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Volumetric muscle loss injury repair using *in situ* fibrin gel cast seeded with muscle-derived stem cells (MDSCs)

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Abstract

Volumetric muscle defect, caused by trauma or combat injuries, is a major health concern leading to severe morbidity. It is characterized by partial or full thickness loss of muscle and its bio-scaffold, resulting in extensive fibrosis and scar formation. Therefore, the ideal therapeutic option is to use stem cells combined with bio-scaffolds to restore muscle. For this purpose, muscle-derived stem cells (MDSCs) are a great candidate due to their unique multi-lineage differentiation potential.

In this study, we evaluated the regeneration potential of MDSCs for muscle loss repair using a novel *in situ* fibrin gel casting. Muscle defect was created by a partial thickness wedge resection in the tibialis anterior (TA)muscles of NSG mice which created an average of 25% mass loss. If

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None.

Authors' contributions

NM carried out experiments and data quantifications and helped writing the manuscript. SDH and LASC performed experiments and helped with gel design/composition. JW and JL helped with experiments and immunostainings. YL and JH provided the MDSCs, helped with experimental design and edited the manuscript. RD designed the project, supervised the experiments and data analysis and wrote the manuscript.

untreated, this defect leads to severe muscle fibrosis. Next, MDSCs were delivered using a novel *in situ* fibrin gel casting method.

Our results demonstrated MDSCs are able to engraft and form new myofibers in the defect when casted along with fibrin gel. LacZ labeled MDSCs were able to differentiate efficiently into new myofibers and significantly increase muscle mass. This was also accompanied by significant reduction of fibrotic tissue in the engrafted muscles. Furthermore, transplanted cells also contributed to new vessel formation and satellite cell seeding.

These results confirmed the therapeutic potential of MDSCs and feasibility of direct *in situ* casting of fibrin/MDSC mixture to repair muscle mass defects.

Keywords

Skeletal muscle; Volumetric muscle loss; Fibrin gel; Muscle-derived stem cells (MDSCs); Muscle repair; Bio-scaffold

1. Introduction

Skeletal muscle consists of contractile units of muscle called myofibers, along with an organized network of vasculature, connective tissue and nerves that allows voluntary activation and contraction of the muscles. In order to maintain its growth and regeneration, muscle is benefiting from the presence of muscle stem cells which are responsible for its growth and maintenance (Lagha et al., 2008; Mauro, 1961; Qu-Petersen et al., 2002; Cossu and Bianco, 2003).

Muscle stem cells provide a tremendous regeneration potential after minor muscle damage such as strains or contusions. Following minor muscle damages, local tissue inflammation triggers the release of different cytokines, chemotactic factors and activation of signaling pathways leading to muscle stem cell recruitment, activation and proliferation (Serrano et al., 2011; Wang and Rudnicki, 2012). This provides a massive source of proliferating myoblasts which eventually differentiate into new myofibers and repair the muscle (Serrano et al., 2011; Wang and Rudnicki, 2012). During this phase of muscle repair, fibroblasts also contribute to regeneration by providing connecting tissue and collagen deposition to replace damaged extra cellular matrix (ECM) and remodel the repaired muscle (Serrano et al., 2011).

Although this innate repair system works well in the case of minor muscle injures (Quintero et al., 2009), in the case of severe injuries such as volumetric muscle defects, the story is quite different (Rivera and Corona, 2016). These types of massive injuries are very common during traumatic incidents such as car accidents, explosive injuries and iatrogenic causes. In these cases, extensive muscle masses are lost leading to the loss of muscle ECM and its structural tissues (Rivera and Corona, 2016; Corona et al., 2015). Severe damage to ECM leads to the lack of appropriate regenerative signaling within the damaged area and subsequently inefficient muscle stem cell recruitment and activation. Therefore, the regeneration will be limited while fibroblast activation leads to massive collagen deposition and formation of scar tissue and fibrosis (Corona et al., 2015). Therefore many of these

patients suffer from severe muscle fibrosis and reduced functionality of the injured muscle (Corona et al., 2015; Garg et al., 2015).

So far, autologous muscle flap transplantation is the only therapeutic option which is only possible in small muscle defects (Juhas and Bursac, 2013). However, in case of extensive tissue losses, this approach is not an option due to limited donor site. Therefore many efforts have been done to use muscle stem cells or bio-scaffolds alone or in combination for skeletal muscle mass replacement (Lutolf and Hubbell, 2005; Sicari et al., 2015).

In this case, using appropriate biocompatible/degradable material along with muscle stem cells allows for a better cell delivery and survival into the muscle defect (Cezar and Mooney, 2015; Han et al., 2016). Therefore, from a theoretical standpoint, while the matrix provides an excellent support for myogenic cells and allows appropriate secretion and activation of myogenic signals, transplanted stem cells can proliferate and differentiate into myofibers and contribute to the repair and remodeling of the muscle. In the ideal scenario, eventually the matrix will be vascularized and remodeled by new myofiber formation and minimal fibrosis (Kim et al., 2016).

