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Chapter 2

# Isolation of Bone Marrow Stromal Cells: Cellular Composition is Technique-Dependent

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## 1. Introduction

Bone marrow contains a colony-forming, fibroblast-like cell population called bone marrow mesenchymal stem cells or bone marrow stromal cells (BMSCs) [1, 2]. Since BMSCs are capable of differentiating into multiple lineages (osteogenic, chondrogenic, adipogenic, neurogenic, and myogenic lineages), they have attracted significant interest as useful somatic stem cells for use in tissue engineering and regenerative medicine [3 - 7]. As BMSCs adhere to tissue culture-treated plastic, they are usually isolated by adherent cultivation of untreated whole bone marrow [8 - 10]. However, this technique may be inefficient for the isolation of BMSCs because untreated bone marrow contains a large proportion of erythrocytes and their presence may interfere with the initial adherence of BMSCs. The removal of unwanted high density blood cells by density gradient centrifugation increases the number of colony-forming units (CFUs) in primary BMSC culture [11]. Removal of erythrocytes by hemolysis treatment is also effective at increasing the number of CFUs [12]. However, recent studies have shown that BMSCs isolated by these techniques are different from those isolated by adherent culture techniques [13]. Since BMSCs consist of a heterogeneous mixture of cells with varying potentials at different stages of differentiation, the characteristics of the cultured cells depend on the initial composition of the cell population [14, 15]. Therefore, the final cellular composition of BMSCs will vary significantly with the isolation technique used. Few studies have focused on the importance of the initial cellular composition of isolated BMSCs. In this chapter, possible differences in the cellular composition of BMSCs isolated from untreated, hemolysed, or density gradient fractionated bone marrow will be discussed. Furthermore, the optimal technique for the isolation of BMSCs for use in tissue engineering and regenerative medicine will be discussed from a clinical point of view.

## 2. Bone marrow stromal cells

BMSCs are a plastic-adherent, non-hematopoietic cell population residing in the bone marrow [16]. As BMSCs are morphologically similar to skin fibroblasts and can be expanded in a culture medium for fibroblasts, they were initially described as stromal fibroblasts [17], though their differentiation potentials are far different from those of skin fibroblasts [18]. While skin fibroblasts are incapable of differentiating into other cell types, BMSCs are capable of differentiating into cells of multiple mesenchymal tissues such as bone, cartilage, fat, tendon, muscle, and marrow stroma [19]. To emphasize this property, BMSCs are also called mesenchymal stem cells or multipotent mesenchymal stromal cells [20], though they can also differentiate into non-mesenchymal (non-mesodermal) cell types such as neurons [21] and insulin-producing cells [22]. Although BMSCs do not possess totipotencies like embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), they are clinically more useful than these totipotent stem cells because they can be easily isolated from a small volume of bone marrow aspirate and do not require gene transfections to demonstrate their differentiation abilities [23]. Thus, BMSCs have attracted significant interest as potent stem cells for use in tissue engineering and regenerative medicine of various tissues. In fact, clinical studies have shown that BMSCs are useful for the treatment of bone, cartilage, heart, and the central nervous system [24-27]. In addition, BMSCs recently attracted attention as immuno-modulatory cells useful for the treatment of immue diseases such as graft versus host disease (GVHD) [28, 29]. Therefore, clinical use of BMSCs should increase over the next few years.

## 3. Animal-derived BMSC as a model of human BMSC

BMSCs are present in the bone marrow of humans as well as other animals such as mice, rats, rabbits, dogs, pigs, sheeps, horses, and cows [4, 8, 30 - 35]. As BMSCs seem to be postnatal stem cells that are common among mammalian species, these animals have been used to investigate the origin and *in vivo* functions of BMSCs [36, 37]. In addition, these animal-derived BMSCs are considered useful as a models of human BMSCs because it is not always easy to recruit a sufficient number of human BMSC donors for experimental use. Furthermore, more reliable results can be obtained by using animal-derived BMSCs because experimental animals have uniform genetic backgrounds and are housed under controlled conditions, eliminating behavioral and environmental variations that could influence BMSC properties. In fact, several studies have reported that the characteristics of human BMSCs varied significantly among donors [15, 38, 39], while such variations are not observed in animal-derived BMSCs. Therefore, animal-derived BMSCs are considered to be useful alternatives to human BMSCs for laboratory experimentation. However, it remains unknown which animal's BMSCs offer the best model system to represent human BMSCs. In general, donor animals of BMSCs are chosen based on their costs and availabilities. However, it has been shown that there are a number of characteristic differences in the BMSCs among species [40]. Therefore, it is important to consider species difference in addition to the costs and availabilities when selecting model systems for human BMSCs.

