs were successfully obtained from fresh human tissues. The epithelial marker CK and the mesenchymal marker Vimentin were used to verify the purity of primary cells by immunofluorescence. CAFs and NFs showed negative staining for CK and positive staining for Vimentin (Figure 6A). In addition, expression of typical CAFs markers, such as α-SMA, FSP-1 and FAP, were analyzed by real-time RT-PCR and western blotting. CAFs showed an increase in α-SMA, FSP-1 and FAP expression (Figure 6B, C). Immunofluorescence analysis also showed that CAFs had higher α-SMA, FSP-1 and FAP expression than NFs, which further confirmed the characteristics of CAFs and NFs (Figure 6A). More importantly, upregulated expression of HSF1 was detected in CAFs by real-time RT-PCR and western blotting (Figure 6D, E, $P < 0.01$), which, in line with the clinical results, suggested the activation of HSF1 in CAFs in vivo.

3.5 | Heat shock factor 1-positive CAFs enhance proliferation, migration and invasion in cancer cells

To verify the function of HSF1-positive CAFs on cancer cells, we treated Cal27 cells with CM from CAFs (Cal27-C) or NFs (Cal27-N).

Cal27-C cells presented fibroblast-like phenotypes, showing a more elongated and spindle-like shape compared with Cal27-N cells (Figure 7A). Western blotting showed an increased expression of mesenchymal markers Vimentin and Snail in Cal27-C cells, whereas no significant change was observed in the epithelial marker E-cadherin (Figure 7B). Meanwhile, Cal27-C cells showed higher proliferation than Cal27-N cells (Figure 7C). Wound-healing assay showed that Cal27-C cells filled the scratched area after 24 hours, whereas larger gaps were observed with control cells, which indicated a significantly higher migratory ability of Cal27-C cells than Cal27-N cells (Figure 7D). Transwell assays also showed a 3.38-fold increase in Cal27-C cell invasiveness over that observed in control cells (Figure 7E, $P < 0.01$). Furthermore, the 3-D coculture system indicated that HSF1-positive CAFs could induce cancer cells to invade collagen; additionally, these cancer cells were more invasive than cancer cells cocultured with NFs (Figure 7F, 3.33-fold, $P < 0.05$). These data indicate that HSF1 expression in CAFs could induce EMT and enhance proliferation, migration and invasion in OSCC.

