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Original Article

YAP down-regulated its target CTGF to maintain stem cell pluripotency in human ovarian cancer stem-like cells

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Abstract: Recent studies have showed Yes-associated protein (YAP) plays a critical role in stem cell pluripotency maintenance. The former studies showed that it involved in ovarian cancer stem cell pluripotency maintenance but how does the downstream genes regulate remain to be explored yet. Here we first demonstrated the role of CTGF in the ovarian cancer stem-like cell (OCSC) self-renewal. We found that YAP down regulated the target gene CTGF to maintain OCSC un-differentiation and only CTGF gene was involved in stem cell pluripotency maintenance and direct regulated by YAP. Taken together, our data indicate that YAP regulated ovarian cancer initiated cell pluripotency by down regulated CTGF expression. This finding will proposed a new idea on hippo pathway regulation mechanism in cancer stem cell and it might serve as therapeutic targets for the treatment of ovarian cancer in clinical.

Keywords: YAP, CTGF, OCSC, stem cell

Introduction

Ovarian cancer represents the most lethal gynecologic malignancy and most patients diagnosed at an advanced stage of disease and the minimal therapeutics treatment efficacy owing to its drug resistance [1, 2]. There are some views that chemotherapeutics resistance of ovary cancer is mainly due to the existing of small populations of cancer stem cell (CSCs) or named cancer stem like initiated cells (OCSC). Some research reported that CSCs organize anchorage-independence, autonomous and spherical structures. CSCs have been detected higher expression levels of the stem cell markers such as OCT-4, Nanog [3]. In this study, the above markers expressed abundantly in the spheroid cells we identified.

Hippo pathway, a highly conserved pathway regulates cell proliferation and differentiation. As its central regulator, YAP gene was also found to play some important roles in the stem cell self-renew and regeneration. Some reports have showed that modulation of Tead4 or Yap activity leads to Cdx2 expression changes in embryonic stem cells and it will also lead to cell fate changes during the trophectoderm formation [4]. YAP and TEAD2 were highly expressed in self-renewing ES cells and they were down-regulated when cells are induced to differentiate [5].

As YAP downstream targets, CTGF, RUNX-2, ErbB4 and ITGB2 *et al.* have participated in some physiological process and homeostasis regulation in Hippo pathway. The report showed that CTGF is sufficient to induce human bone marrow mesenchymal stem/stromal cells (MSCs) to differentiate into fibroblasts [6].

Although the above researches have studied the roles of YAP and its downstream targets in stem cell, there is rare to know how YAP regulates the targets and their definite regulation mechanism in ovarian cancer stem cell. Here we first demonstrated that YAP gene maintains OCSCs stemness condition through down-regulated its target CTGF gene and CTGF involved in the process of YAP regulating and maintaining

OCSCs property and the whole process completed through CTGF down-regulation. On the other hand, as its downstream targets RUNX-2, ITGB2 and ErbB4 all have little regulated by YAP gene.

Materials and methods

Cell culture

Primary ovarian cancer cells which isolated from ascites of patients were grown in serum-free DMEM-F12 (Invitrogen) supplemented with 5 μg/ml insulin (Sigma), 20 ng/ml human recombinant epidermal growth factor (EGF; Invitrogen), 10 ng/ml basic fibroblast growth factor (b-FGF; Invitrogen), and 0.4% bovine serum albumin (BSA; Sigma), followed by culturing in Ultra Low Attachment plates (Corning). Control cells were cultured in the DMEM-F12 supplemented with 10% fetal bovine serum (FBS). All the cells were added with penicillin (10 units/ml) and streptomycin (10 ng/ml) and grown in a humidified incubator at 37°C with 5% CO_2. The medium was changed every 3-4 days.

