

# Unveiling Paclitaxel-Induced Mesenchymal Stem Cells: orchestrating Nrf2 Modulation and Apoptosis in CD44+/CD24- Cancer Stem Cells

Dedy Hermansyah<sup>1</sup>, Siti Syarifah<sup>2</sup>, Adi Muradi Muhar<sup>3</sup>, Agung Putra<sup>4,5</sup>

<sup>1</sup>Department of Surgery, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia; <sup>2</sup>Department of Pharmacology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia; <sup>3</sup>Department of Doctoral Degree Program, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia; <sup>4</sup>Stem Cell and Cancer Research Indonesia, Semarang, Central Java, Indonesia; <sup>5</sup>Department of Pathology Anatomy, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang, Central Java, Indonesia

Correspondence: Dedy Hermansyah, Department of Surgery, Faculty of Medicine, Universitas Sumatera Utara, Jl. Dr. Mansyur No. 5, Padang Bulan, Kec. Medan Baru, Kota Medan, Sumatera Utara, 20155, Indonesia, Email dedi.hermansyah@usu.ac.id

**Background:** Mesenchymal Stem Cells (MSCs) and Cancer Stem Cells (CSC) play pivotal roles in cancer progression and therapeutic responses. This study aimed to explore the effect of MSCs induced by paclitaxel on CSC expressing the CD44+/CD24- phenotype, focusing on Nrf2 modulation and apoptosis induction.

**Methods:** MSCs were characterized for adherence, differentiation potential, and surface markers via standard culture, staining assays, and flow cytometry, respectively. CSCs isolated from MDA-MB-231 using MACS and were characterized based on morphology and CD44+/CD24- expression. Co-culture experiments evaluated the cytotoxic effect of Paclitaxel-induced MSCs on CSC viability using MTT assays. Flow cytometry analysis assessed apoptosis induction via annexin V-PI staining and Nrf2 and Caspase-3 gene expression were measured by qRT-PCR analysis.

**Results:** MSCs exhibited typical adherence and differentiation capabilities, confirming their mesenchymal lineage. CSCs displayed an elongated morphology and expressed CD44+/CD24-, characteristic of stem-like behavior. Paclitaxel induced dose-dependent Nrf2 gene expression in MSCs. Co-culture with Paclitaxel-induced MSCs reduced CSC viability in a dose-dependent manner, with a significant decrease observed at a 5:1 MSCs:CSC ratio. Co-culture decreased the Nrf2 gene expression and increased apoptosis in CSCs, with higher caspase-3 gene expression compared to solitary paclitaxel treatment.

**Conclusion:** Paclitaxel-induced MSCs decreased Nrf2 expression and significantly decreased CSC viability while enhancing apoptosis. This suggests a potential strategy to mitigate paclitaxel resistance in CD44+/CD24- CSCs. Leveraging Paclitaxel-induced MSCs presents a promising avenue for targeting Nrf2 and promoting apoptosis in CSCs, potentially improving the efficacy of chemotherapy and addressing resistance mechanisms in cancer treatment.

**Keywords:** MSCs, paclitaxel, CSC, apoptosis, Nrf2

## Introduction

Breast cancer is estimated to be responsible for 19 million disability-adjusted life years cases in women.<sup>1</sup> Globocan 2022 data revealed that breast cancer is a significant contributor to cancer-related deaths in women, accounting for up to 6.9% of cases.<sup>2</sup> The primary factor contributing to increased breast cancer mortality is the emergence of recurrence and chemoresistance, attributed to the presence of resistant apoptosis in the cancer stem cell (CSC) population.<sup>3,4</sup> Currently, first-line chemotherapy for breast cancer includes doxorubicin and paclitaxel.<sup>5</sup> Numerous research findings have shown that the existence of CSC groups in individuals with breast cancer contributes to treatment ineffectiveness, causing the cancer to come back and increasing the risk of death.<sup>6</sup> Paclitaxel acts by binding to the tubulin subunit  $\beta$ , inducing tubulin polymerization, and stabilizing microtubules.<sup>7</sup> Stabilized microtubules disrupt the mitosis process, activate the p53 gene, and phosphorylate Bcl-2, leading to cancer cell apoptosis.<sup>8</sup> However, the ability of CSCs to induce efflux pumps for

paclitaxel compounds via ATP-binding cassette transporters leads to chemotherapy failure.<sup>9</sup> CSCs exhibit increased glycolysis, oxidative phosphorylation, glutamine metabolism, and lipogenesis compared to bulk tumor cells. Inhibiting key enzymes/pathways like PKM2, LDHA, GLS1, or fatty acid synthase could selectively target the metabolic requirements of CSCs.<sup>10</sup> Therefore, a novel and more effective treatment strategy targeted at CSCs is required.

