RESEARCH ARTICLE

Editorial Process: Submission:04/04/2023 Acceptance:08/12/2023

Mesenchymal Stem Cell-Derived Extracellular Vesicles Increase Human MCF7 Breast Cancer Cell Proliferation associated with *OCT4* Expression and *ALDH* Activity

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Abstract

Objective: The aim of this study was to investigate the effect of mesenchymal stem cells-derived extracellular vesicles (MSC-EVs) on the human MCF7 breast cancer cell proliferation that have been considered to contain limited CSC population and its association with the expression of *OCT4* and *ALDH1* stemness markers. **Methods:** EVs were successfully isolated from the conditioned medium of umbilical cord MSCs using size exclusion chromatography. The isolated EV fraction was verified under a transmission electron microscope (TEM). Five and ten percent (v/v) concentration of MSC-EVs were then co-cultured with MCF7 cells. To investigate MSC-EV uptake by MCF7 cells, we performed confocal microscopy analysis. Subsequently, the proliferation of co-cultured MCF7 cells was determined using trypan blue exclusion assay, while their mRNA and protein expression of *OCT4* as well as ALDH activity as the marker of stemness properties were analyzed using quantitative reverse transcription polymerase chain reaction, Western Blot, and Aldefluor™ assays, respectively. **Result:** MSC-EVs were detected as round-shaped, ~100 nm sized particles under TEM. We also demonstrate that MSC-EVs can be internalized by MCF7 cells. Notably, MSC-EVs of 5% concentration increased *OCT4* mRNA expression and *ALDH1* activity in MCF7 cells. At 10% concentration, MSC-EVs reduced the *OCT4* expression and *ALDH1* activity. **Conclusion:** MSC-derived EVs modulate the stemness of MCF7 cells, either *OCT4* expression or *ALDH1* activity, in a concentration dependent manner along with the increase of cell proliferation.

Keywords: Extracellular vesicles- mesenchymal stem cells- breast cancer cells- stemness

Asian Pac J Cancer Prev, **24 (8)**, 2781-2789

Introduction

Breast cancer is the most frequent cancer and one of the deadliest diseases in the world (Sung et al., 2021). Generally, breast cancer cells are classified into luminal A, luminal B, HER2+, and triple negative subtypes based on its aggressiveness (Dai et al., 2017). MCF7 cells that express estrogen and progesterone receptor belong to luminal A subtypes (Dai et al., 2017). Among other breast cancer subtypes, MCF7 cells are considered to contain less cancer stem cell (CSC) population that has been reported to be responsible for therapy resistance and metastasis leading to poor prognosis on patients (Li et al., 2022). Like mesenchymal stem cells (MSCs), CSCs highly express *ALDH1* and *OCT4* which are responsible for pluripotency and self-renewal. Currently, these markers are widely known as detection tools for stemness properties present both in normal and cancerous stem cells (Zhao et al., 2017; Vassalli, 2019). These days, most of the conventional therapies for breast cancer less considering the role of tumor microenvironment (TME) which are able to communicate reciprocally with cancer cells through paracrine signaling (Walker et al., 2018).

The cellular components of TME, also known as stromal cells, comprise MSCs, immune cells, endothelial cells, and cancer-associated fibroblasts (Walker et al., 2018). Whereas fibronectin, collagen, laminin, proteoglycan, cytokines, chemokines, growth factors, metabolites, and extracellular vesicles (EVs) constitute the non-cellular components (Walker et al., 2018). EVs are subcellular structures enclosed with phospholipid bilayer and classified into microvesicles, exosomes, and

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apoptotic bodies (Abels and Breakefield, 2016). EVs transfer proteins, mRNAs, miRNAs and lipids from donor to recipient cells which in turn affect the phenotype of cells received via activation of several cellular signaling pathways (Abels and Breakefield, 2016). The intercellular communication between cancer and stromal cells could be mediated by EVs released by both cells (El-Saghir et al., 2015).

MSCs are pluripotent cells recruited to TME to promote immunomodulation (El-Saghir et al., 2015). MSCs within TME may originate from many sources, such as from umbilical cord, bone marrow, and adipose tissue (El-Saghir et al., 2015). Nowadays, MSCs are widely used for therapy of many degenerative diseases including for the prevention and regeneration of aging cells, as well as for cancer treatment (Hmadcha et al., 2020; Fraile et al., 2022). Study of Zamani et al. has reported that MSCs expanded in medium containing low concentration of human basic fibroblast growth factor are protected from any morphological and chromosomal aberrations, suggesting the safety of MSCs for regenerative medicine and cancer treatment (Zamani et al., 2022).