In order to achieve this goal, different stem cell types and scaffolds have been tested in animal models to evaluate the therapeutic response. Among the cells, satellite cells, myoblasts and mesenchymal stem cells (MSCs) are common cell types which have been tested alone or in combination of bio-scaffolds (Cezar and Mooney, 2015; Han et al., 2016). Hydrogels including collagen, hyaluronic acid, polyethylene glycol (PEG), and fibrin are also among common biomaterials that have been tested (Lutolf and Hubbell, 2005; Cezar and Mooney, 2015; Kamelger et al., 2004). So far, *In vitro* generation of bioengineered cell/gel construct and subsequent transplantation of the construct is the common approach for these applications. Unfortunately, *in vitro* culture of the bioengineered muscle is not an easy task and often the cells suffer from poor survival and differentiation within the bio-scaffold due to hypoxic condition and lack of perfusion leading to massive cell death within the construct.

Therefore, in the current study we decided to use a different approach and test the feasibility of direct *in situ* defect casting using a bio-degradable scaffold seeded with muscle stem cells. In this case, instead of making the bioconstruct *in vitro*, it will be directly casted in the muscle defect *in vivo*. This method will provide a more practical and precise implanting and avoids above-mentioned *in vitro* problems. So, one of the aims of the current study was to demonstrate the feasibility of this approach (*in situ* casting) and evaluating its outcome in a mouse model for volumetric muscle loss.

For the scaffold, we chose fibrin due to its important role in wound healing and its fibrillary structure which promotes myoblast survival, proliferation and differentiation (Duong et al., 2009; Page et al., 2011; Chung et al., 2016). Furthermore, fibrin gel also supports vascularization and will be completely degraded in few weeks which will allow gradual tissue replacement and integration with host muscle.

For muscle stem cells, in this study we chose muscle derived stem cells (MDSCs) (Lee et al., 2000; Deasy et al., 2001). MDSCs can be isolated easily and in abundance from skeletal

muscles by their slow adhering characteristics using preplating methods. These cells can be expanded exponentially *in vitro* so there is no source limitation (such as satellite cells) and have sustained proliferation, self-renewal and differentiation potential. Furthermore, MDSCs have great survival in hypoxic conditions, are resistant to oxidative stress and have superior in vivo engraftment potential compared to other muscle stem cells (such as satellite cells or myoblasts) (Deasy et al., 2001; Vella et al., 2011; Usas and Huard, 2007). In addition, MDSCs enhance muscle regeneration by stimulation and contribution in new vessel formation as well as promoting neural regeneration in engrafted regions, which are crucial elements needed for a successful engraftment (Ota et al., 2011; Lavasani et al., 2014). These characteristics make them a very attractive option to test for muscle mass defect repair. Therefore the main goal of the current study was to evaluate the efficacy of MDSCs combined with fibrin gel for volumetric muscle loss repair.

2. Materials and methods

2.1. MDSC isolation

MDSCs were isolated from newborn mice muscles using a preplating (PP) technique as described before (Lavasani et al., 2013; Gharaibeh et al., 2008). Briefly, hindlimb muscles were extracted and after mincing into small pieces, were enzymatically dissociated using a serial digestion by collagenase, dispase and trypsin. Dissociated cells were then processed through serial preplating using collagen-coated plates. MDSCs were expanded from PP6 and labeled with a membrane LacZ lentivirus for *in vivo* experiments.

2.2. In vitro gel casting

For *in vitro* cell/gel casting, MDSCs (1×10^6) were suspended in 200 μl of fibrinogen solution (4mg/ml, Sigma, F4753) and gel formation was induced by addition of thrombin (5IU, Sigma, T4648) in a 48 well plate. The plate was transferred for 15 min in CO_2 incubator to allow proper casting of the gel. Gel constructs were then detached and transferred into bigger size plates (24 or 12well plates) for culture. Growth medium (15% fetal bovine serum-FBS, 10% horse serum in IMDM supplemented with 10 ng/ml basic FGF) was then added to the well to allow cell survival and expansion in casted gel. For differentiation of the cells, the medium was replaced with myotube induction medium (5% horse serum in DMEM) for a week to allow formation of multinucleated myotubes.

2.3. Volumetric muscle loss model generation

Three month old immunodeficient NSG mice NOD. Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice were purchased (Stock number: 005557, The Jackson Laboratory, Bar Harbor, ME) and maintained at the barrier facility of the Brown Foundation Institute of Molecular Medicine at the University of Texas Health Science Center at Houston. Mice were group-housed in ventilated cages, given acidified water and irradiated rodent diet (Purina, St Louis, MO) ad libitum, and maintained on a 12:12-h light: dark cycle. All experimental studies were carried out in accordance and approved by the Institutional Animal Care and Use Committee (IACUC) guidelines of The University of Texas Health Science Center at Houston. Mice were divided in three experimental groups (non-treated control, gel alone and gel/MDSC groups, $n = 5$ each).

For generation of the muscle defect, after induction of general anesthesia, hindlimb was disinfected using chlorhexidine followed by 70% ethanol. A small incision (5 mm) was made over the tibialis anterior (TA) to expose the muscle. The muscle defect was created by removing a 4 × 2 × 2mmpartial thickness wedge resection using iris scissors (FST, Foster City, CA). If needed, hemostasis was achieved by gentle pressure with sterile cotton swaps. Then the fascia and skin were sutured using a 5-0 PDSII suture (Ethicon, Cincinnati, OH).