The utilization of exosomes, a small membrane-bound extracellular vesicle that typically ranging from 30 to 150 nanometers in size and nanovesicles, a broader term that encompasses various type of membrane-bound vesicles released by cells and sourced from mesenchymal stem cells (MSCs) can package chemotherapy drugs effectively and function as a reliable delivery system that specifically targets cancer cells, bypassing efflux pumps.<sup>11</sup> Additionally, MSCs secrete growth factors and cytokines with anti-inflammatory properties, tissue regeneration capabilities, and function as therapeutic payloads for cancer therapy.<sup>12</sup> Chemotherapy-induced MSCs inhibit stromal cell line proliferation, followed by angiogenesis prevention and migration inhibition.<sup>13</sup> Paclitaxel-induced MSCs have also been shown to reduce adenocarcinoma cell proliferation and induce apoptosis.<sup>14</sup> Furthermore, MSCs possess homing abilities, allowing them to deliver drugs into target cells without affecting normal cells.<sup>15</sup> Recently, a clinical study is investigating the use of MSCs engineered to express the cytokine IL-12 for treatment of gastrointestinal cancer.<sup>16</sup> Previous research also conducted a trial using MSCs modified to express the TRAIL gene, which induces apoptosis in lung cancer cells.<sup>16</sup> Current MSC-based approaches may not effectively target and eliminate the CSC population, which is responsible for therapy resistance, tumor recurrence, and metastasis.<sup>10</sup> Developing strategies to enhance MSC tropism and targeting of CSCs within the tumor microenvironment is a critical gap. However, an effective curative therapy targeted at CSCs using an MSC-based approach has yet to be developed. Therefore, this study aims to develop a nanovesicle-based MSC therapy modified with paclitaxel chemotherapy induction, targeting the CSC population to prevent recurrence and apoptosis resistance.

## Methods

### Ethics Approval and Consent to Participate

This study was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Universitas Islam Sultan Agung Semarang (Central Java, Indonesia) under number 41/AEC/Biomedik/2022. All participants provided informed consent prior to their inclusion in the study. The research protocol adhered strictly to the guidelines outlined in the Declaration of Helsinki.

### MSCs Isolation

MSCs were obtained from human umbilical cord tissue with explicit informed consent, following established procedures. In summary, the cords were washed with phosphate-buffered saline (PBS) (Gibco™ Invitrogen, NY, USA) to remove cord blood. The cleaned cords were then cut into smaller sections, placed into a T75 culture flask (Corning, Tewksbury, MA, USA), and cultured in low glucose Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco™ Invitrogen, NY, USA), 1% penicillin-streptomycin (Gibco™ Invitrogen, NY, USA), and 0.25% amphotericin B (Gibco™ Invitrogen, NY, USA). The cells were then incubated at 37°C with 5% CO<sub>2</sub>. MSCs derived from passage 5 were used for subsequent experiments.<sup>17</sup>

### MSCs Differentiation Capability Assay

Adipogenic differentiation and osteogenic assay were evaluated using Oil Red O staining and Alizarin Red staining, respectively. To elaborate, adipogenesis was induced in an adipogenic induction medium comprising ADS medium supplemented with 0.5 mM isobutyl methylxanthine, 1 μM dexamethasone, and 200 μM indomethacin. Experiments were conducted at both 15 and 28 days, and confirmation of adipogenic differentiation was achieved through Oil Red O staining.<sup>18</sup>

To prompt osteogenic differentiation, passage 3 MSCs were plated in 6-well plates at a density of  $5 \times 10^4$  cells/well, using osteogenic induction medium comprised of DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma-Aldrich), 50 μg/mL ascorbic acid (Sigma-Aldrich), and 100 nM dexamethasone (Sigma-Aldrich). After 21 days of incubation, mineralization was evaluated through Alizarin Red S staining. Cells were fixed with 4% paraformaldehyde

for 15 minutes, washed with distilled water, and subsequently stained with 2% Alizarin Red S solution (pH 4.2) for 30 minutes at room temperature. Excess stain was removed, and images were captured using a phase-contrast microscope.<sup>19</sup>

## MSCs Surface Marker Analysis

Surface markers of MSCs were validated via flow cytometry analysis following the manufacturer's guidelines. The isolated cells were trypsinized and separated by centrifugation at 1900 rpm for 8 minutes. About  $1 \times 10^5$  cells were suspended in 100  $\mu$ L staining buffer (BD Bioscience, San Jose, CA, USA). These cells were then incubated with fluorescein isothiocyanate-, allophycocyanin (APC)-, peridinin-chlorophyll-protein (perCP)-CyTM5.5.1, and phycoerythrin (PE)-conjugated anti-human CD90, CD73, CD105, and Lin (CD45/CD34/ CD11b/CD19/HLA-DR) antibodies (BD Bioscience, San Jose, CA, USA) for 30 minutes at room temperature. Additionally, an appropriate isotype-specific conjugated anti-IgG (BD Bioscience, San Jose, CA, USA) and unstained cells were used as negative controls. The analysis was conducted using a BD Accuri C6 Plus flow cytometer (BD Bioscience, San Jose, CA, USA).