The impact of MSCs supplementation on the aggressiveness of cancer cells is still debatable (Hmadcha et al., 2020). Several studies have also reported that MSCs induced tumorigenesis including cancer cell proliferation, angiogenesis, and metastasis which are related to immunomodulation (Akimoto et al., 2013; Galland and Stamenkovic, 2020; Mostafa et al., 2022). In addition, MSC-derived EVs (MSC-EVs) have been suggested to exhibit either pro-tumorigenic or anti-tumorigenic activity (Han et al., 2017; Lindoso et al., 2017). Our previous studies have demonstrated that the secretomes of MSCs from umbilical cord could increase the stemness of cancer cells (Hardiany et al., 2018; Purnamawati et al., 2018). Besides EVs, MSC secretomes also contains growth factors and cytokines that may affect the characteristics of cancer cells (Pawitan, 2014). The specific role of EVs within MSC secretomes in regulating the stemness of cancer cells remains unsolved. Therefore, the present study was aimed at investigating the impact of MSC-EVs from umbilical cord on the proliferation and stemness of human MCF7 breast cancer cells.

Materials and Methods

The isolation and verification of EVs from mesenchymal stem cells

Mesenchymal stem cells (MSCs) were derived from umbilical cord specimens that was provided and characterized by Stem Cell and Tissue Engineering cluster, Indonesian Medical Education and Research Institute. Ethical clearance has been approved by the Health Research Ethics Committee, Faculty of Medicine Universitas Indonesia and Cipto Mangunkusumo Hospital, according to Helsinki Declaration of ethical principles.

MSCs were grown in complete alpha MEM (Gibco, USA) under standard conditions (5% CO_2, 37°C) supplemented with 10% platelet rich plasma (PRP) obtained from PMI (the Indonesian Red Cross Society) until 70-80% confluency. Cell morphology was observed under inverted microscope (Nikon eclipse, USA) with 100x magnification. To obtain the conditioned medium (CM) of MSCs, the complete medium was replaced with serum-deprived alpha MEM and cells were subsequently incubated for 24 hours. Collected CM was concentrated using Amicon® Ultra-4 Centrifugal Filter (Merck, Darmstadt, Germany) and centrifuged for 20 minutes according to the manufacturer's protocol. Concentrated CM was placed on SEC column (IZON® qEVoriginal / 35nm, New Zealand). About 0.5 mL of each fraction were collected in tubes. EV fractions (#7-9) were pooled to be used for following experiments. About 100 µL of pooled EV fractions were stained using 1% PKH26 Red Fluorescent Cell Linker (Sigma-Aldrich, St. Louis, USA) for 10 minutes and observed under confocal microscope (ZEISS LSM700, Germany). Furthermore, we performed transmission electron microscopy analysis (JEOL JEM 1010, Japan) to observe EV ultrastructure. For future use, EVs were stored at -80°C.

Culture of human MCF7 breast cancer cells

MCF7 cell line was obtained from American Type Culture Collection (ATCC). Human MCF7 breast cancer cells were grown in a complete medium containing DMEM high glucose medium with 10% FBS, 1% penicillin, and 1% amphotericin under standard conditions (5% CO_2, 37°C). Cells were passaged when 70-80% confluency was reached.

Co-culture of EVs and MCF7 cells

To examine whether human MCF7 cells can uptake MSC-EVs, about 1x104 MCF7 cells per well were grown with PKH26-stained EVs of 10% concentration in a 8-well slide chamber containing 100 µl serum-deprived medium under standard conditions. After overnight co-culture, cells were stained using 50 µg/mL 4′,6-diamidino-2-phenylindole DAPI (Sigma-Aldrich, St. Louis, USA), then observed using confocal microscope (ZEISS LSM700, Germany).

To analyze the effect of MSC-EVs on the human MCF7 cells proliferation, about 1x105 MCF7 cells per well were seeded in triplicate in a 12-well plate using serum-deprived medium and cells were co-cultured with unstained EVs of 0, 5 and 10% concentration and incubated for 72 hours under standard conditions. Subsequently, cells were harvested and viable cell number was counted using trypan blue exclusion assay.

Analysis of OCT4 mRNA and protein expression

To assess *OCT4* mRNA expression, we firstly extracted total RNA from co-cultured cells using TRIpure™ RNA isolation kit (Roche, Switzerland). Analysis of *OCT4* gene expression was performed using one step quantitative reverse transcription polymerase chain reaction (qRT-PCR) SensiFAST™ SYBR® No-ROX kit (Bioline, London, UK) in a 7500 Fast Real-Time PCR System (Applied Biosystem, Massachusetts, USA). 18S rRNA was used as an internal control. Primer sequences for *OCT4* and 18S rRNA were obtained from our previous studies (Wanandi et al., 2019). The level of *OCT4* mRNA expression was analyzed using Livak formula.

