

# Culturing in serum-free culture medium on collagen type-I-coated plate increases expression of CD133 and retains original phenotype of HT-29 cancer stem cell

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## Abstract

**Background:** A sub-population of tumor cells termed cancer stem cells (CSCs) has an important role in tumor initiation, progression, and recurrence. Selecting a suitable procedure for isolation and enrichment of CSCs is the biggest challenge in the study of CSCs. In the present study, the role of the combination of stem cell culture medium and collagen type-I was evaluated for successful isolation and enrichment of HT-29 CSCs.

**Materials and Methods:** HT-29 cells were cultured in serum-containing medium (parental culture medium: Medium + 10% fetal bovine serum) and serum-free medium (stem cell culture medium); both on collagen-coated plates. Spheres forming ability and CD133 expression, as a potential marker of colorectal CSCs, were evaluated in two culture mediums.

**Results:** The results show spheroids usually give rise completely within 15 days in the stem cell culture medium on the collagen-coated plate. CD133 expression in spheroid cells (84%) is extensively higher than in parental cells (25%). Moreover, relative to parental cells, spheroid cells were more radioresistance.

**Conclusion:** Finding of this study suggested that CSCs derived from colon cancer cell line (HT-29) can be propagated and form colonospheres in serum-free culture medium on collagen type-I. According to maintenance of their original phenotype in these conditions, it seems serum-free culture medium on collagen type-I is a suitable way to drug screening of HT-29 CSCs.

**Key Words:** Cancer stem cell, CD133 expression, collagen type-I, spheroid

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## INTRODUCTION

Colorectal cancer is the third leading cause of

cancer-related mortalities in the world. Despite the development in many therapeutic modalities, tumor

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recurrence and metastasis are still the major problems in the colon cancer treatment.<sup>[1]</sup> Increasing evidences suggest that a small sub-population of cancer cells is responsible for tumor initiation and metastasis. As this sub-populations' features are parallel to stem cells, it was termed cancer stem cell (CSC). Self-renewal, high potential to differentiate to other bulk tumor cells, and high proliferation are the main characteristics of CSCs.<sup>[2,3]</sup>

Lapidot, *et al.*<sup>[3]</sup> were the first researchers who demonstrated the existence of CSCs in leukemia cancer in 1994, and later in solid tumors including colon cancer, pancreatic, breast, head and neck, glioblastoma (GBM), and lung.<sup>[4]</sup> Insensitivity of CSCs to conventional therapies supports the hypothesis of CSCs contribution to tumor recurrence. According to CSCs' characteristics, therapeutic targeting and elimination of these cells represent novel opportunities to eradicate cancer and prevent the recurrence.<sup>[5,6]</sup> To achieve this aim, the first step is isolation, characterization, and cultivation of CSCs *in vitro* in order to examine the anti-CSC activity of each individual therapeutic approaches.

Commonly, isolating CSCs involve cell sorting of a sub-population based on cell surface markers expressing on CSCs. This procedure is followed by confirmation of their tumor-initiating potential in xenograft transplantation assays. Many studies have presented CD133 as a specific marker for GBM,<sup>[7,8]</sup> prostate,<sup>[9]</sup> and liver carcinoma<sup>[10]</sup> CSCs. Furthermore, this marker exclusively has been reported as a cell surface marker for colorectal cancer.<sup>[11-14]</sup> Besides these evidences, results of some studies do not agree with CD133 as a single marker in colon CSCs. Such studies recommend that cells with a combination of CD133<sup>+</sup> and CD44<sup>+</sup> markers have the highest tumor initiating potential rather than cell populations with either CD133<sup>-</sup> or CD44<sup>-</sup>.<sup>[15,16]</sup> On the other hand, Du, *et al.*<sup>[17]</sup> demonstrated that CD44, not CD133, is the robust marker for colorectal CSCs. Therefore, there is a doubt about these cell surface markers and the ideal specific markers for colon CSCs.

