Lactate Promotes Cancer Stem-like Property of Oral Sequamous Cell Carcinoma*

Hui ZHAO†, Chuan-yu HU†, Wei-min CHEN, Ping HUANG#
Department of Stomatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Summary: Accumulation of lactate in tumor has been linked to poor prognosis of oral squamous cell carcinoma (OSCC), but the underlying mechanism remained largely uncertain. Previous studies have suggested that presence of cancer stem cells (CSCs) closely correlated with cellular malignancy of OSCC. Here, using 3D organoid culture model, we investigated whether lactate promoted CSCs phenotype in primary OSCC cells. We generated organoids using fresh OSCC specimens and verified that organoids recapitulated histopathology and cellular heterogeneity of parental tumor. Organoids were then transfected with a Wnt reporter to visualize Wnt activity. The sphere forming assay demonstrated that high Wnt activity functionally designated CSCs population in OSCC cells. Further investigations indicated that lactate treatment promoted Wnt activity and increased the expression of CSCs (i.e. $CD133^+$ cells) in organoids. Moreover, silencing monocarboxylate transporter 1 (MCT1), the prominent path for lactate uptake in human tumor with siRNA significantly impaired organoid forming capacity of OSCC cells. Together, our study demonstrated that lactate can promote CSCs phenotype of OSCC, and MCT1 may be a therapeutic target against OSCC growth.
Key words: oral squamous cell carcinoma; lactate; cancer stem cells; organoid; monocarboxylate transporter 1

Tumor cells are prone to display glycolysis and release lactate, called the Warburg effect (i.e., aerobic glycolysis)[1]. For decades, whether lactate promotes tumor progression remains controversial. Traditionally, lactate is believed as a kind of metabolic waste in tumor microenvironment (TME)[2]. On the other hand, there are also studies indicating that accumulation of lactate in tumor was associated with high frequency of chemo- and radio-therapy resistance and metastasis of patients[3, 4]. Recent evidence suggested that monocarboxylate transporter 1 (MCT1), the prominent path for lactate uptake, was highly expressed in tumor sample compared with their normal counterpart. However, how lactate contributes to tumor growth, particularly the oral squamous cell carcinoma (OSCC), is still waiting to be elucidated[5].

OSCC is heterogeneous, harboring cellular hierarchy with cancer stem cells (CSCs) at the apex[6]. In OSCC, it has been verified that CSCs obtained unlimited self renewal and inherent chemo-resistant capacity[6, 7]. Clinical statistics also suggested that presence of CSCs in tumor is closely correlated to poor prognosis of OSCC patients, which renders CSCs becoming promising therapeutic target[8]. Furthermore, latest studies indicated that CSCs properties were plastic and largely affected by the components in TME including lactate[9], one of the most common metabolic byproducts of both tumor and stromal cells. But until recently, whether lactate promotes cancer stem-like property of OSCC remained totally uncertain.

In preclinical models of cancer research, the primary culture closely resembles the original human malignancy and thereby is a convincible platform to investigate tumor physiopathology[10, 11]. Recently exploited 3D organoid culture model exhibited considerable efficiency to support long term expansion of primary tumor cells, which has also been applied in OSCC[12, 13]. Here, using 3D organoid culture model, we aimed to explore whether lactate can promote CSCs phenotype in primary OSCC cells.

Hui ZHAO, E-mail: 269412536@qq.com; Chuan-yu HU, E-mail: 40724003@qq.com
†The authors contributed equally to this work.
#Corresponding author, E-mail: huangping_hust@hotmail.com
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1 MATERIALS AND METHODS

1.1 Collection of OSCC Specimens and Preparation of Primary OSCC Cells

Four human OSCC tumor specimens under IRB-approved guidelines and informed consent of participants were obtained at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (China), according to the provisions of the Declaration of Helsinki. For isolation of primary cells, briefly, fresh specimens were minced into small pieces with scissors, then incubated in serum-free DMEM/F12 medium (Life Technologies, USA) containing 1.5 mg/mL collagenase IV (Gibico, USA), 20 μg/mL hyaluronidase (Sigma-Aldrich, USA), 1% penicillin/streptomycin (Life Technologies, USA) at 37°C for 1 to 2 h. After digestion, tissues were washed with PBS and filtered through a 40 μm mesh (BD Falcon, USA). To eliminate red blood cells, the cells were incubated in red blood cell lysis buffer (eBioscience, USA) on ice for 10 min and washed twice with PBS. The cells were then resuspended in PBS, and counted for cells density for further experiments.

