Gli1 defines a subset of fibroadipogenic progenitors that promotes skeletal muscle regeneration with less fat accumulation

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Introduction

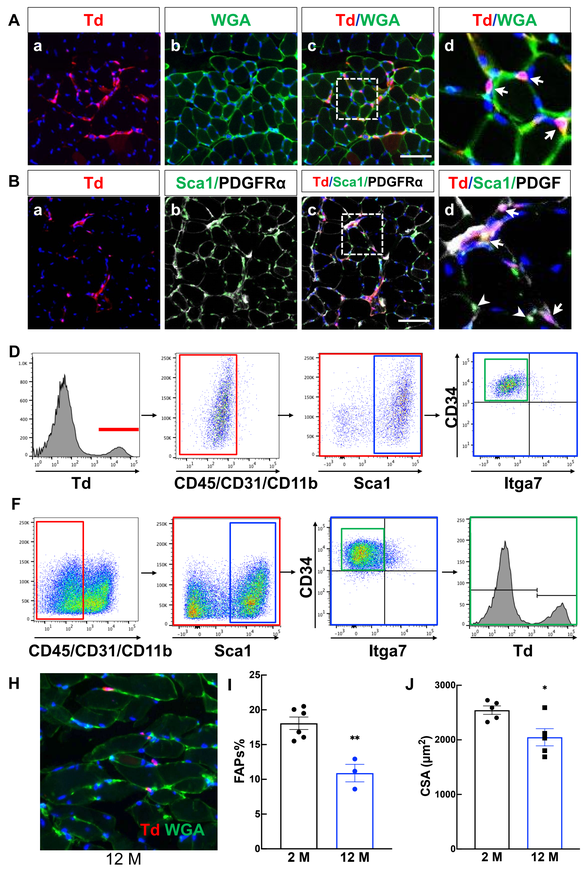
Skeletal muscle has a remarkable capacity for regeneration after injury. Recently, a new type of muscle-resident progenitor cell, referred to as fibro-adipogenic progenitors (FAPs), was identified to be critical in supporting the process of injured muscle regeneration. To date, FAPs remains a poorly defined, heterogeneous population without any specific genetic markers. Gli1 was recently recognized as a marker for bone marrow and periosteal mesenchymal progenitors. In this study, we used *Gli1-CreER* to label FAPs and characterized their changes in healthy, aged muscle, and muscle injury regeneration.

Methods

Animals- All animal work performed in this report was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. *Gli1-CreER Rosa-tdTomato* (*Gli1ER/Td*) mice were generated by breeding *Rosa-tdTomato* mice with *Gli1-CreER*. *Gli1ER/Td/DTA* mice were generated by breeding *Gli1ER/Td* mice with *DTA* mice. To induce *CreER* activity, mice received tamoxifen (Tam) injections (75 mg/kg/day) at 2 months of age for 5 days. Acute muscle injury was induced by injection of 10 μl Notexin (10 μg/mL) into Tibialis Anterior (TA) muscle. *Histology-* TA muscle samples were fixed in 4% PFA for 1 day, and then immersed into 30% sucrose at 4°C overnight. They were processed for cryosections followed by H&E, WGA, Sca1 and PDGFRα antibody staining. *FAP cell isolation-* Hindlimb muscles (quadriceps, gastrocnemius, and tibialis anterior) were dissected and enzymatically dissociated with 0.1% collagenase and 4.8 units/mL dispase in DMEM using the gentleMACs system. The cell slurry was pulled through a 21-gauge needle until all remaining muscle tissue was broken apart, after which the cell solution was filtered through a 40 μm cell strainer. After red blood cell lysis, cells were stained with lineage cell markers (CD45, CD31, CD11b), Sca1, α7-integrin (Itga7), and CD34 antibodies for flow analysis. *Statistics-* Data are expressed as means±SEM and analyzed by unpaired, two-tailed Student's t-test.

