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The enhancement of cancer stem cell properties of MCF-7 cells in 3D collagen scaffolds for modeling of cancer and anti-cancer drugs

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ABSTRACT

Three-dimensional (3D) culture could partially simulate *in vivo* conditions. In this work, we developed a 3D collagen scaffold to investigate cellular properties of MCF-7 cells. The porous scaffolds not only induced the diversification of cell morphologies but also extended cell proliferation. The expression of pro-angiogenic growth factors and the transcriptions of matrix metalloproteinases (MMPs) were significantly increased in cells cultured in 3D collagen scaffolds. In addition, 3D collagen scaffolds could generate a cell population with the properties of cancer stem cells (CSCs). The upregulation of EMT markers and the downregulation of the epithelial cell marker were observed in cells cultured in collagen scaffolds. The expression of stem cell markers, including OCT4A and SOX2, and breast cancer stem cell signatures, including SOX4, JAG1 and CD49F, was significantly unregulated in 3D collagen scaffolds. The proportion of cells with CSC-like $CD44^{+}/CD24^{-/low}$ phenotype was notably increased. High-level expression of CSC-associated properties of MCF-7 cells cultured in 3D was further confirmed by high tumorigenicity *in vivo*. Moreover, xenografts with 3D cells formed larger tumors. The properties of MCF-7 cells in 3D may have partially simulated their *in vivo* behaviors. Thus, 3D collagen scaffolds might provide a useful platform for anti-cancer therapeutics and CSC research.

1. Introduction

Traditional two-dimensional (2D) cell culture system was a convenient way to study cancer cells *in vitro*, but it failed to mimic the tumor structures such as cell–cell communication and cell-extracellular matrix (ECM) interactions, which played a key role in cancer cells [1,2]. Cells cultured in 2D were forced to adopt sheet-like morphology and change cell growth, migration and apoptosis [3,4]. Meanwhile, the deposition of ECM, which was essential for cell proliferation and gene expression, was decreased in cancer cell monolayer culture [5]. The malignant phenotypes of cancer cells were also dramatically reduced when cells were transferred from *in vivo* conditions to 2D cell culture plates. Thus the actions of drugs functioned on cell–cell interactions, epithelial to mesenchymal transition (EMT) and cancer stem cells (CSCs) were reduced [6]. These findings indicated that cancer cells cultured in 2D poorly represented their *in vivo* physiological conditions.

The gap between 2D cell culture system *in vitro* and tumors *in vivo* was bridged by the three-dimensional (3D) cell culture system *in vitro* [7]. A number of matrix materials and synthetic polymers were utilized to develop 3D cell culture scaffolds [8–10]. 3D chitosan-alginate scaffolds were fabricated to mimic glioma microenvironment [11] while PLGA porous scaffolds were utilized to study the relationship between the microenvironment and tumor malignancies *in vitro* [12]. Hydrogels including Matrigel [13,14], alginate [15] and hyaluronic acid [16] were also explored as 3D models for cancer research. Cancer cells in 3D culture displayed high malignancies including overexpression of pro-angiogenic growth factors, enhancement of tumorigenicity and insensitivity to drug treatment.

Cancer cell metastasis was another important phenotype of malignant tumors *in vivo*, which was induced by the process of EMT. The well-polarized epithelial cells were converted into no-polarized mesenchymal cells with properties of invasion and

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motility [17]. Environmental factors, including growth factors, proteases, hypoxia and cell-ECM interactions, could induce cancer cells to undergo the process of EMT. EMT resulted in acquiring properties of invasion and drug insensitivity, and triggering the reversion to CSCs [18]. CSCs had been demonstrated to retain cancer-initiating potential and self-renewal capability, and lead to tumorigenesis and drug resistances in malignant tumors *in vivo* [17]. The cell-surface marker profile of $CD44^{+}/CD24^{-/low}$ was normal way to enrich the population of CSCs [19].

Collagen was the major component of ECM and could provide proper surface for cell adhesion and migration [20]. It had been widely utilized to culture cells for its excellent characteristics, including biocompatibility, mechanical strength, degradability and limited immunogenicity [21,22]. For 3D cancer cell culture systems, most of the studies focused on collagen hydrogels [23–25]. In this study, we developed a porous 3D collagen scaffold for MCF-7 cell culture. Morphology and proliferation of MCF-7 cells in 3D collagen scaffolds were examined. Malignant phenotypes, such as pro-angiogenic growth factor secretion, matrix metalloproteinas (MMP) transcriptions, EMT phenotype expression and CSC property acquisition, were also studied by enzyme-linked immunosorbent assay (ELISA), Quantitative Real-Time reverse transcription-PCR (qRT-PCR) and Fluorescence Activated Cell Sorting (FACS) *in vitro*. The *in vivo* tumor formation was assessed by xenograft implantation and cell suspension injection into athymic nude mice.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) if not mentioned. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, Dulbecco's phosphate buffered saline (D-PBS) and fetal bovine serum (FBS) were purchased from Hyclone. Breast cancer cell line (MCF-7) was purchased from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). Vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) ELISA kits were purchased from Boster Biological Technology., Ltd. (Hubei, China) and basic fibroblast growth factor (bFGF) ELISA kit was got from NeoBioscience Technology Co., Ltd. (Beijing, China).

2.2. Preparation of 3D collagen scaffolds

Collagen scaffolds were fabricated from collagen membranes obtained from Zhenghai Biotechnology Ltd. (Shandong, China). Briefly, the collagen membranes were immersed in 0.5 M acetic acid solution for 8 h at 4 °C. And then the solution was mixed in a blender for 15 min to obtain homogeneous collagen solution and neutralized by 4 M NaOH. The homogeneous solution was dialyzed in deionized water for 5 days and lyophilized. The obtained porous collagen scaffolds were cut into 0.1 × 0.5 × 0.5 cm pellets and crosslinked by 1 mg/ml 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 0.6 mg/ml N-hydroxysuccinimide (NHS) as before [26]. After crosslinking, the pellets were lyophilized again, sterilized by C_o 60 and stored at 4 °C for utilization.

