



OCT4 Affects Gastric Cancer Progression by Promoting Proliferation, Migration, and Inhibiting Differentiation

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Abstract

Background: The Octamer-binding transcription factor 4 (OCT4), a driving factor widely involves in cellular pluripotency of cancer stem-like cells, the roles in cell differentiation became an increasing interest nowadays.

Methods: The mRNA expression of OCT4 in Gastric Cancer (GC) tissues or GC cell lines were determined by Gene Expression Profiling Interactive Analysis (GEPIA) database or quantitative reverse transcription PCR (qRT-PCR). Survival analysis of OCT4 was also identified by Kaplan-Meier plotter. Further, the relative protein expression of OCT4 and BMP-SMAD pathway in GC cell lines was analyzed using Western blot. Functionally, cell proliferation, migration, and differentiation were detected by Cell Counting Kit-8 (CCK-8), 5-Ethynyl-2'-Deoxyuridine (EDU) assay, Wound healing assay and Western blot, respectively. In addition, tumor xenograft model was established to investigate this OCT4 functions *in vivo*.

Results: In this study, high-expressed OCT4 was found in GC tissues and GC cell lines and exhibited a poor prognosis. Functionally, knockdown of OCT4 inhibited cell proliferation and migration in HGC-27 cells, whereas overexpressed OCT4 facilitated cell proliferation and migration in MKN-28 cells. The mRNA expression of CD133 and E-cadherin were increased in HGC-27 and MKN-28 cells silencing OCT4. It was found that OCT4 silencing stimulated a decrease on the tumor weight and volume as compared with control group *in vivo*. HE staining and immunohistochemistry suggested that knockdown of OCT4 promoted tumor differentiation. On the mechanism, BMP-SMAD signaling-related genes expression changed abnormally in HGC-27 cells.

Conclusion: OCT4 could promote cell proliferation, migration and differentiation, which was involved in BMP-SMAD signaling in human GC.

Keywords: OCT4; Gastric cancer; Proliferation; Migration; Cell differentiation

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Introduction

Gastric Cancer (GC) is thought to be one of the common malignancies globally as more than one million new cases per year [1,2]. In the last few years, researchers have gradually reached a consensus in which a complicated interaction between host and environmental factors is the major responsible for GC carcinogenesis, especially an undesirable lifestyle [3]. Although the concern for several therapeutic strategies have been currently considered for patients with GC, including surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, the cancer recurrence and mortality of GC remain a main challenge for cancer management [4]. Moreover, clinical observations have divided GC into three major subtypes based on the Lauren Classification System: Well-differentiated, poorly differentiated and mixed disease [5]. It is found that well-differentiated GC occurs most often in elderly patients and the prognosis is better than other subtypes [6]. The poorly differentiated subtype is characterized by poor prognosis and is mainly present within younger woman [7]. The mixed subtype tends to behave in highly aggressive malignancies, such as highly invasive or metastatic [8]. Thus, it is imperative to better disclose the potential mechanism of GC progression in order to develop a new strategy for GC therapy in future.

The Octamer-binding transcription factor 4 (OCT4) is a typical stemness indicator to maintenance of pluripotency that mediates gene transcription during embryogenesis [9,10]. Recent evidence suggested OCT4 exhibited a high expression in multiple cells, which exerts important regulation in stem cell properties and tumor progression [11-14]. It has been identified that OCT4 facilitated cell proliferation, migration, invasion, and epithelial-mesenchymal transition in colon cancer via targeting SCF/c-Kit pathway [15]. Furthermore, a cox regression analysis showed that OCT4 has

a significant correlation with tumor differentiation, lymph node metastasis and Dukes stage, which acted as an independent prognosis factor in colon cancer [16]. However, the functions and mechanisms of OCT4 in GC are still controversial. Bone Morphogenetic Proteins (BMPs) is a member of the TGF- β superfamily and responsible for differentiation in multiple cells in embryonic development [17]. It is found that BMP signaling was involved in early stages of cellular reprogramming [18]. The implication of BMP signaling activation increased upregulation of pluripotency core genes (OCT4 and Nanog) that is strongly closed to reprogramming. In addition, within the BMP family, BMP-2, BMP-4 and BMP-6 were suggested to be most critical for tumor progression *via* distinct mechanisms [19-21]. For example, BMP-2 could repress cell proliferation by arresting the cell cycle in BGC-823 and SNU-216 cells, which attributed to BMP-mediated EZH2 and H3K27me3 reduction [20]. miR-765 contributed to cell proliferation, migration, and invasion by targeting BMP6 in non-small cell lung cancer [21].

