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RETRACTION

Retraction: MicroRNA-96-5p promotes proliferation, invasion and EMT of oral carcinoma cells by directly targeting FOXF2

Haiyan Wang, Ning Ma, Wenyue Li and Zuomin Wang

The journal has retracted *Biology Open* (2020) **9**, bio049478 (doi:10.1242/bio.049478) because of unreliable data and author fraud. This notice updates and replaces the previous Retraction and Expression of Concern notices.

Cell images in this paper were reported to be duplicated in two other articles by a commenter on PubPeer. Our analysis found that many of the western blots and several cell images were unexpectedly very similar to those in other articles, where they are presented for completely different cell lines and different experimental conditions. There is no overlap in authorship of the three papers and several other flags indicative of outsourced 'papermill' papers were also noted. The journal therefore retracted the article. The corresponding author listed, Zuomin Wang, contacted the journal after seeing news coverage on the retraction to say that she had no knowledge of this paper.

An investigation was carried out by a team at the corresponding author's institute, Beijing Chaoyang Hospital, Capital Medical University. The team report that:

1. Without their knowledge, Wenyue Li and Zuomin Wang were added as authors of the paper by Haiyan Wang using fake email addresses that were used for submission and correspondence with Biology Open.
2. The study was conducted in Qingdao Municipal Hospital and was approved by the ethics review committee of Qingdao Municipal Hospital. No application was received by the ethics review committee of Beijing Chaoyang Hospital. Haiyan Wang participated in this study as an independent investigator without authorization from Beijing Chaoyang Hospital.
3. Haiyan Wang and Ning Ma collected tissue samples together at Qingdao Municipal Hospital, then Ning Ma outsourced the analysis to a commercial company. The investigation team's review of the original data and images received by Ning Ma showed several signs of forgery, including inconsistency between sample sizes and incomplete data, etc.

The BCH investigation team concluded that: 'the practices of preparation and submission of this study for publication clearly violate academic integrity and ethics. We also strongly question the reliability of the data reported.'

The first author, Haiyan Wang, has contacted the journal admitting supplying fake email addresses and ORCiDs, which prevented Zuomin Wang and Wenyue Li from receiving any correspondence. Biology Open requires ORCiDs and/or non-institutional emails for all authors and apologises to Wenyue Li and Zuomin Wang that, despite our checks on this paper, it was published fraudulently in their names.

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RESEARCH ARTICLE

MicroRNA-96-5p promotes proliferation, invasion and EMT of oral carcinoma cells by directly targeting FOXF2

Haiyan Wang[1], Ning Ma[2], Wenyue Li[1] and Zuomin Wang[1,*]

ABSTRACT

Recently, microRNA-96-5p (miR-96-5p) has been reported to function as both a tumor suppressor and oncogene in several cancer types, including gastric cancer, hepatocellular cancer and lung cancer. However, the biological function of miR-96-5p and its precise mechanisms in oral squamous cell carcinoma (OSCC) have not been well clarified. The aim of this study was to study the roles of miR-96-5p/FOXF2 axis in OSCC. In this study, the miR-96-5p level was dramatically enhanced in OSCC tissues and cell lines, and the FOXF2 expression was significantly reduced. In addition, the FOXF2 expression was negatively related to the miR-96-5p level in OSCC tissues. Furthermore, downregulation of miR-96-5p obviously restrained OSCC cell proliferation, invasion and EMT. We confirmed that miR-96-5p could directly target FOXF2 by luciferase reporter assay. Moreover, knockdown of FOXF2 also could markedly promote the proliferation, invasion and EMT of OSCC cells. Finally, overexpression of FOXF2 in OSCC cells partially reversed the promoted effects of miR-96-5p mimic. Knockdown of miR-96-5p restrained OSCC cells proliferation, invasion and EMT via regulation of FOXF2.

KEY WORDS: Oral squamous cell carcinoma, MicroRNA-96-5p, FOXF2, Proliferation, Invasion

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the [6]th most common cancer worldwide. It is reported that 1.6 million new cases of HNSCC are diagnosed each year, and [half] of HNSCC is oral squamous cell carcinoma (OSCC) with [300,000] deaths (Warnakulasuriya, 2009). Although there are several therapeutic treatments such as chemotherapy combined with radical surgery and surgery combined with radiation, the 5-year survival rate of OSCC is only approximately 50% (Leeman et al., 2017). The pathogenesis of OSCC is complex, and many genes and pathways are involved in it. However, the mechanism of OSCC development remains unclear.

MicroRNAs (miRNAs) are a family of small, endogenous noncoding RNAs. They regulate the translation or induce degradation of specific protein coding genes through binding to the 3′-untranslated regions of the mRNA (Ambros, 2004). According to bioinformatic analysis, it was predicted that miRNAs targeted more than 60% of human genes (Xu et al., 2014). Previous reports demonstrated that altered miRNA expression participated in tumorigenesis and the development of various cancers (He et al., 2005, 2007; Feng et al., 2011). Thus, miRNAs are thought to be markers of cancer diagnosis, progression and prognosis (Bartels and Tsongalis, 2009). Many human miRNAs have been confirmed to be dysregulated in OSCC, including miR-543, miR-4513, miR-31, miR-21 and miR-125b (Wang et al., 2019; Xu et al., 2019; Kao et al., 2019; Jia et al., 2019; Chen et al., 2019). Up until now, miR-96-5p had been reported to function as an oncogene in ovarian cancer, HNSCC, hepatocellular carcinoma (Liu et al., 2019; Valeri et al., 2019; Iwai et al., 2018), or function as a tumor suppressor in colorectal cancer (Ress et al., 2015), the functions of miR-96-5p in OSCC were rarely explored previously. Therefore, we investigated the functional roles and mechanisms of miR-96-5p in OSCC.

Forkhead transcription factors are characterized by a winged helix DNA-binding domain and are essential for embryogenesis (Kaufmann and Knöchel, 1996). Some of them, such as FOXQ1, FOXQ3 and FOXO3, have been identified as regulating tumorigenesis and tumor progression (Mottok et al., 2018; Saito et al., 2016; Chae et al., 2019). It has been reported that the Forkhead box F2 transcription factor (FOXF2) functions as tumor suppressor in breast cancer, gastric cancer, colorectal cancer, lung cancer and hepatocellular carcinoma (Cai et al., 2015; Higashimori et al., 2018; Zhang et al., 2015; Kundu et al., 2016; Shi et al., 2016). However, the expression of FOXF2 and its functional roles in OSCC are still unknown.

Here, in order to investigate the functional role of miR-96-5p in OSCC, we detected the miR-96-5p level in OSCC tissues and cell lines. Next, we predicted that miR-96-5p directly targeted FOXF2 according to the online database TargetScan 7.2. For further study, we explored the relationship between miR-96-5p and FOXF2 in OSCC tissues. Lastly, the effects of miR-96-5p or FOXF2 overexpression on proliferation, invasion and EMT of OSCC cells were determined.

RESULTS

High level of miR-96-5p in OSCC tissues and cells

In this study, the miR-96-5p level in OSCC tissues and cells were detected by using qRT-PCR. Our findings demonstrated that the miR-96-5p level in the OSCC tissues was higher than that in the adjacent tissues (Fig. 1A). Next, the data further confirmed that the miR-96-5p level was higher in Tca8113 and Cal-27 cells than that in the other three OSCC cell lines (Fig. 1B). Therefore, Tca8113 and Cal-27 cells were used in the following experiments.