2.3. Cell culture in 2D and 3D

Cells were maintained in DMEM media supplement with 10% FBS and 1% penicillin-streptomycin at 37 °C and 5% CO_2. Following immersed in DMEM with 50% FBS for 0.5 h at room temperature (RT), the collagen pellets were loaded with cell suspension and maintained at 37 °C for 1 h. DMEM with 10% FBS was added to immerse all the cell seeded pellets for 24 h. And then, the scaffolds together with seeded cells were transferred to 30 cm cell culture plates on shaking table and cell culture media was changed every day.

2.4. Cell morphology analysis

The morphology of cells seeded in 3D collagen scaffolds was shown by Scanning Electron Microscopy (SEM, S-3000N; Hitachi, Tokyo, Japan) and confocal analysis. Firstly, samples were washed with cold D-PBS for 3 times and fixed with cold Karnovsky's fixative for 8 h at 4 °C. And then, dehydration of the cell was followed in a series of ethanol (50%, 75%, 85%, 95%, 100% and 100%). Samples were critical point dried and sputter coated with gold platinum prior to SEM imaging. For confocal analysis, 3D collagen scaffolds with cells were immersed into FDA solution for 2 min, washed with D-PBS for twice and viewed by Confocal Microscopy (Leica TCS SP5, Germany). The specimens were fixed in 10% formalin solution and embedded in paraffin for 4 μm sections. Hematoxylin and eosin (H&E) staining was adopted for morphologic analysis.

2.5. Cell proliferation analysis

Cell proliferation in 2D cell culture plates and 3D collagen scaffolds was assayed using hemocytometer. Briefly, 0.1% collagenase I and 0.2% dispase II solution diluted by cell culture media was added into 2D cell culture plates and 3D scaffolds for 1.5 h at 37 °C. The cell suspension was washed with D-PBS for twice and centrifuged at 1000 rpm, then digested with 0.25% trypsin for 2 min and resuspended in DMEM with 10% FBS. The cell number was calculated on hemocytometer at special time point.

2.6. Growth factor expression

After cultured in 3D collagen scaffolds for 9 days, cells were incubated in DMEM containing 2% FBS for 24 h. The media was collected and the secretion of VEGF, bFGF and IL-8 in media was determined according to the manufacturer's protocol. The values of secretion were calculated based on the cell number and normalized to 2D culture conditions.

2.7. Quantitative real-time reverse transcription-PCR (qRT-PCR)

Total mRNA was isolated from MCF-7 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instruction. cDNA was synthesized using SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Power SYBR Green RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) was utilized to perform qRT-PCR using the Bio-RAD CFX96™ Real-Time system (Bio-RAD, Hercules, CA, USA). GAPDH gene transcript was measured as a normalizer to determine the other gene relative transcripts (ΔΔCt). Primer sequences were listed in Table 1.

2.8. Analysis of $CD44^{+}/CD24^{-/low}$ phenotype by FACS

Cells cultured in 2D plates and 3D collagen scaffolds were digested as above to single cell suspension, washed with cold D-PBS and resuspended in cold D-PBS with 10% FBS at 1×10^6/100 ul. Cells were incubated with PE conjugated CD24 and APC conjugated CD44 (eBioscience, 120247 and 170441) at 4 °C for half an hour according to the protocol. After washed with cold D-PBS twice, cells were fixed with cold D-PBS with 10% FBS and analyzed on FACS Calibur (BD Biosciences). Cells unstained and stained with single antibody were analyzed.

2.9. In vivo study of MCF-7 cells cultured in 2D and 3D

All experiments on animals were subjected to Chinese Ministry of Public Health (CMPH) Guide and US National Institute of Health (NIH) Guide.

The tumor-forming capability, one of the most important CSC characteristics, was examined by subcutaneous injection. Cells cultured in 2D and 3D were

Table 1
Sequences of primers used for qRT-PCR.

Gene name	Forward	Reverse
MMP-2	CATCTGTTTTAGCA	GAGCAGAGATTCGGA
	GAGCCTAGACAA	CTTTTTAAGTT
MMP-9	CGCTGGGCTTAGATCATTCC	GTGCCGGATGCCATTCAC
SNAIL	GTTTCCCGGGCAATTTAACA	CCCGACAAGTGACAGCCATT
SLUG	CAGCTACCCAATGGCCTCTCT	GGACTCACTCGCCCCAAAG
ZEB1	TGTGAATGGGCGACCAAGA	GTGGGACTGCCTGGTGATG
ZEB2	GCCGAGTCCATGCGAACT	CCATGATCGGCTGCTTCAT
TWIST1	GCGCTGCGGAAGATCATC	GGTCTGAATCTTGCTCAGCTTGT
TWIST2	GCCCAGTGGAGCATGCA	GGTGGGACACCGAGATGGT
LEF1	CAGGAGCCCTACCACGACAA	TTGTAGAGGCCTCCATCTGGAT
FOXC2	CACGCCGCCTCTCTATCG	CGTCAGTATTTCGTGCAGTCGTA
DDR2	GTCTATGATGGAGCTGTTGGATACA	GACACACCATCGGTCAATTGG
ITGA5	CAGTGCCGAGTTCACCAAGA	GCCTTGCCAGAAATAGCTTCCT
CDH1	ACAGCCCCGCCTTATGATT	TCGGAACCGCTTCCTTCA
VIM	AATGACCGCTTCGCCAACT	ATCTTATTCTGCTGCTCCAGGAA
β-catenin	AAGGCTACTGTTGGATTGATTCG	CCCTGCTCACGCAAAGGT
SPARC	CAGACACACGATGTTGTCAAGGA	CACAGCCCCAGAATCTTTCTCT
SOX2	CCCCTTTATTTTCCGTAGTTGTATTT	GATTCTCGGCAGACTGATTCAA
OCT4A	CCCCTGGTGCCGTGAAG	GCAAATTGCTCGAGTTCTTTCTG
SOX4	CCCGAGTGGGCACATCA	ATCTTGCGCCGCTCGAT
CD49F	GATCCCGGCCTGTGATTAATATT	CTGGCGGAGGTCAATTCTGT
JAG1	CAAACCTTGTGTAAACGCCAAA	CGGGAAGACAGTCGCAGTAGT
GAPDH	ATGGAAATCCCATCACCATCTT	CGCCCCACTTGATTTTGG

