Undifferentiated Stage	Cells	Advantages	Problems
Highly undifferentiated	ES	Pluripotent	Teratoma formation Ethical issue
	iPS	Pluripotent Autologous	Teratoma formation
	MSCs	Autologous	Heterogenicity Limited differentiation lineage
Less undifferentiated	HSP EPC	Autologous	Limited differentiation lineage

Scheme 1. Cell sources for regenerative medicine.

of the ES cells is a valuable feature, but at the same time they may differentiate into cells of unexpected types and may form teratomas after transplantation. Therefore, it is better to prepare target cells by inducing ES cell differentiation and to purify the cells thoroughly in vitro. In addition, since ES cell establishment is accompanied by the death of the fertilized human embryo, the use of this practice raises ethical issues.

2.2. Induced pluripotent stem cells (iPS cells)

Yamanaka et al. originally reported that human fibroblasts can be reprogrammed by transformation with four genes (Oct3/4, Sox2, Klf4, c-Myc), giving ES cell-like immature cells [4]. These pluripotent cells are called induced pluripotent stem cells (iPS cells) and have been studied worldwide. Recently, iPS preparation procedures that do not use the Myc gene, which is one of the oncogenes, and that use viral vectors for gene transfer have been reported [9]. Regarding cell transplantation, iPS cells possess the same teratoma formation issue as ES cells have, but unlike the case with ES cells, autologous iPS cells can be prepared from the patient's cells.

2.3. Mesenchymal stem cells (MSCs)

In 1999, Pittenger et al. reported the existence of MSCs that can differentiate into osteoblasts, chondrocytes, and adipocytes, in the adherent cell fractions of the bone marrow cells [2]. This research team's purification method is very easy and then even now is considered the gold standard for MSC preparation. However, only a few percent of the adherent cell fractions possess the differentiation capacity of the stem cells. Due to the plasticity of MSCs [2,10], they may differentiate into the other types of cells. Since MSCs do not form teratoma and can be easily isolated from the patient, the autologous cell transplantation have been studied widely in preclinical and clinical stages.

2.4. Hematopoietic stem cells (HSCs) and endothelial progenitor cell (EPCs)

In 1960, hematologist Ernest McCulloch and physicist James Till reported the existence of hematopoietic stem cells (HSCs) [11]. After that, Nakauchi's group and Ziegler's group identified mouse and human HSCs, respectively [12,13]. Because the surface marker of the HSC has been strictly identified, it is possible to purify them using a fluorescence activated cell sorter (FACS). There are two subpopulations of HSCs, called short-term HSCs and long-term HSCs. Long-term HSCs function for a long period of time as stem cells, while short-term HSCs do not. The separation of these two populations based on the cell surface marker is being studied [14,15]. In addition, in 1997, endothelial progenitor cells (EPCs) were discovered by Asahara et al. and identified as the precursor cells for forming blood vessels [16,17]. These less undifferentiated stem cells do not form teratomas.

3. Stem cell separation

One of the biggest issues in using stem cells is the establishing the technique for purifying and maintaining their undifferentiated state of stem cells. For highly efficient cell transplantation therapy, a stem cell population with high therapeutic efficacy must be prepared. There are two processes in stem cell separation. One is cell isolation from tissues, organs, blood, or bone marrow. The former requires that the bulk tissue be treated mechanically (homogenization) or enzymatically (digestion) to provide the cell suspension. These treatments can greatly affect the stem cell viability and functions. In contrast, MSCs or HSCs can be easily isolated from the peripheral blood or the bone marrow using a syringe. The other mode is to purify stem cells from cells with different phenotypes or at various differentiation stages.

