

Research Article

CD90- (Thy-1-) High Selection Enhances Reprogramming Capacity of Murine Adipose-Derived Mesenchymal Stem Cells

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Background. Mesenchymal stem cells (MSCs), including adipose tissue-derived mesenchymal stem cells (ADSC), are multipotent and can differentiate into various cell types possessing unique immunomodulatory features. Several clinical trials have demonstrated the safety and possible efficacy of MSCs in organ transplantation. Thus, stem cell therapy is promising for tolerance induction. In this study, we assessed the reprogramming capacity of murine ADSCs and found that CD90 (Thy-1), originally discovered as a thymocyte antigen, could be a useful marker for cell therapy. **Method.** Murine ADSCs were isolated from B6 mice, sorted using a FACSaria cell sorter by selection of CD90^{Hi} or CD90^{Lo}, and then transduced with four standard factors (4F; Oct4, Sox2, Klf4, and c-Myc). **Results.** Unsorted, CD90^{Hi}-sorted, and CD90^{Lo}-sorted murine ADSCs were reprogrammed using standard 4F transduction. CD90^{Hi} ADSCs showed increased numbers of alkaline phosphatase-positive colonies compared with CD90^{Lo} ADSCs. The relative reprogramming efficiencies of unsorted, CD90^{Hi}-sorted, and CD90^{Lo}-sorted ADSCs were 100%, 116.5%, and 74.7%, respectively. CD90^{Hi} cells were more responsive to reprogramming. **Conclusion.** CD90^{Hi} ADSCs had greater reprogramming capacity than CD90^{Lo} ADSCs, suggesting that ADSCs have heterogeneous subpopulations. Thus, CD90^{Hi} selection presents an effective strategy to isolate a highly suppressive subpopulation for stem cell-based tolerance induction therapy.

1. Introduction

Induced pluripotent stem (iPS) cells can be directly generated from fibroblast cultures by expression of four factors (4F), including octamer-binding transcription factor 4, sex-determining region Y-box 2, Kruppel-like factor 4, and myelocytomatosis viral oncogene homolog [1]. Although these factors present attractive sources for stem cell therapy, the mechanisms by which they are generated are not

fully understood. The inefficiency of iPS cell generation has prompted the development of two contending models, namely, the stochastic and elite models. A recent multilineage-differentiating stress-enduring cell study suggested the greater utility of the elite model over the stochastic model [2]. In either model, stem cells or progenitor cells isolated for experimental or therapeutic research are usually heterogeneous populations; therefore, a more favorable subfraction for use in stem cell therapy may

exist. Indeed, several reports have suggested that the differentiation stage of the starting cell line has a critical influence on the efficiency of reprogramming into iPS cells in hematopoiesis [3]. Immature cell populations, such as lin⁻c-Kit⁺Scal⁺CD48⁻CD150⁺CD34⁻ (lack of lineage/Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog/spinocerebellar ataxia/cluster of differentiation 48⁻/150⁺/34⁻) hematopoietic stem cells, in general give rise to iPS cells at higher efficiencies than terminally differentiated cell types. Furthermore, a recent study demonstrated that CD90^{Hi} adipose tissue-derived mesenchymal stem cells (ADSCs) are more capable of forming bone both *in vitro* and *in vivo* than in CD105^{Lo} cells [4]. CD90 (Thy-1) is a 25–37-kDa glycosylphosphatidylinositol-anchored glycoprotein expressed mainly in leukocytes that have been used as murine pan T-cell markers, like CD2, CD5, and CD28.