1.2 Organoid Culture and Sphere Forming Assay

Major procedures of 3D culture were referenced as previsouly described[13, 14]. For organoid culture, single primary cells were mixed with Matrigel (growth factor reduced; BD Biosciences) and embedded into 24-well culture plates (30 μL Matrigel/1000 cells/well), and incubated by organoid culture medium. The composition of OSCC organoid culture medium included DMEM/F12 supplemented with 1× N2, 1× B27, 50 ng/mL human EGF, 10 nmol/L Gastrin, 500 nmol/L A83-01 (All purchased from Sigma, USA). For sphere-formation assay, cells were resuspended in standard sphere-forming medium (1000 cells/mL medium/well): DMEM/F12 supplemented with 1× B27, 20 ng/mL human recombinant epidermal growth factor and 20 ng/mL basic fibroblast growth factor. Organoids and spheres were passaged every 7 days using TrypLE at 37°C for 10 min. For lactate treatment, sodium lactate (5 mmol/L) and CHC (5 mmol/L) were purchased from Sigma-Aldrich (USA)[15]. For measurement of organoid and sphere formation efficiency, the organoids or spheres with diameters >50 μm were scored and shown as clonogenicity (%) in the figures.

1.3 Immunofluorescence Assay

Organoids or spheres were fixed in 4% PFA at 4°C for 20 min before staining. For immunofluorescence staining, the following antibodies were diluted at 1:100 in 5% BSA to detect antigens: CD133 (MiltenyiBiotec, Germany)[16], CK18 (Biogenex, USA)[17], MCT1 (Chemicon, USA)[15]. Briefly, sections were blocked with 5% (w/v) bovine serum albumin in PBS, then incubated with primary antibodies at 4°C overnight, followed by staining with secondary antibodies conjugated to streptavidin-Cy3 (Thermo Fisher, 438315; diluted at 1:100 in 5% BSA) or Alexa Flour 488 (Jackson ImmunoResearch Laboratories, USA; diluted at 1:100 in 5% BSA) at room temperature for 2 h. Finally, nuclei were stained with DAPI (Sigma, USA) for 10 min at room temperature. Organoid and sphere sections were visualized by a fluorescence microscope (TRRFM, Olympus, Germany) or a confocal microscope (FV1000, Olympus, Germany).

1.4 Lentiviral Reporter Assay and Flow Cytometry

TCF/LEF reporter driving expression of GFP (TOP-GFP) lentivirus was purchased from SBO Medical Biotechnology Company [China (SY10100)]. Primary OSCC cells were infected with TOP-GFP lentivirus at MOI = 25 for 72 h. Tumor cells infected with TOP-GFP were used to process organoid and sphere formation assay, and the expression of TOP-GFP was detected by flow cytometry. Fluorescence-activated cell sorting (FACS) was performed according to the manufacturer's instructions using a FACS Aria II Cell Sorter (BD Biosciences, USA). The intensity of TOP-GFP was detected for separation, only the top and bottom 10% cells were respectively isolated as GFP^+ and GFP^- cells population; the frequency of GFP^+ cells was detected to evaluate Wnt activity[18].

