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MiR-1294 acts as a tumor suppressor in clear cell renal cell carcinoma through targeting HOXA6

W. PAN, L.-J. PANG, H.-L. CAI, Y. WU, W. ZHANG, J.-C. FANG

Department of Urinary Surgery, RenMin Hospital of Huangpi District, the Third Hospit
Jianghan University, Huangpi District, Wuhan, P. R. China.

Abstract. – OBJECTIVE: Renal cancer represents about 3% of all human cancers. Clear cell renal cell carcinoma (ccRCC) is the main type of renal cancer. MicroRNAs (miRNAs) have been reported to play crucial roles in the carcinogenesis of human cancers. This study was aimed to investigate the expression of miR-1294 and the mechanisms underlying miR-1294-mediated ccRCC progression.

MATERIALS AND METHODS: The miR-1294 expression levels in ccRCC cell lines were analyzed by quantified real time-PCR (qRT-PCR). The effect of the miR-1294 expression on the overall survival of ccRCC patients was analyzed by the Kaplan-Meier Plotter. Cell proliferation, colony growth, and cell invasion were examined b ll counting kit-8 assay, colony formation ass transwell invasion assay, respectively. The ir- erase activity reporter assay and Western t assay were conducted to validate the connec between miR-1294 and homeobox A6 (HOXA6).

RESULTS: MiR-1294 was ulated i ccRCC cell lines and correl w he pool overall survival of ccRCC ents. T overex- pression of miR-1294 in ccRC olif- eration, colony growth an in was validated as a t of 4 and nega- tively regulated by -1294. Th expression of HOXA6 atten he miR-129 liated ef- fects on ccRC unctions.

CONCLUSIONS: Our ts indicated that miR-1294 funct s as a tumo pressor in ccRCC. MiR-129 ppressed cell eration, colony forma and asion in c CC partially via targ g HOX

Key Wo
R-12 OXA6 ar cell renal cell carcinoma, ation,

Introduction

ar cell renal cell carcinoma (ccRCC), represents 70-80% of all renal cell carcinoma cases and is characterized by high morbidity and poor prognosis[1]. The conduction of surgical resection has a good treatme ect f cal ccRCC[2]. However, the trea ne s for a nced ccRCC remain ed, the p under- standing of th tailed me hind the carcinogene RCC[3].

Micro As NAs) were non-coding RNAs and firstly i ed in *Caenorhabditis eleg* 1993 at the h of 19 to 24 nu- cl des[4]. MiRNAs were wildly recognized as ial modulato to regulate multiple cellu- ioprocesses inly through complementary b g with the untranslated region (3'-UTR) of d g . In addition, miRNAs were shown dual roles in the carcinogenesis of human cancers to function as tumor suppres- oncogenic miRNA at a cancer-specific

Abnormal expression of miR-1294 has been found in various human cancers including glioma, oral squamous cell carcinoma, ovarian cancer, gastric cancer, and osteosarcoma[9-13]. MiR-1294 was found to reduce the expression in glioma and promote the chemosensitivity of glioma cells to temozolomide by regulating the expression targeting protein for Xenopus kinesin-like protein 2[9]. Besides that, the growth of oral squamous cell carcinoma cell could be inhibited by miR-1294[10]. In addition, miR-1294 was shown to regulate the response of ovarian cancer cell to cisplatin via regulating IGF1R[11]. Moreover, it was found that miR-1294 was associated with the prognosis of patients with gastric cancer and osteosarcoma, which highlighted the importance of miR-1294 in cancers[12,13]. However, we still did not understand the role of miR-1294 in ccRCC until now.

A total of 39 Homeobox (HOX) genes located at chromosomes 7p15, 17q21.2, 12q13, and 2q31 have been identified in human to date[14]. These genes were further classified into four sub-families, named HOXA, HOXB, HOXC, and HOXD[14]. Of note, these genes were found to be involved with multiple cell behaviors and have a role in tumor pathogenesis and progression[14,15]. Recently,

Corresponding Author: Wei Pan, MD; e-mail: panwei_wh@163.com

HOXA9, a member of the HOX family, was identified as a direct target of miR-1294[13]. Therefore, it is reasonable to suspect that miR-1294 may have a connection with HOXA6.

