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Optimization of *in vitro* cell culture conditions suitable for the cholangiocarcinoma stem cell study

Krittiyabhorn Kongtanawanich[a], Pornlada Likasitwatanakul[b,c], Methichit Wattanapanitch[d], Siwanon Jirawatnotai[a,e,f,g,*]

[a] Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700 Thailand
[b] Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University 10700 Thailand
[c] Department of Medicine, University of Minnesota, Minnesota 55414 USA
[d] Siriraj Center for Regenerative Medicine, Research Department, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700 Thailand
[e] Siriraj Center of Research Excellence for Precision Medicine and Systems Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700 Thailand
[f] Division of Hematopoiesis, Joint Research Center for Human Retrovirus Infection, Kumamoto University, Kumamoto 860-8555 Japan
[g] Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000 Thailand

[*]Corresponding author, e-mail: siwanon.jir@mahidol.ac.th

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ABSTRACT: Currently, cancer stem cells (CSCs) are extensively studied due to their roles in tumorigenesis, metastasis, and drug resistance. However, the low quantity, and its transient nature present a challenge for their study. Therefore, a culture system supporting high CSC quantity and flexible for high-throughput drug testing is needed. In this study, we used cholangiocarcinoma (CCA) cell lines with a CSC-specific live-cell biosensor system, which expresses green fluorescence protein (GFP) in CSCs, to study factors/conditions affecting CCA stem-like cell quantity in culture. We investigated culture media, seeding density, and compared 2-dimension (2-D) vs 3-dimensional (3-D) culture methods. The effects of anti-CCA drug dosages on CSC numbers were also studied. By comparing three media recipes, a defined serum-free CCA medium was selected for its high CSC yields. We found seeding density and time significantly influence CCA CSC percentages in both 2-D and 3-D settings. Notably, 3-D culture markedly increased CSC percentages compared to 2-D, supporting its application in CSC studies. In 3-D culture, a higher CSC index correlated with higher anti-CCA drug doses, suggesting loss of differentiation as a prominent characteristic under standard therapy. Thus, we report culture conditions influencing CCA CSC quantity, useful for CSC study and anti-CSC drug discovery in CCA.

KEYWORDS: cholangiocarcinoma, cancer stem cell culture, 3-dimensional multicellular spheroid, functional precision medicine, drug screening

INTRODUCTION

CSCs are a small subset of cancer cells with the ability to self-renew and generate various cell types that comprise a tumor. These cells are believed to play pivotal roles in tumor initiation and maintenance, analog to the function of normal stem cells in tissue regeneration. They significantly contribute to tumor growth, metastasis, and therapeutic resistance [1]. Because of these properties, targeting CSCs is a focus in developing new cancer therapies [2]. However, studies of CSC are challenging, i.e. CSCs are a rare subset of cells within a tumor, making them difficult to isolate in large numbers, and intratumor complexity makes it hard to pinpoint and study CSCs accurately. CSCs interact with their surrounding microenvironment, which can influence CSC properties and behavior under varied cultural conditions or treatments. These challenges undermine the development of anti-CSC therapy. Therefore, drug testing platforms/culture conditions that preserve the key characteristics of CSCs and enrich for CSCs are highly desirable.

The 2-D culture system is a predominant platform in cancer research. In the 2-D platform, several factors could potentially affect the number of CSC in the culture, such as type of culture media, cell density, and culturing duration [3, 4]. We selected CCA cell lines as the model for our study, because CCA is an aggressive form of liver cancer with a poor prognosis, making it a critical area of study [5]. Secondly, CCA is considered a cancer with a significant presence of CSCs. These CSCs contribute to the tumor's aggressiveness, resistance to treatment, and potential for metastasis [6, 7]. Therefore, targeting CSCs in CCA is a focus of ongoing research, with therapies aimed at surface markers, signaling pathways, and the tumor microenvironment being investigated [3].

