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ORIGINAL RESEARCH

In vitro spontaneous differentiation of human breast cancer stem cells and methods to control this process

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Abstract—Breast cancer is said to originate from breast cancer stem cells (BCSCs). Previous published studies showed that BCSCsexhibited a high degree of multi-drug resistance. This study aimed to evaluate the spontaneous differentiation of human BCSCs and investigate various *in vitro* conditions that could be used to control this process. BCSCs were sorted from primary cultures of breast malignant tumors based on expression of CD44 and CD24. The *in vitro* spontaneous differentiation of BCSCs was evaluated using standard DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. There were six different methods tried to control the spontaneous differentiation of BCSCs including culturing in serum-free medium, mammosphere medium, basic fibroblast growth factor and epidermal growth factor supplemented medium with serum, and culturing under hypoxic conditions. The results showed that BCSCs always spontaneously differentiated *in vitro* in standard DMEM/F12 plus 10% FBS culture medium. All investigated culture conditions could not completely inhibit the spontaneous differentiation of BCSCs. Serum-free culture medium under hypoxic conditions (mammosphere culture) had the strongest inhibitory effect on this process. These results demonstrated that spontaneous differentiation is a natural process of BCSCs; therefore this process may be somewhat controlled depending on the culture conditions.

Keywords—Breast cancer stem cells, Breast cancer cells, Spontaneous differentiation, Stemness, Stem cell culture

INTRODUCTION

Breast tumors are thought to originate from breast cancer stem cells (BCSCs) that were first discovered by Al-Hajj and colleagues (Al-Hajj et al., 2003). Because BCSCs have been shown to be multi-drug resistant, radiation-resistant, and involved in metastasis and cancer relapse (Al-Ejeh et al., 2011; Liu et al., 2010; Liu and Wicha, 2010; Xie et al., 2012), they are considered to be the target for all new breast cancer therapies (Ginestier et al., 2010; Liu and Wicha, 2010). Some preclinical studies and clinical trials that have targeted BCSCs have had promising results (Ahmadipour et al., 2015; Bostad et al., 2015; Gupta et al., 2009; Hu et al., 2015; Jiang et al., 2015; Kai et al., 2015; Luo et al., 2015; Montales et al., 2012; Wu et al., 2015; Zhang et al., 2014). The data confirm that BCSCs play a critical role in the initiation oftumors. Therefore, BCSCs are an important and valuable model for drug discovery as well as for the development of novel therapies.

As of 2003, BCSCs have been isolated using different methods by several groups. Culturing BCSCs, as described in previous studies, has involved the use of various culture media and techniques, including the use of standard DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) for adherent cultures (Pham et al., 2011; Van Phuc et al., 2011), serum-reduced medium for mammospheres(Carmody et al., 2012; Gupta et al., 2011), and more recently, serum-free medium to maintain BCSCs in culture (de la Mare et

al., 2013; Grimshaw et al., 2008; Lombardo et al., 2015; Manuel Iglesias et al., 2013; Montales et al., 2012; Wang et al., 2014).

In some recent publications, oxygen concentration has been shown to also affect drug resistance (Crowder et al., 2014; Samanta et al., 2014) and stemness of BCSCs (Mimeault and Batra, 2013; van den Beucken et al., 2014). Therefore, in this study we investigated the effects of components of media (FBS and growth factors) and oxygen concentration on spontaneous differentiation of BCSCs *in vitro*. Our results suggest that there are suitable cell culture conditions in which to maintain the stemness of BCSCs.

Material and Methods

Isolation of BCSCs

BCSCs were isolated according to previously published research (Pham et al., 2011; Van Phuc et al., 2011). $CD44^+CD24^-$BCSCs were sorted from a breast cancer cell population using flow cytometry (FASCjazz, BD Bioscience). Briefly, BCSCs were stained with monoclonal antibodies CD44-FITC and CD24-APC. $CD44^+CD24^-$ BCSCs were sorted and re-confirmed using a FACSCalibur flow cytometer. $CD44^+CD24^-$ BCSCs with 100% purity were used in further experiments.

