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研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Chapter 33

Heterogeneous Responses of Human Bone Marrow Stromal Cells (Multipotent Mesenchymal Stromal Cells) to Osteogenic Induction

Hideaki Kagami, Hideki Agata, Yoshinori Sumita, and Arinobu Tojo

Abstract Tissue engineering is a novel technology developed for the regeneration of tissue using cultured cells, scaffolds, and osteogenic inductive signals. Bone marrow stromal cells (also designated as multipotent mesenchymal stromal cells, mesenchymal stem cells, or MSCs) have been the most commonly used cell source for bone tissue engineering. For efficient bone tissue engineering, the cells must be expanded in vitro and induced into osteogenic cells with an osteoinductive reagent such as dexamethasone. Recently, physiological factors such as bone morphogenetic proteins have been shown to induce the osteogenic lineage of bone marrow stromal cells. Osteogenic reagents have been widely used in both basic and clinical studies. However, it is apparent that the cellular responses to those reagents have been heterogeneous in human cells compared with animal cells, which possess a more uniform genetic background. Since the clinical use of those factors will increase further in the cases of orthopaedic applications and in the context of tissue engineering, these responses could be a serious problem in the future. In this chapter, the heterogeneous response of human bone marrow stromal cells to those inductive factors is discussed with reference to possible underlying mechanisms.

Keywords Tissue engineering · Mesenchymal stem cells · Osteogenic reagents · BMSCs · Dexamethasone · Chondrogenesis · TGF-β

H. Kagami (✉)
Tissue Engineering Research Group, Division of Molecular Therapy, The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
e-mail: kagami@ims.u-tokyo.ac.jp

M.A. Hayat (ed.), *Stem Cells and Cancer Stem Cells, Volume 2*,
DOI 10.1007/978-94-007-2016-9_33, © Springer Science+Business Media B.V. 2011

Introduction

In the field of regenerative medicine, the use of biological materials in place of artificial substrates and chemical reagents is gaining acceptance. Growth factors are now in the pharmaceutical market and have attracted much attention. However, one of the ultimate biological materials is cells that can supply a variety of biological reagents such as growth factors and cytokines. More importantly, cells can produce matrices, may repair and moderate cell functions and play important roles during tissue regeneration.

Cultured cells have been used to treat various diseases including severe burns, joint cartilage degeneration, and bone defects. Since the cells can be expanded in vitro, those treatments require only a small amount of donor tissue and even autologous transplantation is feasible. Accordingly, this concept is considered a future therapeutic option in the treatment of various pathologic conditions. Tissue engineering, one of the most well recognized technologies, focuses on the regeneration of lost or damaged tissues using cultured cells, biodegradable scaffold materials, and biological factors. The initial clinical application of cultured cells was the skin substitute for severe burn cases. Subsequently, cultured cells from cartilage have been applied to repair cartilage defects of arthritis patients. Those cells were committed to specific lineages such as keratinocytes or chondrocytes. More recently, the presence of multipotent stem/progenitor cells in adults has been reported. The potential of those stem/progenitor cells for tissue engineering has been explored, since those cells possess higher proliferating capability and can differentiate into various cell lineages.

In terms of tissue engineering, one of the most important differences between committed (differentiating/differentiated) cells and multipotent (undifferentiated) stem/progenitor cells is the requirement for induction during cell culture. Multipotent stem/progenitor cells usually require induced differentiation to a specific lineage to regenerate a specific tissue. On the other hand, committed cells only require expansion prior to transplantation. Although cultured cells have been used clinically for more than 20 years, clinical application of multipotent stem/progenitor cells has a shorter history and information is still limited. Bone marrow stromal cells (BMSCs) are one of the most widely used cell types for this purpose. Although the results from preliminary clinical studies using BMSCs show the usefulness of this population, the heterogeneity of the population is a shortcoming (Phinney et al., 1999; Mendes et al., 2002; Mizuno et al., 2010). The heterogeneity of human BMSCs was much broader than that of animal cells and may affect the efficacy of the treatment.

