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Pseudogene OCT4-pg4 functions as a natural micro RNA sponge to regulate OCT4 expression by competing for miR-145 in hepatocellular carcinoma

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The POU transcription factor OCT4 is a pleiotropic regulator of gene expression in embryonic stem cells. Recent studies demonstrated that OCT4 is aberrantly expressed in multiple types of human cancer; however, the underlying molecular mechanism remains largely unknown. In this study, we report that OCT4-pg4, a pseudogene of OCT4, is abnormally activated in hepatocellular carcinoma (HCC). The expression level of OCT4-pg4 is positively correlated with that of OCT4, and both gene transcripts can be directly targeted by a tumor-suppressive micro RNA miR-145. We find that the non-coding RNA OCT4-pg4 is biologically active, as it can upregulate OCT4 protein level in HCC. Mechanistic analysis revealed that OCT4-pg4 functions as a natural micro RNA sponge to protect OCT4 transcript from being inhibited by miR-145. In addition, our study also showed that OCT4-pg4 can promote growth and tumorigenicity of HCC cells, thus exerting an oncogenic role in hepatocarcinogenesis. Furthermore, survival analysis suggests that high OCT4-pg4 level is significantly correlated with poor prognosis of HCC patients. Taken together, our finding adds a new layer of post-transcriptional regulation of OCT4 and sheds new light on the treatment of human HCC.

Introduction

Hepatocellular carcinoma (HCC) is among the most prevalent and lethal cancers in the human population (1). It arises from modulation of multiple genes by mutations, epigenetic regulation, non-coding RNAs and post-translational modifications of proteins. Although >40% of HCCs are clonal and thought to arise from cancer stem cells (CSCs), the underlying molecular mechanisms remain poorly understood (2,3).

OCT4, a transcription factor in the POU family of proteins, expressed in both embryonic and adult stem cells, has been associated with the pluripotency, proliferative potential and self-renewal properties observed in embryonic stem cells (ESCs) and germ cells (4). It is well known that, *OCT4*, together with three other reprogramming factors, is essential for generating induced pluripotent stem cells (5,6). To date, a large body of evidence has demonstrated that *OCT4* participates in the initiation and progression of various malignancies, such as germ-cell tumors, bladder cancer and liver cancer (7–10). Moreover, expression of *OCT4* is required for maintaining the self-renewal and survival of cancer stem-like cells (11,12). Nevertheless, relatively little is known about how *OCT4* is dysregulated during carcinogenesis.

Abbreviations: CSC, cancer stem cell; ESC, embryonic stem cell; HCC, hepatocellular carcinoma; miRNA, micro RNA; qRT-PCR, quantitative real-time PCR; UTR, untranslated region.

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Non-coding RNAs have recently gained significant attention in delineating the molecular pathogenesis of cancers. Among them, micro RNAs (miRNAs) constitute an evolutionarily conserved class of pleiotropic small RNAs that suppress gene expression post-transcriptionally. Through sequence-specific interactions with the 3′-untranslated regions (UTRs) of cognate mRNA targets, miRNAs usually contribute to translational inhibition or mRNA degradation of large amounts of genes (13). Interestingly, a recent study showed that, in ESCs, the pluripotency factors *OCT4*, *SOX2* and *KLF4* are direct targets of a tumor-suppressive miRNA miR-145 (14), suggesting that miR-145 might play a critical role in *OCT4* dysregulation in cancer (15,16). However, the precise relationship between *OCT4* and miR-145 in HCC remains largely unknown.

Apart from miRNAs, pseudogenes represent another class of non-coding RNAs, which are defined as genomic elements that resemble real genes. Pseudogenes are previously considered to be biologically inconsequential because they harbor premature stop codons, deletions/insertions and frameshift mutations that abolish their translation into functional proteins. Nevertheless, nucleotide sequences within pseudogenes are well conserved probably due to evolutionary selection pressure, suggesting that they may indeed have an important cellular role (17). Pseudogenes exhibit tissue-specific expression patterns (18) and are aberrantly activated in malignancies (19), indicating that pseudogenes may contribute to tumorigenesis, although the mechanisms still remain elusive. Recently, an elegant study conducted by Poliseno *et al.* (20) showed that pseudogenes could function as competing endogenous RNAs to regulate other RNA transcripts by competing for shared miRNAs. That finding added a new transregulatory dimension to mRNA biology besides the protein-coding function (21). Interestingly, bioinformatic analysis revealed a higher-than-expected frequency of pseudogenes for ESC-specific genes such as *OCT4*, *Nanog* and *Stella* (22), suggesting that pseudogenes may coordinate with their parental genes in maintaining the stemness of ESCs/CSCs.

In this study, we report that *OCT4* and its pseudogene *OCT4-pg4* are aberrantly expressed in HCC and that their expression levels displayed a direct correlation. Moreover, we found that *OCT4-pg4* functions as a competing endogenous RNA to protect *OCT4* transcript from being inhibited by miR-145, thus playing an oncogenic role in hepatocarcinogenesis. The present work provides the first evidence for the extraordinary crosstalk among miR-145, *OCT4* and *OCT4-pg4* and sheds new light on the treatment of HCC.

Materials and methods

Patients and specimens

Fresh-frozen and paraffin-embedded primary HCC tissues and corresponding adjacent non-tumorous liver samples were obtained from Chinese patients at Xijing Hospital (Xi'an, China). The use of clinical specimens in this study was approved by the Ethic Committee of Xijing Hospital in Fourth Military Medical University. All cases were reviewed by pathologist and histologically confirmed as HCC. For stratification analysis of survival, the relative expression levels of OCT4-pg4 in HCC ($n = 54$) and normal liver ($n = 10$) samples were determined by quantitative real-time PCR (qRT-PCR). The expression data across all samples were calibrated by the median OCT4-pg4 expression level of normal liver tissues. Then, the relative expression values of 54 HCC samples were sorted by descending order and dichotomized by a median split to define high/low groups of OCT4-pg4 expression.

Cell culture

Human embryonic kidney cell line HEK293 and HCC cell line HepG2 were purchased from American Type Culture Collection. SMMC-7721 was obtained from the Type Culture Collection of the Chinese Academy of Sciences. MHCC97-L and MHCC97-H were obtained from Liver Cancer Institute, Zhongshan Hospital. HEK293, HepG2, MHCC97-L and MHCC97-H were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum. SMMC-7721 cells were grown in RPMI-1640

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medium (Gibco BRL) supplemented with 10% fetal bovine serum. All cell lines were maintained in a humidified atmosphere at 37°C with 5% CO_2.

