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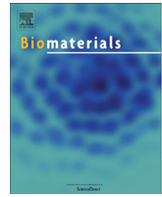

# The effect of mesenchymal stem cell sheets on structural allograft healing of critical sized femoral defects in mice

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ABSTRACT

Structural bone allografts are widely used in the clinic to treat critical sized bone defects, despite lacking the osteoinductive characteristics of live autografts. To address this, we generated revitalized structural allografts wrapped with mesenchymal stem/progenitor cell (MSC) sheets, which were produced by expanding primary syngenic bone marrow derived cells on temperature-responsive plates, as a tissue-engineered periosteum. *In vitro* assays demonstrated maintenance of the MSC phenotype in the sheets, suggesting that short-term culturing of MSC sheets is not detrimental. To test their efficacy *in vivo*, allografts wrapped with MSC sheets were transplanted into 4-mm murine femoral defects and compared to allografts with direct seeding of MSCs and allografts without cells. Evaluations consisted of X-ray plain radiography, 3D microCT, histology, and biomechanical testing at 4- and 6-weeks post-surgery. Our findings demonstrate that MSC sheets induce prolonged cartilage formation at the graft-host junction and enhanced bone callus formation, as well as graft-host osteointegration. Moreover, a large periosteal callus was observed spanning the allografts with MSC sheets, which partially mimics live autograft healing. Finally, biomechanical testing showed a significant increase in the structural and functional properties of MSC sheet grafted femurs. Taken together, MSC sheets exhibit enhanced osteogenicity during critical sized bone defect repair, demonstrating the feasibility of this tissue engineering solution for massive allograft healing.

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## 1. Introduction

Massive bone grafting is commonly used in both military and civilian orthopedic reconstruction surgeries to repair critical sized defects due to trauma or tumor resection. Both experimental and clinical studies have demonstrated that live autologous grafts are superior to processed or devitalized allografts when analyzing bone incorporation, repair, and remodeling [1,2]. However, due to the limited availability of autologous bone grafts, and problems with chronic pain at the donor site [3,4], processed allografts remain an attractive substitute for bone grafting. It has long been recognized that there are several fundamental differences between a live autograft and a processed allograft including: i) autografts contain living cells that can produce new bone [5,6]; ii) autografts contain growth factors and osteogenic substances; and iii) the host can effectively remodel the autograft but does not resorb the processed allograft [7,8]. Clinically, more than 500,000 Americans require bone allografts annually, although due to the lack of appropriate osteogenesis, angiogenesis and remodeling of structural allografts, the 10-year post-implantation failure rate is 60% [9]. Thus, the major challenge to the field of bone grafting is to identify the central factors, cells, and/or environmental cues that govern normal autograft healing and to devise a method to achieve the same healing results when utilizing processed allografts.

There are at least three experimental approaches that have been devised to enhance the osteogenic response of structural allografts. The first strategy involves the delivery of recombinant protein growth factors locally with the allograft (i.e. bone morphogenetic proteins; BMPs) [10,11] or systemically (i.e. parathyroid hormone; PTH) [12] via treatment of the host. Despite the applicability of these approaches, sustaining local growth factor delivery and regulating the influence of growth factors on non-targeted cell populations remains a substantial barrier to tissue engineering and has presented significant safety concerns and complications in some clinical settings [13–15]. While treatments with osteogenic factors show promise, they also rely heavily on the host to provide the appropriate number of cells with which to mount an adequate osteogenic healing response. These host requirements exist

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regardless of the treatment regimen, dose, or delivery method. The second strategy utilizes viral and/or non-viral targeted osteogenic or angiogenic gene delivery approaches [16,17]. While a few of the gene transfer methods for critical defect healing have reached orthopedic pre-clinical trials, there remain significant regulatory, efficacy, and safety concerns with the use of viral agents or genetically altered cells for implantation into patients. The third and possibly the most exciting strategy to promote or enhance devitalized allograft incorporation and critical sized bone defect healing involves the use of the patient's own mesenchymal stem/progenitor cells (MSCs). This approach, currently in experimental phases, entails the isolation of bone marrow derived MSCs from a patient and seeding the cells on a graft or scaffold prior to transplantation. While this approach has demonstrated some success with marrow derived MSCs, experimental strategies have primarily been focused on ensuring the appropriate engraftment of MSCs into host bone when using scaffold or allograft carriers [18–21].

