Non-hematopoietic essential functions of bone marrow cells: a review of scientific and clinical literature and rationale for treating bone defects

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Abstract

Hematopoiesis as the only essential function of bone marrow cells has been challenged for several decades through basic science (*in vitro* and *in vivo*) and clinical data. Such work has shed light on two other essential functions of bone marrow cells: osteopoiesis and angiogenesis/vasculogenesis. Clinical utility of autologous concentrated bone marrow aspirate (CBMA) has demonstrated both safety and efficacy in treating bone defects. Moreover, CBMA has been shown to be comparable to the *gold standard* of iliac crest bone graft (ICBG), or autograft, with regard to being osteogenic and osteoinductive. ICBG is not considered an advanced therapy medicinal product (ATMP), but CBMA may become regulated as an ATMP. The European Medicines Agency Committee for Advanced Therapies (EMA:CAT) has issued a reflection paper (20 June 2014) in which reversal of the 2013 ruling that CBMA is a non-ATMP has been proposed. We review bone marrow cell involvement in osteopoiesis and angiogenesis/vasculogenesis to examine EMA:CAT 2013 decision to use CBMA for treatment of osteonecrosis (*e.g*, of the femoral head) should be considered a non-ATMP. This paper is intended to provide discussion on the 20 June 2014 reflection paper by reviewing two non-hematopoietic essential functions of bone marrow cells. Additionally, we provide clinical and scientific rationale for treating osteonecrosis with CBMA.

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Introduction

According to section 2.2.3. number 2 of the EMA:CAT 20 June 2014 reflection paper, marrow cells that are aspirated, centrifuged, and *re-administered to fulfill their same essential function will generally be regarded as homologous use. In case no substantial manipulation of the cells takes place, the classification is based on the essential function of the cells. Such non-substantially manipulated cells used for the same essential function are not considered ATMPs*.[1] However, in section 2.3.1 it is stated that products once considered a non-ATMP have been classified as an ATMP. As an example, it is specifically listed that *injection of concentrated bone marrow at the site of bone injury with the aim of healing a bone lesion can be considered as non-homologous use.*[1] According to EU Regulation 1394/2007 a *tissue engineered product* (an ATMP) is defined as one that consists of cells or tissues that have been subject to i) substantial manipulation, or ii) are not intended for the same essential function(s) in the recipient and the donor. Since, bone marrow aspirate is centrifuged to produce concentrated bone marrow aspirate (CBMA), it is not substantial manipulation (Annex I of EU Regulation 1394/2007). The issue under discussion is whether or not autologous bone marrow cells active in healing a bone lesion constitute an essential function, and consequently, homologous use of the cells.

The hematopoietic stem cell (HSC) is the most characterized somatic stem cell in the adult and has been utilized in treatment of patients following myeloablation therapy.[2] Multipotent stem cells from adult bone marrow have been used for decades in these patients because HSCs contribute to long-term and complete haematopoiesis.[3,4] Bone marrow is primarily a hematopoietic organ; however, it has long been recognized that hematopoiesis is not the only essential function of bone marrow cells.[5-8] In addition to blood cell replacement, bone marrow cells are active in replacing and building bone and endothelial cells.[9-12] Moreover, iliac crest bone graft (ICBG), a gold standard therapy used in orthopedic surgery, is not considered an ATMP and is not used for hematopoietic reconstitution. Rather, ICBG is used clinically for osteopoiesis and vasculogenesis obtained from the osteoblasts on the graft surface as well as the bone marrow stem and progenitor cells contained within the graft material.[6,13,14]

In this paper, we review data demonstrating that bone marrow cells, in addition to hematopoiesis, have the essential functions of osteopoiesis and vasculogenesis/angiogenesis. As essential functions, to utilize bone marrow cells for treating bone defects represents a homologous use of the cells. With autologous CBMA, the donor and the recipient are the same individual. The homologous use of the cells is preserved by aspirating bone marrow in order that the acquired bone marrow stem and progenitor cells will form the native tissue of the environment in which they are placed via the normal physiological functions of autocrine and paracrine activity.[15,16] Autologous ICBG and CBMA function in the same manner for the treatment of bone defects and consequently, they should both be considered a non-ATMP (Figure 1).

Two essential non-hematopoietic functions of bone marrow cells

Hematopoiesis is a crucial function of bone marrow cells. The bone marrow hematopoietic compartment produces approximately 500 billion cells per day that use the bone marrow vasculature as a conduit to the systemic circulation.[17] Such high cellular turnover demands

upon the bone marrow hematopoietic system certainly demonstrate the importance of the function of hematopoiesis of bone marrow. However, hematopoiesis is not the sole function of bone marrow cells.