In this work, we analyzed the expression level of miR-1294 in ccRCC cell lines using quantified real time-polymerase chain reaction (qRT-PCR). The effect of the miR-1294 expression on the overall survival of ccRCC patients was analyzed by the Kaplan-Meier Plotter website. The association of miR-1294 and HOXA6 was validated by luciferase activity reporter assay and Western blot assay. The effects of miR-1294 or HOXA6 expression on cell proliferation, colony growth, and cell invasion were examined by cell counting kit-8 assay, colony formation assay, and transwell invasion assay, respectively.

Materials and Methods

Cell Lines and Cell Culture

Human ccRCC cell lines (Caki-1 and Caki-2) and normal human renal tubular epithelial cell (HK-2) purchased from Cell Bank of C Academy of Sciences (Shanghai, China) were maintained in Dulbecco's Modified Eagle's dium (DMEM, Invitrogen, Thermo Fisher S entific, Inc., Waltham, MA, US plemente with 10% fetal bovine serum BS, trogen), 100 U/ml penicillin, and 1 g/ml st tomycin at a humidified 37°C inc r 5%

Overexpression miR-12 and HOXA6 in CC Cell Li

To manipul ression of mi 294, the selected ccR cell li ere transfected with miR-1294 nic and neg control (miR-NC) purcha rom Sangon Bio nology Co., Ltd (Sha i, Chi In addition, the pcDNA3.1 expr vect ntaining the open reading frame of HO HOXA tained from GenScript jing na) w mployed to regulate the sion 6. The lipofectamine 2000 (In gen) w sed for the synthetic miRNAs or ion vectors transfection according to rer's instructions.

ation of Total RNA and qRT-PCR

zol reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to isolate total RNA from cultured cells according to the supplier's protocol. First-strand complementary DNA was synthesized from the extracted RNA sample using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The qRT-PCR was conducted using SYBR Gree Mix (TaKaRa, Dalian, China) at ABI7500 PCR System (Applied Biosystems ter City, CA, USA) with the following pr re: 1 cycle of 94°C for 5 min; 30 cycles of for 45 s, 55°C for 45 s, and 72°C fo min, a cycle of 72°C for 10 min. U6 s nuclear R snRNA) was used as a dogen s contr normalize the expres level R-1294. The primer sequences for were ward: 5'-TATGATCTC GAC -3', everse: 5'-CACCTTC ATCCTC snRNA were forwa TCGCTTC GCACA-3', reverse: 5 ACG CACGAATTTGCGT-3'.

Isol of Total Pr a Western Blot

he cells wer collected and lysed in Radioi unoprecipita Assay buffer plus protease i tors and p phatase inhibitors (Beyotime, Hai Jian China). After quantified by bicinch cid (BCA) kit (Beyotime, Haimen, Jiangsu, China), an equal amount of protein sample was separated at 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with the corresponding primary antibodies (anti-HOXA6: ab74064, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH): ab181602; Abcam, Cambridge, MA, USA) at 4°C for overnight following fat-free milk incubation. After that, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ab6721; Abcam, Cambridge, MA, USA) at 37°C for 4 h. The protein bands were developed by BeyoECL Star (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's protocol.

Cell Counting Kit-8 (CCK-8) Assay

The cell proliferation was measured with CCK-8 assay. The cells were seeded into 96-well plate at the density of 3×10^3 cells/well. At indicated time points, CCK-8 reagent (Beyotime, Haimen, Jiangsu, China) was added to the plate and incubated for another 4 h. Then, the optical density at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Colony Formation Assay

Approximately 5,000 cells were seeded in 6-well plates filled with serum-free medium. After incubation for 2 weeks, the cells were washed with phosphate buffered solution, fixed with 4% paraformaldehyde, and then stained with 0.1% crystal violet. The number of colonies generated was counted using Image J 1.42 software (National Institutes of Health, Bethesda, MD, USA).

Transwell Invasion Assay

Cell invasive capability was measured using the transwell invasion assay. The insert was coated with the matrigel (BD Biosciences, San Jose, CA, USA). The cells were seeded in the upper chamber filled with serum-free DMEM, while the bottom chamber was filled with DMEM containing 10% FBS. After incubation for 24 h, the invasive cells were fixed by 4% paraformaldehyde and stained with 0.1% crystal violet.