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Considering their costs and availabilities, mice are more attractive candidates than other laboratory animals. However, rat BMSCs are used as a model of human BMSCs in our laboratory because mouse BMSC characteristics differ from those of human BMSCs. For example, mouse BMSCs need the support of feeder cells for their stable growth, while human BMSCs are able to grow in a feeder cell-independent manner [40]. Responses to differentiation stimuli are also different. While human BMSCs are readily induced to differentiate into the osteogenic lineage by dexamethasone, mouse BMSCs are less responsive to dexthamethasone treatment [41]. Although the reasons why mouse BMSCs differ from human BMSCs remain unknown, it has been suggested that mouse BMSCs are very rare in the bone marrow and need support by other cells for their growth and differentiation [40]. On the contrary, rat BMSCs can be easily isolated from bone marrow and they are able to grow without feeder cells, as do human BMSCs [13]. In addition, rat BMSCs are able to differentiate into multiple lineages under induction protocols used for human BMSCs [42]. Therefore, we believe that rat BMSCs offer a more appropriate model of human BMSCs, though fewer reagents and antibodies are available for rat cells than for mouse cells.

## 4. Isolation of BMSCs

Since BMSCs form adherent colonies in plastic culture vessels, BMSCs are generally obtained from adherent cultures of untreated whole bone marrow [2 - 4]. However, it has been suggested that this technique is inefficient for the isolation of BMSCs because untreated bone marrow contains a large proportion of erythrocytes and their presence may interfere with the initial colony formation of BMSCs [11 - 13]. As human BMSCs are a rare population in the bone marrow (0.01 - 0.1% of whole marrow), it is possible that the efficacy of initial colony formation directly affects the total yield of BMSCs. Inefficient colony formation may also lead to the reduced potentials of BMSCs because previous studies have shown that BMSCs lose their differentiation abilities depending on the duration of *ex vivo* culture [39]. Accordingly, it is important to investigate whether BMSCs can be more efficiently isolated by the removal of unwanted cells. Both density gradient centrifugation and hemolysis (red blood cell lysis) treatment remove erythrocytes for the efficient isolation of the mononuclear cell fraction of bone marrow. Although both these techniques were originally developed for the isolation of white blood cells such as lymphocytes, they can also be used for the isolation of BMSCs because they are contained within the mononuclear cell fraction. In fact, several studies have used either or both of these techniques for the isolation of BMSCs; they reported that BMSCs were more efficiently isolated by these techniques (Table 1) [12, 32, 34, 43].

However, it remains unknown whether BMSCs isolated by these techniques are identical to those isolated from untreated whole bone marrow because BMSCs are composed of heterogeneous cells with varying growth and differentiation potentials [15]. Thus, the cellular composition of BMSC populations could be dependent upon the isolation technique. Although it remains unknown how many different types of cells constitute the BMSC fraction, at least committed osteogenic cells as well as uncommitted stem cells are present when BMSCs are

| Target cells | Compared isolation techniques | The most efficient isolation technique | Reference |
|---|---|---|---|
| Human BMSCs | • Hemolysis (red blood cell lysis)<br>• Density gradient centrifugation<br>• Adherent culture of whole bone marrow | Hemolysis | [12] Horn *et al.*, 2008. |
| Pig BMSCs | • Hemolysis<br>• Dextran sedimentation<br>• Density gradient centrifugation | Hemolysis | [32] Peterbauer-Scherb *et al.*, 2010 |
| Rat BMSCs | • Density gradient centrifugation<br>• Adherent culture of whole bone marrow | Density gradient centrifugation | [43] Polisetti *et al.*, 2010 |
| Equine BMSCs | • Density gradient centrifugation<br>• Adherent culture of whole bone marrow | Density gradient centrifugation | [34] Bourzac et al., 2010 |