harvested as above and resuspended in 200 μl DMEM media supplement with 10% FBS and injected into the mammary fat pad of athymic nude mice (BALB/c-nu) with 4–5 weeks of age. Six mice per experimental group were used in four independent experiments, respectively. All tumors were harvested after 8 weeks. Animals were killed when the tumors were over 1.0–1.2 cm in the largest diameter, avoiding tumor cell necrosis.

In order to further examine the increasing malignant phenotype of cells cultured in 3D collagen scaffolds, cell-collagen scaffolds were implanted into subcutaneous space of athymic nude mice (BALB/c-nu). Same number of cells cultured in 2D were loaded into the collagen scaffolds and implanted as above. The mice were feed with 1 mg estrogen per liter of water [9] and monitored twice a week for signs of tumor growth. After 5 weeks implantation, mice were sacrificed and the tumor weight was calculated.

2.10. Statistical analysis

All data were shown as the mean ± s.d. Statistical analysis was performed by Student's *t*-test. Significant difference was considered for $p < 0.05$.

3. Results

3.1. The morphology and proliferation assay

Cells cultured in porous collagen scaffolds were examined via SEM analysis, confocal imaging, H. E. staining and cell proliferation analysis. MCF-7 cells seeded in 2D cell culture plates exhibited sheet-like trigonal or polygonal morphologies (Fig. 1a), whereas those in 3D collagen scaffolds displayed diversified morphologies (Fig. 1b–c). Cells in 3D showed round, shuttle shape-like and spread-out appearances. 3D collagen scaffold was filled with cells and formed a multi-layer cell structure (Fig. 1d). MCF-7 cells successfully expanded and propagated in 3D collagen scaffolds (Fig. 2). At the time point of the 5th day, cells proliferated to 9.17 ± 0.0.62-fold in 2D and 8.04 ± 2.32-fold in 3D relative to the first day. No statistically significant differences in proliferation rates of cells were observed between 2D and 3D culture systems during the first 5 days. However, MCF-7 cells persisted proliferating in 3D collagen scaffolds to the 13th day with 46.70 ± 8.01-fold increment, while cells cultured in 2D decreased to 4.86 ± 0.98-fold at the 7th day.

3.2. Upregulation of growth factors and MMPs in 3D cultured cells

High expression levels of pro-angiogenic growth factors and MMPs were indicators of malignant tumor *in vivo*. The secretion of pro-angiogenic growth factors, including VEGF, bFGF, and IL-8, was evaluated by ELISA kit (Fig. 3a). The transcriptions of MMP-2 and MMP-9 were examined by qRT-PCR analysis (Fig. 3b). VEGF was considered to play a key role in tumor angiogenesis [27]. More VEGF was secreted by cells cultured in 3D collagen scaffolds than those in 2D (5.04 ± 1.08-fold, $p < 0.01$). Overexpression of IL-8 could promote new blood vessel recruitment and tumor cell invasion [15]. Increased secretion of IL-8 by cells in 3D was observed by 10.65 ± 1.82-fold as compared to cells in 2D. Upregulation of bFGF could induce endothelial cell migration and stimulate the expression of ECM remodeling enzymes *in vivo* [28]. The 3D collagen scaffolds dramatically enhanced bFGF secretion by 6.93 ± 0.92-fold relative to 2D condition. MMP-2 and MMP-9, which were considered to function on ECM degradation for tumor cell invasion and metastasis, were highly expressed in malignant breast tumors [29]. Both MMP-2 and MMP-9 transcriptions of MCF-7 cells in 3D scaffolds were significantly improved to 1.58 ± 0.08-fold and 1.95 ± 0.27-fold, respectively, relative to 2D compartments (Fig. 3b). 3D collagen scaffolds significantly improved the expression of pro-angiogenic growth factors and MMPs *in vitro*.