It is well recognized and described in the literature that bone marrow is responsible for tissue/bone repair as well as enhancing neovascularization. In 1997, it was first demonstrated that CD34+ cells (human hematopoietic stem and progenitor cells are both CD34+ cells) are the precursors to osteoblasts.[18] Recently, it was found that a single bone marrow cell contributed to hematopoietic reconstitution and drives robust osteopoiesis.[19] In this same study, it was found that long-term repopulating HSCs yield progeny that differentiate into osteoblasts following bone marrow transplantation (BMT). Therefore, a means for hematopoietic and osteopoietic maintenance was found involving cells historically thought active in hematopoiesis but not osteopoiesis. Moreover, bone marrow is home to mesenchymal stromal cells (MSCs) that do not directly contribute to blood cell production. Limiting the essential function of bone marrow cells to hematopoiesis alone does not acknowledge the functions of MSCs.

Osteopoietic potential

Within bone marrow, there is significant heterogeneity. Three main regenerative cell types exist in bone marrow: HSCs, MSCs, and endothelial progenitor cells (EPCs).[20] MSCs, also called skeletal stem/progenitor cells or medicinal signaling cells,[21-25] do not directly contribute to hematopoietic reconstitution.[26] CBMA MSC population placed into osteogenic media demonstrated osteoblastic differentiation in the same manner as commercially available MSCs (Figure 2).

In 1869, it was first documented that bone marrow cells have osteogenic potential.[5] It is well known that donor-derived osteopoiesis in both mice and humans occurs following BMT, even from a single bone marrow cell. This suggests that osteopoiesis may be an essential function of bone marrow cells and confirms the earlier findings of osteogenicity of bone marrow.[5,6,19]

ICBG, a non-ATMP, is considered a gold standard in orthopaedic surgery because it is i) osteogenic (contains cells that contribute to osteopoiesis), ii) osteoinductive (contains growth factors, cytokines, and chemokines active in osteopoiesis), and iii) osteoconductive (provides a three dimensional matrix for cells to provide osteopoiesis). Like ICBG, CBMA is also osteogenic and osteoinductive; however, it is not osteoconductive.[13,16,27] Within bone marrow, there are at least two compartments of stem and progenitor cells that provide osteogenic cells,[28-31] with more mature pre-oesteoblastic cells localizing to the marrow space adjacent to trabecular bone.[13]

Bone repair via cells only of bone marrow mesenchymal origin has also been questioned. That is, a single marrow cell can have both hematopoietic and osteopoietic progeny, depending upon environmental clues.[26,31-33] Bone marrow non-MSCs were shown to have a 10-fold greater bone repopulating activity than MSCs in situations of stress and trauma.[10,26] CD34+ cells have been shown to differentiate into functional osteoblasts and fibroblasts *in vitro*[18,34,35] and an *in vivo* study revealed that fibroblasts are of hematopoietic origins.[36] To be sure that the cell investigated *in vivo* is a hematopoietic stem cell and not a progenitor, secondary BMT assays are used. Via secondary BMT assays, it was found that long-term HSCs contribute to hematopoietic reconstitution and drive osteopoiesis, revealing that HSCs have hematopoiesis and osteopoiesis as essential functions. In bone defects, bone formation was shown to be dependent upon the number of bone marrow cells present because differentiation of stem and progenitor cells toward osteogenesis requires high cell-cell interaction.[7] CBMA provides a method to produce and ensure high concentrations of bone marrow cells for treating bone defects.

Bones are dynamic organs in which replacement of cells occurs through recruited precursor cells. Bone remodeling takes place in the endosteum region, the site that contains HSC niches.[37,38] Marrow-derived stem and progenitor cells enter the circulation as part of host

Table 1. Colony forming units-hematopoietic data by donor and sample type.

Donor	Sample	Total colonies per 5×10^4	CFU-E	CFU-GM	CFU-GEMM
1	Mean	235	109	101	21
	Std Dev	21.7	23.9	28.5	7.8
2	Mean	194	63	117	15
	Std Dev	29.6	11.6	20.7	3.4
3	Mean	153	60	86	7
	Std Dev	22.7	7.1	16.9	3.8

Colony counts per 5×10^4 nucleated cells plated. Each donor was analyzed 4 times for a total of 12 samples. Colony forming units-hematopoietic (CFU-h); colony forming units-erythroid (CFU-E); colony forming units-granulopoietic (CFU-GM); colony forming units-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). Analysis performed by Kevy, Jacobsen (Harvard Medical School) and Mandle (BioSciences Research Associates).