2.4. In situ cell/gel casting

For *in situ* casting of the MDSCs with fibrin gel, freshly harvested MDSCs (1×10^5) were resuspended in 20 μl of fibrinogen solution and the muscle defect was filled with the solution. Casting was induced by addition of thrombin solution (5 IU/2 μl). After 2 min waiting period to ensure gel solidification, the wound was carefully covered with the fascia and skin and secured using sutures. For gel alone group, the damage was casted with the fibrin gel alone without incorporation of MDSCs. For the non-treated control group, the wound was closed without any treatment. The mice were sacrificed 4 weeks later and the TAs were isolated. The muscles were dehydrated using increasing sucrose solutions (5 to 30%) before embedding in OCT and frozen in precooled isopentane. Sections of 8–12 μm were used for Immuno-histological analysis.

2.5. Histology

Casted gel constructs or dissected muscles were washed in PBS and embedded in OCT compound and were frozen using precooled isopenthane. Embedded tissues were then sectioned at 8–12mmthickness and fixed in 4% paraformaldehyde, rehydrated and stained with Lac Z (β-Gal).

For immunostaining, the slides were washed with PBS, treated and permeablized with 0.03%Triton X-100 (Sigma, St. Louis, Mo), and blocked with 3% BSA (Amresco, Solon, OH) before applying the primary antibodies. Primary antibodies include rabbit anti-von Willebrand factor (Dako, Carpipenteria, CA), rabbit anti-laminin antibody (Sigma, St. Louis, MO), mouse anti-Pax7 antibody (R&D), mouse anti-Myf5 antibody (SCBT), mouse anti-myogenin (BD Pharmingen) and mouse antimyosin heavy chain-MHC (MYH1) antibody (MF20, DSHB). After incubation, the slides were all washed and stained with appropriate secondary antibodies (goat anti-rabbit Alexa Fluor 555 for the vWF stain, goat anti-mouse Alexa Fluor 555 for the PAX7, Myf5, myogenin and MHC and goat anti-rabbit Alexa Fluor 488 for the Laminin- Life technologies, Carlsbad, CA). The slides were then washed and mounted with Pro–long Gold anti-fade DAPI mounting media (Life Technologies, Grand Island, NY). For the evaluation of the muscle/fibrosis, slides were stained for Lac-Z, H&E or mason trichrome staining.

2.6. Muscle and fibrosis quantification (planimetric assay)

Stained sections were further assessed for the quantification of muscle/fibrosis measurements using a planimetric assay. Microphotographs were taken at 4× magnifications and the analysis was performed with Amira software (FEI, Hillsboro, Oregon) in 10 representative sections per group (2 per muscle). Thresholds for the muscle and fibrosis were adjusted, marked and surface area was calculated using the software. The percentage of

the muscle and of the fibrosis surface area was then determined by the threshold of the muscle/fibrosis divided by the entire area × 100. Muscle fiber cross section area (CSA) and fiber nuclei quantification were done using Image J software (NIH). Data were statistically analyzed using analysis of variance (ANOVA) method.

3. Results

3.1. In vitro characterization of MDSCs

In order to characterize the cells, MDSCs were expanded on Matrigel-coated plates and stained for myogenic transcription factors. As Fig. 1a demonstrates, when MDSCs expanded in myogenic medium, cells uniformly express early myogenic gene of Myf5 which indicates their specification to the myogenic lineage. As demonstrated in the immunostaining images and quantification graph (Fig. 1b), at this stage, majority of the cells do not express Pax7 (a marker for satellite cells) or late myoblast markers (such as Myogenin or myosin heavy chain).

Next, in order to identify their surface markers, MDSCs were stained for common stem cell and myogenic surface markers and analyzed using flow cytometry. As shown in Fig. 1c, MDSCs express high levels of CD29 (beta-1 integrin) and alpha7-integrin, known muscle stem cell/progenitor markers. Interestingly, 50% of the MDSCs are also express Sca-1, a known stem cell marker which also is expressed in early myogenic progenitors. Another muscle stem cell marker of CD34 is barely expressed in a small fraction of these cells. These data are in consistent with previous report of the MDSC surface marker characterization indicating the presence of early myogenic progenitor/stem cells within these cells (Jankowski et al., 2001).

In the next step and in order to prove differentiation potential of MDSCs in the fibrin gel construct, MDSCs were incorporated into fibrin gel constructs *in vitro* and transferred into the culture medium for differentiation in the gel structure. These data is presented in Fig. 1d. Following one week of culture in the gel and after differentiation induction using myotube medium, elongated myotubes can be detected in the 3D gel constructs at day 10 as shown in the brightfield image.

To confirm final maturation of MDSCs within the gel constructs, gels were sectioned and stained for myotube maturation marker (myosin heavy chain-MHC). As demonstrated in Fig. 1d, multinucleated myotubes can be detected within the gel construct expressing terminal differentiation marker of myosin heavy chain-MHC. As indicated, high magnification image of mature myotubes confirmed the presence of multinucleated myotubes with peripherally-located elongated nuclei which is a hallmark of mature myotubes. These data together, confirmed the suitability of MDSC for myogenic differentiation within the fibrin construct.

3.2. Volumetric muscle loss injury leads to bio-scaffold loss and severe fibrosis

In order to generate a mouse model for volumetric muscle loss injury and evaluate the repair process, we have used a wedge excision injury model in TA muscle of the mice. In this model, after general anesthesia and exposing the TA muscle, a partial thickness wedge

resection was performed using iris scissors. By using this approach, a half thickness muscle bio-scaffold loss was created as demonstrated in Fig. 2.