The other way to isolate the CSCs from the bulk of the tumor is using serum-free medium (SFM). Lack of serum in medium causes anchorage-dependent cells to detach from the extracellular matrix so that they will be negatively screened and die through anoikis. However, CSCs as anchorage-independent cells escape from anoikis.<sup>[2]</sup> Emerging studies have presented that in SFM, isolated CSCs from GBM,<sup>[18]</sup> breast,<sup>[19]</sup> melanoma,<sup>[20]</sup> ovarian<sup>[21]</sup> and colon cancers,<sup>[12,22]</sup> frequently grow as nonadherent and form three-dimensional (3D) spheres that are efficiently enriched with CSCs.

Despite these evidences, retaining of CSCs' phenotype is of high importance in the study of CSCs in *in vitro* conditions. In other words, culture condition not only should cause isolating and enriching CSCs but also should allow CSCs to retain their original phenotype during the experiments. Moreover, differentiation of CSCs is one of the complications that may occur during the experiment.<sup>[5,23]</sup>

Kirkland,<sup>[24]</sup> suggested that adherent culture on type-I collagen in serum-free stem cell medium, not only enriches CSCs population, but also retains CSCs' characteristics and increases the expression of CD133. In another study, it has been shown that culturing of CD44<sup>+</sup>/CD133<sup>+</sup> cells in the stem cell medium on type-I collagen-coated plate increases *in vivo* tumorigenic capacity and sphere forming potential of this cell phenotype.<sup>[23]</sup>

In colon cancer cells, collagen type-I inhibits cell differentiation and promotes the expression of both CD133 and Bmi-1 stem cell markers. Furthermore, culturing the CSCs in SFM as adherent condition using collagen-coated plate can provide an opportunity for preliminary estimation of the CSCs-focused drug response.<sup>[23]</sup>

According to advantages of isolation and expansion of CSCs *in vitro* under serum-free culture medium using adherent condition on type-I collagen, in the present study, we evaluated the possibility of isolation, sphere formation and the variation of CD133 expression of CSCs (HT-29) in SFM on collagen-coated plate. Furthermore, as reported in previous research,<sup>[25]</sup> floating cells have strong stem cell properties, therefore, in this study, CD133 expression on CSC s in SFM on collagen-coated plate was compared with CD133 expression on floating cells. Moreover, emerging evidence suggests that normal CSCs had a higher proportion of G0/G1 phase cells and a lower proportion of G2/M phase cells compared with non-CSCs and progressing slowly through the cell cycle. Therefore in this study, cell cycle distribution was investigated for CSCs-HT-29 and non CSC-HT-29.<sup>[26-28]</sup>

## MATERIALS AND METHODS

### Parental ht-29 cell culture

Colorectal (HT-29) cell line was purchased from Pasteur Institute (Tehran, Iran). Cells were cultured in RPMI 1640 medium (Gibco-Invitrogen) supplied by 10% fetal bovine serum (FBS), (Gibco-Invitrogen), 1% penicillin/streptomycin (Sigma-Aldrich): Parental cell medium (PCM). The cells were stored at humidified atmosphere in 37°C with 5% CO<sub>2</sub>. The

cells' medium was changed approximately every 2 days. When the cells reached more than 80% of confluency, were split with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) and sub-cultured for more passages.

### Cancer stem cells culture (adherent-sphere culture)

HT-29 cells which have been grown in PCM detached with trypsin and seeded on type-I collagen-coated dishes: Becton Dickinson (BD) in serum-free DMEM/F12 medium (SFM) containing 6 mg/ml glucose; 1 mg/ml NaHCO<sub>3</sub>; 5 mM HEPES; 2 mM l-glutamine; 4 mg/ml heparin; 4 mg/ml bovine serum albumin (BSA); 10 ng/ml basic fibroblast growth factor; 20 ng/ml epidermal growth factor; 100 mg/ml apotransferrin; 25 mg/ml insulin; 9.6 mg/ml putrescin; 30 nM sodium selenite anhydrous; 20 nM progesterone (Sigma-Aldrich), and 2 ml 50 × B27 supplement (Invitrogen)<sup>[2,29]</sup> other portion of cells were cultured in PCM on type-I collagen-coated dishes (BD).

### Effect of collagen on sphere formation in serum-free medium

A single cell suspension of parental cells was seeded into the collagen-coated plate and control uncoated plate in SFM. After 5 days, the effect of collagen on adhering of floating cells and sphere formation was evaluated.