Prediction of MiR-1294 Targets

The potential targets of miR-1294 were predicted and analyzed using the online bioinformatics algorithms including TargetScan and mi[illegible]. The results indicated that HOXA6 con[illegible] binding site for miR-1294 in its 3'-UTR.

Dual-Luciferase Activity Reporter Assay

The wild-type HOXA6 3'-U[illegible]he mutat[illegible] HOXA6 3'-UTR containin[illegible]edic[illegible] binding sites of miR-1294 were c[illegible]d into [illegible]L3 vector (Promega, Madison, [illegible]USA) [illegible]d as wt-HOXA6 and m[illegible]HOX[illegible]5[illegible] cells [illegible]e co-transfected with [illegible]-HOXA[illegible]mt-HOXA6, and miR-1294 mimic or NC-miR using Lipofectamine 2000. After 48 h of transfection, the luciferase activity was measured using a Dual-luciferase assay system (Promega) according [illegible]the manufacturer's instructions.

Kaplan-Meier Survival Analy[illegible]

The Kaplan-Meier plotter (ww[illegible]lot.com) was used to assess the effe[illegible]of the [illegible]1294 expression on the overa[illegible]urvival of [illegible] patients. The cut-off v[illegible]was a[illegible]e-select[illegible] the algorithm.

Statistical Ana[illegible]s

The data w[illegible]presented [illegible]a[illegible] Standard Deviation a[illegible]ed at SPSS [illegible]ware (SPSS Inc., Chic[illegible]IL, [illegible] The one-way ANOVA followed by Tukey's post[illegible]est was used to measure the [illegible]ce among i[illegible]e groups, while the S[illegible]t's *t*-test was used t[illegible] assess the difference [illegible]een the two [illegible]ups. A log-rank test was used t[illegible]alculate the [illegible]ival difference between low o[illegible]h expressio[illegible]roups. $p<0.05$ was considered stati[illegible]si[illegible]ant.

Results

Low Expression of MiR-1294 in ccRCC

First, we detected the expression of miR-1294 in ccRCC cell lines using RT-qPCR. We found that miR-1294 expression was markedly downregulated in ccRCC cell lines (Caki-1 and Caki-2) compared with normal human renal tubular epithelial cell (HK-2) (Figure 1A). In

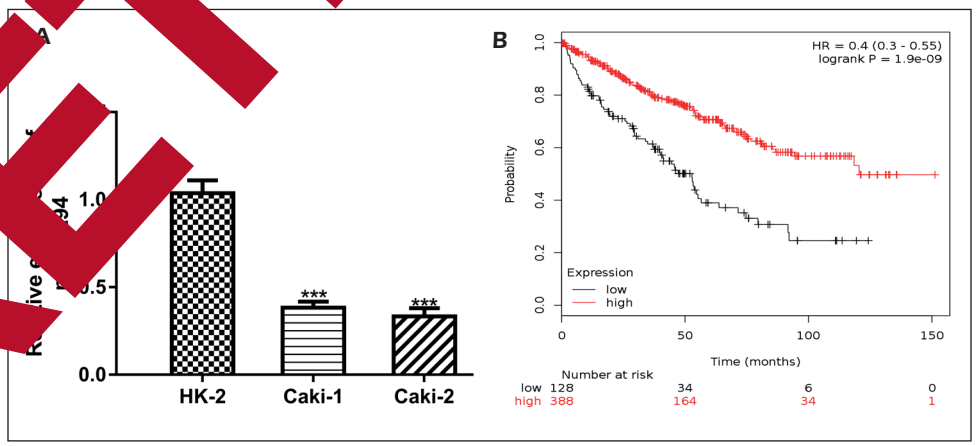

Figure 1. Downregulation of miR-1294 in ccRCC. **A**, miR-1294 expression level in ccRCC cell lines (Caki-1 and Caki-2) and normal human renal tubular epithelial cell (HK-2). **B**, Low miR-1294 expression level predicts poor overall survival of ccRCC patients. ***$p<0.001$. miR-1294: microRNA-1294; ccRCC: clear cell renal cell carcinoma.

addition, the Kaplan-Meier survival curve analyzed through the KM Plotter website indicated that patients with low miR-1294 expression tend to have a poorer overall survival compared to those with high miR-1294 expression (Figure 1B; p = 1.9e-09). Together, our findings revealed that miR-1294 expression was downregulated in ccRCC.