By using laminin immunostaining to mark the muscle bio-scaffold, the damaged zone (Fig. 2b) demonstrates the absence of normal muscle scaffold as compared to the intact control muscle in upper panel (Fig. 2a).

In order to evaluate the outcome of volumetric muscle loss, injured muscles were harvested for histology evaluation one month post-injury and stained for fibrosis. As demonstrated (Fig. 2b,c), injured muscles failed to self-repair and the damaged area was replaced by massive fibrosis. This data indicates the inability of natural muscle repair mechanism in the case of severe muscle loss injuries.

To quantify the amount of muscle mass defect, a test group of TA muscles ($n = 6$) were undergone wedge resection and the damaged muscles were quantified by weight measurement. As shown in Fig. 2d, the wedge resection creates an average of 25% muscle mass loss compared to intact muscle (from average weight of 41.6±0.3 mg in control TAs to 30.9 ± 0.4 mg after wedge resection, mean ± SE).

3.3. In situ MDSC/fibrin gel casting can repair volumetric muscle loss injury

Next we tested the *in situ* casting of the MDSCs with fibrin gel. This method allows excellent shaping of the implant and its proper adherence to the defect and avoids hurdles associated with *in vitro* bioconstruct generation. Therefore, the muscle defect was filled with a fibrinogen solution containing freshly harvested MDSCs (or a fibrin gel alone as control). Then the gel formation was induced by addition of thrombin solution into the cell/fibrinogen mixture. This data is presented in Fig. 3a. As demonstrated, few minutes after the thrombin addition, the muscle defect is completely casted with the solid fibrin gel containing MDSCs. As control, a group of mice were also treated with gel alone casting without MDSCs.

As the final goal of the study was to evaluate the therapeutic potential of the MDSCs, mice were analyzed one month post implantation for histological evaluation of engraftment. Fig. 3b demonstrates sagittal and cross sections of transplanted muscles stained for LacZ (which marks donor MDSCs) which indicates significant contribution of MDSCs in muscle defect repair. Bio-scaffold immunostaining using a laminin antibody along with LacZ staining also indicated bio-scaffold restoration within the transplanted region (Fig. 3c). As demonstrated, muscle defects were reconstructed with LacZ positive (membrane LacZ) myofibers indicating significant contribution of the transplanted MDSCs in muscle repair with minimal fibrosis (Fig. 3d).

Also, in order to demonstrate maturation of donor-derived fibers, sections were stained for expression of striated myosin heavy chain 1 (MYH1). As demonstrated (Fig. 3e), donor-derived LacZ positive fibers uniformly express MYH1 which indicates their terminal striated myofiber maturation *in vivo*.

Finally, total muscle and fibrosis area percent in muscle cross sections were evaluated using a quantitative planimetric assay. A representative image of each group is presented in Fig. 4A. As demonstrated in Fig. 4b, while non-treated (control) and gel alone treated muscles

did not show any significant difference in muscle mass or fibrin area, MDSC/fibrin gel treated group demonstrated significant muscle mass restoration and fibrosis reduction in the transplanted muscles. In addition, engrafted regions demonstrated significant increase in the number of donor-derived small to medium sized myofibers with centrally located nuclei which is the hallmark of skeletal muscle regeneration (Fig. 4c).

3.4. MDSCs contribute to new vessel formation and restoration of satellite cells

Restoration of blood supply is one of the most important requirements for tissue repair. Since MDSCs endow great multi-lineage differentiation potential and were shown to be able to induce new vessel formation (Lee et al., 2000; Deasy et al., 2001; Usas and Huard, 2007), next we looked for their possible contribution in new vessel formation.

This data is presented in Fig. 5a. As demonstrated, after staining for vWF and LacZ to mark any donor derived vessels, we were able to identify contribution of donor-derived cells ($LacZ^+$) in new vessel formation in the regenerated area. Quantification of vWF and LacZ stains revealed abundant contribution (mean of 26%) of donor-derived cells in formation of new vessels (Fig. 5b).

Finally, we evaluated the potential of MDSCs for restoration of adult muscle stem cells (satellite cells) which is crucial for longevity of transplant. This data is presented in Fig. 5b as sporadic donor-derived $Pax7^+$ $LacZ^+$ cells were detected within the engrafted region confirming contribution of MDSCs in satellite cell restoration.

4. Discussion

The results of the current study confirm the therapeutic potential of *in situ* casting of stem cell/bio-scaffold for repair of partial muscle loss defects. As demonstrated MDSCs combined with fibrin gel are able to provide appropriate bio-scaffold and niche for muscle stem cell survival, terminal differentiation and self-renewal in a muscle loss injury model in mice. Furthermore, the results of this study also demonstrate the effectiveness of this approach in significant reduction of scar formation which often leads to function loss and patient disability (Serrano et al., 2011; Rivera and Corona, 2016; Corona et al., 2015).

Regarding the biomaterials, fibrin gel is one of the most studied materials due to its natural bio-compatible/degradable nature and its physiological role in supporting wound healing. Indeed, many *in vitro* and *in vivo* studies have demonstrated its role in supporting cell survival, migration and differentiation in wound healing and minor muscle damages (Duong et al., 2009; Page et al., 2011; Chung et al., 2016). However, as mentioned above, in severe muscle injury models, fibrin gel alone treatment did not show significant improvement compared to non-treated animals indicating the restoration of bio-scaffold alone is not enough for muscle repair in severe cases.

