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Original Article

OCT4 expression maintained cancer stem-like properties in hepatoma cell line

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Abstract: Recent evidence suggests that some solid cancers originate from cancer stem cells (CSCs) and CSCs are responsible for metastasis and disease recurrence. Therefore, targeting CSCs has the potential to significantly improve outcomes for cancer patients. Here we used limited dilution method to selected cancer stem cell like clones and found Holoclone may be a promising candidate for its superior proliferation and anti-apoptosis activity. We found the viability of Holoclone was nearly same as that of HepG2 cell while it showed higher than its parental cell after 96h. And with ultraviolet light for 1 min, Holoclone exhibited lowest percentage of apoptosis cell. Moreover the protein and gene level of Oct4 in the clones were higher than those in their parental cell line, HepG2. In summary, we report Holoclone as a cancer stem cell like clone with better proliferation and anti-apoptosis activity as well as high protein and gene level of Oct4. Thus Holoclone may represent even a Drug Screening Model Cell for tumor drug screening.

Keywords: Cancer stem cells (CSCs), holoclone, HepG2, OCT4

Introduction

As the sixth most common diagnosed cancer and the third leading cause of cancer death in the world, hepatocellular carcinoma (HCC) induces livers damaged by chronic viral infections or metabolic disorders [1, 2]. In the past decades many improvements in treatment have been made in HCC, surgical resection and liver transplantation are the primary effective approaches to treat HCC, however the overall 5-year survival rate of HCC patients remains very poor [3, 4]. Accordingly investigation to elucidate the mechanism of tumorigenesis of HCC is needed in order to improve the patient survival rate.

There is a hypothesis that only small subsets of cells within tumors, termed cancer stem cells (CSCs), are responsible for tumor growth, maintenance, metastasis and recurrence, due to their stem-cell-like self-renewal capacity and unlimited proliferative potential [5]. CSCs have a high level of resistance to chemotherapy and radiotherapy, a few of which may survive and lead to recurrence of the disease [6]. Thus this may explain the extensive heterogeneity, high rates of recurrence in mortality, and the high level of resistance to conventional anticancer therapy observed in hepatocellular carcinoma (HCC) [1]. Therefore, the identification of CSC in HCC is significant for the development of new therapeutic approaches to tumor recurrence.

Recent data showed there are a few transcription factors responsible for the maintenance of self-renewal and pluripotency of both ESCs and ECCs [7, 8]. Among them, Oct4 which is encoded by POU5F1 and also known as Oct3/4 has been labeled as a master regulator [9-11]. Oct4, a member of the family of POU domain transcription factors, is expressed in pluripotent embryonic stem and germ cells [12-14]. Growing evidence demonstrates that Oct4 is found in somatic tumors such as bladder carcinoma, ovarian carcinoma and lung adenocarci-

noma [15-17]. These findings indicate that Oct4 plays a role in tumorigenesis and appeal for more studies of regulation of Oct4 in various cell types [5].

Here we reported we selected the cancer stem cell like clones derived from hepatoma cell line, HepG2 and that the selected clones exhibited superior proliferation and anti-apoptosis activity. Moreover the protein and gene level of Oct4 in the clones were higher than those in their parental cell line, HepG2. These results not verified only that the expression of Oct4 played a crucial role in maintaining cancer stem-like in liver cancer but also suggested the selected clones may share the cancer stem cell like characteristics which may be applied as a model to testify the chemoradioresistant properties in the future.

Materials and methods

Reagents and antibodies

The Anti-Akt (Thr308) (Rabbit, 2965S, CST), Anti-Akt (Ser473) (Rabbit, 4060S, CST), Anti-Akt (pan) (Rabbit, 4691S, CST), Anti-Sox2 (Rabbit, 2748S, CST) were purchased from Cell signaling Technology. The Anti-Oct4 antibody (Mouse, sc-5279) were purchased from Santa Cruz Biotechnology. The polyclonal anti-Oct4-pT235 antibody was custom-made by GenScript (order ID:134164-1) and verified as described previously 17. The peroxidase-conjugated anti-mouse secondary antibody (7076), peroxidase-conjugated anti-rabbit secondary antibody (7074), anti-biotin antibody (7075) and the biotinylated protein ladder detection pack (7727) were all purchased from Cell Signaling Technology. Anti-GAPDH (horseradish peroxidase, HRP; A00192) was from GenScript, and anti-GAPDH (AG019) was from Beyotime Institute of Biotechnology. The dilutions of the above antibodies ranged between 1:1,000 and 1:2,000 when used for immunoblotting.