### Flow cytometry analysis of cd133 expression

Singled cells derived from PCM culture and cells derived from SFM culture were subjected to direct immunofluorescence staining followed by flow cytometry analysis after 5 days' culturing. The cells were washed two times with phosphate buffered saline (PBS) and suspended in the sample buffer (PBS, 0.5% BSA and 2 mM Ethylenediaminetetraacetic acid, EDTA). Then FCR blocking reagent and anti-CD133 (AC133, mouse IgG1, Miltenyi) were added. The samples were mixed well and incubated in the dark for 30 min at 4°C. The analysis was performed with FACS caliber (BD Biosciences, USA) using the Cell Quest software. Furthermore, CD133 expression on floating cells was investigated.

### Cell cycle analysis

Cancer stem cells-HT-29 (cells in SFM culture) and non-CSCs HT-29 (cells in PCM culture) after 5 days' culturing, trypsinized and washed twice in PBS, then 10<sup>6</sup> cells fixed overnight in 70% ethanol at -20°C. After this time, the cells were centrifuged, re-suspended in 0.5 ml propidium iodide (PI) staining solution (containing 50 µg/ml PI and 10 µg/ml RNase) for 30 min at RT. Sample was analyzed using an FACS Caliber flow cytometer (BD Biosciences, USA).

## RESULTS

### Effects of serum-free medium on HT-29 cells

In CSC medium, the primary differentiated carcinoma cells die after 5 days, and the cells with stem cell features survived and grew as 3D spheres.<sup>[2]</sup> As parental HT-29 cells exposed to CSC medium SFM, some black dots appeared on the plate on day 5 of culture. Such dots represent the dead differentiated colon carcinoma cells. Other cells grow as clusters, termed tumor spheres [Figure 1a and b]. Figure 1c and d shows that on the 10<sup>th</sup> day, in SFM, spheres were formed completely, but in PCM, parental cells grew as an integrated surface.

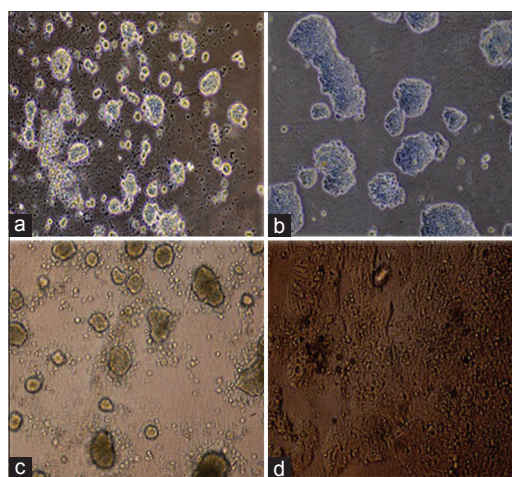
Formation of spheres was observed from day 5 to 15; the sphere size increased during this period. In Figure 2, the morphology of sphere growth in free FBS medium on collagen type-I, has been shown for up to day 15.

### Effect of collagen on sphere formation

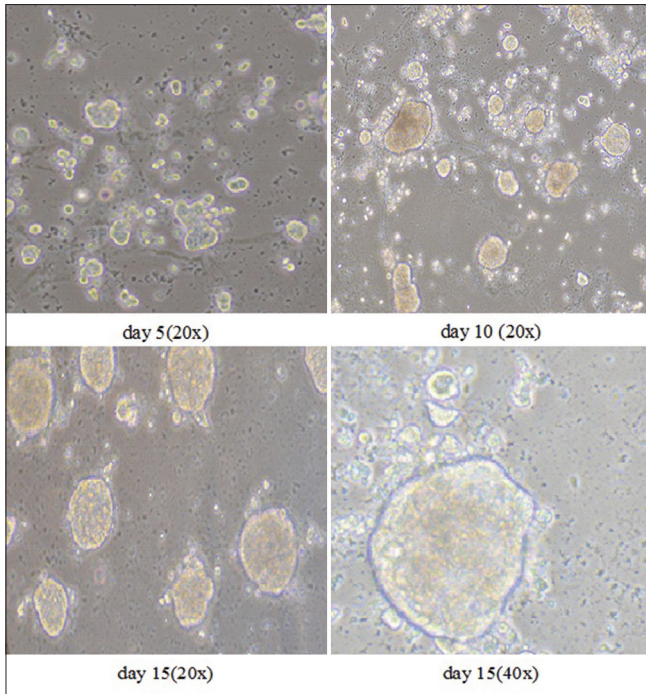
Parental HT-29 cells were seeded onto a collagen-coated plate and un-coated plate in SFM. After 5 days, on collagen-coated plate against of un-coated plate, floating cells was not seen, sphere formed and enlarged in following days [Figure 3].