The effect of miR-96-5p on proliferation of OSCC cells

After transfection with miR-96-5p mimic or inhibitor, the results showed that the miR-96-5p level was significantly upregulated or downregulated in a miR-96-5p mimic or inhibitor group compared to a negative control (NC) group (Fig. 2A), respectively. To study

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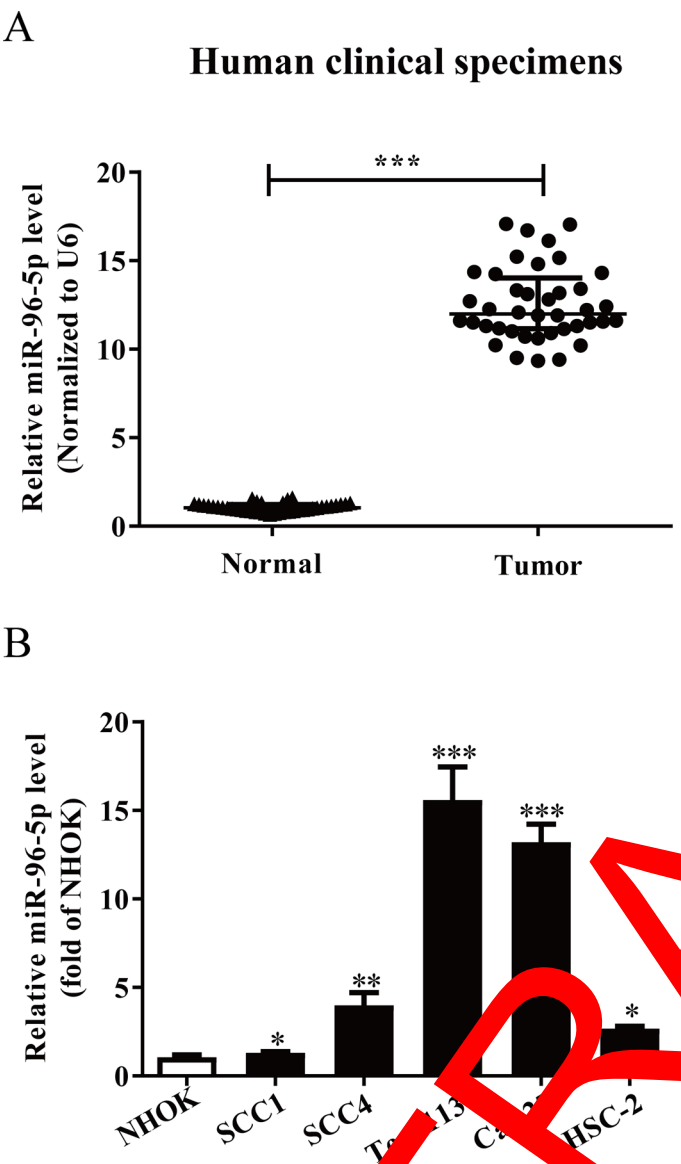

Fig. 1. The levels of miR-96-5p in OSC[illegible]ues and cell lines. (A) Quantitative RT-PCR analysis of m[illegible]96-5p[illegible]el in OSCC tissues and adjacent normal tissues (n=40). Tran[illegible]pt levels w[illegible]ormalized to U6 level. (B) Relative miR-96-5p level analyzed via quanti[illegible]RT-PCR in five OSCC cell lines normalized to U[illegible]=6). All data are prese[illegible]d as means ±s.e.m. *P<0.05, **P<0.01, ***[illegible]0.001 versus normal tissues or NHOK.

the role of miR-96-5[illegible]a reg[illegible]ting Tca[illegible]13 and Cal-27 cell proliferation, our results su[illegible]ed that th[illegible]troduction of miR-96-5p significantly p[illegible]oted Tca[illegible]3 an[illegible]al-27 cells proliferation (Fig. 2B). H[illegible]ever, transfectio[illegible]ith miR-96-5p inhibitor suppressed t[illegible] cell p[illegible]iferation of Tca8113 and Cal-27 cells compared [illegible]h the N[illegible]2B). Next, overexpression of miR-96-5p[illegible]hanc[illegible]expression of CDK4 and cyclin D1 and reduced the exp[illegible]n of p27 at mRNA level (Fig. 2C). In addition, downregulation of [illegible]-96-5p had the opposite effect on regulating expression of these cel[illegible]le genes (Fig. 2C).

The effects of miR-96-5p on invasion and EMT in OSCC cells

Compared to the NC group, the results from the Transwell assays showed that increased miR-96-5p level significantly enhanced the number of invading OSCC cells (Fig. 3A). In addition, we detected MMP-2, MMP-9 and TIMP-1 expression by ELISA and qRT-PCR assays. The results indicated that MMP-2 and MMP-9 expression were markedly increased by enhancing miR-96-5p level in Tca8113 and Cal-27 cells (Fig. 3B,C), while expression of TIMP-1 was dramatically decreased (Fig. 3B,C). Finally, our results suggested that the epithelial marker E-cadherin[illegible]nificantly decreased after overexpression of miR-96-5[illegible] By cont[illegible] introduction of miR-802 increased the mesen[illegible]mal markers [illegible]cadherin and Vimentin expression (Fig. 4[illegible]while the miR-9[illegible]5p inhibitors showed the opposite effect ([illegible]4). Taken[illegible]ogether, we demonstrated that miR-96[illegible]p could p[illegible]mote th[illegible] progression of OSCC by repressing pr[illegible]feration, invas[illegible] EMT of OSCC cells.

miR-96-5p directly [illegible]eted [illegible]OXF2 3′U[illegible]R

We found a mi[illegible]96-5p[illegible]ng site i[illegible]e 3′UTR of FOXF2 by using TargetS[illegible]7.2 online d[illegible]base[illegible]g. 5A). Then, we found that FOXF2, a c[illegible]al oncogene, is [illegible]rect target of miR-96-5p by luciferase [illegible]po[illegible]assay. Introduction of miR-96-5p significantly suppres[illegible]WT FO[illegible]2 reporter activity but not the activity of the mutated reporter constr[illegible]in Tca8113 and Cal-27 cells, suggesting that [illegible]R-96-5p could sp[illegible]ifically target the FOXF2 3′UTR b[illegible]inding to the seed sequence (Fig. 5B). Upregulation of [illegible]R-96-5p could [illegible]ignificantly reduce FOXF2 expression, whereas [illegible]ownregulation [illegible]miR-96-5p enhanced FOXF2 expression [illegible]g. 5C). These [illegible]results suggested that miR-96-5p directly ta[illegible]ted FOXF2 [illegible]ough 3′UTR sequence binding.

Ne[illegible]FOX[illegible]expression was detected by qRT-PCR in OSCC tissues. Our results demonstrated that FOXF2 expression was [illegible]iously downregulated in OSCC tissues compared with the a[illegible]acen[illegible]ssues (Fig. 5D). Next, we also determined the FOXF2 [illegible]xpression in five OSCC cell lines (such as SCC1, SCC4, Tca8113, Cal-27 and HSC-2) and a human normal oral keratinocyte cell [illegible]ulture (NHOK). The FOXF2 expression in Tca8113 and Cal-27 [illegible]lls was lower than that in the other three cell lines (Fig. 5E). [illegible]inally, Pearson's correlation analysis revealed a significant inverse correlation between FOXF2 and miR-96-5p in OSCC tissues (Fig. 5F). From the above data, we predicted that miR-96-5p might negatively regulate FOXF2 expression.