Fibrin gel also has been used as a delivery vehicle for cell transplantation in few muscle injury models (Page et al., 2011; Heher et al., 2015; Gigante et al., 2012). One of the successful applications of fibrin gel was through *in vitro* fibrin microthread formation seeded with primary muscle stem cells/myoblasts from muscle biopsy (Page et al., 2011).

However, the need for large biopsy samples to derive enough myogenic cells and complicated procedure of *in vitro* fibrin microthread generation and transplantation are among limitations of this approach. Here, we have introduced a novel approach based on direct *in situ* casting of the fibrin gel along with MDSCs which provides a straightforward and feasible approach for direct bio-scaffold/stem cell delivery into the muscle defect. This allows efficient material (biomaterial, stem cells) delivery into the wound with excellent casting and superior adherence to the defect. Considering the relative big size of muscle loss in our cases (25% mass loss), this approach looks very promising for small to medium sized muscle mass loss defects.

Furthermore, another advantage of the current study is based on using MDSCs for muscle injury repair. Regarding satellite cells or muscle progenitor cells, few recent efforts have been done using freshly isolated progenitor cells combined with different hydrogels or *in vitro* engineering artificial muscle fibers to preserve their quiescence which looks promising (Rossi et al., 2011; Quarta et al., 2016). However, as one of the major limiting factors for muscle stem cell therapy is limited source of the satellite cells (Cao et al., 2005; Sohn and Gussoni, 2004), MDSCs have the unique advantage in this aspect as can be expanded exponentially *in vitro* without losing their proliferation and differentiation potential (Deasy et al., 2001; Usas and Huard, 2007). Another advantage of MDSCs for this application is their unique potential for survival and engraftment in hypoxic and ischemic conditions which has been demonstrated before (Jankowski and Huard, 2004; Urish et al., 2005). Indeed, MDSCs great survival in hypoxic conditions and their resistance to oxidative stress makes them a very attractive candidate for cell therapy in muscle loss injury patients (Qu-Petersen et al., 2002). Our *in vivo* result clearly demonstrates their great survival and subsequent engraftment in this model.

Another aspect of the tissue injury repair is the restoration of blood circulation through revascularization. Therefore, identification of a stem cell population with myogenic and vascular differentiation potential would be ideal. Considering these potentials, vascular-derived myogenic cells would be the most reasonable candidates. Indeed, MDSCs might have the similar origin and potential and many studies have shown their multi-lineage differentiation abilities into different tissues including muscle and vessels (Lee et al., 2000; Usas and Huard, 2007; Peng et al., 2002; Nishimori et al., 2012). As our *in vivo* results also indicate, when MDSCs are transplanted along with appropriate bio-scaffold material, they are able to actively contribute in muscle tissue regeneration by formation of mature fibers as well as their contribution in the formation of new capillaries. As our quantification results demonstrated, we were able to identify significant donor cell contribution (26%) in neovascularization in engrafted regions. This is another important outcome of the current study which confirms the appropriateness of MDSCs for this application.

Finally, for longevity of engraftment, identification of the appropriate stem cell with self-renewal and differentiation after transplantation is the ideal goal of stem cell therapies (Cao et al., 2005; Sohn and Gussoni, 2004). As our results suggest, MDSCs are not only able to generate mature muscle fibers, they are also able to restore muscle satellite cells after engraftment which is a remarkable potential. Although the frequency of donor-derived

satellite cells in current *in vivo* study was limited to sporadic cells, nevertheless, it confirms the ability of MDSCs to contribute in restoration of satellite cell pool *in vivo*.

5. Conclusions

In conclusion, current study highlights a novel approach for treatment of sizable muscle loss injuries using *in situ* MDSCs/fibrin gel casting. This approach allows significant muscle mass restoration as well as fibrosis reduction with active contribution of transplanted cells in the muscular and vascular regeneration. Considering the ease and abundance derivation of large quantities of MDSCs (no source limitation), their superior survival in hypoxic condition and multi-lineage differentiation potential, this novel stem cell based delivery approach can be considered as a potential therapeutic option in muscle mass loss injuries such as accidents and combat wounds. Eventually, in order to move forward with this approach, further studies using larger animal models are necessary to optimize it for future therapeutic applications.