In this chapter, we focus on BMSCs and their heterogeneity, which was noted from basic and clinical studies using human cells. In particular, we focus on the response to osteogenic induction using dexamethasone and BMP-2 with reference to possible underlying mechanisms.

Bone Marrow Stromal Cells (BMSCs)

BMSCs are fibroblast-like cells that can be cultured as an adherent cell fraction from bone marrow aspirates (Friedenstein et al., 1970). BMSCs possess high proliferating potential and include osteogenic stem/progenitor cells. Since BMSCs differentiate into various mesenchymal tissues, BMSCs have also been designated as multipotent mesenchymal stromal cells, mesenchymal stem cells or simply MSCs. Although BMSCs likely contain multipotent stem/progenitor cells, the population is heterogeneous. In fact, only a small portion of cells can form a secondary (osteogenic) colony (Sacchetti et al., 2007) and self-renewal capability, an essential criterion for stem cells, is difficult to confirm. Accordingly, we prefer to use the term "bone marrow stromal cells" (BMSCs).

Although various specific markers for BMSCs have been suggested, there is still no established marker to define BMSCs. The minimum criteria for MSCs were proposed by The International Society for Cellular Therapy as follows: positive for CD105, CD73 and CD90 and negative for CD34, CD45, CD11a, CD19, and HLA-DR (Dominici et al., 2006). More recent studies suggested that MCAM/CD146$^+$, CD271, mesenchymal stem cell antigen-1 (MSCA-1), CD56, SSEA-4, STRO-1, and platelet-derived growth factor receptor-beta (PDGF-RB; CD140b) might be used to enrich the stem/progenitor cells in culture (reviewed by Salem and Thiemermann, 2010).

Osteogenic Induction of BMSCs for Bone Tissue Engineering

As stated above, one of the major differences between committed (or relatively differentiated) and multipotent (or less differentiated) stem/progenitor cells is the requirement for induction. However, cultures of multipotent stem/progenitor cells such as BMSCs contain some differentiated cells (Mendes et al., 2002; Mizuno et al., 2010). Accordingly, the induction process is not always mandatory for bone regeneration, but considered favorable, especially in cases of BMSCs with relatively low osteogenic ability (e.g., elderly patients) (Mendes et al., 2002). Furthermore, the ability of BMSCs to differentiate into osteoblast-like cells diminishes during culture and passage (Agata et al., 2010). Thus, osteogenic induction might be important to increase the probability of in vivo bone formation. The steroid dexamethasone has been widely used for osteogenic induction for human and most other mammalian BMSCs. More recently, members of the TGF-super family, the bone morphogenetic proteins (BMP), have been used as potent inducers of osteogenesis.

Effect of Dexamethasone on Human BMSCs

Glucocorticoids, small lipophilic hormones that are secreted from the adrenal gland, are important regulators of various physiological functions such as carbohydrate and lipid metabolism, immune function and stress responses in mammals. Because of their strong

anti-inflammatory and immunosuppressive properties, synthetic glucocorticoids such as dexamethasone have been widely used as therapeutic reagents for a variety of diseases (McCulloch and Tenenbaum, 1986; Harrison et al., 2002). However, excessive exposure to glucocorticoids, such as long-term usage of dexamethasone or Cushing's syndrome (hypercorticism) results in the disruption of physiological functions and may lead to osteoporosis (McCulloch and Tenenbaum, 1986; Harrison et al., 2002; Tamura et al., 2004). Accordingly, the inhibitory effects of glucocorticoids on bone formation have been investigated for more than 40 years (Birkenhäger et al., 1967).

