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Original Articles

FOXA1 reprograms the TGF-β-stimulated transcriptional program from a metastasis promoter to a tumor suppressor in nasopharyngeal carcinoma

Junjun Li[a,b,c], Wei Wang[d], Shengnan Chen[a,b,c], Jing Cai[a,b,c], Yuanyuan Ban[a,b,c], Qian Peng[a,b,c], Yin Zhou[a,b,c], Zhaoyang Zeng[a,b,c], Xiaoling Li[a,b,c], Wei Xiong[a,b,c], Guiyuan Li[a,b,c], Mei Yi[c,e], Bo Xiang[a,b,c,*]

[a] Hunan Provincial Cancer Hospital and Tumor Hospital Affiliated to Xiangya Medical School, Central South University, Changsha, Hunan, 410013, China
[b] The Key Laboratory of Carcinogenesis and Cancer Invasion of the Chinese Ministry of Education, Cancer Research Institute and School of Basic Medical Sciences, Central South University, Changsha, 410078, Hunan, China
[c] The Key Laboratory of Carcinogenesis of the Chinese Ministry of Health, Xiangya Hospital, Central South University, Changsha, 410008, Hunan, China
[d] Department of Pathology, Affiliated Hospital of Jining Medical University, Jining, 272029, Shandong, China
[e] Department of Dermatology, Xiangya Hospital of Central South University, Changsha, 410008, China

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is a unique subtype of head and neck squamous carcinoma that is notorious for its high metastatic potential. In this study, we reported that FOXA1 protein was decreased in NPC cells. Loss of FOXA1 is associated with lymph node metastasis and poor prognosis. Silencing FOXA1 in NP69 and C666-1 NPC cells accelerated cell proliferation and migration, while re-expression of FOXA1 has opposite effects. Microarray and RNA-seq analysis revealed that re-expression of FOXA1 in NPC cells reprogrammed the TGF-β-stimulated transcription program, which is characterized by promotion of TGF-β-inducible tumor-suppressive targets but repression of TGF-β-inducible oncogenes expression in NPC cells, leading to restoration of NPC cell sensitivity to TGF-β's growth-inhibitory effect. BAMBI, a TGF-β responsive tumor suppressor, was induced by FOXA1 in NPC cells. FOXA1 binding on the BAMBI gene facilitated SMAD2/3 binding to the BAMBI promoter via increasing BAMBI associated H3K4me1 and H3K27ac modification. Enforced expression of BAMBI in NPC cells suppressed cell proliferation and invasiveness. Our data suggested that FOXA1 is a master factor in controlling the TGF-β-stimulated transcriptome and a regulator of TGF-β biological functions in NPC oncogenesis.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a unique type of head and neck cancer with a high prevalence in Southern China and South East Asia but rare in other areas worldwide [1,2]. Due to the distinctive anatomical structure of nasopharyngeal cavity, NPC has a high propensity to metastasize to cervical lymph nodes at the early stage. More than 70% of NPC patients have already developed cervical lymph node metastasis at the initial diagnosis [3–8]. However, the underlying mechanisms of NPC metastasis are not well understood.

Transforming growth factor (TGF)-β family proteins are multifunctional cytokines in controlling cell physiology, proliferation, and growth, as well as directing cell differentiation [9]. TGF-β is well known for its dual functionality in cell biology. TGF-β acts as a tumor suppressor at the early stage but stimulates epithelial-mesenchymal transition (EMT) to favor tumor cell migration and invasion in the late phases of cancer development. It remains unclear how tumor cells develop mechanisms to overcome the inhibitory effects of TGF-β but exploit the metastasis-promotive effect of TGF-β. SMAD proteins are primary downstream effectors of TGF-β signaling activities. Upon TGF-β binding with the type II receptor, the type I receptor was recruited to the type II receptor, and both were sequentially phosphorylated, which in turn phosphorylates receptor-regulated SMADs (R-SMADs). This phosphorylation enables R-SMADs (SMAD2/3) to bind to coSMAD SMAD4 and translocate to the nucleus where they act as transcription factors and participate in the regulation of target gene expression [10]. The biological outcomes of TGF-β are highly determined by its target genes.

The pioneer factor FOXA1 family plays a critical role during embryonic differentiation, organogenesis and cancer development [11]. FOXA1 and FOXA2, as typical representative pioneer factors, are well known for their ability to open condensed chromatin and facilitate the

* Corresponding author. Cancer Hospital Affiliated to Xiangya School of Medicine, Central South University, Tongzipo Road, Changsha, Hunan, 410013, China.
E-mail address: xiangbolin@csu.edu.cn (B. Xiang).

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binding of other tissue-specific protein factors [12]. It has been demonstrated that the overexpression of FOXA1 alters the estrogen receptor (ER) α transcriptome and contributes to endocrine resistance in breast cancer [13]. Additionally, FOXA1 functions as a pioneer factor for androgen receptor (AR) to regulate prostate-specific gene expression. Jin et al. depicted that FOXA1 defines the AR cistrome by retaining AR at excessive open chromatin regions [14]. These studies suggest that FOXA1-mediated chromatin remodeling is instrumental for other factors. The human FOXA1 gene is located at chromosome 14q21, a region where frequent chromosomal deletions are common in somatic NPC [15,16]. Our previous work indicated that the FOXA1 mRNA and protein levels were decreased in NPC samples [17]. The loss of FOXA1 contributes to the EMT process and tumor invasiveness in NPC. However, neither the association of FOXA1 expression with clinical variables nor its impact on TGF-β dual functionality is clearly understood. In this study, we demonstrated that the downregulation of FOXA1 protein is common in somatic NPC samples. The loss of FOXA1 is associated with lymph node metastasis states and predicts the poor clinical outcome of NPC patients. The depletion of endogenous FOXA1 in NPC cells by RNAi promotes cell proliferation, migration and invasiveness, whereas re-expression of FOXA1 in NPC cells exerts the opposite effect. Additionally, exogenous FOXA1 suppresses NPC cell tumorigenicity and metastasis in vivo. The transcriptomics assay unveiled that FOXA1 expression broadly affects the TGF-β-stimulated transcription program in NPC cells, facilitating tumor-suppressive TGF-β target gene expression but impairing tumor-supportive TGF-β target gene expression. Functionally, the re-expression of FOXA1 re-sensitizes NPC cells to TGF-β-mediated inhibitory effects. However, silencing FOXA1 relieves TGF-β inhibition on cell growth. Bone morphogenic protein and activin membrane-bound inhibitor homolog (BAMBI), a well-known inducible pseudoreceptor for TGF-β, is directly regulated by FOXA1. The FOXA1 binding on BAMBI enhancers leads to increased H3K4me1 and H3K27ace levels associated with BAMBI, which, in turn, promotes TGF-β-stimulated SAMD2/3 binding on the BAMBI promoter. Silencing of BAMBI in FOXA1 expressing NPC cells rescues the cell proliferation and invasiveness of NPC cells. Thus, our data indicate, for the first time, that FOXA1 suppresses NPC progression by switching the TGF-β stimulated transcriptional program from metastasis promoter to tumor suppressor.

2. Materials and methods

2.1. Tumor tissue samples, immunohistochemistry and immunofluorescence staining

A cohort of 114 NPC cases, 64 samples of non-cancerous inflammatory nasopharyngeal epithelial (NPE) tissues, 38 samples of dysplastic NPE, and 14 samples of lymph nodes metastasis samples, was recruited between January 2001 and October 2004 from the Pathology Department of the Affiliated Hospital of Jining Medical University (Shandong, PR China). The FOXA1 protein level was measured by immunohistochemical staining. Immunohistochemistry was performed according to methods described in previous studies [18–21]. A staining index (values, 0–6) obtained as the staining intensity (scores: negative = 0, weak = 1, moderate = 2, or strong = 3) and % of staining of tumor cells (scores: < 10% = 1, 10%–50% = 2, > 50% = 3) were calculated. The sum of these two scores was used as the final immunoreactive score (0–6)—i.e., low expression (0–2 scores) and high expression (3–6 scores). This study was approved by the Institute Research Ethics Committee for the use of clinical samples, and each patient signed a consent form to participate in the study. A polyclonal anti-FOXA1 antibody was obtained from Epitomics Inc. (Burlingame, CA, USA).

2.2. Cell lines and culture

NPC cell lines HK1, C666-1, CNE1 and CNE2 and a hypopharyngeal carcinoma cell line FaDu were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (GIBCO, Grand Island, NY) in a humidified incubator at 37 °C with 5% CO_2 and 95% air.

2.3. Immunofluorescence staining

F-actin was visualized by using CytoPainter Phalloidin-iFluor 594 reagent (Abcam; Cat No. ab 176757). Immunofluorescence staining was performed as described previously [22] and immunostained cells were imaged using a Laser Scanning Confocal Microscopy (UltraView, Perkin Elmer, Cambridge, UK).

2.4. siRNA, shRNA and gene transfection

The siRNAs for the knockdown of human FOXA1 and BAMBI and nonspecific siRNAs (scrambled sequences) were purchased from GenePharma (Shanghai, China) and were used for transfection into NPC cells using Lipofectamine® RNAiMAX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The siRNAs sequences used in this study are listed in Table S1.

