

Review Article

Stem cells approach for bone regeneration

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Abstract

Lack of bone volume and bone loss from pathologies remains problematic for clinicians especially in large bone defect. Grafting with autogenous bone or bone substitutes has been utilized. Currently, advance cellular strategies in stem cell research and tissue engineering play an important role in regenerative medicine including bone regeneration. These technologies have been developed to be an alternative therapeutically options to autogenous bone. This paper provides a brief background of bone biological aspect and cell-based bone regeneration using stem cell. Sources of mesenchymal stem cell (MSC) and characteristics of stem cell were also discussed. Culture-expanded MSCs method and immediate MSCs approach using autologous bone marrow aspirate (BMA) or bone marrow aspirate concentrate (BMAC) transplantation were mentioned in the role and effectiveness in bone regeneration. Autologous BMA and BMAC supply total nucleated cells including MSCs and vascular progenitor cells. The co-existing of these two cell types in culture has been confirmed for their synergistic effect in tissue regeneration. Particularly, BMAC has been evidenced for its clinical effectiveness and cost-effectiveness in bone regeneration. Moreover, BMAC preparation procedure is simple, in-expensive, minimal time consuming, and minimal risk of contamination without a need for complicated laboratory support. Therefore, immediate stem cell application using BMAC is likely to be a milestone for future development in regenerative medicine.

Key words: Bone regeneration, Culture-expanded stem cell, Immediate stem cell application, Stem cell

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Introduction

Autogenous bone has long been accepted as a gold standard grafting material. It possesses an ideal property for bone regeneration including osteogenesis, osteoinduction and osteoconduction. However, donor site surgery is required and an endurance of its related morbidities is the foremost concern of the patients. Therefore, bone substitutes have been developed to be an alternative to autogenous bone. However, they are cell-free materials which mostly provide only osteoconductive property. Hence, a strategy in combination of cell-based therapy to bone substitutes has been proposed. Mesenchymal stem cells (MSCs) supplement is benefit in further providing the osteogenesis and osteoinductive properties to bone substitutes.

Bone tissue and osteogenesis

Bone tissue is composed of bone cells and bone matrix. There are two major types of bone cells: bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts). Osteoblasts are derived from osteoprogenitor cells or MSCs whereas most osteoclasts are from monocyte-macrophage precursor cells of hematopoietic stem cells in the bone marrow. Bone matrix is comprised of 90 - 95%

collagen type I and 5 - 10% of non- collagenous proteins such as osteocalcin, osteonectin, bone sialoproteins and proteoglycans.¹⁻²

Typically, bone is a dynamic tissue in nature. Bone growth takes place in the continuous processes of bone apposition and bone resorption causing bone modeling and remodeling. Bone modeling is the process of bone formation by osteoblasts that increase the bone mass and maintain bone strength. Bone remodeling is the process to optimized bone structure.² Basically, bone remodeling starts with osteoclasts activation by hormone, cytokines and growth factors and then follows by osteoclast-mediated resorption phase. The mineralized bone matrix is later dissolved resulting in Howship's lacunae resorption. Subsequently, reversal phase occurs by the proliferation and differentiation of osteoblastic precursor cells which then activates osteoblasts. Thereafter, new bone is formed by deposition and mineralization of extracellular osteoid matrix from the active osteoblasts and osteoblasts are differentiated into osteocytes in the terminal phase. Likewise, the repeated cycles of bone remodeling are continued throughout the life time.³