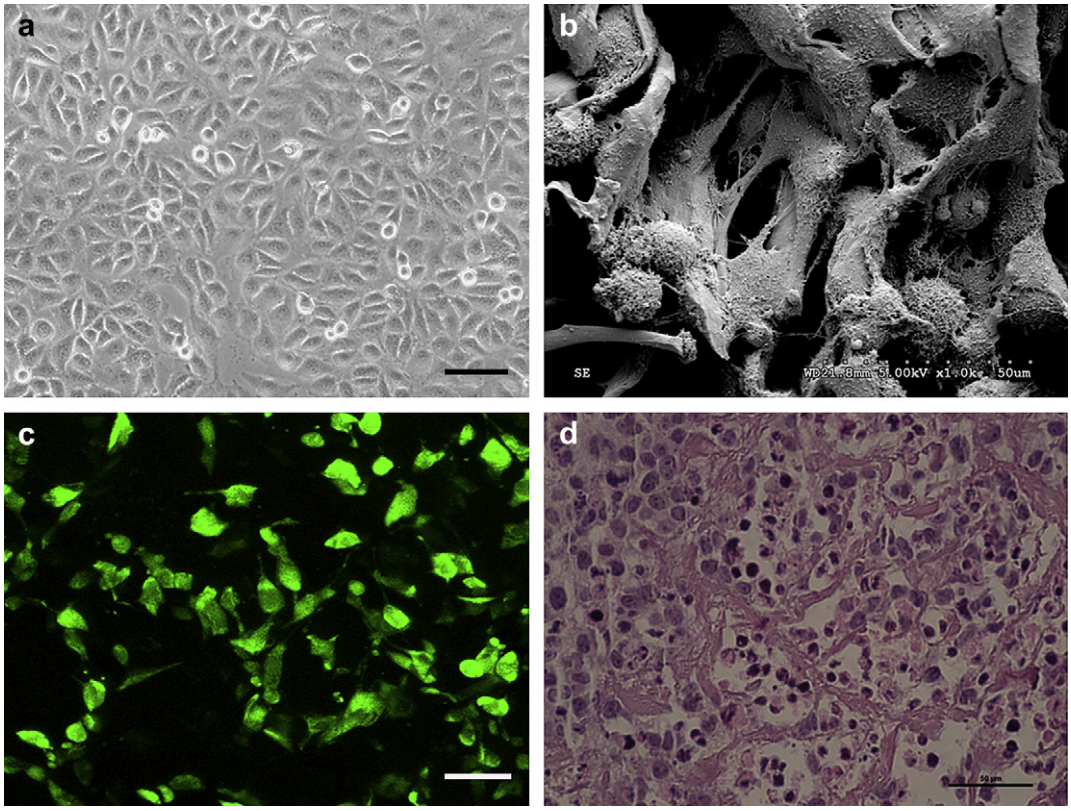


Fig. 1. Morphology images of MCF-7 cells cultured under different conditions. (a) Cells cultured in 2D showed sheet-like trigonal or polygonal morphologies. Scale bar was 50 μm. (b) SEM and (c) confocal images indicated diversified morphologies of cells in 3D collagen scaffolds. Scale bar was 50 μm. (d) H. E. staining of cross-sections of 3D collagen scaffolds cultured with cells showed a multi-layer cell structure. Scale bar was 50 μm.

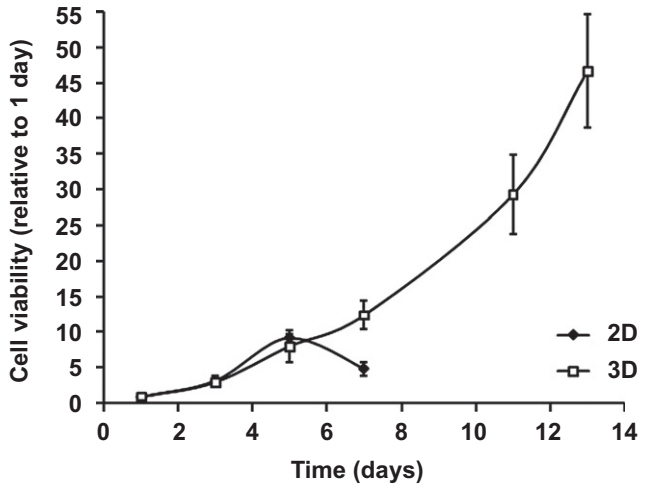


Fig. 2. Proliferation kinetics of MCF-7 cells in 2D and 3D were assessed at indicated time points. Results were shown as mean ± s.d.

3.3. The improvement of EMT-associated properties in 3D cultured cells

The enhanced migration of tumor cells, an important characteristic of malignant tumors, was associated with the process of EMT *in vivo*. Upon reseeded to 2D culture plates, cells from 3D culture system showed a mesenchymal morphology (Fig. 4a). We next examined the transcriptions of EMT markers in cells from the two culture systems (Fig. 4b). The expression of transcription factors, including SNAIL, SLUG, ZEB1, ZEB2, TWIST1, TWIST2, LEF1 and FOXC2, was significantly upregulated in cells cultured in 3D collagen scaffolds with 3.25 ± 0.15-fold, 1.70 ± 0.07-fold, 6.94 ± 0.43-fold, 12.47 ± 1.00-fold, 1.81 ± 0.16-fold, 2.04 ± 0.06-fold, 1.53 ± 0.19-fold and 1.65 ± 0.09-fold, respectively. Additionally, other EMT markers, including discoidin domain receptor tyrosine kinase 2 (DDR2), vimentin (VIM), β-catenin, α5-integrin (ITGA5) and osteonectin (SPARC), were also strikingly upregulated with 2.64 ± 0.17-fold, 1.71 ± 0.03-fold, 13.30 ± 1.60-fold, 26.90 ± 2.16-fold and 1.94 ± 0.15-fold, respectively. However, the epithelial marker E-cadherin (CDH1) was downregulated to 0.64 ± 0.03-fold in 3D cultured cells. The upregulation of EMT markers and the downregulation of epithelial marker suggested that cells cultured in 3D collagen scaffolds were induced to undergo EMT.