Therefore, in this study, we aimed to explore the roles of OCT4 in human GC progression. The results showed that OCT4 exerts its function for GC *in vivo* and *in vitro*. In particular, high-expressed OCT4 in GC facilitated cell proliferation, migration, tumor growth, and reduced differentiation via the BMP signaling pathway. Based upon these results, this study will provide a new insight for GC therapy in clinic.

Methods

Online tools

The mRNA expression level of OCT4 in GC tissues was obtained from Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn/>). Meanwhile, the survival analysis of the OCT4-low and OCT4-high expression subgroups was identified according to Kaplan-Meier plotter.

Cell culture

The human gastric mucosal epithelial cell (GSE-1) and gastric cancer cell lines (BGC-823, AGS and MKN-28) were all purchased from the American Type Culture Collection (ATCC). These cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% of penicillin and streptomycin as well as incubated at 37°C with 5% CO₂.

Plasmid construction and transfection

To construct a plasmid overexpressing OCT4, the CDS sequence of human OCT4 gene was cloned into pcDNA3.1(+) vector (Clontech, USA). Similarly, the shRNA sequence of OCT4 was cloned into pRNAT-U6.1/Neo vector (Clontech, USA) to decrease OCT4 expression. The sequence of shRNA is as follows: 5' - A A A A G C T G G G G A G A G T A T A T A T T T T G G A T C C A A A A T A T A T A C T C T C C C C A G C - 3'. The synthesis and gene sequencing were all accomplished by GENEWIS (***) . Positive clones expressing the interest protein were successfully screened after G418 selection. Then, these stable transfected cells were expanded by culture *in vitro*.

qRT-PCR

To examine the relative mRNA expression level of genes, total RNA was extracted from GC cells by RNeasy Mini Kit (Qiagen, Germany). The cDNA then was amplified using reverse transcription kit (Takara, Japan). Briefly, the synthesis of cDNA was performed at 37°C for 30 min, then 85°C for 5 sec and 4°C forever. Next, the

mRNA relative expression in this study were tested by qRT-PCR using Qiagen QuantiTect SYBR Green PCR Kit (Qiagen, Germany). The primers were designed and synthesized by GENEWIS (***) , as follows:

OCT4

Forward: 5'- AGCAAAACCCGGAGGAGT -3'

Reverse: 5'- CCACATCGGCCTGTGTATATC -3'

CD133

Forward: 5'- AGTCGGAAACTGGCAGATAGC -3'

Reverse: 5'- GGTAGTGTGTACTGGGCCAAT -3'

E-cadherin

Forward: 5'- CGAGAGCTACACGTTACCG -3'

Reverse: 5'- GGGTGTGAGGGAAAAATAGG -3'

GAPDH

Forward: 5'- AGTGCCAGCCTCGTCTCATA -3'

Reverse: 5'- GACTGTGCCGTTGAACTTGC -3'

The mRNA expression of GAPDH was applied as internal control. The qRT-PCR was performed for 40 cycles. Notably, three independent experiments were performed to obtain the valuable data.

Western blot

To collect the protein samples, cells were firstly lysed on ice for 30 min using RIPA lysis buffer (Beyotime, China) supplemented with cocktail inhibitor (Roche, USA). Following, proteins were separated through electrophoresis on 12% SDS-PAGE gels and transferred onto activated PVDF membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk for 30 min. Also, the primary antibodies were incubated overnight, including OCT4, GAPDH (Cell Signaling Technology, China). Next, membranes were incubated with the secondary antibodies (Cell Signaling Technology) at 37°C for 1 h. After washing with TBST at least three times, protein bands were visualized using ECL kit (Proteintech, USA), and determined using ImageJ software.

