Exploring Adipogenic-Myogenic Signaling in Engineered Human Muscle Grafts to Treat Volumetric Muscle Loss

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INTRODUCTION: Volumetric Muscle Loss (VML) is the loss of a large portion of skeletal muscle that overwhelms the natural repair mechanisms and results in functional impairment. Tissue engineered muscle grafts (TEMGs) provide a promising therapeutic strategy. We have previously developed grafts comprised of electrospun fibrin microfiber bundles[1] with C2C12s[2] or stem cells[3,4] to create TEMGs. Human primary myogenic progenitors (hMPs) are myogenic and can be patient specific, making them a promising myogenic cell source for TEMGs. However, skeletal muscle is a complex tissue and contains a diversity of cells that are non-myogenic. Due to their great potential for clinical translation, we investigate human adipose-derived stem cells (hASCs) as a non-myogenic support population for our TEMGs. Our objectives were to (i) assess the effectiveness of hMP-seeded fibrin microfiber bundles as TEMGs (hMP TEMGs) for the treatment of VML, and (ii) assess the effectiveness of hMPs co-cultured with hASCs i.e. (hMP-hASC TEMGs) for treating VML.

METHODS: To accomplish the first objective, 900,000 hMPs (passage 3; Lonza) were seeded on electrospun fibrin microfiber bundles to create hMP TEMGs. The hMP TEMGs were implanted into critical-sized VML defects in which ~30–50% of the tibialis anterior (TA) of Male NOD-SCID IL2rγ^{null} (NSG) mice aged 8–10 weeks was removed. We harvested at 28 days post-injury (DPI) and compared histologic sections with 'No Treatment' and contralateral 'Uninjured' groups. To accomplish the second objective, Passage 2 hASCs were seeded onto hMP TEMGs 7 days post-hMP seeding at 0.5% (4,500 hASCs), 5% (45,000 hASCs), and 50% (450,000 hASCs) of the hMPs concentration and compared to hMP TEMGs without hASCs to assess the effects of dosing at 21 days post-hMP seeding. Subsequently, hASCs were seeded (at 5% the concentration of hMPs) at 0 (6 hours), 3, and 7 days post-hMP seeding (D0, D3, D7) to assess the effects of co-culture timing. To assess the treatment potential of hMP-hASC TEMGs, grafts were implanted into the NSG TA VML model and compared to hMP TEMG treatment and no treatment histologically at 28 DPI. All animal and surgical procedures were performed in accordance with the Institutional Animal Care and Use Committee. All experiments have n = 4 except for qPCR which had n = 3-4. Statistical significance was determined using GraphPad Prism 5 software by either a one-way ANOVA with multiple comparisons and Bonferroni post-test or for qPCR unpaired two-tailed t-tests. Error bars represent standard error of the mean; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

RESULTS: TAs treated with hMP TEMGs were harvested, sectioned, and stained with DAPI/Myosin Heavy Chain (MHC)/Desmin or H&E **(Fig. 1A,B)**. Cross-sectional areas showed a significant increase between the no treatment and hMP TEMG-treated VML, and no significant difference between the hMP TEMG treated VML and uninjured TA **(Fig. 1C).** There was a slight increase in the number of new or regenerating myofibers in the hMP TEMG treated VML compared to the no treatment VML, and both had significantly more than the uninjured TA **(Fig. 1D)**. For the in vitro co-culture studies, the normalized MHC+ showed increasing stains up to 5% (hASCs), then a sharp decrease at 50% indicating a threshold in which hASCs exhibit pro-myogenic effects on hMPs TEMGs **(Fig. 2A)**. Similarly, the normalized Desmin+ areas showed the highest stains at 0.5% and 5% **(Fig. 2B)**. Delaying the seeding of hASCs until Day 7 significantly increased the MHC+ and Desmin+ staining **(Fig. 2C, D)**. To assess the impact of co-culture, we assessed gene expression of hMP TEMGs co-cultured with hASCs. Co-cultured hMP TEMG expressed decreased MYOG (as well as MYF5, MYF6, and MYOD1; $p>0.05$), decreased MYH1, and increased MYH7 **(Fig. 2E)**. The hMP TEMGs co-cultured with hASCs also expressed higher MKI67 **(Fig. 2F**; $p>0.05$**)**. Following VML treatment, TAs were weighed, sectioned, and stained with Masson's Trichrome to visualize collagen (scar tissue) area. The groups with hASCs showed significant overgrowth of the TAs **(Fig. 3A)**. The weights or the injured TAs were normalized by dividing the weight of the contralateral TAs of the same mice **(Fig. 3B)**. The percentage of Col IV+ area (relative to the TA cross-section area) increased when treated with hMP-hASC TEMGs **(Fig. 3C)**. Quantification of the number of hLamin AC+ cells showed an increase in survival of human cells at 28 DPI when treated with hMP-hASC TEMGs **(Fig. 3D)**. In sections stained with F4/80, iNOS, CD206, and DAPI, F4/80+/CD206+ signal was classified as M2 and F4/80+/iNOS+ signal was classified as M1. Quantification of the M2 area divided by the M1 area showed an increase in M2/M1 at 28 DPI when treated with hMP-hASC TEMGs **(Fig. 3E)**.

