A Potential Translational Approach for Bone Tissue Engineering through Endochondral Ossification

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Abstract— **Bone defect repair is a significant clinical challenge in orthopedic surgery. Despite tremendous efforts, the majority of the current bone tissue engineering strategies depend on bone formation via intramembranous ossification (IO), which often results in poor vascularization and limited-area bone regeneration. Recently, there has been increasing interest in exploring bone regeneration through a cartilage-mediated process similar to endochondral ossification (EO). This method is advantageous because long bones are originally developed through EO and moreover, vascularization is an inherent step of this process. Therefore, it may be possible to effectively employ the EO method for the repair and regeneration of large and segmental bone defects. Although a number of studies have demonstrated engineered bone formation through EO, there are no approaches aiming for their clinical translation. In this study, we propose a strategy modeled after the U.S. Food and Drug Administration (FDA) aproved Autologus Chondrocyte Implantation (ACI) procedure. In its implementation, we concentrated human bone marrow aspirate via a minimally manipulated process and demonstrated the potential of human bone marrow derived cells for *in vitro* pre-cartilage template formation and bone regeneration *in vivo*.**

I. INTRODUCTION

Engineering bone tissue is approached through either intramembranous or endochondral ossification [1]. However, long and axial bones, are developed through an intermediate hypertrophic cartilage template that is subsequently remodeled into bone. During this process, mesenchymal cells condense and differentiate into an avascular chondrocyte aggregate, which in turn deposits a matrix composed of sulfated glycosaminoglycans and collagen II. As chondrocytes terminally differentiate into hypertrophic chondrocytes, collagen II is replaced with collagen X and the produced ECM is calcified [2, 3]. Endochondral ossification (EO), which is a cartilage-mediated process, is advantageous over the intramembranous (IO) or direct ossification method for bone tissue engineering because of its inherent ability to form vascularized bone, while IO methods need subsequent vascularization strategies for the same results [4-8]. In vitro and in vivo studies, involving pluripotent stem cells, bone marrow or fat derived stromal cells, with or without a biomaterial scaffold, have proven the possibility of bone tissue engineering via EO [9-11]. However, no attempts were made to translate this technology to the clinic. This abstract presents a clinically relevant strategy for bone tissue engineering through EO. Our approach, as shown in Figure 1, involves the steps of bone marrow aspiration, marrow concentration for bone marrow stromal cells (BMSCs), in vitro cell expansion, and culture to form a pre-cartilage template. BMSCs are proposed since their use is well accepted and clinically approved for bone defect repair [11, 12]. In vitro cell expansion and pre-cartilage template formation steps are modeled based on a clinically practiced therapy, an autologous chondrocyte implantation (ACI) [13-15].

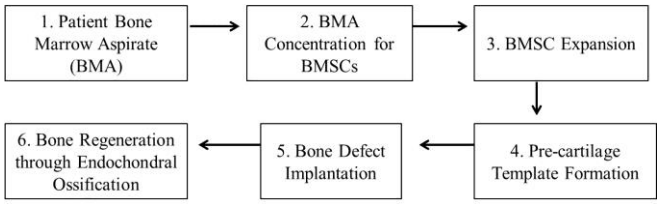

Figure 1. Diagram illustrating the steps involved in the proposed translational approach for bone tissue engineering through endochondral ossifcation.

The objective of this pilot study is to introduce a clinically relevant tissue engineering strategy for bone regeneration through EO and prove the efficacy of the strategy for a critical-sized bone defct repair.

