Stroma derived from breast cancer tissue increases breast cancer stem cells population in vitro

Novi Silvia Hardiany,1 Septelia Inawati Wanandi,1 Kanã Tachi,2 Akira Shiraishi,2 Toshiki Kato,2 Osamu Ohneda2

1Department of Biochemistry & Molecular Biology, Faculty of Medicine Universitas Indonesia, Indonesia
2Laboratory of Regenerative Medicine & Stem Cell Biology, Faculty of Medicine University of Tsukuba, Japan

Corresponding Author:
Dr. dr. Novi Silvia Hardiany, MBiomed
Dept. Biochemistry & Molecular Biology, Faculty of Medicine Universitas Indonesia
Telp. +6221-3910734
Email: novi.silvia@ui.ac.id

Abstract

Breast cancer is the most frequent cancer causing death in woman population in the world. Accumulating evidence found that cancer stem cells (CSCs) are responsible for high tumor recurrence, therapy resistance and metastasis. The behavior of breast CSCs are influenced by their microenvironment. It is remain unclear whether stroma derived from primary breast cancer tissue can increase breast CSCs population. Therefore, this research was a pilot study to analyze the effect of breast cancer stroma towards breast CSCs population and pluripotency characteristic in vitro. Stroma was identified from primary breast cancer tissues obtained from 2 patients, while breast cancer cells were isolated from pleural effusion of breast cancer patient (BC#1). The BC#1 cells were directly co-cultured with breast cancer stroma (BCS) and also treated by 50% normoxic and hypoxic conditioned medium (CM) of BCS for 48 hours. After that, breast CSCs population using aldefluor assay, mammosphere assay as well as pluripotency characteristic using qPCR were observed. The result exhibited that breast CSCs population was 2.9-fold up regulated after co-culture with BCS, 1.7-fold up regulated after treated by hypoxic CM and 1.5-fold up regulated by normoxic CM. Moreover pluripotency characteristic (OCT-4, SOX-2 and NANOG mRNA expression) were also increased. Hypoxic CM increased more mammosphere number of BC#1 cells compared to normoxic CM. We concluded that stroma derived from breast cancer tissue increased breast CSCs population in which cell to cell interaction confers more CSCs population compared to CM treatment. Meanwhile hypoxic CM also stimulates more breast CSCs compared to normoxic CM. Further research is needed to analyzed secreted molecule from stroma which is responsible in enhancement of CSCs population.

Key Words: breast cancer cells, stroma, cancer stem cells, co-culture, conditioned medium
Introduction

Breast cancer is the most frequent cancer causing death in woman population in the world. Accumulating evidence found that cancer stem cells (CSCs) are responsible for high tumor recurrence, therapy resistance and metastasis (Tang et al, 2007; Dalerba et al 2007). Therefore, targeted breast cancer stem cells research is really crucial. Cancer stem cells are small-scale sub population of cancer cells which own stemness and tumorigenic. Breast CSCs arise from mammary multipotent stem cells due to genetic defects in pathways controlling self-renewal and differentiation (Shipitsin et al., 2007). Identification of breast CSC could be detected by its surface markers. Breast CSCs express the hyaluronan receptor CD44 (CD44+) and lack of the expression of CD24 (CD24-/low), an endogenous inhibitor of the chemokine receptor CXCR (Al-Hajj et al., 2003). Other marker for breast cancer stem cells is aldehyde dehydrogenase 1 (ALDH1+) activity. ALDH is an enzyme catalyzed intracellular retinoic acid that connected to cellular differentiation and stem cells protection. ALDH1 is better as a marker for breast CSCs compared to CD44+/CD24- (Tanei et al, 2009).