Plasmids, antagomiRs and small interfering RNAs

The full-length OCT4 3′UTR and OCT4-pg4 were generated by PCR amplification from HepG2 cDNA and cloned into pGL3 (Promega, Madison, WI) and pcDNA3.1 (Invitrogen, Carlsbad, CA). The primers used are as follows—OCT4 3′UTR: forward 5′-TGAGGTGCCTGCCCTTCTAG-3′ and reverse 5′-AAGT GATACATGATGTGGGA-3′; OCT4-pg4: forward 5′-CCATGGCGGGACAC CTGGCT-3′ and reverse 5′-GAATGCATGGGAGAGCCCAG-3′. Before cloning, PCR products were run on a 1.2% (w/v) agarose gel to check for size and PCR specificity. Only PCR products of the correct size were excised from the gel and purified using the gel purification kit (Qiagen, Hilden, Germany). Then, the purified PCR products were ligated into the pMD-18T vector (TaKaRa) by TA cloning and sequenced to verify correct insertion of the fragments. For site-specific mutagenesis, DNA sequences were custom synthesized (GenScript Co., Nanjing, China). For miRNA depletion, antagomiRs were purchased from Dharmacon. For gene knockdown, OCT4-pg4-specific siRNA (#SASI_Hs01_00273527; Sigma–Aldrich) and OCT4-specific siRNA (#SASI_Hs01_00193932; Sigma–Aldrich) were purchased, and siRNA against a common region of OCT4 and OCT4-pg4 was custom synthesized (RiboBio Co., Ltd, Guangzhou, China) with the following sequence: 5′-AGCAGCUUGGGCUCGAGAAdTdT-3′.

Western blotting

Total cell lysates were obtained using Triton X-100 lysis buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Twenty microgram total protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membrane was then probed with primary antibodies as follows: OCT4 (POU5F1) (#2876-1, 1:1000; Epitomics) and β-actin (#A1978, 1:4000; Sigma–Aldrich).

RNA extraction and qRT-PCR

Total RNA was isolated by TRIzol (Invitrogen) and treated with RQ1 RNase-free DNase (Promega) to eliminate DNA contamination. Complementary DNA was synthesized from 1 µg of total RNA using miScript Reverse Transcription Kit (Qiagen). Relative expressions of different genes were detected using Roche SYBR Green PCR Kit on the iCycler real-time detection system (Bio-Rad, Hercules, CA). The $2^{-\Delta\Delta C_T}$ method was used in data analysis, with mRNA levels normalized to glyceraldehyde 3-phosphate dehydrogenase and miRNA levels normalized to U6. The normalized level of target gene expression is calculated by using the formula as follows: ΔC_T (sample or calibrator) = C_T (target gene) − C_T (reference gene); $\Delta\Delta C_T = \Delta C_T$ (sample) − ΔC_T (calibrator); normalized target gene expression level in sample = $2^{-\Delta\Delta C_T}$. For detecting miR-145, miScript Primer Assay (#MS00003528; Qiagen) and miScript Universal Primer were used. The primer sequences for other transcript analyzed are as follows—OCT4: forward 5′-GATGGCGTACTGTGGGCCC-3′ and reverse 5′-TGGGACTCCTC CGGGTTTTG-3′; OCT4-pg4: forward 5′-CAGAAACCCTCTTGCAGGCT-3′ and reverse 5′-GAACCACACTCGGACCACAT-3′; glyceraldehyde 3-phosphate dehydrogenase: forward 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′-GGCATGGACTGTGGTCATGAG-3′. To make primer sets more specific and avoid PCR artifacts, both OCT4 sense primer and OCT4-pg4 sense primer were designed to carry a polymorphism at the 3′ end, respectively (Supplementary Figure S1, available at *Carcinogenesis* Online). The specificity of these qPCR primers was experimentally verified by sequencing the amplified products obtained in the qPCR assay (Supplementary Table S3, available at *Carcinogenesis* Online).

Sequencing analysis

The homology analysis of OCT4 transcript and its pseudogenes was carried out by using the BioEdit software. The universal primer set (forward 5′-TTGCCAAGCTCCTGAAGCAG-3′ and reverse 5′-GCAGATGGTCG TTTGGCTGA-3′) was designed based on the most homologous region that made the primers perfectly complementary to the OCT4 mRNA and all six pseudogenes (Supplementary Table S6, available at *Carcinogenesis* Online). RT-PCR assay using the universal primer set was performed for all samples. PCR products were run on a 1.5% (w/v) agarose gel and the positive electrophoretic bands were purified using the gel purification kit (Qiagen). Then the purified PCR products were ligated into the pMD-18T vector (TaKaRa) by TA cloning and sequenced via automated sequencing. The sequencing results were analyzed by using the Nucleotide BLAST program and the BioEdit software.

Luciferase reporter assay

Cells of 60–90% confluence in 48-well plates were transfected using Lipofectamine 2000 (Invitrogen). One hundred nanograms of firefly luciferase reporter gene construct and 5 ng of the pRL-TK Renilla luciferase construct (for normalization) were cotransfected per well. Cell extracts were prepared 48 h after transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The experiment was carried out in triplicate wells for at least three times.

Cell viability assay

Cells, seeded in 96-well plates, were stained at the indicated time points with 100 µl sterile 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide dye (0.5 mg/ml; Sigma) for 4 h at 37°C. The supernatant was then aspirated and 150 µl dimethyl sulfoxide was added into each well. The intensity of formazan formed was measured at 570 nm using Victor Wallac microplate reader (PerkinElmer, Waltham, MA). The experiment was carried out in quintuplicate wells for at least three times.

Colony formation assays

Cells were seeded at the density of 1×10^3 cells/well in 12-well plates and cultured for 10 days. Following paraformaldehyde fixation and crystal violet staining, the cultures were photographed using GelDoc System (Bio-Rad) and stained cell colonies were counted by Quantity One software (Bio-Rad). The experiment was carried out in triplicate wells for at least three times.

Xenograft tumor model

Six-week-old BALB/c nude mice were purchased from the Model Animal Research Center of Nanjing University. All animal procedures were performed in accordance to the protocols approved by the Institutional Animal Care and Use Committee at the Fourth Military Medical University. For xenograft models, 5×10^6 HepG2 cells stably expressing OCT4-pg4 or transfected with 2′-O-Me-modified si-OCT4-pg4 were injected subcutaneously in the right flank of BALB/c nude mice (five mice per group). Tumor growth was monitored every 5 days for a total period of 30 days. Tumor volumes were calculated by using the following formula: V (mm^3) = $a \times b^2/2$, where a is the largest diameter and b is the perpendicular diameter.