CCK-8 assay

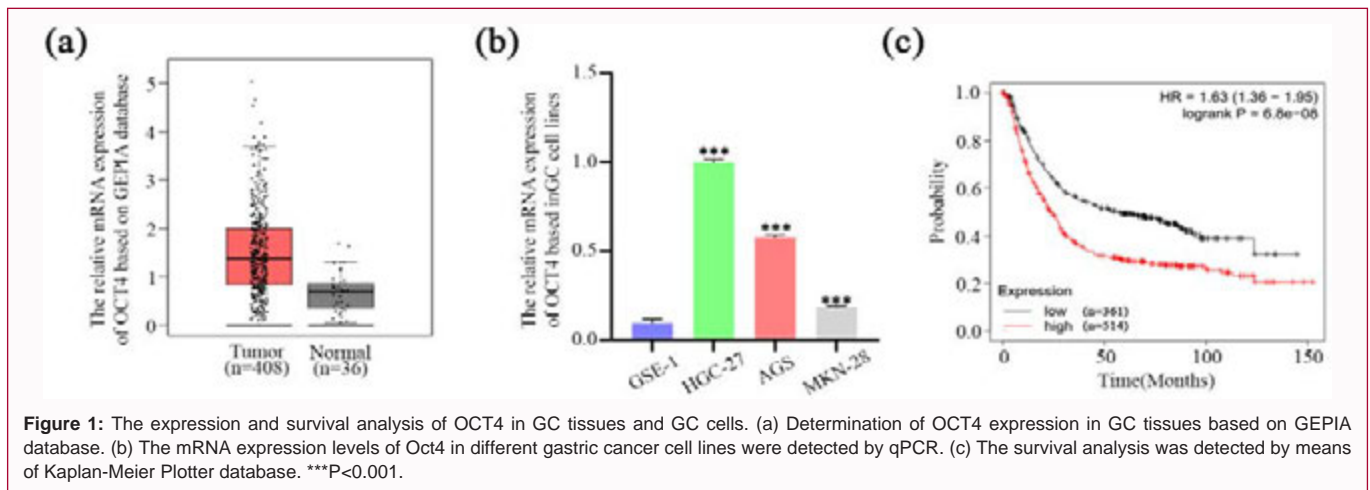
To examine the ability of proliferation, 2×10^3 cells were seeded into 96-well plates. These cells were incubated with CCK-8 for 2 h at different time point, including 0 h, 24 h, 48 h, 72 h and 96 h. The cell growth curve was described by absorbance at 450 nm.

EdU assay

To measure the percentage of proliferative cells, the EdU Kit (RiboBio, Guangzhou, China) was used according to the manufacturer's protocol. 3×10^5 cells were seeded into 24-well plates. These cells were incubated in medium with EdU for 2 h before fixation. Then, the nuclei were stained with Hoechst 33342 for 30 min. The number of proliferative cells was calculated by EdU- and Hoechst-stained cells.

Wound healing assay

To examine the ability of migration, cell wound healing assay was completed. 2×10^5 cells were seeded into 6-well plates at 37°C. Then, a horizontal line was drawn by yellow tips. After washing in PBS, cells were incubated for 24 h at 37°C. The Gap width was recorded at 0 h and 24 h.



Tumor xenograft model

To identify the role of OCT4 *in vivo*, the xenograft model was established. Ethical permission was obtained from Ethics Committee of *** (SYXK20180101), and all methods were performed in accordance with ARRIVE guidelines and regulations. BALB/c nude mice (6-week-old, female, n=6/group) were bought from ***. All mice were randomly divided into vector group and sh-OCT4 group. After that, 4×10^6 stably transfected cells were subcutaneously injected in BALB/c nude mice to develop xenograft tumors. All mice were sacrificed by cervical dislocation under pentobarbital sodium anesthesia at the completion of 30 days. The tumor size and volume were recorded every 2 days. Tumor Volume (V) was calculated by measuring the Length (L) and Width (W) according to the formula $(L \times W^2) \times 0.5$.