Sorting CSCs with limited dilution method

HepG2 cell line were cultured in High glucose DMEM (12430, GIBCO, Auckland, NZ) supplemented with 10% FBS (10099, GIBCO, Grand Island, NY) and 1% penicillin/streptomycin (GIBCO, Auckland, NZ) at 37°C in a 95:5 air/CO_2 water-saturated atmosphere. At confluence, cells were treated with 0.05% trypsin-EDTA (25300; GIBCO) and gradually diluted proportionally 64, 32, 16, 8, 4, 2, 1 and 0.5 cells to each 100μL DMEM medium, respectively adding in 96-well plates. The plates were placed in incubator and cultured, changing the medium every 5 days. After 2 weeks, cells from a colony of a hole were cultured and passaged, called HepG2 Holoclone/HepG2 Meroclone.

Cell culture

HepG2, Holo, Mero cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (12430, GIBCO, Auckland, NZ) supplemented with 10% fetal bovine serum (FBS) (10099, GIBCO, Grand Island, NY) as well as 1% penicillin/streptomycin (GIBCO, Auckland, NZ) at 37°C in a 95:5 air/CO_2 water-saturated atmosphere.

MTT assay

Cell viability was determined using the MTT assay (Roche, Basel, Switzerland; 1146500-7001) according to the manufacturer's instructions. Briefly, cells were cultured with 100 μL medium per well in 96-well microplates. Plates were incubated for 4 h in a humidified atmosphere at 37°C and 5% CO_2 followed by adding 10 μL of the MTT labeling reagent (final concentration 0.5 mg/mL) to each well. Plates were incubated overnight with 100 μL of the solubilization solution in each well. Then cell viability was measured using a microplate reader (Beckman Coulter, Brea, CA; DTX880) at a wavelength of 570 nm with the formation of purple formazan crystals, proportional to the number of metabolically active viable cells.

Immunofluorescence assay

For immunofluorescence assays, cells seeded at $5*10^4$ in 24-well plates. After being incubated for 24 h, each well were fixed 4% paraformaldehyde for 20 min. Cells were permeabilized with 0.25% Triton X-100 for 30 min at room temperature. Then each well was washed with phosphate-buffered saline (PBS) for three times and blocked with PBS containing 5% (w/v) bovine serum albumin (BSA) 1 h at room temperature. Cells were treated with indicated antibodies overnight at 4°C. A negative control (without primary antibody) was conducted. In the second day, each well were washed with PBS for three times and incubated with either 2 U/ml of Alexa Fluor® 488 AffiniPure Goat Anti-