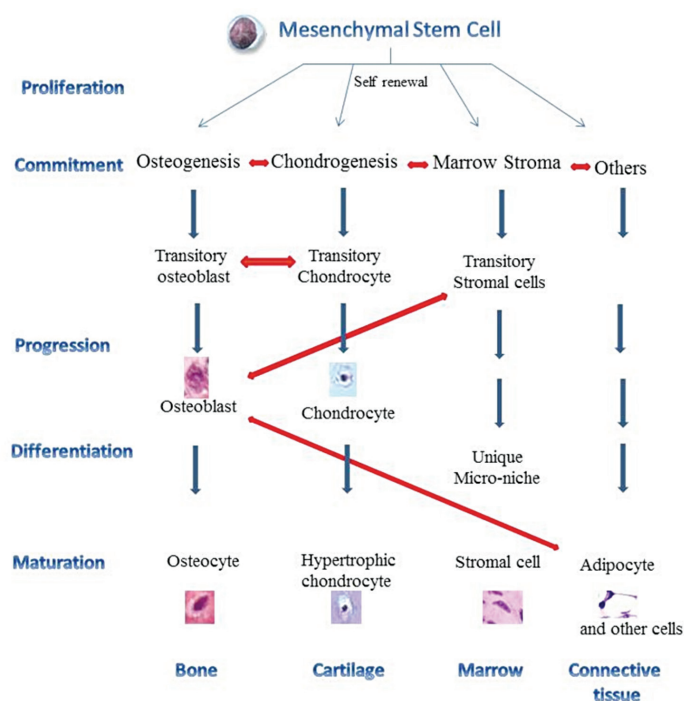


Figure 1 Model of the osteogenic cell lineage from MSCs under dexamethasone induction *in vitro*, modified diagram.⁴

Mesenchymal stem cells (MSCs)

Stem cell refers to non-specialized cell which possesses a long term self-renewal and ability to differentiate into other specialized cell types (Fig. 1). MSC was first described by Friedenstein and called osteogenic stem cell. It was isolated from bone marrow using plastic-adherent capability.⁵ Bone marrow-derived mesenchymal stem cells (BM-MSCs) are fractions of non-hematopoietic cells residing in the bone marrow. These fibroblast-like cells are able to form colony, proliferate and differentiate into other tissue types. There are other synonyms such as stromal cells or mesenchymal stem cell, skeletal stem cell, etc.⁶ Current notion of MSCs are extended from the origin of bone marrow to other sources such as adipose tissue, dental pulp, periodontal ligament etc.

However, the plastic-adherent cell population is not homogenous. The International Society for Cellular Therapy (ISCT) had proposed the minimal criteria for stem cells as follows; 1) obtaining plastic adherence capability in culture, 2) present of specific group of CD markers (expression of CD105, CD73, CD90 and coincident absence of CD34, CD45, CD14 or CD11b, CD97 or CD19, HLA-DR) and 3) possessing a multi-differentiation capacity *in vitro*.⁷ ISCT recommended the term using for cell fractions fitting to these minimal criteria as “mesenchymal stem cell”. Those do not fit to these criteria are suggested to call in a boarder term as “multipotent mesenchymal stromal cells” so that their biologic properties can also be reflected.⁸ In this paper, MSCs refers to both mesenchymal stem cell and mesenchymal stromal cell because the biological aspect of MSCs from previous literatures had not been actually identified.

Cell-based therapy in bone regeneration

Therapeutic technology of cell-based therapy for bone regeneration aims to promote local bone formation by cell transplantation. MSCs, MSCs derived osteoblast or osteoblasts are cultured and expanded before transplantation into bone defects. Another method, MSCs are immediate transplanted using bone marrow aspirate (BMA) or bone marrow aspirate concentrate (BMAC).

Culture-expansion of MSC

MSCs are cultured and expanded *in vitro* to increase cell number aiming for effective bone formation after transplantation. With this method, a large quantity of cells can be proliferated from a small amount of harvested cells. Undifferentiated MSCs or MSC-derived osteoblasts are utilized. The procedure starts from cells isolation, identification, culturing and expansion. This method provides enormous benefits not only for bone but also other hard tissue and soft tissue regeneration. Feasibility of allogeneic MSC therapies has also been claimed because MSCs possess a special advantage of being free from immunoreaction by affording immunosuppression and immunoprivilege *in vivo*.⁹ Nevertheless, the controversies of this issue has also been existed and the role of MSCs in immune response has to be further determined and investigated. Likewise, advanced technology merging with this method has been proposed including gene therapy or cell painting technology. Gene therapy has been applied to treat conditions or diseases that lack certain factors or proteins. In principle, this method delivers cytokines, proteins or growth factors by triggering a target cell in the tissue. Therefore, it can generate that specific factor or protein in adequate amount at the proper time. The Challenge of this method is to target the right location of the right gene in the right cell by possessing a minimal adverse effect.¹⁰ Cell painting technology is performed by coating MSCs with primer. The primer is later bind to antibody and then affords a tissue repair. This procedure was claimed to have no effect on cell viability, activity, and cell differentiation capacity.¹¹

Besides, culture-expanded cells transplantation can be applied either alone or incorporated with bioactive molecules and/or scaffold. However this method does carry with some disadvantages which include: complicated laboratory processing, which can be time consuming, expensive and require a rigid quality control. Other drawbacks include the risk of contamination and chance of tumour transformation. Hence continuous development in cell-based therapy is on-going to provide more efficient and safe options.