H&E staining

For histological examination, H&E staining was utilized in this study. The tumor tissues were fixed with 4% paraformaldehyde for 48 h at least. A series of gradient ethanol were applied for dehydration. The tissues were subsequently subjected to immersion in xylene for 30 min and paraffin embedding. Tumor tissues were continuously sliced (5 μ m). After deparaffinization, HE staining was performed and histopathology was also observed under light microscope (Leica, Wetzlar, Germany).

Immunohistochemistry

To perform the antigen retrieval, the slides with tumor tissue (5 μ m thickness) were incubated in citrate buffer (pH=6) for 20 min. Subsequently, the tissues were incubated in 3% H₂O₂ for 5 min at room temperature. The tissues were rinsed by PBS and cultivated with E-cadherin antibody at 4°C overnight. Next, the tissues were cultured for 30 min at room temperature with anti-mouse IHC antibody (PAB160022, 1:200; Bioswamp). After hematoxylin staining, dehydration and mount, the results were analyzed using a bright-field microscope (Leica, Wetzlar, Germany).

Statistical analysis

Statistical analysis was performed using *t* test in GraphPad software (version 8.0). The data were expressed as mean \pm SEM. Briefly, the comparison between two groups is considered to be significantly different when P<0.05. All experiments were performed at least three times.

Results

OCT4 is highly expressed in GC tissues and GC cell lines and is associated to poor prognosis

In this study, the abundance of OCT4 was retrieved based on Gene Expression Profiling Interactive Analysis (GEPIA). Results showed that OCT4 expression was upregulated in GC tissues compared to normal tissues (Figure 1a). Similarly, the abundance of OCT4 was also elevated in GC cells (BGC-823, AGS, and MKN-28) in contrast to human gastric mucosal epithelial cells (GSE-1), especially in BGC-823 cells (Figure 1b). Likewise, it is found that high-expressed OCT4 was a vital factor for poor prognosis in GC (Figure 1c). These data suggested that OCT4 might play functions as oncogene in GC, which is closely associated to poor prognosis.

OCT4 overexpression might promote cell proliferation and migration in human GC cells

To find the effect of OCT4 on GC, OCT4 expression was decreased by siRNA technology in two of GC cell lines and further applied to the following experiment. Next, the qRT-PCR analysis and western blot were utilized to detect the expression level of OCT4 after cell transfection in order to detect the plasmid function. As displayed in Figure 2a, the expression level of OCT4 was obviously decreased in BGC-823 cells and the expression level of OCT4 was obviously increased in MKN-28 cells. Functionally, CCK-8 assay and EDU assay showed that si-OCT4-treated BGC-823 cells exhibited the proliferation of low capacity in a time-dependent manner, whereas OCT4-treated MKN-28 cells showed an increased cell viability (Figure 2b, 2c). Besides, cell wound healing assay further disclosed that OCT4 knockdown resulted in a decrease of the gap width in BGC-823 cells. Nevertheless, the gap width of MKN-28 cells with high-expressed OCT4 was significantly increased with respect to control group (Figure 2d). It implied that OCT4 could enhance the migration rate of GC cells. Taken together, these findings indicated that OCT4 expression was involved in the progression of GC cells.

OCT4 expression could affect cell differentiation in human GC cells

Previous study showed the high-expressed OCT4 could predict worse prognosis in the right-sided colon cancer which hinted at the presence of relationship between OCT4 expression and tumor differentiation [16]. However, it's still unclear that whether OCT4 is involved in cell differentiation in human GC. To explore the impact of OCT4 expression on tumor differentiation, qRT-PCR were used