1.5 Quantitative RT-PCR

Total RNA was extracted by using RNAiso Plus (Japan), and then complementary DNA (cDNA) was synthesized using the Mixima First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Quantitative RT-PCR analysis was carried out through an ABI PRISM 7300 Sequence Detection System instrument with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

1.6 Knockdown of Human MCT1 in OSCC Organoid Cells

Primary OSCC organoid cells were transfected with siRNA using lipoJetTM (SignaGen, China) following the manufacturer's instructions. Human MCT1-target specific small interfering RNA (siRNA) was synthesized by ViewSolid (China). The sense sequence of MCT1-siRNA (siMCT1) was 5′-AAGAGGCTGACTTTTCCAAAT-3′, and that of control-siRNA (siNC) was 5′-AACTCGCTGAGATTT-CGTTAT-3′[15]. After transfection with siRNA for 48 h, alterations of MCT1 expression level were verified by Western blotting.

1.7 Western Blotting

Purified cells were lysed in NP40 protein lysis buffer containing a protease inhibitor mixture (Sigma, USA). The total proteins were separated using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes. After blocking with 5% BSA in 0.01

mol/L TBS (pH 7.5), supplemented with 0.5% Tween 20 (TBST), the membranes were incubated with specified primary antibodies (CD133; MCT1) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit-IgG or goat anti-mouse IgG secondary antibodies for 2 h at room temperature. Finally, the protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA).

1.8 Statistical Analysis

All data were presented as mean±standard deviations (SD) on triple experiments. In general, unpaired two-tailed Student *t*-test was performed to compare differences between different treatment groups on IBM SPSS Statistics 18. $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Characterizations of OSCC Patient Derived Organoids

Organoid culture is a novel 3D culture model which exhibits high efficiency to support long term expansion of primary cancer cells. It has been verified that patient derived organoids (PDOs) are capable of recapitulating clinical characterizations of parental tumor[12]. To investigate whether organoid culture can be applied in OSCC, we generated PDOs using freshly isolated OSCC cells. Four independent line of PDOs were successfully established from parental tumor (table 1). As shown in our data, HE staining indicated that OSCC organoids harbored similar structure complexity to primary cancer (fig. 1A). To interrogate whether PDO can recapitulate cellular heterogeneity of OSCC tumor bulk, we employed immunofluorescence staining of CD133, which is a marker of OSCC CSCs[16], and CK18, the differentiated cell marker of OSCC epithelial cells[17], in organoid and its original tumor specimen. The results demonstrated that the expression of $CD133^+$ and $CK18^+$ cells in OSCC organoid closely resembled to primary cancer, implying that organoid *in vitro* can preserve cellular heterogeneity of clinical tumor specimens (fig. 1B).

2.2 High Wnt Activity Functionally Designated CSCs Population of OSCC

Primary organoid culture closely resembles the original human malignancy and is a convincible platform to investigate tumor physiopathology[10, 11]. The presence of CSCs closely correlated to poor prognosis of OSCC[6–8]. Besides, it has been verified that the CSCs phenotype were largely mediated by the self renewal Wnt pathway[19]. To visualize Wnt activity in OSCC organoid, we transfected organoid cells with a TCF/LEF reporter that directs the expression of enhanced green fluorescent protein (TOP–GFP), and then selected the single GFP^+ cells which cloned organoid for expansion to exclude variation in lentiviral integration site and copy number between cells[18]. As shown in fig. 2A, following organoid induction (day 1–day 7), the single GFP^+ (Wnt^+) cells (day 1) would

Table 1 Clinical features of patients with OSCC and their corresponding quantifications of organoids forming efficiency

Case no.	Age (years)	Gender	Tumor location	TNM grade	Organoid (‰)
1	52	Male	Tongue	II	10.24±1.47
2	64	Male	Tongue	IV	12.21±0.89
3	68	Female	Cheek	IV	6.77±1.98
4	66	Female	Tongue	IV	5.14±1.17

Tumor specimens were obtained from OSCC patients and processed into single cell suspensions. Cells were then seeded in 3D culture conditions to generate organoids *in vitro*. After 7-day incubation, the organoids with diameter >50 μm were counted to measure forming efficiency.