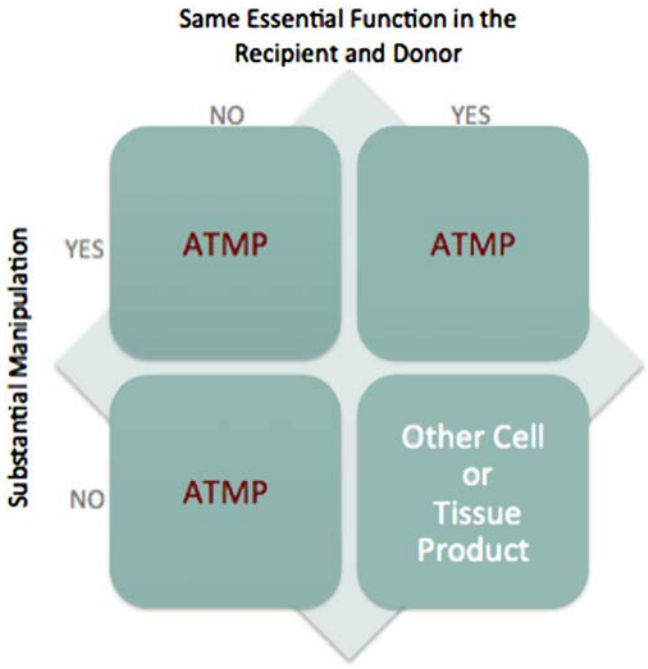

Figure 1. EU regulation 1394/2007. Bone marrow aspirate cells should be classified as a non-advanced therapy medicinal product (ATMP). Concentrated bone marrow aspirate (CBMA) is not (A) a gene therapy medicinal product, (B) a somatic cell therapy medicinal product, (C) a combined ATMP, (D) nor does it fit into the category of a tissue engineered product that has had substantial manipulation and/or the cells/tissues are not used for the same essential function in the recipient as in the donor.

defense, homeostasis, as well as repair and regeneration of injured tissue.[12] The majority of osteoblasts in the human skeleton are found on endocortical surfaces or in intracortical remodeling sites, far from red marrow and trabecular surfaces.[10] This location raised questions as to how the marrow stromal cells can be a source of osteoblasts until it was understood that bone and bone marrow are anatomically and functionally contiguous. Via human cell therapy donor osteoblast studies, it was found that engraftment occurred in transplantation of undifferentiated bone marrow,[39] confirming the reports of osteopoiesis following BMT and that marrow cells are a natural source of osteopoiesis.

Bone and bone marrow are hosts to cells that are interrelated functionally.[7,40,41] The stroma system within the bone marrow is not directly involved in hematopoiesis; however, the hematopoietic system and stroma system of the marrow are intertwined.[40] The idea of bone, bone marrow, and blood being intertwined can be dated to at least 1763,[8] and was expanded with the Chronheim hypothesis of 1867 which stated that the bloodstream, and consequently the bone marrow, was the source of the cells involved in healing, including bone.[16] Further evidence of the interrelated functionality of these systems and cells was obtained from data demonstrating that osteoblasts produce many factors required for HSC survival, renewal, and maturation.[37,40] Additionally, many bone disorders are hematopoietic disorders or disorders of the relationship of hematopoietic and stromal cells (*e.g.*, myelofibrosis with osteosclerosis, Paget's disease).[28,30]

Bone marrow cells in the body, ICBG, and CBMA are involved in the essential function of osteopoiesis. To limit the only essential function of bone marrow cells to hematopoiesis does not take into account the non-hematopoietic cells found within bone marrow nor the osteopoietic role of HSCs found within bone marrow, ICBG, and CBMA. Additionally, such a limitation on bone marrow cells does not allow for the intertwining relationship of bone, bone marrow, and blood. Finally, a restrictive view on the essential functions of bone marrow cells does not address the scientific data supporting clinical use of ICBG in orthopedics, which is for treatment of bone defects and not for hematopoietic reconstitution.[42]

Angiogenic and vasculogenic potential

In addition to hematopoiesis and osteopoiesis, bone marrow cells are also active in supporting the vasculature and differentiating into endothelial cells.[43-47] Bone marrow cells respond to wound healing via various signaling pathways that lead to mobilization of bone marrow EPCs and other cells involved in