It has been shown that glucocorticoids have bimodal effects on bone formation (Harrison et al., 2002). The pharmacological dose ($>10^{-6}$ M) of glucocorticoid suppresses the generation and survival of osteoblasts, while physiological doses (10^{-8} to 10^{-7} M) selectively stimulate proliferation and differentiation of osteoprogenitors, suggesting that the effect of glucocorticoid on bone formation is dose- and target-specific (McCulloch and Tenenbaum, 1986; Weinstein et al., 1998; Harrison et al., 2002). To support this interpretation, previous studies have shown that the physiological dose of dexamethasone can efficiently induce osteogenic differentiation of human and other mammalian BMSCs (Kadiyala et al., 1997; Diefenderfer et al., 2003; Osyczka et al., 2004), while a similar dose suppresses the activities of mature osteoblasts (Harrison et al., 2002). For these reasons, physiological doses of dexamethasone treatment have become the current gold standard for the induction of osteogenic differentiation of human BMSCs (Phinney et al., 1999; Siddappa et al., 2007; Agata et al., 2010).

Alkaline phosphatase (ALP) activity is an early marker for osteogenic differentiation and is required for the initiation of matrix mineralization (Fedde et al., 1999). Accordingly, ALP activity analysis is frequently performed to investigate the osteogenic ability of BMSCs. When non-human BMSCs are exposed to a physiological dose of dexamethasone, they differentiate into the osteogenic lineage with elevated levels of ALP activity (McCulloch and Tenenbaum, 1986; Kadiyala et al., 1997; Aubin, 1999). Human BMSCs are also responsive to dexamethasone. The results from our own experiments showed that the levels of ALP activity increased among all five volunteer donors after exposure to dexamethasone (Fig. 33.1a, b). However, it is noteworthy that huge differences in the basal levels were already present among the donors (ALP activity of non-induced cells). Similarly, the ALP activity levels in induced BMSCs (after exposure to dexamethasone) also showed significant variations (Fig. 33.1a, b). Consequently, these variations led to differences in average ALP activity between induced and non-induced cells, which failed to achieve statistical significance (Fig. 33.1c). Similarly, several groups reported that the responses of human BMSCs to dexamethasone varied significantly among donors (Phinney et al., 1999; Siddappa et al., 2007). On the other hand, it may not be true in non-human BMSCs, such as rat BMSCs, which show relatively consistent responses to dexamethasone regardless of the origin of donor animals (Diefenderfer et al., 2003; Osyczka et al., 2004). Thus, significant donor variation in dexamethasone-responsiveness might be a specific problem for human BMSCs. Therefore, it is quite important to take the influence of donor variation into account when evaluating the osteogenic ability of human BMSCs when using dexamethasone. As suggested elsewhere (Siddappa et al., 2007), compensation for donor variations in basal ALP activity by calculating the rising ratio of ALP activity (ALP activity of induced/non-induced cells) might be a better approach to evaluate the osteogenic ability of human BMSCs.

It has been suggested that human BMSCs are composed of a heterogeneous mixture of cells at various stages of differentiation and that the proportions of non-osteogenic, osteoprogenitors, and committed osteogenic cells vary significantly among donors (Phinney et al., 1999). At least two classes of osteoprogenitor cells are present in BMSC populations: those differentiating without glucocorticoid and those requiring glucocorticoid to differentiate (Aubin, 1999). Thus, there may be differences in the proportions of dexamethasone-responsive osteoprogenitors in human BMSC populations among donors. These differences might explain the significant donor variations in dexamethasone-responsiveness of human BMSCs. To better understand the heterogeneous responses of human BMSCs to dexamethasone, specific markers of dexamethasone-responsive osteoprogenitors and differences in the proportion of dexamethasone-responsive osteoprogenitors among donors should be further investigated.

