FAP Beige Adipogenesis in Volumetric Muscle Loss

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Research

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Abstract

Background Volumetric muscle loss (VML) often leads to chronic muscle weakness, impaired limb function, and even permanent disability. Recent studies suggest muscle residential fibro/adipogenic progenitors (FAPs) can adopt novel beige fat differentiation promoting muscle regeneration. The goal of this study is to define the role of FAP beige adipogenesis in muscle regeneration after VML in a mouse model.

Methods Three months old male C57BL/6J mice, PDGFR α -GFP reporter mice, UCP-1 reporter mice, PDGFR α -CRE^{ERT}/DTA inducible FAP depletion mice and NSG immune-deficient mice were used in this study. Volumetric muscle loss (VML) was created on tibialis anterior (TA) muscle with a punch. To induce FAP beige adipogenesis, Amibegron, a beta 3 adrenergic receptor (B3AR) agonist was administered to mice with I.P. injection. In a separate group, murine and human beige adipogenic FAPs was transplanted to mouse muscle after VML. Limb function was measured with gait analysis at 2 and 6 weeks after VML. Muscle histology and FAP gene expression analysis was also conducted at 2 and 6 weeks after VML.

Results After VML, we observed robust proliferation of FAPs in PDGFR α -GFP reporter mice. PDGFR α -CRE^{ERT}/DTA mice inducible FAP depletion mice showed reduced muscle regeneration after FAPs are depleted, suggesting a positive role of FAP in muscle regeneration after VML. Both Amibegron treatment and beige FAP transplantation significantly improved muscle regeneration and limb function after VML.

Conclusion Stimulating FAP beige adipogenesis with B3AR agonists or transplantation of beige adipogenic FAPs could serve as new strategies in treating VML.

Introduction

Skeletal muscle possesses excellent regeneration potency in responding to mild or moderate injuries. However, loss of a critical size of muscular tissue (known as volumetric muscle loss (VML)) often results in chronic muscle weakness, impaired limb function, and even permanent disability in effected patients due to insufficient muscle regeneration [1–3]. VML is particularly common among military musculoskeletal injuries, including blast injuries and gunshot injuries. It has been recognized as one of the leading causes of long-term disability of discharged military service members [4].

Satellite cells are a group of muscle endogenous progenitor cell populations. After activation, satellite cells undergo expansion and give rise to myogenic progenitors, which eventually differentiate into mature myofibers [5–7]. Appropriate environment (the “cue”) is critical for satellite cells proliferation and myogenic differentiation. Actually, resuming appropriate cues for satellite cell migration and myogenesis remains a major challenge in treating VML [8–9].

Fibro/adipogenic progenitors (FAPs) are a newly discovered group of residential non-myogenic progenitors in muscle [10]. Recently, there is increased interest in FAPs regarding their role in the regulating muscle homeostasis. In unperturbed skeletal muscles, FAPs are typically present in a steady

state; however, they rapidly expand in response to muscle injury to facilitate muscle repair [11]. However, the detailed mechanism of FAP differentiation and its role in muscle regeneration after VML remains unknown up to date.

We recently discovered that FAPs can adopt a novel brown/beige adipose tissue (BAT) differentiation in a mouse rotator cuff muscle injury model [12]. Transplantation of BAT-differentiated FAP significantly improved muscle quality and shoulder function after rotator cuff tendon injury and repair [13, 14], suggesting a beneficial role of BAT differentiated FAP in muscle regeneration. In this study, we sought to define the functional role of FAPs and their BAT differentiation in muscle regeneration after VML. We hypothesize that inducing FAP BAT differentiation can improve muscle regeneration after VML.

Materials And Methods

All reagents used in this study were purchased from Fisher Scientific Corp. (Waltham, MA) unless otherwise indicated.