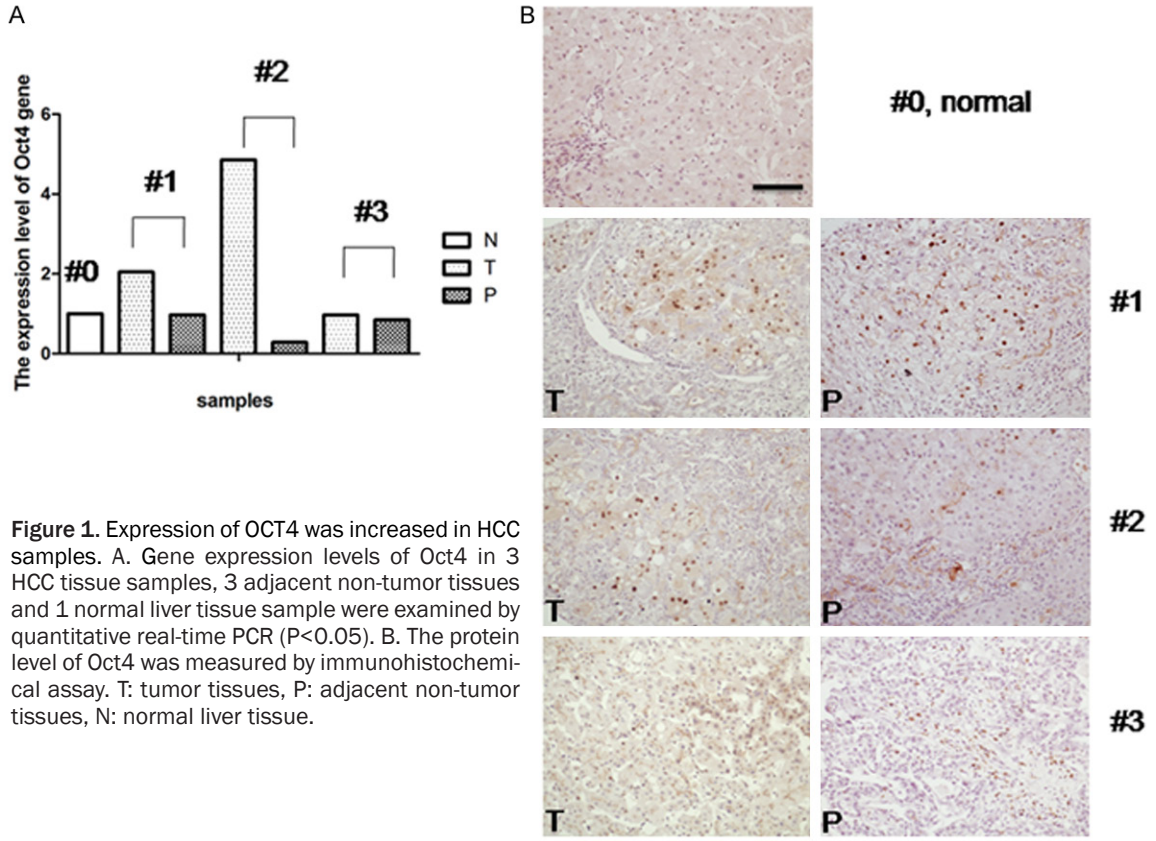

Figure 1. Expression of OCT4 was increased in HCC samples. A. Gene expression levels of Oct4 in 3 HCC tissue samples, 3 adjacent non-tumor tissues and 1 normal liver tissue sample were examined by quantitative real-time PCR ($P<0.05$). B. The protein level of Oct4 was measured by immunohistochemical assay. T: tumor tissues, P: adjacent non-tumor tissues, N: normal liver tissue.

Rabbit IgG (H+L) (711-485-152, Jackson ImmunoResearch Inc., PA, USA) or Alexa Fluor® 594 AffiniPure Goat Anti- Mouse IgG (H+L) (715-515-150, Jackson ImmunoResearch Inc., PA, USA) for 1 h at room temperature. Nuclei were stained with Hoechst 33342 (Hoechst 33342 Trihydrochloride, Trihydrate - FluoroPure™ Grade, H21492, Thermo Fisher Scientific Inc., USA) at room temperature in the dark for 20 min, rinsed with PBS for three times, and mounted in Vectashield (Vector, UK) prior to examination with the confocal microscope.

Western blotting

After washing three times with phosphate buffered saline (PBS), cells were lysed in lysis buffer (#9803; Cell Signaling, Danvers, MA) containing protease (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland) and phosphatase (PhosSTOP; Roche, Basel, Switzerland) inhibitors. The protein content of cellular extracts was quantified by the Bradford assay (Thermo, Rockford, IL). Proteins in the lysates boiled with SDS-PAGE sample loading buffer were separated by SDS-PAGE, blotted on PVDF membranes and probed with the indicated antibodies. The signals were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Millipore WBKLS0100).

Real-time PCR

After washing with PBS twice, total RNA isolated from cells or human samples was extracted using the RNeasy Mini kit (15596026, Qiagen) according to the manufacturer's instructions. One microgram of the resulting RNA was used for reverse transcriptase (RT)-PCR. cDNA was synthesized using oligo-primers and a reverse transcription kit (037A; TAKARA, Shiga, Japan). The Bio-Rad universal SYBR Supermix (72-5121, Bio-Rad, Hercules, CA) was used to perform real-time quantitative PCR assays on a Bio-Rad Cycler (C1000, Bio-Rad, Hercules, CA) with various sequences. PCR was run for 39 cycles with 5 s per 95°C denaturation, 30 s/60°C annealing and 20 s/72°C elongation. All the PCR amplification was conducted in triplicate and repeated in three independent experiments. The relative quantities of selected mRNAs were normalized to that of GAPDH, and the derived values of human liver tumor samples and human adjacent liver tumor