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded tissue sections. A rabbit monoclonal antibody against OCT4 (#2876-1; Epitomics) was used and isotype-matched rabbit immunoglobulin G antibody served as a negative control. Chromogen development was performed with ultraView Universal DAB detection kit (Ventana Medical Systems). The percentage of positive cells and staining intensity were multiplied to produce a weighted score for each case.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 for Windows. All data were presented as mean ± SD. Disease-free and overall survival was analyzed with the Kaplan–Meier method and the statistical probability (P-value) was generated by log-rank test. The significance of associations between OCT4 and OCT4-pg4 expression values was judged via a test statistic based on Pearson product–moment correlation coefficient. Two-tailed Student's t-test was used to evaluate the statistical significance of differences between two groups of data in luciferase assay, qRT-PCR and cell growth assay. Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

Results

OCT4 is aberrantly expressed in HCC and directly targeted by miR-145

To investigate the expression of OCT4 protein in HCC, we performed immunohistochemical staining in 54 HCC tissues and 10 normal liver tissues. Among the 54 HCC tissues, 21 (38.9%) cases were positive for OCT4; however, none of the normal liver tissues exhibited obvious expression of OCT4 (Figure 1A). To study the clinical significance of *OCT4* accumulation in HCC progression, we examined the correlation between *OCT4* level and disease stage. Results revealed that the expression levels of *OCT4* were remarkably increased during HCC progression, indicating that upregulation of *OCT4* is clearly related to HCC stage (Figure 1A and B).

It is known that miR-145 can regulate *OCT4* expression and repress pluripotency in human ESCs (14). To test whether miR-145 plays a functional role in suppressing *OCT4* during liver carcinogenesis, we performed a reporter gene assay in HCC cells. Results showed that miR-145 significantly repressed the luciferase activity of reporter vector harboring wild-type 3′UTR of *OCT4*, whereas mutation of the

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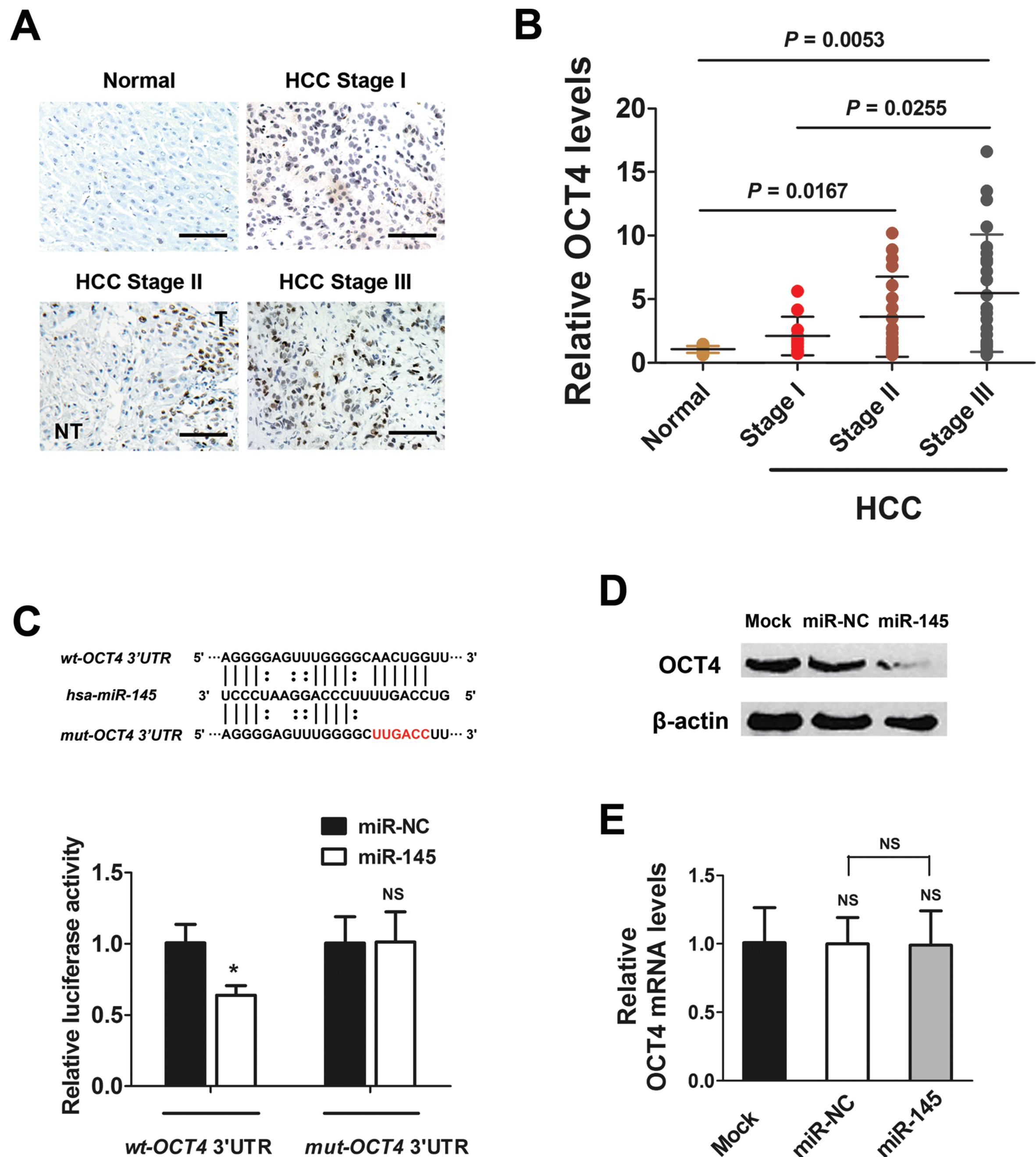

Fig. 1. OCT4 is upregulated during HCC progression and targeted by miR-145. (**A**) Immunostaining of OCT4 in HCC (T) and adjacent non-tumorous (NT) liver tissues. Representative pictures are shown from normal, stage I, stage II and stage III HCC tissues. (**B**) Assessment of OCT4 protein levels by quantitative immunoblotting in 54 HCC tissues and 10 normal liver tissues. (**C**) Relative luciferase activity (mean ± SD) mediated by reporter constructs harboring the wild-type or mutated 3′UTR of OCT4 upon transfection with 100 nM miR-NC or miR-145. (**D**) Western blotting analysis of OCT4 protein levels in HepG2 cells treated with 100 nM miR-NC or miR-145. (**E**) Real-time PCR analysis of OCT4 mRNA levels (mean ± SD) in HepG2 cells treated with 100 nM miR-NC or miR-145.

putative miR-145 binding site in *OCT4* 3′UTR abrogated the inhibitory effect of miR-145 (Figure 1C). Western blotting analyses further confirmed that the OCT4 protein level was markedly decreased upon miR-145 transfection (Figure 1D). However, miR-145 had no effect on the mRNA level of *OCT4* as determined by qRT-PCR (Figure 1E), suggesting that miR-145 regulates *OCT4* expression via translational inhibition in HCC cells.