BCSC culture

In this study, three kinds of media were used: DMEM/F12 supplemented with 10% FBS; DMEM/F12 supplemented with 10% FBS, with 10 ng/ml EGF and 10 ng/ml FGF; and mammosphere medium (**Table 1**). In addition, two oxygen concentrations, 10 and 21%, were tested. Six groups, made up of a combination of three kinds of medium and two oxygen concentrations, were investigated as shown in Table 1. BCSCs were cultured in 96-multi-well plates at 10^4 cells/well. All plates were cultured in a tri-gas incubator at 37°C and 5% CO_2.

Flow cytometry analysis

Cells were washed twice in phosphate-buffered saline containing 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO). Fc receptors were blocked by incubation with immunoglobulin G (Santa Cruz Biotechnology, CA) on ice for 15 min. Cells were stained with anti-CD44-FITC and anti-CD24-APC monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ, USA) at 4° C for 30 min. After washing, cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest Pro software (BD Biosciences) using data acquired from 10,000 events.

Table 1. Culture conditions of the six groups.

Group	Medium	Oxygen (%)
N1	DMEM/F12 supplemented with 10% FBS and 1% antibiotic-mycotic	21%
N2	DMEM/F12 supplemented with 10% FBS, 1% antibiotic-mycotic, 10 ng/ml EGF, and 10 ng/ml bFGF	21%
N3	DMEM/F12 supplemented with 1% (v/v) PSA, 2% (v/v) B-27 supplement, 20 ng/ml EGF and bFGF, 4 ng/ml heparin and 10 μg/ml insulin	21%
H1	DMEM/F12 supplemented with 10% FBS and 1% antibiotic-mycotic	10%
H2	DMEM/F12 supplemented with 10% FBS, 1% antibiotic-mycotic, 10 ng/ml EGF, and 10 ng/ml bFGF	10%
H3	DMEM/F12 supplemented with 1% (v/v) PSA, 2% (v/v) B-27 supplement, 20 ng/ml EGF and bFGF, 4 ng/ml heparin and 10 μg/ml insulin	10%

Doxorubicin resistance assay

BCSCs were cultured as shown in Table 1 and then seeded at a density of 0.4 ×10^4 cells per well in 24-well plates (Nunc) in DMEMF12/10% FBS. After 24 h, cells were treated with 0, 1, 3, and 6 μg/ml doxorubicin (Sigma-Aldrich, St. Louis, MO, USA) for 48 h. Cells were then subjected to apoptotic analysis.

Annexin V/PI assay

Apoptosis was investigated using flow cytometry with annexin V and propidium iodide (PI; BD Biosciences). Cells that had been grown under the six different culture conditions were washed twice in PBS and fixed in cold 70% ethanol for at least 3 h at 4°C. Subsequently, cells were washed twice in PBS and stained with 1 ml of PI (20 g/ml). A 50 μl volume of RNase A (10 μg/ml) was added to samples and incubated for 3 h at 4°C. Stained cells were analyzed using flow cytometry and CellQuest Pro software.

*In vivo*tumorigenesis assay

Female (5–6 weeks old) NOD/severe-combined immunodeficient (SCID) mice (NOD.CB17-Prkdcscid/J; Charles River Laboratories, Wilmington, MA) were subcutaneously injected with 10^5cells/mouse, with three mice per group. Mice were visually monitored for tumor formation at 7, 14 and 21 days post-injection. The Institutional Animal Care and Use Committee of Stem Cell Research and Application Laboratory, University of Science, VNU-HCM approved all animal experiments. s

Statistical analysis

All experiments were performed in triplicate. The significance of differences between mean values was assessed using the *t*-test and ANOVA. A *p*-value less than 0.05 was considered to be significant. Data were analyzed using Prism 6 software.