To further characterize HSF1 expression in CAFs, cell transfection was carried out to knock down HSF1 in CAFs. Indeed, HSF1 expression was decreased in CAFs-H (Figure 8A, B, $P < 0.01$). Meanwhile, mRNA expression of three CAFs markers was decreased in response to HSF1 silencing (Figure 8C). Western blotting also showed that the protein level of α-SMA, FSP-1 and FAP was downregulated in CAFs-H (Figure 8D). Then, we treated Cal27 cells with CM from CAFs-G or CAFs-H. On the contrary, knockdown of HSF1 in CAFs caused fibroblast-like Cal27 cells to become "cobblestone-shaped"
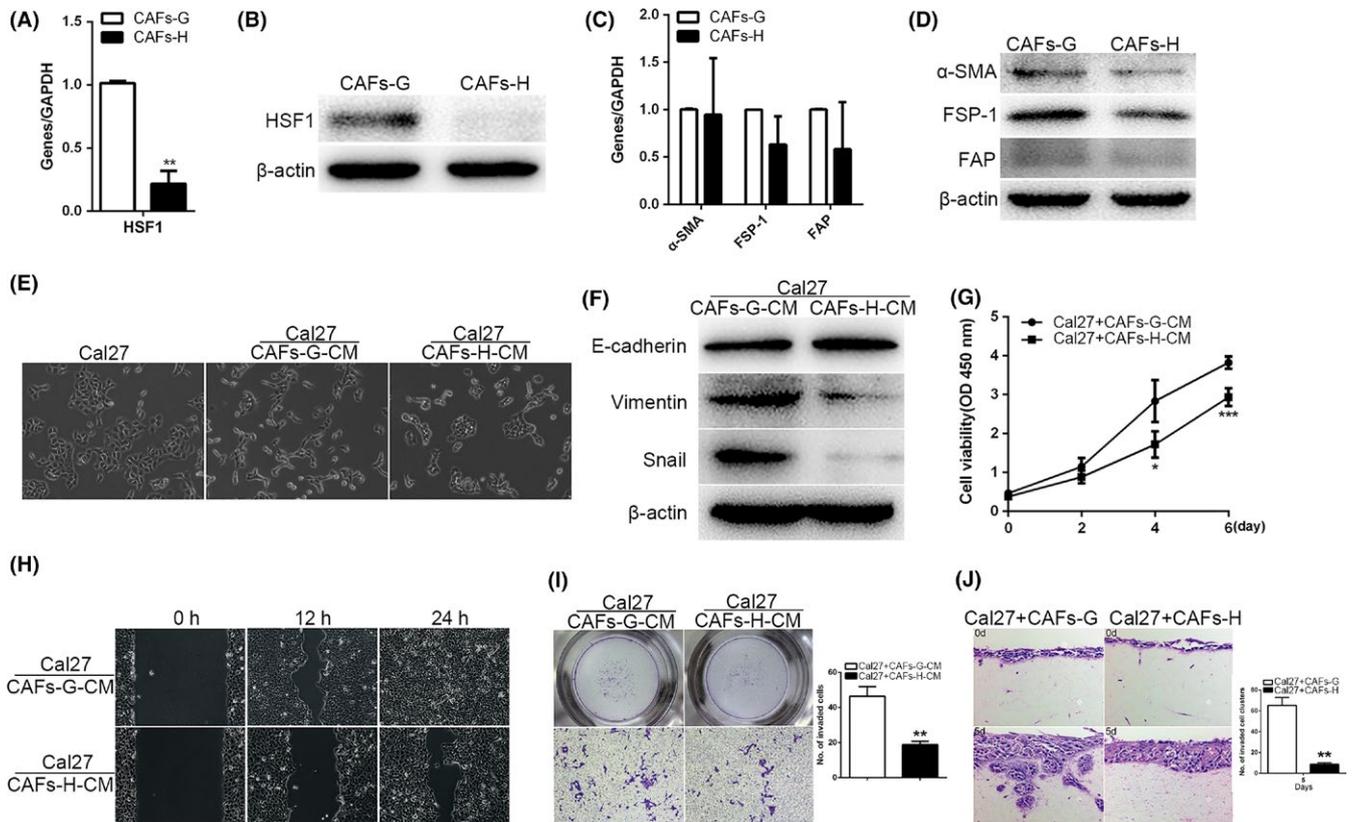
FIGURE 8 Heat shock factor 1 (HSF1) knockdown in cancer-associated fibroblasts (CAFs) abrogates CAFs characteristics, reverses the epithelial-mesenchymal transition (EMT) process and reduces the proliferation, migration and invasion of Cal27 cells. A,B, HSF1 expression based on real-time RT-PCR and western blotting in CAFs-GV248-shRNA-HSF1 (CAFs-H) or CAFs-GV248-NC (CAFs-G). C,D, Quantitation of α-SMA, fibroblast-specific protein-1 (FSP-1) and fibroblast activation protein (FAP) mRNA levels by real-time RT-PCR and protein level by western blotting in CAFs-H or CAFs-G. E, Morphological changes of Cal27 cells treated with CAFs-H-CM or CAFs-G-CM (100×). F, Expression of E-cadherin, Vimentin and Snail was examined by western blotting; β-actin served as loading control. G, CCK-8 analysis of the proliferation of Cal27 cells treated with conditioned medium (CM) derived from CAFs-G or CAFs-H. H,I, Representative images of migration and invasion in Cal27 cells treated with CM from CAFs-H or CAFs-G. J, Representative images of Cal27 cells cocultured with CAFs-H or CAFs-G in a 3-D coculture system, and quantification of invaded cell clusters (400×). Data represent the mean ± SD in A, C, G, I and J; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

and decreased intercellular separation (Figure 8E). Meanwhile, silencing HSF1 in CAFs significantly downregulated Vimentin and Snail expression and slightly upregulated E-cadherin expression in Cal27 cells, together with a markedly decreased proliferative, migratory and invasive ability (Figure 8F, G, H, I). More importantly, the 3-D coculture system showed that the HSF1-silenced CAFs could reduce the invasive ability of cancer cells; the Cal27 cells proliferated and were stable but could not invade collagen (Figure 8J, 7.54-fold, $P < 0.01$). These results further confirm the role of HSF1 expression in CAFs to promote EMT, proliferation, migration and invasion in OSCC.