3.4. CSC phenotype expression in MCF-7 cells cultured in 3D

Given the close relationship between EMT and CSCs, we examined whether the 3D collagen scaffolds possessed the capability to induce the expression of CSC phenotypes. Properties of breast cancer stem cells in MCF-7 cells were analyzed by FACS, qRT-PCR and orthotopic injection. Transcriptions of stem cell markers, including SOX2 and OCT4A, and breast cancer stem cell signatures, including SOX4, JAG1 and CD49F, were enhanced in cells cultured in 3D by 2.45 ± 0.40-fold, 2.80 ± 0.50-fold, 1.55 ± 0.17-fold, 2.53 ± 0.13-fold and 3.62 ± 0.17-fold (Fig. 5a), respectively, relative to those in 2D. The proportion of MCF-7 cells with phenotype of $CD44^{+}/CD24^{-/low}$ configuration was 34.1% in 3D, whereas it was just 3.5% in 2D (Fig. 5b). This data showed that 3D collagen scaffolds strikingly highlighted the CSC property of $CD44^{+}/CD24^{-/low}$ fraction. Single cell suspensions were injected into the mammary fat pad of athymic nude mice to examine the tumorigenicity *in vivo* (Fig. 5c). After 8 weeks, cells cultured in 2D and 3D could form new tumors when the number of injection cells was 1×10^6 (suspended in 200 μl medium). However, when the cell number was less than 1×10^6, the difference of tumorigenic capability was shown between 2D and 3D culture systems. When the injection number of cells was 1×10^5, three in five recipient animals could form tumors in 3D group. In contrast, just one in six animals formed tumor in 2D group. In addition, when 1×10^6 cells were injected into mice, cells cultured in 3D collagen scaffolds formed larger tumors than those cultured in 2D (Fig. 5d). Overall, the data suggested that 3D culture of MCF-7 cells improved the expression of CSC-associated phenotypes.

3.5. High tumorigenic capability of xenografts with 3D cultured cells

Xenografts cultured with cells were implanted into the athymic nude mice to examine the tumorigenicity. The scaffolds loaded with the same number of cells from 2D were implanted as control. After 5 weeks, the tumors were harvested and weighted. Xenografts cultured with MCF-7 cells formed larger tumors at the sites of implantation than those loaded with cells from 2D, with weight of

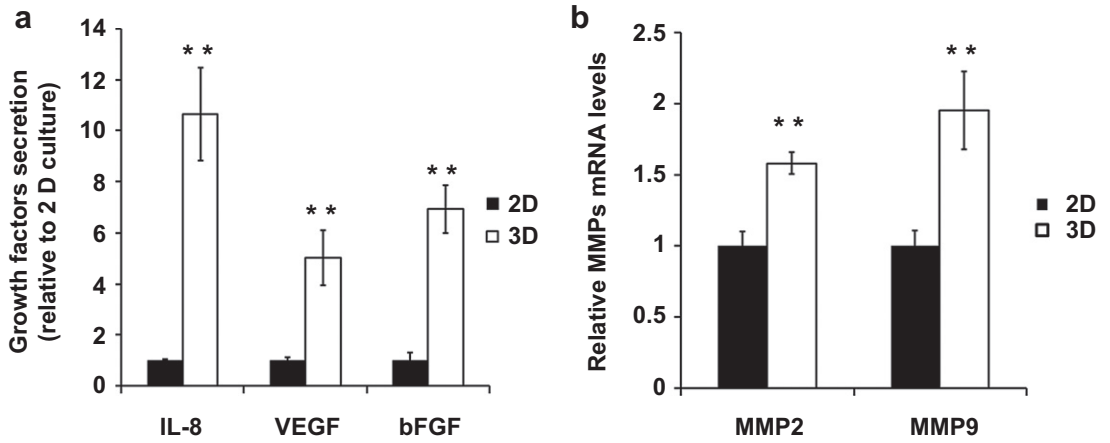
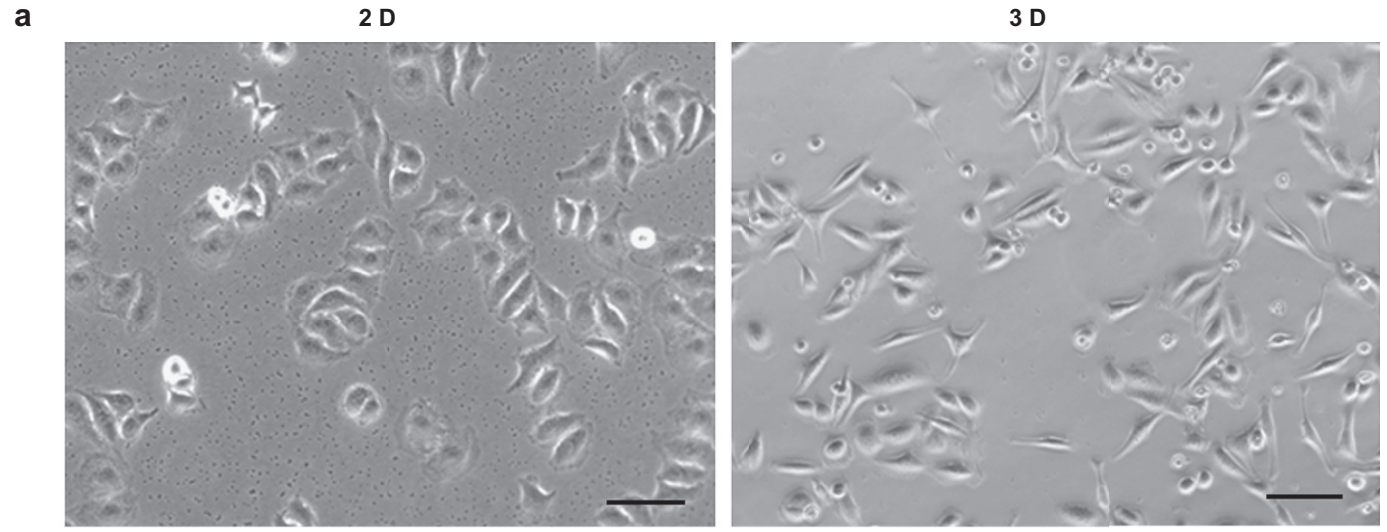
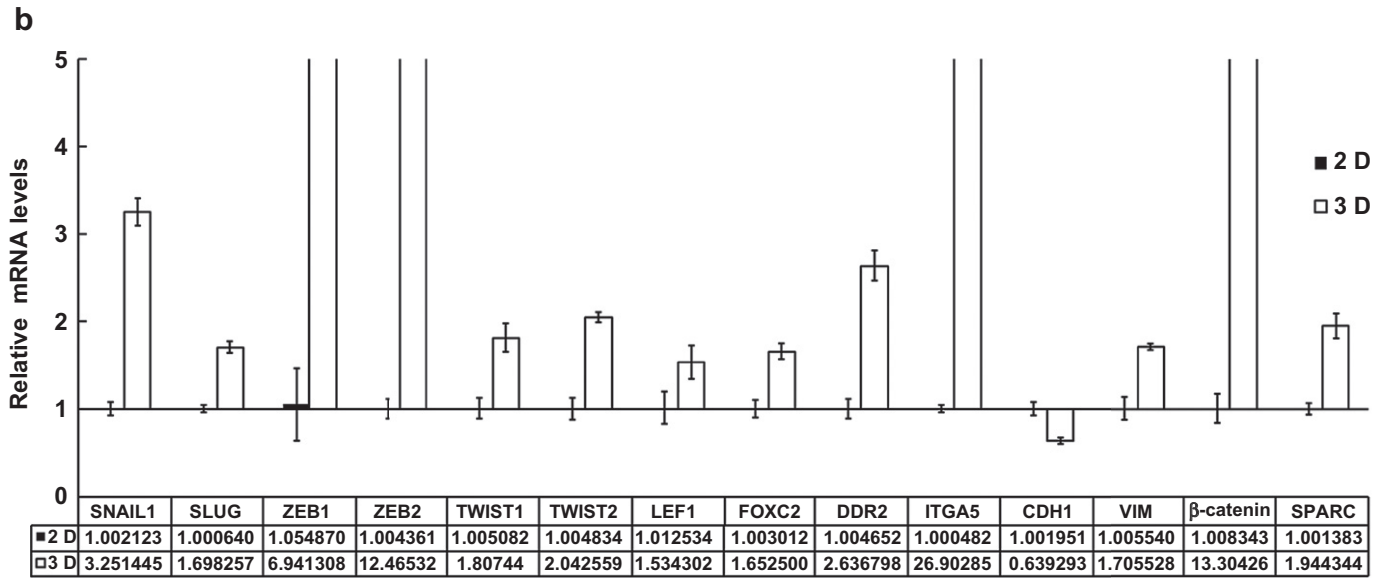


