Introduction: Heterogeneous differentiation of human embryonic stem cells (hESC) limits the potential use of hESCs for cell-based therapies and studies of specific differentiation programs. Here, we describe an in vivo commitment to the osteogenic lineage of mesenchymal cells from hESCs. The process of endochondral ossification was identified by cartilaginous tissue formation followed by blood vessel recruitment and calcification in poly(lactic-co-glycolic acid)/poly(L-lactic acid) (PLGA/PLLA) polymer scaffolds. Direct bone tissue formation was induced in hydroxyapatite (HA) containing PLGA/PLLA composite scaffolds. In addition, hESC-MSCs displayed enhanced osteogenic potential compared to the bone marrow–derived mesenchymal stem cells and contributed healing of critical-size mouse calvarial defects in 8 weeks as shown by X-ray analysis and histology. In view of the limited available cell sources for bone regeneration applications, our results show the significant potential of hESCs for musculoskeletal tissue engineering applications. Moreover, our results indicate that the ossification mechanisms of these cells can be regulated by the scaffold mediated microenvironments.

Materials and Methods: Mesenchymal Cell Derivation
hESC-MSCs were generated as previously reported[1].

Cell staining, Histology, and Immunostaining
Tissue were cut into 5 μm sections and stained with hematoxylin and cosin or alcin blue. For immunostaining, the sections were blocked with 5% normal goat serum in PBS, and incubated with rabbit polyclonal antibodies against types I, II, and X collagen (RDI, Flanders, NJ), and osteocalcin (Biogenesis). Sections were then incubated with either FITC- or Texas Red-conjugated goat anti-rabbit secondary antibody (all 1:100 dilutions) for 1 hour. Immunohistochemistry was performed with Histostain-SP kit (Zymed Laboratories) with rabbit anti-Runx2 and rabbit anti-β-catenin (Cell Signaling) primary antibodies with 1:50 and 1:100 dilutions, respectively.

Polymer Scaffold Preparation
HA composite scaffold composed of PLLA and PLGA were fabricated by dissolving PLGA and PLLA in chloroform to yield a solution of 5% (wt/vol) polymer with or without HA particles (1% or 5% wt/vol).

Transplantation into Athymic Nude Mice
hESC-MSCs were expanded (P9), seeded (3×10^6) onto the polymer sponges, and cultured for 10 days in osteogenic condition. The cell seeded scaffolds were implanted subcutaneously into the dorsal region of 6-week-old athymic nude mice. Small electrical Dremel hand-drill will then be used to gently bore a 4 mm-wide cranial perforation up to the level of the dura mater in the right, non-suture associated parietal region. Cell-scaffolds were implanted and the skin was closed with a running 7-0 nylon suture. Calvaria were X-rayed using a Faxitron X-Ray and further processed for histology.

Results: We investigated in vivo response of hESC-MSCs in ectopic bone regeneration model. Scaffolds seeded with cells were incubated in osteogenic medium for 10 days to allow cell adhesion and growth before implantation. The cell laden scaffolds were then implanted subcutaneously in the dorsal region of 6-week-old athymic nude mice and analyzed after 4 and 8 weeks (1A). Histological examination of the implants revealed formation of connective tissues within the scaffold (1B). At the 4-week time point, the extent of osseous tissue formation was HA-concentration dependent. The 5% HA-PLGA/PLLA composite scaffold showed maximum osseous tissue area, followed by 1% HA-PLGA/PLLA composite scaffolds. In contrast, cross sections of the PLGA/PLLA scaffold showed largely cartilaginous tissue, with minimal bone tissue formation (1B, C). After 8 weeks complete bone formation with no evidence of teratoma or marked inflammation around the site of implant was observed in all scaffolds.

Bone formation of hESC-MSCs after 4 weeks indicated two distinct processes of bone formation depending on the scaffold composition (1C). The capacity to develop a mineralized matrix and bone formation has been implicated in β-catenin activity and transcription factor, Runx2. Immunostaining results showed minimal levels of β-catenin and Runx2 in the PLGA/PLLA scaffold, while the presence of HA in the scaffold caused an HA concentration-dependent stabilization of β-catenin and Runx2 transcription activity (1D).

We further investigated whether hESC-MSCs can contribute to the healing in critical-size defect model mouse calvaria (1E). After eight weeks, X-ray analysis showed that the hESC-MSCs seeded onto the 5% HA PLGA/PLLA scaffolds produced high density mineralization in the area of defect (9A). Histology of cross-section showed characteristic of neo-bone morphology in all implants with hESC-MSCs, indicating that the cells contributed healing of critical-size calvarial defects (1F).

Discussion: The extracellular microenvironment plays a significant role in controlling cellular differentiation. In addition to providing structural stability for developing tissues, scaffolds with desirable biochemical and biophysical cues can direct cellular function differentiation commitment. Recent literatures indicate possible roles of Wnt/β-catenin in determining differentiation and commitment of mesenchymal cells [2]. In the present study, we demonstrated that the mechanisms of endochondral or intramembranous bone formation are modulated by scaffold properties, indicating the importance of scaffold parameters on the modulation of hESC-MSCs in ectopic bone regeneration model. Strategies employing biomaterials to direct the differentiation commitment of hESC-MSCs will further elucidate functional capabilities of these cells.


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Figure 1. Developmental potential of hESC-MSCs in vivo. (A) Gross image of in vivo engineered tissues after 8 weeks implantation. (B) H&E staining of the implanted scaffolds after 4 weeks and 8 weeks. (C) Cells seeded on PLGA/PLLA scaffold show positive for alcain blue staining for negatively charged proteoglycans and while 5% HA-PLGA/PLLA composite scaffolds show direct bone formation by alizarin red mineral staining after 4 weeks of implantation. (D) HA-concentration dependent β-catenin and Runx2 activity: hESC-derived mesenchymal heal the critical size cranial-defect of mouse skull. (E) X-ray and (F) H&E staining. HA-0: PLGA/PLLA scaffold, HA-1: PLGA/PLLA scaffold with 1% w/v HA; HA-5: PLGA/PLLA scaffold with 5% w/v HA. * P< 0.05, ** P<0.01.