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The expression of cancer stem cell markers in human colorectal carcinoma cells in a microenvironment dependent manner

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ABSTRACT

Numerous lines of evidence support the hierarchical model of cancer development and tumor initiation. According to the theory, cancer stem cells play a crucial role in the formation of the tumor and should be targeted for more effective anticancer treatment. However, cancer stem cells quickly loose their characteristics when propagated as 2D cell culture, indicating that the 2D cell culture does not provide the appropriate settings to maintain an in vivo environment. In this study we have investigated the expression of self-renewal, cancer stem cell and epithelial to mesenchymal transition markers after the transfer of human colorectal carcinoma cell DLD1 and HT29 lines from 2D cell cultures to scaffold-attached laminin rich extracellular matrix and scaffold-free multicellular spheroid 3D culture models. Based on the up-regulated expression of multipotency, CSC and EMT markers, our data suggests that human colorectal carcinoma cells grown in 3D exhibit enhanced cancer stem cell characteristics. Therefore, in order to design more efficient targeted therapies, we suggest that 3D cell culture models should be employed in cancer stem cell research.

1. Introduction

According to the hierarchical model of tumor development, tumors are initiated and maintained by a small population of slow cycling cells that possess stem cell-like characteristics. These cells are considered as cancer stem cells (CSCs) and are defined by an undifferentiated cell status, plasticity and self-renewing, like normal somatic stem cells [1,2]. CSCs display a highly heterogeneous population, which sustains dynamic clonal modifications during tumor progression. Thus, CSCs are involved in the resistance to conventional therapeutic agents leading to the repopulation of tumor cells following initial reduction [3]. The resilience of CSCs is typically maintained by an elevated expression of drug efflux proteins or increased DNA repair activity. In addition, the plasticity of CSCs might reduce the therapeutic efficiency as non-stem like tumor cells are capable of a spontaneous conversion to CSC and vice versa [4]. Furthermore, recent studies have linked CSCs with the epithelial-mesenchymal transition (EMT) [5] and tumor metastasis [6] indicating that metastasizing cells could possess or gain CSC features during tumor invasion, eventually leading to the initiation of secondary tumors. However, the CSC-driven molecular mechanisms, which orchestrate tumor progression and resistance to anti-cancer therapies, still remain unclear. Therefore, a better

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understanding of CSC biology could lead to a better optimization of new cancer treatment strategies.

In order to study the biological functions and the response to various types of therapeutic treatment, an appropriate cell model for CSCs is required. In most in vitro studies, two-dimensional (2D) cancer cell culture models are applied. However, such cell culture models poorly represent the complexity of the tumor, as cancer cells grown in 2D predominantly interact with the plastic surface in a growth medium enriched with concentrated nutrients, lacking the appropriate cell-ECM and cell-cell interactions [7]. As tumor initiation is one of the main characteristics defining CSCs, the orthotopic transplantation of tumor cells remains a central model for the CSC research [8]. However, the tumor microenvironment and growth factor background in the xenograft model may not be adequate to the conditions found in patients' tumor. In addition, this technique requires harsh experimental procedures testing for the presence of the most robust growing tumor cells. All these limitations could alter tumor cell growth in the xenograft assay.

By contrast, three-dimensional (3D) cell models provide conditions more akin to those that exist in vivo. These models exhibit regions of hypoxia, zones of quiescent and proliferating cells, chemical gradients and 3D cell-cell and cell-ECM interactions, all of which are maintained in a solid tumor microenvironment [9]. In addition, the response of cancer cells grown under 3D conditions to therapeutic agents is thought to be more representative of actual tumor cells [10]. Furthermore, it was demonstrated that hypoxic niches formed under 3D cell culture conditions could promote CSC conversion or at least self-renewal of already existent CSCs, increase the enrichment of cancer cells positive for CSC markers and enhance cellular tumorigenicity in mice compared to cells grown in a monolayer [11–13]. These observations indicate that increased drug resistance observed in cancer cells grown in a 3D microenvironment could be related to the expansion of CSCs. Therefore, the characterization of cell culture models that better maintain the CSC niche could promote the development of new anti-cancer therapy strategies based on targeted treatment against CSCs.

In this study we examined the changes in gene expression related to the maintenance of CSCs including genes implicated in hypoxia, CSC marker, multipotency and EMT in human colorectal carcinoma (CRC) cell lines DLD1 and HT29 grown in 3D versus 2D cell culture conditions. In order to elucidate the changes in gene expression we employed two 3D cell-culturing methods: 3D laminin enriched extracellular matrix (ECM) and multicellular spheroid (MCS) culture formed by liquid overlay technique. Our present data indicate that CRC cells grown in 3D culture models exhibit enhanced expression of genes regulating CSC properties.