OCT4-pg4 is positively correlated with OCT4 in expression level and can be targeted by miR-145 in HCC

Given that *OCT4* is a direct target of miR-145, we speculated that there would be an inverse correlation between the expression levels of miR-145 and *OCT4* in HCC. However, no obvious correlation ($r = -0.0671$, $P = 0.6299$) between miR-145 and OCT4 protein levels was observed (Figure 2A), indicating that other mechanisms might be involved in regulating *OCT4* expression. The human *OCT4* has six pseudogenes, of which *OCT4-pg1*, *OCT4-pg3* and *OCT4-pg4* were found to be transcribed in somatic cancers (23,24). To investigate the expression of *OCT4* pseudogenes in HCC, we performed DNA sequencing analysis following RT-PCR. Results showed that *OCT4-pg4* represents the majority of *OCT4* pseudogenes and is expressed along with *OCT4* in HCC cells (Supplementary Table S1, available at *Carcinogenesis* Online). Furthermore, we found a positive correlation between *OCT4* and *OCT4-pg4* levels in HCC clinical

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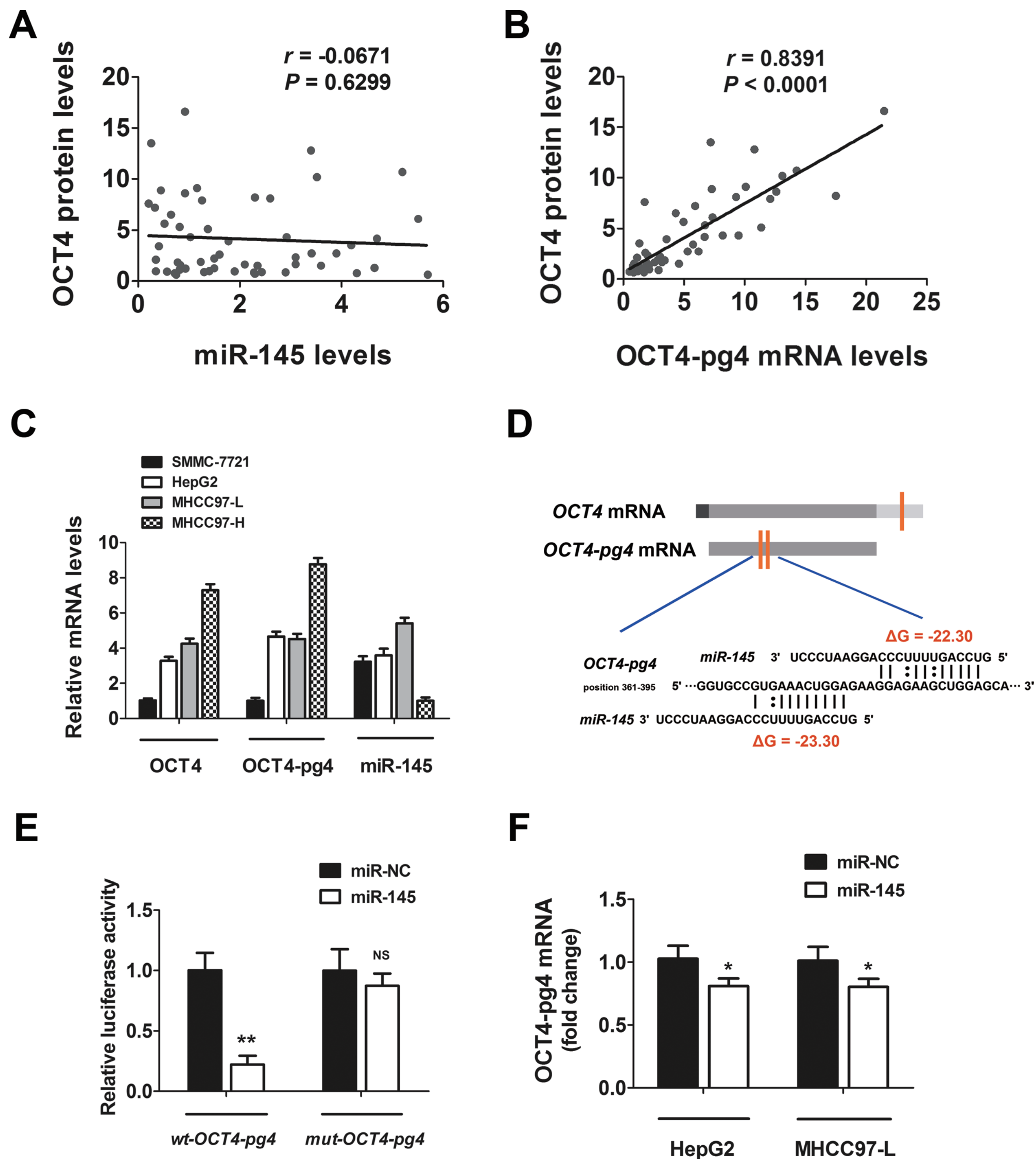

Fig. 2. OCT4-pg4 level is positively correlated with that of OCT4 in HCC. (**A**) Correlation between OCT4 protein level and miR-145 expression level. (**B**) Correlation between OCT4 protein level and OCT4-pg4 expression level. Each data point represents an individual HCC tissue sample, and a correlation coefficient (r) is shown. (**C**) Real-time PCR analysis of expression levels (mean ± SD) of OCT4, OCT4-pg4 and miR-145 in multiple HCC cell lines. (**D**) Bioinformatic analysis of OCT4 and its pseudogene OCT4-pg4. OCT4-pg4 transcript is highly homologous to the open reading frame of OCT4, and sequence variations contribute to the formation of a novel tandem seed matches for miR-145. Orange vertical lines represent miR-145 binding sites. (**E**) Relative luciferase activity (mean ± SD) mediated by reporter constructs harboring the wild-type or mutated OCT4-pg4 upon transfection with 100 nM miR-NC or miR-145. (**F**) Real-time PCR analysis of OCT4-pg4 levels (mean ± SD) in HepG2 and MHCC97-L cells treated with 100 nM miR-NC or miR-145.

samples ($r = 0.8391$, $P < 0.0001$) (Figure 2B) and in several HCC cell lines (Figure 2C), suggesting that *OCT4-pg4* may play a functional role in *OCT4* upregulation.

OCT4-pg4 (*POU5F1P4*) is a processed pseudogene located at 1q22. It is highly homologous to the coding sequence of *OCT4* (*POU5F1*), with only 33 mismatches throughout the RNA body; however, a frameshift mutation near the AUG initiator codon impedes the translation of *OCT4-pg4* transcript (Supplementary Table S2, available at *Carcinogenesis* Online). Although *OCT4-pg4* lacks the *OCT4* 3′UTR, which can be targeted by miR-145, the sequence variations of it coincidently contribute to the formation of a novel tandem seed matches for miR-145 (Figure 2D). Strikingly, the free energy

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predicted for hybridization with *OCT4-pg4* and miR-145 at these sites is Δ = −23.30 and −22.30 kcal/mol, respectively (Figure 2D), indicating that *OCT4-pg4* is a strong candidate target for miR-145. Then, we cloned the full-length *OCT4-pg4* downstream of the luciferase gene. Reporter assays revealed that the luciferase activity was reduced by ~80% upon miR-145 transfection, and this effect was eliminated through mutation of the putative miR-145 binding sites in *OCT4-pg4* (Figure 2E). Further qRT-PCR analysis demonstrated that overexpression of miR-145 has only a slight effect on the mRNA stability of *OCT4-pg4* (Figure 2F). Overall, these data raised the possibility that *OCT4-pg4* might function as a miRNA decoy for miR-145 in HCC.