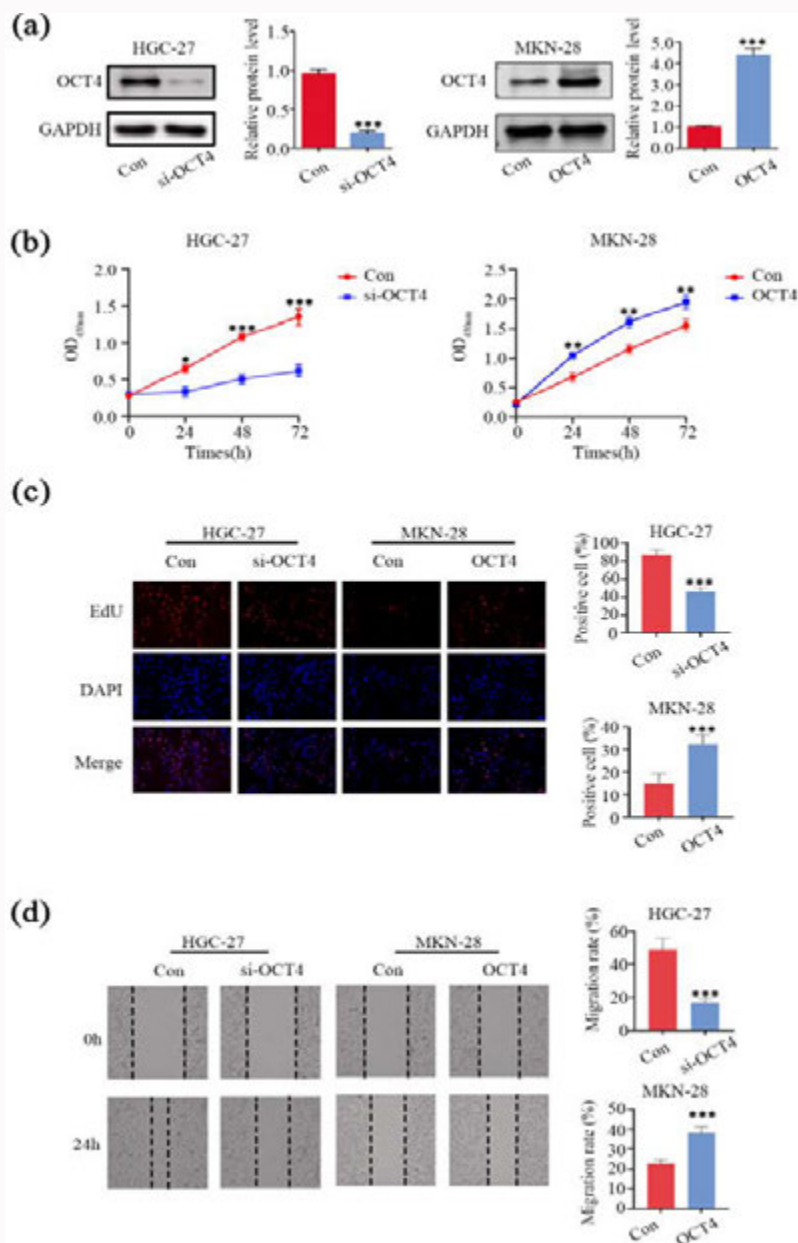


Figure 2: OCT4 affects proliferation and migration, and differentiation of GC cells *in vitro*. (a) The protein expression of OCT4 was performed by Western blot 48 h after transfection. (b) CCK-8 assay. (c) EDU assay. (d) Cell scratch assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

to identify the roles of OCT4 in cell differentiation through detection the expression level of the known differentiation-associated markers, such as CD133 and E-cadherin. These results showed that OCT4 knockdown increased CD133 and E-cadherin expression, whereas overexpression OCT4 reduced CD133 and E-cadherin expression in BGC-823 and MKN-28 cells (Figure 3a, 3b). Therefore, OCT4 overexpression inhibited tumor differentiation in human GC cells, whereas OCT4 knockdown resulted in well differentiation. All in all, these findings indicated that OCT4 expression negatively affected cell differentiation in GC cells.