2.5. RNA isolation and real-time reverse transcription PCR (RT-qPCR)

Total RNA extraction was performed as described previously [22] using TRIzol Reagent (Invitrogen, San Diego, CA). For reverse transcription, 1 μg of total RNA sample treated with DNase I (Roche Diagnostics, Rotkreuz, Switzerland) was reverse-transcribed into cDNA using the M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's protocol. Real-time PCR using SYBR Green I technology was then performed using the CFX96 Touch™ Real-Time PCR Detection System (BioRad). The PCR primers used in this study are listed in Table S1.

2.6. Microarray and RNA-seq

Total RNAs were extracted using TRIzol reagent (Invitrogen). The integrity of the RNA was monitored using Bioanalyzer 2100. Preparation of cDNA and cRNA was conducted following instructions in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA). Microarray profiling was performed using Affymetrix 3′ IVT microarray (Affymetrix). A twofold cutoff was used to identify differentially expressed genes between two cell populations. GO terms enrichment was analyzed using DAVID (https://david.ncifcrf.gov/) [23]. RNA-SEQ was performed by using BGISEQ-500 sequencer. Differentially expressed genes between two groups was identified by NOISeq method [24]. GSEA35 was performed as described previously [25].

2.7. Chromatin immunoprecipitation (ChIP) and qPCR

For ChIP analysis, cells grown on a 10-cm plate were processed as described in the ChIP Assay kit protocol from Millipore (17–295). The chromatin was immunoprecipitated using the following antibodies: anti-FOXA1 (Abcam; Cat No. ab5089), anti-H3K4 me1 (active motif; Cat No. 61634), anti-H3K27ace (active motif; active motif; Cat No. 39685) and anti-SMAD3 (Cell Signaling Technology; Cat No. 8685S). The precipitated DNA fragments were purified and measured by qPCR under the conditions described above. Primers specific to each segment of interest are listed in Table S1.

2.8. Protein extraction and western blotting

The cultured cells were lysed with a lysis buffer (Beyotime, Jiangsu, China). Equal protein amounts from different extracts were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA). The membranes were immunoblotted with the following antibodies: polyclonal anti-FOXA1 (Abcam; Cat No. ab173287), polyclonal anti-BAMBI (Sino Biological Inc.; Cat No. 10890-RP02), anti-E-cadherin (Cell Signaling Technology; Cat No. 3195P), anti-vimentin (Cell Signaling Technology; Cat No. 5741P), anti-SMAD2 (Cell Signaling Technology; Cat No. 5339P), anti-SMAD3 (Cell Signaling Technology; Cat No.9523P), anti-SMAD4 (Cell Signaling Technology; Cat No. 9515P), and anti-GAPDH (BBI Life Sciences; Cat No. D190090-0100). The methods used were in accordance with those reported in previous studies [24,40].

2.9. Cell viability assay

C666-1 and NP69 cells transfected with FOXA1 or BAMBI siRNA or scrambled siRNA were seeded into 96-well plates at a density of 2×10^3 cells per well and were grown for 24, 48, 72 and 96 h. Cell growth was measured by using Cell Counting Kit-8 (CCK-8).

2.10. Cell migration, invasion and adhesion assays

Tumor cell migration and invasion assays were performed as described previously. Briefly, cell suspensions in serum-free medium were seeded onto 8-μm-pore Transwell inserts (Corning-Costar, Cambridge, MA) at a density of 50,000–100,000 cells/well, and then the inserts were held in a lower chamber with 600 μl of culture medium containing 15% FBS. The Transwells were incubated for 6–24 h at 37 °C. Cells on the inside of the Transwell inserts were removed with a cotton swab. Next, cells that migrated to the lower surface of the membrane were fixed and stained. Photographs of five random fields were taken, and the cells were counted to calculate the average number of cells that had transmigrated. For the tumor cell invasion assay, the membrane was pre-coated with 15 μl Matrigel (BD Biosciences, Bedford, MA), and the rest of the method was identical to the tumor cell migration assay except for cell incubation, which was conducted for 24–48 h at 37 °C. Cell adhesion assays were performed as described previously. Briefly, cells suspension (5×10^5 cells/2 ml) was added to collagen I pre-coated 6-well plates and incubated for 30 min at 37 °C, then the non-adhered cells were removed by washing with serum free RMPI 1640 medium. The adhered cells were incubated with RMPI 1640 medium containing 10% FBS at 37 °C for 4 h for recovery. Then the relative cells number from five random fields was counted to calculate the average number of adhered cells.

2.11. In vivo tumorigenesis and metastasis studies

An animal protocol was approved by the ethical review committee of The Central South University of China. To determine the in vivo tumorigenicity of NPC cells, cells were harvested using trypsin-EDTA, washed with RMPI-1640, and re-suspended in serum-free RMPI-1640. The suspended cells (1×10^6/0.2 mL) were subcutaneously injected into the inguinal mammary fat pad of 6- to 8-week-old male BALB/c nude mice. For the nude mouse tumor cell metastasis model, tumor cells were harvested using trypsin-EDTA, washed with RMPI-1640, and re-suspended in serum-free RMPI-1640. The suspended cells (1×10^6/0.2 mL) were injected into the lateral tail vein of 6-week-old male BALB/c nude mice (Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, China), and they were sacrificed 60 days after tumor cell inoculation or when the tumor became moribund. At the end of the experiments, lung tissues were fixed in 4% saline-buffered formalin, embedded in paraffin, sectioned at 5 μm, and then stained with H&E. A minimum of 15 sections was examined per mouse under a light microscope. Experiments with animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Central South University.

2.12. Statistical analysis

Differences in the quantitative variables between groups were analyzed by Student's *t*-test. Pearson's χ^2 test was used to analyze the association of FOXA1 expression with clinicopathological characteristics using the SPSS 13.0 software package (SPSS, Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Frequent loss of FOXA1 protein in NPC (loss of FOXA1 protein is associated with lymph node metastasis and an unfavorable clinical outcome)

We measured the FOXA1 protein level in 114 NPC cases, 64 samples of non-cancerous inflammatory nasopharyngeal epithelial (NPE) tissues, 38 dysplastic NPE samples and 14 lymph nodes metastasis samples. Strong nuclear staining for FOXA1 protein was observed in 62 out of 64 (96.9%) non-cancerous NPE cases, especially in well-differentiated multiciliated epithelial cells at the luminal side of NPE tissues (Fig. 1A). However, FOXA1 protein was significantly decreased in the dysplastic epithelium (26 of 38; 68.4%, $P < 0.001$) (Fig. 1B&C), NPC samples (80 of 114; 70.2%, $P < 0.001$) (Fig. 1D&E) and metastatic lymph nodes (9 of 14, 64.3%, $P < 0.001$) (Fig. 1F) (Table 1). Thus, our data clearly indicate that the loss of FOXA1 protein is common in NPC progression, consistent with our previous observation [17].

We then analyzed the association of FOXA1 expression with clinicopathological variables (Table 2). Although the expression of FOXA1 is not associated with patient ages, gender, WHO classification and clinical stages, the loss of FOXA1 is associated with the lymph node metastasis status. Importantly, our data showed that high expression of FOXA1 protein predicts a more favorable 5-year overall survival (OS) or disease-free survival (DFS) than does lower FOXA1 expression ($P < 0.01$ and $P < 0.05$, respectively; Fig. 1G&H).

3.2. Depletion of endogenous FOXA1 induces NPC cell proliferation and invasion capacity in vitro

To determine the role of FOXA1 protein in NPC cells, endogenous FOXA1 expression was depleted in NP69 or C666-1 cells by siRNA (Fig. 2A&B). Silencing FOXA1 in NP69 and C666-1 cells accelerated cell growth in vitro (Fig. 2C). We also demonstrated that transient depletion of FOXA1 in NP69 or C666-1 cells promotes cell migration and invasiveness in vitro (Fig. 2D&E).

3.3. Expression of FOXA1 suppresses NPC cell growth, migration and invasiveness in vitro

FOXA1 was stably introduced into HK1, CNE1 and CNE2 NPC cell lines using a FOXA1-expressing lentivirus. The exogenous FOXA1 protein was measured by qPCR and western blotting, and the data revealed that the exogenous FOXA1 protein levels in lentivirus-infected cells were comparable to the endogenous FOXA1 protein level in C666-1 cells (Fig. S1A&B). The effect of FOXA1 expression on NPC cell growth, motility and invasiveness was measured. As evidenced by the cell counting kit-8 (CCK-8) assay, re-expression of FOXA1 in NPC HK1, CNE1 and CNE2 cells suppresses cell proliferation (Fig. 3A). The scratch wound-healing assay revealed that FOXA1-expressing NPC cells exhibited significantly reduced mobility compared with vector control cells (Fig. 3B). We also performed the Matrigel-free Boyden chamber migration assay and Matrigel-coated Boyden chamber invasion assay. The results clearly demonstrated that FOXA1 expression suppresses NPC cell migration and invasiveness in vitro (Fig. 3C and D). Cell