Overexpression of MiR-1294 Inhibits ccRCC Cell Proliferation, Colony Formation, and Invasion

Then, we investigated the effects of miR-1294 expression on ccRCC cell behaviors. We introduced synthetic miRNAs into ccRCC cell lines, and we found that miR-1294 level was significantly elevated by miR-1294 mimic compared with NC-miR (Figure 2A). To examine the effect of miR-1294 on cell proliferation, CCK-8 assay and colony formation assay were co[illegible]ed. CCK-8 assay revealed that the intr[illegible] miR-1294 mimic impaired the prol[illegible]tion rat[illegible] when compared with NC-miR ([illegible] 2B). The inhibitory effect of miR-1294 o[illegible]feration was further confirmed by t[illegible]colony [illegible]ation assay. The results indicat[illegible]at the ect[illegible] pression of miR-1294 [illegible]aired [illegible]ony gr[illegible] (Figure 2C). Transw[illegible]nvasio[illegible]ssay showed that the overexpr[illegible]sio[illegible]1294 i[illegible]bited cellular invasion [illegible]gure [illegible]ollecti[illegible], these results demo[illegible]ted the i[illegible]ffects of miR-1294 o[illegible] cell behav[illegible]

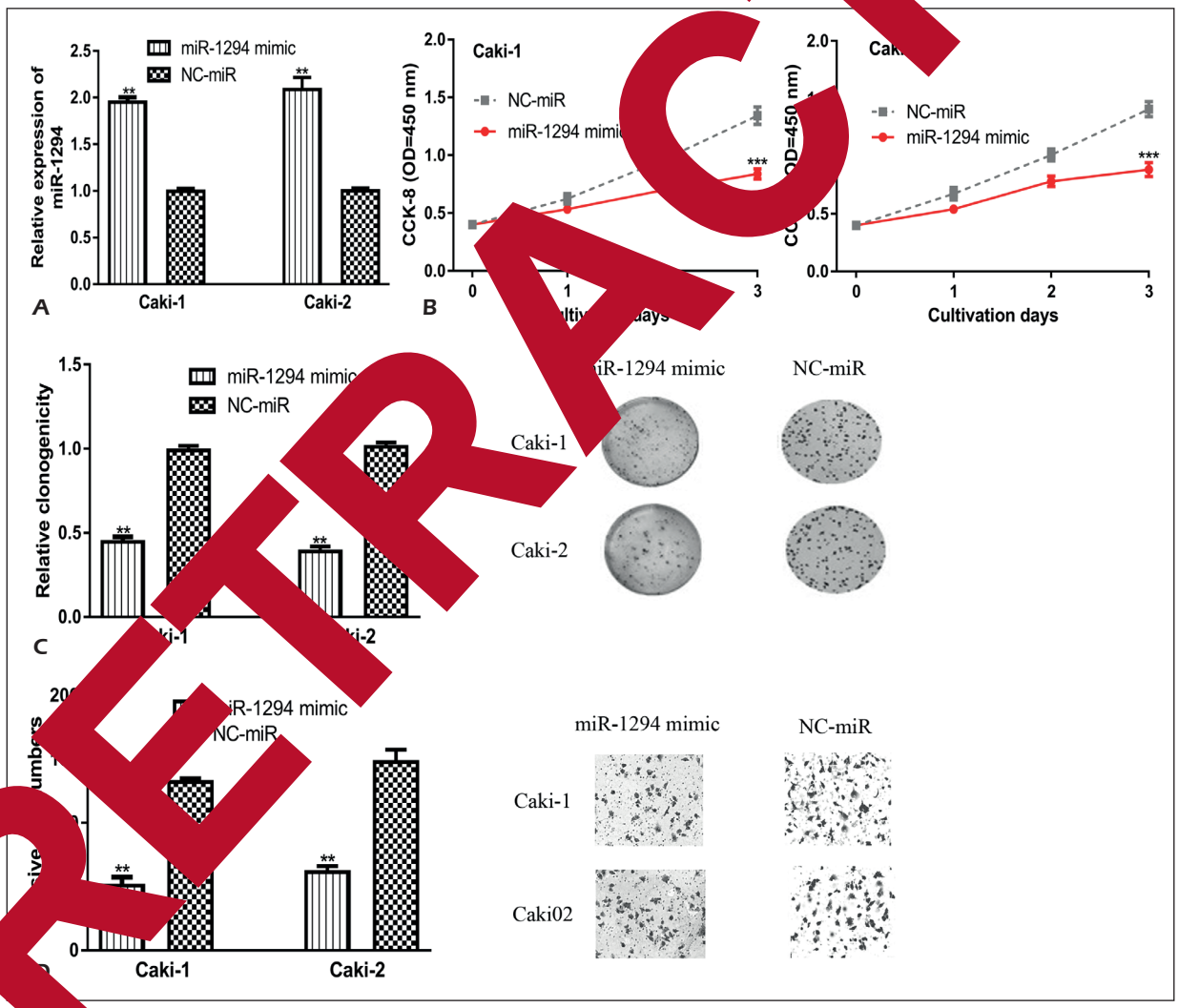

Figure 2. Overexpression of miR-1294 inhibits ccRCC cell proliferation, colony formation, and cell invasion. **A**, miR-1294 expression; **B**, cell proliferation; **C**, colony formation, and (**D**) cell invasion in ccRCC cells transfected with miR-1294 mimic or NC-miR. Magnification 200x. **p<0.01, ***p<0.001. miR-1294: microRNA-1294; ccRCC: clear cell renal cell carcinoma; NC-miR: negative control miRNA.

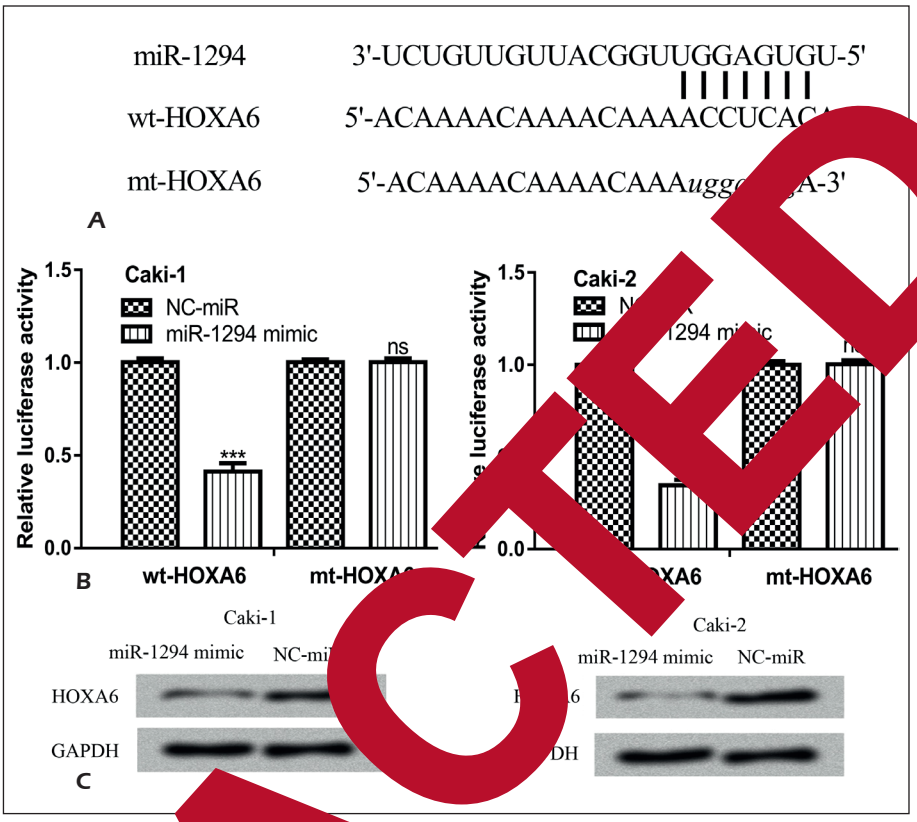

Figure 3. HOXA6 was a direct target of miR-1294. **A**, Binding site between miR-1294 and the 3'-UTR of HOXA6. **B**, Relative luciferase activity of cells transfected with luciferase activity reporter vector and synthetic miRNAs. **C**, HOXA6 expression in cells transfected with synthetic miRNAs. ns not significant, ***$p<0.001$. miR-1294: microRNA-1294; UTR: untranslated region; wt: wild-type; mt: mutant; NC-miR: negative control miRNA; HOXA6: homeobox A6.