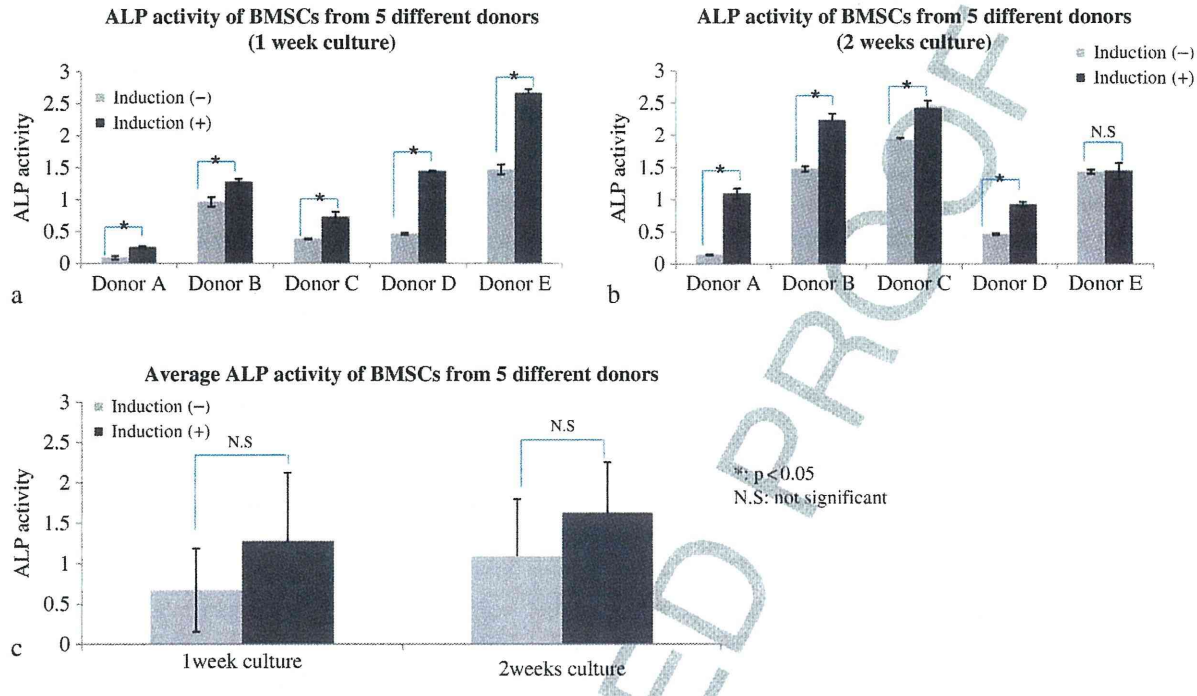


Fig. 33.1 Heterogeneous responses of human BMSCs to dexamethasone. Each graph shows the ALP activity of human BMSCs from five different donors after 1 or 2 weeks of osteogenic induction with 10 nM dexamethasone, 100 μM ascorbic acid, and 10 mM β-glycerophosphate (**a**, **b**). Control represents the results from hMSCs without induction. Graph (**c**) shows the average ALP activity of human BMSCs after 1 or 2 weeks of osteogenic induction, which were compared with that of the control without induction. $^{*}p < 0.05$

Effect of Recombinant Bone Morphogenetic Protein-2 on Human Bone Marrow Stromal Cells

Bone morphogenetic proteins (BMPs) constitute a group of conserved signaling molecules, which belong to the transforming growth factor-β (TGF-β) superfamily. BMPs were originally identified by their capacity to induce ectopic bone formation, which naturally exists within the bone matrix (Urist, 1965). Subsequent studies have shown that BMPs have a variety of functions as pleiotropic regulators for chemotaxis, mitosis, differentiation, stimulation of extracellular matrix synthesis, binding to matrix components, maintenance of phenotype, and apoptosis. The general role of BMPs in the process of bone formation during the development, regulation of bone volume and repair of fractures has been well established (Reddi, 1998). However, only selected BMPs can induce bone formation in ectopic sites.