Experimental Animals and Groups

Forty female wildtype (WT) C57BL/6J mice purchased from Jackson laboratory Corp. (Sacramento, CA, stock # 000664), fifty PDGFR α -GFP reporter mice, twenty UCP-1 reporter mice (JAX stock # 026690), twenty PDGFR α -CRE^{ERT}/DTA inducible FAP depletion mice (generated by crossing B6N.Cg-Tg(PDGFR α -CRE^{ERT}) 467Dbe/J (JAX stock # 018280) with B6.129P2-Gt(ROSA)26Sortm1(DTA)Lky/J (JAX stock # 009669) were used in this study. fifteen NOD.Cg-Prkdc^{scid}>Il2rg^{tm1Wjl}/SzJ immune-deficient mice (JAX stock # 005557) were used for human FAP transplantation experiment. All mice were 3 months old. With $\beta = 0.80$ and $\alpha = 0.05$, we found that a minimum of four mice per group is required to testify a difference of 18% in the cross sectional area of muscle between groups of our pilot experiment. Animals were randomly divided into sham and VML groups, and detailed grouping information is summarized in Supplement 1.

Surgery Procedures and Drug Administration

VML surgery was performed on mice under general anesthesia with 1–5% isoflurane inhalation. After 0.5 cm skin incision was made on the right leg, a full-thickness defect of 4 mm in diameter was removed from the tibialis anterior (TA) muscle with biopsy punch as described previously [15]. In the sham group, we performed skin incision, exposed the TA muscle, and then closed skin. In order to deplete FAPs before surgery, PDGFR α -CRE^{ERT}/DTA mice underwent I.P. Tamoxifen injection (2 mg/d in corn oil) [16] for 2 weeks (five days per week). In the control group, PDGFR α -CRE^{ERT}/DTA mice received I.P. injection with corn oil instead.

To stimulate BAT activity, Amibegron (10 mg/kg in 5% DMSO in saline) [17, 18] (SR-58611A, SML1070-25MG, Sigma-Aldrich, Burlington, MA) was administered to mice at the same time of the VML through daily I.P. injection for 2 weeks or 6 weeks (five days per week). Control groups were injected with 5%

DMSO in saline. All procedures were approved by our Institutional Animal Care and Use Committee (IACUC).

FAPs Isolation and Cell Preparation

In order to collect FAPs, TA samples were harvested after animals were sacrificed and digested using collagenase II (0.2% in DMEM) for 90 minutes at 37 °C sterile water bath. The mixture was filtered through a 40 µm cell strainer, then pelleted and re-suspended in FACS buffer as described previously [19]. The cells were incubated with anti-CD31-FITC, anti-CD45-FITC, anti-integrin α7-APC, anti-PE-Cy7-Sca1 and anti-PDGFRα for 30 minutes before sorted with FACS Aria™ II (BD bioscience). FAPs were counted and collected from the CD31-/CD45-/ITGA7-/Sca1+/PDGFRα + population.

FAP Transplantation

In order to collect UCP-1 positive (+) and negative (-) FAPs for cell transplantation, hindlimb muscles, including quadriceps, gastrocnemius and TA muscles from UCP-1 reporter mice were harvested. FAPs were then isolated with FACS as described above and incubated with Amibegron (final concentration at 10 µM) to induce BAT differentiation for 2 weeks. UCP1(+) and UCP1(-) FAPs were then separated with FACS according to UCP1-driven dtTomato signal. Both UCP-1 (+) and UCP-1 (-) FAPs were cultured in pre-Matrigel coated 24-well plates for 1 week with standard cell culture medium (Ham's F-10, 10% fetal bovine serum, 10 ng/ml bFGF (Thermo Fisher Scientific, MA USA) and 1% antibiotic-antimycotic solution. At the time for cell transplantation, 1×10^6 FAPs were suspended in 30 µL of Matrigel (1:1 diluted in PBS) for each WT mouse.