Immunofluorescence staining

OCSC spheroids were harvested and fixed in paraformaldehyde (4°C, 30 min), permeabilized with PBS that contained 0.3% Triton X-100 (PBST), and incubated with blocking buffer (PBST containing 5% bovine serum albumin). Spheroids were sequentially probed with the primary antibodies and Alexa Fluor 594-or 488-conjugated secondary antibodies (Molecular Probes). Slides were mounted using VectaShield with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Digital images were acquired using an epifluorescence microscope (Nikon Eclipse 80i) with 4-100X objectives.

Quantitative real-time PCR (qPCR)

Spheroid cells, differentiated spheroid cells, or bulk tumor cells were collected in RNAase microtubes. Total RNA extraction and reverse transcription were performed with RNAiso plus and reverse transcription mix kit according to the manufacturer's instructions (Takala). Reverse-transcribed into cDNA using, amplified for 30 cycles in 25 μl reactions with 10 pmol primers. The primers were used as follows: YAPF: TAGCCCTGCGTAGCCAGTTACC, R: GCTGCTCATGCTTAGTCCACTGTC, RUNX-2F: GACACTGCCACCTCTGACTTCTG, R: GGGATGAAATGCTTGGGAACTG, ITGB2F: ATGCTTGATGACCTCAGGAATG, R: AGCACGGTCTTGTCCACGAA, ErBb-4F: GGGACTACAACCATAGCACTCCAAG, R: TCAAAGTTAGCTGCCCTCACACA, CTGFF: CTTGCGAAGCTGACCTGGAA, R: AAAGCTCAAACTTGATAGGCTTGGA. The final PCR products were diluted 10 folds and mixed with forward and reverse primers with manufacturer's instructions. Real-time PCR reaction was performed using CFX96™ Real-Time PCR Detection System (Bio-Rad). Each cDNA sample was tested in triplicate. For quantification of gene expression changes, the ΔΔCt method was used to calculate relative fold changes normalized against b-actin expression.

Protein lysate and western blot analysis

Cells were lysis with cell lysis buffer (Beyotime Co.) added with appropriated volume cocktails (Roche). All the proteins from lysed cells were fractionated by SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Milipore, USA). Nonspecific binding sites were blocked with 5% milk (Cell signal, USA) in TBST for 1 h at room temperature. Blots were incubated with a specific antibody overnight at 4°C. Antibodies were used as follows: anti-YAP (1:1000, Cell Signal), anti-actin (1:1000, Cell Signal), CTGF (1:500, Santa Cruz). Proteins were visualized using a Dura SuperSignal Substrate (Milipore, USA).

Lentiviral shRNA infection assay

Lentivirus that encoded for *Yap* short hairpin RNAs (shRNAs) or non-target oligonucleotides as a control were from GenePharma (Shanghai, China). Cells in spheres were infected with *Yap* and/or *Tead4* shRNA lentivirus for 48 h. *Yap*-specific shRNAs were: 5'-CCCAGTTAAATGTTCACCAAT-3' (shYAP#1) and 5'-GCCACCAAGCTAGATAAAGAA-3' (shYAP#2). shTEAD4#1 (5'CCGGCCGCCAAATCTATGACAAGTTCTCGAGAACTTGTCATAGATTTGGCGGTTTTTG-3), shTEAD4#2 (5'CCGGGCTGAAACACTTACCCGAGAACTCGAGTTCTCGGGTAAGTGTTTCAGCTTTTTG-3'). Interference efficiency was verified by RT-PCR and Western blotting (data not shown).

Statistical analysis

All the assays were performed in triplicate. Groups were compared by two tailed unpaired t test using GraphPad Prism statistical programs (GraphPad Prism, San Diego, CA). Differences were accepted as significant when $P<0.05$.