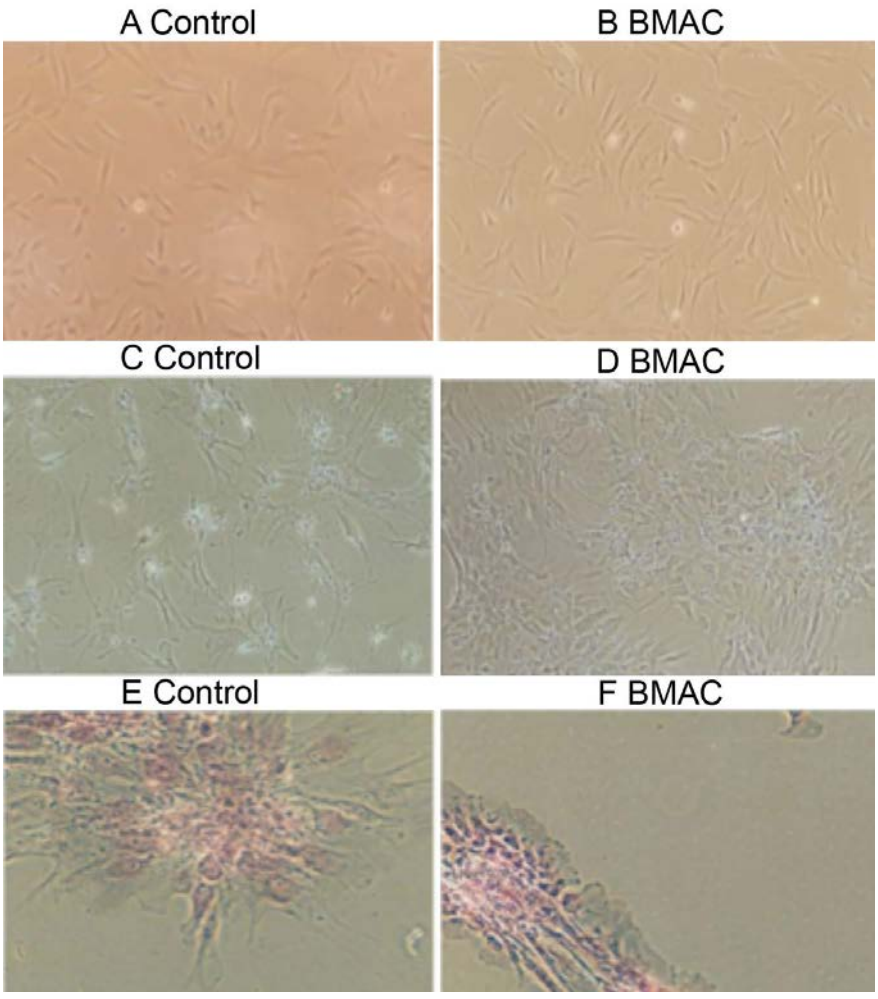

Figure 2. Comparison of harvest bone marrow aspirate cells (BMAC) to commercially available mesenchymal stromal cells (MSCs). A,B) BMC sample culture compared with control hMSC's from known hMSC sample obtained from commercial lab; C,D) Cell morphology changed rapidly when transferred to osteogenic differentiation medium (ODM). Deposition of mineral evident by light microscopy confirmed cells retained potential for osteoblastic differentiation; E,F) Mineral deposition is an indicator of differentiation from the plural potent MSC toward osteogenic cells. Slides of MSC control and BMC cells after 10 days in ODM and stained with Von Kossa silver stain.

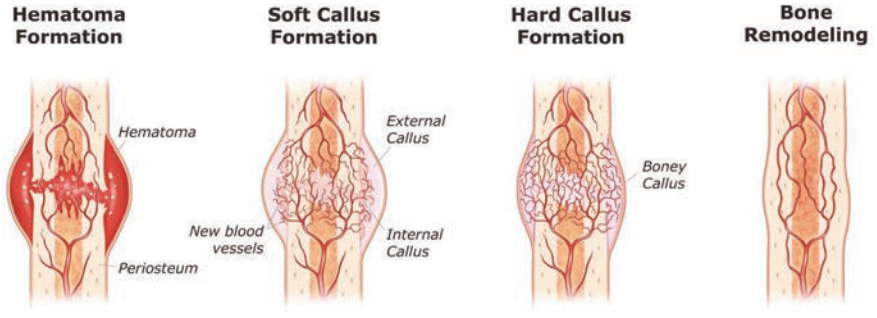

Figure 3. Bone marrow cells are involved in wound healing. Bone marrow cells respond to bone defects via the vasculature. The ischemia at the onset of the defect initiates the healing cascade involving bone marrow cells active in the essential functions of osteopoiesis and angiogenesis/vasculogenesis.