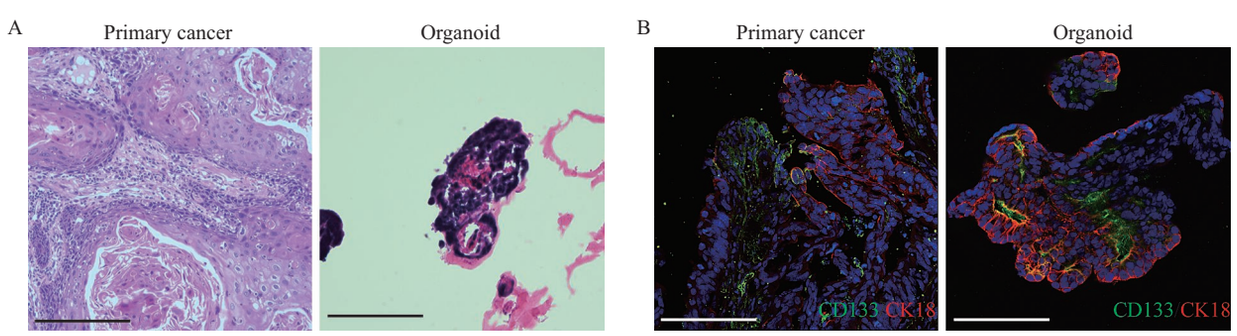

Fig. 1 Characterizations of OSCC patient derived organoids
A: representative HE staining images of primary OSCC specimen (case 1) and the corresponding organoid. Scale bars=50 μm; B: representative images showing immunofluorescence staining of CD133 and CK18 in primary OSCC specimen (case 1) and the corresponding organoid. Scale bars=50 μm