OCT4-pg4 functions as a miRNA decoy for miR-145 to upregulate OCT4 in HCC

To evaluate the role of *OCT4-pg4* in regulating *OCT4* expression, we performed reporter assays using the reporter construct harboring wild-type 3′UTR of *OCT4*. We found that *OCT4-pg4* overexpression remarkably increased the luciferase activity in both HEK293 and HepG2 cells, and the inhibitory effect of miR-145 on *OCT4* was reversed upon *OCT4-pg4* transfection (Figure 3A). However, mutation of the miR-145 binding sites in *OCT4-pg4* entirely abrogated these effects (Figure 3B), suggesting that *OCT4-pg4* could be a competitive antagonist of miR-145 in HCC cells.

Next, we employed RNAi-mediated knockdown to study the loss-of-function phenotypes of *OCT4-pg4*. Gene-specific siRNAs were designed against the variable regions of *OCT4-pg4* to distinguish it from *OCT4* (Supplementary Table S2, available at *Carcinogenesis* Online). The qRT-PCR analysis revealed that transfection of siRNA against *OCT4-pg4* reduced *OCT4-pg4* levels to <20% but had no effect on *OCT4* levels, whereas overexpression of siRNA against a common region of *OCT4-pg4* and *OCT4* remarkably inhibited both genes simultaneously (Figure 3C). In addition, the siRNA specificity was further confirmed via sequencing analysis following the qPCR assay (Supplementary Table S4, available at *Carcinogenesis* Online). Reporter assays in HCC cell lines showed that *OCT4-pg4* knockdown significantly repressed the luciferase activity of reporter vector harboring wild-type 3′UTR of *OCT4* (Figure 3D). Notably, this effect could be completely abolished by antagomiR-mediated miR-145 inhibition (Figure 3D), suggesting that *OCT4* repression mediated by *OCT4-pg4* knockdown was largely dependent on endogenous miR-145 expression in HCC. Western blotting analyses confirmed that OCT4 protein level was decreased upon *OCT4-pg4* knockdown and could be

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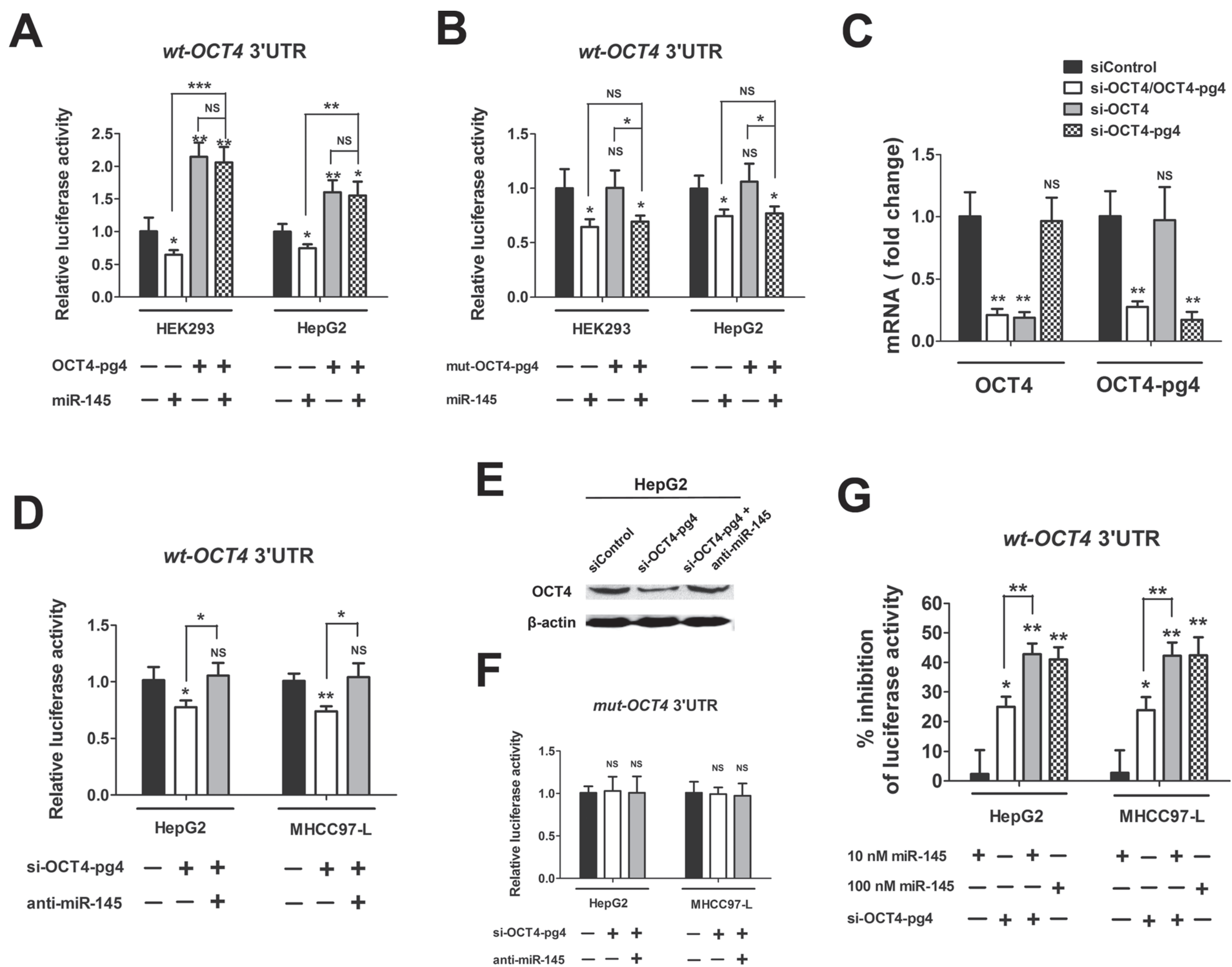