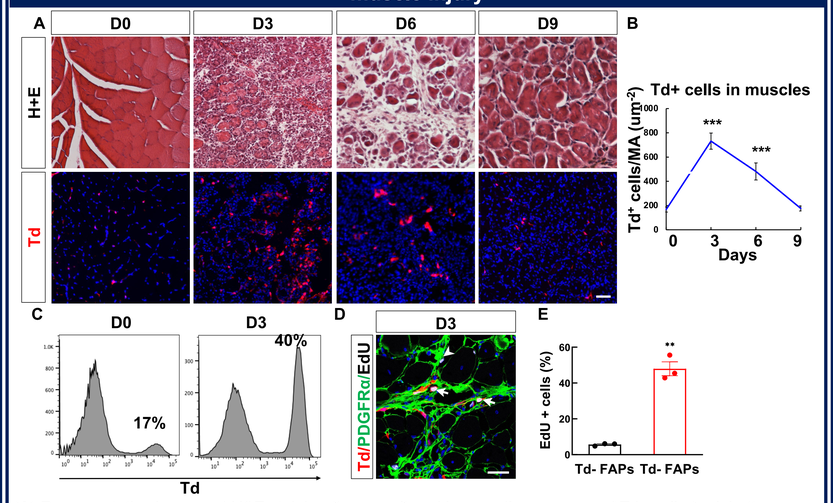
Results

Gli1 marks a small subset of resident FAPs within skeletal muscles and their number is declined with age



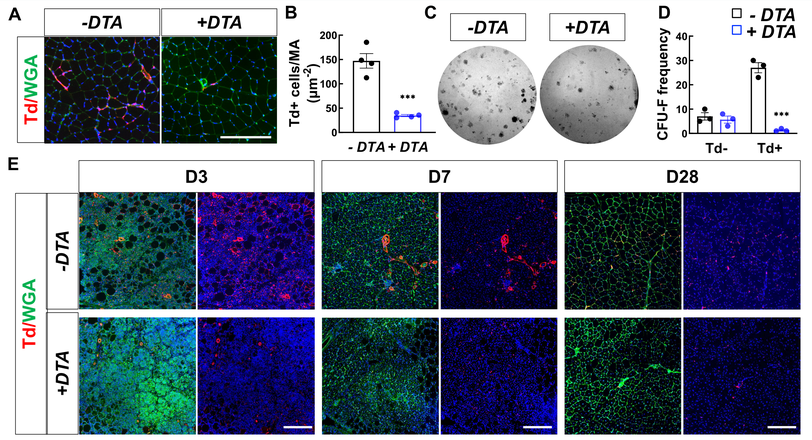
(A) Representative immunofluorescence images of TA muscles of *Gli1ER/Td* mice show Td+ cells are located in the interstitial area. Mice at 2 months of age received Tam injections for 5 days and muscle was collected 1 day later. Panel D is a magnified image from the outlined area in panel C. Td+ (Gli1+) cells are shown in red, DAPI (nuclei) in blue and WGA (muscle fibers) in green. Arrows point to Td+ cells within interstitial area of myofibers. Scale bar: 50 μm. **(B)** Td+ cells co-express FAP markers Sca1 and PDGFRα. Panel D is a magnified image from the outlined area in panel C. Arrows point to PDGFRα+Sca1+Gli1+ cells (Td+ FAPs) and arrow heads point to PDGFRα+Sca1+Gli1- cells (Td- FAPs). Scale bar: 50 μm. **(C)** Venn diagram of Sca1+, PDGFRα+, and Td+ cells in TA muscles. Quantification reveals that Td+ cells constitute a small portion of FAP cells (Td+ FAPs highlighted with a red grid). n=5 mice. **(D)** Gating strategy of flow analysis studying the percentage of FAPs in Td+ cells. **(E)** Quantification revealed that the majority of Td+ cells are FAPs (Lin-Sca1+CD34+Itga7-). n=4 mice/group. ***P<0.001. **(F)** Gating strategy of flow analysis studying the percentage of Td+ cells in FAPs. **(G)** Quantification revealed that a small subset of FAPs (Lin-Sca1+CD34+Itga7-) are Td+. n=4 mice/group. ***P<0.001. **(H)** Td⁺ cells in TA muscle of 12-month-old *Gli1ER/Td* mice. Tissues were harvested 1 day after Tam injections. **(I)** Flow assay shows that the percentage of FAP cells in hindlimb muscles decreases during aging. **P<0.01 vs 2 M. n=5 mice/group. **(J)** Quantification of Td⁺ cells in TA muscle shows that *Gli1-CreER* labeled cells also decreases during aging. MA: muscle area. *P<0.05 vs 2 M. n=3 mice/group.