Knockdown of OCT4 could inhibit the progression of GC *in vivo*

To investigate the roles of OCT4 on tumor growth *in vivo*, we established a xenograft model using BGC-823 cells. It is observed that OCT4 knockdown suppressed tumor volume and weight in

xenograft model (Figures 4a-4c). Furthermore, H&E staining showed the degree of differentiation in sh-OCT4 group was higher than that in vector group (Figure 4d). Likewise, OCT4 knockdown increased E-cadherin expression in sh-OCT4 group (Figure 4e). Combined, these results indicated that OCT4 knockdown inhibited tumor growth and promoted differentiation *in vivo*.

OCT4 mediates in BMP sig-SMAD signaling

To clarify the possible mechanism of OCT4-mediated differentiation in GC, BMP-related signaling was fully considerable since it has been identified BMP signaling was able to control OCT4 expression in previous study [18]. Through qRT-PCR, we identified the mRNA and protein expression of BMP2, BMP4, BMP7, SMAD1, SMAD5 and SMAD9 were different in poorly differentiated GC cells BGC-823 and well-differentiated GC cells MKN-28 cells (Figure 5a, 5b). it implied that BMP-SMAD signaling might be closed to cell

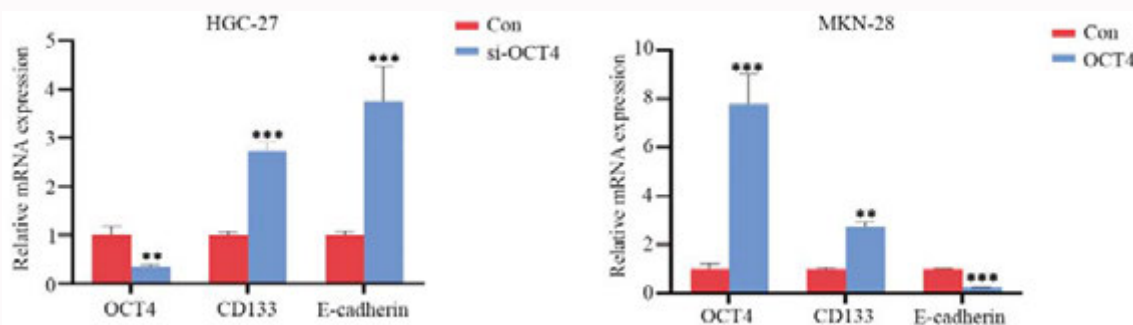


Figure 3: The qPCR was used to analyze mRNA expression levels of cell differentiation-related markers CD133 and E-cadherin in BGC-823 and MKN28 after transfection. **P<0.01, ***P<0.001.

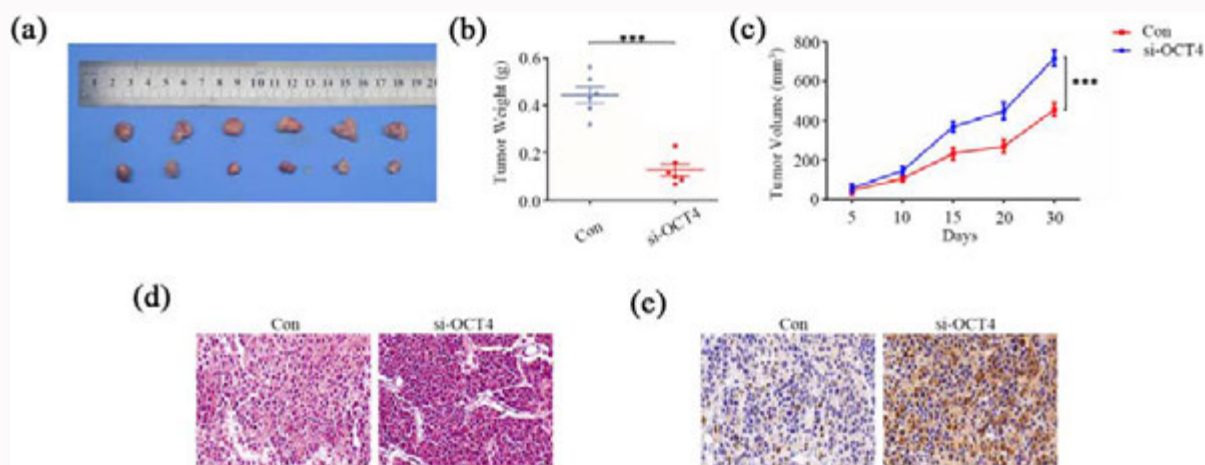


Figure 4: Knockdown of Oct4 inhibited the GC progression *in vivo*. (a) Schematic representation of GC xenograft tumors. (b) tumor weight. (c) tumor volume. (d) H&E staining. (e) CD133 expression. ***P<0.001.