Fig. 3. Malignant phenotype expression of MCF-7 cells cultured in 2D and 3D was examined *in vitro*. (a) The secretion of VEGF, bFGF and IL-8 in MCF-7 cells was tested by ELISA. (b) Relative transcriptional levels of MMP-2 and MMP-9 in 3D cultured cells were detected by qRT-PCR. GAPDH mRNA was utilized to normalize gene expression data. Results were mean ± s.d. $^{**}P < 0.01$, by student's *t*-test ($n = 3$).





	SNAIL1	SLUG	ZEB1	ZEB2	TWIST1	TWIST2	LEF1	FOXC2	DDR2	ITGA5	CDH1	VIM	β-catenin	SPARC
■2 D	1.002123	1.000640	1.054870	1.004361	1.005082	1.004834	1.012534	1.003012	1.004652	1.000482	1.001951	1.005540	1.008343	1.001383
□3 D	3.251445	1.698257	6.941308	12.46532	1.80744	2.042559	1.534302	1.652500	2.636798	26.90285	0.639293	1.705528	13.30426	1.944344

Fig. 4. 3D collagen scaffolds promoted MCF-7 cells to undergo EMT. (a) MCF-7 cells from 3D culture system displayed a mesenchymal morphology after being replated in cell culture plates. Scale bar was 50 μm. (b) Relative expression of SNAIL, SLUG, ZEB1, ZEB2, TWIST1, TWIST2, LEF1, FOXC2, DDR2, ITGA5, CDH1, VIM, β-catenin and SPARC in 3D cultured cells were determined by qRT-PCR. Results were mean ± s.d. GAPDH mRNA was utilized to normalize gene expression data.

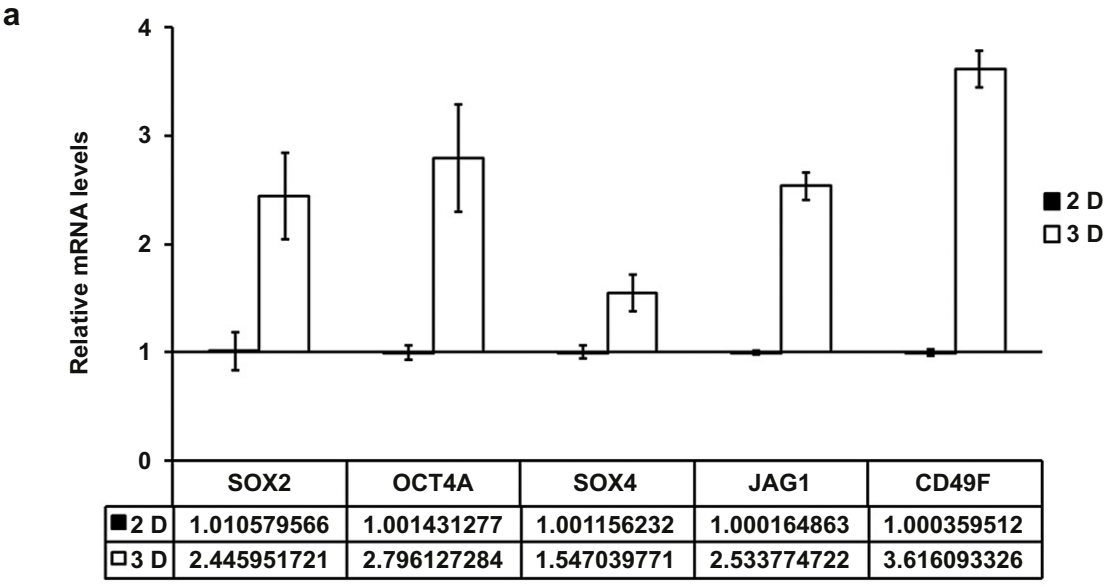
0.7 ± 0.26 g and 0.17 ± 0.27 g, respectively (Fig. 6). 3D cultured cells strikingly improved the tumorigenicity of xenografts *in vivo*.