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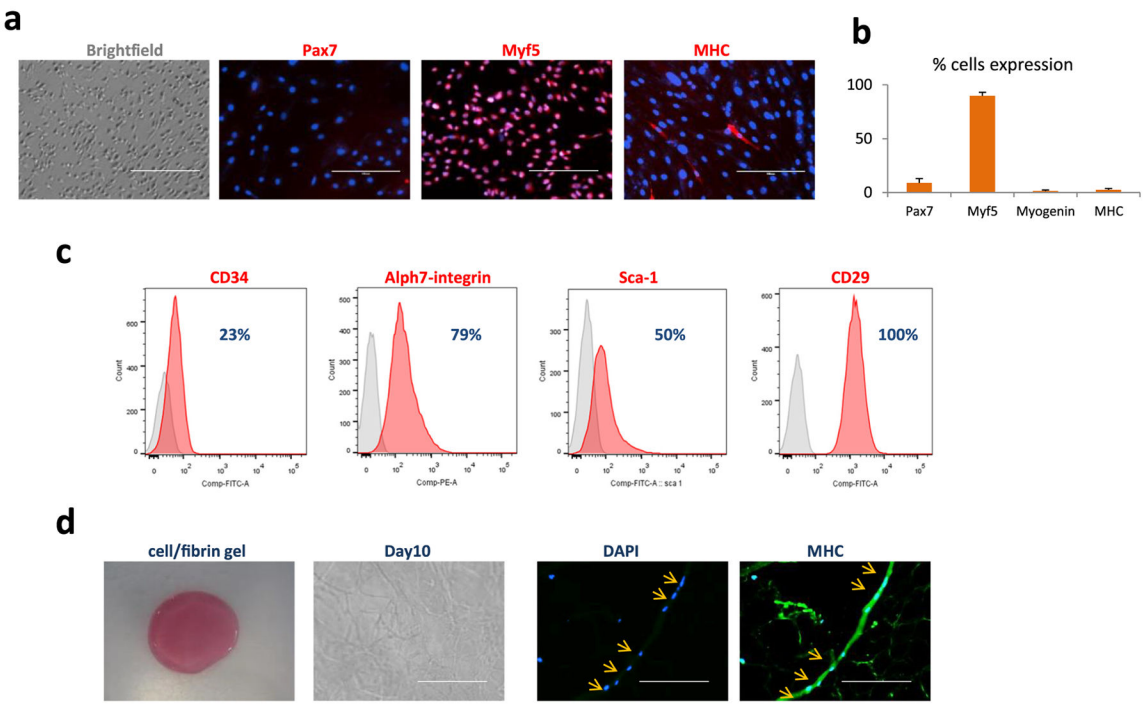

Fig. 1.

In vitro characterization of MDSCs and fibrin gel construct. (a) Monolayer expansion of MDSCs in myogenic induction medium. Brightfield image on left shows MDSCs morphology during expansion. Immunostaining for Myf5 shows uniform nuclear expression of Myf5 and lack of Pax7 and MHC expression during expansion phase. (b) Quantification of immunofluorescent images stained for myogenic markers shows percent of the cells positive for each marker (Mean + SD, $n = 5$ image for each marker). (c) Surface marker characterization of MDSCs indicates the levels of expression of myogenic progenitor (alpha 7 and beta-1 integrin-CD29) as well as stem cell markers (Sca-1, CD34) during *in vitro* expansion. (d) Characterizations of a fibrin gel construct. Left image shows a MDSC/fibrin gel construct. Brightfield image shows MDSCs' differentiation within the gel construct and formation of myotubes 10 days after seeding into the gel construct *in vitro*. Immunostained section of the gel on left panels indicates a multinucleated MHC^{+} myotube formed within the gel construct. Nuclei (marked by arrows) are elongated and located peripherally similar to a mature myofiber. Scale bars are 100 μm.

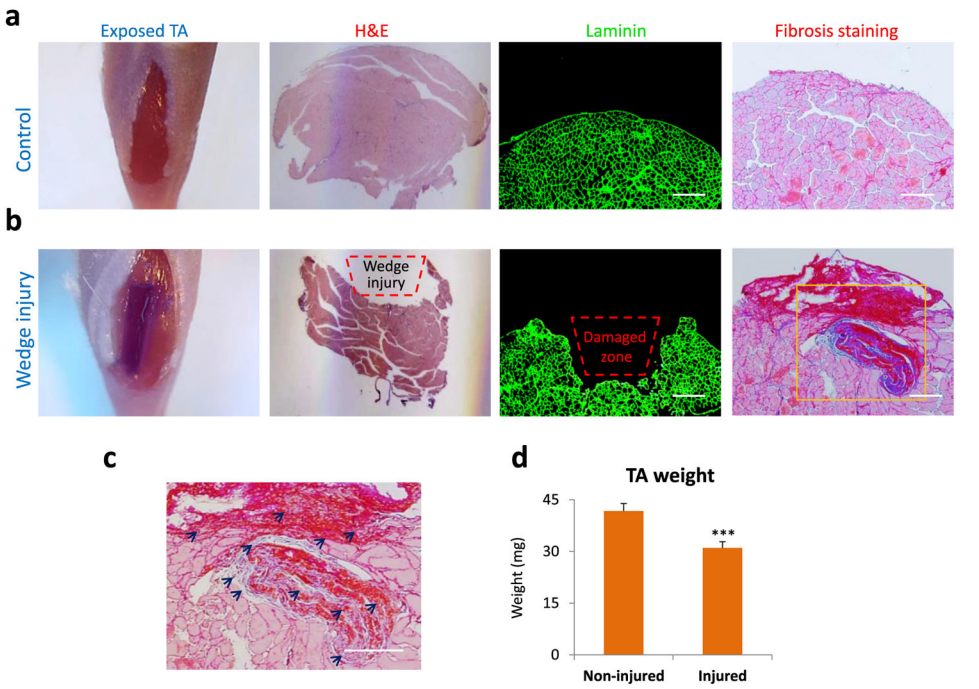

Fig. 2.