By using the CSC-specific live-cell biosensor containing CCA cell lines [8, 9], three cell lines were selected base on their known drug response, cancer stem cell profiles, and their easy-to-handle nature [9–12]. We assessed the influence of culture conditions, including culture media, seeding density, and culture duration to the quantity of CSCs. We also compared

the numbers of CSC grown in 2-D vs 3-D systems. We also tested the influence of chemotherapy on the number of CSC in the 3-D CCA culture.

MATERIALS AND METHODS

Cell culture

The CCA cell lines KKK-D068, HuCCT-1, and TFK-1 were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). For the 2-D culture conditions, KKK-D068 was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA). HuCCT-1 and TFK-1 were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI 1640) (Gibco). Common culture media are culture media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco). CCA stem-like cell enriched medium is composed of DMEM/F12 medium (supplemented with 1X B27™ supplement minus vitamin A (without vitamin A in the formulation) (Gibco), 20 ng/ml of human recombinant epidermal growth factor (EGF) (STEMCELL Technologies, Vancouver, BC, Canada), and 20 ng/ml of basic fibroblast growth factor (bFGF) (STEMCELL Technologies) [13]. The hepatocellular carcinoma (HCC) stem cell enriched medium is composed of DMEM/F12 media with 1X B27™ supplement (Gibco), 20 ng/ml of EGF, 20 ng/ml of bFGF, and 10 ng/ml of hepatocyte growth factor (HGF) (STEMCELL Technologies) [14]. All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Generation of CCA cell lines containing a CSC-specific live-cell biosensor

Generation of CCA cell lines containing the CSC-specific live-biosensor was previously described by our group [9]. In brief, CCA cell lines were transduced with the lentivirus expressing short half-life GFP, dsCopGFP, under the SOX2/OCT4-responsive promoter (SORE6-dsCopGFP) [15]. The cell lines transduced with the lentivirus dsCopGFP without the SOX2/OCT4-responsive promoter (mCMV-dsCopGFP) were used as a fluorescence/background control.

Formation of 3-D multicellular spheroids (MCS) and drug treatment

TFK-1 and KKK-D068 cell lines containing the CSC-specific live-biosensor were seeded onto a 96-well Ultra Low Attachment (ULA) round-bottom plate (Corning, NY, USA) at experimental specific seeding densities per 200 µl of common culture medium. The plate was then centrifuged at 1,000×*g* for 10 min and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 3 days [16]. After 3 days of culturing, the 3D-MCS formed, and were observed to aggregate into a single large cluster. The chemotherapies including gemcitabine (#S1714; Selleckchem, Houston, TX, USA) plus cisplatin (#HY-17394; Medchem, Monmouth Junction, NJ, USA), cisplatin, and 5-fluorouracil (#S1209; Selleckchem) were added for 3 days at varying concentrations indicated in Fig. 4a. 0.5% of dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) was used as vehicle control. After drug incubation, ATPlite™ Luminescence Assay System (PerkinElmer, Waltham, MA, USA) was used for cell viability measurement following the manufacturer's protocol. The cell viability was analyzed and normalized to the vehicle control. Data were presented as a dose-response curve.

Flow cytometric analysis

Flow cytometric analysis was performed on a Cytoflex™ flow cytometer (Beckman Coulter, Brea, CA, USA) as previously described [9]. For 2-D culture, cells were trypsinized and then washed twice with pre-chilled FACS buffer (3% FBS in 1X PBS). The cells were stained with Live/Dead Zombie NIR (BioLegend, San Diego, CA, USA) for dead cell elimination. Detection was performed on a Cytoflex™ flow cytometer (Beckman Coulter), while analysis was performed using FlowJo software (version 10.8.1).

For 3-D MCS, spheroids were collected from 96-well ULA round-bottom plates. Cell dissociation was performed using 0.25% trypsin and mechanical shearing by pipetting until a complete single-cell suspension was obtained. The cells were then washed twice with the pre-chilled FACS buffer and stained with Live/Dead Zombie NIR. The cells were detected and analyzed by flow cytometry.