2. Materials and methods

2.1. Cell lines

Human colorectal carcinoma DLD1 and HT29 cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were maintained in an RPMI-1640 (DLD1) or DMEM (HT29) cell culture medium (Gibco, Germany), respectively. Both media were supplemented with 10% fetal bovine serum (Gibco, Germany), 2 mM glutamine (Gibco, Germany), 1 mM sodium pyruvate (Gibco, Germany), 100 UI/mL penicillin (Sigma) and 0.1 mg/mL streptomycin (Sigma). Cell culturing was carried out in 25 cm^2 plastic cell culture flasks at 37 °C in a 5% CO_2 humidified incubator.

2.2. 3D cell culture experimental design

For the evaluation of changes in gene expression between 2D and 3D, cells, initially grown as monolayer, were transferred to 3D ECM and MCS cultures. All experiments were performed following 2 days or 6 days of cell growth and repeated at least 3 times. For the 2D cell culture, DLD1 and HT29 cells were plated in 9.5 cm^2 plastic 6 well cell culture plates and cell density was as follows: 2.52×10^5 and 1.26×10^4 cells for 2 and 6 days of cell growth, respectively. For 3D ECM cell culture, 0.5×10^5 of DLD1 and HT29 cells were embedded in 1 mL of 0.5 mg/mL lr-ECM protein mixture Geltrex (ThermoFisher Scientific, USA) in culture medium in 24 well plates as described previously [14]. To avoid cell attachment to the well bottom, each well was pre-coated with 1% agarose in sterile water. Multicellular spheroids (MCS) were formed using liquid-overlay technique as described previously [15]. Briefly, 0.7×10^4 DLD1 cells and 3.5×10^4 HT29 cells were suspended in 200 μL cell culture medium and plated in each well of 96 round-bottom well plates pre-coated with 1% agarose in water gel and centrifuged at 1000 × g for 10 min. Cells were photographed every second day with inverted optical microscope Eclipse TS100 and digital camera DS-Fi2 (Nikon, Japan). MCS size was evaluated using SpheroidSizer 1.0 [16].

2.3. RNA extraction

After 2 days and 6 days of cell growth, cells grown in 2D, lr-ECM 3D and MCS were harvested. Total RNA was isolated from approximately 2×10^6 cells using Quick RNA MiniPrep (Zymo Research, USA) according to manufacturer's instructions. The quantity and quality of RNA were measured with Nanodrop (ThermoFisher Scientific, USA).

2.4. RT-qPCR

cDNA were synthesized using Revert Aid RT Kit (ThermoFisher Scientific, USA) according to manufacturer's instructions. A total of 1 μg RNA was used for cDNA synthesis. Quantitative real-time PCR was performed using Realplex4 Mastercycler thermocycler (Eppendorf, USA) and Kapa SYBR Fast qPCR Master Mix (2×) (Kapa Biosystems, USA) according to manufacturer's instructions. Briefly, for each reaction in 96 well plate 1 μL of 5 times diluted cDNA, 5 μL Kapa SYBR FAST qPCR Master Mix, 3.8 μL nuclease-free water and 2 μM forward and reverse primer was used. PCR cycling conditions included polymerase activation at 95 °C for 3 min, followed by 40 2-step amplification cycles consisting of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. The relative changes in gene expression were calculated by ΔΔCt method using HPRT1 as the housekeeping gene for sample normalization [17]. Primer sequences are shown in supplementary file 1.

2.5. Statistical analysis

Each data point is displayed as the mean ± standard deviation of three independent biological experiments. Student's *t*-test was applied to determine the differences between groups and statistical significance was considered at $p < 0.05$.

3. Results

3.1. Three-dimensional cell culture growth conditions induce hypoxia related gene expression

In order to compare cells grown under 2D and 3D cell culture conditions, CRC, DLD1 and HT29 cells were grown as a plastic-attached monolayer (2D), scaffold-attached cells in laminin-rich extracellular matrix (3D ECM) and scaffold-free multicellular spheroids (3D MCS).

2 days following plating, we observed colonies of flat, laterally adjacent cells in the monolayer culture. After 6 days of growth, colonies reached approximately 80% confluence. Whereas cells embedded in a 3D ECM gelatinous protein scaffold formed irregular shaped cell aggregates of 79 ± 33 μm and 42 ± 21 μm in diameter for DLD1 and HT29 cells (Fig. 1A) after 2 days of incubation. The aggregates further reached diameters of 141 ± 51 μm and 145 ± 46 μm for DLD1 and HT29, respectively, on the 6th day of culturing in 3D ECM. Otherwise, cells cultured in MCS culture formed a single aggregate per well. MCS reached diameters of 415 ± 10 μm for DLD1 and 410 ± 10 μm for HT29 cells (Fig. 1A) after 2 days of incubation. MCS continuously increased in size and reached diameters of 548 ± 9 μm for DLD1 and 582 ± 9 μm for HT29 cells, respectively, after 6 days of cell growth.