Generation of a mouse model for volumetric muscle loss injury. (a) TA muscle exposure and cross section of the muscle demonstrates normal muscle histology, intact bio-scaffold (as stained by laminin in green) and lack of fibrosis (mason trichrome staining). (b) A partial thickness wedge resection in the TA muscle creates a significant volumetric muscle loss and lack of bio-scaffold. This defect, if left untreated, will lead to severe fibrosis and scar formation at the injury site as demonstrated in the right image. (c) A magnified image from fibrotic area (from yellow box in panel b right image) demonstrates massive fibrosis and deposition of fibrotic tissue/scar formation (marked by black arrows). (d) Quantification of the defect size by weight indicates average muscle weight loss of 25% in damaged TA muscles (mean + SD, $n = 6$). Scale bars are 100 μm.

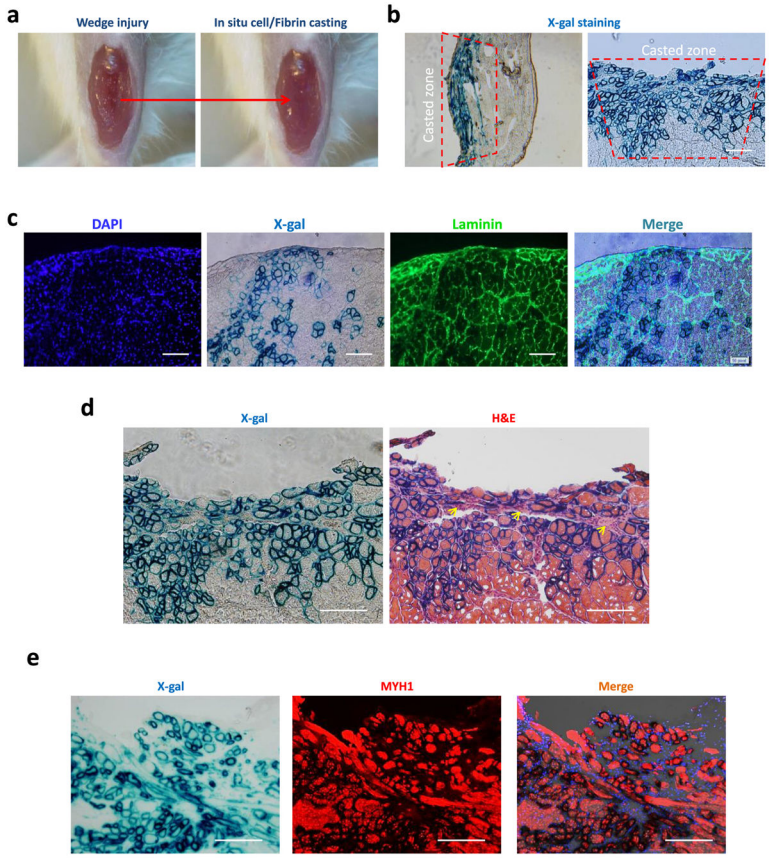

Fig. 3.

In vivo regeneration potential of MDSC/fibrin gel for muscle loss repair. (a) Images demonstrate *in situ* casting of fibrin gel seeded with MDSCs. The defect is completely filled with the casted material and the gel is solidified after addition of thrombin (right panel). (b) One month after implantation, muscles were sectioned for engraftment. Images demonstrate sagittal and cross sections of the treated TA muscles indicating significant contribution of MDSCs in new myofiber formation as labeled with a membrane LacZ. (c) Cross section of a repaired muscle indicates deposition of new bio-scaffold and restoration of muscle ECM as stained for laminin (green) and LacZ. LacZ positive donor-derived cells contribute to new myofiber formation as well as deposition of bio-scaffold in the engrafted region. (d) A high magnification image of engrafted region indicates full repair of muscle defect by donor-derived myofibers with minimal fibrosis (arrows). (e) Immunostaining for MYH1 indicates uniform expression of striated myofiber marker (MYH1 in red) in LacZ positive donor derived fibers. Scale bars are 100 μm.

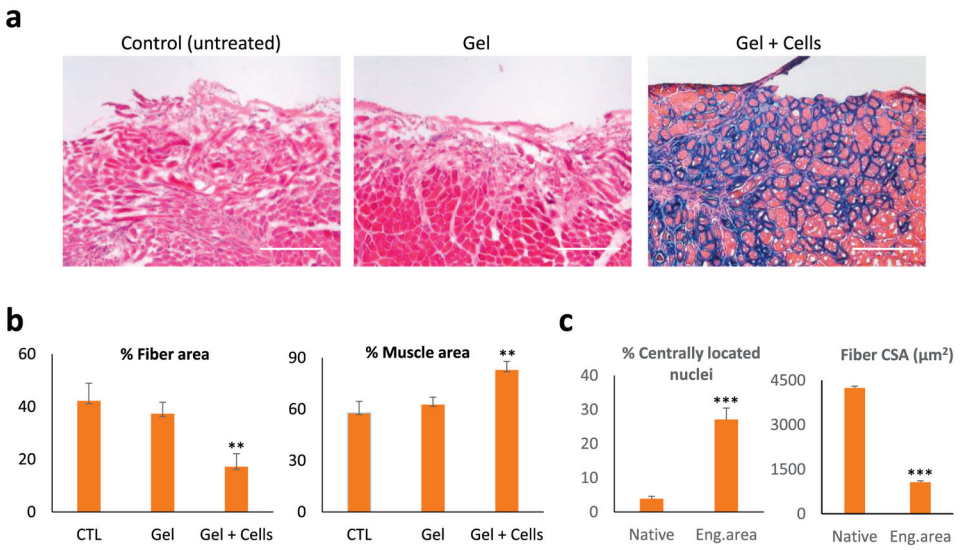

Fig. 4.