DISCUSSION: These data show that hMPs seeded on fibrin microfiber bundles and cultured for 21 days create TEMGs with mature myogenic cells and near full fiber coverage. These hMP TEMGs promote almost complete volume retention and regeneration *in vivo* in NSG mice TA VML. These data also suggest that increasing the concentration of hASCs to hMPs increases their promyogenic effects, however, high concentrations of hASCs in hMP-hASC TEMGs inhibit myogenesis. It was seen that temporal delay in co-culture of hASCs with hMPs promoted hASCs' pro-myogenic effects. Therefore, dosing and timing of co-culture play critical roles in promoting myogenesis. Specifically, hASCs promote a shift towards a more mature myogenic phenotype with higher proliferation (MKI67). In vivo, hMP-hASC TEMGs were seen to increase ECM deposition, increase cell survival, and promote a skew towards an M1 phenotype. These benefits were accompanied by a large tumor-like growth. This was not observed in prior studies with hASCs only[3] or with hMPs only suggesting an interesting interplay in the cell-cell signaling. One caveat is that these studies were performed in immunocompromised NSG mice. Further work will investigate the impact of this interplay in immunocompetent animals.

SIGNIFICANCE/CLINICAL RELEVANCE: This work indicates a possible composition of TEMGs for the treatment of VML that is translatable and an alternative to autologous tissue graft that does not results in donor site morbidity.

REFERENCES: 1. Zhang, S. et al. Biomaterials, 35:3243–3251 (2014). **2.** Gilbert-Honick, J. et al. Biomaterials 164:70–79 (2018). **3.** Gilbert-Honick, J. et al. Cell Transplant 27:1644–1656 (2018). **4.** Somers, S. M. et al. Bioengineering 9:693 (2022).

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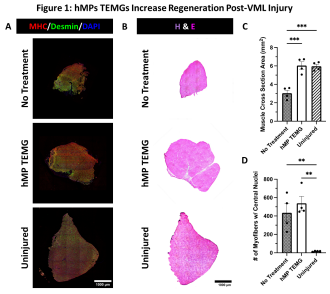

Figure 1. (A) Representative TA cross-sections at 28 DPI stained for DAPI, MHC, and Desmin. (B) Representative TA cross-sections stained with H & E. (C) Quantification of muscle cross section area in mm^2. (D) Quantification of the number of myofibers with centrally located nuclei.

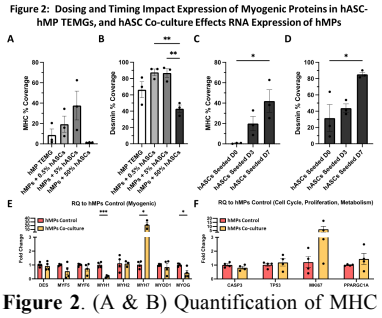

Figure 2. (A & B) Quantification of MHC and Desmin on hMP TEMGs co-cultured with hASCs at various concentrations. (C & D) Quantification of MHC and Desmin on hMP TEMGs seeded with 5% hASCs on D0, D3, or D7, then cultured for 21 days post-hMP seeding. (E) qPCR quantification for myogenic transcripts. (F) qPCR quantification for cell cycle transcripts.

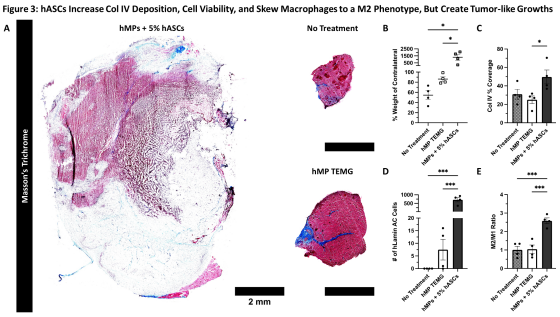

Figure 3. (A) Representative TA cross-sections at 28 DPI stained with Masson's Trichrome. (B) Quantification of weight % of contralateral (uninjured) TA. (C) Quantification of TAs Col IV+ area as a percentage of each TA cross-section. (D) Quantification of the number of hLamin AC+ cells in a cross-section. (E) Quantification of the M2/M1 ratio (based on DAPI, F4/80, CD206, and iNOS staining) in a cross-section.