High-content imaging and image analysis for 3-D MCS

3-D MCS forming by TFK-1 and KKK-D068 cell lines containing the CSC-specific live-biosensor were fixed with 4% PFA for 15 min, then stained with 10 µg/ml of Hoechst 33342 for 1 h. The spheroids were washed 3 times with 1x PBS using an on-top technique and then moved to PhenoPlate 96-well microplates (PerkinElmer) for background reduction purposes. High-content imaging was taken at 10× and 20× water lens using the HTS microplate reader Operetta CLS™ (PerkinElmer). The Columbus Image Data Storage and Analysis System (PerkinElmer) was used to process and analyze the data. The maximum fluorescent intensities of each pixel in the same z-axis were collected from 3-D confocal images to be the maximum projection image as a representation of 3-D confocal imaging. The area of 3-D MCS was defined by a nuclear staining dye (Hoechst 33342-positive cells). In the defined 3-D MCS area, the GFP intensity of the 3-D MCS was measured from every pixel. Meanwhile, the GFP intensity of the background was also measured in the same way. The median of the GFP intensity of 3-D MCS subtracted by the background was calculated to be the representative of the GFP intensity of the 3-D

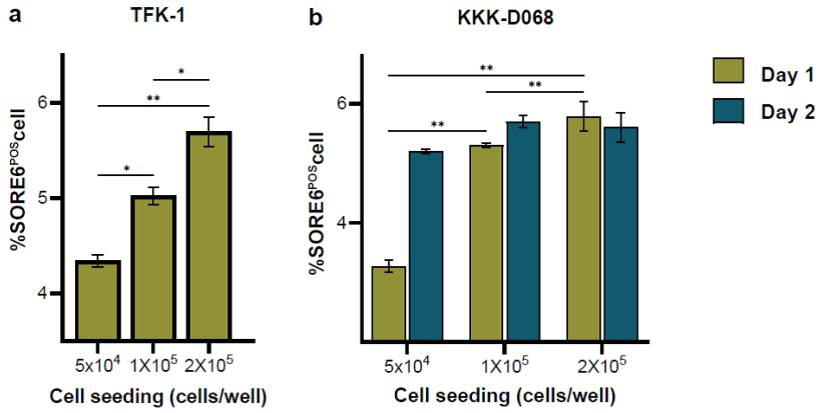

Fig. 1 Cell seeding density and culture duration affect the percentage of CCA stem-like cells in the 2-D culture. Percentages of CSCs in TFK-1 (a) and KKK-D068 (b) cells cultured in the 2-D common culture medium. The percentages of SORE6^{POS} cells were determined by flow cytometry. Data was analyzed by one-way ANOVA with Tukey's HSD post-hoc test.

MCS in each drug concentration. The GFP intensities were measured and calculated across drug treatment concentrations. To account for cellular plasticity and ensure fair comparison across drug concentrations, the normalized CSC index calculated by the GFP intensity of each drug concentration divided by the vehicle control was used as representative information.

Real-time quantitative reverse transcription PCR (RT-qPCR)

The Total RNA Purification Kit (GeneMark, Hsinchu City, Taiwan) was used for RNA extraction. One µg of RNA was converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). KAPA™ SYBR® FAST qPCR Master Mix Kit (Kapa Biosystems, Basel, Switzerland) was used for qPCR on the CFX96™ Real-Time PCR detection system (BioRad, Hercules, CA, USA). Primer sequences were: SOX2-F 5′-GAGCTTTGCAGGAAGTTTGC-3′; SOX2-R 5′-GCAAGAAGCCTCTCCTTGAA-3′, OCT4-F 5′-TCGA GAACCGAGTGAGAGG-3′; OCT4-R 5′-GAACCACACT CGGACCACA-3′, NANOG-F 5′-AAGGTCCCGGTCAAG AAACAG-3′; NANOG-R 5′-CTTCTGCGTCACACCATTG C-3′, GAPDH-F 5′-GTCAACGGATTTGGTCGTATTG-3′; GAPDH-R 5′-CATGGGTGGAATCATATTGGAA-3′. Gene expression was analyzed by fold change relative to the conventional medium condition.