**Table 1.** Removal of erythrocytes by hemolysis or density gradient centrifugation may enable the efficient isolation of BMSCs.

isolated from untreated whole bone marrow [44]. Changes in the relative sizes of these two cell populations greatly influence the characteristics of BMSCs. In other words, a greater number of committed osteogenic cells makes the BMSC fraction more osteogenic, while a greater number of uncommitted stem cells makes them more stem-cell like. Thus, we investigated differences in the cellular composition of BMSCs isolated from untreated, density-gradient-centrifuged, and hemolysed bone marrow, with a special reference to committed osteogenic cells and uncommitted stem cells. For these experiments, rat bone marrow was used instead of human bone marrow to avoid the influence of variations among donors.

## 5. The number of committed osteogenic cells contained in BMSCs varies with the isolation technique

Committed osteogenic cells can be defined as a cell population that is capable of forming bone without osteogenic induction. Because of the presence of this cell population, *in vivo* transplantation of untreated whole bone marrow to ectopic sites usually results in the formation of new bone [45]. If this cell population is decreased or lost by the hemolysis or density gradient centrifugation steps, new bone formation may not be observed in the transplants. On the contrary, if this cell population is enriched by these techniques, more significant bone formation should be observed. Therefore, we investigated the *in vivo* bone-forming ability of three

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populations: marrow that was untreated, marrow that was hemolysed with ammonium chloride, and marrow that was fractionated by density-gradient-centrifugation over Ficoll® (Ficoll-treated). As shown in Figure 1A, the percentage of bone-forming transplants (transplants containing ectopic bone/ total transplants) was the lowest in the Ficoll-treated group. The amount of new bone formation, which was scored on a semi-quantitative scale from zero to three (Table 2), was also lowest in the Ficoll-treated group (Figure 1B). The hemolysed group also showed less bone-forming ability than did the untreated group, though its ability was still greater than that of the Ficoll-treated group (Figure 1 A and 1B).

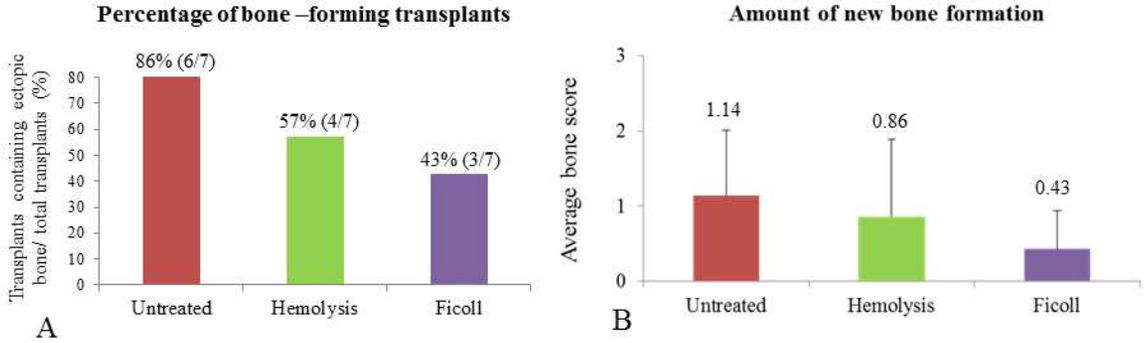


**Figure 1.** *In vivo* bone-forming ability of untreated, hemolysed, or Ficoll-treated bone marrow. (A) The percentage of bone-forming transplants (transplants containing ectopic bone/ total transplants), which was calculated from the results of seven independent experiments, was greatest in the untreated group, followed by the hemolyzed group, and lowest in the Ficoll-treated group. (B) The amount of new bone formation (total bone score/ total transplants) was greatest in the hemolyzed group, followed by the untreated group, and lowest in the Ficoll-treated group (n = 7). (Modified from Agata et al., 2012 [13] with permission)

| | **Bone score (Percentage of new bone area in the transplant)** |
|---|---|
| 0 | No bone evident |
| 1 | Bone area < 5% |
| 2 | 5% < Bone area < 10% |
| 3 | Bone area > 10% |