Multipotent mesenchymal stem cells (MSCs), which represent a nonhematopoietic cell population, can differentiate into mesenchymal tissues (i.e., bone, cartilage, or fat) [5, 6]; they were first isolated from bone marrow and later from non-marrows tissues, including umbilical cord blood and adipose tissue [5, 6]. Immunologically, MCSs are known to be less immunogenic because of, at least in part, the lack of surface-expressed human leukocyte antigens [5]. Moreover, they have been shown to possess anti-inflammatory and immunosuppressive effects both in murine and human models of diabetes [7, 8]. Thus, the immunoregulatory properties of MSCs are appealing, because they inhibit T-cell proliferation and differentiation of monocytes to dendritic cells, modulate B-cell functions, and suppress natural killer cell cytotoxicity. Solid organ transplantation is an established and useful treatment option for end-stage organ failure. Despite excellent short-term results, long-term survival of transplanted grafts has not improved accordingly because of graft rejection, graft fibrosis, or side effects of immunosuppressants. The aim of stem cell therapy for solid organ transplantation is to prevent or treat acute rejection and to involve autologous or allogeneic stem cell transplantation into patients, either through local delivery or systemic infusion. Currently, there are over 100 ongoing clinical trials to evaluate the utility of MSCs, especially in kidney transplantation [9–11].

ADSCs have been shown to consist of heterogeneous subpopulations; therefore, we hypothesized that CD90^{Hi} ADSCs might exhibit increased reprogramming capacities and subsequently have better immunoregulatory effects. Hence, the aim of the present study was to compare the reprogramming capacity of sorted murine CD90^{Hi} ADSCs. Our data demonstrated that CD90^{Hi} selection improved the reprogramming capacity of murine ADSCs, suggesting that CD90^{Hi} ADSCs could help prevent graft rejection.

2. Materials and Methods

2.1. ADSC Isolation. Murine ADSCs were isolated from B6 mice as previously described but with slight modifications [7]. Briefly, adipose tissue was obtained from the inguinal fat pads, washed with Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen, Carlsbad, CA, USA) containing 50 U/mL of

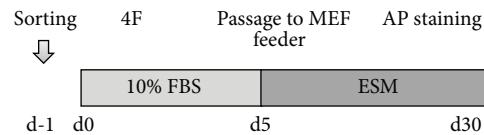


FIGURE 1: Lentiviral-mediated transfer of four iPS cell factor genes in murine ADSCs. A schematic representation of the experiment is shown. Cells were transduced with four factors (4F) after 24 h of incubation. Then, 4F-transduced cells were passaged to MMC-treated MEF feeder cells on day 5. The number of reprogrammed iPS colonies was assessed on posttransduction day 30 by AP staining.

penicillin and 50 µg/mL of streptomycin (PSM; Invitrogen), and then cut into fine pieces, which were incubated with D-PBS containing 1.0 mg/mL of *Clostridium histolyticum* collagenase (Sigma-Aldrich, St. Louis, MO, USA) in a 37°C shaking incubator for 1 h. The digested tissue was filtered through sterile 70-µm nylon mesh, centrifuged at 430 × g for 5 min, and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone; Thermo Scientific, Waltham, MA, USA) and PSM.

2.2. Cell Sorting. Murine ADSCs were washed with D-PBS and treated with Accutase reagent (EMD Millipore, Billerica, MA, USA) in a 37°C incubator for 2–3 min to dissociate the cells, which were then resuspended in staining media [D-PBS supplemented with FBS (1%) and EDTA (2 mM)]. Next, the cells were stained with fluorescein isothiocyanate-conjugated anti-mouse CD34 (eBioscience, San Diego, CA, USA), phycoerythrin-conjugated anti-mouse CD31, eFluor450 anti-mouse CD45, and allophycocyanin-conjugated anti-mouse CD90.2 for 60 min at 4°C. The cells were then washed with staining media, stained with 7-aminoactinomycin D to exclude dead cells, and sorted using the FACSAria cell sorter (Bio-Rad, Hercules, CA, USA) [12]. The particular phenotypic subsets of CD90^{Hi} and CD90^{Lo} ADSCs, based on CD31, CD45, and CD90 expression, were evaluated by multicolor analysis and sorted by adequate gating.