The most well studied and clonogenic MSC populations commonly express a number of cell surface markers including CD105, CD90, CD106, CD146, CD29, and CD166, and lack the expression of hematopoietic lineage markers, including CD34, CD11b and CD45 [22,23]. Cell surface markers such as SSEA4, CD105, Sca1, and Stro-1 have been successfully used to enrich MSC populations [24–26]. MSC populations, however, tend to lose their multipotency and capacity to proliferate with increasing passages in culture, suggesting senescence [27]. Growth factors such as FGF2 and FGF4 have been utilized to promote the expansion of MSC populations [28,29], while some of the well known stem cell transcriptional regulators (*SOX2*, *OCT4*, *NANOG*) have also been used to promote the maintenance and multipotency of MSCs in culture [30,31]. In this study, we utilized a set of these stromal cell markers to evaluate changes in cellular behavior during cell isolation and generation of MSC sheets.

Cell sheet technology has been applied in tissue engineering for several years to regenerate damaged soft tissues, including corneal epithelia [32], periodontal ligament cells [33], bladder epithelia [34], kidney glomeruli [35], oesophageal epithelia [36], myocardial cells [37] and hepatocytes [38]. Cell sheet technology consists primarily of a "thermo-responsive culture dish" which enables reversible cell adhesion to and detachment from the dish surface by controlling the hydrophobicity of the surface [39]. This allows for a non-invasive harvest of high-viability cells in an intact monolayer that includes any deposited extracellular matrix (ECM). The ECM provides the necessary structural and adhesive properties for maintaining cell sheet integrity and resisting deforming forces during transplantation. Through implementation of this technology, MSC sheets can be easily generated and transplanted to the site of large bone defects, acting as a tissue-engineered periosteum surrounding the implanted graft. We expect that the utilization of MSC sheets will enhance allograft incorporation into the host bone without necessitating the use of a biodegradable scaffold.

In this study, we first utilized temperature-responsive culture dishes in the generation of mouse MSC sheets *ex vivo*. Second, we used these cells to test the hypothesis that allografts used in conjunction with MSC sheets possess superior osteogenic properties and enhanced bone defect healing during skeletal repair in a pre-clinical mouse model of critical sized femoral allograft.

## 2. Materials and methods

### 2.1. Study design

Experiments were designed to include six male mice samples per group at different time-point. Host mice carrying allografts were randomly and equally assigned to either control, MSC-seeding or MSC-sheet groups. The sample size for Micro-CT and biomechanical testing was determined by power analysis based on our pilot experiment data.

### 2.2. Mouse strains

C57BL/6J mice were purchased from Jackson Laboratory. Allogeneic bone grafts were obtained from mice of the 129/J strain for implantation into C57BL/6J mice. The University of Rochester Committee of Animal Resources approved all animal surgery procedures.