The behavior of breast cancer stem cells is influenced by their microenvironment. There are complex interactions between breast cancer stem cells with stromal as the component of microenvironment such fibroblasts, mesenchymal stem cells, adipocytes, endothelial cells, and immune cells through growth factor and cytokines networks. Previous study reported that mesenchymal stem cells (MSCs) were recruited from bone marrow and homing around the cancer cells to support tumor growth and metastasis (Chaturvedi et al, 2013). Liu et al elaborated the effect of human bone marrow MSCs toward breast cancer stem cells population in SUM-159 cell line. Those MSCs secrete chemokine which is responsible for increasing ALDH1 positive cells in SUM-159 cell line. They proposed that IL-6 and CXCL-7 loop serve as regulator pathway for breast cancer stem cells self renewal (Liu et al, 2011). Moreover MSCs were already isolated and identified from various cancers such as human gastric cancer, ovarian cancer, including breast cancer and those cells could stimulate proliferation of cancer cells (Cao et al, 2009; Lis et al, 2011; Zhang et al; 2013). However, it still remains unclear whether stromal derived from cancer tissues can induce the enhancement of cancer stem cells. Therefore, this research was a pilot study to analyze the effect of primary breast cancer stroma toward breast cancer stem cells population and pluripotency characteristic.

Material and Methods

Cell culture

Primary breast cancer cells (BC#1) were isolated from metastatic pleural effusion of breast cancer patient as described previously (Tachi et al, 2016). Those cells were cultured in DMEM high glucose medium (Gibco) supplemented with 10 % FBS (Thermo Fisher Scientific), L-glutamine, MEM-non essential amino acid and penicillin/streptomycine at 37°C in a humidified atmosphere of 5 % CO2 and 95 % air. Meanwhile stromal cells were isolated from breast cancer patients (n=2) who undergone mastectomies at University of Tsukuba hospital (Japan) with ethics approval from Tsukuba University. Fresh cancer tissues were cut into small pieces (< 1 mm) and washed with phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Afterward, they were treated by 0.1 % collagenase and incubated at 37°C for 1 hour. Dissociation of tissues was also assisted by syringe 20G followed by filtering it into cells strainer 100 μm-sized (BD Falcon) and centrifuged at 1000 rpm to get single cell suspensions. Those cells were floated in 24-well plate contains DMEM high
glucose medium (Gibco) supplemented with 10 % FBS (Thermo Fisher Scientific), L-glutamine, MEM-non essential amino acid, bFGF and penicillin/streptomycin at 37°C in a humidified atmosphere of 5 % CO2 and 95 % air. After adherent fibroblast-like cells confluent, those cells were trypsinized and cultured into 25 mm2 TC-Flask (Corning) for expansion.

**Identification of breast cancer stem cells population**
Breast cancer stem cells population was detected by ALDH1 activity using ALDEFLUOR kit (Stemcell Technology). Approximately 3 x 10^5 of breast cancer cells were harvested and diluted in 300 µL of Aldefluor buffer. 1.5 µL of Aldefluor reagent was added to cells and incubated in 37°C for 30 minutes subsequently analyzed using FACS sorting (MoFlo XDP; Beckman Coulter, Tokyo, Japan).

**Identification of stromal cells**
Flow cytometry analysis (MoFlo XDP; Beckman Coulter, Tokyo, Japan) was performed in order to identify stromal cells. Cells were stained with monoclonal antibodies against CD90, CD31 (FITC-conjugated); CD73 (PE-conjugated) as well as CD105, CD45 (APC-conjugated) (Becton Dickinson, San Jose, CA, USA). FITC-IgG, PE-IgG and APC-IgG were used for gating and compensation. (Becton Dickinson, San Jose, CA, USA). Differentiation assay was also conducted in order to characterize the type of stromal cells. Stromal cells were maintained in osteogenesis [Iscove’s Modified Dulbecco’s Medium (IMDM) + 1%FBS + dexamethasone (DEX) + Epidermal Growth factor (EGF) + β-glycerophosphate+Ascorbic acid] and adipogenesis [IMDM + 10%FBS + DEX + insulin + isobutyl methylxanthine (IBMX) + indomethacine] condition for 28 days. Each medium was replaced every 4 days.