the healing cascade. The typical course of response to injury involves posttraumatic ischemia and edema, local inflammation and removal of damaged tissue via phagocytosis, activation of cellular precursors, revascularization of the traumatized region, extracellular matrix as a substrate, increased numbers of regenerating cells, differentiation of the regenerating tissue, morphogenesis of regenerating tissue, and finally, functional restoration.[48] These naturally occurring processes involve bone marrow cells.

Bone marrow contains various progenitor and endothelial cells that are incorporated into sites of reduced vascularity to stimulate angiogenesis and vasculogenesis.[43-46,49,50] Within the injury site, vascularization occurs through local factors stimulating adjacent cells (angiogenesis) and through bone marrow recruited EPCs (vasculogenesis).[51] Consequently, enhancing angiogenesis and vasculogenesis is another essential function of bone marrow cells.[48,52] To focus upon bone, fracture healing relies upon angiogenesis and vasculogenesis to facilitate callus formation and remodeling; the harmatoma is inherently angiogenic in composition (Figure 3).[53] These processes involve cells of the bone marrow and it is known that circulating bone marrow hematopoietic stem and progenitor cells differentiate into endothelial cells at sites of vascular remodeling.[43-45]

All bone maintenance, formation, and healing is linked to vascular supply. Osteoblasts and osteocytes must be within 0.1 mm of a vascular structure to survive and function.[54] Additionally, trabecular bone formation rates have a positive correlation with blood vessel area. Moreover, vascularity and bone are tightly linked in the processes of endochondral ossification, callus formation, and bone regeneration.[55] Bone formation and repair develops heavily upon vascular growth, involving endothelial cells and bone marrow EPCs.[56,57]

Human $CD34^+$ cells originating from the bone marrow are involved in angiogenesis and vasculogenesis. In a study investigating bone marrow mononuclear cells (BMMNCs) and the $CD34^+$ cell fraction of the BMMNCs, it was found that $CD34^+$ cells significantly enhanced neovascularization and bone repopulation, confirming work that BMMNCs and CBMA demonstrate enhanced neovascularization and functional restoration of induced localized ischemic injury.[58,59] Blood vessel formation occurs commonly in adults through bone marrow cells and is a major factor in regulating bone healing and several other processes.[43] The essential functions of bone marrow cells must therefore also include enhanced angiogenesis and vasculogenesis.

Rationale for treating osteonecrosis of the femoral head with concentrated bone marrow aspirate

Treating bone healing disorders with bone marrow is not a new therapy,[60] nor is the osteogenic nature of bone marrow new knowledge.[5] Phemister presented the idea of *creeping substitution* in 1930 to describe old bone being gradually absorbed and replaced by new bone.[61] Phemister demonstrated that when there are limited osteogenic elements surviving about the periphery of the necrotic bone, bone absorption may be greatly in excess of bone formation resulting in cavitation. Additionally, Phemister recognized the importance of vessels for the bone and demonstrated that vessel injury may be the cause of some cases of osteonecrosis of the femoral head (ONFH). Vessel injury interferes with blood supply, leading to massive osteonecrosis. Treatment of ONFH has included core decompression since the 1960s when core biopsies were found to immediately reduce pain.[62] Newer approaches may also include vascularized grafting as a means of introducing vascularity.[63] These clinical approaches suggest that improved vascularity may improve core decompression outcomes.