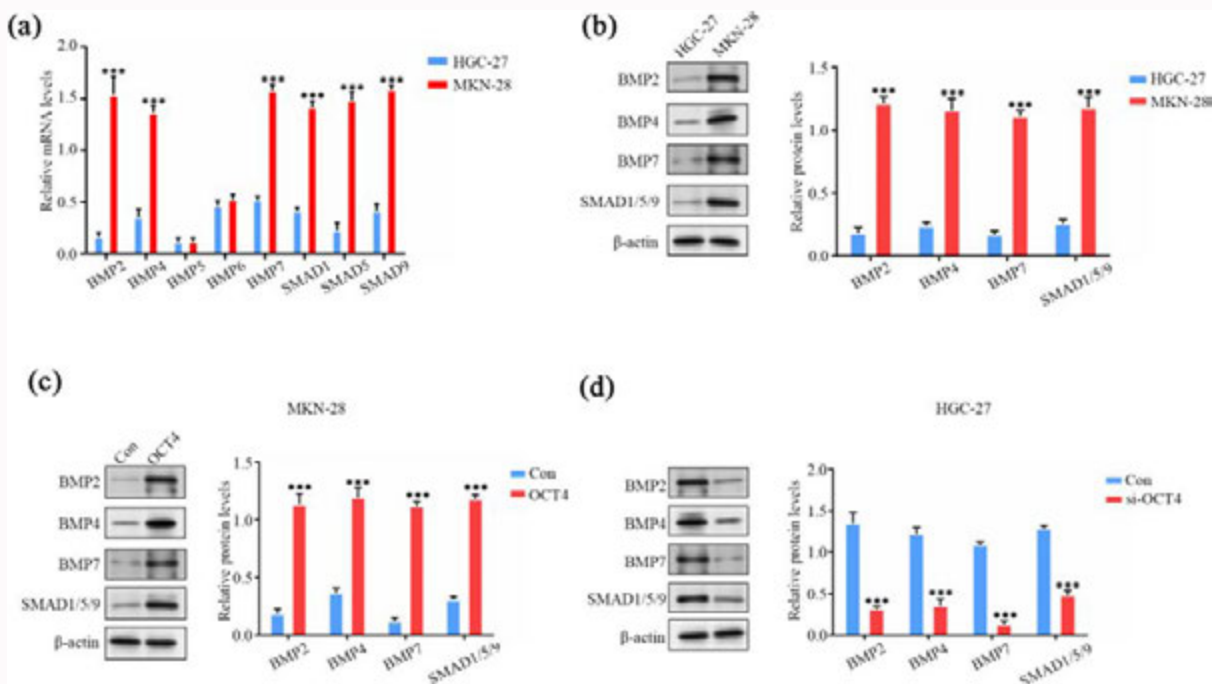


Figure 5: Oct4 affects gastric cancer cell differentiation by mediating BMP-SMAD pathway. (a) The qPCR was used to determine the BMP-SMAD signaling-related gene expression in HCG-27 and MKN-28 cells. (b) Western blot was used to determine the BMP-SMAD signaling-related gene protein expression in HCG-27 cells with OCT4 overexpression. (c) Western blot was used to determine the BMP-SMAD signaling-related gene protein expression in BGC-823 and MKN-28 cells when OCT4 expression changed. ***P<0.001.

differentiation in GC. Next, it was found that OCT4 knockdown in MKN-28 cells decreased the protein expression of BMP2, BMP4, BMP7, SMAD1, SMAD5 and SMAD9, whereas OCT4 overexpression in BGC-823 cells increased the protein expression of BMP2, BMP4, BMP7, SMAD1, SMAD5 and SMAD9 (Figure 5c). In conclusion, OCT4 expression could mediate the mRNA and protein expression of BMP-SMAD signaling-related genes in BGC-823 cells, which is related cell differentiation in GC.