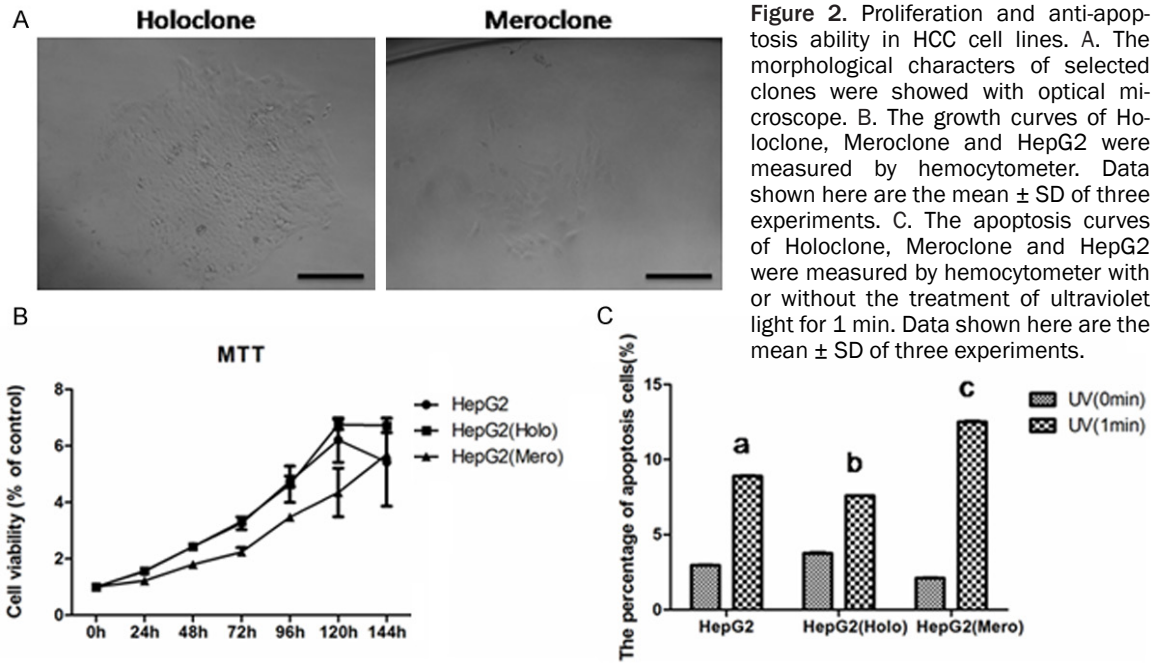

Figure 2. Proliferation and anti-apoptosis ability in HCC cell lines. A. The morphological characters of selected clones were showed with optical microscope. B. The growth curves of Holoclone, Meroclone and HepG2 were measured by hemocytometer. Data shown here are the mean ± SD of three experiments. C. The apoptosis curves of Holoclone, Meroclone and HepG2 were measured by hemocytometer with or without the treatment of ultraviolet light for 1 min. Data shown here are the mean ± SD of three experiments.

samples were further normalized to those of human normal liver tissue samples, with the latter being set as 1. Or in other cases, relative quantities of selected mRNAs in the 3 cell lines were normalized to that of GAPDH, and the derived values of 2 cell lines were further normalized to that of HepG2 cells, with the latter being set as 1. Mostly, the data were expressed as mean ± s.d. of triplicate measurements from one of three independent experiments that gave similar results. The statistical analyses were performed with either the Student's t-test or analysis of variance using the SPSS 20 software, and $P<0.05$ was considered statistically significant (*$P<0.05$).

Results

Expression of OCT4 was increased in HCC samples

Oct4 expression levels in 3 HCC tissue samples, 3 adjacent non-tumor tissues and 1 normal liver tissue sample were examined by quantitative real-time PCR. Oct4 levels in cancer samples were significantly higher than those in the noncancerous samples, among which the gene level of Oct4 in #1 and #2 tissue samples showed twice and eight times than their adjacent non-tumor tissues, respectively ($P<0.05$) (**Figure 1A**). Furthermore, the immunohistochemical results revealed that the tumor samples demonstrated higher Oct4 expression when compared with their adjacent non-tumor tissues (**Figure 1B**).

