DLK1 Is Necessary and Sufficient to Suppress Human Muscle Fatty Infiltration

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Introduction: Fatty infiltration impairs muscle regeneration and function after chronic injury. Multiple mechanisms of skeletal muscle fatty degeneration have been proposed including fibroadipogenic progenitor (FAP) mediated pathways. It is still unknown what the main adipogenic driver is within human skeletal muscle after injury. Our previous work identified a subpopulation of FAPs which express Delta Like Non-Canonical Notch Ligand 1 (DLK1). We hypothesized that DLK1 modulates human FAP adipogenic potential.

Methods: Under IRB approval and informed consent, primary human FAPs were isolated from deltoid and supraspinatus muscle from both male and female patients (age 42-66) undergoing rotator cuff repair via flow cytometry with the surface marker combination: CD31-/CD45-/CD56-/CD34+/PDGFRa+. FAP DLK1 expression was characterized with flow cytometry (n=4). FAPs were cultured *in vitro* in adipogenic medium to analyze adipogenesis with or without recombinant DLK1 (rDLK1) treatment (n=3). To test whether DLK1 protein can alter FAP lineage commitment, we created three human FAP cells lines that 1) overexpress a GFP-tagged DLK1 protein (DLK1 OE), express shRNAs targeting DLK1 (DLK1 KD), or express GFP to serve as a control (GFP CTRL) (n=3). Adipogenic differentiation in these lines was quantified by perilipin immunofluorescence (fat marker) after 14 days. Under IACUC approval, we then tested DLK1 function with a xenotransplant model of human FAPs into mice looking at fatty infiltration in mice treated with 1) recombinant DLK1 versus PBS (n=3) and 2) DLK1 overexpressing FAPs versus control FAPs (n=3).

Results: We compared FAPs isolated from healthy deltoid muscle to FAPs from injured full thickness tears supraspinatus muscle with full spectrum flow cytometry for DLK1 protein expression (**Figure 1a**). FAP specific DLK1 protein expression was significantly decreased in injured compared to healthy muscle (**Figure 1b**). We tested *in vitro* culture of FAPs in adipogenic media with recombinant DLK1 protein compared to PBS control (**Figure 1c**). Addition of DLK1 reduced adipogenic differentiation by nearly 3-fold (**Figures 1d, 1e**). We tested the three human FAP cells lines that 1) overexpress a GFP-tagged DLK1 protein (DLK1 OE), express shRNAs targeting DLK1 (DLK1 KD), or express GFP to serve as a control (GFP CTRL) in adipogenic culture (**Figure 1f**). The GFP tag on the DLK1 OE line was located on the intracellular domain (**Figure 1g**). The respective overexpression and knockdown of DLK1 protein was confirmed with western blot staining for expression of DLK1 (**Figures 1h, 1i**). We quantified adipogenesis by perilipin staining (**Figure 1j**). DLK1 overexpression both reduced adipogenic differentiation and lipid production per cell by greater than 2-fold (**Figures 1k, 1l**). In contrast, DLK1 knockdown increased adipogenic differentiation and lipid production per cell by over 2.5-fold (**Figures 1k, 1l**). These findings demonstrate the robust mediation of FAP adipogenesis through FAP specific expression of DLK1 (**Figure 1m**). To study the effect of DLK1 treatment on fatty infiltration after muscle injury we employed xenotransplantation of human FAPs along with glycerol injury (**Figure 2a**). We quantified the amount of fat with immunofluorescence staining as above. The rDLK1 treated mice had more than a 5-fold reduction in fat compared to PBS treated controls (**Figures 2b and 2c**). We transplanted our DLK1 overexpression (DLK1 OE) and GFP control (GFP CTRL) human FAP cell lines in the same mouse model of fatty infiltration (**Figure 1d**). The mice transplanted with DLK1 overexpressing cells developed nearly 3-fold less fat than those that received control cells (**Figures 2e, 2f**). In addition, we quantified the amount transplanted human cells which differentiated into human adipocytes within the mouse muscle through co-localization with GFP (**Figures 2g, 2h**). The mice transplanted with DLK1 overexpressing FAPs had over 2-fold less human FAPs which differentiated into adipocytes compared to those mice transplanted with control cells (**Figure 2h**).