Although more than 20 BMPs have been discovered, only BMP-2, -4, -6, -7, and -9 have been proven capable of driving multipotent cells into osteoblastic phenotypes in culture (De Biase and Capanna, 2005). Among them, BMP-2 is considered the most potent osteoinductive agent in the TGF-β superfamily. Since the expression of BMP-2 is correlated with the differentiation of osteoblasts and chondroblasts from mesenchymal stem cells, it is considered a strong inducer of bone formation and chondrogenesis (Reddi, 1998). However, the exact cellular and molecular mechanisms of BMP-2 are not fully understood.

The osteoinductive property of BMP-2 has been clearly shown with animal cells (Chen et al., 2002). BMP-2 target genes include several homeodomain proteins, the bone-related runt homology domain factor *RUNX2* (*CBFA1/AML3*), and an SP1 family member, *OSTERIX*, which may co-operatively work to promote cell differentiation into osteoblasts (Li et al., 2011). When rodent BMSCs were treated with BMP-2, they committed to the osteogenic lineage, produced bone

matrix proteins and expressed ALP (Reilly et al., 2007; Difenderfer et al., 2003). For human BMSCs, BMP-2 upregulates bone matrix proteins and mineralization, and enhances dexamethasone-induced osteogenic differentiation (Lecanda et al., 1997). However, the efficacy of rhBMP-2 on human BMSCs might be less consistent than that observed with rodent cells and limited and/or conflicting effects have also been reported (Diefenderfer et al., 2003; Osyczka et al., 2004; Reilly et al., 2007; Mizuno et al., 2010) (Fig. 33.2). Among donors from more than a dozen patients, only the cells from one donor showed significantly elevated alkaline phosphatase activity after exposure to BMP-2 (Difenderfer et al., 2003). Interestingly, the responsiveness was partially affected by the choice of serum. When five different sera were used for cultivation and induction with rhBMP-2, ALP activities increased in two of them, but not in the others (Mizuno et al., 2010). Although the reason for those controversial results is not clear, it may reflect the heterogeneous responsiveness of human BMSCs to rhBMP-2.

The in vivo efficacy of rhBMP-2 was also evaluated using animal models. RhBMP-2-coated natural bone mineral (NBM) accelerates regeneration in a rat calvarial defect model (Schwarz et al., 2009). When rhBMP-2 was applied to critical sized craniotomy defects in rhesus macaque, it facilitated the osseointegration of rectangular bone flaps. After 6 months, the BMP-2-treated craniotomy defects were on average 71% covered with calcified material versus an average of 28% coverage in empty control defects (Sheehan et al., 2003). Thus, BMP-2 has been shown as a strong osteogenic inducer in vivo. In clinical studies, rhBMP-2 combined with allograft dowels increased the rate of interbody fusion in patients who have undergone anterior lumbar fusion surgery (Burkus et al., 2003). The addition of rhBMP-2 to the treatment of type-III open tibial fractures reduced the frequency of bone-grafting procedures and other secondary interventions (Swiontkowski et al., 2006). Currently, rhBMP-2 is commercially available as an osteoinductive material (Infuse, Medtronic, Sofamar Danek, TN, USA). Although most of the results from clinical studies showed the usefulness of rhBMP-2, high doses of the factor are required for in vivo efficacy. Some researchers reported that the efficacy in human study