Proliferation of HCC cell lines

We examined the selected clone cells with optical microscope, finding morphological characters of selected clones different from each other (**Figure 2A**). And the viability assay demonstrated that the Holoclone and Meroclone exhibited different viabilities in vitro and the Holoclone may obtain better growth ability for the Holoclone showed higher viability since 24 h in contrast with Meroclone and the latter's viability always was slower. The viability of Holoclone was nearly same as that of HepG2 cell while it showed higher than its parental cell after 96 h (**Figure 2B**).

Anti-apoptosis ability in HCC cell lines

In order to study the anti-apoptosis ability in our selected HCC cell lines, we carried out the apoptosis assay to find that the Holoclone showed higher anti-apoptosis ability than others. After treated with ultraviolet light for 1 min, Holoclone exhibited lowest percentage of apoptosis cell while Meroclone showed higher (**Figure 2C**).

Expression level of OCT4 in HCC cell lines

In order to study the Oct4 level in our selected HCC cell lines, we conducted the Immu-

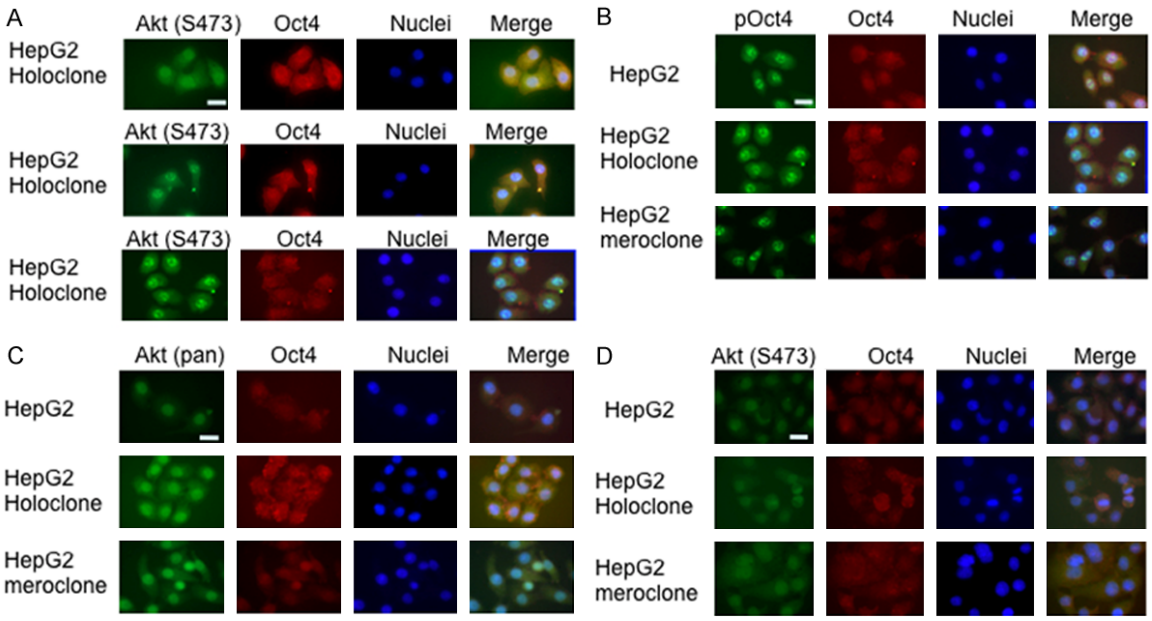

Figure 3. Protein expression level of OCT4 was measured in HCC cell lines by Immunofluorescence Assay. A-D. Immunofluorescence Assay was conducted to measure Oct4 expression in HepG2, Holoclone and Meroclone. Moreover Western blot assay showed that both Holoclone and Meroclone expressed the OCT4 which was higher than that of HepG2 cells (**Figure 4A**). Furthermore, the gene level of Oct4 was higher in both Holoclone and Meroclone when compared with HepG2 cells (**Figure 4B**).

nofluorescence Assay and found that Holoclone manifested Oct4 expression higher than the Meroclone and HepG2 cells (**Figure 3A-D**). Moreover Western blot assay showed that both Holoclone and Meroclone expressed the OCT4 which was higher than that of HepG2 cells (**Figure 4A**). Furthermore, the gene level of Oct4 was higher in both Holoclone and Meroclone when compared with HepG2 cells (**Figure 4B**).

