Human Bone Grafts Seeded with Induced Pluripotent Stem Cells-Mesenchymal Progenitors for Spine Fusion in a Rat Model

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INTRODUCTION

- Posterolateral lumbar arthrodesis is a common fusion procedure, with a ~40% failure rate.[1]
- Bone grafts and substitutes are widely used, with nearly 2.2 million bone grafting procedures performed annually worldwide.[2]
- Autologous bone remains the "gold standard", but it is associated with significant donor morbidity and supply can be limited in patients with poor bone quality secondary to comorbidities.

- Allograft bone is more easily obtained, but has limited osteogenic and angiogenic potential due to decellularization and demineralization.
- One strategy to convert allograft bone to an osteogenic equivalent to autograft is to seed it with stem cells capable of bone formation.
- Induced Pluripotent Stem Cells (iPSCs) are a novel stem cell source that is:
  - generated from the patient's own cells (autologous),
  - has unlimited proliferation potential, and
  - the ability to become any cell in the body, including bone-forming cells

The aims of this study were to determine whether:

(1) human iPSC-mesenchymal progenitors (iPSC-MPs) can adhere, remain viable, and undergo osteogenic differentiation on human cancellous bone allograft *in vitro*; and

(2) iPSC-MPs seeded bone allografts would lead to greater bone formation in a rat spine fusion model compared to an unseeded graft.

METHODS

Generation of iPSC-MPs: Human skin fibroblasts (50-year-old female, ATCC) were reprogrammed under low oxygen (5%) via mRNA-transfection (Oct4, Sox2, Klf4, Lin28A, Nanog and c-Myc).[3] iPSCs were characterized for pluripotency, expanded, and induced to mesenchymal progenitors (iPSC-MPs) using a published protocol.[4]

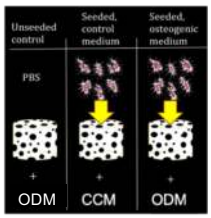
Characterization of iPSC-MPs:

- Flow cytometry for CD34, CD45, CD90, CD73, and CD105 surface markers was performed and compared to bone marrow derived MSCs (BM-MSCs).
- Monolayer osteogenic differentiation with Alizarin Red stain at 28 days in different culture media: Complete Culture Media (**CCM** (α-MEM with 16.5% FBS, 2 mM L-glutamine, and 1% penicillin streptomycin) or Osteogenic Differentiation Media (**ODM,** CCM with 10 nM dexamethasone, 20 mM β-glycerophosphate, and 50 μM L-ascorbic acid 2-phosphate). 50,000 iPSC-MPs/well were plated in 6-well plates and incubated at 37°C, 5% CO_2 in either CCM or ODM.

Bone allograft preparation

- Cancellous bone cores (4.8mm^2 ~400mg)
- were obtained from the cadaveric humeral head of a 51 year-old female (Lonetree Medical Donation, LLC).

- Cores were decellularized, freeze-dried, and sterilized.[5]
- Cores were divided in three groups and conditioned overnight in respective culture media before seeding with ± 1.2 x 10^6 iPSC-MPs (passage 5 or 8).



***In vitro* Analysis:**

- Live/Dead stain: Viability and cell attachment at 3 hours, 3 days, and 28 days after cell seeding.
- Alkaline phosphatase (ALP) activity: measured at 28 days.
- Histology: Hematoxylin & Eosin (H&E) and immunohistochemical staining for osteocalcin at 28 days.

***In vivo* Analysis**
(Rat Spinal Fusion Model):

Twelve 12-wk-old, male, Nude Outbred Immunodeficient rats underwent a 1-level posterolateral intertransverse fusion procedure of L4-L5 lumbar vertebrae with bone graft placed (**Figure 1**).
These were equally divided in three treatment groups (n=4/group): **A- iPSC-MPs in CCM; B- iPSC-MPs in ODM; C- unseeded in ODM.**

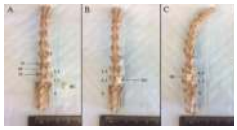


Figure 1. (A-B) Dorsal and (C) lateral cephalocaudal view of cadaveric adult rat spine and bone graft positioning. TP, transverse process; SP, spinous process; FJ, facet joint; S, sacrum; BG, bone graft.

- At 12 weeks, rats were euthanized.
- MicroCT analysis (Siemens Inveon): spine fusion masses between L4-L5 evaluated for BV/TV.
- Histology: H&E and Alcian-Blue-Hematoxylin (ABH) were performed.
- Immunohistochemistry (IHC): Osteocalcin (Abcam) and Human Nuclei (Millipore) were performed.

RESULTS

IPSC-MPs characterization and *In vitro* analysis of iPSC-MPs on bone cores

- iPSC-MPs had >95% expression of mesenchymal cell surface markers CD90 and CD73. Interestingly, CD105 expression was 22.6 ± 8.48%.
- At 28 days, iPSC-MPs cultured in ODM stained positive for Alizarin-Red, indicating mineralization.
- iPSC-MPs were found attached to the bone cores at 3 hours and were still viable at 28 days post-seeding. Live cells were most abundant at the periphery compared to the center of the cores. No live cells were seen on the unseeded bone cores (**Figure 2**).
- At 28 days, ALP activity was higher in the ODM seeded group (P=0.0205) (**Figure 3**) and showed greater tissue formation (**Figure 4**).