## Cancer Cell Culture

The MDA-MB-231 human breast cancer cell line was sourced from the American Type Culture Collection (#HTB26 ATCC, Manassas, VA, USA). MDAMB-231 cells were maintained in high glucose Dulbecco's modified Eagle's Medium (DMEM) (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA), 12.5  $\mu$ g/mL amphotericin B (Gibco, USA), 150  $\mu$ g/mL streptomycin, and 150 IU/mL penicillin (Gibco, USA). The cells were cultured at 37°C with 5% CO<sub>2</sub>.

## CSC Isolation and Validation

Flow cytometry was employed to investigate cancer stem cells (CSCs) in MDA-MB-231 cells. CSCs were isolated from MDA-MB-231 cells using CD44 and CD24 antibodies linked to magnetic microbeads (Miltenyi Biotec Inc, CA, USA). CSCs were identified as the CD44+/CD24- cell population. A magnetic-activated cell sorting (MACS) system, with anti-CD44 and anti-CD24-biotin coupled anti-biotin microbeads (Miltenyi Biotec Inc, CA, USA #Catalog: 130-095-194), was utilized to separate the CSC population based on CD44 and CD24 surface expression. Magnetic separation (MS) and low dead column (LD) columns (Miltenyi Biotec Inc, CA, USA #Catalog: 130-095-194) were used for positive and negative selection, respectively. The phenotype of CD44+/CD24- was confirmed using anti-CD44-FITC and anti-CD24-PE monoclonal antibodies with flow cytometry (BD Biosciences, Franklin Lakes, New Jersey). Furthermore, the CSC population was validated through a mammosphere capability assay. Mammospheres derived from CSCs were cultured in ultra-low attachment well plates at a density of  $1 \times 10^5$  cells/mL. The number of cell clusters (diameter > 50  $\mu$ m) in each well was assessed morphologically under a microscope on days 0, 3, and 7.<sup>20</sup>

## MSCs Induction with Paclitaxel

Passage 4th MSCs, which had reached 70% confluence, were induced with paclitaxel at a concentration of 2000 ng/mL for 24 hours. After incubation, the medium was removed and replaced with fresh MSC medium, followed by a 24-hour incubation period. Subsequently, the cells were ready for co-culture.

## Cytotoxic Assay

The impact of Paclitaxel-induced MSCs on CSC cells' viability was assessed using the MTT assay, following a methodology similar to that outlined by Mosman,<sup>21</sup> with minor adjustments. MDAMB-231 cells ( $2 \times 10^3$  cells/well) were plated in a 96-well microplate and allowed to adhere overnight before exposure to Paclitaxel-induced MSCs (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. Variations in concentrations were applied to establish the IC<sub>50</sub> value, indicating the concentration needed to reduce cell viability by 50% compared to untreated cells. Negative controls comprised untreated cells. Following treatment, MTT solution (0.5 mg/mL in medium, BioVision Inc, SF, USA) was added to each well and incubated for 4 hours under 37°C with 5% CO<sub>2</sub> in a fully humidified environment. Subsequently, dimethyl sulfoxide (DMSO) was used to dissolve the resulting MTT formazan crystals, with further incubation in the dark for 4 hours. Absorbance was measured at 595 nm using an ELISA plate reader (Biorad). Each treatment was performed in triplicate, and cytotoxic activity was quantified as IC<sub>50</sub>.<sup>22,23</sup>

## Apoptosis Analysis

The cells were exposed to Paclitaxel-induced MSCs at various ratios of MSCs to CSCs (1:1, 2:1, 3:1, 4:1, 5:1) for 24 hours. Apoptosis was quantified using the Annexin V-PI assay (BD Biosciences, USA). Following incubation, the cells were collected and treated with 5 $\mu$ L Annexin V-FITC and 5 $\mu$ L PI (50 $\mu$ g/mL) for 30 minutes at 4°C in the absence of light. Subsequently, flow cytometry analysis (BD Accuri C6 plus, BD Biosciences, USA) was conducted to evaluate the cells.<sup>24,25</sup>

## Nrf2 and Caspase-3 Gene Expression Analysis

Total RNA was extracted from CSC cell cultures using TRIzol following the manufacturer's instructions (Invitrogen, Shanghai, China). First-strand cDNA was synthesized using SuperScript II (Invitrogen, Massachusetts, USA) and 1 ng of total RNA. Real-time PCR was performed using SYBR No-ROX Green I dye (SMOBIO Technology Corp, Hsinchu, Taiwan) for reverse transcription on a PCR max Eco 48 instrument. The mRNA levels of the Nrf2 and Caspase-3 genes were then assessed using specific primers. The thermocycler conditions were set as follows: an initial step at 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for one minute. The cycle threshold (Ct) was used to quantify gene expression. Data were analyzed using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA) employing the  $2^{-\Delta\Delta Ct}$  method (Livak method). Each reaction was performed in triplicate to ensure accuracy.