To prove the concept that human BAT differentiating FAP can improve muscle regeneration, we collected human FAPs (hFAPs) from medical waste of hamstring muscle in a 26 years old female patient with autographic Anterior Cruciate Ligament (ACL) reconstruction using the cell surface markers of CD31-/CD45-/CD29-/CD56-/CD184-/PDGFRα + with FACS as describe previously [20]. hFAPs were then expanded in vitro and treated with 10 µM Amibegron for two weeks to induce BAT-differentiation. A portion of the hFAPs were treated with DMSO to serve as the control. BAT-differentiated and control hFAPs were suspended in 30 µL of Matrigel (1×10^6 cells per 30 µL of Matrigel) and transplanted to NSG mice TA muscle after VML as described above.

Gait Data Analysis

We conducted DigiGait™ (Mouse Specifics Inc., Quincy, Massachusetts) analysis as described previously to measure the hind limb function at 2 weeks or 6 weeks after surgery [21]. Mice walked at 10 cm/s for 10 s on the DigiGait system. Stride length, stance width and paw area at the peak stance were chosen for assessing limb function [12, 22].

Muscular Harvesting and Histology Analysis

Mice were sacrificed at 2 or 6 weeks after surgery and bilateral TA muscles were harvested. The wet weight TA muscles was measured, and the muscle weight loss was assessed using the equation: $([TA_{right} - TA_{left}]/TA_{left}) \times 100\%$. Muscles were subsequently flash-frozen and then cryosectioned at a thickness of

10 µm. Masson trichrome (American MasterTech, Lodi, CA) staining was conducted as described previously [12, 22]. For immunostaining, sections of muscle were fixed with 4% paraformaldehyde, rinsed with Phosphate Buffer solution (PBS), and then incubated in blocking solution (0.3% Triton X-100, 5% bovine serum albumin in PBS) for 1 hour followed with incubation with the primary antibodies at 4° C over night. The sections were incubated with secondary antibodies (1: 250 diluted) for 2 hours, rinsed with PBS, counterstained with DAPI and mounted using VectaShield.

Primary antibodies included anti-Laminin (L9393, Sigma-Aldrich, 1:500 diluted), and anti-UCP-1(sc-6529, Santa Cruz Biotechnology, 1:50 diluted) and anti-PDGFR α (sc-398206, Santa Cruz Biotechnology, 1:200 diluted). The secondary antibodies included Alexa Fluor 594- conjugated anti-rabbit IgG (ab150076, Abcam Inc. 1:1000 diluted) and Alexa Fluor 647- conjugated anti-goat IgG (ab150131, Abcam Inc. 1:1000 diluted), and Alexa Fluor 488- conjugated anti-mouse IgG (ab150113, Abcam Inc. 1:1000 diluted).

Image Capture and Data Quantification

Histologic images were obtained with the Axio Imager 2 microscope (Zeiss) and analyzed using ImageJ (National Institutes of Health) as described previously [23]. All images were evaluated by two researchers blinded to treatment group. Cross sectional area (CSA) was measured for TA muscle fibers from the injured site on 200 × picture. Five pictures (containing about 300–500 myofibers) were measured for each sample. The collagen area fraction was evaluated as percentage of Aniline Blue stained area over the entire sample area [21, 23]. Percentage of cell populations was calculated as the cell number of specific cell population/total cells number x 100%. The UCP-1 area fraction was calculated as UCP-1 positively stained area / total area of the sample x 100% [12, 22].

RT-PCR

RT-PCR was performed to examine the cell proliferation (Ki67), fibrosis (α -SMA, Collagen-1, Collagen-3), pro-myogenic cytokine (IGF1, Follistatin) and BAT related (UCP-1, PRDM16) gene expression in the FAPs. Total RNA was extracted using Trizol reagent according to the manufacture instruction. Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Bioscience Inc., Indianapolis, IN.) was used to synthesize cDNA. RT-PCR was performed to quantify gene expression of using SYBR Green Detection and an Applied Biosystems Prism 7900HT detection system (Applied Biosystems, Inc., Foster City, CA). Sequences of the primers for target genes were showed in Supplement 2. The expression level of each gene was normalized to that of the house-keeping gene of S26. Fold changes relative to sham controls were calculated by $\Delta\Delta CT$.