Results

Spontaneous differentiation of BCSCs *in vitro*

The percentage of sorted $CD44^+CD24^-$BCSCs gradually decreased after 1, 2, 4 and 8 weeks of culture in DMEM/F12 supplemented with 10% FBS and 1% antibiotic-antimycotic, although cell morphology was not significantly different. The percent of $CD44^+CD24^-$ BCSCs decreased from 100% at start to 65.43±15.21%, 47.39±17.31%, 33.87±9.81%, and 12.31+4.52% after 1, 2, 4 and 8 weeks, respectively. Moreover, doxorubicin resistance also dramatically decreased after 4 and 8 weeks of culture. In fact, the percent of apoptotic cells significantly increased after 4 and 8 weeks of culture compared with after 1 and 2 weeks (20.41±43%, 32±9.41%, 54.78±14.76%, and 68.41±14.39% apoptotic cells after 1, 2, 4 and 8 weeks, respectively). The *in vivo*tumorigenesis assay also showed that tumorigenesis of cultured cells decreased after 8 weeks of culture (100% of mice bearing tumors per group after 1, 2 and 4 weeks vs. 75 % mice bearing tumors per group after 8 weeks).

Effects of culture conditions on the $CD44^+CD24^-$ BCSC population

To optimize culture conditions for BCSCs, a combination of three different culture media and two oxygen concentrations were used. BCSCs were cultured in these six different conditions (**Table 1**) for 8 weeks. The $CD44^+CD24^-$ cell population was isolated using flow cytometry and cell shape changes were monitored using an inverted microscope. The results are presented in **Figs. 1** and showed that cell morphology showed little change between week 1 and week 8 in all groups (see **Table 1**).

As presented in **Fig. 1**, the cell population with the BCSC phenotype was different between groups. In the DMEM/F12 supplemented with 10% FBS and a normal level of oxygen, after 4 weeks, there were only 21.57±4.41% $CD44^+CD24^-$ BCSCs, while this percentage increased when cells were grown in the same medium but under hypoxic conditions (39.57±2.60%). For all media, the hypoxic condition always resulted in a greater percentage of $CD44^+CD24^-$BCSCsthan under normoxic conditions (in DMEM/F12 + 10% FBS, 21.67±4.41% vs. 39.67±2.60%; in DMEM/F12 plus FGF and EGF, 42.00±2.52 vs. 68.33±4.41%, and in mammosphere medium 61.67±6.01% vs. 78.00±2.08%, under normoxic and hypoxic conditions, respectively).

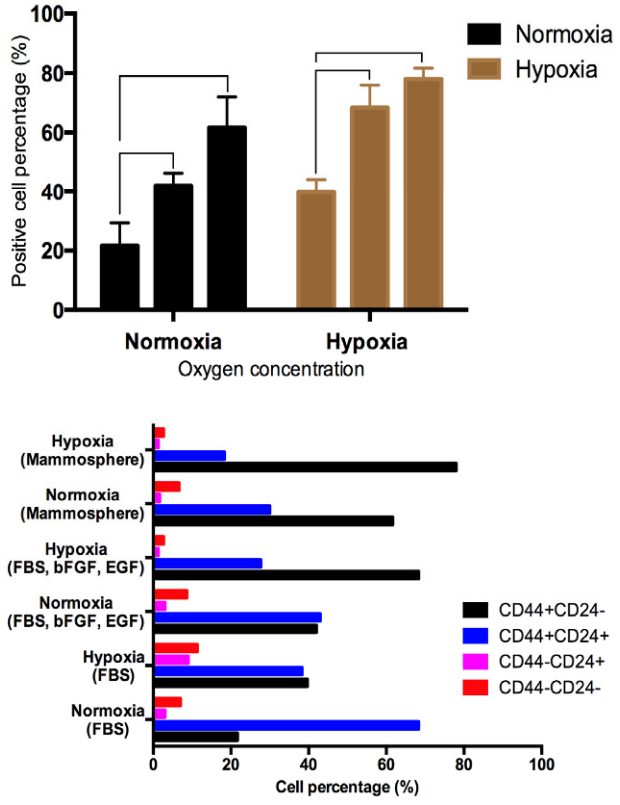

Figure 1. Effect of oxygen concentrations and media on percentage of $CD44^+CD24^-$ BCSCs after 4 weeks (A); Effect of oxygen concentrations and culture media on spontaneous differentiation of BCSCs into different phenotypes (B).