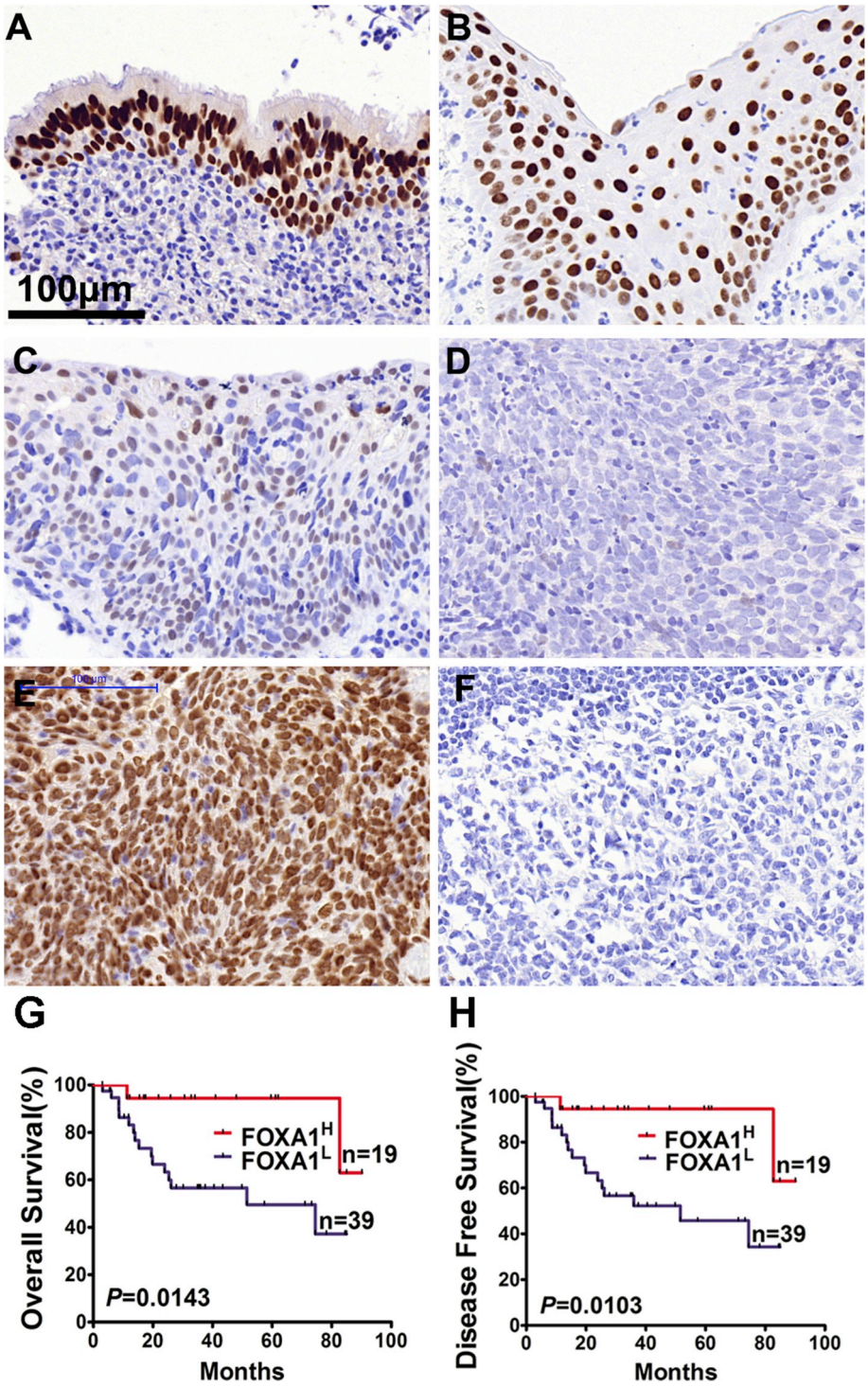

Fig. 1. Loss of FOXA1 protein in NPC predicts an unfavorable prognosis.
A, strong immunostaining of FOXA1 protein in normal NPE. B, strong immunostaining of FOXA1 protein in hyperplastic NPE. C, weak staining of FOXA1 in dysplastic NPE. D, negative staining of FOXA1 in NPC. E, moderate to strong staining of FOXA1 in NPC samples. F, negative staining of FOXA1 in NPC lymph node metastasis. G, overall survival of NPC patients stratified according to FOXA1 protein expression. H, event-free survival of NPC patients stratified according to FOXA1 protein expression.

adhesion assays revealed that expression of FOXA1 reduced NPC cells adhesion on collagen I pre-coated plates (Fig. S2).

3.4. Forced expression of FOXA1 reduces mesenchymal properties in NPC cells

Stable expression of FOXA1 leads to increased E-cadherin, β-catenin and epithelial CK4 but decreased vimentin mRNA levels in HK1, CNE1 and CNE2 cells (Fig. S3A). A western blot assay also showed the up-regulation of E-cadherin and β-catenin but downregulation of vimentin protein in FOXA1-expressing HK1, CNE1 and CNE2 cells (Fig. S3B). Immunofluorescence assay confirmed increased membrane E-cadherin but decreased cytoplasmic vimentin in FOXA1-expressing HK1 and CNE2 cells (Fig. S3C). Phalloidin-iFluor staining revealed that FOXA1

Table 1
Loss of FOXA1 expression is common in NPC samples.

	$FOXA1^{L}$	$FOXA1^{H}$	Total	χ^2	*P value*
	case (%)	case (%)			
Normal tissues[a]	2(3.1)	62(96.9)	64		
Hyperplasia[b]	26(68.4)	12(31.6)	38	51.044	< 0.01 (P^{a-b})
NPC[c]	80(70.2)	34(29.8)	114	74.169	< 0.01 (P^{a-c})
Metastatic lymph nodes[d]	9(64.3)	5(35.7)	14	35.472	< 0.01 (P^{a-d})

A value of $P < 0.05$ was considered statistically significant.

Table 2
Association of the FOXA1 protein level with the clinicopathological data of NPC patients.

Clinical information	$FOXA1^{L}$	$FOXA1^{H}$	Total	X^2	*P*
	N = 80	N = 34			
	case(%)	case(%)			
Age (yrs)					
≤54	36(67)	18(33)	54	0.604	0.437
> 54	44(73)	16(27)	60		
Gender					
Female	22(76)	7(24)	29	0.601	0.438
Male	58(68)	27(32)	85		
WHO					
I	0	0	0	0.615	0.433
IIa	18(64)	10(36)	28		
IIb	62(72)	24(28)	86		
Clinical stage					
I + II	8(62)	5(38)	13	0.163	0.686
III + IV	12(55)	10(45)	22		
Lymph node metastasis (N)					
N_0	1(20)	4(80)	5	5.182	0.023
N_{1-3}	38(70)	16(30)	54		
Distant metastasis (M)					
M_0	26(59)	18(41)	44	2.532	0.112
M1	13(81)	3(19)	16		

Abbreviations: WHO I, keratinizing squamous cell carcinoma, WHO IIa, Non-keratinizing differentiated carcinoma, WHO IIb, Non-keratinizing undifferentiated carcinoma.

expressing cells showed mild to moderate morphological changes, including less formation of membrane protrusions and decreased length of intracellular F-actin stress fibers compared with control HK1 and CNE2 cells (Fig. S3C). Thus, our data indicated that forced expression of FOXA1 reduces mesenchymal properties of NPC cells. However, depletion of FOXA1 failed to trigger EMT in C666-1 cells (data not shown), suggesting the loss of function of FOXA1 alone is insufficient to accomplish cell identity transition.

3.5. FOXA1 overexpression decreases in vivo tumor formation and metastasis

We then asked whether FOXA1 suppresses NPC malignant behavior in vivo. Human tumor xenograft models revealed that FOXA1 suppresses the tumorigenicity of HK1 cells in nude mice. Xenografts from FOXA1-expressing cells were much smaller than the vector control cells (Fig. 4A). Hematoxylin & eosin (H&E) staining suggested that FOXA1 promotes NPC cell differentiation with a decreased nucleus-to-cytoplasm ratio, well-organized tumor architecture and cilia formation in some tumor cells (Fig. 4B). Immunohistochemical staining demonstrated fewer Ki67-or vimentin-positive cells in tumors from FOXA1/HK1 cells but increased membrane-localized E-cadherin and β-catenin protein in tumors from FOXA1/HK1 cells (Fig. 4C). To determine the FOXA1 effect on the metastatic potential of NPC cells, we constructed a lung metastasis model by tail vein injection. The data indicated that FOXA1 expression inhibits metastatic tumor formation in the lungs of nude mice (Fig. 4D and E). Thus, our data indicated that FOXA1 promotes epithelial differentiation and suppresses NPC cell tumorigenicity and metastatic potential in vivo. These data indicate that FOXA1 plays a role as a bona fide tumor suppressor in NPC.

3.6. FOXA1 reprograms the TGF-β-stimulated transcription program in NPC HK1 cells

To address the downstream effects of FOXA1 expression, we performed microarray analysis on control versus'FOXA1 high' asynchronous HK1 cells. Using three biological replicates, we found 298 differentially regulated genes (including 211 upregulated and 87 downregulated genes) with a false discovery rate (FDR) of $P < 0.05$ in FOXA1-expressing HK1 cells (Supplementary dataset 1). Pathway analysis of the FOXA1-upregulated genes revealed the top 5 signaling pathways affected by FOXA1, including differentiation pathways (retinol metabolism) and the TGF-β signaling pathway, which is perturbed in NPC (Fig. S4). Gene Set Enrichment Analysis (GSEA) revealed the data from FOXA1 high microarray were correlated with the response to TGF-β, TGF-β signaling pathway, TGF-β receptor signaling pathway and EMT identified by gene ontology (GO) or KEGG (Fig. 5A). Impressively, several TGF-β-responsive genes were differentially expressed in FOXA1/HK1 cells. Among the TGF-β-responsive genes upregulated by FOXA1 are several tumor suppressor genes, including BAMBI, PMEPA1, LMO7 and HPGD. However, CD44, vimentin, L1CAM, CCND2 and PTHLH, TGF-β-inducible oncogenes, were decreased in FOXA1/HK1 cells (Fig. 5B) (Fig. S4). We measured the SMAD protein level in FOXA1-expressing or FOXA1-deficient NPC cells. Our data revealed that FOXA1 expression led to a weak to moderate increase in SMAD2, SMAD3 and SMAD4 protein levels in HK1, CNE1 and CNE2 cells. By contrast, depletion of FOXA1 resulted in a weak reduction of the SMAD protein levels in C666-1 cells (Fig. S5).