Statistical analysis and data visualization

All graphs and statistical analyses were determined and created with the GraphPad Prism software and R (http://www.R-project.org/, version 4.3.3). All experiments were analyzed from at least 3 replicates, except for the effect of 2-D cell seeding densities, which was from a representative experiment of 2 replicates. Statistical significance was determined between each group using a two-tailed t-test for all experiments with 3 replicates. For the 2-D cell seeding density experiment (Fig. 1), statistical analysis was performed using a one-way analysis of variance (ANOVA) to determine if there were significant differences between the various seeding densities in the same culturing duration. Where significant differences were identified, Tukey's Honestly Significant Difference (HSD) post-hoc test was employed to perform pairwise comparisons and determine which specific seeding densities differed significantly from one another. A p-value < 0.05 was considered statistically significant for all tests. Statistical marks in all figures were abbreviated as follows: ns: $p > 0.05$, *: $p \leqslant 0.05$, **: $p \leqslant 0.01$, ***: $p \leqslant 0.001$, and ****: $p \leqslant 0.0001$.

RESULTS

Cell seeding density and duration of culture affect the percentage of CCA CSC

To investigate the impact of cell seeding density on CCA CSC percentages, the TFK-1 and KKK-D068 cells containing the CSC-specific live-cell biosensor [9] were seeded in a 6-well plate at various cell densities of 5×10^4, 1×10^5, and 2×10^5 cells/well, for 1 to 2 days, in common culture medium. We analyzed and found that CSC percentages were higher at high cell seeding densities, i.e. 5.80% in 2×10^5, 5.30% in 1×10^5, and 3.27% in 5×10^4 cells/well, in both TFK-1 and KKK-D068 cells (Fig. 1a,b). We found that after 2 days in culture, KKK-D068 cells grew and increased in cell densities, resulting in higher percentages of CSC (Fig. 1b). These findings suggested that cell density and culturing duration positively influenced the steady number of CSC in CCA culture. Interestingly, at the highest seeding density (2×10^5 cells/well), there was no increase in the CSC percentage from day 1 to day 2, suggesting a threshold exists beyond which increased cell density no longer enhances CSC numbers. From