We found that culture medium and oxygen concentration affected the BCSC phenotype in culture, as shown in **Fig. 1**. Effects of FBS, high oxygen concentrations as well as a lack of FGF and EGF resulted in the differentiation of BCSCs with a $CD44^+CD24^+$ phenotype. In medium with 10% FBS in DMEM/F12 and under normoxic conditions, there were up to 70% $CD44^+CD24^+$ cells after 4 weeks. This result also showed that BCSCs underwent spontaneous differentiation during *in vitro* culture.

Effect of culture medium on drug resistance of BCSCs

BCSCs cultured under different conditions also exhibited different levels of drug resistance. Fig. 4 shows that BCSCs cultured in DMEM/F12 supplemented with 10% FBS were more sensitive to doxorubicin than BCSCs cultured in DMEM/F12 supplemented with 10% FBS and EGF and bFGF or mammospheres under both normoxic and hypoxic conditions. Under normoxic conditions, doxorubicin treatment of BCSCs cultured in DMEM/F12 plus 10% FBS, DMEM/F12 plus 10% FBS, EGF and FGF, or in mammosphere culture medium resulted in a gradual decrease in the percentage of apoptotic cells from 63.00±8.4 to 44.50±12.96 to 22.50±8.25%, respectively. Similarly, under hypoxic conditions, these percentages were 63.00±8.485, 44.50±12.96, and 22.50±8.250%, respectively. A decrease in the drug sensitive cell population resulted in an increase in the drug resistant cell population (**Fig. 2**).

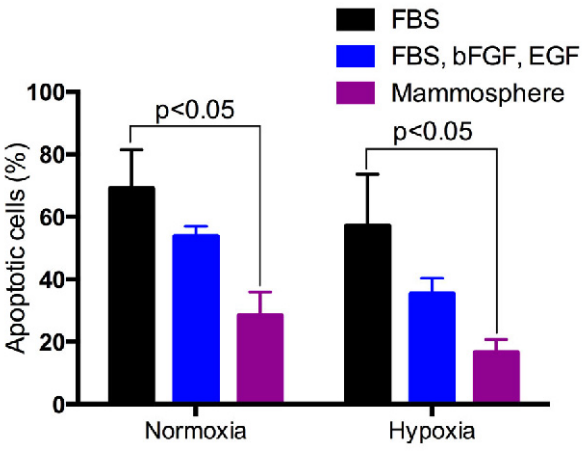

Figure 2. Doxorubicin resistance of BCSCs was decreased in medium with FBS and under normoxic conditions. (A) Flow cytometry was used to determine the percent apoptotic BCSCs after being treated with doxorubicin for 28 h; (B) Percent apoptotic BCSCs cultured under different conditions and treated with doxorubicin for 48 h.

Table 2. Appearance of tumors in nude mice after 4 weeks following injection of 10^5 BCSCs cultured under different conditions.

Time of transplantation (days)	Fresh BCSC	Normoxia			Hypoxia		
		FBS	FBS, bFGF EGF	Mam	FBS	FBS, bFGF EGF	Mam
0	0	0	0	0	0	0	0
7	3/3	0	0	3/3	2/3	3/3	3/3
14	3/3	1/3	2/3	3/3	3/3	3/3	3/3
21	3/3	2/3	3/3	3/3	3/3	3/3	3/3

BCSC: Breast Cancer Stem Cells; FBS: Fetal bovine serum; bFGF: Basic fibroblast growth factor, EGF: Epidermal growth factor; Mam: Mammosphere

Culture conditions also affected tumorigenesis*in vivo*. Based on the results shown in Table 2, BCSCs in mammospheres under both normoxic and hypoxic conditions retained their tumorigenicity when 10^5 cells were injected into mice, while cells cultured under adherent culture conditions in DMEM/F12 plus 10% FBS or 10% FBS with EGF and FGF showed reduced tumorigenicity (**Table 2**).