To better define the FOXA1 effect on the TGF-β-stimulated transcriptional program, we treated control or FOXA1/HK1 cells with TGF-β (5 ng/mL) for 24 h; then RNA-seq was utilized to compare the transcriptome difference. Total RNA was isolated, and strand-specific paired-end RNA BGI sequencing with reads of 50 bp in length was performed. Next, we utilized NOISeq method [24] to determine FOXA1-dependent TGF-β-responsive transcripts in HK1 cells. The following comparisons were performed: (1) Control versus Control + TGF-β; (2) Control versus FOXA1; (3) Control + TGF-β versus FOXA1 + TGF-β; and (4) FOXA1 versus FOXA1 + TGF-β. We obtained 852, 1,726, 1,422, and 288 differentially expressed transcripts, respectively (Supplementary dataset 2–4). A supervised heatmap revealed five major categories as follows: (a) TGF-β-inducible genes activated by FOXA1, (b) TGF-β-inducible genes repressed by FOXA1, (c) TGF-β-induced genes, (d) TGF-β-repressed genes, and (e) a set of transcripts induced by either TGF-β or FOXA1 alone but decreased in FOXA1/HK1 cells treated with TGF-β (Fig. 5C). This supervised heatmap clearly depicted that FOXA1 expression reprograms the TGF-β-stimulated transcription program, as evidenced by FOXA1 facilitating TGF-β-inducible genes belonging to the category (a) but abolishing the induction effect of TGF-β on genes belonging to the category (b). We also compared the differentially expressed genes (DEGs) between FOXA1/HK1 + TGF-β vs Control/HK1 + TGF-β. Functionally, these DEGs were categorized into keratinocyte differentiation, cell cycle, glycolysis and gluconeogenesis (Fig. 5D). FOXA1 expression in HK1 suppressed the CCNA1, CCNB1, hexokinase 1, and LDH1A levels when cells were treated with TGF-β, suggesting that FOXA1 blocks TGF-β-stimulated cell cycle progression and glycolysis. Detailed information concerning the DEGs is listed in supplementary dataset 4. We also performed GSEA analysis on the RNA-seq data, and the results revealed that data from FOXA1 high RNA-seq were correlated with SMAD binding even under serum-free

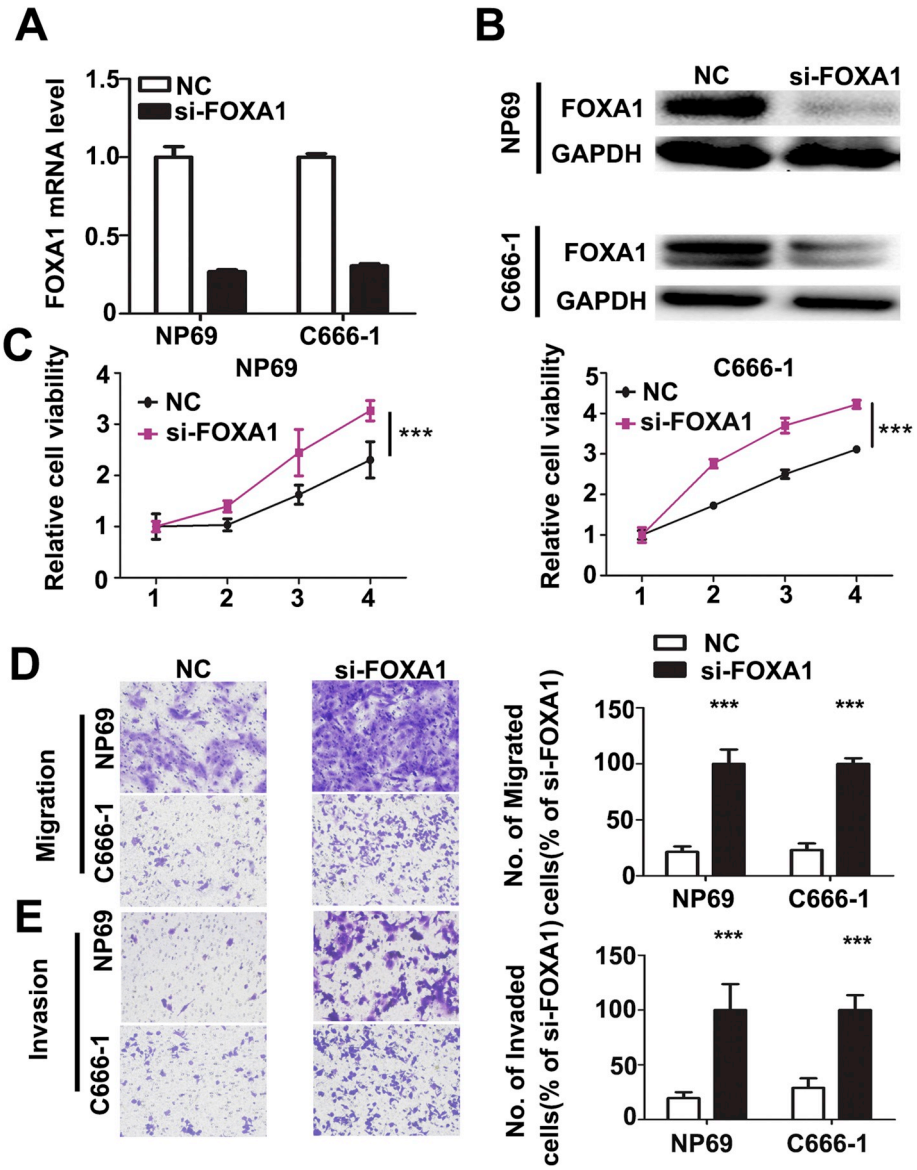

Fig. 2. Loss of FOXA1 protein promotes NPC cell proliferation, migration and invasiveness in vitro.
A, FOXA1 mRNA levels in NP69 and C666-1
cells transiently transfected with FOXA1 siRNAs were determined by qPCR. B, the FOXA1 protein levels in NP69 and C666-1 were measured by western blotting. C, the FOXA1 protein level in Cas9-mediated knockout C666-1
cells was measured by western blotting. D, CCK8 assay showed that FOXA1 deficiency promotes cell growth. E, migration assay. F, invasion assay. ***$P < 0.01$.

conditions (Fig. 5E). GSEA also revealed that FOXA1 high RNA-seq data correlated with cell cycle progression, glycolysis and gluconeogenesis and keratinocyte differentiation (Fig. 5E).

We further evaluated the RNA-seq data by RT-qPCR. As shown in Fig. 5F, recombinant TGF-β stimulated the expression of metastasis-(L1CAM, PHLDA1, PTHLH, HMGA2, THBS2, and vimentin) effect was abolished by FOXA1. However, FOXA1 expression facilitates the expression of recombinant TGF-β-induced tumor suppressor genes (BAMBI, LMO7, PMEPA1) (Fig. 5F). Similar effects were observed in FOXA1 expressing CNE1 and CNE2 cells (Fig. S6). We also treated cells with SB431542, an ALK5 inhibitor that blocks TGF-β signaling. We observed that SB431542 treatment led to decreased expression of representative TGF-β-responsive tumor suppressor genes (BAMBI, PMEPA1, LMO7) tumor suppressors by FOXA1. However, SB431542 treatment led to the reduction of TGF-β-responsive oncogenes, such as CD44, L1CAM, PHLDA1, PTHLH, and HMGA2, in control cells but showed little oncogene expression in FOXA/HK1 cells (Fig. 5G). Functionally, FOXA1 expression sensitizes HK1 cells to TGF-β mediated growth inhibition (Fig. 5G). Thus, our data clearly demonstrated that FOXA1 switches the TGF-β-stimulated transcriptome from a metastasis-promoting program to a tumor-suppressive program.