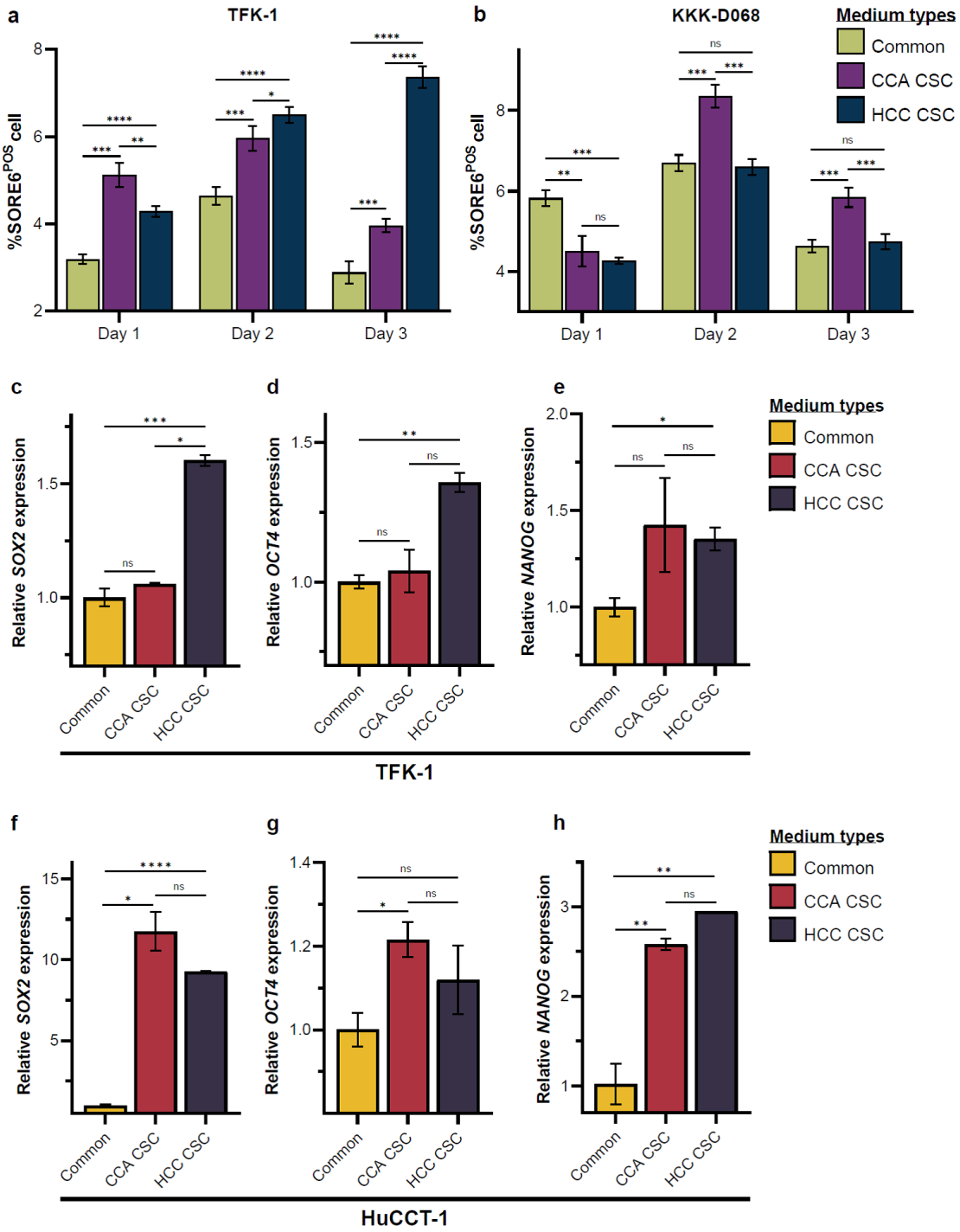

Fig. 2 Medium types affect the percentages of CCA stem-like cells and the expression of core pluripotent genes in the 2-D culture. Percentages of the CCA TFK-1 stem-like cells (a) and KKK-D068 stem-like cells (b) under different culture media. The mRNA expression of core pluripotency genes, including *SOX2* (c, f), *OCT4* (d, g), and *NANOG* (e, h) in TFK-1 (c–e) and HUCCT-1 (f–h) cell lines cultured in each type of medium for 3 days. The percentages of SORE6^{POS} cells were determined by flow cytometry. Common: common culture medium; CCA CSC: cholangiocarcinoma stem-like cell enriched medium; HCC CSC: Hepatocellular carcinoma stem-like cell enriched medium.