Statistical Analysis

All data were presented as mean $\pm$ SD. Analyses of variance (ANOVAs) with Tukey post hoc comparisons were applied to determine the statistical difference among all groups of each experiment. Statistical difference was indicated when $p < 0.05$.

Results

Increased of FAPs after VML

Significant atrophy of TA was observed after VML as evidenced of significantly reduced muscle weight and muscle fiber cross-section area (CSA) of TA muscle (vs. sham, $p < 0.05$). Immunofluorescent staining showed that number of PDGFR α positive FAPs were significantly increased at 2 weeks ($50.4\% \pm 6.11\%$) and 6 weeks ($34.2\% \pm 4.66\%$) after VML surgery (vs. sham, $p < 0.01$) (Fig. 1).

Gene Expression of FAPs

Expression level of genes related to proliferation (Ki67), fibrosis (α SMA, Collagen 1 and Collagen 3) and fat (PPAR γ) significantly increased in FAPs after VML at two and six weeks compared to those in the sham (vs. sham, $p < 0.05$). However, no difference of pro-myogenic cytokine gene (IGF1 and Follistatin) expression was found between the sham and VML groups at both time points ($p > 0.05$). Gene expression level of CEBP increased at 2 weeks but decreased at 6 weeks in FAPs after VML compared to the sham (Fig. 2).

Depletion of FAP Impairs Muscle Regeneration after VML

PDGFR α -Cre/DTA mice received Tamoxifen (TM, to induce FAPs depletion group) has significantly more severe muscle atrophy, inferior limb function but less fibrosis compared to those received corn oil injection (vehicle for TM, no FAP deletion) ($p < 0.05$). Immunofluorescent staining showed significantly increased PDGFR α positive FAPs and UCP-1 expression after VML in the control groups; however, few PDGFR α and UCP-1 positive cells were found in the FAPs depletion mice after VML. Depletion of FAPs also significantly increased muscle atrophy and worsened hindlimb gait compared to non-depleted controls (Fig. 3).

FAPs Differentiate into BAT in TA Muscle after VML

Immunofluorescent staining showed that $18.75\% \pm 3.09\%$ and $16.50\% \pm 1.42\%$ of PDGFR α -GFP positive FAP has positive UCP-1 staining at 2 weeks and 6 weeks after VML surgery (vs. sham, $p < 0.01$). BAT related genes (UCP-1 and PRDM16) were also up-regulated in FAPs after VML at two and six weeks (vs. sham, $p < 0.05$). (Fig. 4).

Amibegron Enhances Muscle Regeneration after VML by Stimulating FAP BAT Differentiation.

10 mg/kg Amibegron and vehicle were administered to PDGFR α -GFP reporter mice immediately after VML surgery for 2 or 6 weeks. We found significantly reduced muscle weight loss with increased muscle fiber CSA in the Amibegron treated group compared to the vehicle group ($p < 0.05$). Animals receiving 10 mg/kg Amibegron had significantly reduced fibrosis and increased UCP-1 expression compared to vehicle treated control ($p < 0.05$). More PDGFR α -GFP and UCP-1 double positive BAT differentiated FAPs were found in the VML + Amibegron group, when compared to the VML + Vehicle group ($p < 0.01$). Gait analysis showed that daily injection of Amibegron also significantly improved limb function after VML (vs. vehicle group, $p < 0.05$) (Fig. 5).

Transplantation of Mouse UCP-1(+) FAP Improves Muscle Regeneration after VML

Muscle atrophy and fibrosis was significantly improved in the VML + FAP(UCP-1(+)) transplantation group, when compared to the VML + FAP(UCP-1(-)) and VML + Matrigel transplantation groups ($p < 0.05$). Gait analysis also showed limb function was significantly improved after UCP-1(+) FAP transplantation, when compared to the VML + FAP(UCP-1(-)) and VML + Matrigel groups ($p < 0.05$) (Fig. 6).

Beige hFAP Transplantation Decreases Muscle Atrophy and Improves Muscle Regeneration after VML Injury.