2.3. Lentiviral Preparation. To prepare the lentiviruses, human embryonic kidney (HEK)-293Ta cells were cultured in DMEM supplemented with 10% FBS and PSM. Then, the cells were seeded at 5.0×10^6 cells per 100 mm dish 1 day before viral transduction. Fugene 6 transfection reagent (22.5 µL; Promega, Madison, WI, USA) was diluted with 500 µL of DMEM and incubated for 5 min at room temperature. Plasmid DNA (2.5 µg) was added to the mixture, which was incubated for an additional 15 min at room temperature. Then, the culture media was replaced with fresh DMEM supplemented 10% FBS and the DNA/Fugene 6 mixture was added dropwise onto the HEK-293Ta cells. The medium was replaced after 24 h. After an additional 48 h, virus-containing supernatants, derived from the HEK-293Ta cultures, were filtered through a 0.22-µL cellulose-acetate filter and analyzed without being frozen.

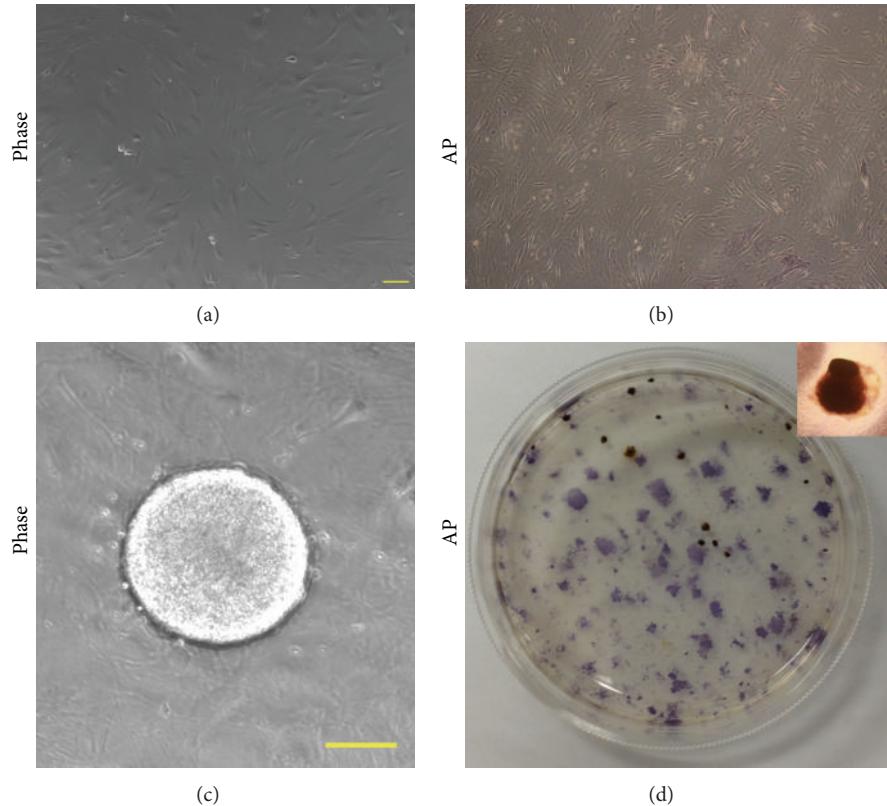


FIGURE 2: 4F transduction resulted in iPS colony formation in unsorted murine ADSCs. Phase (a and c) and AP staining (b and d) of pre- and post- (c and d) transduction were shown.

2.4. Lentiviral Transduction. Sorted CD90^{Hi} and CD90^{Lo} cells were plated onto 6-well culture plates at a density of 1.0×10^5 cells/well, respectively. After incubation for 24 h, the culture medium was retrieved and viral supernatants were added to each well. The next day, the cells were washed with complete medium three times. Mouse embryonic fibroblasts (MEFs) were mitotically inactivated by the addition of 10 µg/mL of mitomycin C (MMC; Nacalai Tesque Inc., Kyoto, Japan) for 90 min and then washed with D-PBS three times. The cells were then cultured with complete medium for at least 2 h, trypsinized, counted, and plated on gelatin-coated 60 mm dishes at a density of 5.0×10^5 cells/well. On posttransduction day 5, the cells were trypsinized; suspended in DMEM supplemented with 15% (v/v) FBS, PSM, 1-mM sodium pyruvate (Invitrogen), 10^{-4} M 2-mercaptoethanol (Nacalai Tesque, Inc.), nonessential amino acid solution, and 1,000 U/mL of leukemia inhibitory factor; after which they were counted. Then, 1,000 cells were transferred to MMC-treated MEF feeder cells. On post-transduction day 30, alkaline phosphatase (AP) staining was performed to assess reprogramming efficiency.