Discussion

Recently, the presence of Cancer Stem Cells (CSCs) are believed as a major reason for progressive tumor phenotypes, such as tumor initiation, self-renewal, metastasis, and treatment failure [22]. Endowed with self-renewal and unlimited proliferation, increasing evidence suggests that CSCs may be responsible for tumor progression, especially Gastric Cancer (GC) [23]. There are many transcription factors have been confirmed to participate in mediation of CSCs formation, including OCT4, KLF4, c-Myc, Nanog, and etc. [23-25]. It is well-known that the coding gene OCT4 (also known as POU5F1), located in the human chromosome at 6p21.3, is the family of POU transcription factors. With the continuous exploration of OCT4, the complex biological function in CSCs has become more and more obvious at present. The forced expression of OCT4 has been reported in many CSCs [26-28]. In the case of colon cancers, the application of Oct4-Sox2 complex decoy contributes to increase the radiosensitivity of CSCs in a differentiation therapy way [29]. In the decades, numerous efforts have been made to uncover the potential molecular mechanisms regarding OCT4-mediated tumor progression with the intent to find a novel therapeutic strategy for GC treatment.

In this paper, we findings highlighted in GC, OCT4 expression was markedly elevated in GC tissue compared to normal tissue using GEPIA database. Moreover, we also demonstrated the high-expressed OCT4 in GC cell lines, but less in human gastric mucosal epithelial cell, suggested that it may play functions act as an oncogene in GC progression. Besides, the survival analysis of OCT4 expression in GC further demonstrated that the high-expressed OCT4 may be closely associated to poor prognosis in human GC. Biological functional studies revealed that OCT4 knockdown caused an inhibition effect of proliferation and migration in BGC-823 cells, whereas elevated OCT4 promoted cell proliferation and migration in MKN-28 cells. These findings provided a clue that OCT4 expression in human GC could respond to a variety of biological behaves in tumor progression. In a recent original article in the journal *Functional & integrative and genomics* published in August 2022, researchers pointed out that the elevated expression of OCT4 was enriched in diffuse, poorly differentiated, and stage-III gastric tumors with poor prognosis, suggesting OCT4 exist in correlation with tumor differentiation in GC. Therefore, we next investigated the effect of OCT4 expression in special GC differentiation -markers patterns, including CD133 and E-cadherin. Through qRT-PCR assay, it is found that OCT4-mediated transcription confers poor differentiation for GC, manifesting as CD133 and E-cadherin expression was repressed in transfected si-OCT4 cells with high expression of OCT4 as well as CD133 and E-cadherin was upregulated in OCT4-activated GC cells. In lines with the results from *in vitro* experiment, we found that knockdown of OCT4 reduced tumor weight and volume compared to vector groups. Also, histological classification showed in si-OCT4 group, GC tissues represented a well differentiated phenotype. In

addition, immunohistochemical examination revealed the protein expression of differentiated marker E-cadherin in human GC also was upregulated, suggested OCT4 expression is strongly link to tumor differentiation for *in vivo*. Notably, we found OCT4 expression may be closely to BMP-SAMD signaling at protein levels that elicit its biological functions. Nevertheless, further studies remain needed to determine how OCT4 activates the BMP-SAMD pathway by molecular cascade reaction and how such activation affects cell differentiation in human GC.

Conclusion

In conclusion, we results showed that OCT4 level was elevated in GC tissues and GC cell lines, it was strongly related to progression of human GC. *In vitro*, OCT4 contributed to cell proliferation, and migration. Also, OCT4 silencing could reduce tumor growth *in vivo*. Most importantly, we firstly found a crosstalk between OCT4 and GC differentiation that OCT4 probably repressed cell differentiation *via* BMP-SAMD signaling pathway. These findings will provide a new approach for target therapy of human GC.

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