Fig. 3. OCT4-pg4 upregulates OCT4 expression by competing for miR-145 in HCC. (**A**) Relative luciferase activity (mean ± SD) in HEK293 or HepG2 cells cotransfected with OCT4-3′UTR reporter construct and a vector expressing OCT4-pg4 or 100 nM miR-145 plus the OCT4-pg4-expressing vector. (**B**) Relative luciferase activity (mean ± SD) in HEK293 or HepG2 cells cotransfected with OCT4-3′UTR reporter construct and a vector expressing mutated OCT4-pg4 or 100 nM miR-145 plus the mut-OCT4-pg4-expressing vector. (**C**) Real-time PCR analysis of expression levels (mean ± SD) of OCT4 and OCT4-pg4 in HepG2 cells treated with siRNA against OCT4 or OCT4-pg4 or both. (**D**) Relative luciferase activity (mean ± SD) in HepG2 or MHCC97-L cells cotransfected with OCT4-3′UTR reporter construct and si-OCT4-pg4 or si-OCT4-pg4 plus antagomiR-145. (**E**) Western blotting analysis of OCT4 protein levels in HepG2 cells treated with si-OCT4-pg4 or si-OCT4-pg4 plus antagomiR-145. (**F**) Relative luciferase activity (mean ± SD) in HepG2 or MHCC97-L cells cotransfected with mut-OCT4-3′UTR reporter construct and si-OCT4-pg4 or si-OCT4-pg4 plus antagomiR-145. (**G**) Relative luciferase activity (mean ± SD) in HepG2 or MHCC97-L cells cotransfected with OCT4-3′UTR reporter construct and 10 nM miR-145 or 100 nM miR-145 or 10 nM miR-145 plus si-OCT4-pg4.

rescued by transfection of antagomiR-145 (Figure 3E). Furthermore, we found that *OCT4-pg4* knockdown had no effect on the luciferase activity of reporter vector harboring mutated-type 3′UTR of *OCT4* (Figure 3F). Taken together, above data demonstrated that pseudogene *OCT4-pg4* functions as a miRNA decoy to protect *OCT4* from being inhibited by miR-145 in HCC.

To test whether *OCT4-pg4* knockdown could enhance the efficiency of miR-145-mediated *OCT4* inhibition, we performed reporter assays in HCC cell lines. The reporter vector containing wild-type 3′UTR of *OCT4* was cotransfected with different concentrations of miR-145 mimics. We found that transfection of 100 nM miR-145 mimics reduced the luciferase activity by ~40%, whereas transfection of 10 nM miR-145 mimics had only a negligible effect (Figure 3G). However, upon *OCT4-pg4* knockdown, miR-145 treatment at the concentration of 10 nM reduced the luciferase activity to a level comparable with that of 100 nM (Figure 3G), strongly supporting the notion that *OCT4-pg4* antagonizes miR-145 function in HCC.

OCT-pg4 promotes cell proliferation and colony formation of HCC cells

To explore the biological role of *OCT4-pg4* in hepatocarcinogenesis, we performed 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assays following gene-specific knockdown of *OCT4-pg4* and *OCT4*. Results showed that *OCT4-pg4* knockdown significantly inhibited cell proliferation of HepG2 cells (Figure 4A). Besides, we found that simultaneous knockdown of *OCT4-pg4* and *OCT4* showed the strongest effect (Figure 4A), indicating that *OCT4* and its pseudogene may have additive roles for growth promotion. Moreover, these observations were further confirmed by a colony formation assay (Figure 4B). Since SMMC-7721 HCC cells exhibit low levels of *OCT4-pg4* and *OCT4* (Figure 2C), we used it for gain-of-function study. Stable transfection of wild-type *OCT4-pg4* in SMMC-7721 cells resulted in remarkable upregulation of OCT4 proteins (Figure 4C), accelerated cell proliferation rate (Figure 4D) and increased colony number (Figure 4E), suggesting that *OCT4-pg4* exerts an oncogenic role during hepatocarcinogenesis.

OCT-pg4 overexpression enhances tumorigenicity of HCC cells and is associated with poorer prognosis of HCC patients

To confirm the *in vitro* findings, we used a nude mouse xenograft model to detect the HCC-promoting effect of *OCT4-pg4*. HepG2 cells stably expressing *OCT4-pg4* or cells treated with siRNA against *OCT4-pg4* were subcutaneously injected into the posterior flanks of nude mice. Analysis of tumor growth curves revealed that *OCT4-pg4*-overexpressing cells grew faster than control cells, whereas *OCT-pg4*