2.5. AP Staining. 4F-transduced ADSCs were stained using an AP staining kit (Muto Pure Chemicals, Tokyo, Japan) following the manufacturer's recommended protocol. Briefly, cultured cells were rinsed twice with D-PBS and then fixed for 5 s in ice-cold methanol. Then, the cells were washed with tap

water, stained with AP solution at 37° for 120 min, and washed again with running water. The number of AP⁺ colonies were manually inspected and counted.

3. Results

Figure 1 describes the experimental protocol used in this study. First, we evaluated the reprogramming capacity of unsorted ADSCs. As shown in Figure 2, parental ADSC showed no colonies and AP staining was negative (negative control). However, 4F transduction resulted in ADSC colony formation. After culturing for 30 days, the cells were stained with AP to test iPS cell pluripotency. AP staining was positive in all colonies. These results clearly demonstrated that murine ADSCs can be reprogrammed using standard 4F. Next we used sorted ADSCs to assess the reprogramming capacities of CD90^{Hi} and CD90^{Low} ADSCs. As shown in Figure 3(a), the CD90 marker was widely expressed on the surfaces of the CD31⁻CD45⁻CD34⁻ ADSCs. After 24 h of incubation, sorted CD90^{Hi} or CD90^{Lo} ADSCs became attached to the culture dishes (Figure 3(b)) and both showed similar morphologies. Next, we assessed the effect of *in vitro* culture. As shown in Figure 4(a), 7-day culture resulted in the reexpression of CD90 even after sorted CD90^{Lo} ADSCs. These results suggest that sorted cells might be transduced as soon as possible.

To show the successful induction of the undifferentiated state, we employed immunocytochemistry of Oct4