Work relating to Phemister from 342 patients with early stages of ONFH found that common features of ONFH include i) reduction in osteogenic progenitors, ii) increased apoptosis, and iii) altered intramedullary vascularity.[64] The anatomical changes and cellular mechanisms of note are increased apoptosis of osteocytes and inadequate vessels.[65] Non-traumatic ONFH continues to represent a significant challenge in orthopedic surgery and climaxes through the final common pathway of decreased blood flow to the femoral head that leads to ischemia and death of the bone.[66] It was concluded that core decompression should be supplemented by CBMA to overcome these cellular and vascular issues.[64]

The understanding of the onset of nontraumatic ONFH to be of cellular origin has been formed gradually.[67-71] Histologic examinations of bone marrow at the site of ONFH demonstrated the pathogenesis of necrosis of hematopoietic cells, endothelial cells, and lipocytes; osteocytes atrophy and die, and a subsequent increase in fatty marrow water content is detectable by magnetic resonance imaging.[66] ONFH represents a gradual degradation of bone marrow; red marrow converts to fatty marrow in the proximal femur and all cell types of bone and marrow are affected.[71] In patients with ONFH, revascularization can occur naturally after the bone dies.[66,72] Bone marrow cells induced efficient neovascularization in rabbit femoral bone defects and enhanced regeneration of the bone defects.[50,58] Utilizing CBMA therapy, patients with ONFH have been treated safely and efficaciously.[60,64,69,73-82] Bone marrow cells are naturally engaged in the formation of new blood vessels[13,43-46,49,53,56,83,84] so that use of CBMA to address reduced vascularity in ONFH also has clinical utility.

Discussion

CBMA is an autologous cell composition that contains the nucleated cells found in bone marrow. Because the process to produce CBMA does not select any subpopulation of bone marrow cells, nor involves any culture expansion steps, it is a total nucleated cell (TNC) composition. As such, CBMA retains its hematopoietic potential (Table 1).

ICBG has three significant disadvantages compared with CBMA.[13] First, the process of ICBG harvesting adds operative time, pain, and blood loss; carries an increased risk of infection, cutaneous nerve damage, and local fracture. Second, the amount of bone available is limited and may be insufficient in many settings. Finally, ICBG has biologic limitation as a cellular graft because the metabolic demands of the graft site often exceed the capacity of the graft delivered since all cells greater than 1 to 2 mm of the graft surface die during transplantation.[85] CBMA overcomes the cellular limitation of ICBG.[27,86]

CBMA results in significant reduction in patient morbidity compared to ICBG because CBMA is obtained through a simple aspiration process rather than surgical resection of the iliac crest to obtain bone and bone marrow and to produce morsels for packing into a bone defect.[13,27,60,87] In contrast, CBMA requires only bone marrow aspiration and centrifugation.[88] It has been shown that CBMA and ICBG are both osteogenic and osteoinductive.[13] Both ICBG and CBMA have been used clinically for the non-hematopoietic essential functions of the cells delivered.

To be regarded as an ATMP requires that cells/tissues be substantially manipulated and/or used for a purpose other than their natural essential function. Osteopoiesis and vasculogenesis have been shown to be natural essential functions of bone marrow cells. Therefore, in accordance with EU regulation 1394/2007, CBMA should be classified and regulated as a non-ATMP.

Conclusions

Since bone marrow cells have the natural function of being involved in neovascularization, it begs the question as to why in addition to core decompression CBMA should be added for treating ONFH. There are two important reasons.

In patients with ONFH, the marrow in the

proximal femur has converted to fatty marrow, unable to sustain the *creeping substitution* of bone noted by Phemister and others. Because CBMA is the cellular component of ICBG, CBMA addresses the acellularity of the necrotic zone.[64]

Moreover, there is a decrease in blood perfusion in ONFH patients which seems to be the major factor in the disease.[89] It has been shown that CBMA significantly improves perfusion in a murine model.[59] Administration of CBMA provides an autologous cellular augmentation of the potential beneficial effects of core decompression and involves whole bone marrow as a composite of osteoprogenitor, endothelial, and hematopoietic progenitor cells in an environment supporting them in normal physiologic conditions.[73]

The essential function and the mechanism of action of the cell population in CBMA includes more than hematopoietic reconstitution. Osteopoiesis is a critically important essential function of bone marrow cells. The scientific and clinical literature support the conclusion that bone marrow cells naturally involved in hematopoietic reconstitution are also involved in osteopoiesis and angiogenesis/vasculogenesis. Additionally, in 2013, EMA:CAT concluded that CBMA was a non-ATMP for the treatment of osteonecrosis because of three key points: i) non-mesenchymal bone marrow stem/progenitor cells are active in osteoblast formation, ii) bone marrow hematopoietic stem/progenitor cells differentiate in both hematopoietic and osteocytic pathways, and iii) certain stromal cells do not contribute to hematopoietic reconstitution.[90] CMBA is the cellular compartment of ICBG and should be regulated in the same manner as ICBG. As such, CBMA should remain classified as a non-ATMP for the treatment of osteonecrosis.

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