MSC differentiation into osteoblast *in vitro*

Basically, MSCs are able to differentiate into mesoderm cell lineages under specific stimulation of a particular tissue type. The standard method for the osteogenic differentiation of MSCs *in vitro* can be carried out by incubating a confluent monolayer of MSCs in an osteogenic induction medium that consists of dexamethasone, beta-glycerophosphate, and ascorbic acid. A combination of supplements such as vitamin D3, transforming growth factor-beta and bone morphogenetic proteins (BMPs) are also evidenced to facilitate osteoblastic differentiation.¹²

The osteoblastic differentiation can be verified by detection of collagen type I, osteocalcin, extracellular calcium deposits, expression of alkaline phosphatase,

and/or expression of osteoblast specific marker genes. The relevance between *in vivo* and *in vitro* osteoblastic differentiation has been confirmed. Still, the best determinant of true differentiation potential should be based on the real physiological condition *in vivo*. It is frequently quoted that osteoblastic differentiation *in vitro* is not a prerequisite for the osteogenic differentiation *in vivo*.¹³ A diagram illustrating the stages of osteogenic differentiation *in vitro* is shown in Fig. 2. Each stage was reported to express a certain cell surface molecule and extracellular matrix molecule. However, the osteogenic differentiation potential of MSCs was reported diminishing with the increase in number of subcultures.¹⁴

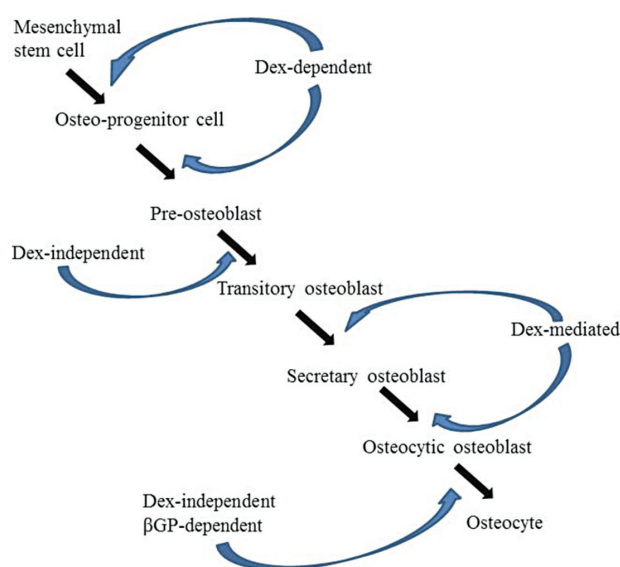


Figure 2 Model of the osteogenic cell lineage from MSCs under dexamethasone induction *in vitro*, modified diagram.¹⁵

MSC differentiation into osteoblast *in vivo*

The determination of the osteogenesis capacity of MSCs *in vivo* is usually assessed after they were transplanted into one or two tissue types, skeletal and ex-skeletal site. In skeletal site, bone defects are usually created on cranium or extremity to accommodate MSCs transplantation. Subcutaneous or peritoneal embedding technique has been used for transplantation in the ex-skeletal site. Thereafter, the amounts of regenerated bone and/or biomechanical and/or biochemical properties will be tested after desired healing periods.

“Niche” hypothesis, proposed by Schofield in 1978, indicated that not only location of stem cells but also cellular components and signaling molecules in the dynamic micro-environment affect the behaviors of MSCs *in vivo*.¹⁶ The dissimilarity of micro-environment *in vivo* may cause limitation of osteoblastic differentiation. The osteogenic response of MSCs is reported to be regulated from biochemical signaling released by osteocytes and osteoblasts. *In vivo*, culture-expanded cell seeding on various bone substitutes such as hydroxyapatite/tricalcium phosphate (HA/TCP) and bovine bone mineral (BBM) have been reported to promote new bone formation.