### CD133<sup>+</sup> Expression in parental cell medium and serum-free medium on collagen type-I

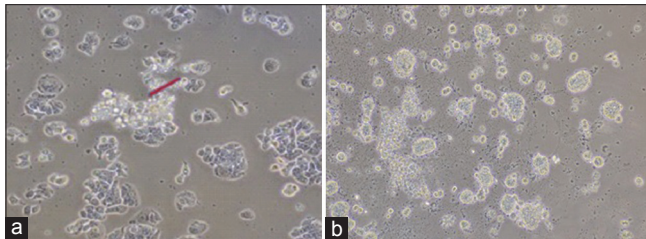
In many studies, CD133<sup>+</sup> introduced as a specific marker for different CSCs such as colon cancer cells. Our results indicated the proportion of CD133<sup>+</sup> in parental HT-29 cells was approximately 25%. However, for serum-free, stem cell culture medium, 84% of these



**Figure 1:** Parental HT-29 cell culture; (a) in serum-free medium (SFM) on the collagen-coated plate at day 5. Dark dots represent dead differentiated cells. Survived cancer stem cells have formed clusters named spheres; (b) in parental cell medium (PCM) on collagen-coated plate at day 5; (c) in SFM on collagen-coated plate at day 10; (d) in PCM on collagen-coated plate at day 10, (x20)



**Figure 2:** Sphere growth generated by incubating parental HT-29 cells in serum-free medium on collagen-coated plate on different days of culture



**Figure 3:** Parental HT-29 cells in serum-free medium (a) onto un-coated plate (b) onto collagen-coated plate, floating cells were shown with arrow onto un-coated plate

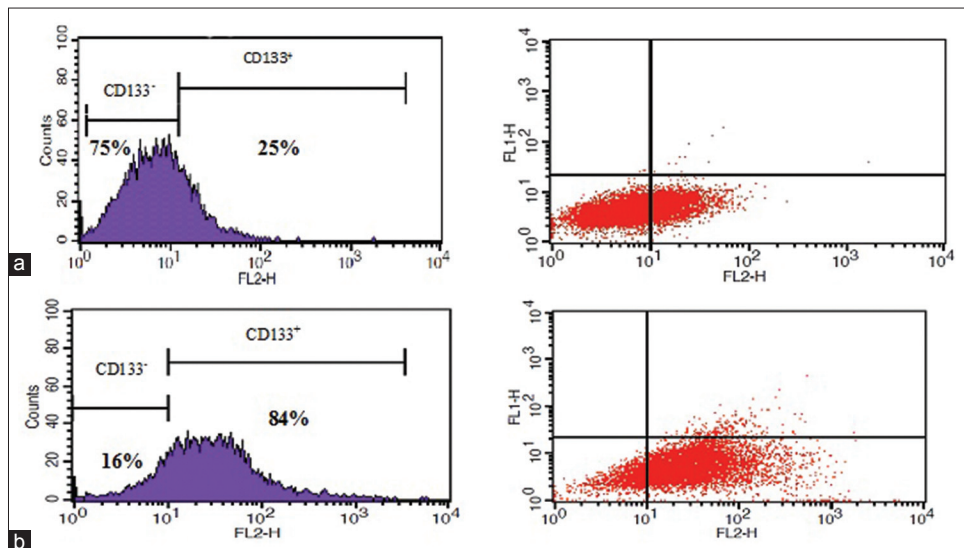
spheroid cells were positive in terms of CD133 expression as demonstrated by flow cytometry [Figure 4]. Furthermore, the proportion of floating cells expressing CD133 was approximately 97%. The expression rate of CD133 on floating cells was close to that in SFM onto collagen-coated plate [Figure 5].