Transplantation of BAT-differentiated human FAPs (beige hFAPs) results in decreased muscle atrophy in mice after VML compared to those received transplantation of un-induced FAPs and matrigel ($60.6\% \pm 4.2\%$ in beige hFAPs vs. $75.2\% \pm 6.3\%$ in un-induced hFAPs vs. $71.4\% \pm 1.8\%$ in matrigel; $p < 0.05$). Muscle fiber cross sectional area (CSA) also increased following transplantation of beige hFAPs compared to the other two groups ($1026.7 \mu\text{m}^2 \pm 143.5 \mu\text{m}^2$ in beige hFAPs group vs. $754.7 \mu\text{m}^2 \pm 170.4 \mu\text{m}^2$ in un-induced hFAPs group vs. $697.6 \mu\text{m}^2 \pm 106.2 \mu\text{m}^2$ in matrigel group; $p < 0.05$). Animals receiving beige hFAPs also exhibited significantly less fibrosis significantly enhanced hindlimb gait at 6 weeks post transplantation ($p < 0.05$) (Fig. 7).

Discussion

Volumetric muscle loss is a devastating muscle injury that beyond the capability of spontaneous muscle regeneration. Treating VML remains a clinical challenge to the effected patients. Current treatments for VML focus on fulfilling the wound with acellular biomaterial with or without combination with a cellular component that promotes muscle regeneration [24]. Cells currently used for VML treatments are mainly myoblasts or other myogenic stem/progenitor cells [25, 26]. In this study, we demonstrated that a non-myogenic progenitor—FAP, when induced to BAT differentiation, can be used in treating VML. BAT-differentiated FAPs, when transplanted into the wound with an acellular material of matrigel, significantly improved muscle regeneration and limb function after VML.

Different than myogenic progenitors that directly differentiate into myocytes and fuse into new myotubes, FAPs are likely to promote muscle regeneration by generating a pro-myogenic environment to facilitate intrinsic myogenic progenitor migration and differentiation. Using inducible FAP depletion mice, we demonstrated a beneficial role of FAPs in muscle regeneration after VML. Supportive role of FAP in muscle regeneration has been reported previously in other injury models. FAPs have been shown to support muscle stem cells (MuSCs) differentiation in vitro [11, 27]. In a recent study, Wosczyzna et al. reported that depletion of FAPs resulted in loss of expansion of muscle stem cells (MuSCs) and CD45 + hematopoietic cells after barium chloride-induced injury and impaired skeletal muscle regeneration in mice [27]. In our current study, we used the same approach of PDGFR α -CRE^{ERT}/DTA mice to deplete FAPs with Tamoxifen after VML. We found significantly reduced muscle regeneration and hindlimb function in

Tamoxifen-induced FAP depletion mice after VML compared to the control. This data provided direct evidence in supporting the concluding that FAPs have a beneficial role in muscle regeneration after VML.

In our PDGFR α -CRE^{ERT}/DTA mice, we observed about 50% FAP depletion in after 2 weeks of Tamoxifen induction. This is similar to 50%-75% FAP depletion rate of FAPs reported by Wosczyzna et al. in their study [28]. Different than satellite cell depletion mouse model, which usually reaches over 90% satellite cell depletion rate [29–31], the same Cre/DTA mouse model only achieves 50%-75% depletion rate for FAPs. Nevertheless, our data suggested that 50% depletion of FAP is sufficient to alter muscle homeostatic conditions and impairs regeneration after VML. It is possible that the surviving FAPs are severely damaged by Tamoxifen-induced DTA toxin thus lost their role in promoting muscle regeneration. Future work is needed to future define the fate of surviving FAPs in this model.