## Statistical Analysis

The presented data are represented as the mean  $\pm$  standard error of the mean (SEM) from three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) as the data met the assumptions of normality and homogeneity of variance, followed by LSD post hoc test with SPSS version 26. Significance was determined at a P value ( $* < 0.05$ ). The 50% inhibitory concentration (IC<sub>50</sub>) was calculated using nonlinear regression curve fitting in Microsoft Office Excel for Windows.

## Results

### MSCs Isolation and Characterization

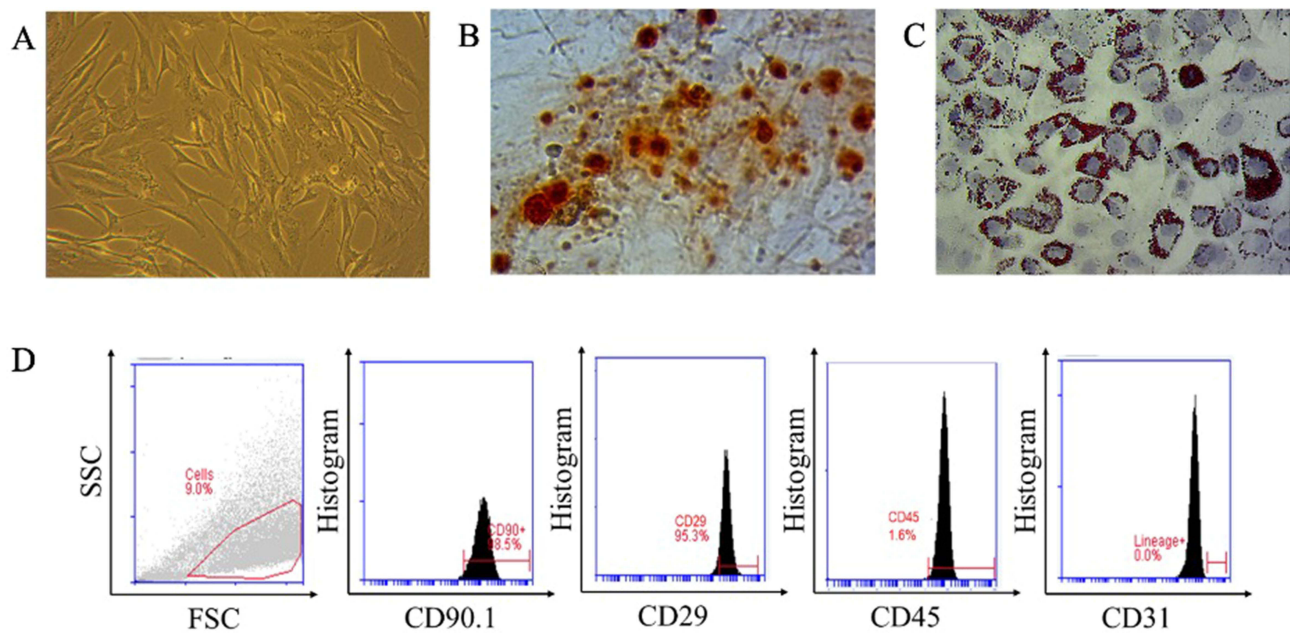
MSCs were assessed for their ability to adhere to plastic surfaces in standard culture conditions. By passage four, MSCs exhibited adherent growth, forming characteristic monolayers of spindle-shaped fibroblast-like cells (Figure 1A). To confirm the MSCs' differentiation potential in vitro, osteogenic and adipogenic differentiation media were employed. Osteogenic differentiation was confirmed by the presence of red staining indicating calcium deposition (Figure 1B), while adipogenic differentiation was evidenced by the accumulation of neutral lipid vacuoles stained with Oil Red O (Figure 1C). Flow cytometric analysis of UC-MSCs showed positivity for CD90 (98.50%) and CD29 (95.30%), and negativity for CD45 (1.60%) and CD31 (0.00%) (Figure 1D). Additionally, MSCs were cultured under hypoxic conditions with 5% O<sub>2</sub> for 12 hours to induce cytokine and growth factor production.