To determine the *OCT4* protein expression, we performed Western Blot assay. Total protein was extracted from co-cultured cells using RIPA lysis buffer (Abcam, USA) according to the manufacturer's protocols. Protein concentration was quantified using Bradford assay (Bio-Rad, USA). Protein was separated by 12% SDS PAGE and transferred into nitrocellulose membranes (Advansta Inc, California, USA). Subsequently, membrane was blocked with 5% skimmed milk at room temperature for 1 hour. Membrane was then incubated with primary antibody against 45 kDa human *OCT4* (Abcam, UK, rabbit polyclonal antibody, 1:500) overnight at cold room and followed by secondary antibody HRP-conjugated IgG anti-rabbit for 2 hours at room temperature. Protein band detection was analyzed using ECL detection kit (Bio-Rad, USA). Subsequently, the membrane was stripped for immunoblotting with primary antibody against 45 kDa human β-actin (CST, USA, mouse monoclonal antibody, 1:8000 dilution) and incubated overnight in a cold room. β-actin was used as an internal control. On the next day, membrane was incubated with secondary antibody HRP-conjugated IgG anti- mouse for 1 hour at room temperature. Finally, membrane was analyzed using ECL detection kit (Bio-Rad, USA). The density of protein bands was quantified by ImageJ software (Gallo-Oller et al., 2018).

Aldefluor assay

ALDH activity was determined using ALDEFLUOR™ kit (Stem Cells Technologies, Canada). Briefly, about 5 x 10^5 harvested cells were suspended in 500 uL ALDEFLUOR™ assay buffer. Cell suspension was added with the activated reagent containing the ALDH substrate (BIODIPY-Aminoacetaldehyde). As a control, half of the cell suspension were immediately transferred into a control tube which has been added with diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH. Then, cell suspension was incubated for 45 minutes at 37°C. The activity of ALDH was recorded using flowcytometry (BD FACSCanto™ II, Canada) and analyzed in BD FACSDiva™ Software.

Statistical analysis

All triplicate data were presented as means ± SD. Statistical analysis was performed using one way ANOVA using SPSS 21, with $p < 0.05$ as a cut-off for determining a significant difference.

Results

MSC-EVs were successfully isolated and identified

We collected CM from 70-80% confluency of MSCs grown in serum-deprived medium for 24 hours (Figure 1). MSCs have spindle shape and adhere to the plastic tissue culture flask when cultured under standard conditions, as reported in the previous study (Dominici et al., 2006).

After EV isolation, we identify the presence of EVs in the pooled fractions (#7-9) under confocal microscope. Red fluorescence dots are clearly present in the PKH26-stained EV fractions (Figure 2A) than those in the 1% PKH26 solution without EVs as a negative control (Figure 2B). Thereafter, we also analyzed the EV ultrastructure and detected round shaped and about 100 nm sized particles (Figure 3).

The uptake of MSCs-derived EVs by human MCF7 breast cancer cells

To examine whether human MCF7 breast cancer cells can uptake MSC-EVs, we performed confocal microscopy by using red and blue channel for imaging PKH26-stained EVs and MCF7 cell nucleus, respectively. In addition, double channels were used to confirm the uptake of EVs by MCF7 cells. Figure 4A-4E demonstrated MCF7 cells co-cultured with PKH26-stained EVs, whereas in figure 4F-4I MCF7 cells were not co-cultured with EVs but supplemented with 1% PKH26 solution as a negative control. Range indicator in Figure 4A and 4F was used to verify cell location. Red fluorescence dots were found in the cytoplasm of MCF7 cells (Figure 4B, 4D, and 4E), indicating a positive signal for MSC-EVs since no red dots were found when cells were stained with PKH26 solution only (Figure 4G and 4I).

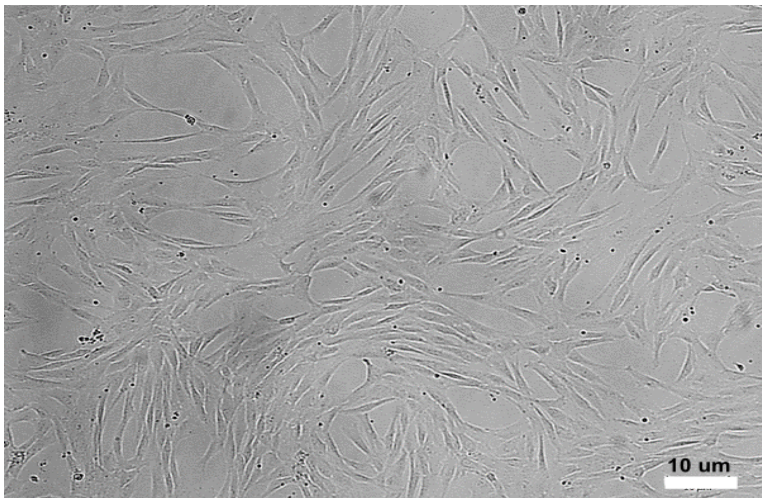

Figure 1. Morphology of MSCs. Cells were grown in serum-deprived medium for 24 hours. Cell morphology was observed under inverted microscope (Nikon eclipse, USA) with 100x magnifcation.