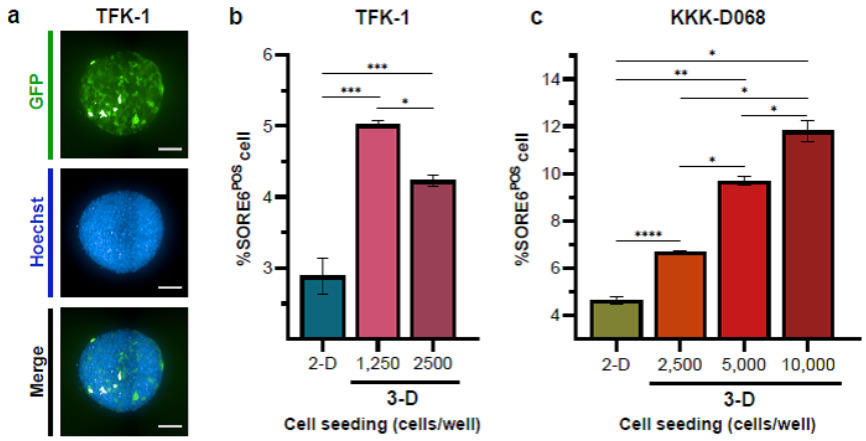

Fig. 3 3-D MCS culture enriches CCA stem-like cells. (a) Representative images of the 3-D spheroids cultured for 3 days at a seeding cell number of 1,250 cells/well. Scale bar = 100 µm. The images were taken using a 20× water immersion objective lens. Percentages of CSCs from different initial cell seeding numbers of TFK-1 (b) and KKK-D068 (c) cells in 3-D culture compared to 2-D culture (3 days culture in 6-well plate: 1×10^5 cells/well of TFK-1, 5×10^4 cells/well of KKK-D068). The percentages of SORE6^{POS} cells were determined by flow cytometry.

these results, the initial cell seeding density and culturing duration affect the steady number of the CSC in CCA culture.

Effect of culture medium on CSC quantity in CCA 2-D culture

To preserve the CSCs in CCA culture, specific culture media have been proposed [17]. There are a limited examples of culture media used in CCA stem-like cell studies [13]. Since, HCC shares lineage with CCA [18], we also included the HCC stem cell preserving medium in our study.

To investigate the effect of CSC-selective media on the 2-D culture of CCA, we compared the CSC percentages in CCA cultured in a common medium to 2 types of selective media, the CCA [13] and the HCC stem cell media [14]. We found that TFK-1 CCA cells cultured in both CCA and HCC stem cell media consistently yielded higher CSC percentages, compared to those cultured in the common medium (Fig. 2a,b).

To validate the increases of CSC by the CSC preserving media, we investigated the expression of core pluripotent factors, including *SOX2*, *OCT4*, and *NANOG* by RT-qPCR in TFK-1 and HuCCT-1 cells. The results showed upregulations of *SOX2*, *OCT4*, and *NANOG* mRNA in both cell lines, cultured in the CCA and HCC stem cell preserving media, compared to the common medium (Fig. 2c–h), confirming that these GFP-positive CCA cells were expressing the core stem cell transcription factors. Therefore, under our culturing conditions, specialized media can be used to increase the percentage of CCA stem-like cells.

The 3-D MCS culture enhances the CSC population in CCA

The 3-D multicellular spheroid culture allows cancer cells to grow in 3-D, in which partial tissue organization/structure formation is permitted and cell-cell and cell-extracellular matrix interaction and communication are preserved [19]. Thus, it has been proposed that 3-D culture may enhance the CSC population *in vitro* relative to 2-D culture [20].

To study the role of 3-D culturing protocol on the number of CSC in CCA, we performed the 3-D MCS cultures of TFK-1 and KKK-D068 cells containing the CSC-specific live-cell biosensor. The 3-D spheroids were labeled with a nuclear staining fluorescent dye, Hoechst 33342. The green fluorescent signals, representing the CSC population from the CSC biosensor in the Hoechst-positive area, were imaged and analyzed (Fig. 3a). We next compared the effect of 3-D and 2-D culturing systems on CSC percentages in the cultures. We found that the 3-D culture of TFK-1 cells displayed significantly higher percentages of CSCs, compared to the 2-D culture, in both cell densities (Fig. 3b). Consistently, the culture of KKK-D068 cells exhibited an increase in the percentage of CSCs from 4.63% in 2-D to 11.8% in the 3-D culture at 1×10^4 seeding density (Fig. 3c). These results establish that the 3-D culture is more permissive to the enrichment of CCA stem-like cells, making it a preferable method for studying CCA stem-like cells *in vitro*.