**Table 2.** Bone score of each sample was determined from the percentage of the area containing bone (new bone area/ total area) (Modified from Agata et al., 2012 [13] with permission)

As these results showed that Ficoll-treated bone marrow contains fewer committed osteogenic cells than either untreated or hemolysed bone marrow, we next investigated whether BMSCs isolated from Ficoll-treated bone marrow actually contains lower numbers of committed osteogenic cells. Untreated, hemolysed, or Ficoll-treated rat bone marrow was plated on cell culture dishes, and adherent colony-forming cells were expanded as BMSCs. Although these BMSCs did not show significant differences in their morphology or their expression of cell-surface CD54 and CD90 (Figure 2), they showed a significant difference in the expression of cell-surface alkaline phosphatase (ALP) (Figure 3A). The difference in ALP expression was also confirmed by quantitative ALP assays (Figure 3B).

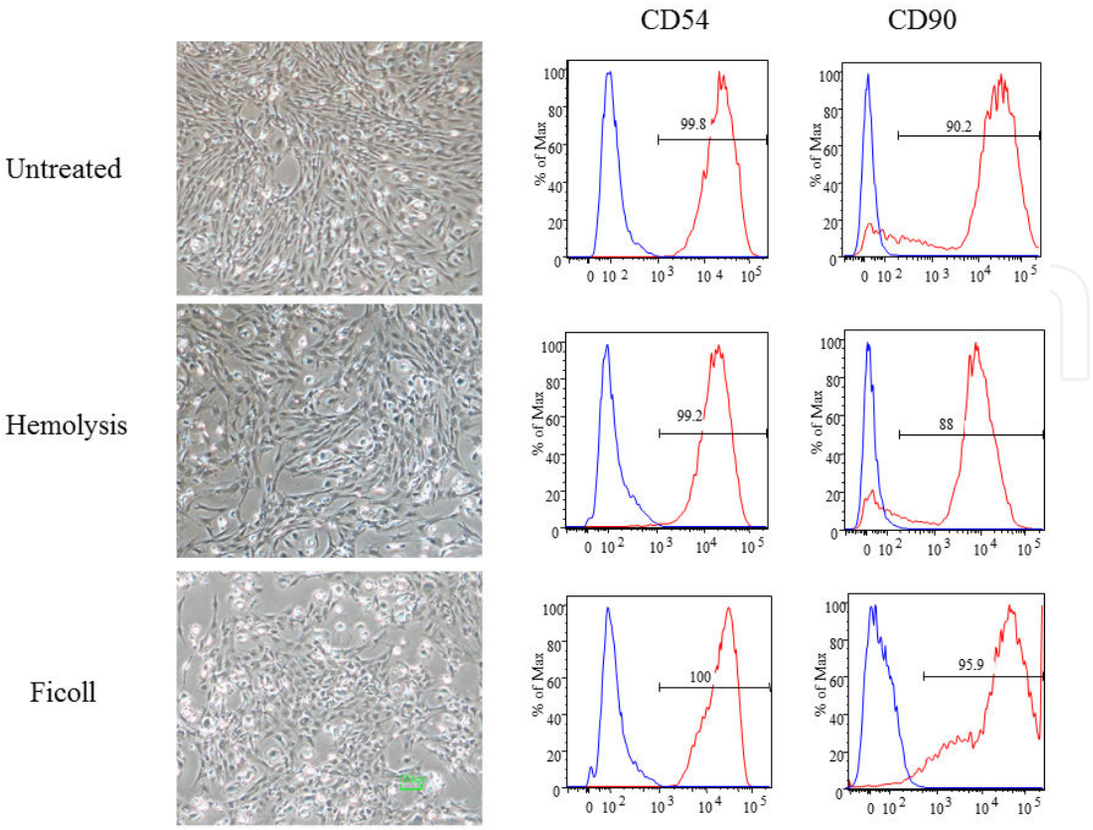


**Figure 2.** Morphology and expression of cell surface CD54 and CD90 of BMSCs that were isolated from untreated, hemolysed, or Ficoll-treated bone marrows. (Modified from Agata et al., 2012 [13] with permission)

Since these BMSCs were simply cultured in non-induction medium, the expression of cell surface ALP directly indicates the number of committed osteogenic cells contained in each BMSC. Therefore, it can be concluded that BMSCs isolated from Ficoll-treated bone marrow contain lower numbers of committed osteogenic cells than those isolated from untreated or hemolysed bone marrow.