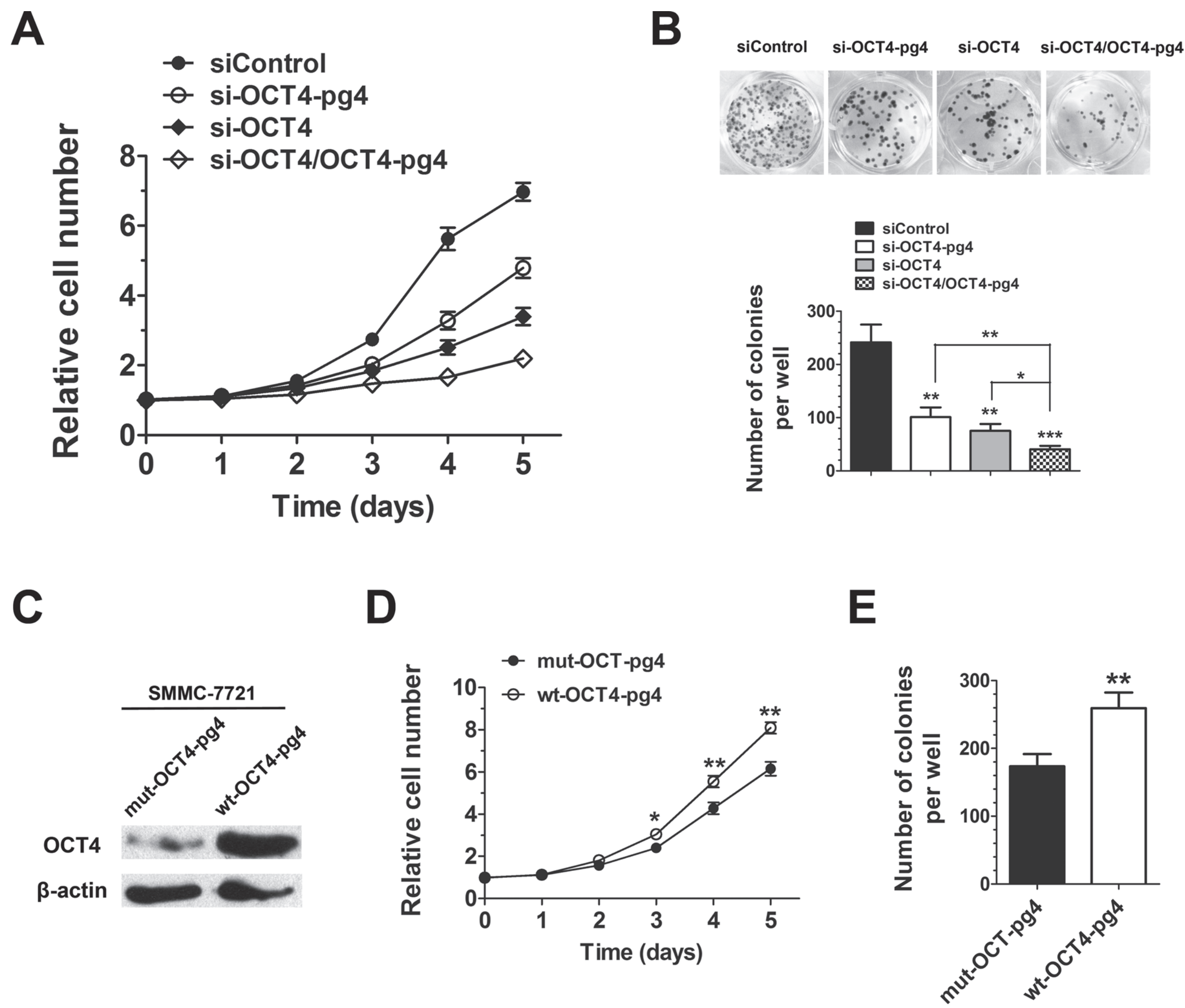

Fig. 4. OCT-pg4 promotes HCC cell proliferation and colony formation. (**A**) Proliferation curve of HepG2 cells transfected with siControl, si-OCT4-pg4, si-OCT4 or si-OCT4/OCT4-pg4. (**B**) Colony formation assay of HepG2 cells transfected with siControl, si-OCT4-pg4, si-OCT4 or si-OCT4/OCT4-pg4. Representative pictures of crystal violet-stained colonies are shown. (**C**) Western blotting analysis of OCT4 protein levels in SMMC-7721 cells stably expressing wild-type or mutated OCT4-pg4. (**D**) Proliferation curve of SMMC-7721 cells stably expressing wild-type or mutated OCT4-pg4. (**E**) Colony formation assay of SMMC-7721 cells stably expressing wild-type or mutated OCT4-pg4.

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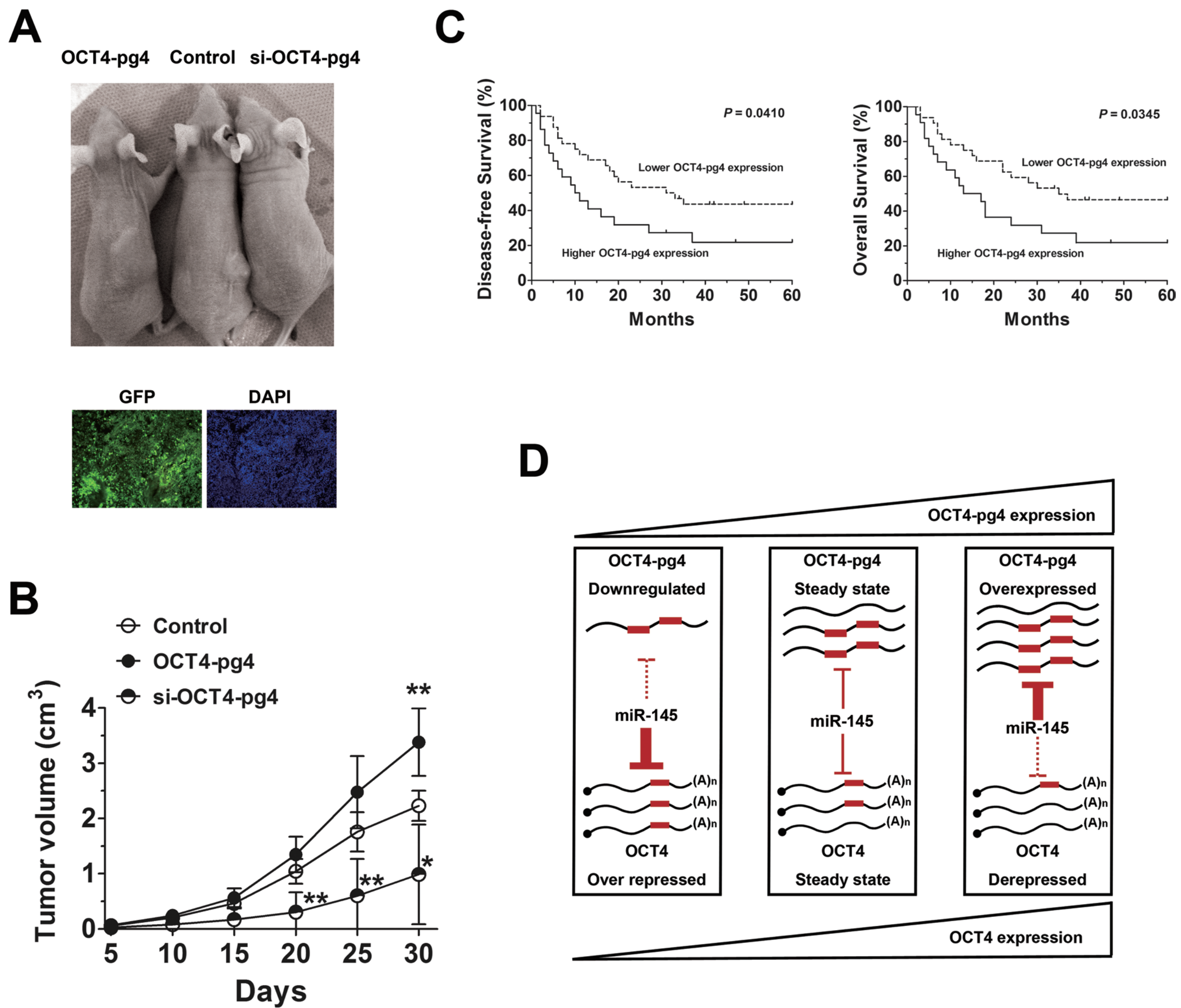

Fig. 5. OCT4-pg4 enhances tumorigenicity and is associated with poorer prognosis. (**A**) Effect of OCT4-pg4 on tumor formation in nude mouse xenograft model. 5×10^6 HepG2 cells stably expressing OCT4-pg4 or transfected with si-OCT4-pg4 were injected subcutaneously in the right flank of BALB/c nude mice (five mice per group). Photographs illustrate representative features of tumors (top) and expression efficiency of OCT4-pg4 in xenograft tissues (bottom; green fluorescent protein-positive cells) 30 days after inoculation. (**B**) Tumor volume (mean ± SD) in mice injected with HepG2 cells stably expressing OCT4-pg4 or transfected with si-OCT4-pg4. (**C**) Kaplan–Meier graphs representing the probabilities of disease-free survival and overall survival in HCC patients stratified according to low/high OCT4-pg4 expression levels. (**D**) Working model. In the steady state (middle), equilibrium exists between miR-145 and its targets OCT4 and OCT4-pg4. Downregulation of OCT4-pg4 (left) leads to increased availability of miR-145 to bind to OCT4 transcripts, thus decreasing its protein level. In contrast, overexpression of OCT4-pg4 (right) leads to fewer miR-145 free to bind to OCT4 transcripts and thus OCT4 abundance increases. Red rectangles represent miR-145 molecules.

knockdown significantly attenuated tumorigenicity of HepG2 cells (Figure 5A and B).