3.7. FOXA1 binding on the BAMBI enhancer recruits SMAD2/3 to its promoter

BAMBI is transcriptionally regulated by the TGF-β/SMAD signaling pathway [26]. Therefore, we attempted to characterize the mechanisms by which FOXA1 induces BAMBI expression. Analysis of the sequence across the BAMBI gene locus revealed the presence of three putative forkhead binding motifs (FKHD) at -1436 - -1421 bp, +1585 - +1599 and + 2212 - +2226 bp, whereas three tandem SMAD binding elements (3 × SBE) at -189 - -149 bp [26] (Fig. 7A). ChIP-qPCR assay performed in FOXA1/HK1 cells using anti-FOXA1 demonstrated that FOXA1 binds to +1585 - +1599 of BAMBI (Fig. 6B). Concomitantly,

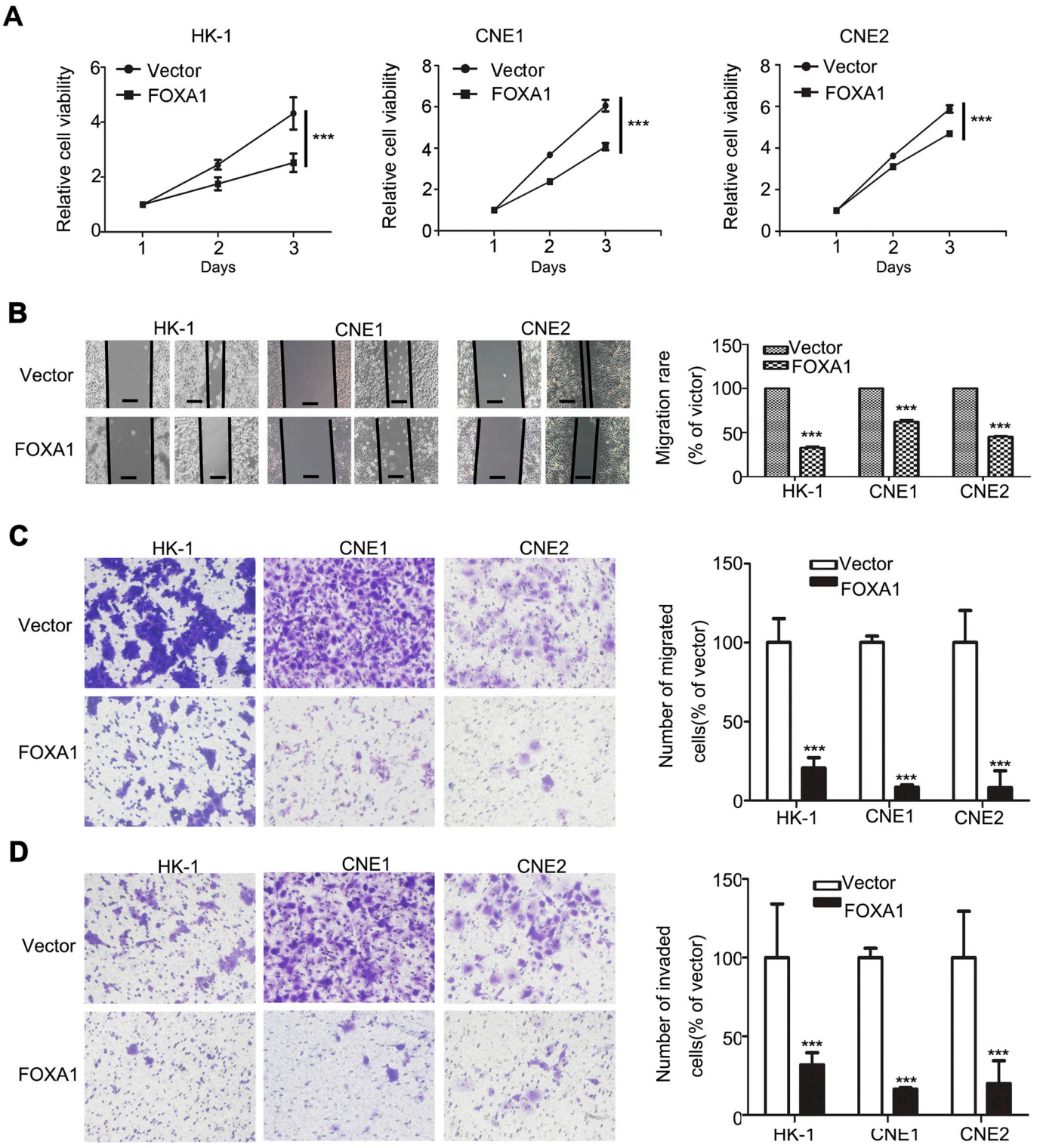

Fig. 3. Re-expression of FOXA1 protein at the physiological level suppresses NPC cell proliferation, migration and invasiveness in vitro.
A, CCK8 assay showed exogenous FOXA1 reduces NPC cell growth and proliferation. B, Wound-healing assay showed FOXA1 inhibits NPC cell migrative ability. C, migration assay. D, invasion assay. ***$P < 0.001$.

the association of BAMBI with H3K4me1 and H3K27ace around FOXA1 binding sites in HK1 cells is induced, suggesting that FOXA1 binding induces H3K4me1 and H3K27ace modifications that spread to nearby regions of the FKHD motif (Fig. 6C). These epigenetic changes facilitate subsequent recruitment of SMAD2/3 binding to the 3 × SBE site at the BAMBI promoter in FOXA1-expressing HK1 and CNE2 cells (Fig. 6D). Consequently, forced expression of FOXA1 led to robust induction of BAMBI mRNA in HK1, CNE1 and CNE2 cells, whereas depletion of FOXA1 in NP69 and C666-1 cells exerted opposite effects (Fig. 6E). Western blot assay also revealed similar results (Fig. 6F). Thus, our data indicated that FOXA1 binding promotes BAMBI transcription, which is mediated by the TGF-β-activated SMAD complex through inducing BAMBI gene-associated chromatin remodeling.

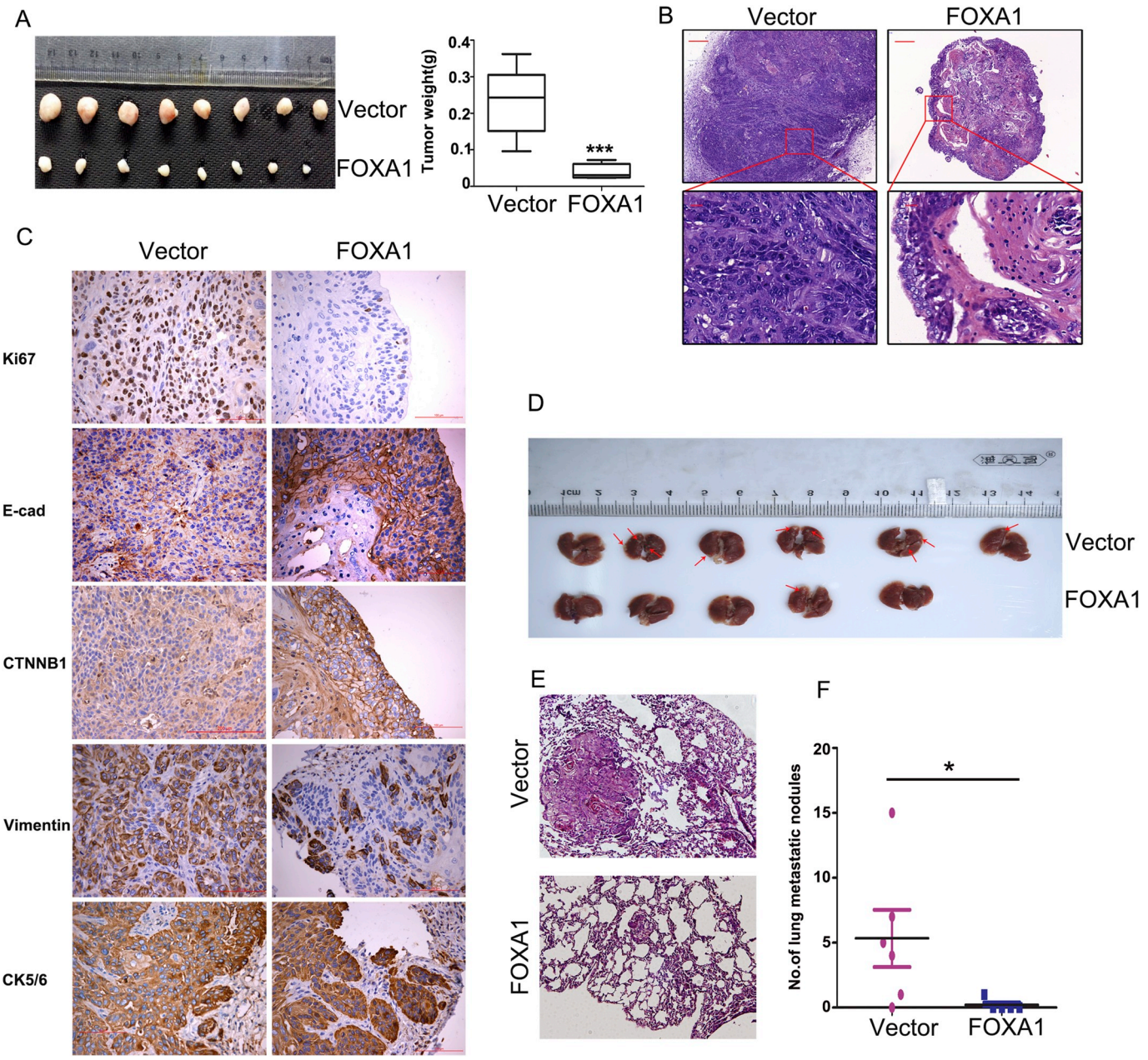

Fig. 4. FOXA1 promotes cell differentiation but suppresses NPC cell tumorigenicity and metastasis in vivo.
A, xenograft assay performed in nude mice showing that FOXA1 suppresses HK1 cell tumorigenicity. B, representative H&E staining of xenografts showed that FOXA1 expression promotes HK1 cell differentiation toward ciliated columnar cells. C, immunohistochemical staining showed that FOXA1 inhibit the NPC cell EMT process in vivo. D, nude mouse metastasis assay showed the macroscopic mouse lung at 8 weeks after tail vein injection of control vector and FOXA1-expressing NPC HK1 cells. E, representative H&E staining of lung metastatic tumors is shown. F, the number of lung metastatic tumor nodules in mice (n = 6 or 5, respectively) was examined under an anatomical microscope and summarized at the end of the experiment. The scatter diagram in the graph depicts the number of metastatic nodules in 15–20 tissue sections from every group. *$P < 0.05$, ***$P < 0.001$.