Since bone marrow-derived MSCs are a useful source for cell transplantation, various methods for purifying the MSCs with expected functions have been developed, as described in Scheme 2. In general, the cell separation is based on the physical properties (size or density) or biological properties (surface markers) of the cells. Some methods allow separation of the stem cells in a continuous manner but others do not. We call the process performed by the latter type of system digital type cell separation. One example of digital type cell separation is the magnetic activated cell sorter system (MACS). The MACS system can divide the cells based on the marker molecule into two populations, the positive and negative populations. In contrast, continuous-type cell separation is the technique with which the weakly positive cells can be separated from the strongly positive cells in a continuous manner. The continuous-type cell separation method is not widely used. Dipole magnet flow fraction (DMFF) can separate the cells depending on how many magnetic beads are bound onto the cell surface, and then the continuous cell separation can be achieved. Since the stem cell surface marker expression is changing continuously depending on the differentiation stages, it is of prime importance to purify the stem cells in a continuous manner.

3.1. Cell sorting by the electric field

The FACS, which is also one of the continuous methods, identifies the target cells carrying the fluorescent probe-labeled antibody against the specific cell surface marker, and can sort the cells using the electric field. It is possible but complicated to sort the cells by using plural cell surface markers. Since the fluorescence intensity of each cell can be measured and picking up the cells with specified intensity by gating is possible, FACS is a very powerful tool for basic stem-cell research. Another method for continuous cell separation, based on detecting the cellular electronic features, has also been reported [18–20]. With this method, since the cells do not have to be labeled by an antibody or a chemical, the purified cells contain no contaminants.

3.2. Cell separation by the magnetic fields

In the MACS system, the magnetic bead-labeled antibody binds only to the target cell surfaces, and the labeled cells can be separated from the unlabeled cells [21–23]. The major advantage of the MACS system is its simple procedure. The cell sample is just mixed with the magnetic bead-labeled antibody against the target cell surface marker, and the target cells carrying the magnetic beads are easily separated from the negative cells using a magnet.

Cell property	Force field	System	Type
Physical Property	Gravity	Density gradient Ultracentrifugation	Digital
	Adhesion	Common Culture	Digital
	Electronic	DEP (Dielectrophoresis)	Continuous
Biological Property		FACS (Fluorescence Activated Cell Sorter)	Digital (Gating)
	Magnetic	DMFF (Dipole Magnet Flow Fraction)	Continuous
		MACS (Magnetic Activated Cell Sorter)	Digital
	Hydrodynamic	Cell Rolling Column	Continuous
		Cell Adhesion Membrane	Digital

Scheme 2. Cell separation systems.

To treat a large number of cells is also easy. Therefore, devices that can be used clinically have already been developed. Using this system, an effect of T cell removal on the graft versus host disease (GVHD) has been reported [24]. Recently, the continuous cell separation using the magnetic bead method under a continuously changing magnetic field has also been reported [25].

3.3. Separation using hydrodynamic force

Cells can be separated by hydrodynamic shear stress in a microfluidic system that includes interaction with the cells. Kato et al. reported the lineage-CD34 cell separation using polyethylene terephtalate filters [26]. Nagrath and Sequist succeeded in catching circulating cancer cells in the bloodstream by using a microchannel with 100-μm pillars modified with the specific antibody in the Circulating Tumor Cells (CTC)-chip diagnosis method [27]. An adhesion-based cell separation chip was also reported by Chang et al. [28]. In related work, our group has been studying a novel system for continuous separation of stem cells with different surface marker expression levels by using the dynamic interaction of the cell surface and a solid surface. In this system, the cells, which are weakly adhering to a solid surface via multiple specific interactions between cell surface marker molecules and the corresponding antibody, received the hydrodynamic force and rolled on the solid surface. Because the rolling speed is determined by the number of interactions as shown in Fig. 1, this system works as a continuous-type cell separation column. HSCs and EPCs are well defined and are useful for cell purification in the FACS system. In contrast, marker molecules for the MSCs whose undifferentiation tendency is much higher than that of HSCs and EPCs are still unclear, and the expression levels of the marker molecules seem to change as they differentiate. Studies have shown that the CD34 level of MSCs or HSCs is not stable [29,30].