In order to investigate whether CRC cell growth in a 3D cell culture results in the formation of response to hypoxia, hypoxia-dependent gene expression changes were evaluated after 6 days of cell culturing and compared to expression levels in cells grown as a 2D monolayer. For the analysis, we have selected hypoxia-related genes of CAIX, GLUT1 and VEGFA. After 6 days of cultivation, DLD1 and HT29 cells grown in 3D lr-ECM environment exhibited up-regulation of CAIX, GLUT1 and VEGFA gene expression (Fig. 1B and C). In addition, CRC cells cultured in 3D ECM, over-expressed hypoxia-related genes in a more vigorous manner than in cells grown in MCS.

3.2. Expression of multipotency regulating genes is activated in multicellular spheroid culture

To determine whether cellular multipotency is regulated in a 3D environment dependent manner, we evaluated the expression of multipotency markers NANOG, LIN28, SOX2, KLF4, OCT4 and c-Myc in DLD1 and HT29 cells grown in 3D models, as compared to expression levels in cells cultured in 2D. RT-qPCR data analysis revealed that after two days of cell growth the expression of NANOG and KLF4 genes was significantly down-regulated in DLD1 cells grown in 3D ECM (Fig. 2A), while LIN28, SOX2, OCT4 and c-Myc showed no considerable changes in expression. On the other hand, DLD1 cells grown in multicellular spheroids exhibited significantly elevated expression of multipotency markers compared to 2D. Interestingly, 6 days after plating DLD1 cells in multicellular spheroids showed no significant changes in gene expression when compared to the 2D culture (Fig. 2C).