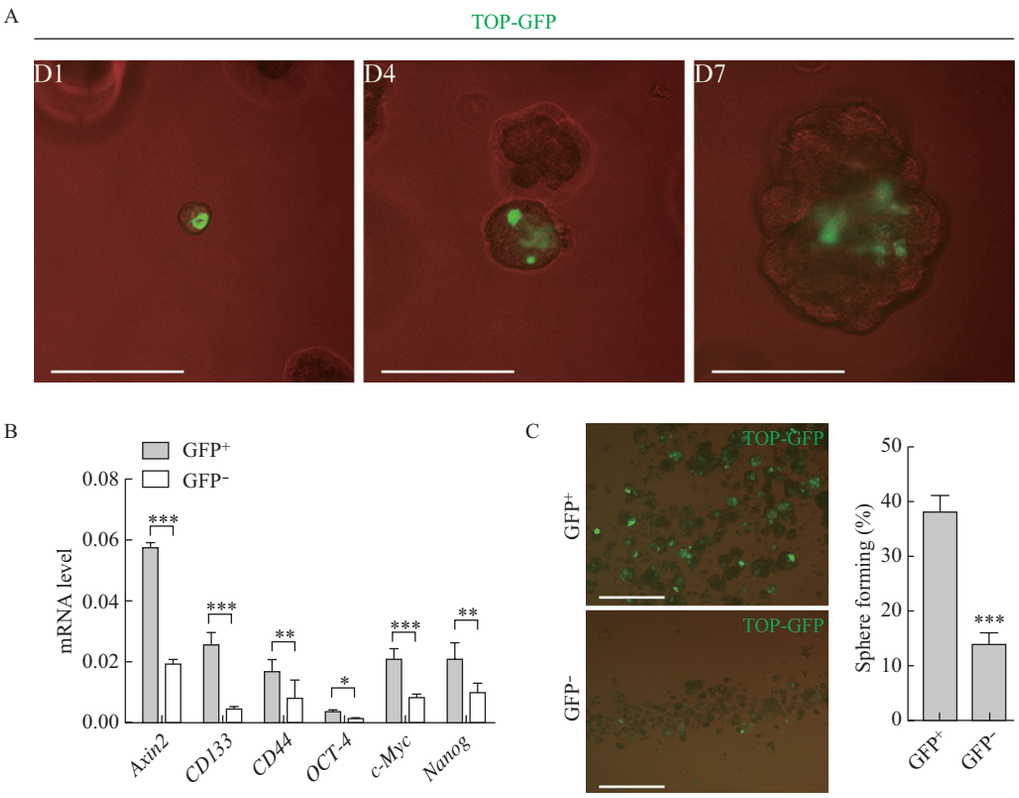

Fig. 2 High Wnt activity functionally designated CSCs population of OSCC

A: representative fluorescence images showing incubation procession of single TOP-GFP$^+$ (Wnt$^+$) cell in organoid culture. Three pictures were respectively captured at day 1 (D1), day 4 (D4), and day 7 (D7) since cells were imbedded. Scale bars=100 μm; B: RT-PCR analysis showed the expression level of Wnt targeted gene Axin2, and several OSCC stem cells associated genes CD133, CD44, OCT-4, c-Myc and Nanogin GFP$^+$(Wnt$^+$) and GFP$^-$(Wnt$^-$) cells population. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001; C: representative fluorescence images (left) and quantification analysis (right) of forming efficiency of sphere forming assay using GFP$^+$(Wnt$^+$) and GFP$^-$(Wnt$^-$) cells population.Scale bars=100 μm. $^{***}P$<0.001

differentiate into GFP$^-$ (Wnt$^-$) cells (day 7). To detect correlation of Wnt activity with CSCs phenotype in OSCC cells, the highest and lowest 10% of TOP-GFP expressing cells were evaluated and separated using flow cytometry (FACS). The RT-PCR analysis demonstrated that GFP$^+$ cells subpopulation (highest 10%) exhibited higher mRNA expression level of Axin2, one of the major Wnt target genes than GFP$^-$ cells subpopulation (lowest 10%), implying that GFP$^+$ cells displayed higher Wnt activity (fig. 2B). Consistent with this, our data indicated that GFP$^+$ cells subpopulation exhibited higher mRNA expression level of several CSCs associated genes including CD133, CD44, OCT-4, c-Myc and Nanog[6] (fig. 2B), and *in vitro* sphere forming capacity (fig. 2C) than GFP$^-$ cells subpopulation. Together, the results suggested that high Wnt activity functionally designated CSCs population of OSCC.

2.3 Lactate Treatment Promoted Wnt Activity and Increased Expression of CD133$^+$ Cells in OSCC Organoids

To investigate whether lactate can promote CSCs phenotype of OSCC, we administrated OSCC cells respectively with PBS (control group), lactate, or lactate plus α-cyano-4-hydroxycinnamate (CHC), which is an inhibitor of lactate uptake[15], in organoid culture model. After 3 days incubation, it was observed that lactate treatment significantly increased organoid forming efficiency of OSCC cells (fig. 3A), which is a functional evaluation of CSCs property. Additionally, immunofluorescence images verified that higher intensity of Wnt$^+$ cells was shown in organoids treated with lactate, when compared with those treated with PBS (control) or lactate plus CHC (fig. 3B). Further FACS analysis demonstrated that lactate treatment increased frequency of Wnt$^+$ cells in OSCC organoids (fig. 3C), impling that lactate can promote Wnt activity in OSCC cells. Consistent with these findings, the immunofluorescence staining and Western blotting analysis of CD133, one of the CSCs markers of OSCC, verified that lactate treatment significantly increased protein expression level of CD133 in OSCC cells (fig. 3D and 3E). Overall, our data suggested that lactate treatment promoted CSCs phenotype of OSCC.