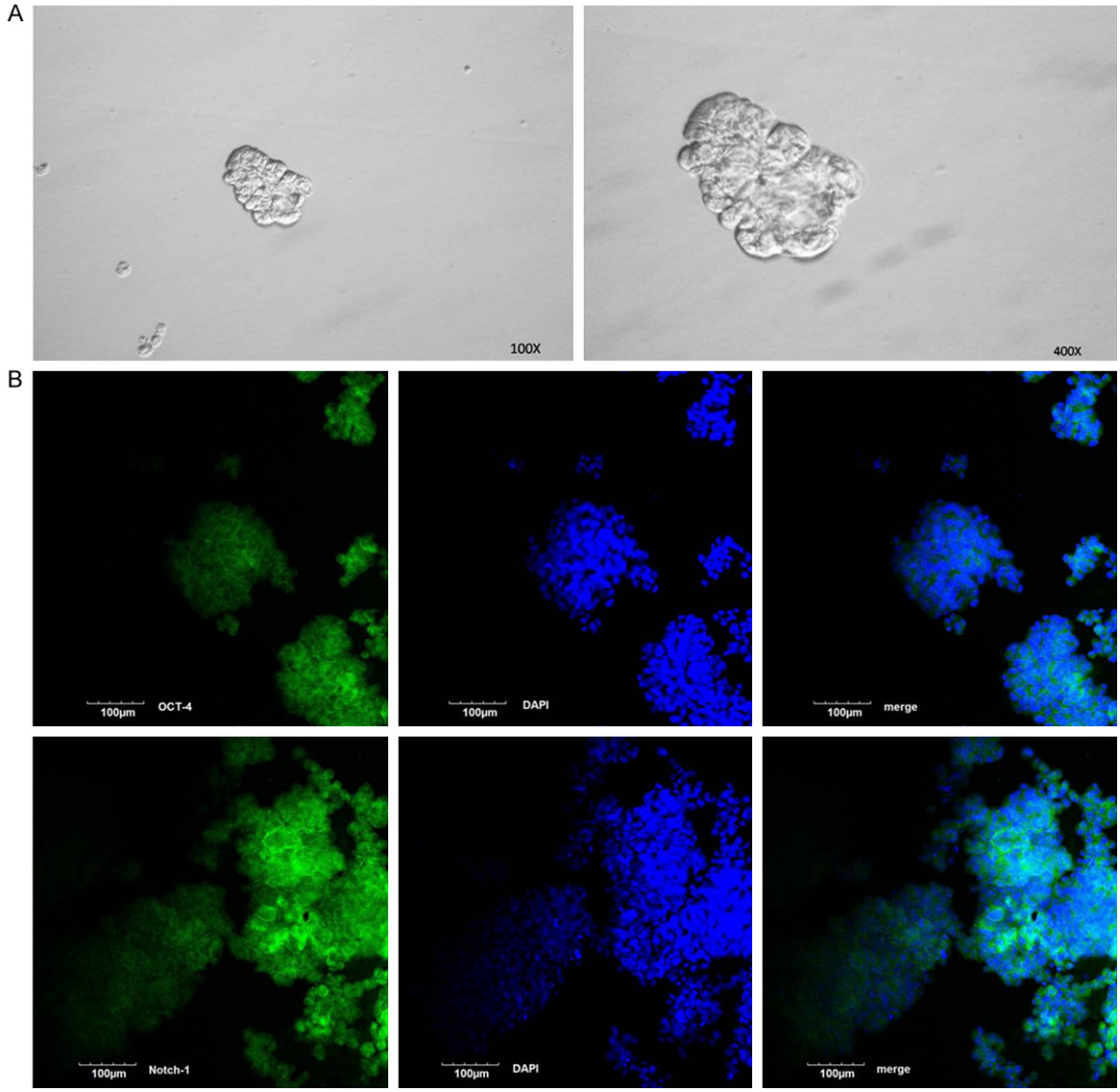

Figure 1. Ovarian cancer stem cell (OCSC) spheroids isolation and culture. A: Images of non-adherent spherical clusters derived from cultured primary ovarian cancer cells. Scale bar = 100 μM for the upper lane, and 250 μM for the bottom lane. B: Immunofluorescent staining results showing the stem cell markers expression in OCICs. Nuclei were stained with DAPI. Scale bar = 100 μM for each panels.