FAPs are proven to be able to differentiate to mature adipocytes (white fat cells) and fibroblasts in vitro and in vivo. Recently, Gorski reported that FAPs can differentiate into brown/beige-like adipocytes (BAT) that express uncoupling protein 1 (UCP-1) [32]. In our currently study, we demonstrated that FAPs differentiate into UCP-1(+) BAT-like cells in muscle after VML. The function of UCP-1(+) BAT-like FAPs in muscle regeneration is not fully understood. Meyer et al. has demonstrated that healthy rotator cuff muscles are encapsulated by epimuscular adipose tissue that appear to adopt a beige phenotype when the RC tendon is torn [33]. The same group further showed that transplantation of brown fat into supraspinatus muscle improved muscle regeneration after cardiotoxin injury, suggesting a positive role for BAT in RC muscle regeneration following injury [34]. In our previous work, we also demonstrated that transplantation of BAT-FAPs promotes muscle regeneration and shoulder function after massive rotator cuff tears and repair [13, 14]. In this study, we demonstrated that both mouse and human BAT-like FAP transplantation improved muscle regeneration and hindlimb function after VML in our mouse model.

The underline of mechanism of BAT-FAP in promoting muscle regeneration remains unknown at this time. In addition to thermogenesis and energy metabolism, BAT has been shown to possess a non-thermogenic metabolite function through with an endocrine/paracrine manor. Insulin-like growth factor 1(IGF1) and follistatin, two important promyogenic growth factors are among BAT derived cytokines (also known as batokines) [35–38]. Previous work has suggested that follistatin is the soluble mediator of functional interactions of FAP and myogenic satellite cells during muscle regeneration [39]. However, we did not see change of IGF1 and follistatin gene expression change in FAPs following VML. It is possible FAP derived cytokines other than IGF1 and follistatin may play an important role of FAP-mediated muscle regeneration after VML. A recent study showed that WNT1 Inducible Signaling Pathway Protein 1 (WISP1) is an important FAP-derived extracellular signal indirectly affects the myogenic differentiation of MuSCs [40]. Future work is needed to define beige FAP-derived promyogenic factors that promote muscle regeneration after VML.

β 3 adrenergic receptors (B3AR) are selectively expressed in adipose tissue and selective β 3 adrenergic receptor agonists can effectively stimulate BAT activity in human without significant cardiovascular system side effects [41, 42]. In this study, we demonstrated that Amibegron treatment significantly

improved muscle regeneration and hindlimb function after VML. Mirabegron, a β_3 adrenergic receptor agonist, with a similar structure to Amibegron, has been approved by the FDA for treating over-reactive bladders [43, 44]. Though it may mildly increase heart rate and blood pressure in patients, Mirabegron could be considered as a potential novel treatment for VML in the future.

There are some limitations in this study. First, we did not measure the direct contractility of TA muscle after VML in this study. However, this may not be a critical flaw of this study since we measure the hindlimb gait as a functional measurement. Our results showed that both Amibegron injection and BAT-differentiated FAP transplantation significantly improve limb function after VML. Second, only one time point of 6 weeks was used to evaluate the functional role of gait and muscle histology in the cell transplantation in order to reduce the number of animals used in this study. We believe 6 weeks after VML is a reasonable time point for gait analysis and muscle histology based on results from FAP reporter mice and FAP depletion mice in this study. However, a single time point excluded the possibility of studying the dynamic change BAT-FAP after transplantation in VML. Last but not least, it should be also noted that only a single donor of human FAPs was recruited in this study. Though it is sufficient to prove the concept that BAT-differentiated human FAPs could be improve muscle regeneration after VML, more donors will be recruited in future human FAP studies.

Conclusion

In conclusion, results from this study demonstrated FAP BAT differentiation after VML. B3AR agonist Amibegron induces BAT differentiation of intrinsic FAPs and improves muscle regeneration after VML. Transplantation of both mouse and human BAT differentiated FAPs significantly improved muscle regeneration and limb function VML. Novel information gain from this study suggests that FAP BAT differentiation may serve as new therapeutic target to treat VML in the future.

Declarations

Ethics approval and consent to participate

All procedures were approved by our Institutional Animal Care and Use Committee (IACUC) (Department of veterans affairs medical center at San Francisco) (No.17-003-01).