4. Discussion

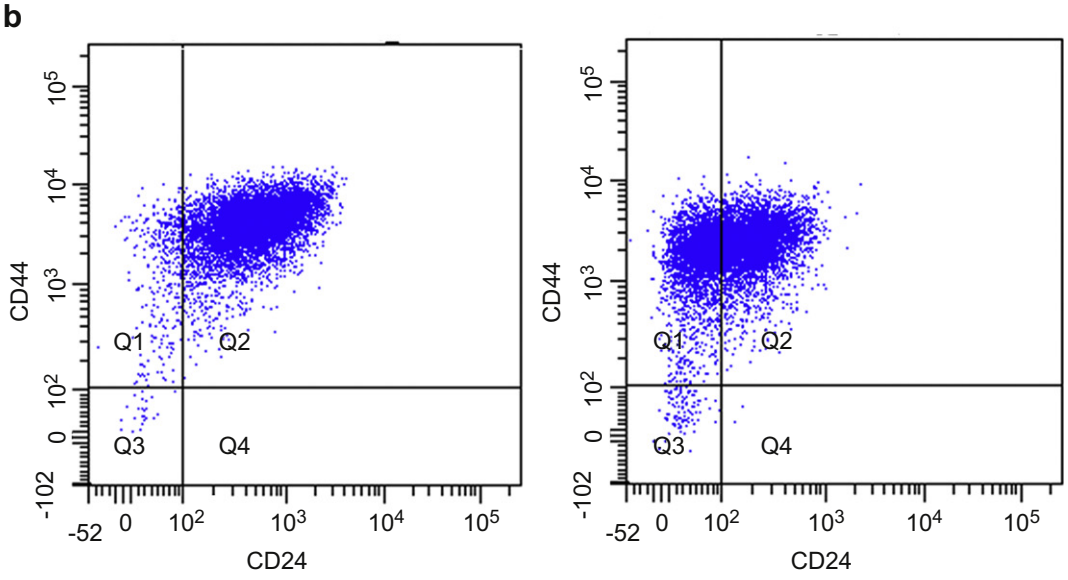
Cancer cells cultured in 3D scaffolds could mimic the tumor architecture *in vitro*. The 3D structures significantly increased malignant phenotypes of cancer cells which had been suppressed in cells cultured in 2D. Recent studies focused on the population of CSCs in 3D *in vitro* [30]. In this study, we developed a 3D collagen scaffold to mimic the structure of the tumor. The MCF-7 cell properties, including morphology, proliferation, secretion and tumorigenicity, were monitored. In addition, the overexpression of CSC-associated properties in MCF-7 cells cultured in 3D collagen scaffolds was found.

As collagen scaffolds provided enough interspaces for cell growth and attachment, MCF-7 cells cultured in collagen scaffolds displayed persistent proliferation and formed a multi-layer cell structure. The newly formed structure significantly improved the communications of cell–cell and cell-ECM, and provided a hypoxic centre [12,31,32]. The hypoxic microenvironment induced cells insensitivity to antigrowth signals and resistance to apoptosis, and thus extended the time period of proliferation. Overexpression of pro-angiogenic factors and MMPs, which participated in cancer ECM degradation, metastasis and angiogenesis *in vivo* [28,33,34], was also induced by cell-ECM interactions and hypoxia in 3D. This was consistent with *in vivo* condition that growth factors and MMPs were significantly upregulated in malignant tumors. Upregulation of growth factors and MMPs promoted xenografts vascularization to provide sufficient nutrients for rapid tumor formation *in vivo*, resulting in the enhanced tumor-forming capability of implants in mice [11,12,35].

3D culture also induced cells to adapt the hypoxic condition by EMT [36]. EMT, transformation from epithelial cells to mesenchymal cells, was required for tumor invasion and metastasis *in vivo* [37]. We found that 3D collagen scaffolds promoted the



	SOX2	OCT4A	SOX4	JAG1	CD49F
2 D	1.010579566	1.001431277	1.001156232	1.000164863	1.000359512
3 D	2.445951721	2.796127284	1.547039771	2.533774722	3.616093326



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Cells	10^6	10^5	10^4	10^3
2 D	6/6	1/6	0/6	0/6
3 D	6/6	3/5	0/6	0/6

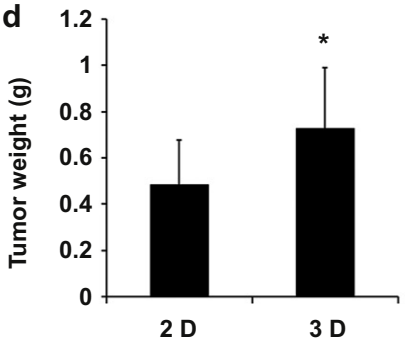

Fig. 5. MCF-7 cells acquired CSC phenotypes after being cultured in 3D collagen scaffolds. (a) Relative transcriptional levels of SOX2, OCT4A, SOX4, JAG1 and CD49F were determined in cells cultured in 3D collagen scaffolds. Results were mean ± s.d. GAPDH mRNA was utilized to normalize gene expression data. (b) Flow cytometric analysis of CD24 and CD44 surface markers in MCF-7 cells from 2D and 3D culture system. Cells in 3D showed higher proportion of $CD44^{+}/CD24^{-/low}$ antigen phenotype than those in 2D. (c) MCF-7 cells cultured in 2D and 3D were injected into the mammary fat pad of athymic mice at indicated numbers. The number of tumors/injections was shown. (d) 3D cultured cells formed larger tumors than 2D cultured cells when the injection number was 1×10^6. Results were mean ± s.d. $^*P < 0.05$, by student's *t*-test.