Knockdown of FOXF2 promoted OSCC cells proliferation, invasion and EMT

To investigate the functional roles of FOXF2 in OSCC cells, the proliferation and invasion of OSCC cells were detected after transfection with si-NC or si-FOXF2. Transfection with si-FOXF2 significantly decreased the FOXF2 expression in Tca8113 and Cal-27 cells compared with the si-NC group (Fig. 6A). Then, we found that silencing FOXF2 evidently promoted the proliferation of Tca8113 and Cal-27 cells (Fig. 6B). Next, a Transwell assay revealed that decreased FOXF2 expression accelerated invasion of Tca8113 and Cal-27 cells (Fig. 6C). Moreover, knockdown of FOXF2 markedly upregulated expression of MMP-2 and MMP-9, and downregulated expression of TIMP-1 in Tca8113 and Cal-27 cells (Fig. 6D). For further study, downregulation of FOXF2 reduced the expression of E-cadherin, and enhanced the expression of N-cadherin and Vimentin (Fig. 6E). Consequently, silencing FOXF2 remarkably restrained OSCC cell proliferation, invasion and EMT.

miR-96-5p markedly promoted the proliferation, invasion and EMT of OSCC cells through regulating FOXF2 expression

To determine whether miR-96-5p regulated the proliferation, invasion and EMT of OSCC cells by directly targeting FOXF2,

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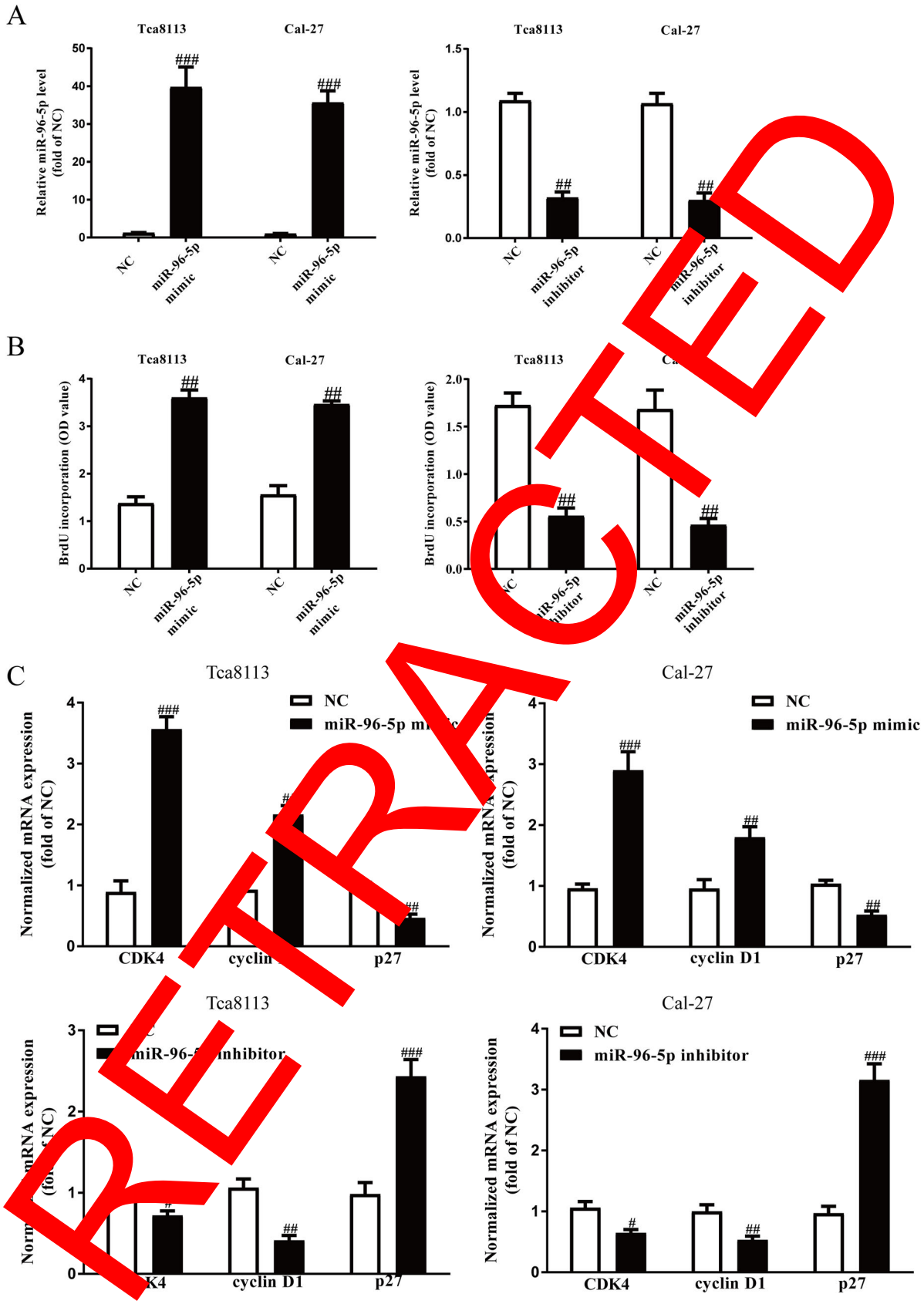

Fig. 2. The effects of miR-96-5p on proliferation and related molecules in OSCC cells. Tca8113 and Cal-27 cells were transfected with miR-96-5p mimic or inhibitor for 48 h. (A) The level of miR-96-5p was detected by quantitative RT-PCR. (B) Cell proliferation was assessed by a BrdU-ELISA assay. (C) The mRNA expression of CDK4, cyclin D1 and p27 were determined by quantitative RT-PCR. All data are presented as means±s.e.m., n=6. $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 versus NC.

