Osteogenic Differentiation of Induced Pluripotent Stem Cells for Musculoskeletal Regenerative Medicine

Feini Qu[1,2,3], Omolabake Oyebamiji[1,2], Farshid Guilak[1,2]
[1]Washington University, St. Louis, MO, [2]Shriners Hospitals for Children, St. Louis, MO, [3]University of Washington, Seattle, WA
feiniqu@uw.edu

Disclosures: F. Qu (N), O. Oyebamiji (N), F. Guilak (Cytex - 3A, 4, Agathos - 1)

INTRODUCTION: Humans have limited regenerative potential of musculoskeletal tissues following limb loss. To this end, successful attempts to regrow missing limbs could significantly improve the prognosis for amputees. The murine digit has been used to study mammalian limb regeneration, where stem/progenitor cells regrow the digit tip after distal amputation [1–4]. Previously, we found that osteoprogenitor cells play a critical role in digit regeneration [5], but the mechanisms controlling skeletal elongation and patterning remain unclear. Recent studies suggest that activating elements of a limb-like developmental program, such as *Hox* genes, may be critical for bone outgrowth [4,6]. We hypothesize that *Hox* gene expression in osteoprogenitors is required for proper bone morphogenesis after digit amputation. To test whether *Hox* genes modulate skeletal patterning *in vitro*, we first sought to develop a procedure to derive osteoblasts from murine induced pluripotent stem cells (iPSCs).

METHODS: Cell Culture: Murine iPSCs were differentiated toward a mesenchymal state using a high-density micromass to produce pre-differentiated iPSCs (PDiPSCs; **Fig. 1A**), a protocol that we previously developed for iPSC chondrogenesis [7,8]. PDiPSCs at passage 3 were plated onto gelatin-coated 24-well plates and exposed to the following media conditions: growth (Control; DMEM-HG with 10% fetal bovine serum [FBS], 1% penicillin/streptomycin [PS], 1% insulin, transferrin, and sodium selenite [ITS+], 1% MEM nonessential amino acids [NEAA], 0.1% β-mercaptoethanol, 50 µg/mL ascorbate, 40 µg/mL proline, 4 ng/mL basic fibroblast growth factor), osteogenic (Osteo; α-MEM with 10% FBS, 1% PS, 50 µg/mL ascorbate, 100 nM dexamethasone, 10 mM β-glycerophosphate), or osteogenic with supplements (Osteo+; Osteo with ITS+, NEAA, β-mercaptoethanol). As a positive control, MC3T3 murine pre-osteoblasts (passage 7; ATCC CRL-2593) were cultured in growth (Control; α-MEM with 10% FBS, 1% PS) and Osteo media. Media was changed every 3–4 days. Evaluation: At 0, 14, 21, and 28 days after osteogenic culture, cells were fixed and stained with Alizarin Red (AR) and Oil Red O (ORO) to assess calcium deposition and lipid accumulation, respectively. Cells were imaged with bright field microscopy using a Cytation5 plate reader and the staining area fraction (%) was quantified using ImageJ (n=3–4/group/time). To evaluate the expression of osteogenic (*Runx2*, *Sp7*, *Bglap*) and adipogenic (*Adipoq*) gene markers, RNA isolation, cDNA synthesis, and real-time polymerase chain reaction were performed for cells in 12-well plates at 0 and 21 days of osteogenic culture (n=3–4/group/time). Analysis was performed with the ΔΔCT method, with all samples compared to their respective Control group at day 0 and *Rn18s* used as the endogenous control gene. BMP-2 Supplementation: To improve osteogenesis, PDiPSCs were cultured for up to 28 days in Osteo+ media with recombinant human/mouse/rat bone morphogenetic protein-2 (BMP-2) at 0 (vehicle), 10, and 50 ng/mL. AR and ORO staining and analysis were performed at 14, 21, and 28 days as previously described (n=3/group/time). Significance was assessed by 1- or 2-way ANOVA with Tukey's post-hoc test ($p<0.05$).