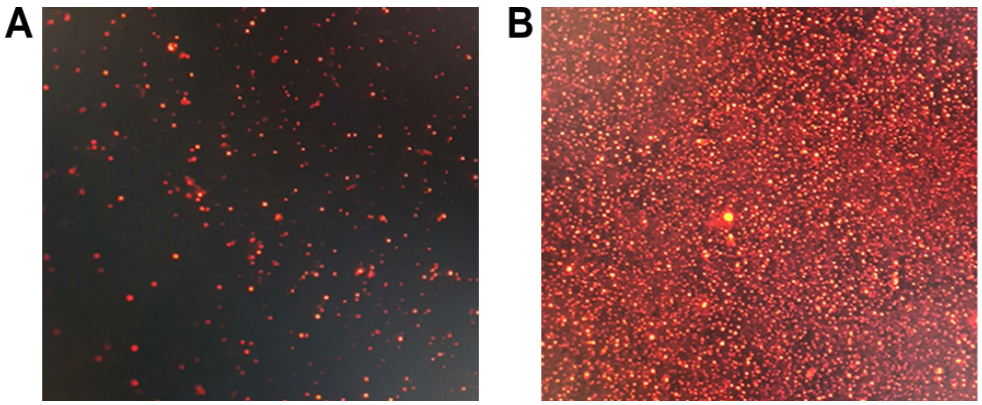

Figure 2. Fresh EVs Isolated from CM-MSCs. Observation was performed using confocal microscopy analysis (ZEISS LSM700, Germany) with 630x magnification. (A) PKH26-stained EVs; (B) 1% PKH26 solution without EVs as a negative control.

The effect of MSC-derived EVs on the MCF7 breast cancer cell proliferation

The present study demonstrates that the viable cell number of MCF7 was significantly increased after 72-hour co-culture with MSC-EVs of either 5% (2.44-folds, $p \leq 0.001$) or 10% concentration (2.89-folds, $p \leq 0.001$) in a concentration-dependent manner (Figure 5). However, there was no substantial change of MCF7 cell morphology.

The effect of MSC-derived EVs on the OCT4 expression in MCF7 breast cancer cells

To investigate whether EVs affect the stemness of breast cancer cells, both in RNA or protein level, we performed qRT-PCR and western blot respectively. We demonstrate that 5% EV supplementation significantly increased (2.72-folds, $p \leq 0.01$) *OCT4* mRNA expression in MCF7 cells (Figure 6A). Surprisingly, 10% EVs reduced the *OCT4* mRNA level in MCF7 cells which was significantly lower (2.56-folds, $p \leq 0.01$) than that with 5% EVs. Conversely, increased concentration of EVs seems to decrease the *OCT4* protein expression (Figure 6B).

The effect of MSC-derived EVs on the activity of ALDH in MCF7 breast cancer cells

ALDH is well-known enzyme regulating pluripotency of stem cells (Vassalli, 2019). To assess the activity of ALDH within co-cultured cells, we performed ALDEFLUOR assay. In this study we found that the pattern of ALDH activity of MCF7 reached the highest at 5% EV supplementation. However, the higher concentration caused the declining ALDH activity even compared to the control (EV 0%) (Figure 7).

Discussion

These days, several methods have been used to isolate EVs (Gardiner et al., 2016). Many researchers utilize ultracentrifugation (UC) to isolate EVs from numerous sources (Gardiner et al., 2016). However, EV aggregation and non-vesicular particle contamination have been thought to be the limitation of this method (Webber and Clayton, 2013; Linares et al., 2015). Several

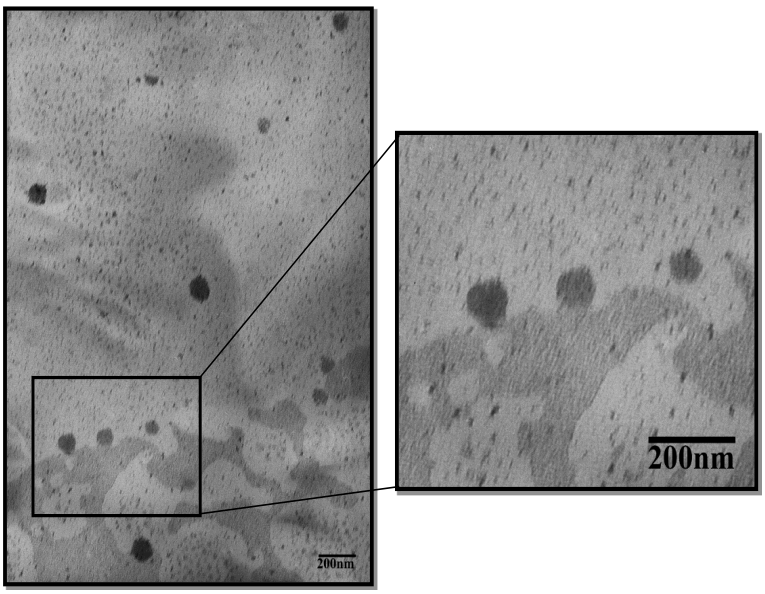

Figure 3. The Ultrastructure of EVs. TEM analysis was performed on SEC-isolated EV fraction using negative staining method (JEOL JEM 1010) with 25.000x magnification. Black round-shaped, ~100 nm sized particles were considered as EVs.