**Cell cycle distribution of cancer stem cells (CSCs)-HT-29 (cells in serum free medium culture) and non-CSCs HT-29 (cells in parental cell medium culture)**

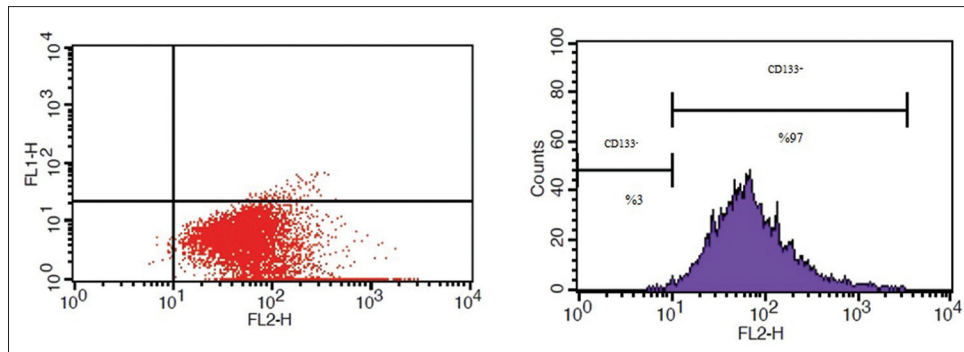
Cell cycle distribution were measured in CSCs-HT-29 and non-CSCs HT-29 cells. As displayed in Figure 6, CSCs-HT-29 had a significantly higher proportion of G0/G1 phase cells compared with non-CSCs HT-29 ( $P < 0.05$ ), whereas G2/M phase proportion was significantly decreased ( $P < 0.05$ ).

**DISCUSSION**

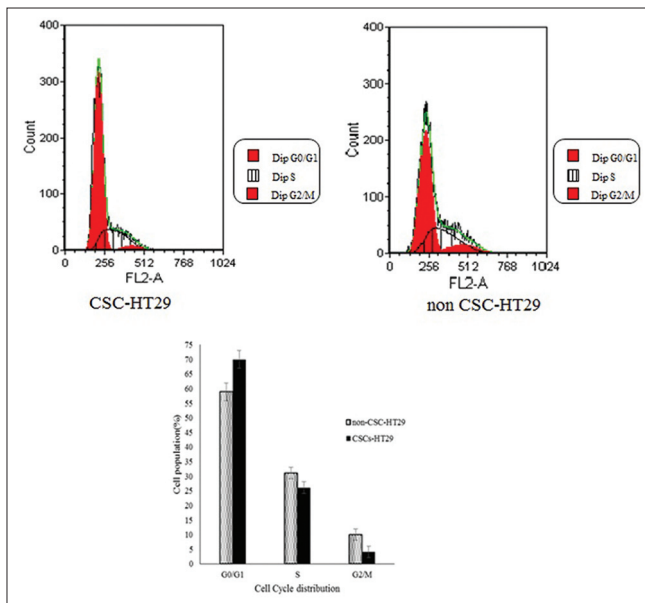
Recently, the role of CSCs in tumor initiation, progression and recurrence, and their resistance to chemotherapy and radiotherapy has been exclusively studied. Regarding this issue, many researchers are interested to understand the biological properties of CSCs to be able to design new therapeutic approaches to eradicate CSCs.<sup>[5,30-32]</sup> Therefore, the first step to proceed toward these aims is to isolate and learn the characteristics of CSCs.<sup>[33]</sup> Commonly, CSCs grow and propagate as unattached floating 3D-spheroids in SFM; supplemented with specific growth factors.<sup>[9]</sup> Cammareri *et al.*<sup>[2]</sup> demonstrated that in SFM differentiated cells die after 5 days of culture, while undifferentiated cells in this medium survive and proliferate. Fang *et al.*,<sup>[5]</sup> reported that in SFM isolated human colon cancer cells form nonadherent spheroids which 90% of these spheroid cells were