Discussion

Oct4 has been identified as one of four major factors that contribute to the reprogramming capability of adult cells into germline-competent-induced pluripotent stem cells [18-20]. Recently the expression of Oct4 has further been shown in ECCs (embryonal cancer stem-like cells), suggesting that threonine 235 of Oct4 was phosphorylated by Akt and that the levels of phosphorylated Oct4 in ECCs correlated with resistance to apoptosis and tumorigenic potential [5]. Thus its expression may be implicated in self-renewal and tumorigenesis via activating its downstream target genes [21]. Herein we reported the selection of clones from HepG2 cell lines. Holoclone showed strong proliferative and anti-apoptosis capabilities in vitro (**Figure 2**). We also demonstrated that Oct4 expression was transcriptionally and translationally up-regulated in both Holoclone and Meroclone (**Figures 3** and **4**). These results implied that Holoclone we selected possess superior proliferation and anti-apoptosis activity and may share the cancer stem cells properties.

Recently, studies suggested that the reduction of Oct4 expression in lung CSCs induced apoptosis and the inhibition of tumor growth partly through the Oct4/Tcl1/Akt1 pathway [22]. In addition, some reports also showed Oct4 expression could enhance features of Cancer Stem Cells in a mouse model of breast cancer. The authors found that $OCT4^{high}$ 4T1 cells have an increased ability to form tumorsphere and a high expression of stem cell markers such as Sca-1, CD133, CD34, and ALDH1, when compared with $OCT4^{low}$ 4T1 cells. And $OCT4^{high}$ 4T1 cells also have greater tumorigenic potential in vivo [23]. In accordance with previous studies, we found Oct4 do play an important role in the Holoclone offering superior proliferation and anti-apoptosis activity, which may convince that Holoclone share the cancer stem cell like characteristics.

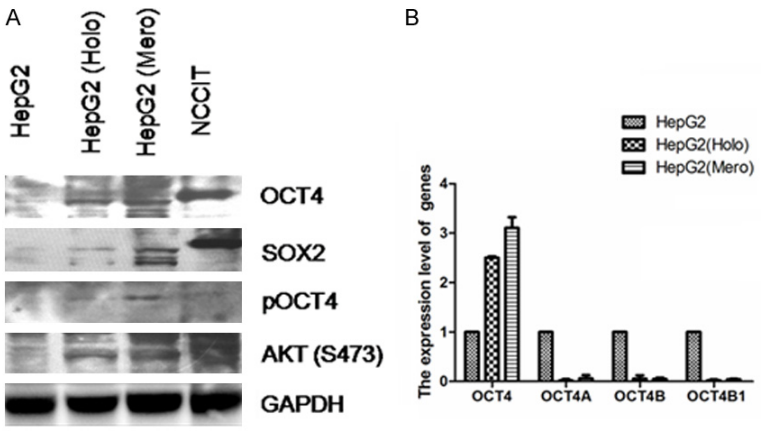

Figure 4. Protein and gene expression level of OCT4 was increased in HCC cell lines. A. The protein expression levels of Oct4 in HepG2, Holoclone, Meroclone and NCCIT cells were measured by Western blot assay. B. The gene levels of Oct4 in HepG2, Holoclone and Meroclone were measured by RT-PCR. Data shown here are the mean ± SD of three experiments.

It should be noted that this study has examined only proliferation and anti-apoptosis activity to evaluate the cancer stem cell characteristics of Holoclone, however, additional studies are necessary to look into downstream molecular mechanisms by which Oct4 regulates the maintenance and expansion of Holoclone.

Notwithstanding its limitation, this study does suggest the selected Holoclone share cancer stem cell characteristics with superior proliferation and anti-apoptosis activity when compared with its parental HpeG2 cell. Moreover the protein and gene level of Oct4 in the clones were higher than those in HepG2. Thus Holoclone may represent even a Drug Screening Model Cell for tumor drug screening.

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Disclosure of conflict of interest

None.

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