Consent for publication

Not applicable

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Zili Wang, Hubert Kim, Brian T. Feeley and Xuhui Liu designed the research; Zili Wang, Carlin S. Lee, Jinshen He, Mengyao Liu and Kunqi Jiang conducted the experiment and analyzed the data. Zili Wang, Xuhui Liu and Brian T. Feeley wrote the manuscript. All authors have read and approved the final submitted manuscript.

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Figures

Figure 1

Immunofluorescent staining ($\times 200$) of tibialis anterior of PDGFR α -GFP reporter mice. Compared with the sham, PDGFR α -GFP positive cells were significantly increased in the tibialis anterior at 2 weeks and 6 weeks after VML surgery (A-D). PDGFR α -GFP is stained for green, laminin in red and DAPI in blue. (E) Muscle weight loss = $([T_{\text{Right}} - T_{\text{Left}}]/T_{\text{Left}}) \times 100\%$. (F) Average cross sectional area of muscle fibers

in each group. (G) Percentage of PDGFR α -GFP positive cells / total cell number. Solid lines indicate $p < 0.05$. Error bars indicate SD.

Figure 2

Ki67, α -SMA, Collagen 1 and Collagen 3-gene expression was up-regulated three to five-fold at 2 weeks and two to three fold at 6 weeks in FAPs after VML. No differences of IGF1 and follistatin gene

expression were found in FAPs from the VML and sham groups at either time point. Solid lines indicate $p < 0.05$. Error bars indicate the standard deviation.

Figure 3

Trichrome staining (original magnification, $\times 200$) of tibialis anterior muscle showed significant fibrosis after VML in Corn oil injected Cre(+)/DTA(+) group and Tamoxifen injected Cre(-)/DTA(+) group) after 6 weeks of VML(A-C); however, fibrosis was obviously reduced in the FAP-depletion group (VML+Tamoxifen Cre(+)/DTA(+) group) (D). Immunofluorescent staining indicated that, compared to sham group, the number of PDGFR α positive cells and UCP1 expression were significantly increased in the Corn oil injected Cre(+)/DTA(+) group and Tamoxifen injected Cre(-)/DTA(+) group) (E-G); however, few PDGFR α

and UCP1 positive cells were found in the FAP-depletion group (H). Laminin was stained in red, PDGFRα in green, UCP-1 in pink and DAPI in blue (E-H). (I) Muscle weight loss = $([T_{\text{Right}} - T_{\text{Left}}]/T_{\text{Left}}) \times 100\%$. (J) Average cross sectional area of muscle fibers in each group. (K) Area fraction of collagen (%) = area of collagen staining /entire sample area. (L) Ratio of PDGFRα positive cells (%) = PDGFRα positive cells number /total cells number. (M) UCP-1 area fraction (%) = area of UCP-1 staining/entire sample area. (N) Gait analysis. Solid lines indicate $p < 0.05$. Error bars indicate the SD. TM = Tamoxifen; VML = volumetric muscle loss; IF = immunofluorescent staining; DTA = diphtheria.

Figure 4

FAP BAT differentiation after VML. Immunofluorescent staining indicated that, compared to sham group, the number of PDGFR α positive cells and UCP1 positive cells were significantly increased in the VML groups after 2 weeks and 6 weeks after VML (A-D). (E) The ratio of PDGFR α -GFP and UCP-1 double positive cells (PDGFR α +UCP1+ cell number / total PDGFR α + cells x100%) significantly increased at 2 and 6 weeks after VML (vs. sham). (F) RT-PCR showed that UCP-1 and PRDM16 gene expression were significantly increased at 2 weeks and 6 weeks in FAPs after VML surgery compared to those in the sham group. (solid line indicates $p<0.05$)