### CD44+ / CD24- Expression Profile in CSC Isolated from MDA-MB-231

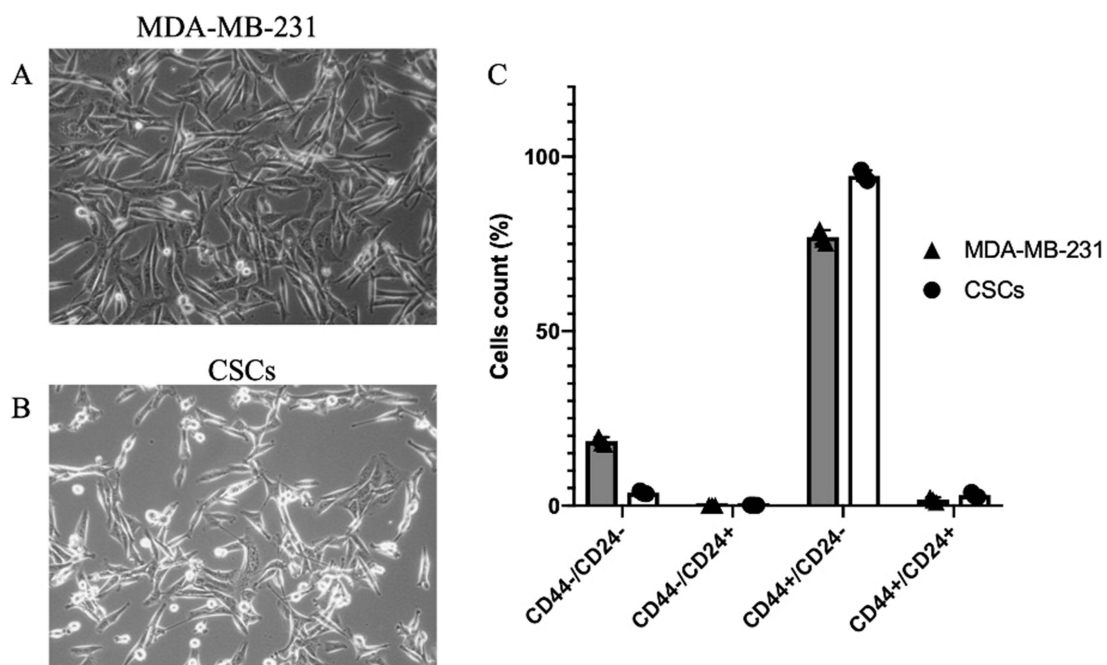
The CSC population was isolated from MDA-MB-231 cells using magnetic cell sorting, based on the CD44+/CD24- expression profile. The morphology of CSCs appeared more elongated or irregular compared to non-stem cancer cells (Figure 2A and B), indicative of stem-like characteristics. Moreover, the purities of the isolated CSCs and MDA-MB-231 cells were determined to be  $94.30 \pm 2.47\%$  and  $79.20 \pm 4.31\%$ , respectively, demonstrating high CD44 expression and lack of CD24 expression (Figure 2C). Elevated CD44 expression is associated with cancer development and poor prognosis of CD24 expression is associated with undifferentiated cells.

### Cytotoxic Effect of Co-Culture Paclitaxel-Induced MSCs on CSC Cell Population

Paclitaxel has been associated with the induction of Nrf2 gene expression in cancer cells. Several studies have highlighted the role of Nrf2 in mediating chemoresistance and its potential as a therapeutic target in cancer treatment.