Similarly to DLD1 cells, the expression of NANOG and KLF4 was

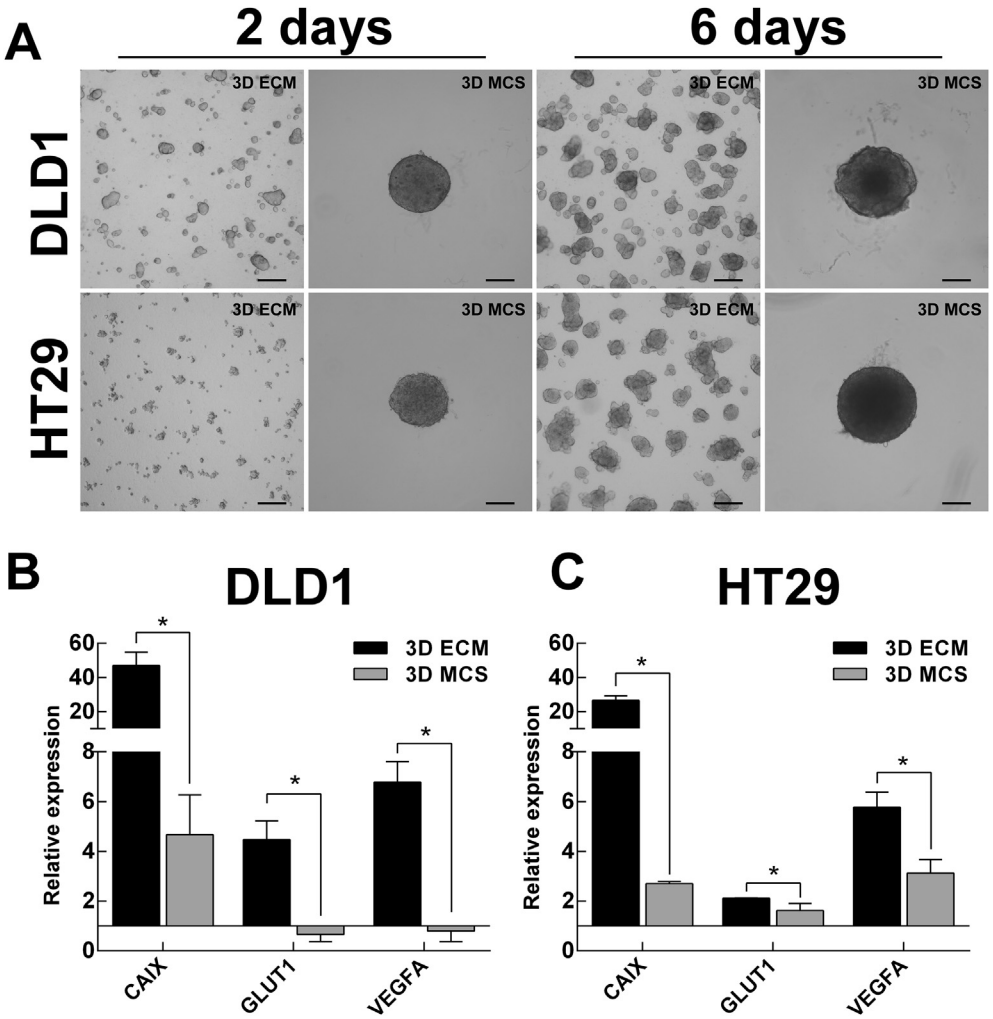

Fig. 1. Three-dimensional cell culture growth conditions induce hypoxia related gene expression. Representative phase contrast images of 3D ECM and MCS cell growth after 2 and 6 days (**A**). Graph showing relative expression of genes involved in cellular response to hypoxia (CAIX, GLUT1 and VEGFA) in DLD1 (**B**) and HT29 (**C**) cells grown in 3D ECM and MCS cell cultures compared to expression levels in CRC cells grown in 2D. Relative gene expression was evaluated following 6 days of cell growth. RT-qPCR data analysis was based on $2^{-\Delta\Delta Ct}$ and HPRT1 was used as the housekeeping gene for RT-qPCR data normalization. Results show mean ± SD (n = 3).

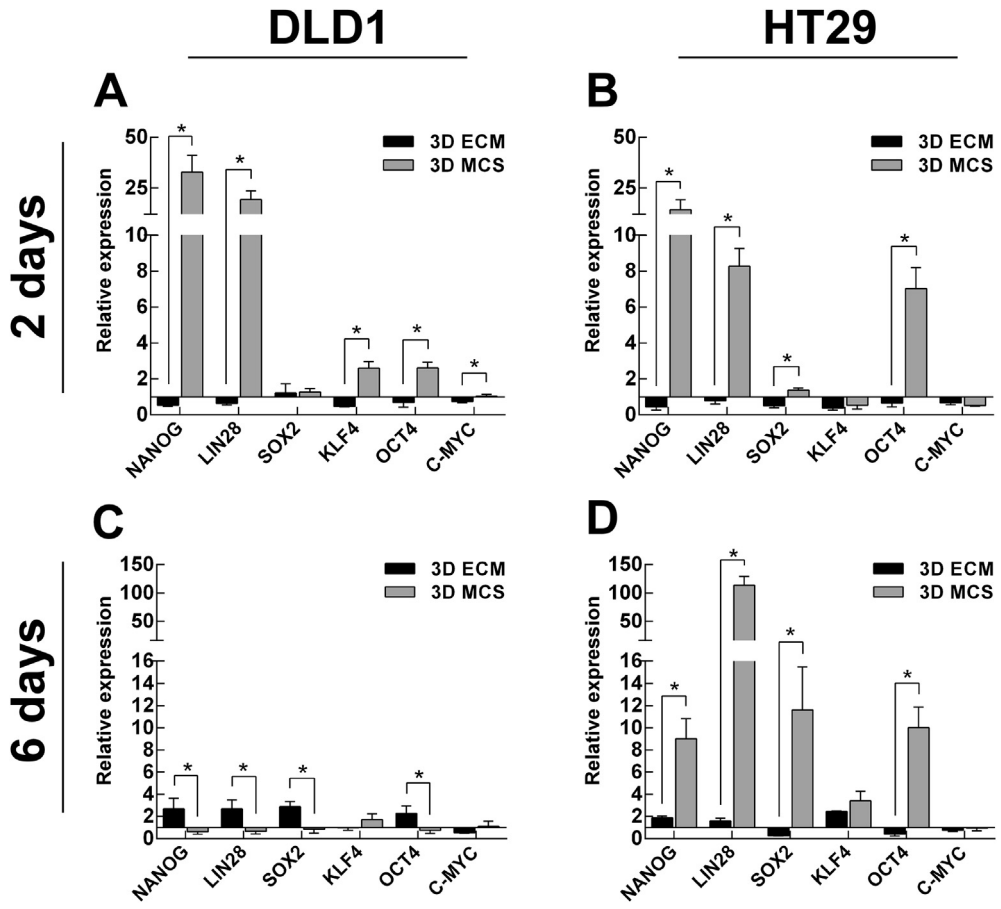

Fig. 2. Cell multipotency marker expression changes in CRC cells grown in lr-ECM and MCS cell cultures. Graph showing relative expression of genes (NANOG, LIN28, SOX2, KLF4, OCT4 and C-MYC) in DLD1 (**A** and **C**) and HT29 cells (**B** and **D**) grown in 3D ECM and MCS cell cultures compared to expression levels in CRC cells grown in 2D. Relative gene expression was evaluated following 2 and 6 days of cell growth. RT-qPCR data analysis was based on $2^{-\Delta\Delta Ct}$ and HPRT1 was used as the housekeeping gene for RT-qPCR data normalization. Results show mean ± SD (n = 3).