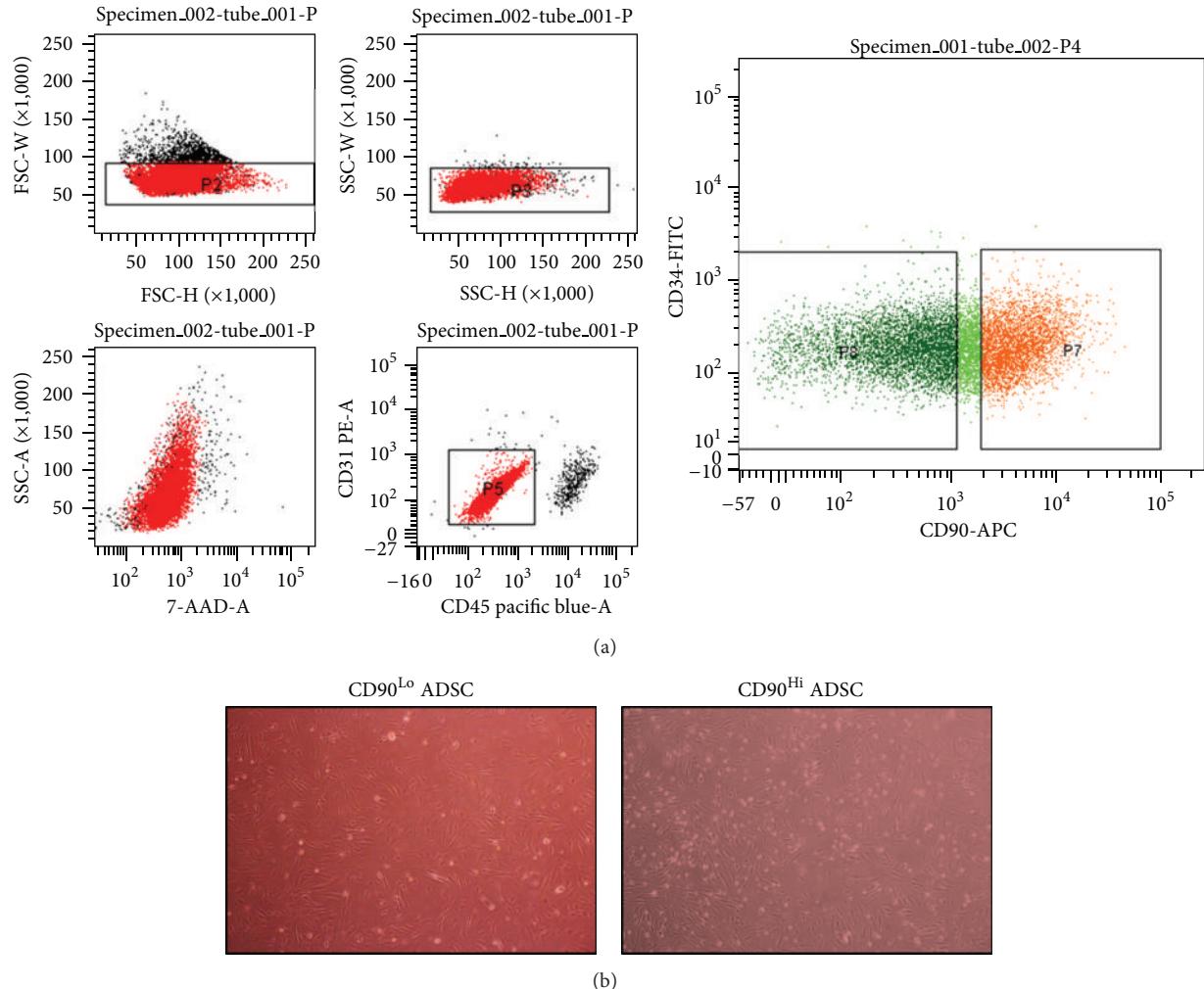


FIGURE 3: CD90^{Hi} and CD90^{Lo} sorting. (a) Gates for CD90^{Hi} (P7) and CD90^{Lo} (P8) are shown. (b) Sorted cells showed similar morphologies 24 h after sorting. Then, the cells were transduced with 4F.

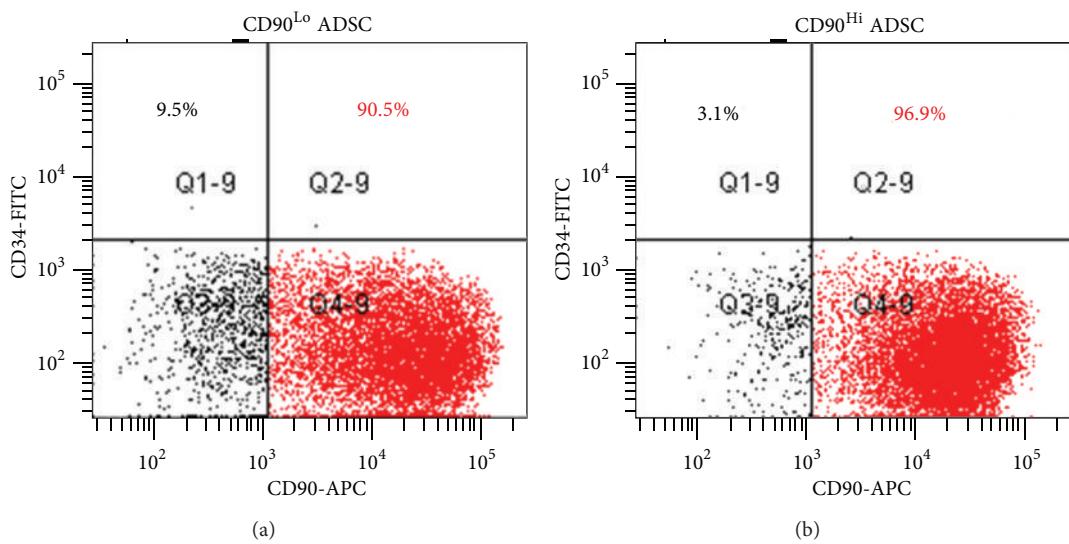


FIGURE 4: Phenotypical analysis after *in vitro* culture of sorted ADSCs.