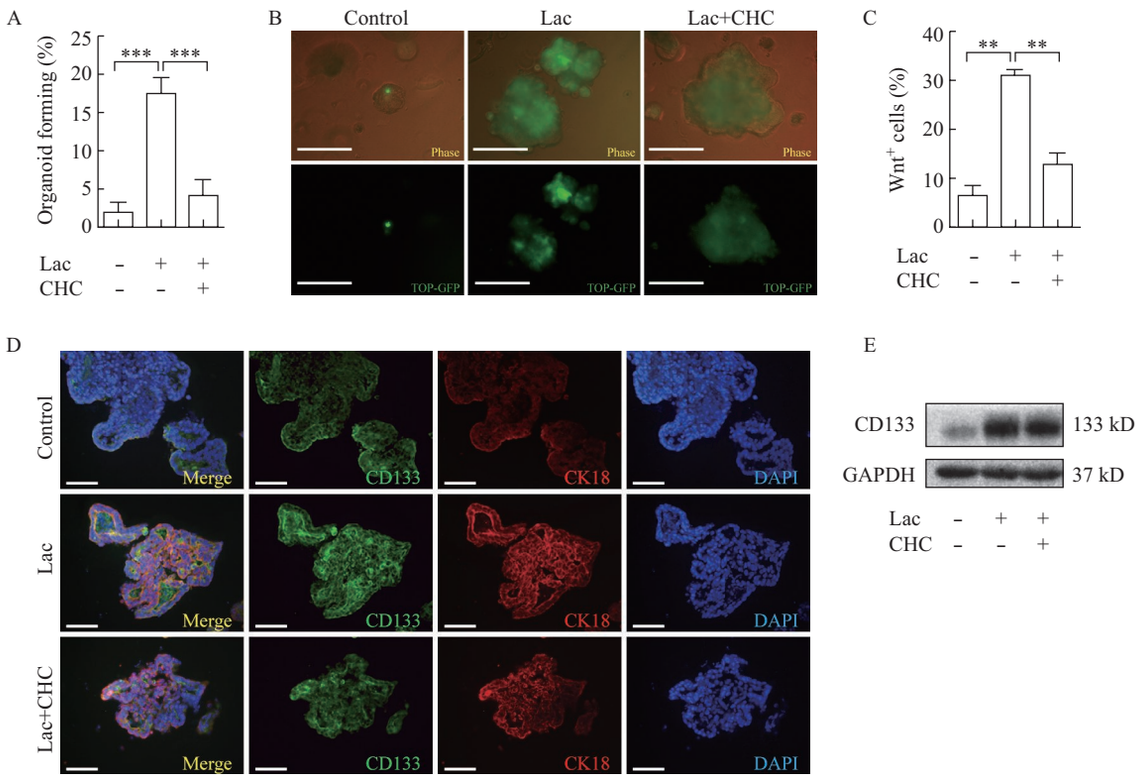

Fig. 3 Lactate treatment promoted Wnt activity and increased expression of CD133+ cells in OSCC organoids
A: Quantification analysis showed organoids forming efficiency of OSCC cells treated with PBS (control), lactate (Lac) and lactate plus CHC (Lac+CHC). $^{***}P<0.001$; B: Representative images showed intensity of $GFP^+(Wnt^+)$ cells in OSCC organoids treated with PBS (control), lactate (i.e. Lac, 5 mmol/L), and lactate plus CHC (5 mmol/L). Scale bars=100 μm; C: FACS analysis showed frequency of $GFP^+(Wnt^+)$ cells in OSCC organoids treated with PBS, lactate, and lactate plus CHC. $^{**}P<0.05$; D: Representative IF images showed expression of $CD133^+$ and $CK18^+$ cells in OSCC organoid treated with PBS (control), lactate (i.e. Lac, 5 mmol/L), and lactate plus CHC (5 mmol/L). Scale bars=100 μm; E: Western blotting assay showed the expression level of CD133 protein in organoids groups treated with PBS, lactate, and lactate plus CHC.