## 6. The number of uncommitted stem cells contained in BMSCs also varies with the isolation technique

Although it remains unknown whether BMSCs contain committed progenitors of other lineages, their multi-lineage differentiation potentials are mainly attributed to the presence of uncommitted stem cells among heterogeneous BMSC populations. Therefore, it is important to investigate whether the number of uncommitted stem cells contained in BMSCs varies with the isolation techniques. Note, however, that it is difficult to calculate their numbers accurately because no specific markers for uncommitted stem cells are currently available. However, the abundance of these cells in BMSCs populations can be determined by analyzing the responsiveness to differentiation-inducing media (induction media), since uncommitted stem cells are highly responsive to differentiation stimuli. Thus, BMSCs that are rich in these cells show

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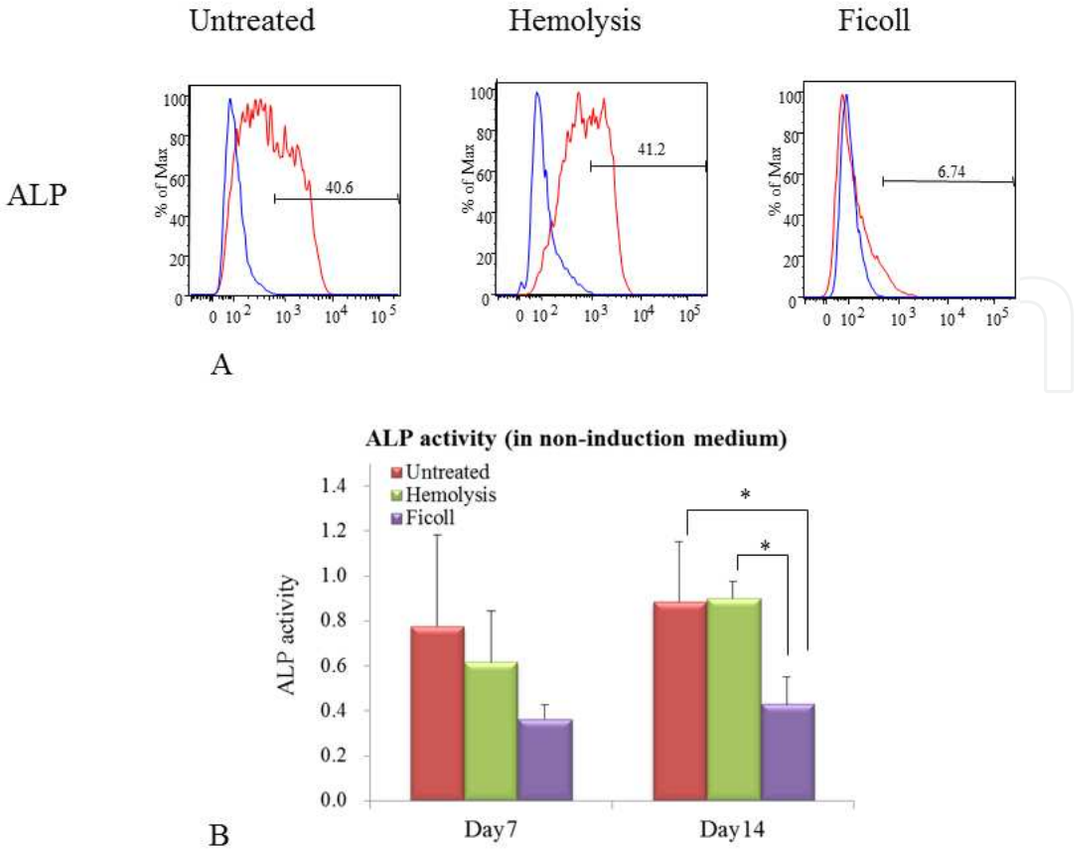