HOXA6 Was a Direct Target of MiR-12

The bioinformatic algorith ested th HOXA6 could bind to mi re 3A). This prediction was valid by th ual-Luciferase Activity Report say. Figure 3B, the transf ion 294 mimic inhibited the lucife activity lls transfected with wt-HOX However, m 94 mimic did not affec erase activ of cells transfected w mt-H 6 (Figure 3B). The Western b assay showe miR-1294 mimic transfe decreased HOX expression compare th NC R (Figure 3C). These results su d th OXA6 was a direct target of miR-

A6 W nctional Downstream Ta t in th R-1294-Mediated ccRCC C haviors Inhibition

ate whether miR-1294 regulates RCC cell behaviors by targeting HOXA6, a e experiment was performed. Western blot s d that HOXA6 expression was significantly upregulated by pcHOXA6 transfection (Figure 4A). The overexpression of HOXA6 increased cell proliferation, colony formation, and cell invasion (Figure 4B-4D). HOXA6 overexpression partially reversed the effects of miR-1294 on these cell behaviors (Figure 4B-4D). These results suggested that HOXA6 was a functional target of miR-1294.

Discussion

The dysregulation of miRNAs and mRNAs can trigger the abnormal status of multiple biological processes to contribute to the carcinogenesis of human cancers[16]. Since miRNAs exert their effects by regulating the expression of mRNAs, it is reasonable to deduct that miRNAs has a crucial role in the tumorigenesis of human cancers[17].

In the current study, miR-1294 expression was revealed to be downregulated in ccRCC cell lines compared with the normal cell line. In addition, low miR-1294 expression was found as a predictor for the poor prognosis of ccRCC patients. These results indicated that miR-1294 functions as a tumor suppressive miRNA in the progression of ccRCC, which is the same as its role in glioma, oral squamous cell carcinoma, ovarian cancer,