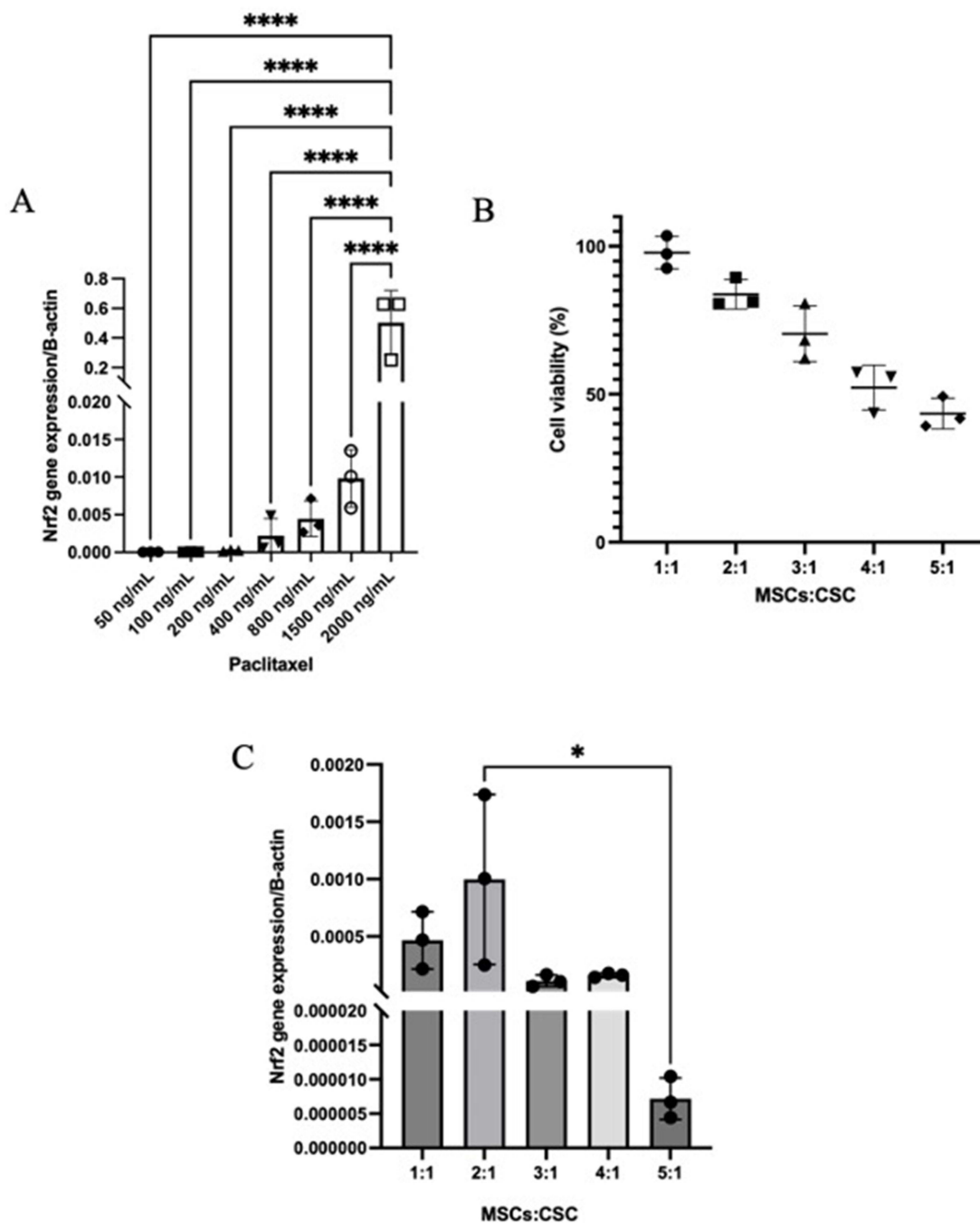


**Figure 1** (A) Morphological observation reveals MSCs with a uniform spindle-shaped appearance. (B) Osteogenic differentiation is indicated by calcium deposition, visualized through Alizarin Red staining. (C) Adipogenic differentiation is evident from the accumulation of neutral lipid vacuoles, stained with Oil Red O. (D) Graphs demonstrate the phenotype of MSCs, depicting expression levels of CD90, CD29, CD45, and CD31.



**Figure 2** (A) the morphological of MDA-MB-231 and (B) CSC cells. (C) the percentage of CD44 and CD24 characterization of the surface marker on MDA-MB-231 cells and CSC. The bar and graph represent the mean  $\pm$  SD of three independent trials with at least 3 replicates.

Research has shown that Nrf2 activation contributes to tumor chemoresistance by promoting the expression of antioxidant and drug efflux genes, thereby inducing the efficacy of chemotherapy. Additionally, Nrf2 has been linked to the enhancement of cancer cell death, as well as the suppression of resistance to various anticancer treatments. This study in line with previous study, the presence of paclitaxel on the MSCs cells significantly increased the Nrf2 gene expression level in doses-dependent manner up to  $0.50 \pm 0.21$  (Figure 3A). This phenomenon maybe suggested that



**Figure 3** Role of Nrf2 in Paclitaxel-Induced Modulation of Cancer and Mesenchymal Stem Cells. **(A)** Nrf2 gene expression level of MSCs in the presence of paclitaxel. **(B)** Cytotoxic activity of Paclitaxel-induced MSCs on the CSC cell population. **(C)** Nrf2 gene expression level of Paclitaxel-induced MSCs on the CSC cell population. Total RNA extraction was performed following the procedures outlined in the materials and methods section, and subsequently utilized for reverse transcription and qRT-PCR analysis. The data are presented as the mean ± standard deviation (SD) of results obtained from three independent experiments. Statistical significance was determined using one-way ANOVA, with \* $p < 0.05$  indicating a significant difference, and \*\*\*\* $p < 0.001$  indicating a highly significant difference.

