Original Article

Isolation and characterization of CD105⁺/CD90⁺ subpopulation in breast cancer MDA-MB-231 cell line

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Abstract: Background: The epithelial-mesenchymal transition (EMT) generates cells with properties of stem cells, if that happened, the stem cell should be with mesenchymal property. This study aimed to identify a group of cells with mesenchymal stem cell (MSC)-like characteristics in breast cancer bone metastatic cell line MDA-MB-231, moreover, the relevance between breast cancer stem cells and the EMT was observed. CD105 and CD90, identified as the standards of MSCs, were used for the identification. Methods: The CD105⁺/CD90⁺ and CD105⁻/CD90⁻ subpopulation of MDA-MB-231 cells were detected and sorted by flow cytometry. MSC-like characteristics in cell proliferation, migration and cell cycle were investigated here by MTT assay, transwell migration assay, and PI staining respectively. The expression profiles of some stem cell-associated genes were also observed by quantitative real time PCR. Results: Around 0.99% and 90.77% of parental cells were identified as CD105⁺/CD90⁺ and CD105⁻/CD90⁻ cell subpopulations respectively. The CD105⁺/CD90⁺ cells exhibited stronger migratory capacity as compared to parental and CD105⁻/CD90⁻ cells, while less CD105⁺/CD90⁺ cells were arrested in the S phase. Besides, pluripotent stem cell factors, like Oct-4, Nanog, Klf4 and Sox-2, were all upregulated in CD105⁺/CD90⁺ cells, with also proliferation increase, as compared with other two populations. Conclusion: The CD105⁺/CD90⁺ subpopulation from breast cancer MDA-MB-231 cells was proven to possess “mesenchymal stem cell-like” characteristics, and its high migratory ability might be associated with EMT. Moreover, using the surface markers of CD105 and CD90 for the identification of MSCs might provide new theoretical basis for the recurrence and metastasis of breast cancer.

Keywords: Breast neoplasm, CD105, CD90, cancer stem cell, epithelial mesenchymal transition, mesenchymal stem cells

Introduction

Cancer stem cells (CSCs) are believed to originate from normal stem cells. Therefore, they possess similar biological characteristics and potential functions for chemoresistance, metastasis, and tumor recurrence. Endoglin (CD105) and thymus cell antigen 1 (Thy1, CD90) have been identified as the standards of mesenchymal stem cells (MSCs) by the International Association for Cell Therapy [1]. CD105 has an important role in angiogenesis [2], and Saroufim had shown that tumoral CD105 is an independent predictive marker for death risk and unfavourable prognosis in patients with clear-cell renal cell carcinomas after curative resection [3]. Moreover, high expression of CD105 in breast tumor is associated with poor overall and disease-free survival, and can be an independent predictor. CD105 is also an important therapeutic target in metastatic breast cancer [4]. Yang showed that CD90 is a potential marker for liver CSCs [5].

Recent studies have demonstrated that epithelial–mesenchymal transition (EMT) has an important function in invasion and metastasis of most types of epithelial malignancies. The EMT is a latent embryonic process converting polarized and adjacent epithelial cells to mesenchymal cells. This process, reversible by definition, transiently confers cell motility, allow-
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Table 1. Primers used for RT-PCR quantitation

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct3/4-forward</td>
<td>GAAAGTTGAGAA GGAGAAGCTG</td>
</tr>
<tr>
<td>Oct3/4-reverse</td>
<td>CAAGGGGCAGGCTACAGATGTC</td>
</tr>
<tr>
<td>Nanog-forward</td>
<td>CAGAAGGTCCGCTAGCAGAAGAAAA</td>
</tr>
<tr>
<td>Nanog-reverse</td>
<td>TGTTTCAGGGCAGCTAGTTT</td>
</tr>
<tr>
<td>Sox2-forward</td>
<td>AGCTCCAAAGGAGAAGCAGAAGAAAA</td>
</tr>
<tr>
<td>Sox2-reverse</td>
<td>GGA AAGTTGAGGATCGAACAA</td>
</tr>
<tr>
<td>Klf4-forward</td>
<td>GAGCTACAGTGGTAAGGTTT</td>
</tr>
<tr>
<td>Klf4-reverse</td>
<td>CAAGAAGGTGGTGAAGCAGG</td>
</tr>
<tr>
<td>GADPH-forward</td>
<td>ATGGTACATGACAAGGTTCG</td>
</tr>
</tbody>
</table>

