Secretomes of Adipose and Umbilical Cord-Derived Stem Cells Affect ALDH1A1 Expression in Breast Cancer Stem Cells

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Introduction: Conditioned media of Mesenchymal Stem Cell's culture containing secretomes has been identified to mediate communication between MSCs and cancer cells which could affect the stemness of cancer stem cells.

Methods: Conditioned media (CM) of Adipose Stem Cells (ASCs) and Umbilical Cord Stem Cells (UCSCs) was collected and supplemented to Breast Cancer Stem Cells (BCSCs) ALDH⁺ cells for 72 hours. The expression of ALDH1A1, Oct4 and Sox2 genes in BCSCs was determined using qRT-PCR technique, while the viability was measured using Tryphan blue exclusion assay.

Results: CM of ASCs (50% v/v) could significantly increase both cell viability and the expression of ALDH1A1, Oct4 and Sox2 genes in BCSCs. In contrast, CM of UCSCs (50% v/v) could significantly decrease the expression of ALDH1A1 gene and decrease the viability of BCSCs without affecting Oct4 and Sox2 expression.

Conclusion: ALDH1A1 was differentially expressed in BCSCs after supplementation with secretomes of ASCs and UCSCs. Further studies are required to confirm the role of MSCs secretomes on the pluripotency and viability of BCSCs.

Keywords: BCSCs, ASCs, UCSCs, Secretomes, ALDH1A1.

1. INTRODUCTION
Mesenchymal stem cells (MSCs) are non-hematopoietic pluripotent cells that give rise to a variety of connective tissue cell types. They are able to differentiate into endodermal, ectodermal, and mesodermal origins when placed in the appropriate environments. Based on the minimal criteria established by the International Society of Cellular Therapy, MSCs were characterized by positive expression of stromal cell markers CD73, CD105 and negative of hematopoietic markers CD14, CD34, and CD45.1 Mesenchymal stem cells express and produce a wide spectrum of biologically active factors known as secretomes. These factors such as fibroblast growth factor (FGF), IL-1 and 6, TGF-β, and VEGF which are vital for paracrine mechanism.¹

Both breast and abdominal derived adipose stem cells (ASCs) have a comparable gene expression profile and similar effects in supporting breast cancer cell line proliferation.² While umbilical cord stem cells (UCSCs) are categorized as perinatal stem cells; therefore, they are less immunogenic and possess pluripotency properties.³

In the tumor microenvironment, MSCs play an important role on the modulation of tumor progression and drug sensitivity. Recent studies found that MSCs homing and reside within the tumor microenvironment with great affinity and alter the tumor condition.¹ However, the dynamic and reciprocal interaction between MSCs and cancer cells remain unclear. Growing evidence has suggested that MSCs produced tumorigenic substances when they are in contact with tumor cells, either by direct cell to cell contact or indirect paracrine release mechanism.³

Cancer stem cells (CSCs) have been identified as a side population within tumors, owing stemness properties like self-renewal and expression of stem cell-related genes. Moreover, interactions among cancer stem cells and their microenvironments as well as with secretomes of MSCs initiate a cascade of growth factors and inducing elements, which in turn influence CSCs function. Among the four major pluripotent factors, OCT4 and SOX2 are two pluripotent transcription factors essential to pluripotent and
self-renewing phenotypes that contribute to the reprogramming of differentiated somatic cells into induced pluripotent stem cells. There is evidence that Oct4 and Sox2 act synergistically in regulating the enhancers of pluripotent stem cell-specific genes.4,5 For years ALDH1 has been used as marker for malignant human mammary stem cells. High ALDH1 activity cell populations correlated with tumorigenesis, poor prognosis, and increased metastasis in xenografted mouse models.6 ALDH1A subfamily (ALDH1A1 and ALDH1A3) are involved in drug resistance by protecting stem cells against toxic endogenous and exogenous aldehydes and of stemness properties through retinoic acid signaling pathway by oxidation of all-trans-retinal and 9-cis-retinal to retinoic acid (RA).5,6 Furthermore, ALDH1A1 expression in breast cancer was found to correlate with advanced disease stage, triple negativity, and poor outcome following neoadjuvant chemotherapy.7,8

Since the role of MSCs derived from various sources on ALDH1A1 expression related to the pluripotency of human BCSCs is still controversial until now. In this study, we aimed to analyze the effect of secretomes contained conditioned media of adipose-derived and umbilical cord-derived MSCs on the ALDH1A1; Oct4 and Sox2 expression of BCSCs (ALDH+ cells).