The Gli1+ FAPs subpopulation preferentially expand in response to acute muscle injury



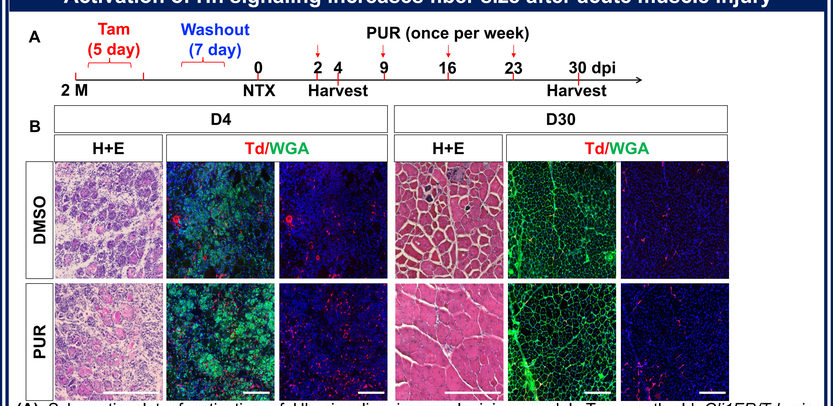
(A) Representative images of H&E staining (top panel) and immunofluorescence of Td+ cells (red, bottom panel) in TA muscles at day 0, 3, 6 and 9 (D0, D3, D6 and D9) post Notexin-induced muscle injury. Scale bar: 50 μm. **(B)** The numbers of Td+ cells in muscle at different time points post injury were quantified from immunofluorescence images. MA: muscle area. n=4 mice/group. ***P<0.001. **(C)** Flow analysis of Td+ cells in total FAPs at day 0 and day 3 after injury. **(D)** *In vivo* proliferation assay. Two-month old *Gli1ER/Td* mice received Tam injections for 5 days, an intramuscular injection of NTX at 7 days later, and an EdU injection at 9 days later. Muscle tissues were harvested at 3 days post injury. Representative TA muscle image at day 3 post injury show PDGFRα staining (green), Td (red), DAPI (nuclei, blue) and EdU staining (white). Arrows point to EdU+PDGFRα+Gli1+ (proliferating Td+ FAPs) cells, arrowhead points to EdU+PDGFRα+Gli1 (proliferating Td-FAPs) cells. Scale bar: 50μm. **(E)** Quantification shows that Td+ FAPs have greater EdU incorporation than Td- FAPs. n=3 mice/group. **P<0.01