**Generation of Green Fluorescens Protein (GFP)-Stromal cells**
Approximately 2 x 10^4 of stromal cells were seeded at 24-well-plate and cultured the cells up to 40-50% confluent (2 days). Afterward, medium was replaced by fresh culture medium containing Polybrene (4 μg/mL) and 10 μL of GFP-lentiviral (Mission TurboGFP™ Sigma) were added to each well. Transfection cells were incubated for 48 – 72 hours. GFP-positive cells were observed under fluorescence microscope. After cells were confluent, cells were harvested and seeded into 10 cm dish. GFP-positive cells were sorted using FACS sorting (MoFlo XDP; Beckman Coulter, Tokyo, Japan).

**Co culture of breast cancer cells and GFP-stroma cells**
Co-culture was performed directly to get cell to cell contact between breast cancer cells and stromal cells. Approximately 4 X 10^5 of GFP positive stroma were co cultured with 4 x 10^5 of breast cancer cells for 48 hours at 37°C in a humidified atmosphere of 95 % air and 5% CO2. After co-culture, breast cancer cells are collected using FACS sorting as the GFP negative cells and then analyzed for ALDH1 activity using flow cytometry (MoFlo XDP; Beckman Coulter, Tokyo, Japan). Moreover, breast cancer cells also collected for RNA extraction.

**Generation of conditioned medium (CM) and treatment**
Four hundred thousand of stromal cells were seeded in 6 cm dish with 10 % FBS DMEM high glucose/L-glutamine/MEM-NEAA/bFGF and allowed to adhere overnight at 37°C, 5% CO2, 21 % O2. The following day, media is removed and cells are washed 3 times using PBS. Serum free- low glucose media were added to the cells and incubated for 24 hours in normoxic (37°C, 20% O2, 5% CO2) and hypoxic (37°C, 1% O2, 5% CO2) condition. After 24 hours, media were collected, centrifuged to remove cell debris and passed through 0.22 µm filter. Concentration of conditioned media was 50 % (according to optimization, 50% CM
confer the highest ALDH1 activity). It was performed by diluting CM in freshly DMEM low glucose/10% FBS. Breast cancer cells were cultured with that freshly 50% CM for 48 hours.

**Analysis of pluripotency**
Pluripotency genes (OCT4, SOX2 and NANOG) as well as ALDH1 mRNA were analyzed using quantitative Polymerase Chain Reaction/qPCR. First, RNA was extracted from breast cancer cells after treatment with conditioned media and co-culture using Sepasol RNA 1 Super G (Nacalai, Tesque, Kyoto, Japan). cDNA synthesis was performed using Revertra Ace qPCR RT kit (Toyobo). Then, cDNA was amplified using qPCR (Applied Biosystem, Foster City, CA, USA) with Thunderbird SYBR qPCR Mix (Toyobo). PCR cycles (40 cycles) for 10 seconds on 95°C; 30 seconds on 60°C; 30 seconds on 72°C, Melt curve analysis for 1 minutes on 95°C; 1 minutes on 55°C; 10 seconds on 55°C (80 cycles, increase 0.5°C every cycles). The primer sequences are shown in table 1.

**Table 1. Primer sequences (Tachi et al, 2016)**

| Human ALDH1 | Sense: 5'−TACCACCAGGGCCAGTGTTGTATAG-3' | Antisense: 5'−GAACACTGTGGGCTGGACAAAGTAG-3' |
| Human OCT4 | Sense: 5’-CTGGGGGTTCTATTTGGGAAGGTA-3’ | Antisense: 5’-CTGCAGGAAACAGATTCTCCAGGTT-3’ |
| Human SOX2 | Sense: 5’-GAGTGGAAACTTTGTCGGAGACG-3’ | Antisense: 5’-CCGGTATTTTATAATCCGGGTGCTC-3’ |
| Hman NANOG | Sense: 5’-ACAGAAATACCTCAGCCTCCAGCA-3’ | Antisense: 5’-CTCCAGGTTGAATTGTCCAGGTC-3’ |

**Mammosphere assay**
Approximately 1 X 10⁴ breast cancer cells (BC#1) after normoxic and hypoxic CM-treatment were cultured in ultra low attachment of 6-well plate (Corning) using DMEM high glucose medium (Gibco) supplemented with 10 % FBS (Thermo Fisher Scientific), L-glutamine, MEM-non essential amino acid and penicillin/streptomycin. The mammosphere size ≥ 700 µm was counted under a microscope.