**Figure 3.** Differences among committed osteogenic cell populations from BMSCs isolated without treatment, or after hemolysis, or Ficoll separation. (A) The expression of cell surface alkaline phosphatase (ALP) of non-induced BMSCs was greatest in the untreated group, followed by the hemolyzed group, and lowest in the Ficoll-treated group. (B) Quantitative ALP assays confirmed the lowest ALP activity in the Ficoll-treated group. Data are presented as the means ± standard deviation (n = 3). *: $P < 0.05$. (Modified from Agata et al., 2012 [13] with permission).

great responsiveness when culture medium is changed from non-induction medium to induction medium. Accordingly, we investigated BMSCs isolated from untreated, hemolysed, or Ficoll-treated bone marrow for their responses to osteogenic induction medium. As shown in Figure 4A, the Ficoll-treated group showed the lowest ALP activity on day seven. However, this group significantly upregulated ALP activity and showed the greatest activity after 14 days of culture in osteogenic medium, though the difference did not reach a statistically significant level.

Since the Ficoll-treated group constantly showed the lowest ALP activity when cultured in non-induction medium (Figure 3B), the ratio of ALP upregulation (ALP activity in osteogenic induction medium/ ALP activity in non-induction medium) was also the greatest in this group. Gene expression analyses of osteopontin and core-binding factor subunit alpha-1 (*Cbfa1*), both of which are indicators of osteogenic differentiation, also showed the greatest responsiveness in the Ficoll-treated group (Figure 3B - 3E). These results indicate that BMSCs isolated from Ficoll-treated bone marrow contain greater numbers or higher concentrations of uncommitted stem cells than those isolated from untreated or hemolysed bone marrow.