Genetic ablation of Gli1+ cells causes delayed muscle regeneration



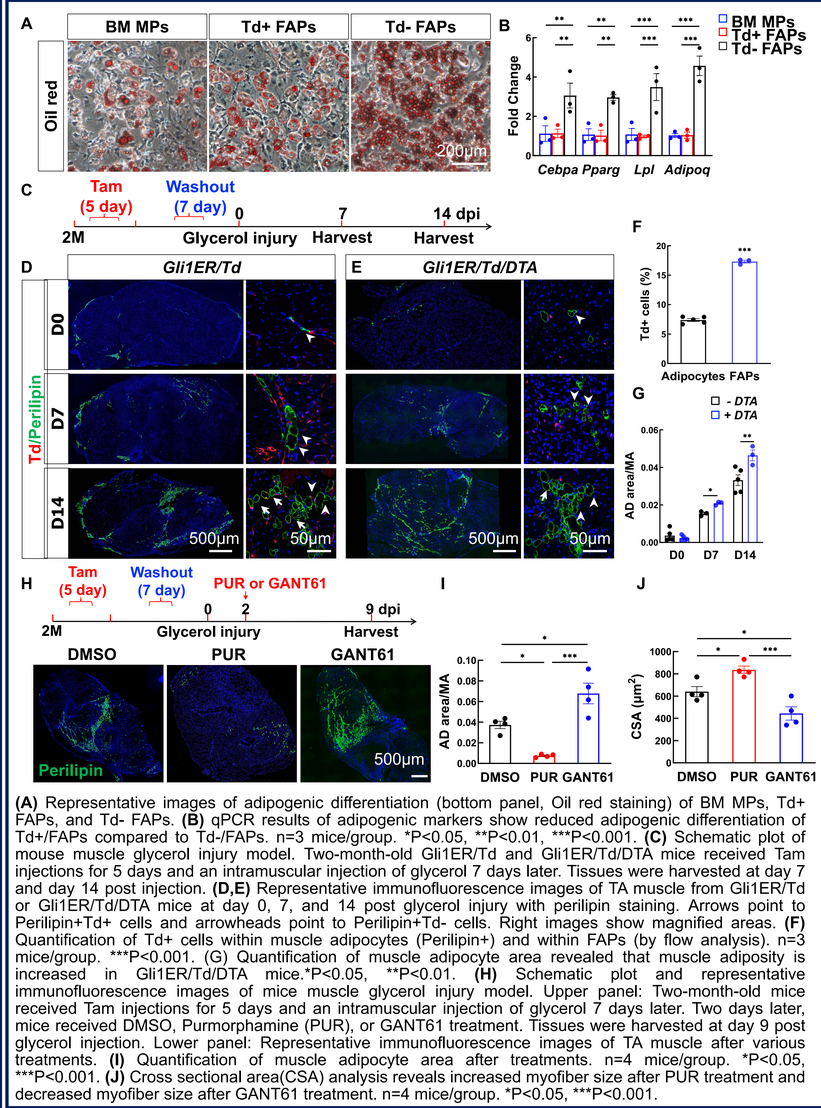
(A) Representative immunofluorescence images of TA muscles from *Gli1ER/Td* and *Gli1ER/Td/DTA* mice show Td+ cells (red), DAPI (nuclei, blue) and WGA (green). Scale bar: 200 μm. **(B)** Quantification revealed a significant decrease of Td+ cells in *Gli1ER/Td/DTA* muscle compared to *Gli1ER/Td* muscle. n=4 mice/group. ***P<0.001. **(C)** Representative image of CFU-F colonies from *Gli1ER/Td* and *Gli1ER/Td/DTA* muscle cells. **(D)** Quantification revealed that Td+ CFU-F number decreases significantly in *Gli1ER/Td/DTA* mice, while Td- CFU-F number remains the same. n=3 mice/group. ***P<0.001. **(E)** Representative immunofluorescence imaging of *Gli1ER/Td* and *Gli1ER/Td/DTA* muscle at day 3, 7 and 28 days (D3, D7 and D28) post NTX injury. Red: Td+ cells; blue: DAPI (nuclei); green: WGA. At each time point, the left panel contain all 3 colors and the right panel contain red and blue signals only. Scale bar: 200 μm.

Activation of Hh signaling increases fiber size after acute muscle injury



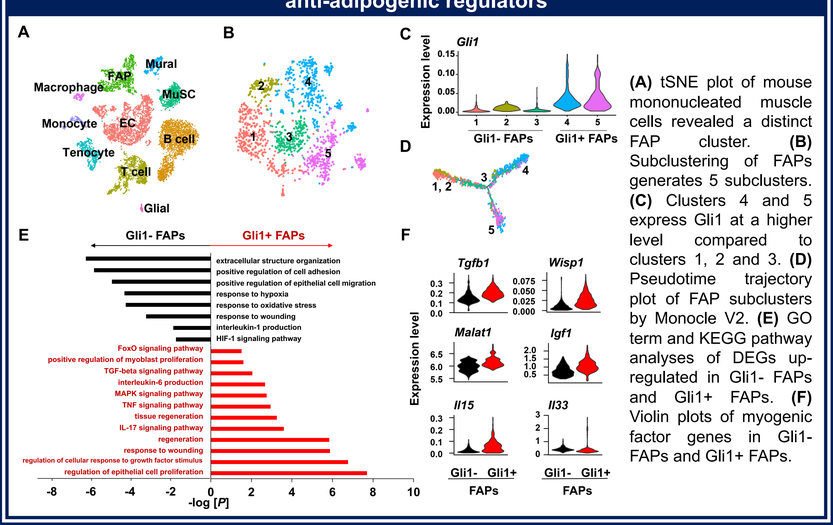
(A) Schematic plot of activation of Hh signaling in muscle injury model. Two-month-old *Gli1ER/Td* mice received Tam injections for 5 days and an intramuscular injection of NTX 7 days later. Two days later, mice received weekly vehicle (DMSO) or Purmorphamine (PUR) treatment. Tissues were harvested at day 4 and day 30 post-NTX injection. **(B)** Representative H&E staining and immunofluorescence images of Td+ (red) cells, combined with WGA (green), in TA muscles of DMSO or PUR treatment group at day 4 and day 30 post NTX injury. Scale bar: 200 μm.