Cell proliferation analysis

The CD105+/CD90+ subpopulation, CD105/CD90 subpopulation, and MDA-MB-231 cell group (parental population) were cultured separately. Each population of cells was seeded in a 96-well culture plate with the same concentration as that of the logarithmic growth phase. The sulforhodamine B (SRB) assay was used to compare and observe the proliferative capacity among the three subgroups [10]. The adherent cells were counted at different times for 9 d after seeding, and the growth curve was plotted.

Cell cycle analysis assay

Because stem cells are quiescent slow-cycling cells, DNA content/cell cycle analysis of each population was performed, and the proportions of G0/G1, S, and G2/M phases were analyzed by flow cytometry [11]. According to formers, cells were trypsinized, washed with cold PBS (phosphate-buffered saline), fixed with 70% ethanol/PBS overnight at 4°C, and centrifuged. The pellets were resuspended in 500 μl PBS in the presence of 50 μg/ml propidium iodide and 1 mg/ml DNase-free RNase A and incubated in the dark for 30 min at room temperature. Stained cells were washed once and resuspended in PBS. The cell cycle data for individual samples were acquired using the BD FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). The proportions of cells in G0/G1, S, and G2/M phases were analyzed using dedicated software.

Cell migration and invasion assay

Cells (1×10^5) were seeded on Boyden chambers with 8.0 μm pore size (Transwell, Corning Life Sciences, Acton, MA, USA) in serum-free medium. Medium containing 20% FBS served as a chemoattractant in the lower chamber. After 24 h (for migration assays), non-invading cells were removed with cotton swabs. Invaded cells were fixed with methanal, stained with Giemsa solution and counted in five microscopic fields for each well. Data are represented as the mean ± SEM from three individual experiments.

RNA extraction, reverse transcription PCR, and quantitative real-time PCR

The expression of stemness genes Oct3/4, Nanog, Sox2, and Klf4 were detected by quan-
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Quantitative real-time PCR in each cell subpopulation. Total RNA from cells was extracted using TRizol (Invitrogen) and chloroform, and further purified with an RNeasy kit (Qiagen). Approximately 500 ng of RNA was used for reverse transcription following the protocol of a SuperScript III First Strand Kit (Invitrogen). The resulting cDNA was diluted 12 times to perform real-time PCR with ABI SYBR Green PCR master mix (Kapa) in ABI Prism 7500 Real-Time PCR System (PE Applied Bio systems). The quantitative amount of stemness gene mRNA of the three populations was analyzed following the manufacturer’s instructions, and acquired data were analyzed by software 7500 version 2.0.6 (PE Applied Bio systems). Error bars represent the standard deviation (SD) of at least three PCR experiments of each sample. Results were obtained in at least three experiments. Primers used for RT-PCR quantitation are listed in Table 1.

Statistical analysis

Statistical data were analyzed by SPSS13.0 software. Data were processed as the mean ± SD. Statistical significance was determined by one-way ANOVA or Kruskal-Wallis H test. Mean differences between groups were compared by LSD and chi-square test ($\chi^2$ test). $P < 0.05$ was considered statistically significant (*represents $P < 0.05$, **represents $P < 0.01$).