3.6 | Heat shock factor 1-positive CAFs promote xenograft formation

A xenograft model was established to further characterize the relevance of HSF1 expression in CAFs. Images of tumor-bearing nude mice and solid tumors are shown in Figure 9A, B. Xenograft tumors

were established in both Cal27 + CAFs-G and Cal27 + CAFs-H groups. The Cal27 + CAFs-H group showed a significantly lower tumor volume than the control group (Figure 9C, $P < 0.01$). There was no significant difference in body weight loss between the two groups (Figure 9D). Silencing HSF1 in CAFs significantly reduced tumor growth. Subsequently, the expression of α-SMA, E-cadherin and Vimentin was analyzed in tumor specimens by immunohistochemistry. The Cal27 + CAFs-H group showed a significant decrease in the expression of α-SMA and Vimentin (Figure 9E). Taken together, these results indicated that HSF1-positive CAFs can form a microenvironment conducive to tumor growth and promote tumor progression.

4 | DISCUSSION

Evidence has shown that the close communication between TME and cancer cells contributes to invasion and metastasis in tumors.[33,34] As
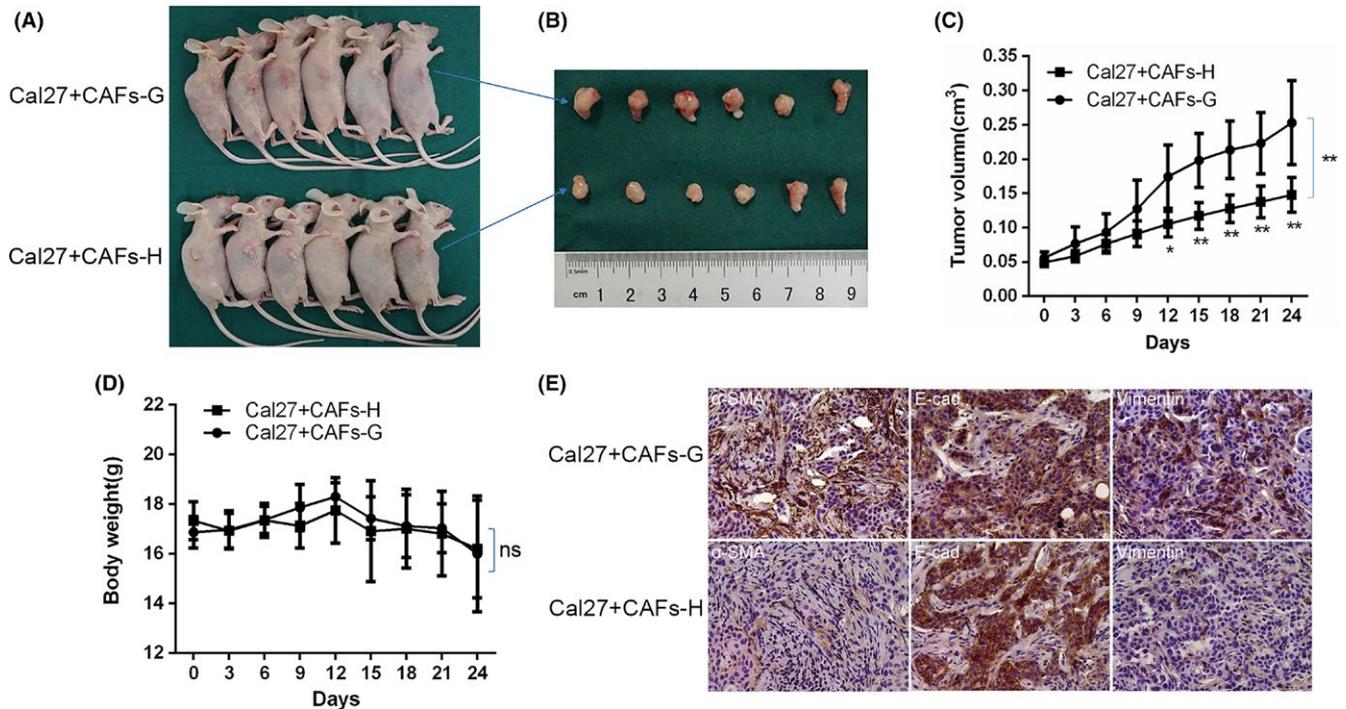
FIGURE 9 Heat shock factor 1 (HSF1) knockdown in cancer-associated fibroblasts (CAFs) decreases tumor growth in vivo. A, General observation of tumor-bearing nude mice s.c. coinjected with Cal27 cells and CAFs-GV248-shRNA-HSF1 (CAFs-H) or CAFs-GV248-NC (CAFs-G) (n = 6). B, Tumor tissues were resected after 24 d. C, Volumes of xenograft tumors in each group. D, Body weight measurements in each group. Data represent the mean ± SD in C and D; *P < 0.05, **P < 0.01 ns, no significance. E, Representative immunohistochemical staining for α-smooth muscle actin (α-SMA), E-cadherin and Vimentin in xenograft tumor tissues from the Cal27 + CAFs-H and Cal27 + CAFs-G groups (400×)