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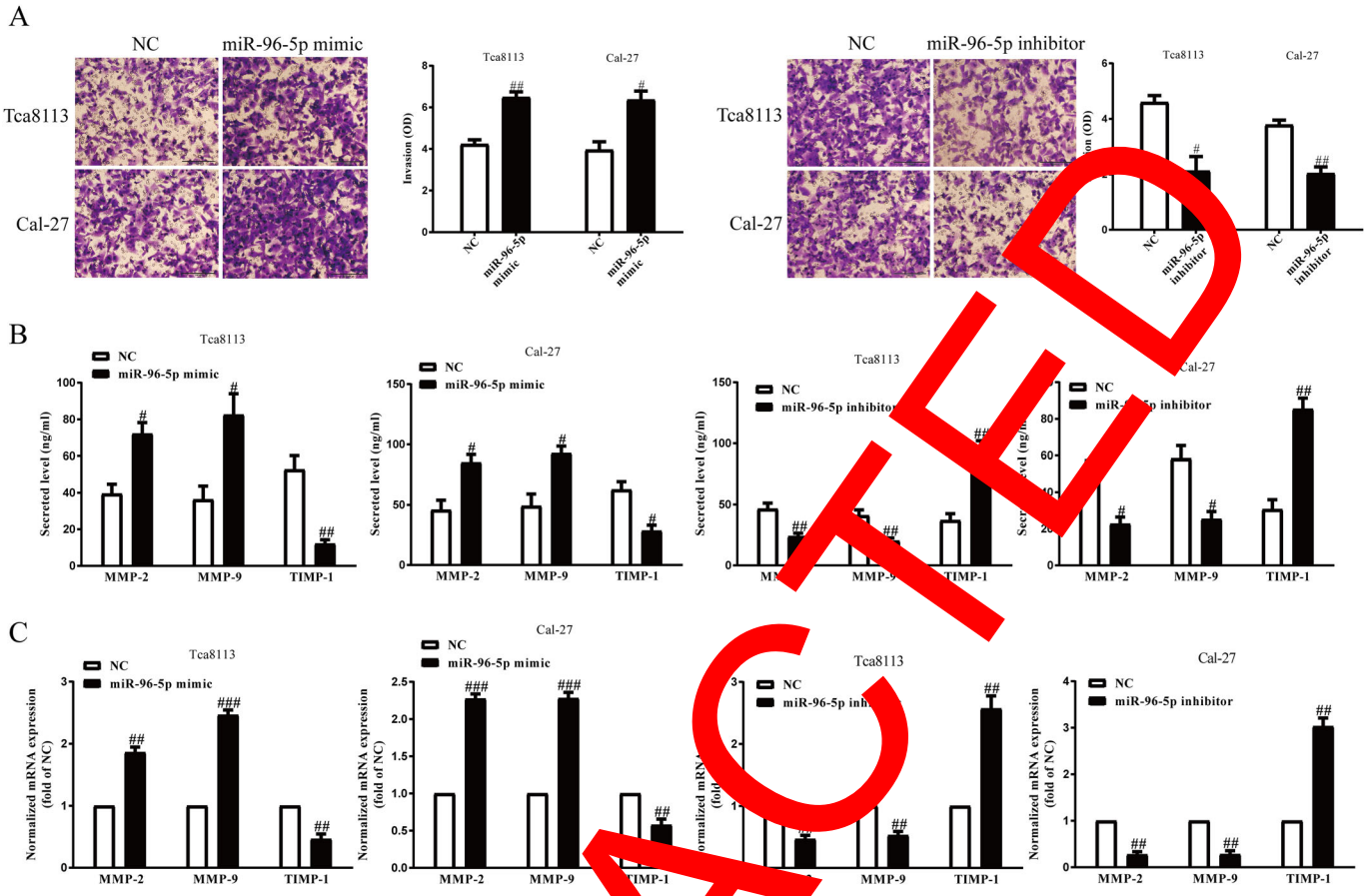

Fig. 3. The effects of miR-96-5p on the invasion and related molecu… exp…sion in OSCC cells. Tca8113 and Cal-27 cells were transfected with miR-96-5p mimic or inhibitor for 48 h. (A) The invasion was assessed by T…ell assay. (B) Total secretions of MMP-2, MMP-9 and TIMP-1 in the culture supernatants were detected by ELISA assays. (C) The mRN… xpression o…P-2, MMP-9 and TIMP-1 were examined by qRT-PCR. All data are presented as means±s.e.m., n=6. $^{\#}P$<0.05, $^{\#\#}P$<0.01 ve… NC

we co-transfected NC or miR-96-5p mimic …h …DNA3.1 or pcDNA-FOXF2 into Tca8113 and …al-27 c… (Fig. 7A). Overexpression of FOXF2 abroga… the promote… ffect of miR-96-5p mimic on cell proliferat… ig. 7B). At the sa… time, expression of cyclin D1 and CD… was …reased and expression of p27 was increased in miR-96-5p-overexp…ing Tca8113 and Cal-27 cells after exogenous …OXF2 upregulatio… ig. 7C). Next, the data showed that FOXF… overexpression partially reversed the invasion of Tca8113 a… Cal-27 …ls promoted by miR-96-5p mimic (Fig. 7D) th…gh dec…asing MMP-2 and MMP-9 expression and increa… TIM…-1 expre…on (Fig. 7E). Next, overexpression of FOXF… …bited mi…96-5p mimic-induced EMT of OSCC c… (Fig. 7… Henc… the promoted effects of miR-96-5p mi…c we… reversed …verexpression of FOXF2. Altogether, …the abo… results suggested that overexpression of miR-96-5p …romoted … …oliferation, invasion and EMT via direct… …wnre…ating FOXF2 expression.

DISCUSSION

miRNAs serve as imp… …t regulatory factors, which affect OSCC progression. Jiang et al. reported that MiR-223 promotes OSCC proliferation and migration by regulating FBXW7 (Jiang et al., 2019). Chen et al. indicated that miR-125b suppresses oral oncogenicity by targeting the anti-oxidative gene PRXL2A (Chen et al., 2019). Numerous studies have found that miR-96-5p not only affected cell proliferation, but was also closely associated with prognosis in cancers including ovarian cancer, HNSCC, hepatocellular carcinoma and colorectal cancer (Liu et al., 2019; Vahabi et al., 2019; Iwai et al., 2018; Ress et al., 2015).

In this study, our results showed that the miR-96-5p level was upregulated in OSCC tissues compared with the adjacent normal tissues. In addition, miR-96-5p level was also upregulated in OSCC cell lines compared with NHOK cells. Moreover, we, for the first time, explored the functional roles of miR-96-5p in OSCC cells. We found that overexpression of miR-96-5p more significantly promoted OSCC cell proliferation than the cells transfected with NC, whereas downregulation of miR-96-5p inhibited OSCC cell proliferation. Cell cycle regulation involved complex events, such events revealed that cell cycle related proteins provided a promising mechanism for the inhibition of growth (Qiu et al., 2017; Wang et al., 2015). An earlier study suggested that the upregulation of cyclin-dependent kinases (CDKs) and cyclin (D1 and E1) participated in cell cycle progression and arrested cells at the G0/G1 phase (Wang et al., 2015). p21 and p27 have been reported as inhibitors of CDK protein with an anti-proliferative effect on mesangial cells (Wang et al., 2011). Here, we also found that introduction of miR-96-5p enhanced expression of CDK4 and cyclin D1, while it also reduced p27 expression.

Invasion is one process of metastasis. Here, the data indicated that an increased or decreased miR-96-5p level significantly inhibited or