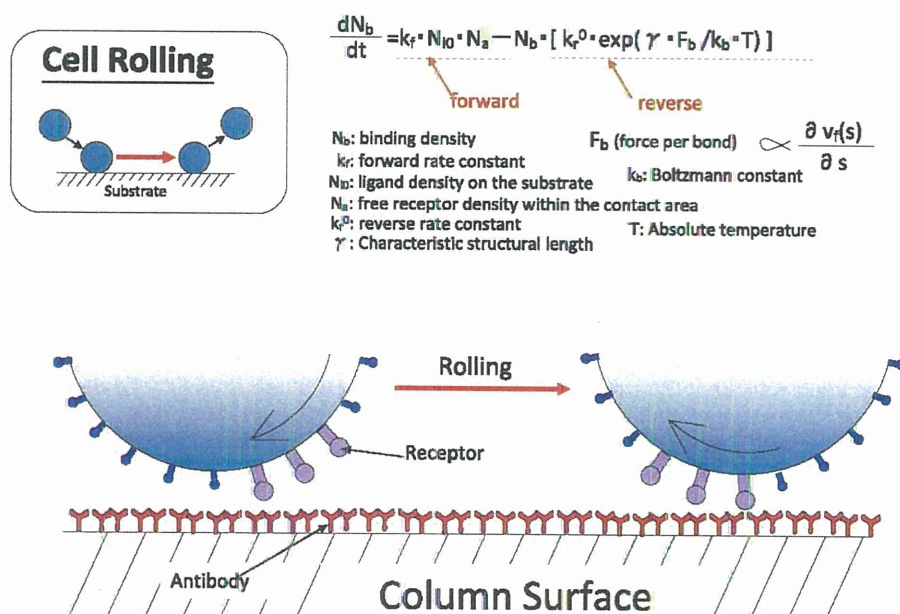

Fig. 1. Cell rolling on the antibody-immobilized solid surface.

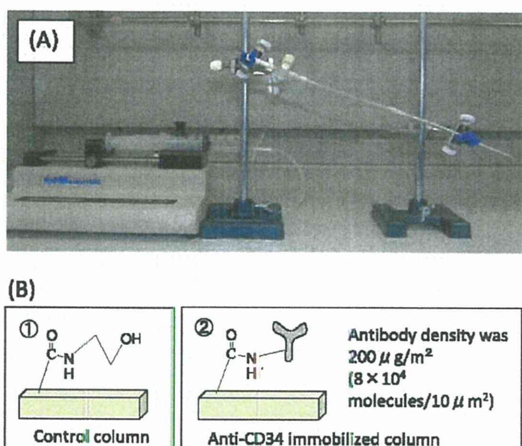

Fig. 2. (A) Appearance of the cell rolling column and (B) surface chemistry of the column lumen.

4. Development of a cell rolling column

We have recently developed an antibody-immobilized column that can separate MSCs on the basis of the CD34 marker expression level [31,32]. The separation mechanism is based on dynamic interaction between the cell surface marker (CD34) and an immobilized antibody and is known as cell rolling [33]. The rolling velocity is regulated by the ligand or cell surface receptor density [34–38]. Silicone tubes with 0.5-mm inner diameter were used as a substrate for the antibody-immobilized column. Graft polymerization of acrylic acid onto the silicone tube surface was conducted as follows [39,40]. The tube was treated with ozone gas for 4 h, dipped in 10% acrylic acid/methanol solution, and incubated at 60 °C. After 4 h, the tube was washed with water. To immobilize anti-CD34 antibody on the tube surface, we preactivated the poly(acrylic acid)-grafted tube with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSC), filled with the anti-mouse CD34 rat IgG antibody solution at a concentration of 10 μg/ml, and incubated the tube at 37 °C for 15 h. The tube was washed with phosphate buffered saline (PBS), treated with 1 mM 2-aminoethanol solution for 1 h, and preserved at 4 °C until experimental use. The column length was 10 cm, and the tilt angle was 20° (Fig. 2). A total of 2×10^4 crude MSCs in 10 μL of PBS was injected into the column. The column was flushed with PBS at a flow rate of 50 μL/min until the flow volume reached 250 μL, and at 600 μL/min thereafter. The eluted cell suspension was collected from the top of the column, and cell suspensions were fractionated by elution volume (12.5 μL per fraction).