Elevated CSC indexes produced by chemotherapies

As we have demonstrated, the 3-D culture can increase the stable levels of CCA stem-like cells in the culture,

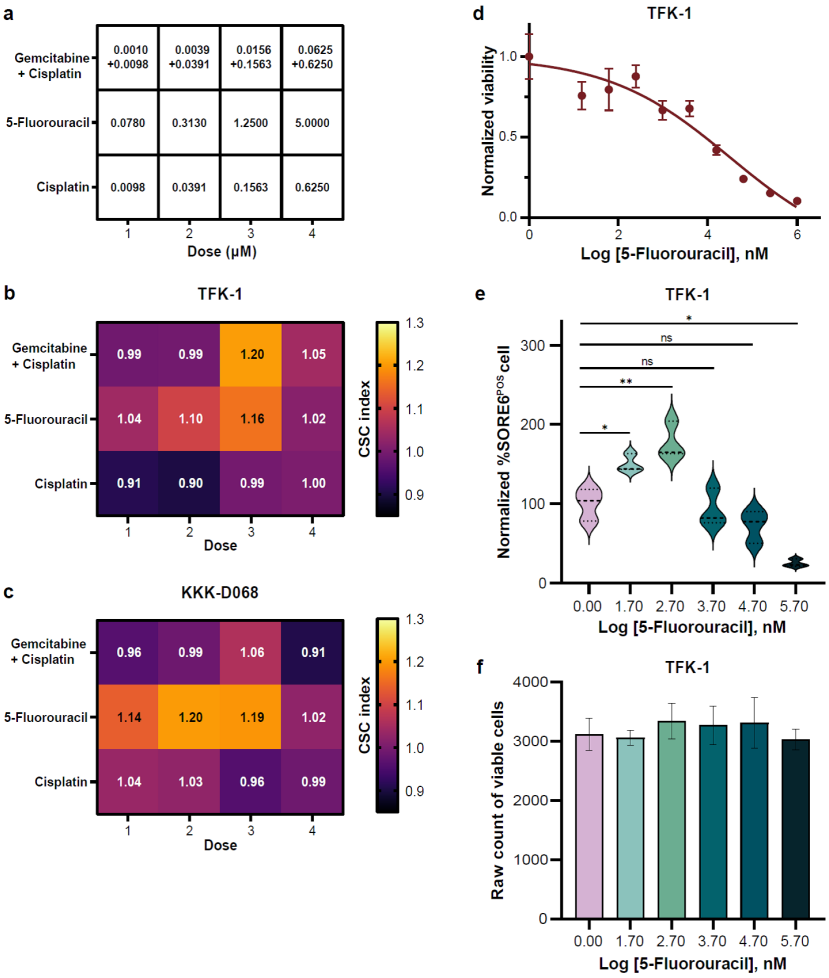

Fig. 4 Chemotherapies elevated CSC content in the 3-D CCA cultures. (a) Doses of the indicated regimens used in these experiments. CSC indices in the 3-D MCS treated by indicated regimens of TFK-1 (b) and KKK-D068 (c). The actual values of the index are as indicated. TFK-1 and KKK-D068 cells were seeded at 2,500 and 5,000 cells/well, respectively. The 3-D MCS was allowed to grow for 3 days before being treated with the drugs for 3 days. The dose-response curve (d), normalized percentage of SORE6^{POS} cells (e), and raw viable cell count (f) of TFK-1 3-D MCS under 3 days of 5-fluorouracil treatment. For (b) and (c), the heatmap color represents CSC indices, which were quantified from high-content imaging data. Normalized viability was determined by ATPlite™ assay. The normalized percentage of SORE6^{POS} cells, which was calculated from raw viable cell count, was determined by flow cytometry.

enabling a sufficient quantity of CSC for relevant research. We then used our system to study the role of standard anti-CCA chemotherapies on the content of the CCA stem-like cell under treatment. We created the 3-D spheroids of TFK-1 and KKK-D068 containing the CSC biosensor as cancer avatar models. These 3-D cultures were treated with various doses of anti-CCA drugs, including 5-fluorouracil, cisplatin, and a combination of gemcitabine and cisplatin (Fig. 4a).