Results

Cells identified with CD105+/CD90+ were isolated from MDA-MB-231

In the present study, cultured cells in the exponential phase were used. Flow cytometry showed that cells of the CD105+/CD90− subpopulation accounted for 0.99%, whereas those of the CD105−/CD90− subpopulation accounted for 90.77% (Figure 1A). Then, two subgroups of cells, namely, CD105+/CD90− and CD105−/CD90+, were sorted by flow cytometry. Cells of the two subpopulations were cultured in 24-well plates. The number of cells sorted was initially very low. Only scattered cells were observed on the plates 4 h after sorting, and the double-negative cells were greater than the double-positive cells (Figure 1B).

CD105+/CD90+ subpopulation showed higher proliferation

After 72 h incubation, it can be found that every single double-positive cell had formed a small clone. These cells started to cover the well at 3 days.
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d, indicating that they were in the exponential phase. After 6 d, the double-negative cells reached the exponential phase. The two subgroups of cells were separately planted on six-well plates at the same conditions and initial cell concentrations. The results show that the time in which the double-positive subpopulation achieved the logarithmic phase was less than that of the double-negative subgroup (Figure 2C). The cell growth curves of the double-positive, double-negative, and parental populations revealed that the proliferation rate of the double-positive population was significantly higher than that of the other two populations.

CD105⁺/CD90⁺ subpopulation possessed slow cycling characteristics

For cell cycle analysis, exponential phase cells of the three populations were collected to show the proportions of cells at G0/G1, S, and G2/M phases. And, proportions in different phases of the three populations were compared, and the results were statistically analyzed. The results indicated that the double-positive population had more cells in the G0/G1 and G2/M phase than the double-negative and parental populations, with less cells observed in the S phase (Figure 2A, 2B). Given that stem cells are quiescent with slow cycling characteristics, this

Figure 2. Cell proliferation assay and cell cycle analysis for CD105⁺/CD90⁺ subpopulation, with CD105⁻/CD90⁻ cell subpopulation and parental populations as controls. A. DNA content histogram obtained after cytometry analysis. B. Mathematical analysis for cell repartition in different cycle phases. C. Cell proliferation curve of the three populations in nine days. *: \( P < 0.05 \); **: \( P < 0.01 \).
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The result showed that the CD105+/CD90+ cells contained much more stem-like cells than the other two subpopulations.

*CD105+/CD90+ cells exhibited stronger migratory capacity and cell adhesion*

When cells of the three populations were attached to the lower chamber after 72 h, the number of adherent cells from the double-positive, parental, and double-negative populations was also counted, while the double-positive population had the highest number of cells that passed through the membranes at 72 h, compared with the other two populations (Figure 3A, 3B). In addition, the number of cells that passed through the polycarbonate membranes after 24 h from the double-positive, parental, and double-negative populations was also calculated. Findings showed that the number of double-positive population cells that passed through the membrane was the highest compared with that of the other two populations (Figure 3C), whereas that of the double-negative population was the least. Moreover, clusters of double-positive cells on the lower chamber were observed to attach well, with normal cell formation. By contrast, only a few scattered cells were observed on the plates of the other two populations, and the cells demonstrated weak adhesion.

*Stem cell-associated genes were overexpressed in CD105+/CD90+ cells*

Stem cell-associated genes, such as Oct-4, Sox2, and Nanog, are recommended for identifying gastric CSCs [12]. Here, the expression of stemness genes Oct3/4, Nanog, Sox2, and also Klf4 were detected by quantitative real-time PCR technology (RT-PCR). Among the three groups at the same imaging conditions, the gra-
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Quantitative real time PCR analysis of stemness genes, Oct3/4, Nanog, Sox2 and Klf4, in the three populations. The expression of Oct3/4, Nanog, Sox2 and Klf4 were shown in A-D respectively. **: P<0.01.

Figure 4.