3.8. BAMBI suppresses NPC cell proliferation, migration and invasiveness in vitro

We next asked whether BAMBI functions as a tumor suppressor in NPC development. The human BAMBI gene was stably introduced into HK1 and FaDu cells using a lentivirus expression system (Fig. 7A and B). The CCK8 assay revealed re-expression of BAMBI in these cells inhibited cell proliferation (Fig. 7C). In addition, BAMBI suppressed tumor cell migration and invasiveness in vitro, as evidenced by the Transwell assay (Fig. 7D). Silencing endogenous BAMBI expression in C666-1 NPC cells not only accelerated cell growth (Fig. 7E and F) but also facilitated its migrative and invasive abilities (Fig. 7G). Finally, we observed that repressing BAMBI in either FOXA1/HK1 or FOXA1/CNE2 cells rescued its invasiveness suppressed by FOXA1 (Fig. 7H and I). Thus, our data clearly indicate that BAMBI is a FOXA1-regulated tumor suppressor gene downstream of the TGF-β/SMAD signaling pathway.

4. Discussion

In this study, we demonstrated that the pioneer factor FOXA1 is frequently decreased in sporadic NPC. Loss of FOXA1 predicts poor clinical outcomes. FOXA1 suppresses NPC cell proliferation and invasiveness in vitro, and tumorigenicity and metastasis potential in vivo. Mechanistically, FOXA1 reprograms the TGF-β-stimulated

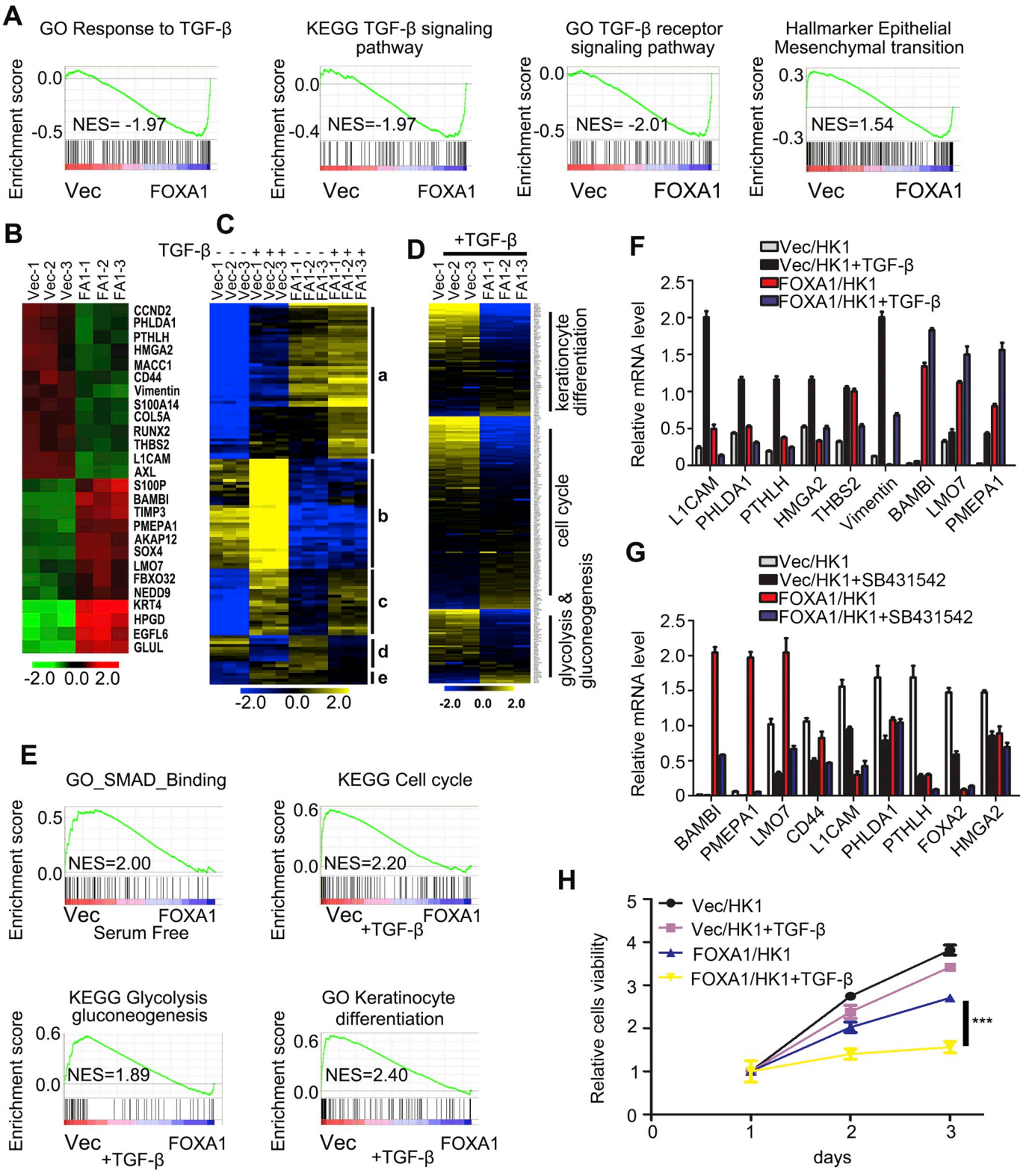

Fig. 5. FOXA1 reprograms TGF-β-stimulated transcription.

A, GSEA analysis of Affymetrix microarray data from FOXA1-deficient or FOXA1-expressing HK1 cells. B, representative heatmap showed differentially expressed TGF-β target genes regulated by FOXA1 expression. C, heatmap showed clustering of differentially expressed genes (DEGs) in HK1 cells expressing FOXA1, untreated or treated with TGF-β. D, heatmap showed expression data for representative DEGs between FOXA1-expressing or FOXA1-deficient HK1 cells treated with TGF-β. E, GSEA analysis showed RNA-seq data from FOXA1-deficient or FOXA1-expressing HK1 cells, untreated or treated with TGF-β. ***$P < 0.001$. F, realtime RT-PCR assay showed FOXA1 differentially regulates TGF-β-responsive genes expression in HK1 cells. G, realtime RT-PCR assay showed the effect of ALK5 inhibitor SB431542 on TGF-β-responsive genes expression in FOXA1-deficient or FOXA1-expressing HK1 cells. H, growth curve measured by CCK8 assay showed that expression of FOXA1 re-sensitize HK1 cells to TGF-β mediated growth suppression. ***$P < 0.001$.