Planimetric quantification of muscle mass and fibrosis indicates therapeutic efficiency of MDSC/fibrin gel casting method for muscle defect repair. (a) Representative images of TA cross sections from different experimental groups show different levels of fibrosis vs. engraftment in experimental groups. (b) Planimetic analysis of muscle vs. fibrosis mass in control (untreated), gel alone and gel + cell (MDSC) treated muscles indicates significant increase in the muscle mass along with significant reduction of fibrosis in the treated muscles. $^{**}P < 0.01$ (Data are mean + SD, $n = 10$ muscle section per experimental group). Scale bars are 100 μm. (c) Quantification of fibers with centrally located nuclei and their cross section area (CSA) indicates significant increase of regenerative fibers in engrafted area of treated muscles compared to non-engrafted native regions.

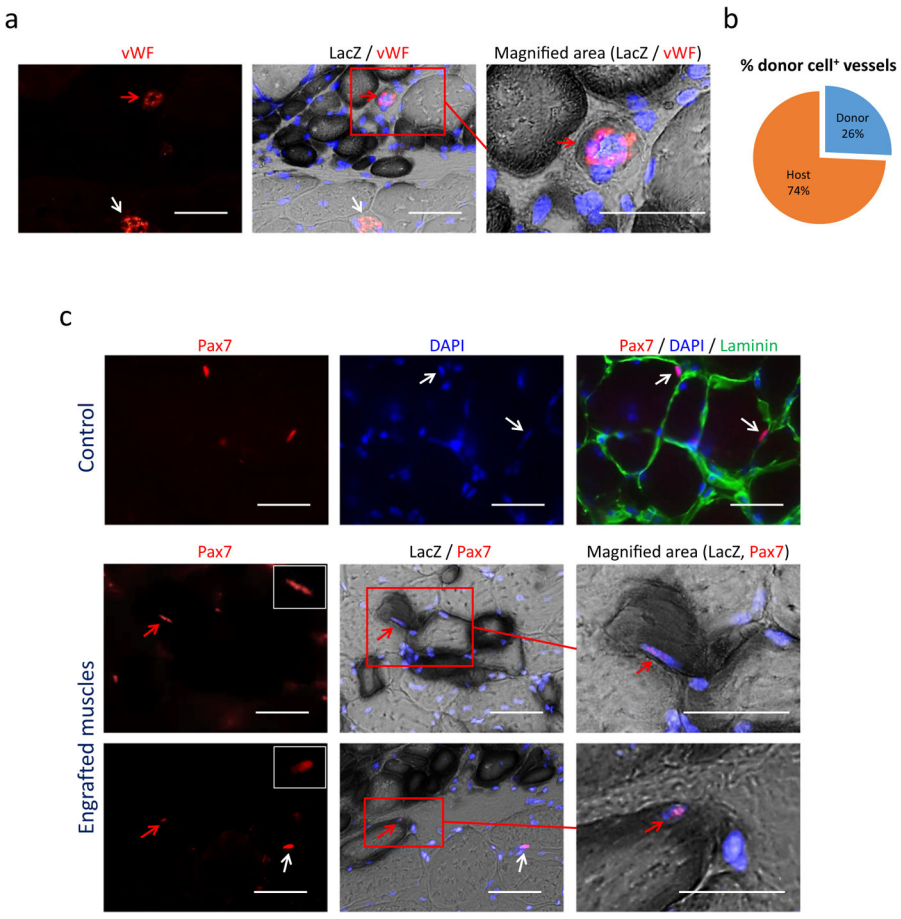

Fig. 5.

MDSCs contribute in new vessel formation and restoration of muscle stem cells in engrafted muscles. (a) A cross section of engrafted muscle indicates contribution of MDSCs in new capillary formation as double positive for vWF and LacZ (marked with red arrow). A host vessel ($LacZ^{-}$ vWF^{+}) is marked with a white arrow at the bottom of image for comparison. Right image demonstrates magnified area (red box). (b) Quantification of donor positive vessels in engrafted muscles. As demonstrated LacZ positive donor derived cells contributed in an average of 26% of the vessels in engrafted regions. (Data are mean of positive and negative vessels for donor cells, $n = 14$ muscle cross sections from treated group). (c) Cross sections of the engrafted muscle indicate contribution of MDSCs in satellite cell seeding as stained for Pax7 and LacZ. Upper panel demonstrates specificity of Pax7 staining (red) to mark satellite cells under basal lamina as stained with the laminin antibody (green). Lower panels show donor-derived satellite cells. Red arrow marks donor- derived satellite cells positive for Pax7 and LacZ. These Pax7 positive (red) nuclei are magnified in left panels (white boxes). Right images demonstrate magnified area with nuclear expression of Pax7/ DAPI in LacZ positive fibers. White arrow marks a LacZ negative host satellite cell in lower panel as a control. Scale bars are 50 μm.