RESULTS: Murine PDiPSCs were successfully differentiated toward the osteoblast lineage, similar to MC3T3 murine pre-osteoblasts. Both PDiPSCs and MC3T3s produced mineralized cultures when exposed to standard osteogenic media (Osteo) as measured by Alizarin Red staining (**Fig. 1**). While additional supplements to the Osteo media (Osteo+) significantly enhanced PDiPSC osteogenesis, Alizarin Red staining remained lower than MC3T3s in Osteo media at 21 and 28 days ($p<0.05$, **Fig. 1C**). Osteo+ media also increased adipogenesis, as evident by Oil Red O staining (**Fig. 1B**). In contrast, there was no mineral detected in PDiPSCs and MC3T3s cultured in their respective Control media over time. However, PDiPSCs exposed to Control media readily accumulated lipid, a feature that was absent in MC3T3s. The osteogenic transcription factors *Runx2* and *Sp7* (osterix) and the adipokine *Adipoq* (adiponectin) were upregulated in PDiPSCs, but not MC3T3s, at 21 days relative to day 0, with *Sp7* significantly increased for PDiPSCs cultured in Osteo+ media compared to all other groups ($p<0.05$, **Fig. 2**). Expression of *Bglap* (osteocalcin) was upregulated relative to day 0 for all groups, with PDiPSCs and MC3T3s exhibiting the greatest fold change when cultured in Osteo+ and Osteo media, respectively ($p<0.05$, **Fig. 2**). BMP-2 supplementation improved PDiPSC osteogenesis in a dose- and time-dependent manner (**Fig. 3**). While 50 ng/mL of BMP-2 was the most effective at stimulating mineralization and reducing lipid accumulation compared to all other groups at early time points ($p<0.05$, **Fig. 3B**), 10 ng/mL of BMP-2 produced similar results after 28 days.

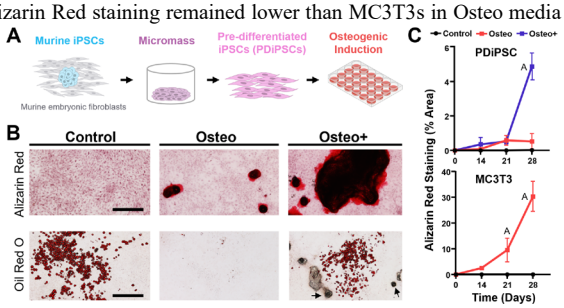

Fig. 1. (**A**) Schematic of iPSC differentiation. (**B**) PDiPSCs stained with Alizarin Red and Oil Red O after 28 days of culture. Arrows show mineralized nodules. Scale: 0.5 mm. (**C**) Alizarin Red staining (% area) of PDiPSCs and MC3T3s over time (n=3–4/group/time, mean ± SD). A: $p<0.05$ vs. all other groups.

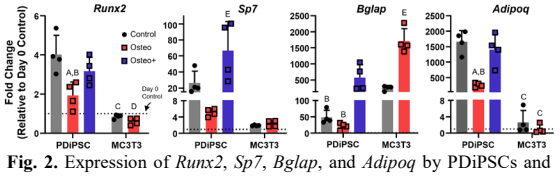

Fig. 2. Expression of *Runx2*, *Sp7*, *Bglap*, and *Adipoq* by PDiPSCs and MC3T3s after 21 days of osteogenic culture, shown as fold change relative to Control at day 0 (dotted line) for each cell type (n=3–4/group, mean ± SD). A: $p<0.05$ vs Control. B: $p<0.1$ vs. Osteo+. C: $p<0.05$ vs. PDiPSC. D: $p<0.1$ vs. PDiPSC. E: $p<0.05$ vs. all other groups.

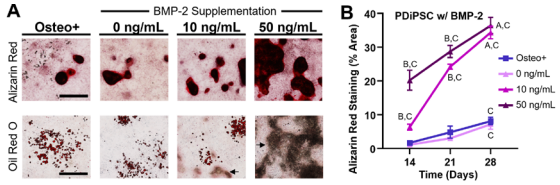

Fig. 3. (**A**) PDiPSCs stained with Alizarin Red and Oil Red O after 14 days of culture in Osteo+ media supplemented with various concentrations of BMP-2. Arrows show mineralized nodules. Scale: 0.5 mm. (**B**) Alizarin Red staining (% area) of PDiPSCs over time (n=3/group/time, mean ± SD). A: $p<0.05$ vs Osteo+ and 0 ng/mL. B: $p<0.05$ vs. all other media conditions. C: $p<0.05$ vs. all other time points.

SIGNIFICANCE: Limb loss affects an estimated 2 million Americans, significantly reducing quality of life. Therefore, strategies that restore the missing limb via the controlled regeneration of composite musculoskeletal tissues can substantially improve patient outcomes.

REFERENCES: [1] Lehoczky+, PNAS 2011. [2] Dawson+, Wound Repair Regen 2018. [3] Storer+, Dev Cell 2020. [4] Qu+, FASEB J 2020. [5] Qu+, ORS 2021. [6] Rux+, Dev Dyn 2017. [7] Diekman+, PNAS 2012. [8] O'Connor+, Tissue Eng Part A 2021. [9] Cignarelli+, Int J Mol Sci 2019. [10] Zubiría+, Life Sci 2020.

ACKNOWLEDGEMENTS: This work was supported by the NIH (K99 HD105203) and the ON/ORS Kick-Starter Grant.