II. MATERIALS AND METHODS

A. Bone Marrow Concentration and Culture

Fresh human bone marrow aspirate (BMA) from a female donor, between the ages of 18-25 years old, was purchased (Lonza, Walkersville MD, USA). 35 ml of BMA was concentrated (cBMA) to a final volume of 5 ml using an automated cell separator, the *Magellan®System* (Arteriocyte Medical Systems, Hopkinton MA, USA). cBMA was recovered and seeded in 150 mm tissue culture plates containing Dulbecco's-modified Eagle's Medium

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supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin (Life Technologies, Carlsbad CA, USA. Human mesenchymal stem cells (hBMSC) cultures grew at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed after 4 days with washes of phosphate buffer solution (PBS) (Life Technologies, Carlsbad CA, USA). The medium was subsequently replaced every 3 days. When the culture reached 90% confluency, the hBMSCs were recovered with 0.25% trypsin containing 0.01% EDTA (Life Technologies, Carlsbad CA, USA), and passaging was performed. All subsequent experiments were performed with passages 1-3.

B. Colony Forming Unit (CFU-f) Assay

Un-processed and concentrated BMA was analyzed for colony forming ability. In brief, CFU-*f* assay was carried out by plating 100 µl of cBMA and BMA into 150 mm culture plates. After incubation for 14 days, cells were washed with PBS and then stained with 0.5% crystal violet for 10 minutes, rinsed several times with distilled and de-ionized (DDI) water and photographs were taken to visualize CFUs of hBMSCs.

C. Flow Cytometry

Flow cytometry was run on a BD LSR II Flow Cytometer (Becton Dickinson, Franklin Lakes NJ, USA). Cells grown in expansion medium were harvested, and separately labeled with PE-conjugated anti-human CD-73, 90, 105, 34, and 45 antibodies (BD Pharmingen, San Jose CA, USA). Unstained cells, live/dead and isotype controls were used [16]. Flow cytometry data was analyzed using FlowJo software (Tree Star, Inc.).

D. In Vitro Pellet Culture and Animal Surgical Procedure

For in vitro pellet culture, cells were plated in phenol-red free alpha-MEM (Life Technologies, Carlsbad CA, USA) with 10% FBS. Medium was changed every 3 days. Cells were passaged at 80% confluence. Pellet culture was performed with passage 2 cells. 500,000 hBMSC cells were pelleted into a polypropylene conical 15 ml tube and cultured for 16 days in chondrogenic media containing 10 ng/ml TGF-β_1, ITS^{+1}, 0.1 mM ascorbate-2-phosphate, 0.4 mM proline, and 100 nM dexamethasone, to form chondrogenic pellet. The 16 day old pellets were placed in the center of a donut shaped Healos scaffold disc, and then implanted in a mouse critical-size calvarial model using NSG/Col3.6tpz transgenic mice created in Dr. Rowe's laboratory [17, 18]. Briefly, the animals were anesthetized with a ketamine (135 mg/kg)–xylazine (15 mg/kg) blend and one 3.5-mm-diameter defect was introduced in the right parietal lobe using a Dremel MultiPro drill with a trephine bit. After construct placement in the defect, the incision was closed with resorbable sutures, and the animals were given analgesic (bupronephrine, 0.08mg/kg). Samples were harvested after 8 weeks of surgery.

E. Histology and X-Ray Imaging

Animals were sacrificed by CO_2 asphyxiation and calvarial samples were harvested and fixed. An incision was made above the defect area for photographs and X-Ray imaging (LX60; Faxitron, Tucson AZ, USA) [18]. Each calvarium was covered with an embedding medium (Cryomatrix; Termo Shandon) and affixed to an aluminum stage for cryosectioning. Thin sections (7 mm) of tissue were transferred via tape (Cryofilm; Section-Lab Co. Ltd., Japan) to a plastic slide. Sections were then rinsed three times in PBS, followed by distilled water, and placed on a glass slide. A 50% glycerol solution was applied to the section and a glass coverslip was placed on top. Images of finished slides were acquired with a fluorescent microscope (ImagerZ1; Zeiss, Germany) equipped with a digital camera (Axiocam; Zeiss, Germany) and filter set (49002 and 31002; Chroma).