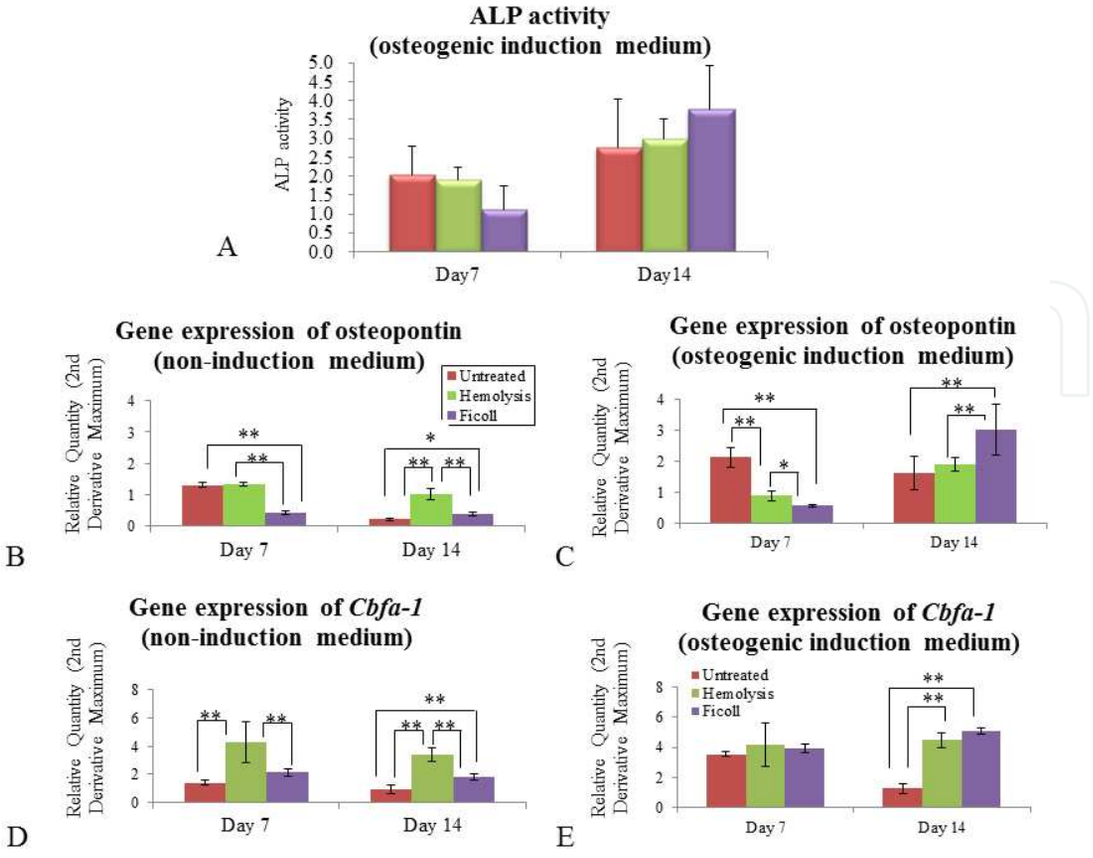


**Figure 4.** Differences in the responses to osteogenic induction medium among BMSCs isolated from untreated, hemolysed, or Ficoll-treated bone marrow. (A) ALP activities in osteogenic induction medium. (B) Gene expression of osteopontin in non-induction medium. (C) Gene expression of osteopontin in osteogenic induction medium. (D) Gene expression of *Cbfa-1* in non-induction medium. (E) Gene expression of *Cbfa-1* in osteogenic induction medium. Data are presented as the means ± standard deviation (n = 3). *: $P < 0.05$, **: $P < 0.01$. (Modified from Agata et al., 2012 [13] with permission)

## 7. Potential merits of hemolysis treatment or density gradient centrifugation of bone marrow to isolate BMSCs

Although hemolysis treatment of bone marrow with ammonium chloride primarily removes only erythrocytes from bone marrow, *in vivo* transplantation experiments indicated that some of the committed osteogenic cells contained in bone marrow are lost or damaged during the hemolysis treatment (Figure 1A and 1B). Thus, we hypothesized that BMSCs grown from hemolysed bone marrow might contain lower numbers of committed osteogenic cells and their cellular composition would differ from that of normal BMSCs (BMSCs grown from untreated bone marrow). However, contrary to the hypothesis, flow cytometric analyses revealed that these BMSCs contained equivalent numbers of committed osteogenic cells (Figure 3A). Since these BMSCs showed similar responses to osteogenic induction medium (Figure 4A), they seem to contain similar numbers of uncommitted stem cells as well. Therefore, it is likely that the cellular composition of BMSCs grown from hemolysed bone marrow is relatively close to that of normal BMSCs. As the cell yield in primary culture (harvested cell number after primary culture/ days of primary culture/ initially seeded cell number) was greater in the hemolysis

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group (0.52 in the hemolysed group and 0.44 in the untreated group), it can be concluded that hemolysis treatment of bone marrow is an efficient approach to the isolation of BMSCs.

After centrifugation over Ficoll®, bone marrow is separated into several fractions such as plasma, mononuclear cells, granulocytes, and erythrocytes. Since BMSCs belong to the mononuclear cell fraction in the bone marrow, it is likely that BMSCs are efficiently enriched in this fraction even though this isolate contains significantly lower cell numbers than untreated or hemolysed bone marrow (Figure 5).