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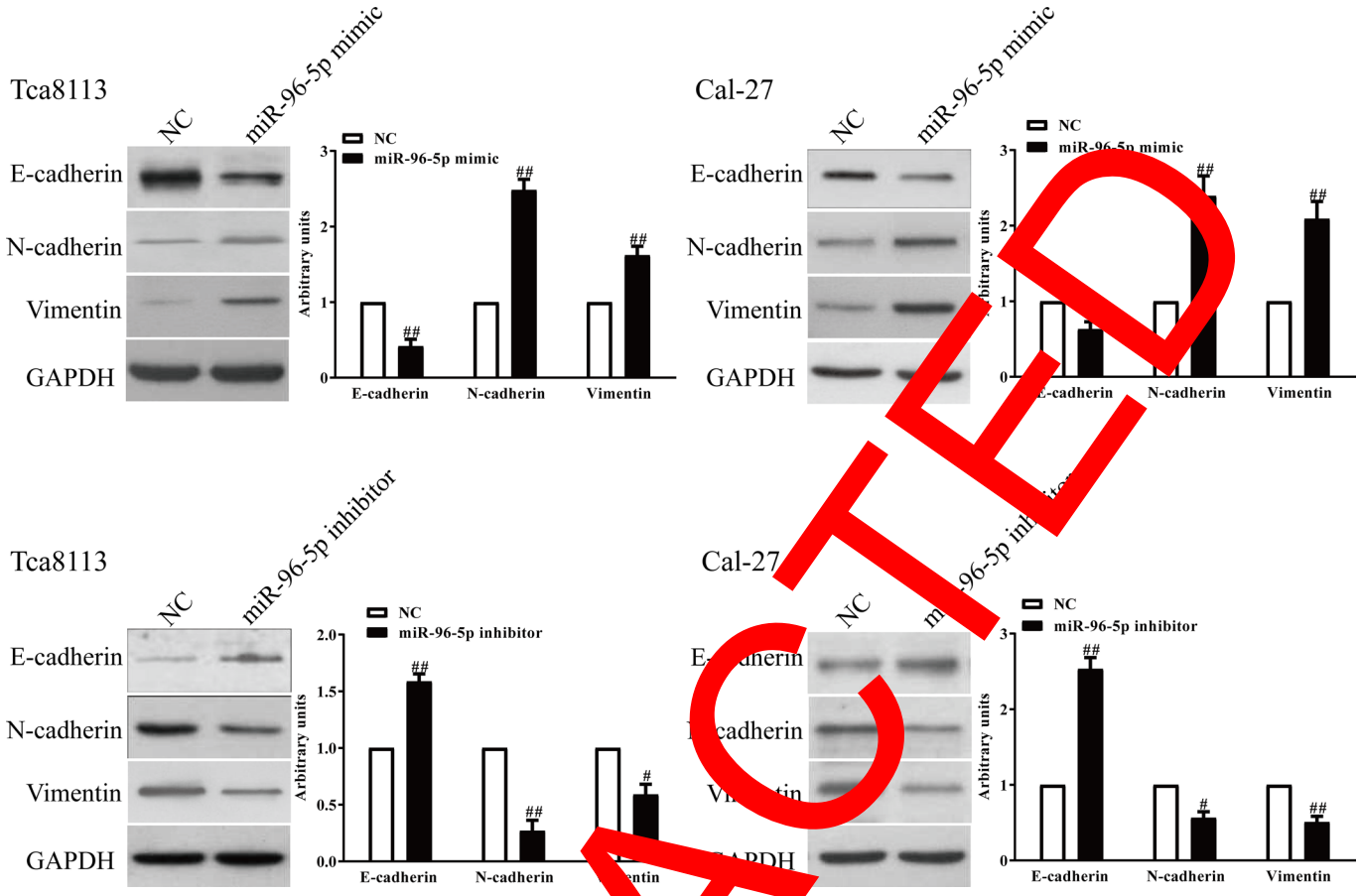

Fig. 4. Effects of miR-96-5p on the EMT of OSCC cells. Tca8113 and Cal-27 cells were transfected with miR-96-5p mimic or inhibitor for 48 h. The expression of E-cadherin, Vimentin and *N*-cadherin were detected by western blot assays. All data are presented as mean±s.e.m., n=6. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus NC.

enhanced, respectively, the invasive ability of CC1 cells compared with the control group. Furthermore, one very important step in cancer cell invasion is that proteolytic enzymes degrade the extracellular matrix (ECM) components (Simpson-Haidaris and Rybarczyk, 2001). Moreover, the MMPs degrading the ECM process contributes to cancer growth, angiogenesis, invasion and metastasis (Bogenrieder and Herlyn, 2003; Vihinen and Kähäri, 2002; Sounni et al., 2003). Both MMP-2 and MMP-9 degrade components of the basement membrane to promote cancer invasion (Vihinen and Kähäri, 2002; Hornebeck et al., 2002; Klein et al., 2004). Moreover, it is reported that the imbalance between TIMPs and MMPs is important in the early stage of tumor progression (Herszényi et al., 2012). Next, upregulation of miR-96-5p significantly decreased expression of MMP-2 and MMP-9, and increased TIMP-2 expression in Tca and Cal-27 cells, whereas downregulation of miR-96-5p enhanced both MMP-2 and MMP-9 expression and reduced TIMP-2 expression. The EMT process has been confirmed to be critical in cell invasion in types of cancer (Christiansen and Rajasekaran, 2006). At the molecular level, EMT is characterized by downregulation of the epithelial marker E-cadherin and cytokeratins, with upregulation of mesenchymal markers like N-cadherin, Vimentin and fibronectin (Yao et al., 2011; Gheldof and Berx, 2013). We found that the introduction of miR-96-5p promoted the EMT of OSCC cells by decreasing E-cadherin expression and increasing N-cadherin and Vimentin expression. All the above results indicated that miR-96-5p restrained cell proliferation, invasion and EMT of OSCC.

It is well known that miRNAs perform their function by regulating the expression of their target gene. Thus, we explore the functional target gene for miR-96-5p, which is involved in OSCC progression. TargetScan and luciferase reporter assay suggested that FOXF2 might be the functional target gene of miR-96-5p. Furthermore, increased expression or knockdown of miR-96-5p significantly inhibited or promoted FOXF2 expression, respectively. FOXF2 is a tumor suppressor and participates in the development and progression of multiple cancers (Cai et al., 2015; Higashimori et al., 2018; Zhang et al., 2015; Kundu et al., 2016; Shi et al., 2016). In our study, we also observed that FOXF2 expression was frequently at low levels in tumor tissue when compared with its paired non-tumor tissue, which agreed with previous studies in breast cancer, gastric cancer, colorectal cancer, lung cancer and hepatocellular carcinoma (Cai et al., 2015; Higashimori et al., 2018; Zhang et al., 2015; Kundu et al., 2016; Shi et al., 2016). Hence, our results indicated that FOXF2 expression might play a cardinal role in tumorigenesis of OSCC. Besides, miR-96-5p level was inversely correlated with FOXF2 expression in OSCC. Moreover, the data showed that silencing FOXF2 could restrain the proliferation, invasion and EMT of OSCC cells. Next, our results showed that overexpression of FOXF2 inhibited the proliferation, invasion and EMT of OSCC cells promoted by upregulation of miR-96-5p. Our findings