### 2. METHODS

#### 2.1. Ethics and Specimens

According to Helsinki Declaration of ethical principles 1964, this study has been approved by the Health Research Ethics Committee Faculty of Medicine University of Indonesia, Cipto Mangunkusumo Hospital.

Mesenchymal stem cell specimens were obtained from Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital Faculty of Medicine Universitas Indonesia, Jakarta Indonesia. Human subcutaneous adipose tissues and human umbilical cord stem cells have been characterized by the expression of stromal cell markers CD73, CD90 and CD105 positive; CD11b, CD19, CD34, CD45 and HLA DR-PE negative. Breast Cancer Stem Cells (ALDH+) obtained from Cell Culture Laboratory for Cancer Stem Cells, Department Biochemistry and Molecular Biology Faculty of Medicine Universitas Indonesia, Jakarta Indonesia. The BCSCs specimen has been characterized by the ALDEFLUOR™ assay for isolation of viable BCSCs for subsequent downstream assessment.

#### 2.2. Cell Culture and Multidifferentiation Capabilities of MSCs

Minimum Essential Medium Alpha Medium (α-MEM), F12 medium (DMEM F12), Phosphate Buffered Saline (PBS), TrypLE select solution, Fetal Bovine Serum (FBS), antibiotic solution containing streptomycin (10 mg/ml) and penicillin (10000 IU/ml) and amphotericin B (25 μg/ml) were used for BCSCs and MSCs culture. The cells were incubated under 5% CO2 and 37 °C condition. The media were changed every 3 days and cells were subcultured when confluence obtained.

Adipogenic, osteogenic, and chondrogenic differentiation experiments were performed. Osteogenic differentiation was evaluated by Alizarin Red-S staining. Oil Red O solution was used for differentiated adipocyte staining, and chondrogenic differentiation was stained with Alcian Blue solution.

#### 2.3. Preparation of MSCs Secretomes

Early passed human ASCs and UCSCs (P3–P5) were first grown to 80% confluence in α-MEM medium with 10% FBS. Culture medium was removed and the cells were washed three times by PBS to remove any residual medium. The cells then grown in non serum α-MEM medium for 24 h after which the MSCs secretomes was collected as 100% ASCs and UCSCs conditioned medium (CM). The CM was centrifuged in 1000 rpm for 10 min to remove cell debris and filtered using 0.22 μm filters. The CM was stored at −80 °C until experiment was performed.

#### 2.4. Supplementation of BCSCs (ALDH+) with MSCs CM and Viability Assay

Breast CSCs (ALDH+) were harvested and counted (Luna automatic cell counter). 5 x 105 of >90% viable cells then seeded in non serum α-MEM F12 medium. 24 hours later, the medium was replaced with non serum F12 medium as background. 50%v/v (1:1) α-MEM:F12 medium as control, and both 50% v/v(1:1) of CM_ASCs:F12 and CM_USCs:F12. After 72 h of supplementation, the cells were harvested, counted using Trypan blue exclusion dye assay and lysed using Tripure Isolation reagent. The experiment was performed three times (=3) with 3 wells per condition each time.

#### 2.5. RNA Isolation and q RT-PCR Assay

Isolation of RNA was performed following the manufacturer’s kit instruction (TriPure Isolation Reagent Roche). The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific Skanlt Software for Varioskan® Flash). Quantitative reverse transcriptase PCR was performed with SYBR Green and reverse transcriptase enzyme (One-Step qRT-PCR Kit KAPA® SYBR® FAST). 18s RNA was used as an internal control. The normalized fold expression was obtained using the 2-CT method. Primers used for q-RT-PCR are in Table I.

#### 2.6. Statistical Analysis

All values are expressed as mean±SE. All analyses were performed with SPSS 20.