Immediate stem cell application

Autologous immediate stem cell application is a strategy to repair tissues by harvesting stem cell at point of care and then transfers it back to patients without need for culture and expansion in laboratory. This approach has been attracted the clinicians' attention for both hard and soft tissue repair. In principle, adult stem cell is utilized for this purpose and its sources include bone marrow, adipose tissue and peripheral blood. BMA is the most promising source which a favorable harvesting site is iliac crest. Bone marrow contains a mixture of cells such as MSCs, endothelial progenitor cells and bioactive molecules such as growth factors, cytokine and fibrin.

MSCs in bone marrow

There is no constant prevalence or fixed ratio of MSC per nucleated cells in the bone marrow has been published. Only a small fraction of nucleated cells in the bone marrow were found to belong to MSC In both human and animal. Prevalence of MSCs was reported as 0.001 - 0.01% of nucleated cells.¹⁷

Factors affecting the number of MSC in the bone marrow correlate with age, gender, systemic condition and volume of bone marrow aspirate. These factors influence both the quantity and quality of MSC. The number of MSC per nucleated cells was shown to decline with age. It was reported to be at 0.002% in young adult and 0.0001% in the elderly.¹⁸ In addition, it decreases after skeletal maturity and diminishes in females.^{19 - 20} Likewise, alcohol and corticosteroid have an adverse effect on the quality of MSC.²¹ Regarding the site and number of aspiration, Eelco et al. recommended an aspiration volume of at least 8 ml because a lower or higher volume did not demonstrate higher cell yield. Repeated aspiration at the same site demonstrated a lower cell concentration and less MSC than the first aspiration. On the other hand, multiple aspirations did not seem to affect the quality of MSCs.²²

The use of bone marrow aspirate (BMA)

The simplest approach is a percutaneous injection of BMA into bone defect. This method was reported in an enhancement of bone healing in treatment of fresh fracture, non-union or delayed union of long bone and

spinal fusion model. Furthermore, BMA incorporated in bone substitutes was also endorsed to generate new bone in both intra- and extra-skeletal sites in animal experiment. However, the scarcity of MSC in BMA is a crucial concern affecting the quantity of bone formation. Hernigou et al. 2005 claimed that more than 1,500 progenitors cells/cm³ or $54,962 \pm 17,431$ progenitors cells was required for effective treatment in human tibia non-union defect.²³

The use of bone marrow aspirate concentrate (BMAC)

Basically, BMAC is a concentrated form of BMA. It is enriched the quantity of nucleated cell, MSCs and vascular progenitor cells which can amplify neovascularization and its osteogenic capacity. The preparation of BMAC or isolation of nucleated cells can be achieved by using various techniques such as centrifugation, density gradient separation, size-sieved cells selection, anti-fibroblast microbeads combined with fluorescence microscopy and bone marrow concentration systems. The centrifugation method separates lighter portion from the heavier portion of fluid using centrifugation force. Human bone marrow can be separated into three layers after single centrifugation. However, a double centrifugation method has been applied for clearer isolation of nucleated cell layer. Density gradient separation using Ficoll or Percoll to separates the pure fraction from the target portion on a preformed gradient upon centrifugation force are recognized as one MSCs isolation method. However, the most convenient device for BMAC preparation in clinic is the bone marrow concentration system which able to extract the nucleated cell portion containing MSCs with high cell recovery. Recently, many bone marrow concentration systems are available in the market such as Marrow-Stim[™], Res-Q[™], Harvest[®] or SmartPReP[™], Sepax[®], Accelerate[®], Cobe 2991, Regenexx[™].

Different designs of bone marrow concentration device have been manufactured. For example, MarrowStim[®] bone marrow concentratration system (Biomet 3i, Biology, USA) provides the special container "MarrowStim[™] tube" that contains additional filter serving nucleated cells extraction. The concentration method requires a single centrifugation for 15 minutes at room temperature. It could achieve separation in three distinct layers, cell poor

plasma, nucleated cell layer (BMAC), and red blood cell layer. Thereafter, separated BMAC layer can be obtained easily by drawing out with 10 ml syringe. The quantity of nucleated cell and MSC were confirmed to be significantly increased, $p < 0.05$.²⁴ The exploration regarding the efficacy of nucleated cell and MSCs enrichment in BMAC in clinical application have also been confirmed by many studies. The BMAC preparation at point of care for immediate use has been shown to be convenient, less time consuming and being user-friendly than other methods. In which, successful treatment of various bone pathologies such as non-union limbs, ischemic limb, femoral head osteonecrosis (ON), avascular necrosis, unicameral bone cyst and fracture have been published.