Discussion: Our data demonstrate that DLK1 is both necessary and sufficient to restrain adipogenesis in human FAPs, and DLK1 overexpression significantly mitigates fatty infiltration after muscle injury in vivo. These findings identify DLK1 as a promising target for preventing maladaptive muscle remodeling.

Significance/Clinical Relevance: Targeting DLK1 expression in FAPs may offer a novel strategy to prevent fatty infiltration and improve muscle healing after injury—a critical unmet need in musculoskeletal medicine.

Images:

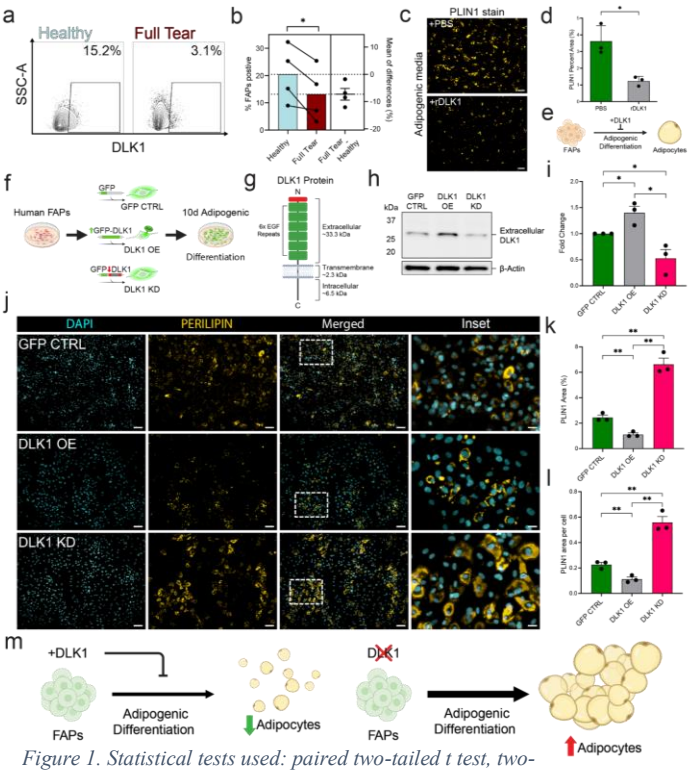

*Figure 1. Statistical tests used: paired two-tailed t test, two-tailed t test, and two-way ANOVA with multiple comparison correction *p<0.05 **p<0.01. All plots are mean±SEM.*

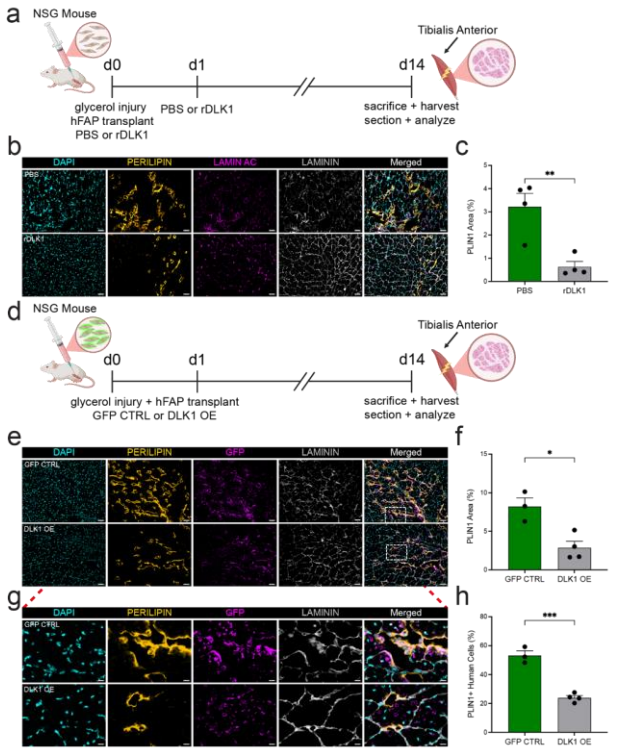

*Figure 2. Statistical test used: two-tailed t test. *p<0.05 **p<0.01 ***p<0.001. All plots are mean±SEM.*