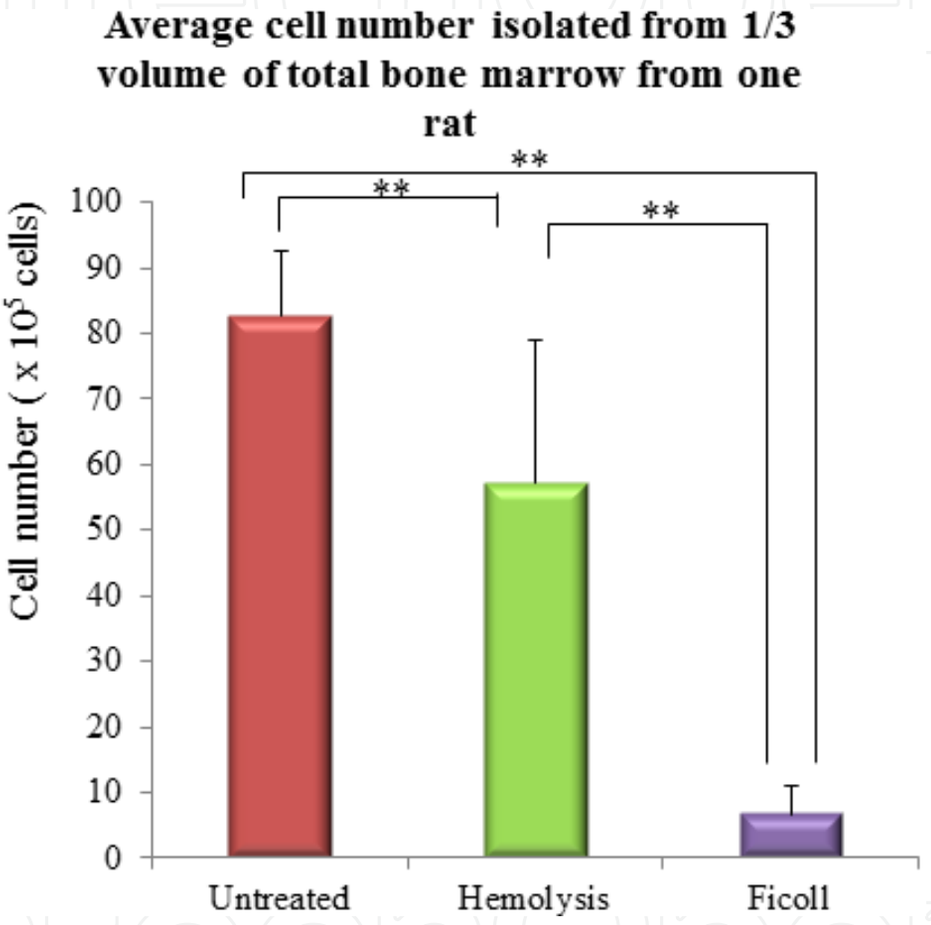


**Figure 5.** Rat bone marrow was divided into three portions and the suspensions were either hemolyzed, or subjected to Ficoll fractionation, or left without treatment (untreated). Significant differences were observed in the average numbers of cells isolated among the groups. Data are presented as the mean ± standard deviation (n=6). **: $p < 0.01$ (Modified from Agata et al., 2012 [13] with permission).

However, in contrast to expectations, the cell yield in primary culture was the lowest in this group (0.13 in the Ficoll-treated group). In addition, the cellular composition of this group's BMSCs seemed to be different from that of normal BMSCs, because these BMSCs showed significant differences in the percentage of cell-surface ALP-positive cells and the responses to osteogenic induction medium (Figure 3A and 4A), though they showed similarities in the morphologies and the expression of cell-surface CD54 and CD90 (Figure 2). Therefore, it can be concluded that density gradient centrifugation of bone marrow is not an efficient approach to the isolation of BMSCs that possess normal characteristics. However, this technique may be

useful for the isolation of more potent (more primitive) BMSCs because BMSCs grown from Ficoll-treated bone marrow seem to contain greater numbers or higher concentrations of uncommitted stem cells.

## 8. Conclusion

As the cellular composition of BMSCs varies significantly with the isolation technique, it is important to select an appropriate isolation technique for the purpose that is intended. For example, if BMSCs are used for bone tissue engineering, it might be better to isolate BMSCs by hemolysis, because BMSCs that contain greater numbers of committed osteogenic cells are efficiently obtained by this technique. On the contrary, if BMSCs are used for the stem cell therapies of non-bone diseases such as stroke, it might be better to isolate BMSCs by density gradient centrifugation, because BMSCs obtained by this technique contain greater numbers of uncommitted stem cells. Flow cytometric or magnetic cell sorting with antibodies might also be useful for the isolation of BMSCs for use in stem cell therapies because BMSCs isolated by this technique possess greater multi-lineage potency. However, most of the current clinical studies still use the conventional adherence technique for the isolation of BMSCs because the fact that the characteristics of BMSCs varies with the isolation techniques remains largely unknown. Since the results of clinical studies are greatly affected by the potentials of the BMSCs used, selection of an appropriate isolation technique may lead to a better outcome. Nonetheless, further investigations are required to use these new techniques in clinical studies because available information concerning the safety, feasibility, and efficacy of these techniques is still limited. Furthermore, the cost effectiveness of these techniques should be investigated, since the conventional technique does not require any special reagents. Continuing investigations are important for the establishment of truly reliable new therapies using BMSCs.

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