Discussion

Cell, scaffold and bioactive molecules are key factors in tissue regeneration. MSCs as a cell component was introduced for cell-based therapy which can be used either alone or in combinations with other components to promote bone regeneration. MSCs should be amplified by culture and expansion or enriched by concentration method to increase in cell number prior transplantation. Well-known sources of MSCs are bone marrow, adipose tissue and periosteum. Recently, MSCs from dental origin have been receiving the attention of research and application which their sources includes dental pulp, exfoliated deciduous teeth, periodontal ligament, dental follicle and apical papilla.²⁵ Culture and expansion of MSCs method requires a complicated laboratory support, laborious, expensive and time consuming. In contrary, BMAC usage offers enriched MSCs for immediate application and it can be used effectively in combination with scaffolds. The later method affords more potential for co-transplantation of vascular progenitor cells with their signaling molecules. Furthermore, new advance tissue engineering such as gene transfer, growth factor delivery, 3D printing and cell sheet technology have also been developed for further promote bone formation.

Conclusion

MSCs possess self-renewal and multipotency and they provide osteogenesis under specific condition *in vitro* and after transplantation into bone defects *in vivo*. Therefore, MSCs supplement to promote bone regeneration has been recommended to provide a cellular-based tissue engineering method. In addition, the strategy of MSCs transplantation using BMAC can be another worthy therapeutic option for bone grafting in alternative to autogenous bone.

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บทคัดย่อ

การใช้เซลล์ต้นกำเนิดเพื่อการสร้างกระดูก

ลัดดาวัลย์ สุนันท์ลิพานนท์

คณะทันตแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

การสูญเสียและการบกพร่องของปริมาณกระดูกเป็นปัญหาที่พบได้ โดยการรักษาจะใช้การปลูกถ่ายกระดูกของผู้ป่วยเองหรือใช้กระดูกเทียม แต่ปัจจุบันการใช้เซลล์ต้นกำเนิดเพื่อการเสริมสร้างเนื้อเยื่อในการแพทย์สาขาเวชศาสตร์ฟื้นฟูภาวะเสื่อมมีบทบาทเพิ่มขึ้น วิทยาการเหล่านี้ได้มีการพัฒนาเพื่อใช้เป็นทางเลือกในการให้การรักษา บทความนี้กล่าวถึงพื้นฐานลักษณะทางชีววิทยาของกระดูกและการใช้เซลล์ต้นกำเนิดในการสร้างกระดูก แหล่งและลักษณะเฉพาะของเซลล์ต้นกำเนิด บทบาทและผลการสร้างกระดูกจากการปลูกถ่ายเซลล์ต้นกำเนิดที่ผ่านกระบวนการเลี้ยงและขยายเซลล์ในห้องปฏิบัติการและการปลูกถ่ายเซลล์ต้นกำเนิดในทันทีโดยใช้ไขกระดูก และไขกระดูกเข้มข้นของผู้ป่วยเอง ไขกระดูกทั้งสองชนิดประกอบด้วยเซลล์ที่มีนิวเคลียสซึ่งมีทั้งเซลล์ต้นกำเนิดมีเซนไคมอลและเซลล์ดั้งต้นของหลอดเลือด ทั้งนี้การมีเซลล์ทั้งสองชนิดอยู่ร่วมกันจะส่งเสริมกันในการสร้างกระดูก อีกทั้งประสิทธิผลและความคุ้มค่าทางคลินิกในการสร้างกระดูก โดยวิธีการใช้ไขกระดูกเข้มข้นยังได้รับการยืนยัน นอกจากนี้ขั้นตอนการเตรียมไขกระดูกเข้มข้นเป็นวิธีที่ง่าย ไม่แพง ใช้เวลาน้อย และมีความเสี่ยงต่อการปนเปื้อนน้อย โดยไม่อาศัยขั้นตอนที่ซับซ้อนทางห้องปฏิบัติการ ซึ่งถือได้ว่าเป็นแนวทางหนึ่งในการพัฒนาทางการแพทย์ในอนาคต

คำสำคัญ: การสร้างกระดูก, การเลี้ยงและขยายเซลล์ต้นกำเนิด, การปลูกถ่ายเซลล์ต้นกำเนิดในทันที, เซลล์ต้นกำเนิด