down-regulated in HT29 after 2 days of cell growth in 3D ECM (Fig. 2B). On the contrary, the expression of multipotency-related genes NANOG, LIN28 and OCT4 genes was significantly increased in HT29 cells grown in MCS. After 6 days of cultivation, HT29 cells grown in a 3D ECM presented a similar gene expression pattern to 2D, whereas cells grown in MCS maintained a considerably elevated expression of multipotency-related markers including NANOG, LIN28, SOX2, KLF4 and OCT4 (Fig. 2D).

3.3. CRC cells grown in three-dimensional cultures up-regulate expression of cancer stem cell markers

Next, we examined the influence of the 3D microenvironment on the expression of cancer stem cell markers ALDH1A1, CEACAM5, LGR5, DPP4, CD133, ALCAM, CD24, CD29 and CD44 in CRC cells grown in 3D versus 2D. RT-qPCR data analysis indicated that the expression of ALDH1A1 and CEACAM5 was up-regulated in DLD1 cells after 2 days of cultivation in a 3D ECM (Fig. 3A). In addition, DLD1 cells grown in multicellular spheroids exhibited a significant over-expression of ALDH1A1, CEACAM5, ALCAM, CD24 and CD29 genes. Furthermore, after 6 days of cell growth, DLD1 cells exhibited an increased expression of ALDH1A1, CEACAM5 in 3D ECM; whereas the expression of ALCAM, CD24 and CD29 was elevated in DLD1 cells cultivated in both 3D models (Fig. 3C). In addition, the expression of LGR5 was significantly up-regulated in cells grown in MCS.

HT29 cells exhibited less dramatic shifts in the expression of CSC markers than DLD1 cells when culturing cells in a 3D microenvironment. After 2 days of cultivation, no significant changes in the expression of most CSC-related genes was observed in HT29 cells grown under both 3D culture conditions, except for the expression of LGR5, which was up-regulated in cells grown in MCS (Fig. 3B). After 6 days of cell growth the expression of DPP4 and CD133 was up-regulated in cells cultivated in a 3D ECM (Fig. 3D); whereas HT29 cell growth in MCS resulted in an up-regulated expression of LGR5, CD133 and CD24.

3.4. EMT markers are up-regulated in CRC cells in a three-dimensional specific manner

We evaluated changes in the expression of ZEB1, ZEB2, SNAIL1, SNAIL2, N- and E-cadherins, which are considered as putative markers of EMT, in CRC cells grown in 3D versus 2D. After 2 days of cultivation in a 3D ECM, DLD1 cells showed no significant difference in expression levels of EMT markers compared to cells cultured in 2D (Fig. 4A). In contrast, DLD1 cells grown in MCS exhibited elevated expression levels of all examined EMT markers with the exception of the expression of SNAIL2 which remained unchanged. The expression of all inspected EMT markers was significantly elevated in DLD1 cells grown in both 3D culture models (Fig. 4C).