the key component of the TME, CAFs are important regulators of tumor growth, angiogenesis, invasion, metastasis, and poor prognosis in many malignancies.[6,7,15,35-37] Recently, HSF1 expression in CAFs has attracted substantial attention regarding its role in tumor progression. HSF1 may be a key factor in the transformation of NFs to CAFs. However, the mechanism has not been fully elucidated in OSCC.

Cancer-associated fibroblasts support invasion and metastasis of cancer cells by remodeling the stroma.[38,39] HSF1 activation has been reported as a crucial regulator in the transcriptional reprogramming of the stroma to form a tumor-supportive environment.[26] Accumulating evidence suggests that HSF1 is overexpressed in various malignant tumors and functions as a potential diagnostic biomarker.[18,19,27-29,40,41] Our data show that HSF1 expression in tumor cells was significantly correlated with pathological grade, recurrence and death, and high HSF1 expression in cancer cells had a significantly poor prognostic impact on survival outcomes. Our findings on 121 OSCC samples are consistent with the results of the prior studies, showing that HSF1 expression was higher in OSCC tissues than in normal tissues and that high nuclear HSF1 expression was significantly related to tumor size and histopathological grade. Importantly, we had more tissue samples than Ishiwata et al.[41] Interestingly, we also found that the high expression of HSF1 in stromal CAFs had a close relationship with recurrence and mortality in OSCC patients, and multivariate regression analysis indicated that HSF1 in CAFs was an independent

prognostic factor. The results were in accordance with the previous studies in breast, lung, and esophageal cancer, showing that HSF1 activation in the stroma is associated with poor patient outcomes.[26,40] Furthermore, the expression of HSF1 in CAFs was significantly and independently correlated with that in tumor cells, which was consistent with the studies of Scherz-Shouval et al.[26] Taken together, our results suggest that HSF1 has an important role in regulating the interaction between the stroma and cancer cells, which go "hand in hand" to facilitate tumor progression. Based on our knowledge, we conclude that HSF1 in CAFs is an independent prognostic factor of OSCC, and high HSF1 expression in both CAFs and cancer cells can improve our ability to predict OSCC patient outcomes. Thus, assessing HSF1 expression in both stroma and tumor cells might help to diagnose and guide treatment choices in OSCC progression.

Results of clinical specimens show that HSF1 in stroma could function as an independent prognostic factor, which reminds us of the vital role of HSF1 in CAFs on tumor progression. Indeed, the results of xenograft models confirm the role of HSF1 in CAFs in promoting tumor growth. In addition, in vitro studies have shown that HSF1 has multiple carcinogenic effects, such as regulating cell proliferation, migration and invasion, and inducing EMT. These results were supported by a recent study which showed that HSF1 is important for tumor progression and that it enhances cell migration and plays a critical role in EMT.[42,43] These data may provide an explanation for

the high expression of HSF1 in CAFs in promoting OSCC progression. However, we have not further studied how HSF1 in CAFs can build a "bridge" between CAFs and cancer cells to promote tumor development in OSCC. It is suggested that CAFs can secrete growth factors, cytokines and chemokines to form a microenvironment conducive to tumor metastasis.[10,11,44] According to studies by Scherz-Shouval et al, transforming growth factor beta (TGF-β) and SDF1, two cytokines secreted by CAFs, play a critical role in stromal HSF1 promoting tumor growth of adjacent cells.[13,16,26,45] However, further study is warranted to confirm the potential mechanism underlying the regulation of CAFs by HSF1 during crosstalk with tumor cells and tumor progression.

In conclusion, our findings show that the infiltration of α-SMA-positive CAFs could promote OSCC progression. Furthermore, the expression of HSF1 in both CAFs and tumor cells was positively associated with poor prognosis and OS. Moreover, HSF1 expression in CAFs induced tumor cells to undergo partial EMT and enhanced the proliferation, migration and invasion of cancer cells, and knockdown of HSF1 in CAFs reduced tumor growth. Our study emphasizes the crucial role of HSF1 in OSCC progression and highlights its potential in preventing and treating OSCC in the future.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

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