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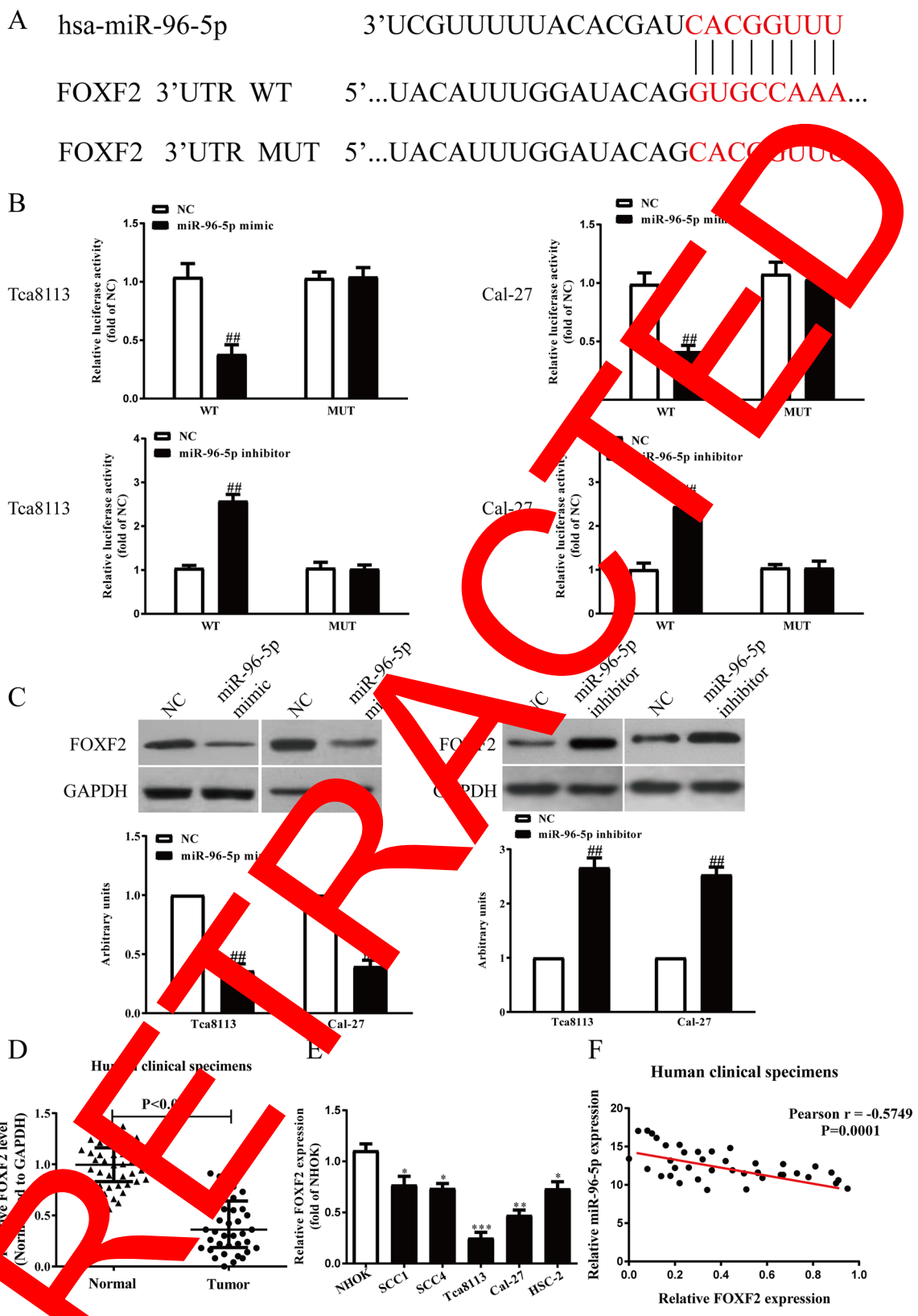

Fig. 5. FOXF2 is a direct target of miR-96-5p. Tca8113 and Cal-27 cells were transfected with miR-96-5p mimic or inhibitor for 48 h. (A) Schematic representation of FOXF2 3'UTRs showing putative miRNA target site. (B) The analysis of the relative luciferase activities of FOXF2-WT and FOXF2-MUT. (C) The protein expression of FOXF2 were determined by western blot assay. (D) Quantitative RT-PCR analysis of FOXF2 expression in OSCC tissues (n=40) and adjacent normal tissues (n=40). Transcript levels were normalized to GAPDH expression. (E) Relative FOXF2 expression analyzed via quantitative RT-PCR in five OSCC cell lines normalized to GAPDH (n=6). (F) Pearson's correlation analysis of the relative expression levels of miR-96-5p and the relative FOXF2 mRNA levels in OSCC tissues. All data are presented as means±s.e.m., n=6. $^{\#\#}P$<0.01 versus NC; $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 versus normal tissues or NHOK.

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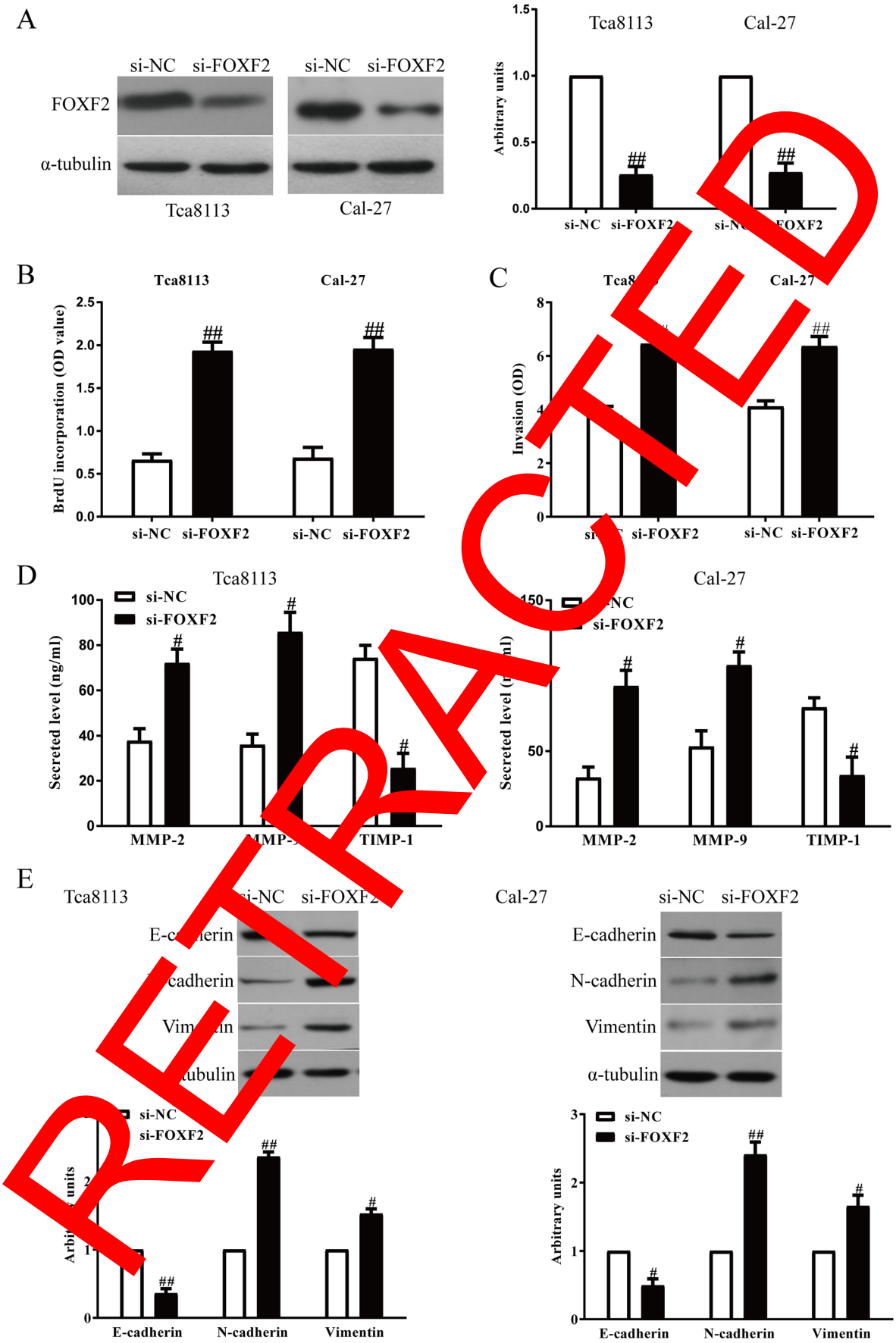

Fig. 6. See next page for legend.

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Fig. 6. The effects of FOXF2 knockdown on the proliferation, invasion and EMT in OSCC cells. Tca8113 and Cal-27 cells were transfected with si-NC or si-FOXF2 for 48 h. (A) The protein expression of FOXF2 was determined by western blot. (B) Cell proliferation was assessed by a BrdU-ELISA assay. (C) The invasion of OSCC cells was assessed by Transwell assay. (D) Total secretions of MMP-2, MMP-9 and TIMP-1 in the culture supernatants were detected by ELISA assays. (E) The expression of E-cadherin, Vimentin and N-cadherin were detected by western blot assays. All data are presented as means±s.e.m., n=6. $^{\#}P$<0.05, $^{\#\#}P$<0.01 versus si-NC.

demonstrated that miR-96-5p might act as an oncogene in OSCC by directly targeting FOXF2.