### 3. RESULT

#### 3.1. Morphologies and Differentiation of ASCs, UCSCs and BCSCs

Both ASCs and UCSCs used in this study displayed spindle fibroblast like morphology. The cells usually adhere within hours after plating and gradually fused into a single layer after their confluence have been reached. Breast CSCs showed sphere

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**Table I. Primer sequences for RT-PCR amplification of target genes.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer forward</th>
<th>Primer reverse</th>
</tr>
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<tbody>
<tr>
<td>ALDH1</td>
<td>5′-TTG GAA GAT AGG GCC GTG AC-3′</td>
<td>5′-GGA GAC CCT GCC GGA TCC-3′</td>
</tr>
<tr>
<td>Oct4</td>
<td>5′-GAG GAG TCC CAG GAC ATC AAA-3′</td>
<td>5′-AGC TTC CTC CAC CCA GTT CT-3′</td>
</tr>
<tr>
<td>Sox2</td>
<td>5′-GGG GAG TAA GAA ACA GCA TGG A-3′</td>
<td>5′-GTT GAT GGG ATT GGT GTT CT-3′</td>
</tr>
<tr>
<td>18S</td>
<td>5′-AAA CCG CTA CCA CAT CGA CG-3′</td>
<td>5′-CCT CCA ATG GAT CCT CGT TA-3′</td>
</tr>
</tbody>
</table>

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Fig. 1. Morphologies of ASCs, UCSCs and BCSCs. Morphological appearance of ASCs (a) and UCSCs (b) exhibited a spindle shaped or fibroblastic morphology in α-MEM media with 10% fetal bovine serum. Inverted microscope, magnification, 40×. (c) Morphological appearance of BCSCs (ALDH+1) showed floating sphere formation (mammospheres) in non-serum F12 media. Inverted microscope, magnification, 100×.

Fig. 2. Differentiation potential of MSCs. (a) Adipogenic differentiation, part of the cells contained numerous oil red O-positive lipid droplets. Magnification, 200×. (b) Osteogenic differentiation, alizarin red staining detected mineral deposit in MSCs cultures. Magnification, 100×. (c) Chondrogenic differentiation of MSCs staining by Alcian blue. Magnification, 100×.

formation two-three days after plating in non serum DMEM F12 media (Fig. 1).
Differentiation potential to mesodermal lineages of MSCs occurred in the early passage of MSCs P3–P5 (Fig. 2).

3.2. Optimization of Concentration and Harvesting Time in Preparation of MSCs Conditioned Media

Analysis of optimal concentration and harvest time in CM preparation were performed to get a significant change in gene expression (Fig. 3).

Fig. 3. Optimization concentration and harvesting time in preparation of CM MSCs. Sig*p value < 0.05 as determined by Mann Whitney U test. Optimization concentration of CM MSCs (a–b) showed dose dependent manner of MSCs secretomes in affecting the expression of ALDH1A1 gene in BCSCs. Different conditioned media concentration of ASCs and UCSCs were incubated to BCSCs for 72 hours, the secretomes of (50% v/v) CM showed significant result in gene expression of BCSCs compared with the secretomes of (25% v/v) CM. Optimization of harvesting time in preparation of MSCs conditioned media by 24 and 48 hours (b–c). The 48 h secretomes harvesting time have shown a decrease in effect on ALDH1A1 gene expression which might due to the reduction of secretomes previously been secreted for utilized by MSC cells after being in α-MEM medium without serum for 48 hours.

3.3. Expression Analysis of ALDH1A1, Oct4 and Sox2 Genes in BCSCs After 72 h Supplementation with CM of ASCs and UCSCs

CM of ASCs (50% v/v) could significantly increase the relative expression of ALDH1A1, Oct4 and Sox2 genes in BCSCs while the CM of UCSCs (50% v/v) showed significantly decrease the expression of ALDH1A1 gene in BCSCs without affecting Oct4 and Sox2 expression (Fig. 4).