**Statistical Analysis**
All data were presented as means ± SD from triplicate experiments. Statistical analysis was performed using Student’s t test with p < 0.05 as a significant difference.
Results

**Morphology of breast cancer cells (BC#1) and stromal cells**
Morphology of primary breast cancer cells (BC#1) were epithelial-like cells which had adhered to the plastic surface (figure 1A), while stromal cells appear spindle shape or fibroblastic and also attached to the plastic surface as shown in figure 1B.

![Morphology of BC#1 and stromal cells](image)

Figure 1. Morphology of breast cancer cells (A) and stromal cells (B) were different. Magnification X100.

**Characteristics of Stromal Cells**
Flow cytometry analysis exhibited that stromal cells expressed positive for CD90, CD73, CD105 but negative for CD31, CD45. Their expression same with mesenchymal stem cells from adipocyte tissue (AT-MSC) as a control (figure 2).

![Flow cytometry analysis](image)

Figure 2. Flow cytometry analysis. A. Stromal cells demonstrated positive for CD90, CD73, CD105, but negative for CD31, CD45. AT-MSC as a control isolated from adipocyte tissue of healthy person.

Differentiation assay also proved that stromal cells were able to differentiate into both osteocytes and adipocytes as shown by positive staining of Oil Red O (figure 3). Therefore, stromal cells resemble mesenchymal stem cells.
Figure 3. Differentiation assay. Stomal cells were capable to differentiate into adipocyte and osteoblast. (AT-MSC: Adipocyte Tissue-Mesenchymal Stem Cells).

**Generation of GFP-stromal cells**

Stromal cells have been labeled by GFP (as shown in figure 4A) in order to distinguish them with breast cancer cells after co-culture. GFP-stromal cells were sorted (figure 4B) using FACS sorting and plated into 100-mm petri dishes (Sumimoto, Bakelite) for expansion.

Figure 4. A. GFP-stromal cells were seen under a fluorescence microscope. Magnification x200. B. FACS sorting was performed to obtain purified GFP-stromal cells.

**Direct co-culture increased breast cancer stem cells population.**

We did direct co-culture between GFP-stromal cells and breast cancer cells to evaluate the effect of stroma cells towards breast CSCs. Breast cancer cells were appear surrounded by stromal cells (figure 5A-B). FACS sorting was performed to sort breast cancer population as GFP-negative cells. Afterwards, ALDH1 activity was observed that representing the population of breast cancer stem cells. The results demonstrated that breast CSCs population significantly increased after co-culture as shown in figure 5C.
Direct co-culture increased relative expression of pluripotency gene.

Pluripotency characteristic were observed by detecting relative expression of ALDH1, OCT4, SOX2 and NANOG using qPCR. ALDH1 and OCT4 expression significantly upregulated in breast cancer cells upon co-cultured with GFP-stromal cells (figure 6A-B). In addition, SOX2 and NANOG mRNA expression were also increased but not statistically significant (figure 6C-D).

Figure 6. Pluripotency characteristic ( * < 0.05; ** p < 0.01)
Stromal-Conditioned Medium (CM) increased breast cancer stem cells population and pluripotency characteristic.
Breast cancer cells were treated by normoxic and hypoxic conditioned medium of stromal cells. The results demonstrated that Aldefluor positive cells which representing breast CSCs significantly increased both after normoxic and hypoxic CM treatment (figure 7A). Moreover, SOX2 and NANOG mRNA expression also significantly increased especially after hypoxic-CM treatment (figure 7B).