Altogether, the miR-96-5p level was dramatically upregulated and the FOXF2 expression was significantly downregulated in OSCC tissues. Introduction of miR-96-5p promoted proliferation, invasion and EMT of OSCC cells by directly downregulating FOXF2 expression. Hence, these findings suggested important roles for miR-96-5p/FOXF2 axis in the OSCC pathogenesis and its potential application in cancer therapy.

MATERIALS AND METHODS

Human tissue samples

Thirty pairs of human OSCC tissues and their adjacent non-cancer tissues were collected from patients at the Beijing Chaoyang Hospital, Capital Medical University between Feb 2018 and Feb 2019. All samples were immediately frozen in liquid nitrogen for subsequent quantitative RT-PCR and western blot analysis. All participants signed a written informed consent form. This study was approved by the Ethical Committee of Beijing Chaoyang Hospital, Capital Medical University (BCH2018011208) and complied with the guidelines and prin[illegible] the Declaration of Helsinki.

Cell culture

The human OSCC cell lines such as SCC1, SCC4, Tca8113, Cal-27, HSC-[illegible] and the normal human oral keratinocyte cell lines (NHO[illegible]) we[illegible]urchased from the American Type Culture Collection (ATCC, U[illegible]). All th[illegible]ells were cultured in the DMEM/F12 medium (GIBCO, US[illegible] containin[illegible]0% FBS (GIBCO, USA) and penicillin/streptomycin (1[illegible]U/ml and [illegible]0 mg/ml, respectively) (GIBCO, USA) at 37°C in a humidified [illegible]sp[illegible]e of 5% CO_2.

Transient transfection

The miR-96-5p mimics, miRNA-NC, [illegible]-96-5p inhibitors, [illegible]C and si-FOXF2 were purchased from Ge[illegible]Ph[illegible] (Shanghai, China). The FOXF2-overexpression plasmid was [illegible]nerated [illegible]serting FOXF2 cDNA into a pcDNA3.1 vector, which was sequence[illegible]d confirmed by Gene-Pharma. The miR-96-5p [illegible]mics, NC, miR-96-5p [illegible]ibitors, si-NC, si-FOXF2, pcDNA3.1 vecto[illegible]nd FOXF2-overexpression plasmid were transfected using Lipofecta[illegible]ne 3000 [illegible]gent (Invitrogen, USA) per the manufacturer's protocols [illegible]ells were [illegible]nsfected for 48 h, and total RNA and protein were collecte[illegible]

Isolation of RNA a[illegible]quantita[illegible]olyr[illegible]rase chain reaction analy[illegible]

Total RNA fro[illegible]OSCC ce[illegible]were extracted using TRIzol (Invitrogen, USA) following th[illegible] manufact[illegible]r's [illegible]ols. MiRNA-specific RT primers (RiboBio, [illegible]angzhou[illegible]hina) for miR-96-5p and random primers (TaKaRa, Da[illegible]C[illegible]na) for mRNAs were synthesized. Quantitative polymerase chain r[illegible]on (qPCR) was used to measure reverse-transcribed cDNA with SYBR Gr[illegible]PCR Kit (QIAGEN, Shanghai, China) under the following conditions: pre-d[illegible]turation at 95°C for 5 min, denaturation at 95°C for 10 s, annealing and extension at 60°C for 30 s, the followed steps were running for 40 cycles. The relative miRNA and mRNA expression levels were normalized by U6 and GAPDH, respectively. The relative expression levels of miR-96-5p, FOXF2, CDK4, cyclin D1, p27, MMP-2, MMP-9 and TIMP-1 were normalized to those of internal control U6 or GAPDH using the comparative delta CT ($2^{-\Delta\Delta Ct}$) method. Prime sequences are shown in Table 1.

Protein extraction and western blot analysis

Transfected cells were lysed with RIPA lysis buffer (Thermo Fisher Scientific, USA) containing protease inhib[illegible]rmo Fisher Scientific, USA). The concentration of extracted [illegible]otein wa[illegible]asured by using a BCA protein assay kit (Thermo Fish[illegible]cientific, USA[illegible]qual amounts of protein were separated with l[illegible] SDS-PAGE an[illegible]transferred to polyvinylidene difluoride (PVDF[illegible]mbranes (Milli[illegible]e, USA). The membranes were then blocked with 5% [illegible]fat milk (BD[illegible]iosciences, San Jose, USA) in TBST for 1 h[illegible]room tempe[illegible]e, follo[illegible]ed by incubation with primary antibodies [illegible]FOXF2 (ab23300[illegible]in D1 (ab16663), CDK4 (ab108357), p[illegible](ab320[illegible], MMP-2 (ab97779), MMP-9 (ab38898) and TIMP[illegible](ab10912[illegible](Abcam, USA) overnight at 4°C. Subsequently, the me[illegible]nes [illegible]e washed [illegible]h TBST three times and probed with th[illegible]corres[illegible]ng horse[illegible]sh peroxidase-conjugated secondary antib[illegible]es (Cell Sig[illegible]ng Te[illegible]ology, USA) for 2 h at room temperature. [illegible]E reagent (Pierce)[illegible]sed to detect the signals on the membranes.

Transwell invasion a[illegible]

After transfection, cells w[illegible]resuspended in serum-free DMEM/F12 me[illegible]d then were seeded into the upper chamber (Corning, New York, [illegible]A) coated with matrigel (BD Biosciences, San Jose, USA), and medium [illegible]ntaining 10% FB[illegible]was added into the bottom chamber. After incubation [illegible]r 24 h, cells rema[illegible]g on the upper membrane were carefully removed, [illegible]d those migrating [illegible]the basal side of the membrane were fixed with 4% p[illegible]ormaldehyde [illegible]d stained with Crystal Violet (Sigma-Aldrich, St Lou[illegible]A) fo[illegible]min. Finally, migrated cells in random three visual fields were photographed and counted under a microscope (Olympus, Tokyo, Japan). Finally, the washing solution was examined at 540 nm for the c[illegible]he number of OSCC cells.

Measurement of MMP-2, MMP-9 and TIMP-1 levels by ELISA assay

According to the protocol, the supernatants of OSCC cells were collected [illegible]fter treatment, and the concentrations of MMP-2, MMP-9 and TIMP-1 [illegible]re measured using a sandwich ELISA kit (USCN, USCN Life Science, Wuhan, China) according to the manufacturer's instructions. Briefly, the primary antibody was coated onto ELISA plates and incubated for 2 h at room temperature. Samples and standards were added to the wells and incubated for 1 h. Then the wells were washed and a biotinylated antibody was added for 1 h. The plates were washed again, and streptavidin conjugated to horseradish peroxidase was added for 10 min. After washing, tetramethylbenzidine was added for color development and the reaction was terminated with 1 mol/l H_2SO_4. Absorbance was measured at 490 nm. Values were expressed as ng/ml.

Luciferase reporter assay

The luciferase reporter vectors (pGL3-FOXF2-3′UTR WT and pGL3-FOXF2-3′UTR MUT) were synthesized by GenePharma. OSCC cells were seeded into 24-well plates and transfected with pGL3-FOXF2-3′UTR WT or pGL3-FOXF2-3′UTR MUT, along with miR-96-5p mimics or miR-NC using Lipofectamine 3000 per the manufacturer's instructions. After transfection for 48 h, luciferase reporter assays were performed with the Promega Dual-Luciferase Reporter Assay System. The relative firefly luciferase activities were measured by normalizing to renilla luciferase activities.