Discussion

Cancer stem cells (CSCs), important targets for therapeutic approaches, are a small subset of cells found in tumors that can self-renew and differentiate, initiate tumors, and induce tumor growth, recurrence, and metastasis [13]. Numerous biomarkers in breast cancer stem cell lines have been analyzed by researchers. Breast CSCs have been described to be immune-phenotypically characterized by CD44+/CD24−/low ESA−Lin− [14], while not all breast CSCs have the same immunophenotype. Another study shows that the high expression of CD34 is associated with the progenitor cell of T-cell acute lymphocytic leukemia (T-ALL) [15]. Similarly, CD34 is not expressed in each cell line and also not essential for the self-renewal and multi-differentiation potential of CSCs [16]. The mechanism about how cells maintain complete systems during long-term culture in vitro, similar to the body, remains unknown.

Diagnostic use of CD105 indicates the important role in controlling the clinical signs of the breast cancer; moreover, it may be used in the field of diagnostic follow-up, determining the response to treatment and prognosis of the disease [17]. CD90, another commonly used stem cell markers, is proven to be differentially expressed in some breast cancer cell lines and might be related to their malignancy grade [18]. Here, we selected this two cell surface markers, CD105 and CD90, that expressing on the surface of MSCs, to analyze MDA-MB-231 breast cancer cells, derived from breast cancer patients with bone metastasis. Then, CD105+/CD90− subpopulation was characterized, and the relevance between breast cancer stem cells and the EMT was also observed.

Long-lived CSCs with indefinite proliferative potential are likely derived from transformed...
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adult stem cells, and are thought to share many characteristics with their parental population, including a quiescent slow-cycling phenotype [19]. Previous studies showed that stem cells have the characteristics of quiescent cells, such as corneal stem cells in mice and hair follicle stem cells. Stem cells in malignant tumor tissues with strong vitality are few and mostly in the quiescent state, but have a key function in regulating tumor growth, and can self-renew and proliferate under special stimulation [20]. This ability can help in protecting the consumption of immature cells in the body to confer malignant tumors with resistance to apoptosis and hinder traditional tumor therapies, such as radiotherapy and chemotherapy. This ability may also be the mechanism of tumor recurrence and metastasis.

Cell cycles analysis of the three populations by flow cytometry showed that most of the CD105+/CD90- cells were detected in the G0/G1 phase, with significant differences observed between the parental group and double-negative group, indicating CD105+/CD90- cells to be quiescent slow-cycling cells. Additionally, as higher rate of angiogenesis and cellular proliferation have been observed in malignant tumors compared to the benign tumors [21], the cell proliferative and migratory abilities were also assessed using cell growth curves and transwell assay. Taken together, results showed that the CD105+/CD90- cells had higher proliferative and migratory abilities than the other two types of cells.

The epithelial-mesenchymal transition (EMT) is a process in which epithelial cells trans-differentiate and acquire an invasive mesenchymal phenotype. It has a central function in embryogenesis and mesoderm differentiation into multiple tissue types during development [22]. The emergence of embryonic stem cell-associated genes in high-grade undifferentiated cancers suggests that aberrant regulations of EMT may have a function in CSC characteristics [23]. Many stem cells are found present in tumor microenvironment such as cancer stem cells (CSCs), mesenchymal stem cells (MSCs), all of which might be the inducers of EMT in tumor cells [24], meanwhile, clinical evidence has demonstrated that regulators of EMT in cancer cells was correlated with poor clinical outcomes and tumor aggressiveness. Quantitative real-time PCR revealed that the CD105+/CD90- cells have highly expressed embryonic stem cell-associated genes, indicating the relevance between the two markers and EMT.

In conclusion, the CD105+/CD90- subpopulation of breast cancer MDA-MB-231 cells possessed "mesenchymal stem cell-like" characteristics, and its high migratory ability may be associated with EMT. Moreover, using the surface markers of CD105 and CD90 for the identification of MSCs might provide new theoretical basis for the recurrence and metastasis of breast cancer.

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Disclosure of conflict of interest

None.

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