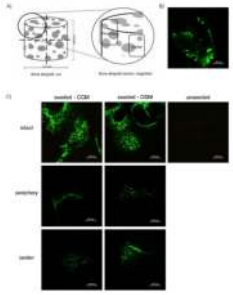


Figure 3. Alkaline phosphatase (ALP) activity at 28 days (nmol p-nitrophenol phosphate/μg DNA).

Figure 2. Live (green)/dead (red) staining of seeded and unseeded bone cores cultured in media at 100X. (A) Bone cores were cut into quarters and halves for analysis. (B) 3 hours culture. (C) 28 days culture.

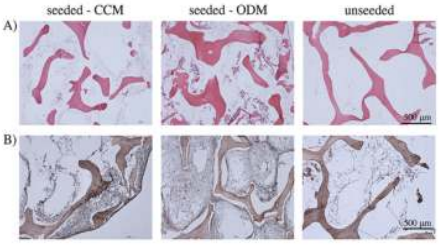


Figure 4. (A) H&E staining and (B) immunostaining for osteocalcin of bone cores at 10X after culture in CCM or OSM for 28 days.

***In vivo* analysis of iPSC-MPs on bone allograft**

- At 12 weeks, all bone grafts were in place and did not display signs of synovial reaction or inflammation.
- Micro-CT showed no intergroup difference for BV, TV and BV/TV: Group A, BV/TV 0.12 ± 0.03; Group B, BV/TV 0.12 ± 0.03; and Group C, BV/TV 0.12 ± 0.02 (P≥0.05).
- H&E, ABH and IHC staining for osteocalcin showed marginally superior intramembranous (IO) and endochondral (EO) ossification in the seeded groups, particularly the ODM group (**Figures 5-6**).
- A small number of implanted human iPSC-MPs were found at the fusion site after 12 weeks (**Figure 7**).

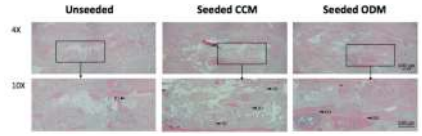


Figure 5. H&E staining at 12 weeks. Lamellar bone is dark pink provided by the bone graft, connective tissue in light pink, and porous trabecular bone graft in white. Areas of intramembranous (IO) show deposition of unorganized collagen fibers. Areas of endochondral ossification (EO) show chondrocytes forming the cartilage framework that will be replaced by mature trabecular bone or lamellar bone.

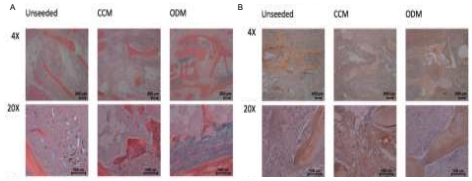


Figure 6. (A) Alcian Blue-Hematoxylin staining at 12 weeks post-implantation. Some endochondral ossification (EO) was present in all groups, but greatest in the seeded ODM group. **(B) Osteocalcin staining at 12 weeks post-implantation.** Positive staining of bone graft and some surrounding tissue.

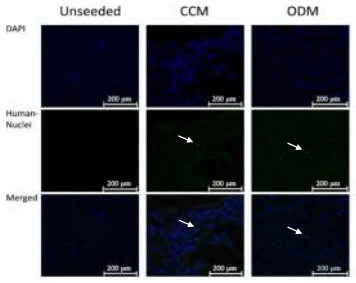


Figure 7. Immunofluorescence for Human-Nuclei at 12 weeks. *Top Row:* DAPI stain, showing nuclei of all the cells in the area. *Middle Row:* Human-Nuclei antibody, showing positive nuclei of iPSC-MPs in the area, found in the CCM and ODM groups. *Bottom Row:* merged images, showing overlapping of DAPI stain and Human-Nuclei antibody. Few human iPSCs-MPs were present after 12 weeks of implantation *in vivo*.

CONCLUSION

IPSC-MPs can attach, remain viable, and undergo osteogenesis when seeded on bone allografts. *In vivo*, at 12 weeks, human bone grafts seeded with iPSC-MPs in CCM or ODM showed slightly higher osteogenic capacity compared to unseeded grafts, as reflected by ongoing IO and EO. Seeded grafts had more cellular connective tissue with trabecular bone in different stages of maturity. Interestingly, the micro-CT data showed no intergroup difference. Scarce human iPSC-MPs survived, but, whether these differentiated or not towards osteoblasts remains unknown. It is possible that iPSC-MPs secrete paracrine factors that enhance tissue regeneration from endogenous cells. This study shows that human iPSC-MPs are a novel candidate stem cell source that could pave the way to create patient-specific bone constructs for orthopedic regenerative medicine, however, further research on their survival and fate *in vivo* is warranted.

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DISCLOSURES:
The authors report no conflicts of interest in relation to this study.

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