Statistical analysis

The data were expressed as the means±standard error of the mean (s.e.m.). The number of independent experiments was represented by 'n'. Correlations between miR-96-5p and FOXF2 levels were analyzed using Pearson's correlation coefficient. Two-tailed Student's t-test was used for other comparisons. The associations between miR-96-5p level and clinicopathological features were analyzed using χ^2 test. P<0.05 was considered statistically significant.

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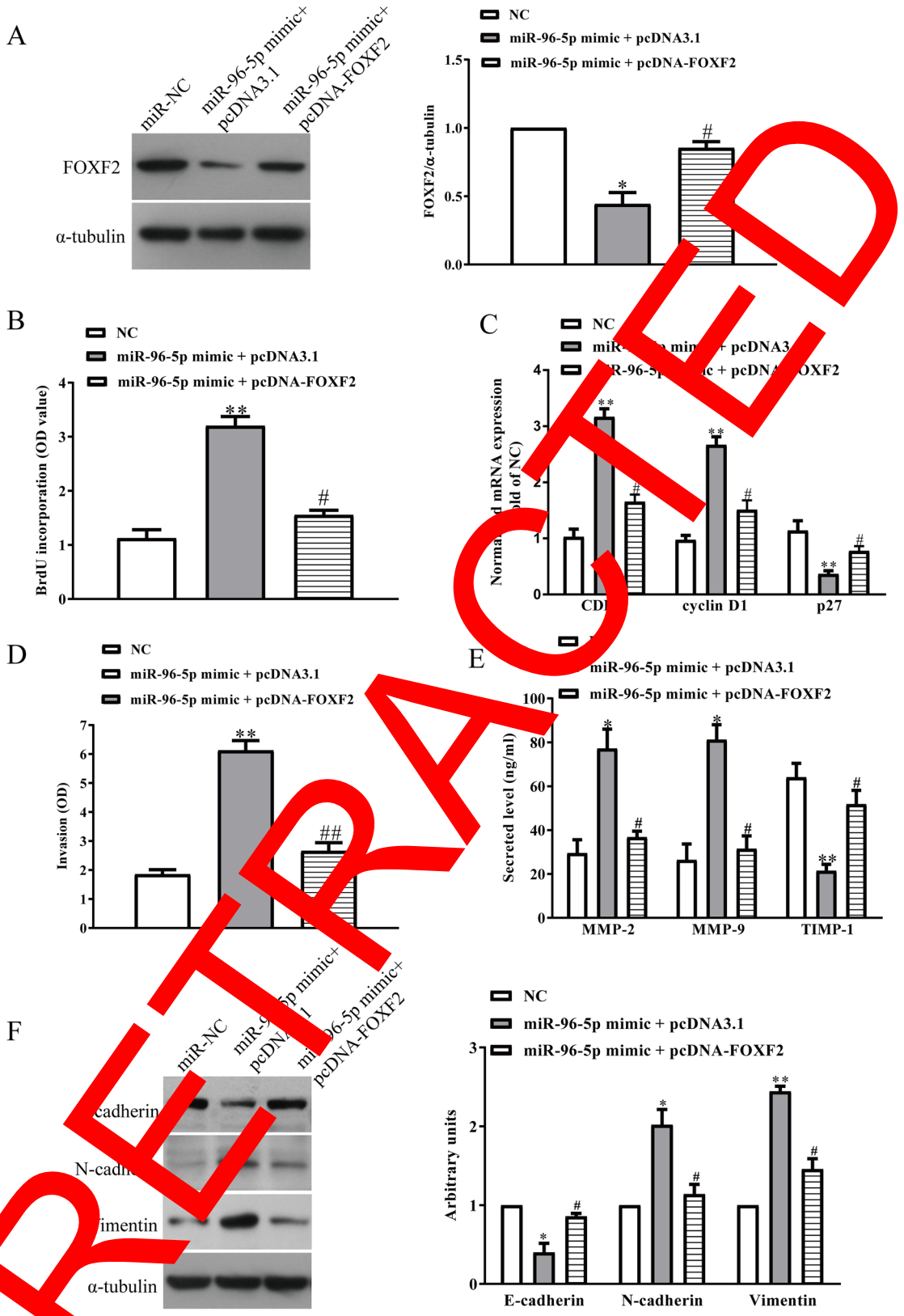

Fig. 7. Introduction of FOXF2 promoted cell proliferation and invasion in miR-96-5p-overexpressing OSCC cells. Tca8113 cells were co-transfected with pcDNA3.1 or pcDNA-FOXF2 with or without miR-96-5p mimic. (A) The protein expression of FOXF2 was determined by western blot assay. (B) Cell proliferation was assessed by a BrdU-ELISA assay. (C) The expression of CDK4, cyclin D1 and p27 were determined by quantitative RT-PCR, respectively. (D) The invasion of OSCC cells was assessed by Transwell assay. (E) Total secretions of MMP-2, MMP-9 and TIMP-1 in the culture supernatants were detected by ELISA assays. (F) The expression of E-cadherin, Vimentin and N-cadherin were detected by western blot assays. All data are presented as means±s.e.m., n=6. *P<0.05, **P<0.01 versus NC; $^{\#}P$<0.05, $^{\#\#}P$<0.01 versus miR-96-5p mimic+pcDNA3.1.

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Table 1. Sequences of primers for qRT-PCR

Gene	Primer sequence
FOXF2	F: 5′-AATGCCACTCGCCCTACAC-3′
	R: 5′-CGTTCTGGTGCAAGTAGCTCT-3′
CDK4	F: 5′-GGGGACCTAGAGCAACTTACT-3′
	R: 5′-CAGCGCAGTCCTTCCAAAT-3′
Cyclin D1	F: 5′-GCTGCGAAGTGGAAACCATC-3′
	R: 5′-CCTCCTTCTGCACACATTTGAA-3′
p27	F: 5′-AACGTGCGAGTGTCTAACGG-3′
	R: 5′-CCCTCTAGGGGTTTGTGATTCT-3′
MMP-2	F: 5′-TACAGGATCATTGGCTACACACC-3′
	R: 5′-GGTCACATCGCTCCAGACT-3′
MMP-9	F: 5′-TGTACCGCTATGGTTACACTCG-3′
	R: 5′-GGCAGGGACAGTTGCTTCT-3′
TIMP-1	F: 5′-CTTCTGCAATTCCGACCTCGT-3′
	R: 5′-ACGCTGGTATAAGGTGGTCTG-3′
miR-96-5p	F: 5′-TTTGGCACTAGCACATTTT-3′
	R: 5′-GAACATGTCTGCGTATCTC-3′
U6	F: 5′-CTCGCTTCGGCAGCACA-3′
	R: 5′-AACGCTTCACGAATTTGCGT-3′
GAPDH	F: 5′-GAGTCAACGGATTTGGTCGTATTG-3′
	R: 5′-CCTGGAAGATGGTGATGGGATT-3′

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z.W.; Methodology: H.W., N.M., W.L.; Software: H.W., N.M., W.L.; Validation: H.W.; Formal analysis: H.W., N.M., W.L.; Investigation: H.W., N.M., W.L.; Resources: Z.W.; Data curation: H.W.; Writing - original draft: H.W.; Writing - review & editing: Z.W.; Supervision: Z.W.; Funding acquisition: Z.W.

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Data availability

The data sets used or analysed in this study are available fr the co sponding author on reasonable request.

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