MSCs that induced by paclitaxel can increased the antioxidant properties and highly proliferated. Furthermore, we analyse the cytotoxic effect of Paclitaxel-induced MSCs on the CSC cell population, in this study we used several ration of MSCs and CSC. Based on MTT assay, we found that the increase of ratio MSCs decrease the cell viability in doses-dependent manner (Figure 3B). To confirm the potential effect of Paclitaxel-induced MSCs on the CSC cell population, we also checked the Nrf2 gene expression after MSCs treatment. The MSCs:CSC 5:1 significantly decreased the Nrf2

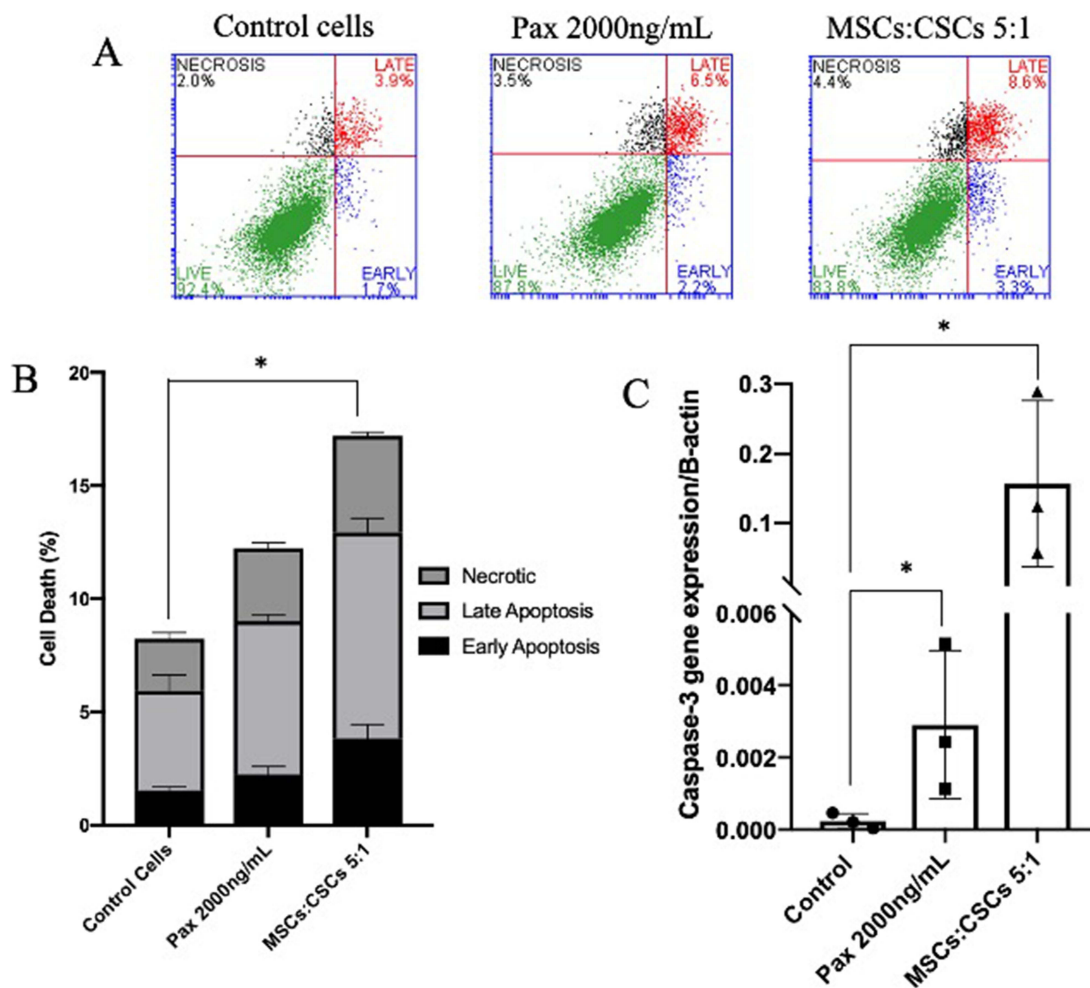
gene expression level (Figure 3C). This findings suggesting targeting Nrf2 has been proposed as a strategy to overcome paclitaxel resistance and improve the effectiveness of chemotherapy in CSC.

## The Effect of Paclitaxel-Induced MSCs on CSC Apoptosis

The effect of paclitaxel-induced MSCs was examined on cell apoptosis using flow cytometry analysis with annexin V-PI staining (Figure 4A). The co-culture of Paclitaxel-induced MSCs and CSC increased the apoptosis cells up to  $17.20 \pm 8.77\%$ , the presence of paclitaxel alone also increased the cell death ( $4.23 \pm 2.49\%$ ), lower compare with co-culture of Paclitaxel-induced MSCs (Figure 4B). Furthermore, the apoptosis induction correlated with caspase-3 gene expression level, the culture of Paclitaxel-induced MSCs and CSC induced caspase-3 gene expression higher than single treatment of paclitaxel (Figure 4C). This apoptosis phenomenon might be triggered by activation of caspase-3 in CSC.