2.4 Knockdown of MCT1 Impaired Organoid Forming Capacity of OSCC

MCT1 has been identified as the prominent path for lactate uptake in human tumor, including OSCC[15, 20]. Using immunofluorescence staining, our data demonstrated that organoid can modulate the expression of MCT1 in primary OSCC (fig. 4A). To further investigate the role of lactate uptake in OSCC tumor growth, siRNA of MCT1 was used to silence the expression of MCT1 in OSCC cells. The result of Western blotting assay demonstrated that OSCC cells with manipulation of siMCT1 exhibited significantly lower protein expression level of MCT1 than their counterpart (si-control) (fig. 4B). Organoid forming assay was then performed to evaluate proliferation capacity of OSCC cells[12]. As depicted in fig. 4C and 4D, the results indicated that silencing MCT1 significantly impaired organoid forming rate of OSCC cells. Besides, our data suggested that along with increased concentration of lactate in medium (0, 1, 5 mmol/L), the organoid forming efficiency of OSCC cells with si-control manipulation increased gradually, otherwise, those with si-MCT1 manipulation showed no alteration. Together, these data suggested that knockdown of MCT1 impaired proliferation capacity of OSCC cells.

3 DISCUSSION

Solid human tumors, including OSCC, are growing in 3D environment[10, 11, 21]. Previous studies indicated that OSCC specimens harbored several 3D characterizations, such as cellular heterogeneity and gradient of hypoxia[7, 16, 22]. In view of this, standard 2D cell line culture failed to recapitulate *in vivo* 3D situation of parental tumor. Besides, the efficiency of 2D primary cell line culture was very low[10, 11, 21]. To overcome this, recently exploited 3D organoid culture model was applied for cancer researches[12]. It has been suggested in extensive studies that the organoid culture model may display a range of practical advantages: (1) organoid model possesses higher efficiency to support long term expansion and retains a stable karyotype of primary tumor cells; (2) PDOs can recapitulate

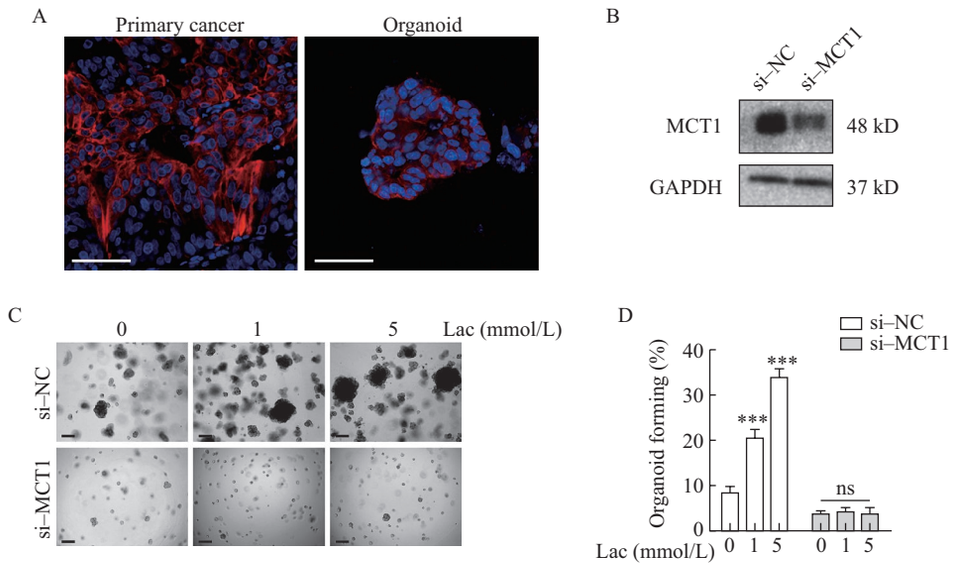

Fig. 4 Knockdown of MCT1 impaired organoid forming capacity of OSCC

A: Representative IF images showed expression of MCT1$^+$ cells in primary OSCC specimens and the corresponding organoid. Scale bars=100 μm; B: Western blotting assay showed the alteration of MCT1 protein expression level in OSCC organoids cells after si-MCT1 treatment; C and D: Representative images (C) and quantification analysis (D) showed organoid forming efficiencyof OSCC cells with manipulation of si-MCT1 (bottom) and its si-control (top) under lactate treatment at doses of 0, 1 and 5 mmol/L. Scale bars=200 μm; $^{***}P<0.001$