Figure 5

Amibegron enhances muscle regeneration after VML surgery by stimulating FAP BAT differentiation. Trichrome staining (original magnification, $\times 200$) of tibialis anterior muscle showed significant fibrosis in the two VML groups at 2 weeks and 6 weeks after VML surgery, when compared to the sham group. However, fibrosis was significantly decreased after Amibegron treatment at both 2 weeks and 6 weeks after VML surgery compared to the vehicle treatment group (A-F). Immunofluorescent staining exhibited

that, compared to sham group, the number of PDGFR α positive cells and UCP1 expression were significantly increased in the two VML groups at 2 weeks and 6 weeks after VML surgery; however, more PDGFR α -GFP and UCP1 double positive cells were found in the VML + Amibegron group, when compared to the VML + Vehicle group. Laminin was stained in red, PDGFR α in green, UCP-1 in pink and DAPI in blue(G-L). (M) Muscle weight loss = $([T_{\text{Right}} - T_{\text{Left}}]/T_{\text{Left}}) \times 100\%$. (N) Area fraction of collagen (%) = area of collagen staining / entire sample area. (O) The ratio of PDGFR α and UCP-1 double positive cells = $\text{PDGFR}\alpha + \text{UCP-1} + \text{cell number} / \text{PDGFR}\alpha + \text{cell number} \times 100\%$. (P) UCP-1 area fraction (%) = area of UCP-1 staining / entire sample area. (Q) Gait analysis of injured limb exhibits that stride length, stance width and paw area at peak stance of the two VML groups had inferior limb function when compared to the sham group; however, limb function was improved in the VML + Amibegron group, when compared to the VML + Vehicle group. Solid lines indicate $p < 0.05$. Error bars indicate the SD. VML = volumetric muscle loss; IF = immunofluorescent staining.

Figure 6

Transplantation of mouse UCP-1(+) FAP improves muscle regeneration after VML. Trichrome staining (original magnification, $\times 200$) of tibialis anterior muscle showed significant fibrosis in the three experimental groups at 6 weeks after VML surgery, when compared to the sham group. However, fibrosis was significantly decreased in the VML + FAP (UCP-1(+)) group, compared to the VML + Matrigel and VML + FAP (UCP-1(-)) groups (A-D). Immunofluorescent staining exhibited that, compared to the sham and VML + Matrigel group, more PDGFR α positive FAPs were found in the transplantation groups. Significantly higher UCP-1 expression was found in the VML + FAP (UCP-1(+)) transplantation group,

compared to the VML + FAP (UCP-1(-)) and matrigel transplantation groups. Laminin was stained in red, PDGFR α in green, UCP-1 in pink and DAPI in blue(G-L). (I) Muscle weight loss = $([TA \text{ right} - TA \text{ left}] / TA \text{ left}) \times 100\%$. (J) Average cross sectional area of muscle fibers in each group. (K) Area fraction of collagen (%) = area of collagen staining / entire sample area. (L) Ratio of PDGFR α positive cells (%) = PDGFR α positive cells number / entire cells number. (M) UCP-1 area fraction (%) = area of UCP-1 staining / entire sample area. (N) Gait analysis of injured limb exhibits that stride length, stance width and paw area at peak stance of the three experimental groups had inferior limb function when compared to the sham group; however, limb function was significantly improved in the VML + FAP (UCP-1(+)) group, when comparing to the VML + Matrigel and VML + FAP (UCP-1(-)) groups. Solid lines indicate $p < 0.05$. Error bars indicate the SD. VML = volumetric muscle loss; IF = immunofluorescent staining.

Figure 7

Beige hFAP transplantation decreases muscle atrophy and improves muscle regeneration. (A) Representative trichrome staining of tibialis anterior (magnification, x200) 6 weeks after VML injury. (B-C). Quantification of fibrosis and fiber CSA at 6 weeks after VML injury ($p < 0.05$). (D). Beige hFAP transplantation improves hindlimb function after VML injury. Mice receiving beige hFAPs transplantation

exhibited significantly improved enhanced propel time and stride length at 6 weeks after VML compared to the other two groups ($p < 0.05$).

Supplementary Files

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- [Supplement1FlowDiagramofExperiment.tif](#)