Results

Ovarian cancer stem cells isolation and culture

Primary ovarian cancer cells which isolated from ascites of patients were grown in serum-free DMEM-F12 (Invitrogen). Single cell suspension was moved to another flask and cultured with serum-free DMEM-F12. The details were seen in the materials and methods section. About four weeks, the spheres were observed at the bottom of these flasks and about more than two hundred cells gathered into a sphere (**Figure 1A**). We also detected the stem markers expression in these cells. Through the immunofluorescence staining, stem markers such as OCT-4 and Nanog were all expressed highly in OCSCs (**Figure 1B**).

YAP and its target genes expression in ovarian cancer stem cells

To investigate YAP expression in OCSCs we then detected its expression in OCSCs by qPCR and western-blot and found that YAP was all expressed highly (**Figure 2A**, **2B**). CTGF gene expression has also been detected by qPCR and the results showed that its expression was decreased both in qPCR and western-blotting (**Figure 2A**, **2B**). The other downstream targets

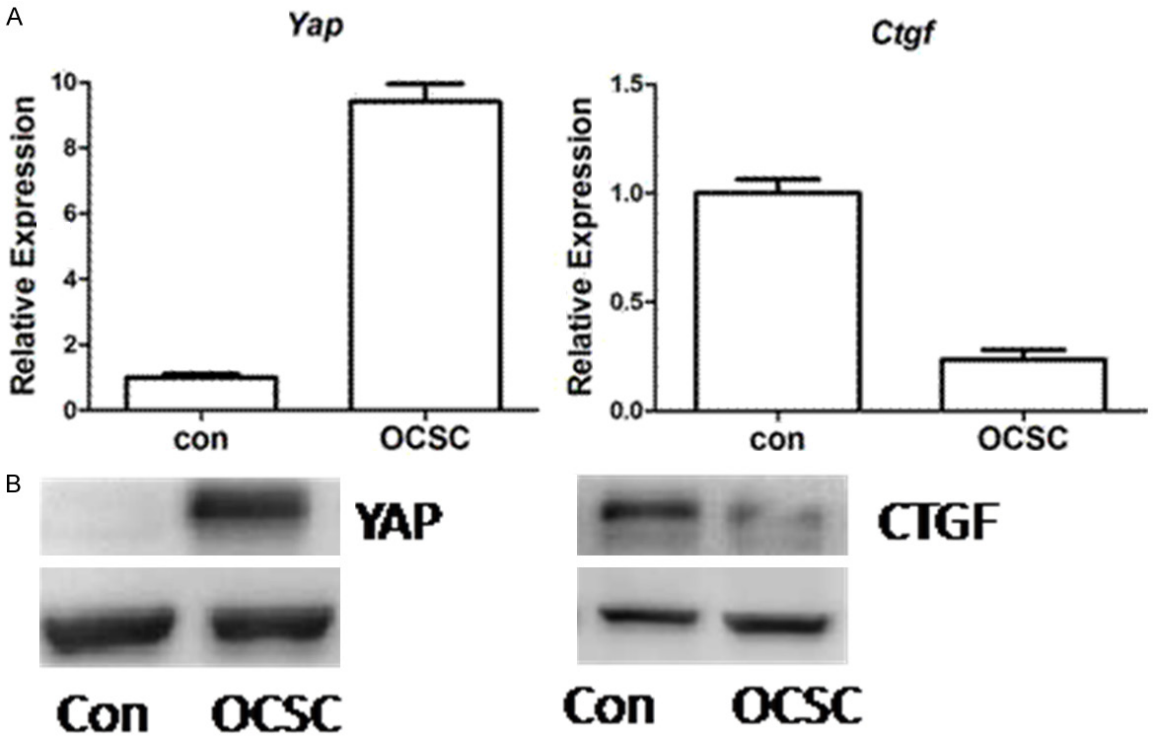

Figure 2. YAP and CTGF expression in OCSCs. A: Real-time RT-PCR results showing YAP and CTGF gene expression in OCSC. B: Western blotting results of YAP and CTGF gene expression in OCSC.