## Discussion

This study's Results highlight the potential of Paclitaxel-induced mesenchymal stem cells (MSCs) to influence the behavior of cancer stem cells (CSCs) and improve cancer cell sensitivity to chemotherapy. The study revealed that paclitaxel exposure led to a dose-dependent increase in Nrf2 gene expression in MSCs, suggesting a potential link between paclitaxel treatment and the enhanced antioxidant properties of MSCs. Nrf2 gene can lead to shifts in MSC



**Figure 4** Paclitaxel-induced MSCs triggered apoptosis of CSC. (A) Flow cytometry in CSC, (B) The quantification of percentage apoptotic cells under Paclitaxel-induced MSCs and single paclitaxel treatment on CSC, and (C) Ration of caspase-3 gene expression under Paclitaxel-induced MSCs and single paclitaxel treatment on CSC. The columns depict the mean  $\pm$  standard deviation (SD) of three independent trials, each with at least 3 replicates. Statistical significance was assessed using one-way ANOVA, where \*P < 0.05 indicates a significant difference compared to the untreated group.

behavior, potentially affecting their differentiation capacity, secretory profile, and interactions with other cell types within the tumor microenvironment leading to tumor apoptosis.<sup>26,27</sup> Paclitaxel that induced in MSCs maybe participate actively in the tumor microenvironment by secreting various factors and interacting with other cells, including CSC.<sup>28,29</sup> Importantly, co-culture experiments demonstrated a dose-dependent decrease in CSC viability upon exposure to Paclitaxel-induced MSCs. Notably, the ratio of MSCs:CSC at 5:1 significantly reduced Nrf2 expression in CSCs, indicating a potential mechanism for overcoming paclitaxel resistance.

The observed impacts of Paclitaxel-induced MSCs on Nrf2 expression and CSC apoptosis align with earlier research emphasizing Nrf2's involvement in chemoresistance and its potential as a target for cancer therapy.<sup>30</sup> The constitutive activation of Nrf2 in various cancers has been shown to induce pro-survival genes, promote cancer cell proliferation, and increase cancer resistance.<sup>31,32</sup> However, the study also revealed that Paclitaxel-induced MSCs significantly decreased the Nrf2 gene expression leading to apoptosis induction. The study's findings are in line with previous research that has demonstrated the potential of MSCs to influence the tumor microenvironment and modulate the behavior of cancer cells. The study's results suggest that Paclitaxel-induced alterations in MSCs might impact the secretion of growth factors, cytokines, or other signaling molecules, thereby influencing the behavior and responses of CSC.<sup>13,33,34</sup> Earlier research has also shown that MSCs can release cytotoxic agents, including TNF-Related Apoptosis-Inducing Ligand (TRAIL), which specifically triggers apoptosis in various cancer types.<sup>35</sup> A recent report has suggested that bone marrow MSCs can stimulate apoptosis and inhibit the growth of glioma U251 cells by downregulating the PI3K/AKT signaling pathway.<sup>36</sup> Likewise, intravenously transplanted MSCs were discovered to hinder tumor growth in a Kaposi sarcoma mouse model by inhibiting AKT activation.<sup>35</sup> In mammary carcinomas, umbilical cord MSCs were found to reduce cell growth and induce apoptosis by inhibiting ERK1/2 and AKT activation.<sup>37</sup> Additionally, the Wnt signaling pathway has been implicated in MSCs' ability to suppress tumor cell proliferation.<sup>38</sup> A mechanistic investigation into MSCs' inhibitory effect on breast cancer cells revealed that the release of the protein Dickkopf-1 (Dkk-1) from MSCs hampers tumor growth by suppressing Wnt signaling.<sup>39</sup>

Our study underscores the potential of Paclitaxel-induced MSCs in modulating Nrf2 expression and sensitizing CSC to apoptosis. Targeting Nrf2 in the context of paclitaxel resistance could serve as a promising strategy to enhance the efficacy of chemotherapy in CSC. However, further investigations are warranted to elucidate the intricate molecular mechanisms underlying the observed effects and validate the translational potential of these findings in clinical settings.

## Conclusion

In Conclusion, Paclitaxel-induced MSCs decreased Nrf2 expression and significantly decreased CSC viability while enhancing apoptosis. This suggests a potential strategy to mitigate paclitaxel resistance in CD44+/CD24- CSCs. Leveraging Paclitaxel-induced MSCs presents a promising avenue for targeting Nrf2 and promoting apoptosis in CSCs, potentially improving the efficacy of chemotherapy and addressing resistance mechanisms in cancer treatment. These results contribute to the growing understanding of the tumor microenvironment and highlight the significance of targeting specific cellular interactions to improve cancer treatment strategies.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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