Among the treatment regimens, we quantified the treated spheroid using high-content imaging and found that 5-fluorouracil consistently elevated the normalized CSC indices across all doses, ranging from 1.02 to 1.16 in TFK-1 cells and from 1.02 to 1.20 in KKK-D068 cells (Fig. 3b,c). The combination of gemcitabine and cisplatin caused a slight increase in CSC content at doses of 0.0156 + 0.1563 μM in both cell lines (Fig. 4b,c). On the other hand, cisplatin treatment showed unchanged CSC indices across all the doses (Fig. 4b,c). Additionally, we validated the CSC enrichment observed with 5-fluorouracil treatment using flow cytometry. We investigated the dose-response of 3-D MCS to the 5-fluorouracil treatment, ranging from 0.0153 to 1000 μM (1.18 to 6 Log_{10} nM) using the Adenosine TriPhosphate (ATP) monitoring system (Fig. 4d). We then explored the CSC percentages of

the normalized viability ranging from 0.853 to 0.116 by flow cytometry. The CSC percentages increased at dosage of 0.05 to 0.5 (1.7 to 2.7 Log_{10} nM) µM 5-fluorouracil concentrations. Notably, 0.5 µM (equal to 2.7 Log_{10} nM) of 5-fluorouracil resulted in the highest CSC percentage (Fig. 4e). The CSC percentages were calculated from $SORE6^{POS}$ cells among the viable population (Fig. 4f). These results indicated that certain regimens, but not all, such as 5-fluorouracil and the combination of gemcitabine plus cisplatin may enrich CCA stem-like cell *in vitro*.

DISCUSSION

We demonstrated here that CCA stem-like cells are presented in the *in vitro* culture systems. CCA is a malignancy characterized by intricate molecular and cellular pathways, necessitating the development of robust and accurate models to facilitate comprehensive research and therapeutic advancements [21–23]. Similar to other types of CSC, we demonstrated that the population of CCA stem-like cells is dynamic and can be influenced by culturing methods [13, 24, 25], including the types of culture media, density of cells in culture, and duration of culture. In addition, we verified that CCA stem-like cells may be significantly enriched when cultured in the 3-D compared to the 2-D cultures [13, 26]. Although this information has been reported in other types of cancer spheroids, or organoids [20, 27], the basic notion of how CCA stem-like cells behave in 2-D and 3-D culture and in varied culture conditions remains limited. Our results suggest that cell line-dependent variations may be influenced by factors such as anatomical subtypes, disease etiology, and epigenetic/genetic background alterations [5, 10]. We found that KKK-D068 cells inherently contain a higher number of CSC than TFK-1 both in 2-D and 3-D settings. Nevertheless, the number of CSCs is highly dynamic and influenced by cell confluence, media types, and the decrease in the media composition, highlighting the need for real-time monitoring.

Based on these results, we recommend conducting optimization before each CSC study. Our results indicated that the 3-D CCA spheroids containing the CSC-specific live-cell biosensor can effectively be used to study CCA stem-like cells under chemotherapy. The combination of the CSC live-cell biosensor in CCA cells and the 3-D culturing protocol allows real-time capturing of the CSC dynamic in response to various treatments or culturing conditions of interest. The GFP intensity represents the functional activity of SOX2/OCT4 proteins and was verified as the stem-like expression in variant cancer types including CCA. Given the high plasticity of CCA stem-like cells, as demonstrated in this study and our previous work [9], thus the expression of CCA stem-like cells should be compared to the identical culture conditions. Therefore, we created the normalized CSC index for these concerns. Drug-induced CSC enrichment might be the mix of either the survival ability of pre-existing or phenotypic conversion from non-CSC to CSC phenotype. Therefore, further elucidation of apoptosis and necrosis following drug treatment is required. Moreover, to further investigate the source of CSC enrichment, single-cell phenotypic tracking in a 3-D culture model might intensively explain this phenomenon. Altogether, the 3-D CCA spheroid platform is more flexible than organoid-based models, yet retains characteristics of tumor *in vivo* [19, 27, 28]. Recently, the results from 3-D drug testing have been considered as highly clinically transferable [29, 30]. We propose that this platform is also applicable for high-throughput drug testing. Therefore, the application of our system for the identification of new anti-CSC drug regimens, especially in CCA, will be performed.

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