In Vivo Characterization of Human Fetal Enthesis Cells

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Disclosures: None

Introduction: One of the most common injuries seen in adults is rotator cuff tears at the supraspinatus tendon enthesis [1-2]. To address this clinical challenge, stem cell therapy has recently emerged as an attractive approach [1]. However, it is unclear what the most appropriate therapeutic stem cell sources for enthesis fibrocartilage healing are. Previous mouse studies have shown that embryonic/postnatal tissue-derived progenitor cells present desired regenerative capacities for musculoskeletal tissue repair [3]. Although mouse work provides some insights into cell types for enthesis repair, there is a knowledge gap as to whether human has similar enthesis progenitors and whether these cells also maintain consistent regenerative potentials. Therefore, the goal of this study is to 1) examine whether human fetal enthesis cells could form fibrocartilage-like tissues *in vivo*, 2) examine how dynamic micro-environments of fetal cells dictate cell function after transplantation.

Methods: Our preliminary scRNA-seq data showed that most of human fetal enthesis cells had progenitor potentials, reflected by self-renewal and multilineage differentiation capacities. Therefore, we isolated fetal enthesis cells, without cell sorting, from human fetal tissues provided by the Developmental Origins of Health and Disease Biorepository at Icahn School of Medicine at Mount Sinai (ISMMS) with full compliance with New York State Laws and ISMMS regulations for human subject protocols. The fetal cells were loaded onto an absorbable gelatin sponge overnight before transplantation. Intramuscular transplantation and renal capsule transplantations were performed in NSG-GFP male and female mice (n=3). For intramuscular transplantations, a 1-cm longitudinal opening was made on the vastus lateralis muscle, and fetal cells carried by sponge were inserted. The muscle and skin were sutured closed separately. For renal capsule transplantation, a 1-cm longitudinal incision was made between the left flank and abdomen of the mouse. A 2-mm pocket was made in the renal capsule, and the sponge carrying the fetal cells was inserted. The kidney was placed back into the body cavity, and the incision was sutured. Histology and immunohistochemistry: vastus lateralis muscles and kidneys with identified transplanted cells were dissected at 4 weeks, 6 weeks, and 9 weeks post surgery. The tissues were fixed, cryosectioned, and stained with H&E and Safranin O to observe tissue morphology. Expression of fibrocartilaginous markers (i.e., SOX9, OSX, COLI, COLII) was evaluated by immunostaining.

Results: Transplanted cells in highlighted regions from 4 weeks to 9 weeks were negative for GFP, demonstrating that fetal cells survived and were maintained in the host tissue (Fig. 1). Histological sections showed clear sponge degradation with time and matrix deposition at 9 weeks post-surgery. Of note, a more organized matrix was observed at week 9 (Fig.1). Fetal enthesis cells are found to express SOX9, but not OSX (osterix). After transplantation, the cells showed decreased SOX9 expression with increased expression of OSX as osteogenic markers over time (Fig. 2A). Consistent with histology, COLI, a core matrix protein of fibrocartilage, showed an obvious increase from 4 weeks to 9 weeks (Fig. 2B). Conversely, COLII showed an obvious decrease from 4 weeks to 9 weeks. This inverse relationship between COLI and COLII further indicates the formation of nascent fibrocartilage tissue. Transplanted cells in muscle and renal capsule displayed similar morphology, new tissue formation, and SOX9 expression, which indicates limited environmental influence on fetal enthesis cells. However, this needs further examination (Fig. 3).

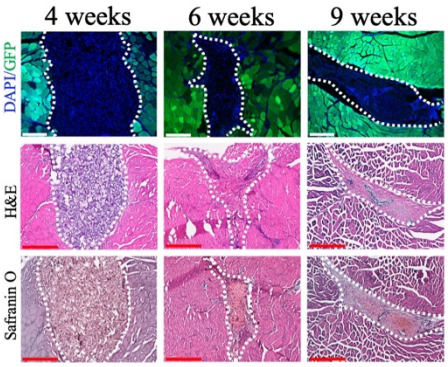

Figure 1: Immunofluorescence, H&E, and Safranin-O staining of muscles transplanted with fetal enthesis cells at 4 weeks, 6 weeks, and 9 weeks after implantation. The regions with transplanted cells are highlighted by dashed lines. Red scale bar, 500um; White scale bar, 250um.

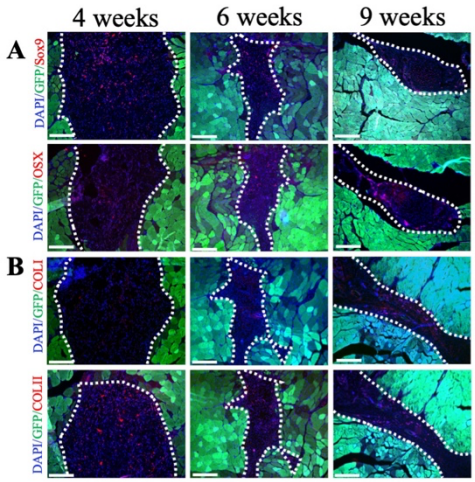

Figure 2: Immunostaining of SOX9, OSX, COLI, and COLII in fetal enthesis cells transplanted to muscles at 4 weeks, 6 weeks, and 9 weeks. White scale bar, 250um

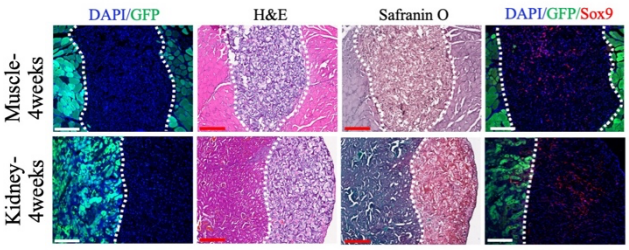

Figure 3: Immunostaining and histological images of kidney regions with transplanted fetal cells. Red scale bar, 500um; White scale bar,

Discussion: Fetal enthesis cells transplanted into muscle formed fibrocartilage-like tissues, which indicates their regenerative capacities. Consistent SOX9 expression in transplanted cells showed their cartilaginous features. COLI in fetal cells was increasingly expressed across the transplantation time, accompanied by decreased COLII expression. This further indicates that fetal cells form a fibrocartilage structure rather than simple cartilage tissue, a conclusion strengthened by the observation of organized fibers in the transplanted regions of interest. An osteogenic marker, OSX, was also found to be more expressed at the transplanted regions at the later time point. As enthesis formation is characterized by fibrocartilage deposition followed by its mineralization, the expression of OSX in implanted fetal cells suggests the occurrence of mineralization. More studies should be conducted to examine whether this process is fibrocartilage mineralization or ectopic ossification. Surprisingly, no obvious difference in the morphology of fetal cells and related matrix/protein expression was found between renal capsule and muscle transplantation at week 4, which could be explained by robust identities of fetal enthesis cells. Our study suggests that human fetal enthesis retains autonomous maturation capacity even when removed from its original environment. The similarities seen from our data with previous mouse data provide a bridge from animal models to clinical translation.

Significance/Relevance: Our study provides a better understanding of human fetal enthesis cell niche and function that could translate to potential use in cell-based therapies for rotator cuff healing.

References: [1] S. Kar et al. *The Journal of Physiology* 2025 [2] H. Lu et al. *Annu Rev Biomed Eng* [3] F. Fang et al. *Cell Stem Cell* 2022