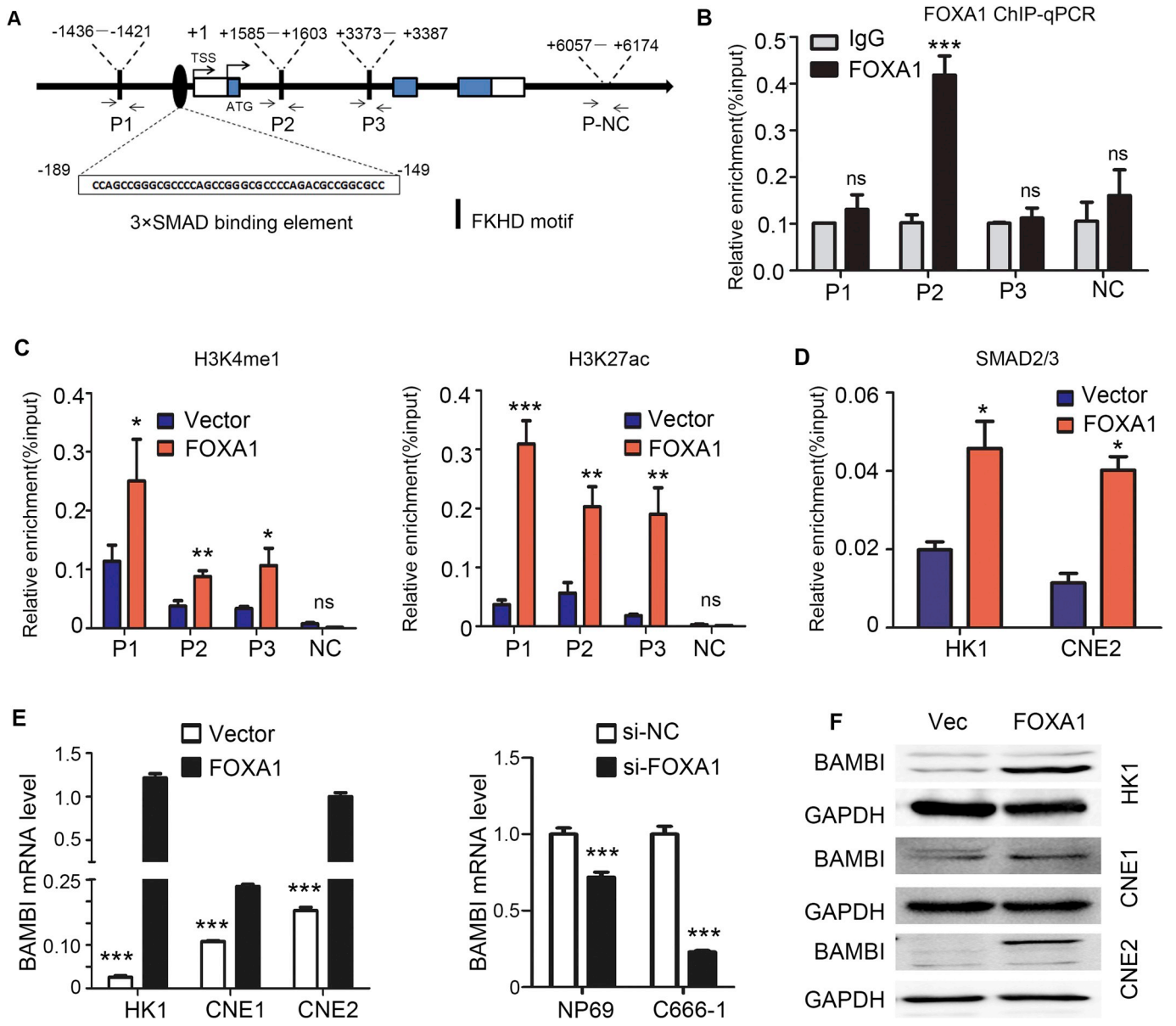

Fig. 6. FOXA1 facilitates SMAD complex binding to the BAMBI promoter and promotes BAMBI transcription.
A, schematic diagram showed the putative forehead (FKHD) motif and SMAD binding elements in the BAMBI gene locus. B, ChIP-qPCR performed to in FOXA1/HK using anti-FOXA1 showed FOXA1 binding to the FKHD motif at +1585-+1599 of BAMBI. C, ChIP-qPCR performed in HK1 cells showed FOXA1 induced H3K4me1 and anti-H3K27ace modification on BAMBI-associated chromatin. D, ChIP-qPCR showed FOXA1 facilitates SAMD2/3 binding to the BAMBI promoter. E, real-time qPCR showed FOXA1 upregulates the BAMBI mRNA level in various NPC cell lines. F, western blotting showed that FOXA1 upregulates BAMBI protein levels. ns, not significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

transcriptome from a metastasis-supportive program to a tumor-suppressive network. We further depicted that BAMBI, a canonical TGF-β target gene, was directly regulated by FOXA1 and functions as a tumor suppressor gene in NPC. FOXA1 binding to BAMBI facilitates the induction of BAMBI expression by TGF-β signaling.

Normal nasopharynx epithelium (NPE) expresses a high level of FOXA1 protein, especially in well-differentiated ciliated columnar cells, suggesting a role of FOXA1 in NPE differentiation. Indeed, re-expression of FOXA1 in HK1 cells promotes cell differentiation in the xenograft assay. FOXA1-expressing HK1 tumor cells were more well-organized and exhibited ciliogenesis. FOXA1 has been recognized as an urothelial differentiation marker. Loss of FOXA1 is associated with enhanced tumor proliferation and invasion in aggressive bladder cancer [27]. Because both the nasopharynx and bladder epithelium are lined with pseudostratified-ciliated columnar cells, we speculate that FOXA1 is a critical luminal differentiation marker for the epithelium from various tissue origins. We also depicted that FOXA1 was decreased in dysplastic epithelium and most NPC samples. Furthermore, loss of FOXA1 is associated with lymph node metastasis and predicted the poor prognosis of NPC patients. Our data are similar to the observation that the loss of FOXA1 expression independently predicted an unfavorable overall survival in bladder cancer [28].

Loss of heterozygosity (LOH) on chromosomal arm 14q was observed in 65%–85.2% of sporadic NPC [29–33], suggesting that putative tumor suppressor genes on chromosomal 14q may contribute to the carcinogenesis of NPC. Among the common deletion regions, 14q21-q24 has been defined as a minimally deleted region [29]. The human FOXA1 gene maps to 14q21.1. In this study, we depicted that the loss of FOXA1 protein was observed in 70.2% of NPC samples, a value that is comparable to the LOH frequency of 14q in NPC. Thus, downregulation

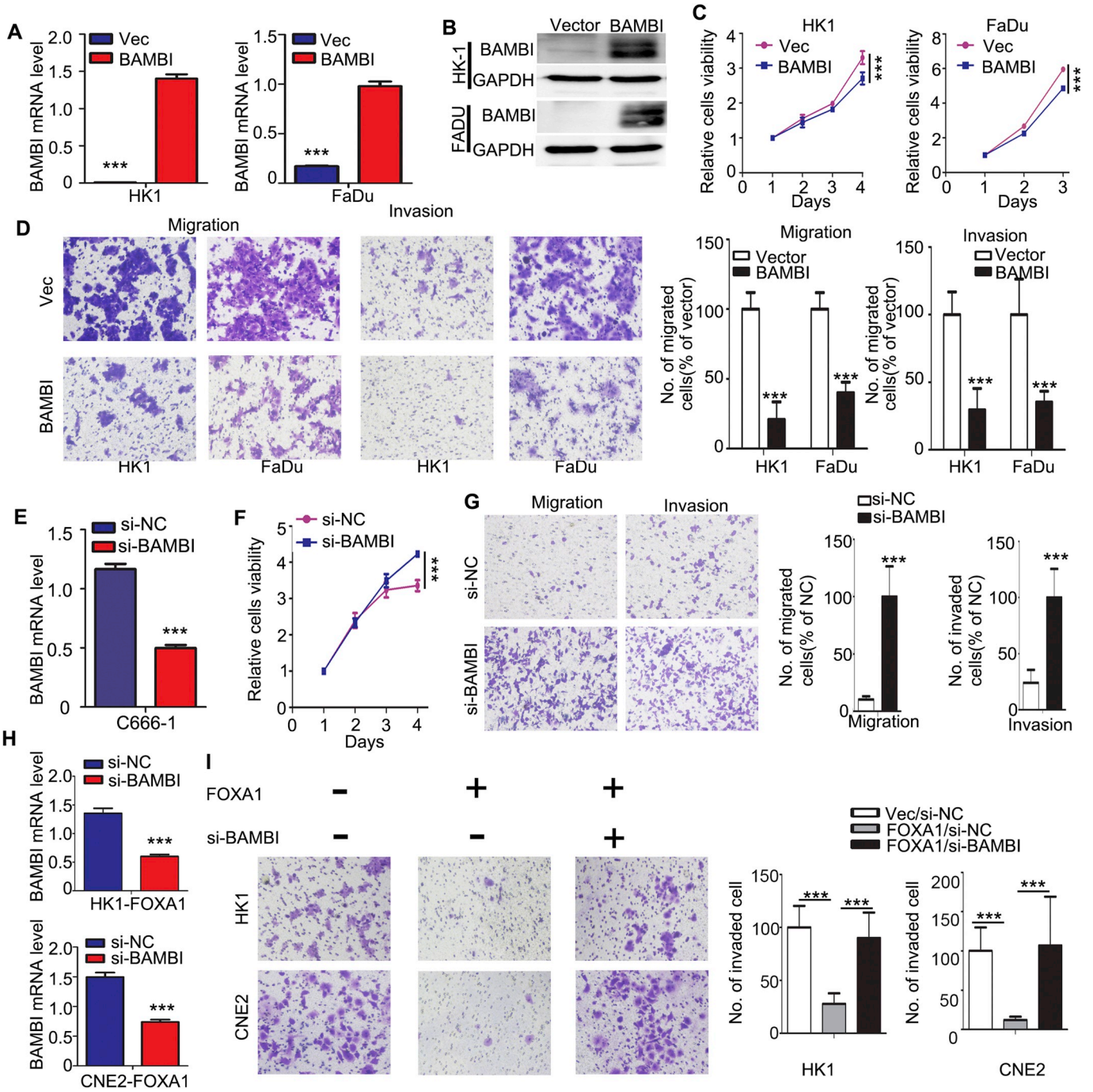

Fig. 7. BAMBI suppresses NPC cell proliferation, migration and invasiveness in vitro.
A, real-time qPCR showed BAMBI mRNA expression levels in lentivirus-infected cells. B, western blotting demonstrated the BAMBI protein levels in lentivirus-infected cells. C, CCK8 assay showed BAMBI overexpression reduced cell growth and proliferation. D, migration and invasion assay performed using Transwell membrane inserts. The data showed BAMBI overexpression inhibited cell migration and invasiveness in vitro. E, real-time qPCR showed BAMBI mRNA expression levels in BAMBI siRNA-transfected C666-1 cells. F, CCK8 assay showed BAMBI silencing accelerated cell growth and proliferation. G, migration and invasion assay showed BAMBI depletion led to enhanced migration and invasion in C666-1 cells. H, real-time qPCR showed BAMBI mRNA expression levels in FOXA1-expressing cells transfected with BAMBI siRNAs. I, migration and invasion assay showed silencing BAMBI rescued migration and invasiveness in FOXA1-expressing cells. $^{**}P < 0.01$, $^{***}P < 0.001$.

of FOXA1 in the NPC sample might result from LOH at 14q. Most NPC cells express a high level of ΔNP63α protein, a p53 family member that exerts a transcription repressor role in many genes [34]. Another possibility is ΔNP63α-mediated transcription repression in FOXA1. We found that ΔNP63α contributed to the loss of FOXA1 expression in NPC, as evidenced by that silencing ΔNP63α restored the FOXA1 mRNA and protein levels in NPC HK1 cells (Fig. S6).