transformation of MCF-7 cells from epithelial to mesenchymal morphologies. Furthermore, upregulation of generous EMT markers, including transcription factors, cell-surface proteins and cytoskeletal proteins, was observed in cells cultured in 3D collagen scaffolds, whereas the epithelial marker E-cadherin was decreased. The probable reason for this was that the hypoxic centre in 3D architecture upregulated the expression of hypoxia-inducible factors (HIFs) [38]. HIFs activated lysyl oxidase (LOX)-SNAIL

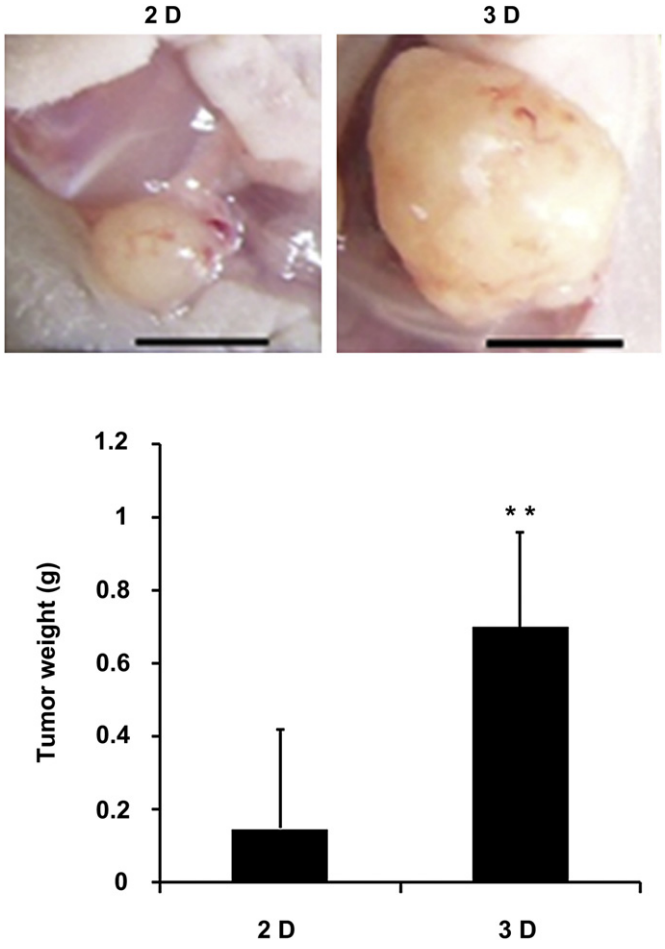

Fig. 6. Tumorigenicity of implants loaded or cultured with MCF-7 cells *in vivo*. The tumor weights were examined. Scale bar was 0.5 cm. Results were mean ± s.d. $^{**}P < 0.01$, by student's *t*-test.

pathway to attenuate E-cadherin expression and initiated the critical step of EMT [39,40]. In addition, the repression of E-cadherin resulted in the liberation of β-catenin which might form β-catenin/TCF/LEF complex to stabilize the high expression level of EMT-associated transcription factors to maintain EMT [41]. Upregulated ZEB1 and ZEB2 were involved in mediation of EMT by repressing E-cadherin transcription during the initial step of EMT [42], while TWIST1 was able to directly repress E-cadherin expression independent of SNAIL pathway [43]. The enhanced expression of EMT-induced markers, including DDR2, ITGA5 and SPARC, reflected the adaptation of cells to 3D architecture as these proteins were receptors of ECM [20]. The high transcriptional level of EMT markers and low level of epithelial marker showed that MCF-7 cells cultured in 3D collagen scaffolds might acquire mesenchymal phenotypes by the process of EMT.

Mani *et al*. had demonstrated that EMT-induced by overexpression of SNAIL1 and TWIST1 in epithelial cells resulted in the acquisition of breast cancer stem cell associated phenotypes [44]. As anticipated, the stemness of MCF-7 cells was improved by upregulation of stem cell markers and breast cancer stem cell signatures in our study. OCT4A and SOX2 were two major stem cell markers which functioned on stem cell self-renewal and pluripotency. Overexpression of these two transcription factors might cause dedifferentiation of cancer cells, leading to the emergence of CSC-like cells [45]. Breast cancer stem cell signatures, including SOX4, JAG1 and CD49F, were related to sphere-forming efficiency and tumor-forming capability [46]. The phenotype $CD44^{+}/CD24^{-/low}$, an important property of breast cancer stem cells, was always utilized to enrich CSCs in luminal cell lines [47]. We found that the population of cells with $CD44^{+}/CD24^{-/low}$ phenotype was significantly increased in 3D. Moreover, the tumorigenic capability of CSCs was also shown by the fact that cells cultured in 3D scaffolds displayed higher tumorigenicity than those in 2D. It suggested that 3D collagen scaffolds promoted MCF-7 cells to generate a population of cells with CSC properties. The enhanced stemness of MCF-7 cells might be another reason for the high tumor-forming capability of xenografts cultured with cells.

The results suggested that cells with CSC phenotypes were generated in 3D scaffolds by the process of EMT. The findings might be useful for the research on new anti-cancer drugs which functioned on EMT and CSCs.

5. Conclusion

In this study, we developed a 3D collagen scaffold to study MCF-7 cells. The scaffolds extended the time period of cell proliferation, promoted overexpression of pro-angiogenic growth factors, MMP transcriptions and enhanced tumorigenicity of cells *in vivo*. In addition, the upregulation of EMT markers was observed in 3D cultured cells. High proportion of cells with CSC properties was also generated in 3D culture. We anticipated that the 3D collagen scaffolds might provide a useful model to evaluate the efficacy of novel anti-cancer drugs and enrich breast cancer stem cells *in vitro*.

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