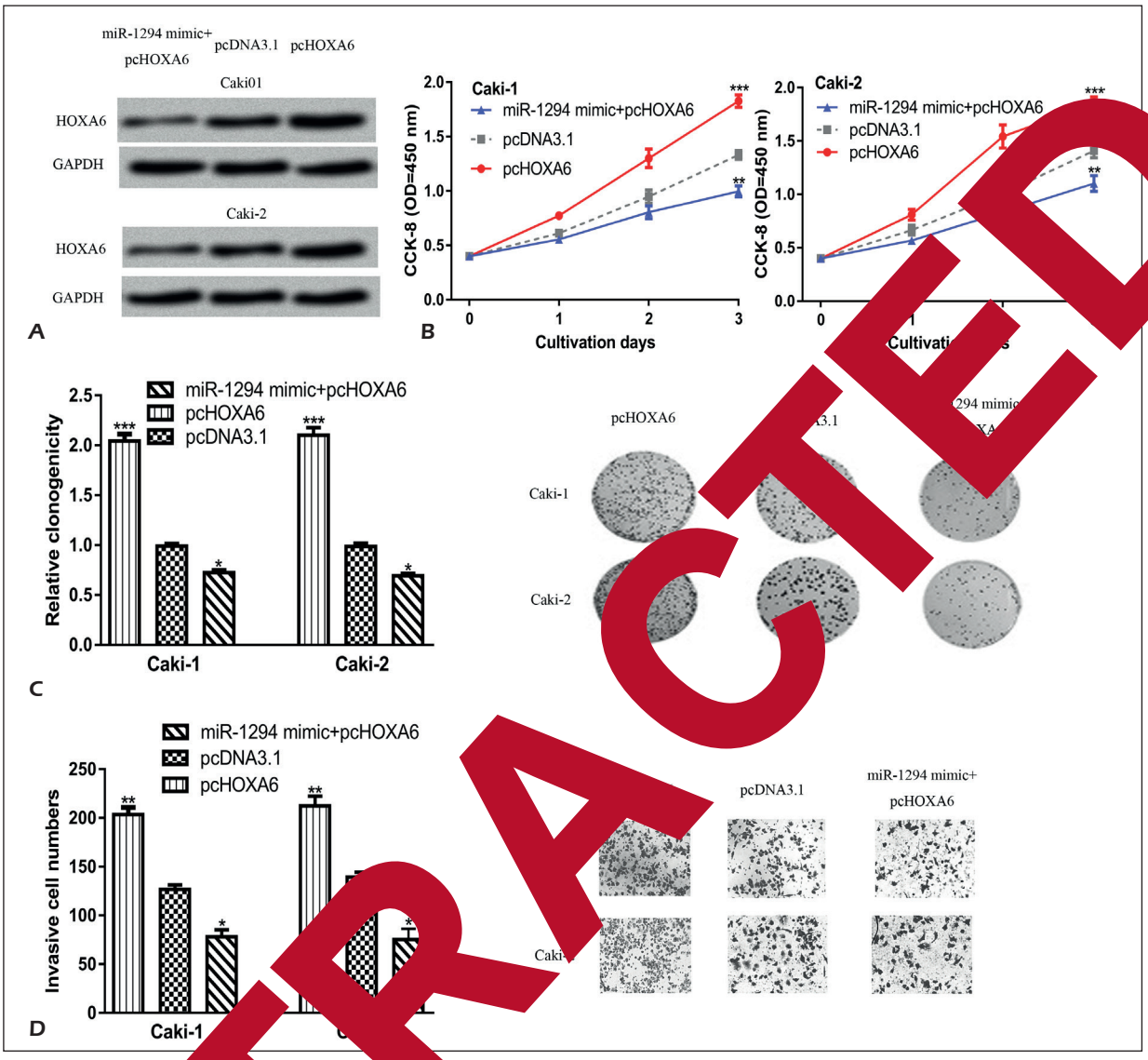

Figure 4. miR-12 s ccRCC cell iors by targeting HOXA6. **A**, HOXA6 expression; **B**, cell proliferation; **C**, colony formatio d (**D**) vasion in ccRCC cells transfected with pcHOXA6, pcDNA3.1, or pcHOXA6 and miR-1294 mimic. Magn cation 200x. 05, $^{**}p<0.01$, $^{***}p<0.001$. miR-1294: microRNA-1294; HOXA6: homeobox A6; ccRCC: clear cell r cell carcinoma.

ga ance d osteosarcoma[9-13]. Then, CCK-8 ass y form assay, and transwell sion wer ducted to investigate the ical r -1294 in ccRCC. CCK-8 assay icated t troduction of miR-1294 mimic im cell proliferation rate. The colony for- confirmed the results of the CCK-8 say. The transwell invasion assay revealed that -1294 overexpression could inhibit cell invasi collectively, these results indicated that the inhibitory effect of miR-1294 on ccRCC cell behaviors *in vitro* and overexpression of miR-1294 hindered cancer progression.

Multiple targets of miR-1294 have been identified in human cancers, including targeting protein for Xenopus kinesin-like protein 2, c-Myc, IGF1R, and HOXA9[9-11,13]. Here, the bioinformatics algorithms showed that HOXA6 was also a putative target of miR-1294. HOXA6 was reported to be upregulated in cancers including cervical cancer, colorectal cancer, and non-small cell lung cancer[18-20]. The overexpression of HOXA6 was shown to promote malignancy behaviors of cancer cell and to be correlated with the poor survival outcome of cancer patients[18-20]. The luciferase activity reporter assay and Western blot

assay together confirmed this prediction. To directly address whether the effects of miR-1294 in regulating ccRCC cell behaviors can be attributed to its regulation of HOXA6, rescue experiments were performed. It was found that the overexpression of HOXA6 resulted in increased proliferation rate and invasive ability compared with the pcDNA3.1 group. HOXA6 overexpression decreased the miR-1294-mediated suppression on cell proliferation and invasion.

Conclusions

The current study provided evidence that miR-1294 functions as a tumor suppressor by negatively regulating HOXA6 to suppress ccRCC cell proliferation, colony formation, and cell invasion, and thereby may be developed as a novel therapeutic target for ccRCC treatment.

Acknowledgments

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Conflict of Interest

The Authors declare that they hav onf interests.

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