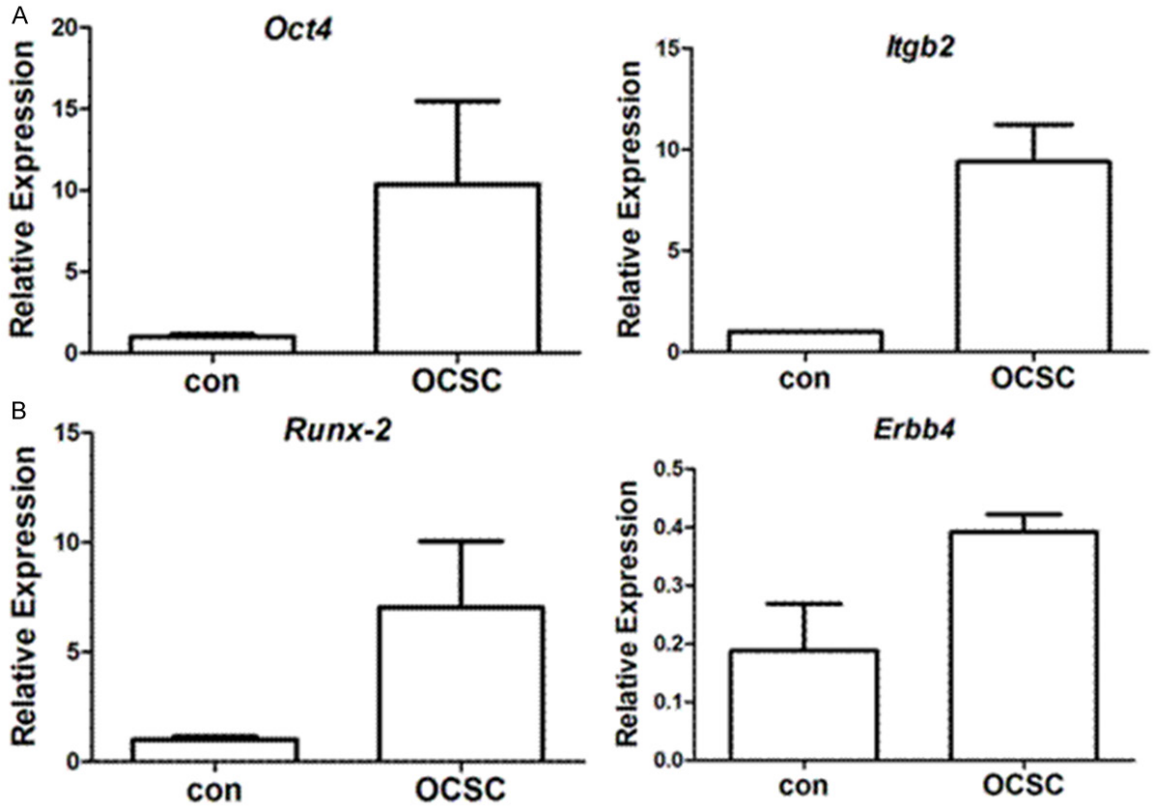

Figure 3. Other YAP downstream targets expressions in OCSCs. Upper (A): Real-time RT-PCR results showing OCT-4 and ITGB2 gene expression in OCSC. Bottom (B): Real-time RT-PCR results showing RUNX-2 and Erbb4 gene expression in OCSC.