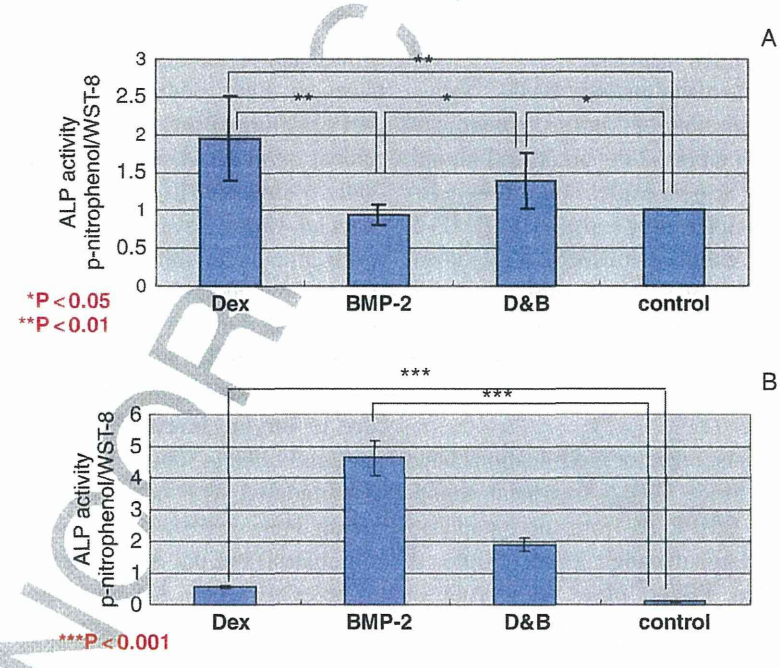


Fig. 33.2 Effects of osteogenic induction media on ALP activities of human and murine MSCs. Graph (**a**) shows the average ALP activity from six human samples after 1 week of osteogenic induction with Dex (D), BMP-2 (B), or D + B. Control represents the results from hMSCs without induction. Graph (**b**) shows the average ALP activity from mouse MSCs after 1 week of osteogenic induction with D, B, or D + B, which were compared with that of the control without induction. In hMSCs, Dex significantly upregulated ALP activity. On the other hand, BMP-2 showed no effect and even reduced the ALP activity of hMSCs with Dex. In mouse cells, BMP-2 significantly upregulated ALP activity (From Mizuno et al., 2010 with permission)

was less remarkable than that from animal studies, which may also imply species differences (Kwong and Harris, 2008).

Factors that Might Affect Responsiveness to Recombinant Bone Morphogenic Protein-2

Although the results from previous studies on the responsiveness of human MSCs to rhBMP-2 were variable, the reasons for these discrepancies are not well understood. Several studies including our own have tried to show underlying mechanisms for the lack of responsiveness.

It was possible that differences in the use of BMP receptors affected the downstream actions of BMP signaling. Although rat BMSCs and differentiated human osteoblasts express mRNA for one of the type I BMP receptors (ALK-6), human BMSCs lack this BMP receptor (Osyczka et al., 2004). However, forced expression of this receptor did not enhance ALP activity. This result suggests that the lack of ALK-6 is not a major reason for the limited response.

Other possible mechanisms include BMP-2 antagonists, since the expression of various antagonists against BMPs has been reported in animal and human cells. It was shown that *noggin* expression was upregulated in most of the samples after exposure to BMP-2, thus the application of exogenous BMP-2 may induce *noggin* expression in hMSCs in a serum-containing environment (Diefenderfer et al., 2003; Mizuno et al., 2010). Although the effect of noggin is plausible, the effects of other antagonists such as follistatin and chordin were not clear.