Discussion

Al-Hajj and colleagues discovered BCSCs more than 10 years ago (Al-Hajj et al., 2003). BCSCs have since become targets for drugs as well as new therapies, and have become central to breast cancer treatment. Therefore, it is essential to have a ready supply of BCSCs to pursue drug discovery as well as therapy development. However, we show in this study that culture conditions including culture medium and oxygen concentration can affect the stemness of BCSCs during their time in culture.

In the first experiment, the results showed that BCSCs readily differentiated into different types of breast cancer cells. In fact, given the particular characteristics of stem cells, BCSCs can differentiate into specific cells (Pham et al., 2011). In this case, BCSCs differentiated into breast cancer cells. The medium containing FBS strongly triggered BCSC differentiation. FBS, which contains a pool of undetermined factors and components, caused the differentiation of BCSCs. Although FBS is an essential component of cell culture medium, it has been shown to cause differentiation of certain kinds of stem cells such as neural and embryonic, as well as germ cells (Bettiol et al., 2007; Franke et al., 2014; Hung and Young, 2006; Tateishi et al., 2008; Zahir et al., 2009).

In the next experiments, results further showed that in

medium supplemented with FBS, a high percentage of BCSCs lost their stemness. This demonstrated that FBS was the differentiating factor. Conversely, BCSCs retained their stemness when cultured in medium lacking FBS. Studies in which proliferation of stem cells was required, therefore, have used serum-free medium.

This study also showed that the oxygen concentration affected maintenance of stemness of BCSCs. A high oxygen concentration triggered the differentiation of BCSCs. In fact, a high concentration of oxygen will increase the amount of radical oxygen species (ROS). ROS also acted as a differentiating factor for stem cells in previous studies. ROS can interfere with certain signaling pathways related to stemness. Hypoxic cultures have been shown in some studies to increase the expression of certain stemness-related genes. Hypoxia-inducible factor (HIF) was found to induce the expression of human embryonic stem cell markers in cancercells(Mathieu et al., 2011). Hypoxia has also been shown to prevent etoposide-induced DNA damage in cancercells through a mechanism involving HIF-1(Sullivan and Graham, 2009). More importantly, hypoxia was found to induce miR-424, which decreases tumor sensitivity to chemotherapy by inhibiting apoptosis (Zhang et al., 2014). Furthermore, hypoxia could also induce Jagged2, promoting breast cancer metastasis and self-renewal of cancerstem-like cells (Xing et al., 2011). Finally, hypoxia was shown to induce expression of HIF-1, which directly enhanced transcriptional activity of stem cell factor (SCF) in response to hypoxia and epidermal growth factor (EGF) (Han et al., 2008).

Conclusion

BCSCs can spontaneously differentiate into breast cancer cells. We showed that both the type of culture medium and concentration of oxygen strongly influenced BCSC differentiation. FBS and ROS were also differentiation factors. BCSCs were also found to differentiate into breast cancer cells in medium supplemented with FBS and under normoxic conditions. Therefore, proliferation of BCSCs requires that they be cultured in serum-free medium under hypoxic conditions. These results hoped that there was a significant contribution to BCSC study. However, in this study we showed that it was difficult to culture BCSCs while maintaining 100% stemness ($CD44^+CD24^-$ BCSC phenotype). Further experiments will need to be performed to improve upon these results.

Abbreviations

BCSCS: Breast cancer stem cells; EGF Epidermal growth factor; FBS: Fetal bovine serum; HIF: Hypoxia-inducible factor; SCF: Stem cell factor

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Competing interests

The authors declare that they have no competing interests.

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