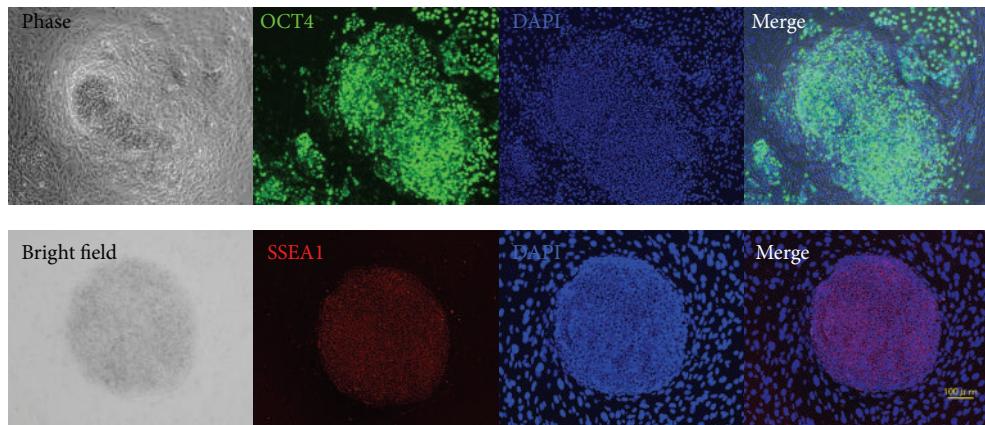
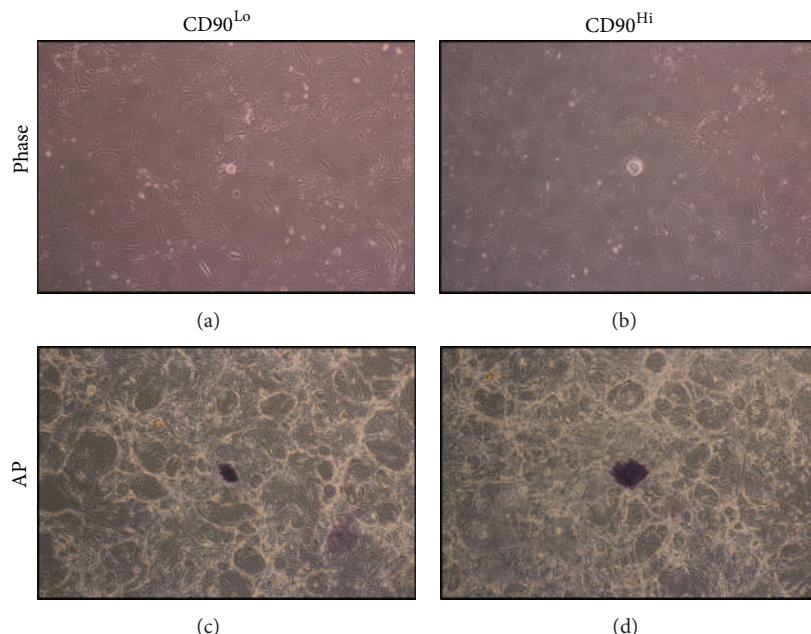


FIGURE 5: Immunocytochemistry of iPS colonies.