The number and surface marker profile of cells in each fraction were analyzed by the FACS system (Fig. 3) [32]. The figure clearly shows that delayed fraction at the fraction numbers 8–10. We then used FACS to evaluate the surface marker expression of the isolated MSCs on the anti-CD34 antibody-immobilized column. MSCs with a high expression of CD34 and Sca-1 were presented in the retarded fractions, and a continuous change in the marker expression level was also confirmed on FACS, suggesting that the antibody-immobilized column could be useful to isolate MSCs continuously on the basis of their surface marker density.

The osteoblastic differentiation capacity of the MSCs in each fraction was evaluated by gene expression analysis (Type 1 collagen, osteonectin, CBFA1, and osteopontin) and alizarin red S staining. Type 1 collagen and osteonectin are constantly expressed during osteoblastic differentiation [41,42], while CBFA1 is expressed during the process of maturation. In the case of CBFA1, the expression level in fractions 3, 5, and 6 was higher than that in other fractions. After the differentiation induction, the MSCs were stained with alizarin red S solution, which is also shown in Fig. 3. Isolated MSCs in early fractions (fractions 2 and 3) or later fractions (fractions 5–7) were strongly positive. This staining pattern in terms of the fraction number was similar to that of the CBFA1 expression pattern. These results suggest that separated MSCs in early fractions or later fractions had a high potential for osteoblastic differentiation. It has been reported that osteoblastic progenitor cells were enriched in the CD34-positive population from bone marrow [43]. Our results clearly showed that there are two possibilities with regard to their origin. First, the osteoblastic progenitor cells in bone marrow were contaminated in culture dish-adherent cell fractions, and second, a fraction of MSCs differentiated into progenitor cells during the cultivation [44].

In nature, cell rolling is mainly observed in blood vessels as an inflammatory response of leukocytes [33], and its mechanism is derived from temporary interaction between the cell surface and ligands. Our separation technique would principally enable a labeling-free process, and the isolated cells would not be contaminated with fluorescent or magnetic-labeled antibody.

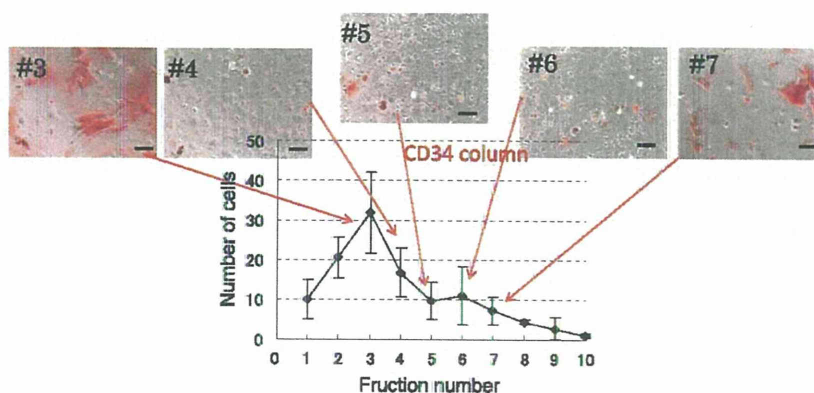

Fig. 3. Osteoblastic differentiation property of isolated MSCs on the CD34 antibody-immobilized column. Scale bar shows 10 μm.

5. Conclusions

An anti-CD34 antibody-immobilized column was developed for separating MSCs based on their surface maker density. We selected the anti-CD34 antibody as the immobilized ligand, and crude MSCs were separated on this column. We succeeded in separating two cell populations with a high ability for osteoblastic differentiation. Not only the cell separation technology but also the other novel technologies, including injectable scaffold, cell sheet technology, and cell tracking technology, will play important roles in the translational research of stem cell-based regenerative medicine in the future.

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