III. RESULTS AND DISCUSSION

Bone marrow concentration to improve connective tissue progenitors (CTPs) or MSCs has been practiced for many years. In this study BMA was concentrated via minimally manipulated process, using a Megallan device. The device is fully automated, closed looped, and has the potential to support rapid concentration of BMA with reproducible results. To examine cBMA enrichment, a colony forming unit (CFU-*f*) assay was performed. While both BMA and cBMA displayed similar number of colonies, cBMA showed greater uniformity and a significantly larger colony area (quantification data not shown here) than the un-processed BMA group. Larger CFU-*f* area indicates significantly higher number of cells/colony for the cBMA than BMA.

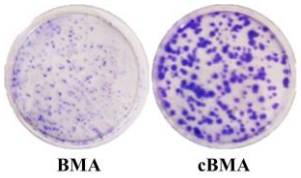

Figure 2. Colony forming unit assay for the Un-processed BMA and Concentrated BMA is shown. Enriched cells display significantly larger colonies than non-enriched cells.

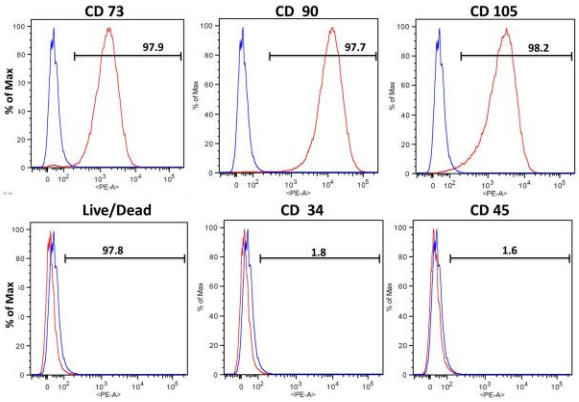

Figure 3: Flow cytometry analysis of the hBMSCs derived from the human bone marrow aspirate concentrated using Magellan® System (Arteriocyte): blue lines indicate fluorescence of isotypic control, and red lines indicate fluorescence signal of specific antigen. Plots are depicted with % maximum on y-axis, and PE fluorescence intensity for the indicated markers on the x-axis.

Flow-cytometry was used to establish the properties of the cultured hBMSCs derived from the concentrated BMA.

Flow-cytometry data demonstrates expression of typical adult MSCs cell surface markers such as CD 90, 73 and 105, as seen in Figure 3. The results show strong, over 97%, expression of MSC positive markers and no expression of hematopoietic cell markers, CD 34 and CD 45.

Thus obtained BMSCs were cultured using pellet culture technology by re-aggregating cells and culturing under chondrogenic conditions for 16 days before implantation in vivo to induce pre-cartilage templeate formation. The pellet culture system has been studied for its potential to regenerate cartilage as reported in several studies, continued culture of the pellet leads to hypertrophic cartilage template formation *in vitro* [19-21].

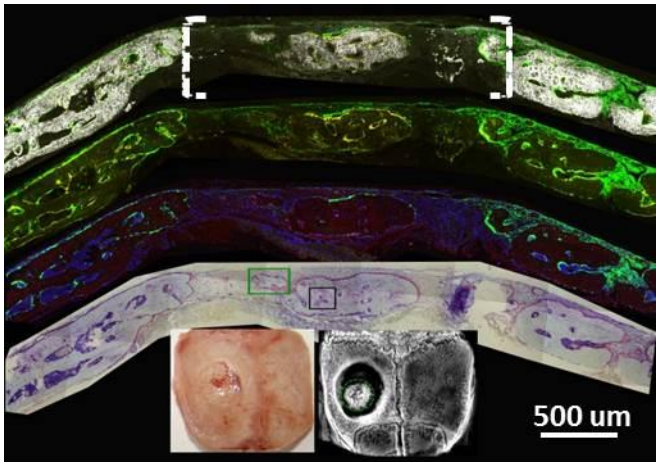

Figure 4: New bone morphogenesis by hBMSCs through endochondral ossification. From top, lanes 1-4 histological sections showing the localization and bone formation of the donor human cells versus mouse host cells. The brackets indicate approximately the original edges of the defect. Lane 1 shows dark field image indicating bone formation; lane 2 is the overlay of the green fluorescence showing mouse osteoblasts and the yellow Demeclocycline mineral stain; Lane 3 is the overlay of human specific nuclear stain and DAPI; and lane 4 showing Toluidine blue stain confirming cartilage formation. Digital and X-Ray images, placed at the bottom, showing bone formation in the central region of the defect**.**