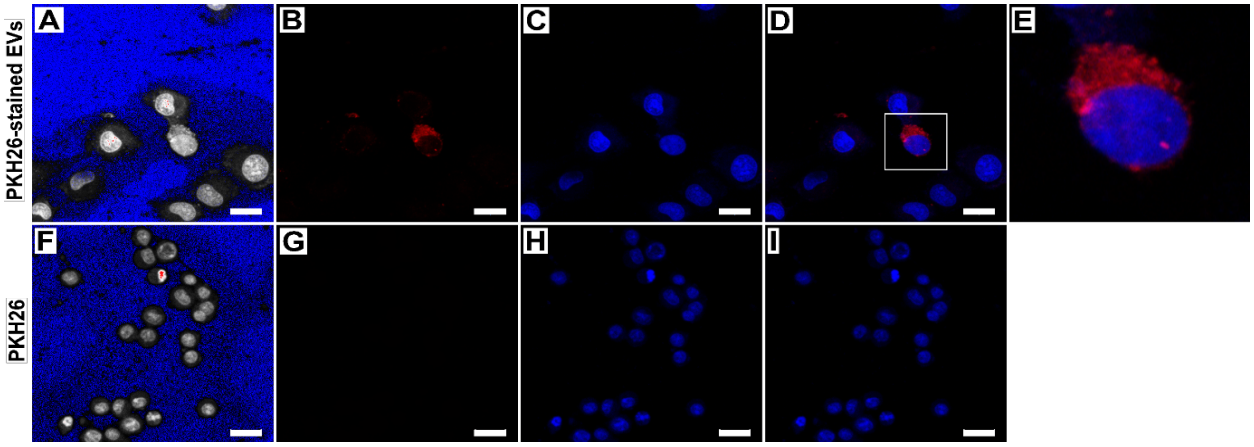

Figure 4. Confocal Microscopy Analysis for Confirmation of EV Uptake in Human MCF7 Breast Cancer Cells. MCF7 cells were co-cultured with 10% PKH26-stained EVs (v/v) overnight, then counterstained with DAPI. Observation was performed using confocal microscopy (ZEISS LSM700) with 400x magnification. Scale bar: 20 µm. PKH26-stained EVs are depicted as red fluorescence dots. The internalization of EVs in the cytoplasm of MCF7 cells is shown in the insert white square. (A-E) MCF7 cells co-cultured with PKH26-stained EVs; (F-I) MCF7 cells supplemented with 1% PKH26 solution only, without EVs; (A, F) range indicator; (B, G) red channel; (C, H) blue channel; (D, I) merged red and blue channel; (E) magnification of white square in (D).

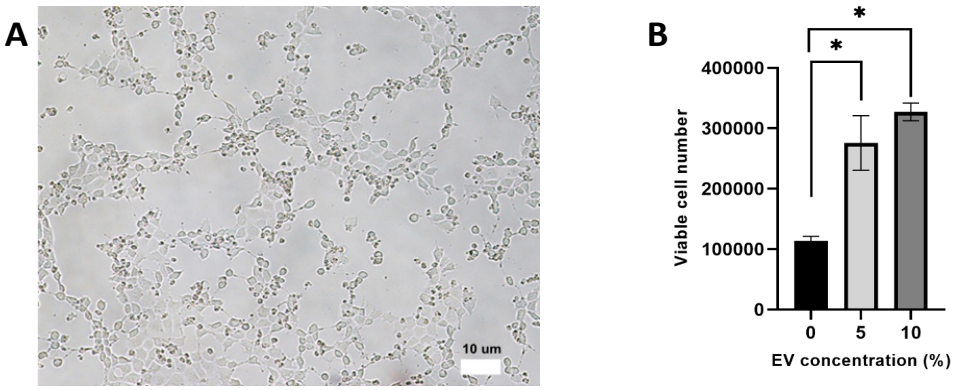

Figure 5. The Effect of MSC-EVs on MCF7 Cell Proliferation. Cells were co-cultured with various concentrations of MSC-EVs under standard condition for 72 hours. About 105 MCF7 cells per well were seeded on a 12-well plate in triplicate and co-cultured with 5 and 10% (v/v) MSC-EVs for 72 hours. PRP-deprived medium (0% EVs) was used as a control. Data was shown as mean ± SD. Statistical analysis was performed using Student's t-test and statistical differences compared to control were indicated as * for $p<0.05$. (A) morphology of MCF7 cells; (B) viable cell number of MCF7 cells after co-cultured with EVs.