histopathology and molecular characterizations of parental tumor[12]. Thus far, organoid culture has been employed in drug screening and disease modeling in various types of tumor, but the application of organoid model in primary OSCC cells remained still uncertain[13]. In the present study, we generated tumor organoids using individual OSCC specimens, and then demonstrated that OSCC PDOs were capable of recapitulating structure complexity and cellular heterogeneity of parental tumor, thus providing an eligible patients derived preclinical model for further investigations (fig. 1A and 1B).

CSCs are a rare subset of tumor cells that harbor principal properties of self renewal, unlimited *in vivo* tumor initiating capacity and long term repopulation potential[6–8]. Latest studies revealed that CSCs resided in niches that consisted of the components in TME[18]. To their largest extent, these niches maintain the principle properties and preserve the phenotypic plasticity of CSCs, protect them from immune system and facilitate their metastatic capacity[9, 18]. Typically, the Wnt signaling, one of the self renewal pathways that mediate a great deal of CSCs properties, are largely affected by the Wnt ligands in TME niche, such as Wnt proteins secreted from Wnt ligand cells[18]. More importantly, the heterogeneity of TME introduces a distribution of heterogeneous Wnt activity in tumor cells, while the level of Wnt signaling has been verified to correlate with tumor-initiating capacity in xenotransplantation assay[9]. Defining how the niches contribute to mediation of CSCs properties helps to design therapeutic targets against these interactions and may take effect on impairing CSCs in tumor[18]. Here, we transfected PDOs cells with a Wnt reporter to visualize Wnt activity in primary OSCC cells; consistent with other's findings, we further demonstrated that high Wnt activity functionally designated CSCs population (fig. 2A–2C). Using this model, we investigated whether lactate, one of the major components in TME and associated with poor prognosis of OSCC patients, may contribute to perseverance of CSCs phenotype. Our results demonstrated that lactate treatment significantly promoted organoid forming capacity, Wnt activity, and CSCs marker (CD133) expression in primary OSCC cells (fig. 3A–3E), implying that lactate may advocate CSCs properties in OSCC. In light of this, it may be possible to explain the correlation of acidic TME with poor prognosis in OSCC patients.

MCT1 is the uptake transporter of lactate and has been verified to highly express in many human tumors compared with their normal counterpart, including OSCC[20]. Recent studies indicated that, through MCT1 the extracellular lactate can be recycled into pyruvatein tumor cells, and then enhance the oxidative phosphorylation (OXPHOS), which is both the energy resource and signal upstream of many stem cells pathways, such as Wnt and p38/MAPK signal[23, 24]. In line with this piece of evidence, there are also studies identifying that the expression of MCT1 and the transportation of lactate in tumor cells may take

effect on mediation of tumor growth. In the present study, using OSCC PDOs, we investigated whether the expression of MCT1 contributed to CSCs property of OSCC cells. At first, our data demonstrated that the expression of MCT1 in OSCC can be modulated in corresponding PDOs *in vitro* (fig. 4A). And furthermore, consistent with other's findings, we observed that OSCC cells with manipulation of silencing MCT1 displayed decreased organoid forming efficiency, which was a kind of functional evaluations of CSCs property (fig. 4C and 4D). More importantly, compared with their normal counterpart (OSCC cells with si-NC manipulation), which exhibited higher organoid forming efficiency as the concentration of lactate in medium gradually increased, the OSCC cells with si-MCT1 manipulation showed no response to the alteration of lactate in medium. Taken together, these results suggested that lactate can, at least partially, promote the CSCs phenotype in OSCC cells, and manipulation of silencing MCT1 may defend this procession and thereby become a therapeutic target.

Conflict of Interest Statement

The authors declare no potential conflicts of interest.

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