Similar to DLD1 cells, HT29 cells demonstrated no significant

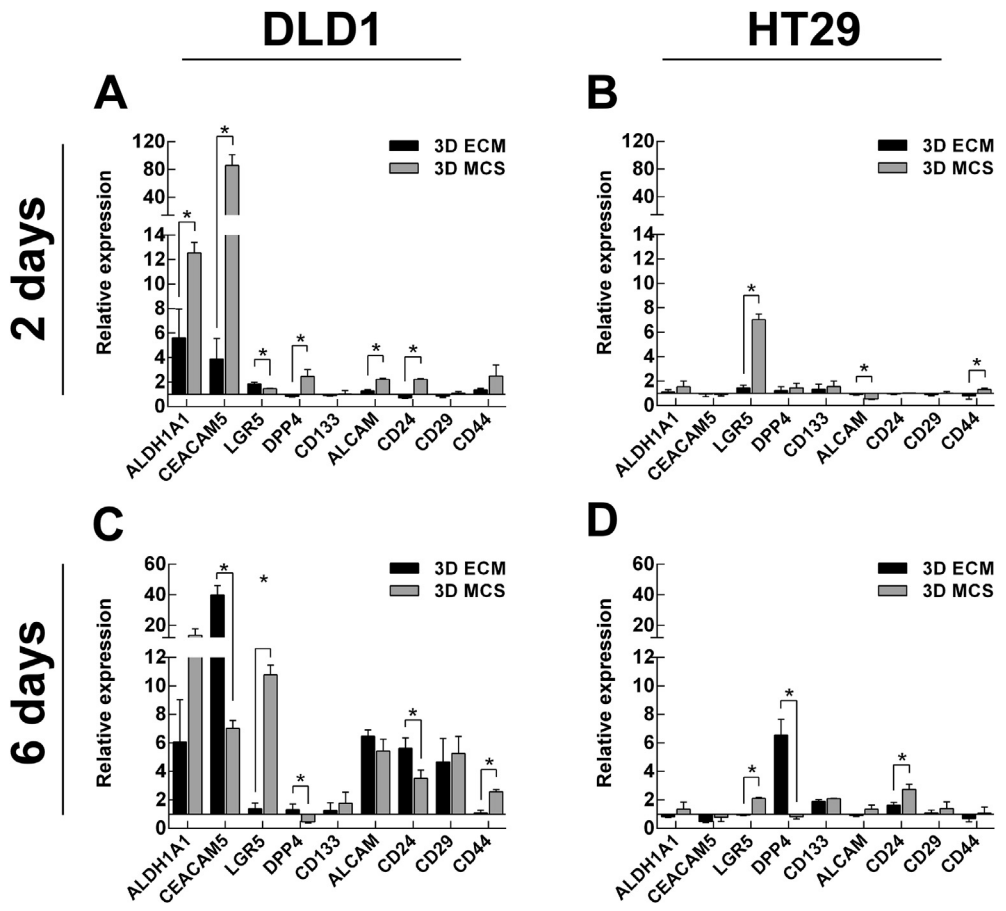

Fig. 3. Cancer stem cell marker expression changes in CRC cells grown in lr-ECM and MCS cell cultures. Graph showing relative expression of genes (ALDH1A1, CEACAM5, LGR5, DPP4, CD133, ALCAM, CD24, CD29 and CD44) in DLD1 (**A** and **C**) and HT29 cells (**B** and **D**) grown in 3D ECM and MCS cell cultures compared to expression levels in CRC cells grown in 2D. Relative gene expression was evaluated following 2 and 6 days of cell growth. RT-qPCR data analysis was based on $2^{-\Delta\Delta Ct}$ and HPRT1 was used as housekeeping gene for RT-qPCR data normalization. Results show mean ± SD (n = 3).

up-regulation of EMT associated gene expression after 2 days of growth in 3D ECM. In addition, HT29 cells grown in MCS demonstrated a similar expression pattern to the one exhibited by DLD1 spheroids (Fig. 4B). In contrast, the expression of EMT markers decreased in HT29 cells after 6 days of cultivation under both of the 3D cell culture conditions. RT-qPCR analysis indicated that the expression of SNAIL2 was notably up-regulated in cells cultivated in a 3D ECM (Fig. 4D); whereas cells grown in MCS demonstrated an increased expression of SNAIL1 and E-cadherin and significant a down-regulation of ZEB2 expression.

4. Discussion

Hierarchical tumor theory is becoming supported by mounting experimental evidence [1,2,18]. According to the theory, cancer stem cells could be targeted to develop a more effective anticancer treatment. However, 2D cell culture models widely applied in cancer research do not provide the conditions needed to maintain the CSC niche, physiologically relevant to in vivo. Our data indicated that cultivating human colorectal cancer DLD1 and HT29 cells in a 3D microenvironment affected the expression levels of multipotency, cancer stem cell and epithelial to mesenchymal transition (EMT) markers. Importantly, the expression patterns in CRC cells grown in 3D ECM and scaffold-free 3D MCS models were different compared to gene expression levels in cells grown in 2D. Therefore, our present data demonstrate that human colorectal carcinoma cells grown in 3D exhibit enhanced cancer stem cell characteristics.

In order to evaluate the cellular plasticity observed in a 3D microenvironment, we further investigated the changes in the expression of genes involved in multipotency maintenance in DLD1 and HT29 cells grown in 3D models compared to expression levels in cells cultivated in a monolayer. Our data showed that the expression of selected multipotency markers was extensively up-regulated in CRC cells grown in 3D cell cultures compared to 2D. In addition, the expression of these markers appeared to be regulated in a time dependant manner in DLD1 cells grown in 2D and 3D cell models, resulting in expression level equalization between these conditions following 6 days of cell growth (data not shown). These observations were consistent with a previous studies showing the enrichment of cells expressing multipotency markers in a 3D culture [19,20]. Our data indicated that the expression of NANOG, LIN28, KLF4 and OCT4 was most robustly elevated in CRC cells grown in 3D cultures. Previous studies showed that the elevated expression of NANOG in tumor cells correlated with poor prognosis, EMT transition and metastasis [21,22]. In addition, cells possessing increased expression of NANOG have a tendency to form spheroids and induce the expression of c-Myc, SOX2 and Lgr5 [23]. Furthermore, NANOG was shown to be regulated by OCT4 and SOX2 indicating regulatory relationships between multipotency markers [24]. Moreover, it was demonstrated that Lin28 positively regulates the CSC marker ALDH1 by blocking let7 miRNA maturation [25].