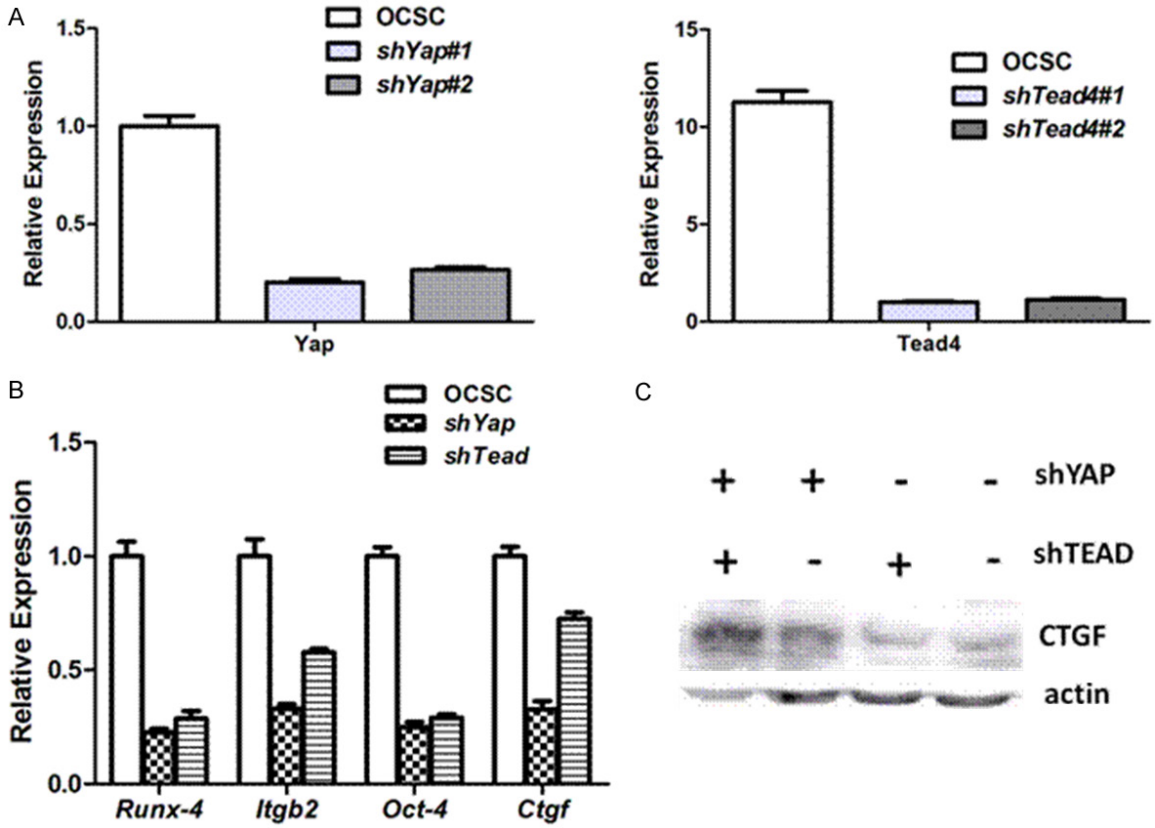

Figure 4. YAP and TEAD have regulated CTGF expression in stem cell pluripotency maintenance. A: RT-PCR results showing YAP and TEAD4 expression in shYap targets expression and shTead4 genes silenced in OCSC. B: RT-PCR results showing YAP targets expressions in YAP and TEAD4 genes silenced OCSC. C: CTGF expression in shYap and shTead4 OCSC cells.

have been detected and showed different expression (**Figure 3**).

CTGF has regulated directly by YAP involved in stem cell pluripotency maintenance

To investigate if CTGF was involved in maintaining OCSC pluripotency, we detected CTGF expression in OCSC cells and the results showed CTGF decreased in OCSC cells. Then we knocked down Yap and Tead4 expression in OCSCs by using shRNAs. PCR results confirmed the knock-down efficiencies of the indicated shRNAs (**Figure 4A**). All the results have presented that CTGF involved in the process of YAP maintaining OCSCs properties and the whole process completed through CTGF down-regulation.

YAP down-regulated CTGF gene expression through TEAD gene to maintain OCSC property

Previous studies have demonstrated some downstream targets include CTGF, RUNX-2, ErbB4 and ITGB2 et al. have involved in some physiological process and homeostasis regulation in Hippo pathway [7, 8]. We want to know if they all regulated by TEAD in the process of YAP maintaining cancer stem cell. In our study, those YAP targets expressions were higher in OCSCs and their expression decreased in YAP/TEAD knockdown OCSCs. It indicated they had direct or indirect regulated by YAP gene in OCSCs (**Figure 4B**). Although CTGF expression was decreased in the OCSCs but RT-PCR results changed little. Then we detected with Western-blotting and the results showed that CTGF expression was higher in shYap/Tead genes in the OCSCs (**Figure 4C**). This indicated that their regulation maybe in the protein level but not RNA level.