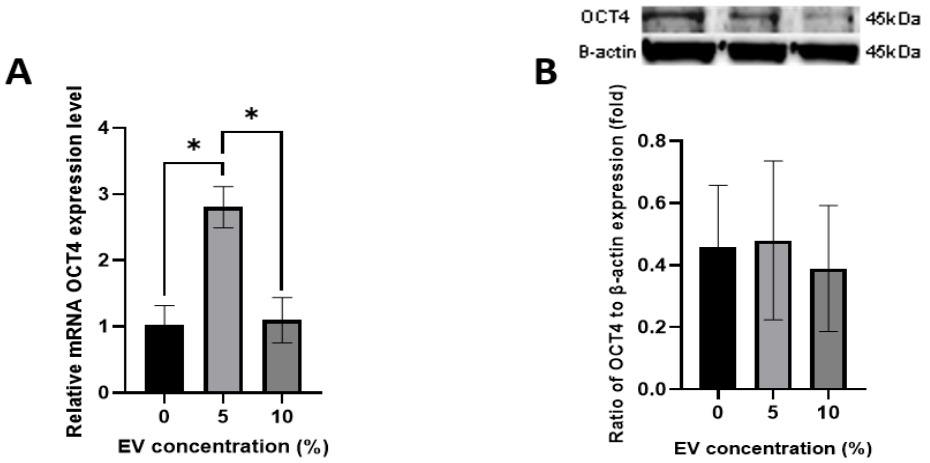

Figure 6. Expression of OCT4 in Response to EV Co-Culture. (A) qRT-PCR analysis. Triplicate experiments were performed to determine the gene expression. The relative mRNA OCT4 expression was analyzed using Livak formula (2-ΔΔCT). (B) western blot. SDS PAGE using 20 µg protein was followed by incubation using Oct-4 (Abcam, UK) and β-actin (CST, USA) primary antibody as well as anti-rabbit and anti-mouse secondary antibody, respectively. β-actin was used as internal control. The analysis was done using ImageJ software. Data are presented as means ± SD. *$p<0.05$.