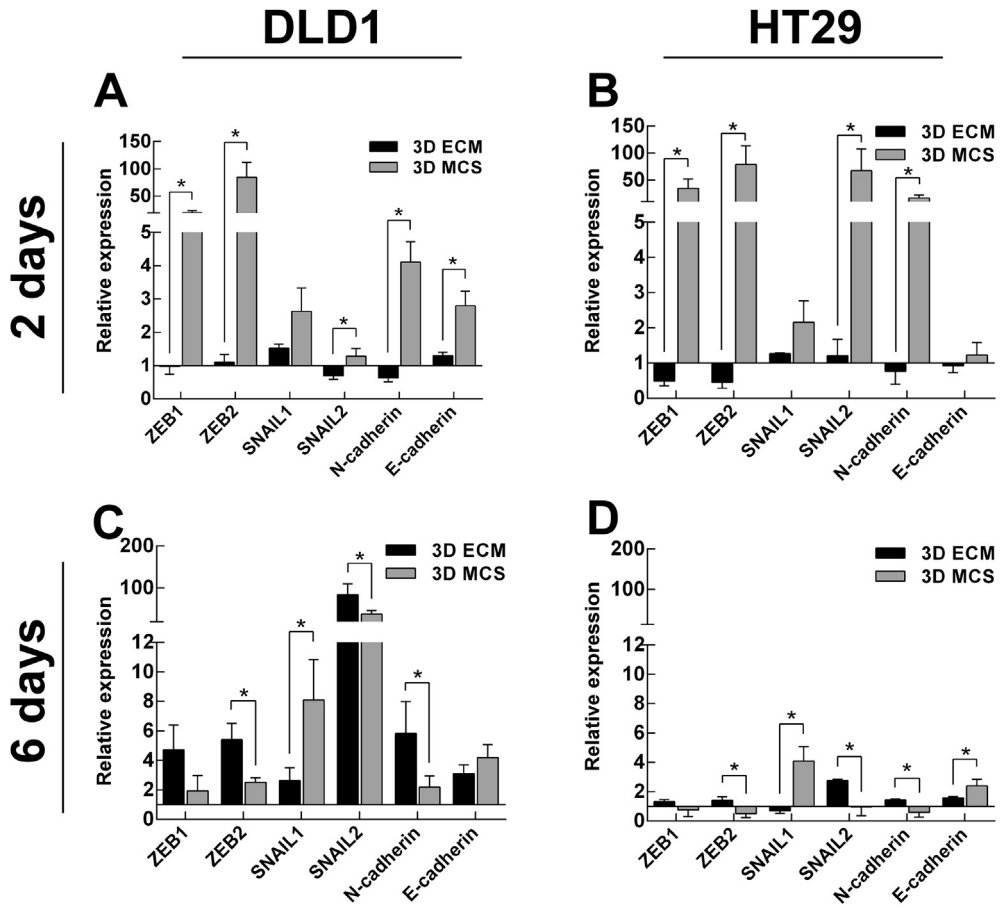

Fig. 4. EMT marker expression changes in CRC cells grown in lr-ECM and MCS cell cultures. Graph showing relative expression of genes (ZEB1, ZEB2, SNAIL1, SNAIL2, N-CADHERIN and E-CADHERIN) in DLD1 (**A** and **C**) and HT29 cells (**B** and **D**) grown in 3D ECM and MCS cell cultures compared to expression levels in CRC cells grown in 2D. Relative gene expression was evaluated following 2 and 6 days of cell growth. RT-qPCR data analysis was based on $2^{-\Delta\Delta Ct}$ and HPRT1 was used as housekeeping gene for RT-qPCR data normalization. Results show mean ± SD (n = 3).

Altogether, these findings indicate the link between the expression of multipotency-related genes and a 3D microenvironment in DLD1 and HT29 cells. Therefore, our observation of the upregulation of cellular multipotency under 3D cell culture conditions is an additional suggestion of the possibility that 3D cell cultures could be applied as a more physiologically relevant model to study multipotency in cancer cells.