Discussion

Cancer stem cell has been a concern recently because some research showed a much smaller number of cancer stem cell populations

indeed exist in some cancer tissues which initiated cancer cell and related with stem cell property maintenance, tumorigenesis, malignant metastasis and recurrence [9]. Recent studies have showed that cancer stem cell that are capable of self-renewal and directional differentiation included ovarian tissue is the main reason that lead to tumor occurrence and progression [10-12]. Hippo pathway has attracted intense research interest recently because it involved some important causes such as cell proliferation, differentiation and tumor formation. As a key coactivatior in Hippo pathway, YAP has also found expressed highly in stem cell and required for self-renewal and suppression of differentiation [13, 14] and our former study has confirmed it well [15].

CSCs have the distinct cell origin and may arise from the differentiated cells that have acquired self-renewing capacity but not the transformation of normal stem cell. This point of view has also been demonstrated in our study. In this study, we cultured ovarian cancer stem-like cells by tumor-bearing mice successfully and identified them through the above markers detection. After about one month culture, OCSCs have been investigated in clusters which gathered with hundreds of thousands of cells and the largest diameter up to 500 um. OCSCs not only have strong clone-forming ability, the stem cell markers are all expression well. Cancer stem cell tumorigenicity has often been seen as one of the characteristics of cancer stem cell. Our former study showed OCSC tumorigenicity has enhanced dramatically (data not shown). The results showed that YAP immunostaining was stronger than that in controls and we also investigated that stem markers expression were stained deeply than the controls.

ErBb4, RUNX-2, CTGF and ITGB2 are all the downstream targets in Hippo pathway [7, 16]. RUNX-2 as a Runx family member plays key roles in regulating mesenchymal stem cell differentiation during bone formation [17]. YAP/TAZ regulates Runx activity in mesenchymal stem cell differentiation [18, 19]. Overexpression of CTGF occurs in many cancer types such as esophageal squamous cell carcinomas, pancreatic cancer, prostate cancer. CTGF also induced hepatic progenitor cells to differentiate into hepatocytes [20]. CTGF has been reported to induce human bone marrow mesenchymal stem cell differentiation into fibroblasts [6]. In our study, we got the similar results in our study that CTGF expression level decreased in OCSCs and increased in the differentiated OCSCs respectively. The previous studies have put forward the view that CTGF promote the proliferation in some biological processes. In our study, we supposed a new idea that CTGF not only acts in cell proliferation but also in regulating differentiation of stem cells. Our study results may suggest that its down-regulation may maintain stem cells under un-differentiation condition. Integrin family mainly medicates stem cell and extracellular matrix adhesion. In our study, RUNX-2, ITGB2 are all expressed high even after YAP and TEAD shRNA their expression changed little. We speculate that these genes as targets in cell proliferation, migration, tumorigenesis didn't involved directly in cancer stem cell maintenance in Hippo pathway. We want to know that if they are regulated by the upstream gene regulation directly or indirectly. It is not clear that if Hippo pathway interacts with TGF-β, Wnt or other signal pathway in stem cell regulation. The specific regulatory mechanism need a continue study.

We found CTGF expression decreased in the OCSCs growth process but increased reversely when knockdown YAP and TEAD and its expression elevated significantly after we both knockdown YAP and TEAD genes by qPCR and western-blot detection. The other two targets RUNX-2 and ITGB2 expression are both high in OCSCs by qPCR assay. However, their expressions decreased very little after YAP/TEAD knockdown that indicated they had little influences on ErbB4 gene in OCSC.

Disclosure of conflict of interest

None.

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