Our preliminary in vivo investigation of the hBMSCs potential to form bone through a hypertrophic-cartilage template was carried out using NSG/Col3.6Tpz reporter-mouse defect model. The transgenic reporter mouse containing a Col3.6 promoter driven green fluorescent protein (GFP) is used for ease of tracking and distinguishing host cells versus implanted hBMSCs. In this study, a 16-day old chondrogenic pellet was implanted on the right side of the parietal lobe in a 3.5 mm size calvarial defect and analyzed after 8 weeks.

Although mineralized tissue formation was limited to the central region of the defect, as seen in the X-Ray image in figure 4, this study show the possibility of regenerating bone from the chondrogenically cultured hBMSCs. The mineralized tissue formation was visualized by dark field imaging of the histological section (Figure 4, lane 1). Mineralization was further confirmed using Demeclocycline mineral labeling (Figure 5A). The lack of green fluorescence within the pellet region is direct evidence that hBMSCs are responsible for the newly formed mineralized tissue. Toluidine blue staining and cellular hypertrophy observed in Figure 6 indicate that the new mineralized tissue formation occurred through endochondral ossification process.

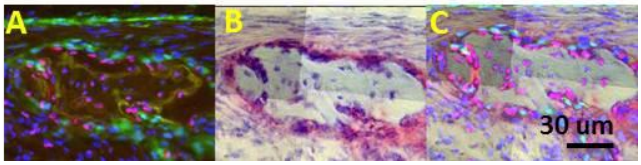

Figure 5: Mineralization and Alkaline Phosphatase (AP) activity of human donor cells versus mouse host cells. (A) Showing yellow Demeclocycline mineral stain in the inner section of implant, both human donor cells (red) and mouse osteoblasts (green) are found on the periphery of implant, while only human donor cells are found in the inner sections of implant. (B) Showing AP staining primarily on the periphery of implant. (C) Is an overlay of lane 1 and 2, both human donor cells and mouse osteoblasts present on the periphery of implant indicating both are involved in AP expression.

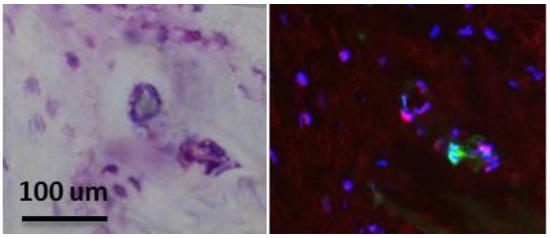

Figure 6: Hypertrophic-cartilage template formation by human donor cells: (left) Toluidine blue staining showing hypertrophic chondrocyte morphology; (right) the presence of human donor cells, in red color, in the hypertrophic region of the implant.

IV. CONCLUSION

Through this pilot study, a potentially clinical strategy for bone regeneration via endochondral ossification has been proposed. As part of the strategy implementation, human bone marrow aspirate was successfully concentrated for BMSCs via a minimally manipulated process. We found that the in vitro formed pre-cartilage template is capable of inducing mineralized tissue formation via a cartilage-mediated process. The in vivo data further suggest donor cell induced bone formation. Although this cell-based approach resulted in partial defect repair, a biomaterial scaffold with high-density cell population may help to obtain bone regeneration throughout the defect area. To our knowledge, this is the first time a translational approach, modeled after ACI, for bone defect repair via the endochondral ossification route is proposed and partially implemented.

Acknowledgment

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