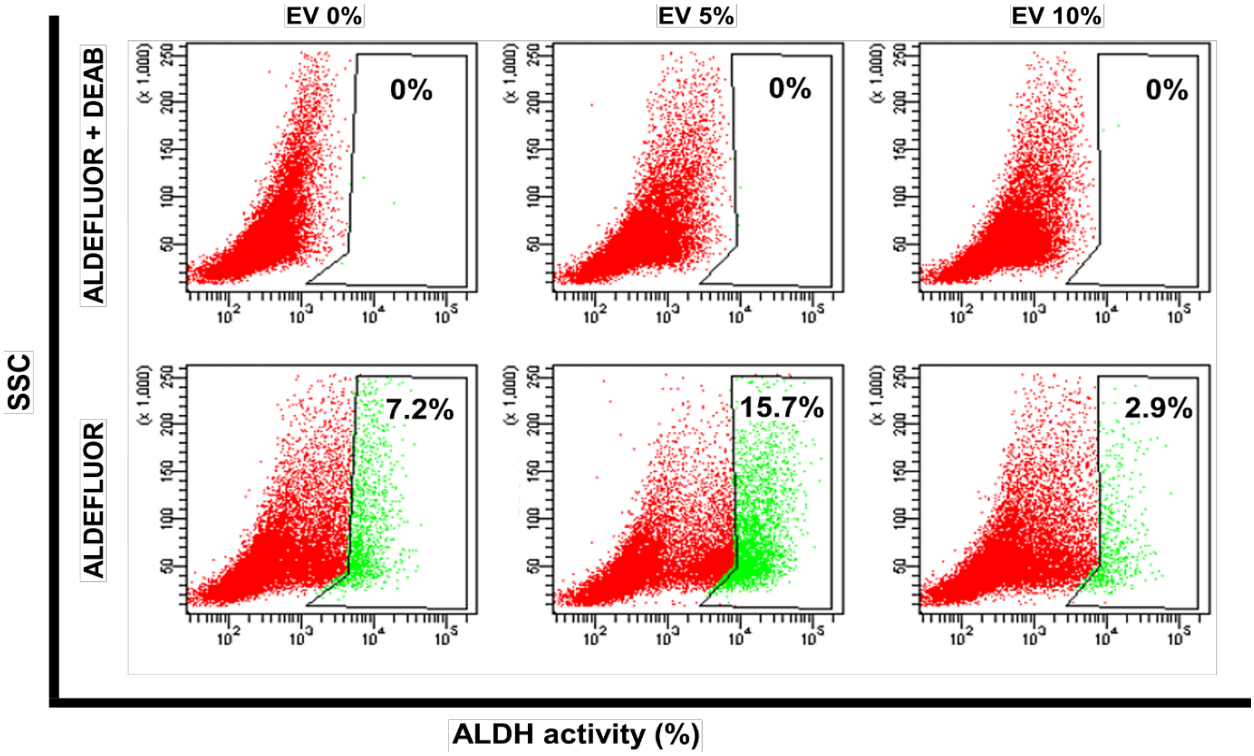

Figure 7. ALDH Activity in MCF7 Cells after Co-Culture with MSC-EVs. Following 72-hour co-culture with 0, 5, and 10% EVs, harvested MCF7 cells were assessed for ALDH activity by employing Aldefluore™ assay. DEAB, a specific inhibitor of ALDH, was added as a negative control. About 2.5 x 105 cells were recorded in BD FACSCanto™ II and analyzed in BD FACSDiva™ Software.

studies comparing the EV purity obtained using UC and SEC elucidated that EVs isolated using SEC have less contamination of macromolecular structures and non-vesicular proteins than those using UC (Benedikter et al., 2017). Therefore, SEC is superior to UC in terms of purity and morphological stability. Moreover, SEC is also preserved the biophysical properties of isolated EVs (Nordin et al., 2015). Based on this evidence, we select SEC method to isolate EVs.

The presence of EVs in the PKH26-stained SEC fractions was successfully demonstrated under confocal microscopy. PKH26 is a lipophilic dye that easily forms strong noncovalent interactions with cells by intercalating into lipid bilayer of cell membrane (Puzar Dominkus et al., 2018). After bound to membrane structure, it will be stable for a certain period and can be detected as red fluorescence dot signal (Puzar Dominkus et al., 2018). Furthermore, we verified the isolated EVs by their shape and size observed under TEM as described in the previous studies (Franquesa et al., 2014; Nordin et al., 2015; Pavani et al., 2020). The size of these particles (~100 nm) also fit to the manufacturer's specification about the size range of isolated EVs which is from 35 to 350nm. The internalization of MSC-EVs into MCF7 cells after co-culture was successfully proven in our study under confocal microscopy analysis (Figure 4D and E). As an intercellular mediator, EVs are taken up by cells to release their cargo (Abels and Breakefield, 2016). Hitherto, it has been proposed that EVs can be internalized by cells through plasma membrane fusion as well as endocytosis (Mulcahy et al., 2014). The internalization of EVs may be dependent on the type of cell and its physiological state, and whether ligands on EV surface recognize receptors on cell surface or vice versa (Abels and Breakefield, 2016).

This result suggests that the supplementation of MSC-EVs induced MCF7 cell proliferation. The higher EV concentration was, the faster breast cancer cell proliferation was. It is well known that cancer cell growth is strongly affected by the cellular interactions with tumor stromal cells, including MSCs, which are facilitated by EVs (Han et al., 2017). Despite the anti-inflammatory effect of MSCs and its ability to regenerate damage tissues, MSC treatment has been also reported to aggravate tumor growth in vivo (Mostafa et al., 2022). Previously, several studies have elucidated that either CM or EVs from MSCs is able to trigger the cancer growth (Purnamawati; et al., 2017; Zhou et al., 2019). The increase of MCF7 cell proliferation was known to be associated with the activation of ERK pathway (Zhou et al., 2019). In addition, the activation of P2X receptor, Wnt/β-catenin and Akt signaling by MSC treatment have been suggested as other underlying mechanisms that stimulate breast cancer cell proliferation(Li et al., 2015; Maffey et al., 2017). In contrary to our result, several previous study have indicated that MSC-EVs had suppressive effects on cell proliferation and migration leading to cell dormancy (Makiko Ono et al., 2014; Casson et al., 2018). Moreover, MSCs derived from different sources may give distinct impacts on cancer progression (Akimoto et al., 2013). Umbilical cord-derived MSCs inhibited proliferation but MSCs from adipose tissue were able to promote cancer cells proliferation (Akimoto et al., 2013; Wanandi et al.,

2018).

OCT4 is a transcription factor that belongs to Yamanaka factors and considered as a major regulator of pluripotency in stem cells and tumorigenesis (Villodre et al., 2016). This transcription factor activates its downstream target genes, such as SOX2 and NANOG that are also known as pluripotent markers (Mohiuddin et al., 2020). The high expression of *OCT4* is suggested to be correlated with the poor prognosis (Rasti et al., 2018). This study resulted in a similar modulation of *OCT4* mRNA and protein expression levels in MCF7 cells co-cultured with EVs. Although MSC-derived EVs of 5% concentration significantly enhanced the expression of *OCT4* mRNA, higher EV concentration (10%) reduced the level of *OCT4* mRNA to the physiological level like control. Nevertheless, the modulation of its protein level has no significant difference between both EV concentrations. This phenomenon might be due to the regulation of *OCT4* gene expression in MCF7 cells at transcriptional and translational levels to preserve cell homeostasis.