In this study, our data demonstrated an altered expression of cancer stem cell marker in CRC cells grown in 3D cultures as it was observed previously in colon, breast and lung cancer cells [26–28]. However, robust gene over-expression was present only in DLD1 cells. On the other hand, the basal expression levels of CSC markers in HT29 cells were higher than in DLD1, indicating that the change in gene expression could be also dependent on the origin of the cell line [29]. Consistent with this finding HT29 cells were shown to have better sphere forming abilities and resistance to chemotherapy - both characteristics attributed to CSCs [30,31]. Moreover, DLD1 cells grown in a 3D ECM showed an increase in a number of over-expressed CSC markers in a time dependent manner whereas the expression of ALDH1A1 and CEACAM5 was more robustly altered in cells grown in 3D. A number of studies observed that ALDH1A1 expression and activity is an indicator of cancer cells with stem cell properties in normal and cancer colon cells and other malignancies [32–35]. The ALDH1 family of dehydrogenases are known to oxidise some anticancer drugs leading to an increased resistance of CSCs to chemotherapy [36]. Furthermore, CEACAM5 was shown to promote migration and invasion of tumor cells indicating its role in epithelial-mesenchymal transition and metastasis [37,38].

A previous report by Mani et al. demonstrated relations between the expression of cancer stem cell markers and EMT-related genes [38]. Therefore, we further elucidated the changes in expression of EMT-related genes in CRC cells grown in 3D compared to expression levels in cells cultivated in a monolayer. Our data indicated an up-regulation of EMT associated gene expression in CRC cells grown in 3D cultures. These results were consistent with previous reports indicating that EMT is induced by the 3D microenvironment [27,28]. However, the expression of genes in CRC cells was time and microenvironment dependent. For instance, a robust up-regulation of gene expression was observed in CRC cells cultured in MCS after 2 days of cell growth, while expression levels peaked in DLD1 cells following cell growth of 6 days in 3D ECM. Furthermore, our data revealed, that the expression of transcription factors SNAIL1/2 and ZEB1/2 was most robustly up-regulated in CRC cells grown in 3D. These EMT-controlling TF families have been previously related to CSC activation and expression of CSC markers [39,40]. In the present study, we also observed up-regulated expression of E- and N-cadherins. Previously it was demonstrated that the expression of E-cadherin is repressed by ZEB1 and SNAIL1 [41,42]. On the other hand, it was demonstrated that E-cadherin promotes spheroid formation [43] indicating that induced expression of E-cadherin in CRC cells

during spheroid formation might be EMT activation independent. Altogether, these observations suggest that EMT gradually proceeds during the growth of MCS culture and therefore this culture model should be considered as a tool for EMT investigations.

In the present study, we observed the up-regulation of gene expression related to cellular response to hypoxia in CRC cells grown under both 3D culture conditions 6 days after cell plating, compared to expression levels in cells grown in 2D. Unexpectedly, the up-regulated expression of hypoxia related genes including CAIX, GLUT1 and VEGFA was more robust in DLD1 and HT29 cells grown in a 3D ECM than in MCS, despite CRC cells forming spheroids of a larger diameter. This suggests that the expression of genes regulated by hypoxic conditions might be dependent on the presence of the extracellular matrix and cell-ECM interactions. On the contrary, in the current study we observed that the changes in expression of CSC markers is higher in CRC cells cultivated in MCS suggesting that the hypoxic response could be related to metabolic rearrangements occurring in CRC cells grown in 3D. This is further supported by the finding that the expression of carboanhidrase CAIX gene was most robustly up-regulated in both cell lines. CAIX has been shown to promote spheroid and xenograft growth by reducing intracellular acidity, increased due enhanced glycolysis [44]. In addition, we have previously shown that the expression of genes involved in metabolic pathways was vigorously altered in LLC1 cells grown under ECM conditions [45].

5. Conclusions

Our current study demonstrates that CRC cells exhibit a more cancer stem cell like phenotype in cells grown under two different 3D cell culture models, when compared to cells grown in a monolayer cell culture. In addition, the changes in expression of multipotency, CSC and EMT markers in CRC cells were time and three-dimensional environment specific. Therefore, our present results suggest that 3D culture models should be considered as an important tool for the further investigation of molecular mechanisms involved in the maintenance of CSCs and for the development of CSC-targeted cancer therapies.

Competing interests

None of the authors have any competing interests.

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List of abbreviations

3D	three dimensional
CRC	colorectal cancer
CSC	cancer stem cells
EMT	epithelial to mesenchymal transition
ECM	laminin rich extracellular matrix
MCS	multicellular spheroids

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.01.111.

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