FIGURE 6: Colony formation of the sorted cells. Phase (a and b) and alkaline phosphatase staining (c and d) of CD90^{Lo} (a and c) and CD90^{Hi} (b and d) are shown.

and SSEA1. As shown in Figure 5, colonies were positive for Oct4 and SSEA1. Sorted and 4F-transduced ADSCs also exhibited colony formation. As shown in Figures 6(a) and 6(b), morphologically distinct colonies were visible in both CD90^{Hi} and CD90^{Lo} 4F-transduced ADSCs on posttransduction day 14. On posttransduction day 30, the colonies were stained with AP. There were AP+ colonies in both groups, although the CD90^{Hi} ADSCs tended to form larger colonies than CD90^{Lo} ADSCs (Figures 4(c) and 4(d)). As shown in Figure 7, CD90^{Hi} ADSCs exhibited more AP+ colonies than CD90^{Lo} ADSCs. The reprogramming efficiencies of unsorted, CD90^{Hi}-sorted, and CD90^{Lo}-sorted ADSCs were 100%, 116.5%, and 74.7%, respectively.

4. Discussion

In the present study, we demonstrated that murine ADSCs can be reprogrammed by standard 4F transduction. Furthermore, iPS cell formation was also observed in CD90-based sorted cells and the CD90^{Hi} sorting resulted in enhanced reprogramming capacity of murine ADSCs compared with CD90^{Lo} ADSCs with regard to colony number. Moreover, there was a trend in the association between CD90 expression level and individual colony size. These results clearly demonstrated that ADSCs have heterogeneous subpopulations and that the CD90^{Hi} ADSCs present favorable candidates for the application of clinical stem cell therapy.

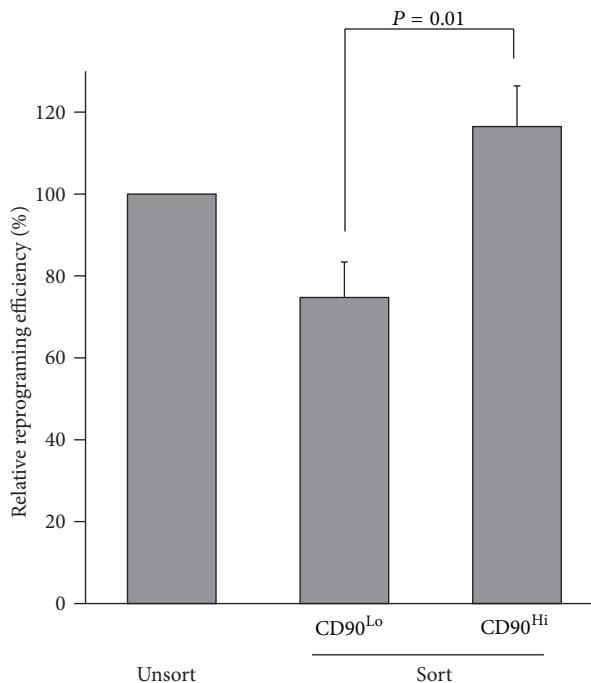


FIGURE 7: Relative reprogramming efficiency of sorted cells compared with unsorted controls.

The importance of CD90 in oncogenesis or properties of cancer stem cells has been reported [13]. CD90 expression reportedly has prognostic values in esophageal squamous cell carcinoma (ESCC) patients, because higher CD90 expression was significantly associated with a strong family history of ESCC and higher incidences of lymph node metastasis. However, the importance of CD90 expression in MSCs has not been fully elucidated; therefore, our next project is to clarify the mechanisms of CD90-mediated immunomodulatory effects. Of note, recent study reported that CD90-expressing niche stromal cells would support hematopoiesis [14].

Stem cell therapy for autoimmune disease is also well described. In a murine inflammatory bowel disease model, ADSCs alleviated experimental colitis by inhibiting inflammatory and autoimmune responses [15]. In mice, Thy-1 is also expressed by thymocytes, peripheral T cells, myoblasts, epidermal cells, and keratinocytes.

We and others previously reported that it is possible to reprogram murine and human cells to pluripotency by direct transfection of mature double-stranded microRNAs without viral vectors [16–18]. These viral-free strategies present effective methods for future stem cell therapy.

Currently, studies are underway to compare the immunoregulatory properties of viral-free strategies both *in vitro* and *in vivo*.

Acknowledgment

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and pCAG-VSV-G-RSV-Rev) were kindly provided by the Hiroyuki Miyoshi (RIKEN; The Institute of Physical and Chemical Research, Tsukuba, Japan).

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