Interestingly, we also detected the modulation of ALDH activity in MCF7 cells co-cultured with EVs which is comparable to *OCT4* mRNA expression. This suggests that both gene expressions might be controlled by the same signalling triggered by EV-carried ligands. Previously, we have also reported that *ALDH1*A1 and *ALDH1A3* – two *ALDH1* isoforms – regulate the stemness of MSCs and CSCs (Purnamawati et al., 2018; Wanandi et al., 2018). As described in our previous in silico study (Wanandi et al., 2018), *ALDH1A1* has a direct protein-protein interaction with OCT4, while *ALDH1A3* indirectly interacts with *OCT4* through *ALDH1A1*. It has been reported that the increase of ALDH activity was correlated with self-renewal ability of BCSCs (Chang et al., 2018). Our previous report (Purnamawati et al., 2018) revealed that the CM of MSCs derived from umbilical cord has elevated the expression of *ALDH1A3* mRNA in human ALDH+ breast CSCs. In contrast to that report, we have also published that the expression of *ALDH1*A1 mRNA has been suppressed in human ALDH+ breast CSCs treated with CM of MSCs (Purnamawati; et al., 2017). Thus, we suggest that the modulation of ALDH activity found in this study might be resulted from the gene expression of different *ALDH1* isoform which may have differential regulation depending on the EV concentration. In addition, the high expression of ALDH protein was associated with high proliferative, metastatic, and colony formation capacity of cancer cells (Chunyan Yu, 2011). Nevertheless, this is not the case for our present data with 10% EV concentration due to the nature of MCF7 cells that comprise more fast dividing with limited self-renewal capacity rather than CSC populations. Hence, EV supplementation could not induce the self-renewal and pluripotent of MCF7 cells more than their nature capacity.

Increasing evidence has suggested that MSC-EVs can shuttle their cargo to facilitate physiological and pathological changes of target cells, including stemness maintenance and renewal either for tissue homeostasis or for cancer development and progression (Melo et al., 2014; Lopatina et al., 2016). MSC-EVs contain numerous lipids, proteins, and nucleic acids such as mRNA and miRNA that are known to play crucial roles in cancer, either as tumor suppressor or promoter, by regulating assorted processes associated with cell proliferation, apoptosis, angiogenesis, migration and tumorigenesis (Lindoso et al., 2017). Figueroa et al. has denoted that miR-1587 contained in exosomes from glioma-associated MSCs induced the proliferation and clonogenicity of glioma stem-like cells (Figueroa et al., 2017). Nonetheless, in our knowledge, there is still limited evidence available for the involvement of MSC-EVs in the stemness expression of CSCs which drives cancer aggressiveness.

Overall, we conclude that MSC-derived EVs modulates either *OCT4* expression or ALDH activity of MCF7 cells, in a concentration dependent manner. It is also noteworthy that MCF7 proliferation linearly increased in line with the increase of EV concentration. Hence, treatment of MSCs and their biological products such as secretomes and EVs for cancer patients needs to be carefully considered with tight monitoring and evaluation of the expression of stemness markers. Further investigations are required to unravel the underlying mechanisms of MSC-EV contents involved in modulating pluripotency and self-renewal of CSCs in cancer.

Author Contribution Statement

Conceptualization: SIW, RDA, and NZ. Data curation: NZ, RAS, and SA. Data analysis: SIW, RDA, and NZ. Funding acquisition: SIW. Investigation: SIW, RDA, NZ, RAS, and SA. Methodology: SIW, RDA, and NZ. Writing original draft, review, and editing: SIW and NZ. All authors contributed to the article and approved the final version of this paper for publication..

Acknowledgements

General

The authors would like to express our gratitude to DRPM-UI for providing the financial support. We also thank the Indonesian Medical Education and Research Institute, Faculty of Medicine Universitas Indonesia for providing research facilities, the Diagnostic and Research Center, Faculty of Medicine Universitas Indonesia for providing confocal microscope facility, and the Eijkman Institute for facilitating TEM analysis.

Funding Statement

This research was financially supported by the DRPM-UI through PUTI Q2 Grant 2020 (NKB-1557/UN2.RST/HKP.05.00.2020].

Approval

This research is part of a student thesis which has been approved by the Master's Programme in Biomedical Sciences, Faculty of Medicine Universitas Indonesia.

Ethical Declaration

This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No. 205/

UN2.F1/ETIK/2016).

Data Availability

The datasets are available from the corresponding author upon request.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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