### 2.3. Bone marrow MSC isolation

Bone marrow derived MSCs were isolated from 6-week old C57BL/6J mice using a modified version of a previously described protocol [40]. In brief, mice were sacrificed by $CO_2$ asphyxiation followed by cervical dislocation. Femurs and tibiae were dissected from the surrounding tissues. The epiphyseal growth plates were removed and the bone marrow was collected by flushing with αMEM culture medium containing 100 U/ml Penicillin, 100 μg/ml streptomycin and 10% FCS (Hyclone) with a 25-gauge needle. Single cell suspensions were prepared by gently mixing the cells with a pipette followed by filtration through a 70-μm strainer. $2–5 \times 10^7$ total bone marrow cells were obtained using 5-10 mice. The EasySep Mouse Mesenchymal Progenitor Enrichment Kit (Stem Cell Technologies) was used as a negative selection strategy to remove unwanted cells of the hematopoietic and endothelial lineages (CD45, TER119 antibodies linked to magnetic beads) according to manufacturer's instructions. Remaining viable cells were counted using trypan blue staining and re-seeded at 500 cells/$cm^2$ and grown in Mesenchymal Stem Cell Growth Medium for expansion. Media were replaced 3 days and refreshed 6 days post adherence. At day 7, cells were harvested for colony forming units-fibroblast (CFU–F) assay and a second *in vitro* expansion. To monitor the colony formation, some of the colonies were fixed using 4% PFA and stained with 0.2% crystal violet solution for 1 h before they were washed with water and images were captured. Colonies consisting of at least 50 cells were counted as CFU-Fs.

### 2.4. Generation of MSC sheets

When cells reached 80% confluence during the second expansion, some of the cells were then harvested for flow cytometry analysis and the rest of the MSCs were re-seeded at 3 different densities (200, 100, 50 cells/$mm^2$) on thermo-responsive 6-well culture plates with UpCell surface (Thermo Scientific, Cat. 174901) to generate cell sheets. Once the cells reach 100% confluence (monolayer cell sheet), cell sheets were harvested for flow cytometry, RNA isolation, and *in vivo* implantation. MSCs at 80% confluence were used for RNA isolation and flow cytometry as a control.

### 2.5. Cell viability assay

After 24 h of culture, newly formed MSC sheets were trypsinized using 0.25% trypsin-EDTA (Invitrogen) and then stained with 0.4% trypan blue solution. Cell viability was estimated by cell counting via hemocytometer in which non-viable cells appear blue, viable cells are unstained.

### 2.6. Flow cytometry analysis

Following the second expansion, cells were trypsinized and stained with 0.2ug of CD105 antibody conjugated to FITC, 0.2ug of CD29 antibody conjugated to PE, and 0.2ug of Sca-1 antibody conjugated to APC (ebioscience). After washing in FACS buffer, the cells were analyzed on an LSR-II (Beckton Dickson) and the data were further analyzed using FlowJo software (Tree Star). MSCs at 80% confluence and cell sheets were also collected for Flow cytometry analysis using the CD105 antibody.

### 2.7. Real-time PCR analysis

Total RNA was isolated from MSCs using RNeasy Mini Kit from Qiagen Inc. One microgram of RNA was subjected to reverse transcription using the iScript cDNA synthesis Kit (Bio-Rad). The obtained cDNA was then amplified via real-time PCR using an ABI 7500 Real-time PCR System (Applied Biosystems) and SYBR® Green Real-time PCR Supermix (Bio-Rad). The primers used for real-time PCR are listed in Table 1, and β-actin was used as the housekeeping gene. Quantification of the relative expression levels of these target genes was achieved by normalizing to β-actin using the ΔΔCt method.

### 2.8. Devitalization of bone allografts

Eight week-old male 129/J mice were used for donation of devitalized allografts. Briefly, mice were euthanized and a 4 mm mid-diaphyseal segment was removed from each femur by osteotomy using a rotary Dremel with custom circular diamond blades. Allograft segments were flushed of the bone marrow using 25-guage needles, the periosteal tissues were manually stripped, and the bone grafts were washed repeatedly in 70% ethanol for at least 4 h. The allografts were then stored in 100% ethanol at $-80$ °C for at least 7 days to complete the devitalization process.

### 2.9. Wrapping of MSC sheets on allografts

Following MSC sheet formation, cell sheets were wrapped onto devitalized allografts. Briefly, the cultured MSC sheets were covered by a cell transfer membrane (Thermo Scientific, Cat.1749016) and kept at 25 °C for 10 min. After the cell layer adhered to the membrane, it was detached carefully from the thermo-responsive culture plate. The cell sheet and membrane were then placed in a new larger dish with the cell layer facing up, the devitalized room temperature allografts were placed

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