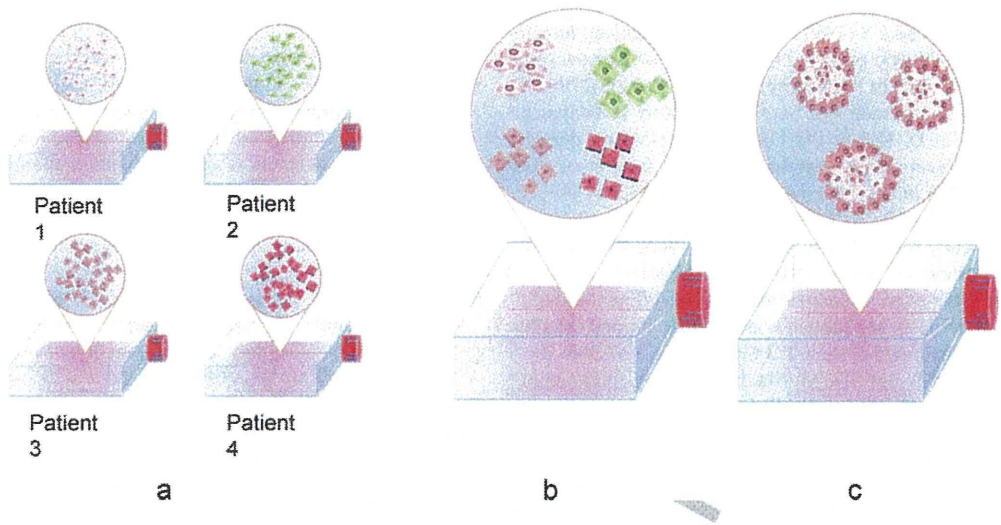
Other than antagonists, receptor modifications have also been suggested. Since TGF-β treatment causes rapid translocation of BMPR-IB from the cytoplasm to the cell surface (Singhatanadgit et al., 2008), it is possible that serum TGF-β plays some role in the responsiveness of human MSCs to rhBMP-2. It has been reported that BMP activates ERK signaling, which in turn decrease nuclear translocation of BMP-activated Smads, thus affecting the responsiveness of MSCs to BMP-2 (Osyczka and Leboy, 2005). However, the results from experiments using the same ERK inhibitor were not consistent and may require further clarification (Mizuno et al., 2010). So far, a simple explanation of the heterogeneous response of human BMSCs to rhBMP-2 is not available. The responsiveness might be determined as a balance of positive stimuli (rhBMP-2) and inhibitory factors, which may include some unknown mechanisms.

Heterogenicity of Human Cells for Therapeutic Use

Interestingly, the heterogenic response to osteogenic induction was observed not only for rhBMP-2 but also for dexamethasone. Since BMP receptors are located on the cell membrane while glucocorticoid receptors are located in the nucleus, the major reason for this heterogeneous responsiveness might not be environmental factors but may depend on the cells themselves.

There is no doubt that human cells are more heterogeneous than those from laboratory animals since various factors (age, gender, general condition, and also genetic background) could affect the properties of BMSCs (Phinney et al., 1999) (Fig. 33.3a). As mentioned above, cultured BMSCs vary in their responsiveness. First, BMSCs are not a uniform population but a mixture of various types of cells. The results from clonal analyses showed that not all BMSC-colonies were osteogenic (Kuznetsov et al., 1997) (Fig. 33.3b). Second, various levels of differentiation were observed even in a single culture flask (or even in one colony) (Ylöstalo et al., 2008) (Fig. 33.3c). Since flow cytometry showed relatively uniform cell surface marker expression by BMSCs, this second hypothesis is more likely and the heterogeneous responsiveness may be due to varying levels of differentiation (stemness) of cultured cells. In support of this interpretation, rhBMP-2 stimulated ALP activities in undifferentiated cells (fresh bone marrow cells and colony-forming units fibroblasts) but not in differentiated osteoblastic cells (Kim et al., 1997). Although the detailed mechanisms require further investigation, those characteristic features of BMSCs as well as human cells should be kept in mind when clinical use of these somatic cells or growth factors are planned.

Preliminary clinical studies have shown the usefulness of somatic cells such as BMSCs for various diseases including bone tissue engineering. It will be



AQ1 **Fig. 33.3** Schematic illustration of the potential heterogeneity in human BMSCs. Various factors (age, gender, general condition and also genetic background) differ among individuals, which should affect the heterogeneity of BMSCs from donors (**a**). The nature of BMSC culture is heterogeneous and BMSC culture consists of a mixture of various types of cells. It is noteworthy that not all BMSC-colonies are osteogenic (**b**). Even in one culture flask (and in one colony), various levels of differentiation can be observed, which may explain another type of heterogeneity (**c**)

important to understand the mechanisms of heterogeneous response, which may contribute to the further development of bone tissue engineering as well as clinical use of BMP-2.

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