3.4. The Viability Analysis of BCSCs After 72 h Supplementation with CM of ASCs and UCSCs

After 72 h supplementation with CM of ASCs (50% v/v) the viability of BCSCs has been increase significantly while

Fig. 4. Impact of MSCs conditioned media on the expression of (a) ALDH1A1 (b) Oct4 and (c) Sox2 genes in human breast Cancer stem cells. Analysis of quantitative reverse transcriptase (q-RT) PCR after 72 hours supplementation of BCSCs with (50% v/v) CM_ASCs or (50%v/v) CM_UCSCs showed the opposite expression of ALDH1A1 gene. Secretomes of ASCs causes significant increase in the expression of ALDH1A1 gene and likewise of Oct4 and Sox2 genes. However the secretomes of UCSCs only caused significant decrease in ALDH1A1 expression without affecting the Oct4 and Sox2 genes expression. Results of the expression of ALDH1A1, Oct4 and Sox2 genes calibrated to 18S rRNA gene expression as house keeping gene and compared with the control (BCSCs in media without secretomes). Data are expressed as the means ± s.e. Sig*p value < 0.003 ; **p value < 0.000 as determined by Mann Whitney U test.

Fig. 5. The viability analysis of BCSCs after 72 h supplementation of CM. The viability of BCSCs showed significant increase after 72 h supplementation with (50% v/v) CM_ASCs while (50% v/v) CM_UCSCs led to non significant decrease. This result had the same characteristics with the differences of ALDH1A1 expression in BCSCs shown at Figure 4. Data were express as the means ± s.e. Compared with the control (BSCs in α-MEM:F12 media (1:1) without secretomes) Sig => p value < 0.002 as determined by Mann Whitney U test.
supplementation with CM of UCSCs (50% v/v) led to non-significant decrease (Fig. 5).

High significant correlation between the viability and ALDH1A1 gene expression in BCSCs after 72 h supplementation with CM_ASCs and CM_UCSCs determined by Spearman correlation test.

4. DISCUSSION AND CONCLUSION
It has become evident that cancer cells do not act in isolation during their progression to malignancy. Cancer as heterogeneous cellular entities, growth in dynamic interactions among the cancer cells themselves, and between cells and the constantly changing microenvironment. In the tumor microenvironment, mesenchymal stem cells (MSCs) play an important role on the modulation of tumor progression and drug sensitivity. These cells produce and release a wide spectrum of biologically active growth factors and cytokines known as secretomes which are involved in the cell–cell and tissue–tissue communication. These secretomes may affect tumor cells and potentiate or inhibit their development.

Recent evidence suggests that enhanced aldehyde dehydrogenase (ALDH) activity is a hallmark of cancer stem cells (CSCs). The ALDH1A1, one of ALDH isoforms expressed in humans, was generally believed to be responsible for the ALDH activity of CSCs due to the ability to detoxify cytotoxic drugs via retinoic acid (RA) signaling pathway.6 Though some studies suggested that ALDH1A3 isofrom was significantly contribute to aldefluor positivity which may be tissue and cancer specific, but in contrast for a highly aggressive breast cancer subtype, inflammatory breast cancer, and Triple negative Breast Cancer, ALDH1A1 expression significantly correlate with development of metastasis and worse outcome.18

The result of this research showed the effect of secretomes of 50% (v/v) CM_ASCs significantly increased ALDH1A1 expression and the viability of BCSCs which may correlates with retinoic acid signaling pathway (RA-RAR/RXR) in tumor progression effect. The Increase of Sox2 and Oct4 showed the ability of ASCs secretomes to upregulate the pluripotency genes expression. In contrary with that, the secretomes of 50% (v/v) CM_UCSCs caused decrease on the BCSCs viability and the significant decrease of the ALDH1A1 expression without affecting the expression of Sox2 and Oct4 genes. This result suggested that UCSCs secretomes might have the suppression effects to BCSCs (ALDH+) without affecting the pluripotency of BCSC. The changing of ALDH1A1 expression and the Viability in BCSCs after supplemented has been shown the same characteristics with high corellation suggesting the same pathway might take a role in this phenomena. Furthermore, the effect of MSC’s secretomes in modulation of gene expression showed a dose dependent manner. Further investigation are required to confirm the role of Secretomes of MSCs on the pluripotency and viability of BCSCs.

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References and Notes

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