**Figure 4:** Expression of CD133 on HT-29 cells in (a) Parental cell medium, (b) Serum-free medium



**Figure 5:** Proportion of floating cell membranes expressing CD133



**Figure 6:** Cell cycle distribution in cancer stem cells (CSCs)-HT-29 and non CSCs-HT-29. Cells were harvested, fixed, and stained with propidium iodide, CSCs-HT-29 showed a significant increase in G0/G1 phase proportion compared with non CSCs-HT-29 while the G2/M phase proportion was significantly decreased

positive for CD133/EPCAM. These tumor spheroids had high proliferation and self-renewal capacity. After exposing the tumor spheroids with serum medium, cells were differentiated and expression of CD133 decreased from 90% in spheroid population to 40.5% in the differentiated adherent population.

Wei, *et al.*<sup>[32]</sup> proposed cells grow as nonadherent spheroids when cultured in SFM. Cells of these spheroids have higher tumorigenic and metastatic potential comparing to their parental cells. Therefore, they possess the properties of CSCs. After 7 days in a serum-free culture, the proportion of CD133 expression on HCT116 and HT-29 spheroid cells were 80–60%, respectively. However, it declined to 1% in serum medium. In another study on HT-29 cells,<sup>[34]</sup> there was no significant difference between the proportion of CD133 positive cells in nonadherent spheroids and

parental cells (6.25% and 5.6%). On the other hand, there are some reports showing that a nonadherent culture may lead to increase the instability of CSCs over multiple passages. Hence, CD133 expression may change as reported. Motegi, *et al.*<sup>[35]</sup> reported that GBM stem cells were unstable when propagated in SFM and floating condition, whereas in SFM and adherent condition on collagen type-I, were stably propagated over 20 passages. The expression of CD133 as one of the major GBM stem cell markers was significantly increased on collagen type-I. Authors have shown that collagen type-I inhibits cell differentiation and promotes the expression of CD133 in colorectal carcinoma.<sup>[23,24]</sup> These reports suggest that collagen type-I seems to be suitable for culture and maintenance of other CSCs. On the other hand, adherent culture provides a suitable condition for drug screening. In nonadherent condition, cells would not be exposed equally to various reagents, also, in this condition carrying out the viability tests such as MTT-assay is difficult. Regarding the advantages of the adherent culture of CSCs on collagen type-I rather than unattached floating spheroid, in the present study, we evaluated the ability of HT-29 cells in forming spheroids in SFM and adherent condition on collagen type-I. In accordance with previous studies,<sup>[2,5,32]</sup> it was observed that differentiated cells died after 5 days of culture in SFM and sphere formed on 15 days. This evidence suggests that tumor spheres from HT-29 by culturing them into the collagen-coated plate in SFM can be achieved. Furthermore, considering the doubt existed on CD133 expression of HT-29 stem cells, we detected a proportional CD133 expression on parental cells and spheroids that formed in stem cell cultures on collagen type-I statistically, CD133 positive cells in parental cells were <25%. However, this was more than 84% on spheroid cells that formed into collagen-coated plate in SFM. Furthermore, CD133 expression on spheroids cells was close to floating cells.

Cell cycle may function as a regulatory process in radioresistance. Cells are the most radiosensitive in

the G2-M phase, less sensitive in the G1 phase, and least sensitive in late S phase. Cell cycle progression between radiation fractions permits redistribution of radioresistant s-phase tumor cells into a more sensitive phase of the cell cycle which cause maximal killing of the tumor cells however CSCs are usually in G0/G1 phase, a relatively stationary phase therefore progressing slowly through the cell cycle and are more radioresistance than non CSCs. Our results showed CSCs-HT-29 had increased G0/G1 phase proportion, decreased G2/M phase proportion compared with its parental cells, indicating they are relatively quiescent. These results were in agreement with the previous stem cell studies, representing the slow-cycling nature of stem cells.<sup>[26,27,36]</sup>

From results of this study, it is concluded that HT-29 stem cells were enriched in these spheroid cells. Therefore, it seems that culturing them in SFM on the collagen-coated plate is a suitable approach for isolating, propagating and deliberating the properties of HT-29 stem cells.

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### Conflicts of interest

There are no conflicts of interest.

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