Gli1+ FAPs have reduced adipogenesis and suppress intramuscular adipogenesis and improve myofiber size in response to glycerol injury



(A) Representative images of adipogenic differentiation (bottom panel, Oil red staining) of BM MPs, Td+ FAPs, and Td- FAPs. **(B)** qPCR results of adipogenic markers show reduced adipogenic differentiation of Td+/FAPs compared to Td-/FAPs. n=3 mice/group. *P<0.05, **P<0.01, ***P<0.001. **(C)** Schematic plot of mouse muscle glycerol injury model. Two-month-old Gli1ER/Td and Gli1ER/Td/DTA mice received Tam injections for 5 days and an intramuscular injection of glycerol 7 days later. Tissues were harvested at day 7 and day 14 post injection. **(D,E)** Representative immunofluorescence images of TA muscle from Gli1ER/Td or Gli1ER/Td/DTA mice at day 0, 7, and 14 post glycerol injury with perilipin staining. Arrows point to Perilipin+Td+ cells and arrowheads point to Perilipin+Td- cells. Right images show magnified areas. **(F)** Quantification of Td+ cells within muscle adipocytes (Perilipin+) and within FAPs (by flow analysis). n=3 mice/group. ***P<0.001. (G) Quantification of muscle adipocyte area revealed that muscle adiposity is increased in Gli1ER/Td/DTA mice.*P<0.05, **P<0.01. **(H)** Schematic plot and representative immunofluorescence images of mice muscle glycerol injury model. Upper panel: Two-month-old mice received Tam injections for 5 days and an intramuscular injection of glycerol 7 days later. Two days later, mice received DMSO, Purmorphamine (PUR), or GANT61 treatment. Tissues were harvested at day 9 post glycerol injection. Lower panel: Representative immunofluorescence images of TA muscle after various treatments. **(I)** Quantification of muscle adipocyte area after treatments. n=4 mice/group. *P<0.05, ***P<0.001. **(J)** Cross sectional area(CSA) analysis reveals increased myofiber size after PUR treatment and decreased myofiber size after GANT61 treatment. n=4 mice/group. *P<0.05, ***P<0.001.

Single cell RNA-seq analysis indicates that Gli1+ cells express myogenic and anti-adipogenic regulators



(A) tSNE plot of mouse mononucleated muscle cells revealed a distinct FAP cluster. **(B)** Subclustering of FAPs generates 5 subclusters. **(C)** Clusters 4 and 5 express Gli1 at a higher level compared to clusters 1, 2 and 3. **(D)** Pseudotime trajectory plot of FAP subclusters by Monocle V2. **(E)** GO term and KEGG pathway analyses of DEGs up-regulated in Gli1- FAPs and Gli1+ FAPs. **(F)** Violin plots of myogenic factor genes in Gli1- FAPs and Gli1+ FAPs.

Conclusions

Gli1 is an effector of Hedgehog (Hh) signaling. We demonstrated that *Gli1-CreER* labels a subpopulation of FAP cells that undergo age-dependent reduction. Interestingly, they show the same response kinetics of FAPs after muscle injury and activating Hh signaling accelerates muscle repair, Gli1+ cells also shown less adiposity potential, suggesting that *Gli1-CreER* labeled subpopulation of FAP cells play a predominant role in the regeneration process of injured skeletal muscles with less fat accumulation.