To evaluate whether the expression level of *OCT4-pg4* correlates with survival of HCC patients, we investigated a cohort of 54 HCC patients. Kaplan–Meier analysis revealed that high *OCT4-pg4* level in HCC tissues is significantly correlated with the markedly reduced disease-free survival and overall survival of HCC patients (Figure 5C), suggesting the important roles of *OCT4-pg4* in pathogenesis of HCC and prognosis of HCC patients.

Discussion

Like other solid tumors, HCCs appear to be sustained by a minority population of CSCs. Dedifferentiated hepatocytes, hepatic oval cells and bone marrow cells are the three major types of liver stem cells (25). Recent studies showed that liver CSCs could be enriched via different cell surface markers, such as CD13, CD24, CD44, CD90, CD133, EpCAM (CD326) and OV6. Liver CSCs could also be identified through functional assays, e.g. selecting cells with high aldehyde dehydrogenase activity or isolating the side population cells by Hoechst dye staining (26). Moreover, accumulating evidence has demonstrated that several signaling pathways that are critical for inducing the HCC stemness are dysregulated during hepatocarcinogenesis, such as Wnt/β-catenin, transforming growth factor-beta and IL-6/STAT3 pathways (27,28). However, the molecular genetics and the mechanisms responsible for the highly aggressive clinical picture of HCC still remain poorly understood.

In this study, we demonstrate that *OCT4*, a pluripotency sustaining factor in embryonic stem cells, is aberrantly expressed in advanced HCC. *OCT4*, also known as *POU5F1*, is initially transcribed as a maternal transcription factor in the oocyte but remains active in embryos throughout the preimplantation period (29). *OCT4* expression is associated with an undifferentiated phenotype (30), and numerous studies point to the fact that *OCT4* is expressed in various types of human cancer but not in normal somatic tissues (31–33). Nevertheless, some studies aroused controversy with the finding that *OCT4* expression is absent in multiple tumor cell lines (34,35). Here, our data showed that OCT4 protein is not expressed in normal liver

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tissues and is nearly absent in early stage HCCs (stage I), whereas in late-stage HCCs (stage II or stage III), OCT4 protein is overexpressed at a high frequency (Figure 1A and B). This finding, in line with previous reports (7,8), supports the notion that *OCT4* expression is dysregulated in HCC. Our functional study further revealed that knockdown of *OCT4* in HCC cells resulted in reduced cell proliferation and colony-forming ability (Figure 4A and B), suggesting an oncogenic role for *OCT4* in hepatocarcinogenesis. In addition, Wang *et al.* (36) demonstrated that *OCT4* could mediate chemotherapeutic drug resistance in HCC through a potential OCT4-AKT-ATP-binding cassette G_2 pathway. Given the critical role of *OCT4* in maintaining the stemness of stem cells, we argue that the OCT4-positive HCC cells, which are characterized by chemoresistance and proliferative potential, are probably derived from liver CSCs.

OCT4 expression must be closely regulated. The precise level of *OCT4* governs three distinct fates of ESCs. A <2-fold increase of OCT4 protein turns ESCs into primitive endoderm and mesoderm, whereas repression of *OCT4* induces loss of pluripotency and dedifferentiation to trophectoderm (37). In ESCs, *OCT4* can be transcriptionally activated by itself (38) and negatively regulated by miR-145 (14). miR-145 is a well-known tumor-suppressive miRNA, which is often downregulated in multiple types of malignancy, including HCC (39–42). Therefore, it seems plausible to reason that *OCT4* overexpression in cancer is caused by miR-145 downregulation. However, we did not observe a reverse correlation between miR-145 and *OCT4* levels in HCCs (Figure 2A). On the other hand, our study demonstrated that a non-coding RNA expressed from the *OCT4* pseudogene *OCT4-pg4* was able to regulate the corresponding OCT4 protein level in HCC by acting as a miRNA decoy for miR-145 (Figure 5D).

In mouse oocytes, processed pseudogenes could be processed into endogenous small interfering RNAs that inhibit the expression of cognate genes through conventional RNA interference (43). However, endogenous small interfering RNA pathway has yet to be identified in human somatic cells (44). Besides, it is notable that only a small percentage of pseudogenes undergo antisense transcription, whereas all transcribed pseudogenes are theoretically capable of competing with cognate mRNAs for miRNA binding. Five years ago, Ebert *et al.* (45) introduced artificial miRNA decoys termed ‘miRNA sponges’ as a means to create loss-of-function phenotypes for miRNAs in mammalian cells. Given the efficacy of stably integrated miRNA sponges to inhibit miRNA seed families, it seems reasonable that endogenous non-coding RNAs might have evolved to be able to sequence-specifically sequester miRNAs. The first such natural miRNA sponge was discovered in plants, in which the trehalose-6-phosphate synthase RNAs can attenuate the miR-399-mediated stress response (46). More recently, Poliseno *et al.* (20) found that the mammalian pseudogene *PTENP1* could function as a competing endogenous RNA to regulate *PTEN* expression by competing for shared miRNAs. The 3′UTR of *PTENP1* is highly homologous to that of *PTEN*, thus playing a critical role in sequestering miRNAs. In our study, a lack of 3′UTR in the pseudogene *OCT4-pg4* was observed, indicating that *OCT4-pg4* might have lost its ability to function as a miRNA decoy. However, as miRNA binding sites are located in open reading frame sequences as well (47), the entire transcript of pseudogenes may possess decoy function. Our subsequent analysis showed that the sequence variations of *OCT4-pg4* coincidently contribute to the formation of a novel tandem seed matches for miR-145. Furthermore, *OCT4-pg4* displayed a much higher affinity for miR-145 compared with *OCT4* transcript, strongly supporting the notion that *OCT4-pg4* functions as a miRNA decoy for miR-145 in HCC. Interestingly, pseudogene *OCT4-pg4* is located at 1q22, a chromosomal region frequently amplified in HCC (48), suggesting that the aberrant expression of *OCT4-pg4* is probably due to gene amplification. Bioinformatic analysis revealed that *OCT4-pg4* sits in the intron of *ASH1L*, a large gene spanning over 220 kb of the human genome. Therefore, the *OCT4-pg4* expression is likely to be influenced by the transcription of its host gene. However, the transcriptional orientations of these two genes are just opposite, with *OCT4-pg4* on the sense strand and *ASH1L* on the antisense strand (Supplementary Figure S2, available at *Carcinogenesis* Online), strongly suggesting that *OCT4-pg4* probably has its own promoter and transcribes independently. It is also noteworthy that *ASH1L* is an activating histone methyltransferase upregulated in HCC (49), so it is still possible that the transcription of *OCT4-pg4* would be influenced by the transacting function of *ASH1L*. Overall, the discovery of pseudogene transcripts that block miRNA activity has revealed a new layer of post-transcriptional regulation with many potential biological roles.

Supplementary material

Supplementary Figures S1–S3 and Tables S1–S6 can be found at http://carcin.oxfordjournals.org/

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