Chromosome 14q harbors multiple crucial tumor suppressor genes for NPC, as evidenced by the monochromosome transfer of chromosome 14 into NPC cells suppressing NPC malignant behavior [35]. We further provided new evidence to support this conclusion because the restoration of FOXA1 in HK1, CNE1 and CNE2 NPC cell lines suppressed cell proliferation and invasiveness in vitro and tumorigenicity and metastasis in vivo. Restoration of FOXA1 also reduced cells adhesion on

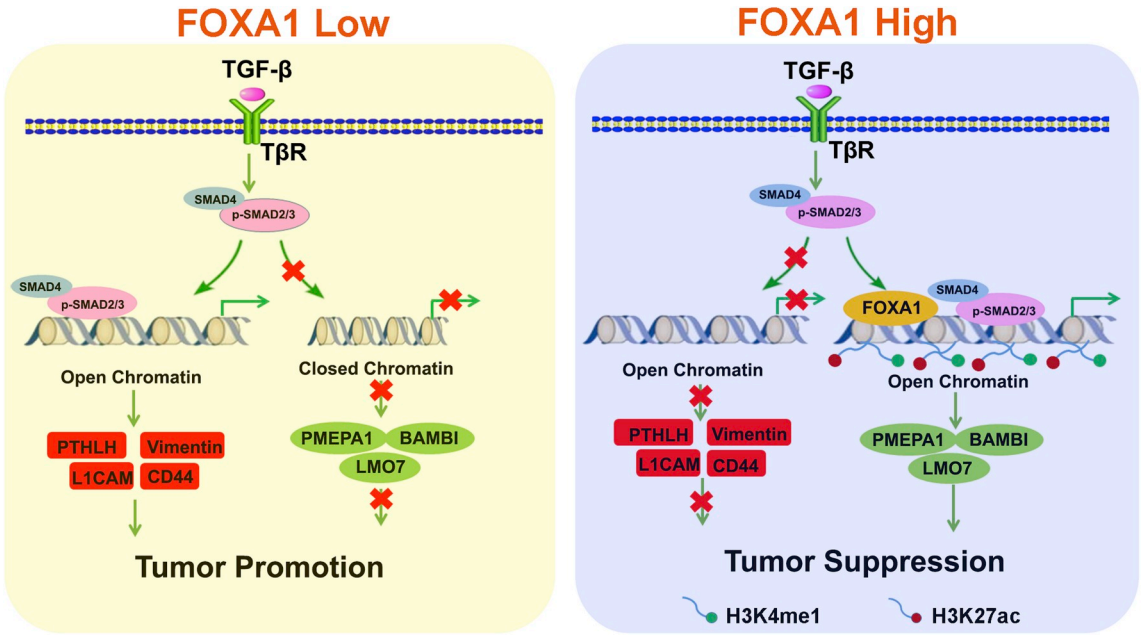

Fig. 8. Schematic diagrams illustrating FOXA1-mediated reprogramming of the TGF-β/SMAD transcription program.
In FOXA1-deficient NPC cells, the TGF-β activated SMAD complex preferentially binds to oncogenic target genes, including PTHLH, HMGA2, and vimentin. In contrast, in FOXA1-sufficient NPC cells, FOXA1 binding to tumor-suppressive target genes such as BAMBI creates excessively open chromatin, siphoning abundant SMAD complexes to distribute to these tumor-suppressive target genes; thus, TGF-β preferentially activate TSGs.

collagen I pre-coated plates (Fig. S2), which is in consistent with downregulation of integrin β2 (ITGB2) mRNA level in HK1 cells by FOXA1 (Supplementary dataset 1). However, the depletion of FOXA1 in C666-1 cells by RNAi accelerated cell growth and invasiveness in vitro, highlighting its tumor-suppressive role in NPC development. The role of FOXA1 in human cancer development is complicated. We demonstrated that exogenous FOXA1 reduced mesenchymal properties in NPC cells, a finding that is consistent with other observations that FOXA1 acts as a repressor of EMT in pancreatic cancer [36,37] and breast cancer [38,39]. However, silencing FOXA1 failed to trigger EMT in C666-1 cells, indicating that the disruption of FOXA1 alone could not initiate EMT in our model, likely due to the compensatory activation of FOXA2 in the absence of FOXA1, because the loss of FOXA1/2 more efficiently induces EMT than either FOXA1 or FOXA2 silencing alone in pancreatic cancer cells [37].

FOXA1 act as a pioneer factor to open condensed chromatin, which, in turn, facilitates lineage transcription factor entry to activate tissue-specific gene expression [40]. In this study, either microarray or RNA-seq revealed that FOXA1 reprogrammed the TGF-β stimulated transcriptome in NPC HK1 cells. In NPC cells, FOXA1 upregulates a set of TGF-β-responsive genes associated with tumor suppression. Among the FOXA1-promoted TGF-β target genes, there are several well-known tumor suppressor genes, including PMEPA1, BAMBI, and LMO7. For example, PMEPA1 is a TGF-β-inducible inhibitor for canonical SMAD signaling [41]. Expression of PMEPA1 halts prostate cancer bone metastases induced by TGF-β/SMAD signaling. Disruption of this negative feedback loop by silencing PMEPA1 accelerates prostate cancer metastases to the bone [42]. LIM domain only 7 (LMO7), a PDZ and LIM domain-containing multifunctional protein, regulates the actin cytoskeleton and assembly of adherent junctions in epithelial cells [43]. LMO7 is induced by TGF-β [44], and mice lacking LMO7 are prone to develop spontaneous lung adenocarcinoma, highlighting its tumor suppressive function [45]. We demonstrated that TGF-β1 preferentially activated tumor suppressive genes expression and suppressed cells growth in the presence of FOXA1, whereas ALK5 inhibitor treatment led to downregulation of tumor suppressive TGF-β responsive genes in FOXA1 expressing cells, suggesting ALK5 targeting therapy would be cautious for those patients within high FOXA1 expression. Given that TGF-β1 is elevated in various human cancer and correlates with tumor metastasis and unfavorable clinical outcome, various therapeutic strategies to block TGF-β pathway in cancer have been developed [46,47]. Based on our observation, we surmise that those NPC patients with high FOXA1 expression would not benefit from TGF-β signaling inhibitors such as ALK5 inhibitors. Thus it is reasonable to stratify NPC patients according to the expression level of FOXA1, when the clinical trials of ALK5 inhibitors for the treatment of NPC.

BAMBI is transcriptionally regulated by TGF-β [26]. We demonstrated that BAMBI is a direct target of FOXA1. FOXA1 binding on BAMBI facilitates SMAD2/3 binding to the BAMBI promoter, in which FOXA1-mediated H3K4me1 and H3K27ace plays a role. Regulation of BAMBI by FOXA1 is very similar to FOXA1-regulated alpha-fetoprotein (AFP) transcription, in which FOXA1 is essential in providing chromatin access for TGF-β-activated Smad2 and Smad4 entry and subsequent DNA binding to the AFP regulatory region during the differentiation of embryonic stem cells [48]. We further provided detailed evidence to show that BAMBI is functionally tumor suppressive in NPC proliferation, migration and invasiveness, a finding that was in accordance with other observations that BAMBI is a tumor suppressor gene silenced in lung cancer [49]. BAMBI is a well-known pseudoreceptor for TGF-β and competitive inhibits TGF-β/SMAD signaling activation. We propose that the regulation of BAMBI by FOXA1 helps to prevent the hyperactivation of TGF-β/SMAD signaling to maintain the homeostasis of nasopharynx epithelial cells.

Although FOXA1 usually interacts with compacted chromatin and increases chromatin accessibility [50,51], it can also promote chromatin inaccessibility and repress gene transcription, in which TLE proteins are involved [52]. For example, FOXA1 binds to the IL-6 promoter and prevents the recruitment of the transcription factor nuclear factor-κB (NF-κB) to this region, thus repressing IL-6 transcription in breast cancer [53]. Indeed, we observed that FOXA1 reduced a set of TGF-β-responsive genes associated with cancer aggressiveness. Among those genes, vimentin, CD44, HMGA2 and PTHLH have tumor-supportive roles in cancer progression. For example, PTHLH is a parathyroid hormone-related protein induced by TGF-β that promotes EMT

and bone metastasis in various cancers [54,55]. We noted that FOXA1 enhanced tumor suppressor gene expression, including that of PMEPA1, LMO7 and BAMBI, even without exogenous TGF-β. By contrast, the induction of oncogenes, including PTHLH, L1CAM, PHLDA1, HMGA2 and vimentin, by TGF-β was abolished by FOXA1. A weak interaction was exhibited between the FOXA1 winged helix and the NH(2)-terminal domains of SMAD3 protein [56]. We proposed that FOXA1 binding on those genes with the FKHD motif creates excessive openness of local chromatin, siphoning many SMAD complexes to redistribute to the promoters of the tumor-suppressive target genes, thus reducing SMAD distribution on oncogenic target gene regulatory regions (Fig. 8). Our hypothesis was supported by a similar mechanism underlying FOXA1 reprogramming androgen receptor transcriptional program [14].

In conclusion, we depicted that FOXA1 is highly expressed in pseudostratified ciliated columnar cells of the nasopharynx. Loss of FOXA1 protein is common in NPC and is associated with lymph node metastasis and an unfavorable outcome. FOXA1 acts as a tumor suppressor master factor in NPC development by controlling the TGF-β-stimulated transcription program. In the absence of FOXA1, TGF-β prefers to activate oncogenic target gene expression. FOXA1 switches the TGF-β-stimulated transcription program from tumor supportive to tumor suppressive, sensitizing NPC cells to the TGF-β growth-inhibitory effect. Our study provides new insight for precise medicine targeting TGF-β signaling in cancer treatment.

Conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.036.

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