![Figure 7A](image1.png)

**Figure 7A. Stromal-hypoxic CM stimulated mammosphere number of breast cancer cells.** Mammosphere number in breast cancer cells after treating by hypoxic CM were significantly increased (p < 0.05) compared to control. Nevertheless, the enhancement of mammosphere number in breast cancer cells upon normoxic CM treatment was not significant (figure 8).

![Figure 7B](image2.png)

**Figure 7B. Stromal-CM significantly increased breast CSCs population (A) and pluripotency characteristics (B). (★ p< 0.05; ★★ p < 0.01)**

**Stromal-hypoxic CM stimulated mammosphere number of breast cancer cells.**
Mammosphere number in breast cancer cells after treating by hypoxic CM were significantly increased (p < 0.05) compared to control. Nevertheless, the enhancement of mammosphere number in breast cancer cells upon normoxic CM treatment was not significant (figure 8).
Discussion

This work was a pilot study to investigate the effect of stromal derived breast cancer tissue toward breast CSCs population. Although there were several researchers who already isolated MSCs from various cancers (Cao et al, 2009; Lis et al, 2011; Zhang et al; 2013), none of them explored the effect of those stromal cells into CSCs population. Nevertheless, Liu et al proved that breast CSCs population in SUM 159 was stimulated by MSCs derived from human bone marrow. They concluded that MSCs derived from bone marrow may promote human breast tumor growth by producing cytokine meshwork that stimulating CSCs population (Liu et al, 2011). Herein, we demonstrated that stromal derived from breast cancer tissues enhanced breast CSCs population detecting by an increase in ALDH positive cells. ALDH activity was firstly measured by Cheung et al to isolate leukemia stem cells (Cheung et al, 2007). The function of ALDH is to convert retinal into retinoic acid (RA) in which RA play a role in stimulating RAR/RXR gene transcription for differentiation, survival and proliferation (Allahverdiyev et al, 2012). Accumulating evidences found that ALDH was successfully detected in various cancers, therefore it is postulated as a universal CSC marker (Marcato et al, 2011). Stemness characters were regulated by transcription factor such as OCT4, SOX2 and NANOG that maintain pluripotency. We demonstrated that pluripotency characteristics were also up-regulated in breast cancer cells upon co-culture and conditioned medium treatment. It means that stromal cells support self renewal capacity of breast cancer cells leading to therapy resistance.

Characterization of our stromal cells exhibited that it seems to MSCs. Previous study proved that mesenchymal cells generate interleukin-6 (IL-6), interleukin-8 (IL-8), CCL5 and CXCL5. IL-6 already demonstrated as direct regulator of breast cancer stem cells self renewal through activation of STAT3 (Korkaya et al., 2011). Potter et al also proved that stromal cells secreted CCL2 which stimulate cancer cell migration, tumor growth and neo-angiogenesis (Potter et al, 2012). In our research, cell to cells interaction between stromal and breast cancer cells perhaps are important. According to our results, breast CSCs population increased higher in direct co-culture compared to conditioned medium (CM) treatment. Breast CSCs population were 2.9 fold up-regulated after direct co-culture; 1.7 fold up-regulated after hypoxic CM while only 1.5 fold up-regulated after normoxic CM treatment. Conditioned medium contain secreted factors which affect breast CSCs population. We consider that hypoxic condition in stromal cells may increase the production of secreted factors that can stimulate CSCs growth. In fact, our results exhibited that hypoxic CM treatment also enriched mammosphere number in breast cancer cells. Taken together we concluded that stromal derived breast cancer tissue could increase breast CSCs populations especially by facilitating cell to cell interactions. Further analysis is required to elucidate the mechanism of this process by exploring secreted molecules which is responsible for the enhancement of CSCs. It may provide new strategy for breast cancer therapy.

Acknowledgements

The authors would like to express our gratitude to Direktorat Riset dan Pengabdian Masyarakat Universitas Indonesia (DRPM-UI) for International collaboration grant, as well as thank to Takeda Science Foundation (TSF) Japan for the fellowship research program.


